DEDIFFERENTIATION AND REDIFFERENTIATION OF CANINE ARTICULAR CHONDROCYTES

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Abstract

Articular cartilage can be damaged directly through injury or osteoarthritis (OA). This tissue is very poor at regenerating itself due to its avascular nature and the immobility of chondrocytes within the tissue. There are a range of surgical techniques to repair cartilage lesions. Cellular therapies such Autologous Chondrocyte Implantation (ACI) and later modifications have been used to repair cartilage lesions for the past two decades. However, there is currently no completely successful treatment of cartilage lesions, with the newly generated cartilage often possessing very poor mechanical properties. Also, cell-based therapies require large numbers of chondrocytes which have to be expanded in monolayer. A consequence of this expansion is a loss of the chondrocyte phenotype (dedifferentiation). The overall aim of this thesis was to develop a greater understanding of chondrocyte dedifferentiation and redifferentiation *in vitro* using canine chondrocytes. Dogs can also suffer with OA and have been used extensively as a model for OA. Firstly, canine chondrocytes were expanded in monolayer up to P5 to confirm dedifferentiation. These cells were shown to have lost their typical chondrocytic phenotype through decreased expression of collagen type II and increased expression of collagen type I and CD44. A considerable part of this element of the thesis also involved identifying antibodies that would cross-react with the target canine antigens. The next aim of the thesis was to

redifferentiate dedifferentiated chondrocytes through three-dimensional (3D) culture. Initial problems with high density pellet culture led to the selection of a supporting material. Alginate was chosen as it is a naturally occurring polymer which has previously been used to culture chondrocytes in 3D. After making several adjustments to the set-up and downstream analysis of the beads, chondrocytes from different passages were seeded into them. Alginate beads seeded with P2 chondrocytes appeared to contain cells with a more chondrocyte-like phenotype compared to P3- and P4-seeded beads. However, expression of collagen type I was still relatively high in P2-seeded beads, indicating 3D culture alone is not enough to induce complete redifferentiation. Therefore, the final aim of this thesis was to enhance the redifferentiation of dedifferentiated canine chondrocytes. Two initial conditions were selected; addition of 25µg/ml ascorbate to the culture medium and incubating the beads under reduced oxygen conditions (2.4%). Culturing the beads under reduced oxygen conditions (2.4%) appeared to enhance redifferentiation. However addition of ascorbate to the culture medium had mixed results. This culture system can now be further adapted and modified to better enhance chondrocyte redifferentiation. This work could include combining the two conditions already tested as well as adding growth factors to the culture medium. More successful maintenance of the chondrocyte phenotype *in vitro*, could potentially lead to better articular cartilage regeneration both *in vitro* and *in vivo*.

Publications

Papers published during the completion of this thesis were as follows:

Lewis R, Feetham CH, Gentles L, **Penny J**, Tregilgas L, Tohami W, Mobasheri A, Barrett-Jolley R. Benzamil sensitive ion channels contribute to volume regulation in canine chondrocytes. *British Journal of Pharmacology* 2013 Apr;168(7):1584-96

Penny J, Harris P, Shakesheff KM, Mobasheri A. The biology of equine mesenchymal stem cells: phenotypic characterization, cell surface markers and multilineage differentiation. *Frontiers in Bioscience (Landmark Edition)* 2012 Jan 1;17:892-908

Declaration

This work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

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Abbreviations

3D	Three Dimensional
ACI	Autologous Chondrocyte Implantation
ANOVA	Analysis of Variance
BCA	Bicinchoninic acid
BMP	Bone Morphogenetic Protein
BSA	Bovine Serum Albumin
$CaCl_2$	Calcium Chloride
CD	Cluster of Differentiation
CH13L1	Chitinase 3-Like 1
CLEC3A	C-type Lectin Domain Family Member 3
CO_2	Carbon Dioxide
COMP	Cartilage Oligomeric Matrix Protein
CRTAC1	Cartilage Acidic Protein 1
CRTL1	Cartilage Link Protein 1
DAB	Diaminobenzidine
DMEM	Dulbecco's Modified Eagle Medium
DMMB	1,9-Dimethylmethylene Blue
DMSO	Dimethyl Sulphoxide
DPX	Distyrene, a Plasticizer, Xylene

ECM	Extracellular Matrix
EDTA	Ethylenediaminetetraacetic Acid
EFEMP1	Endothelial Growth Factor-Containing Extracellular Matrix Protein 1
FBS	Fetal Bovine Serum
FGF	Fibroblast Growth Factor
G	Gauge
GADPH	Glyceraldehyde 3-phosphate Dehydrogenase
GAG	Glycosaminoglycan
H&E	Haematoxylin and Eosin
H_2O_2	Hydrogen Peroxide
HBSS	Hank's Balanced Salt Solution
HCl	Hydrochloric Acid
Hh	Hedgehog
HMG	High Mobility Group
hTERT	human Reverse Transcriptase Telomerase
IBSP	Integrin-Binding Sialoprotein
IGF	Insulin-like Growth Factor
IL-1β	Interleukin-1β
IMS	Industrial Methylated Spirit
iPS	Induced Pluripotent Stem Cell

kDa	kiloDalton
LECT1	Leukocyte Cell-Derived Chemotaxin 1
MACI	Matrix-Induced Autologous Chondrocyte Implantation
MAGP2	Microfibril-Associated Glycoprotein-2
MW	Molecular Weight
NaCl	Sodium Chloride
NBF	Neutral Buffered Formalin
OA	Osteoarthritis
OCT	Optimal Cutting Temperature
P	Passage
PAGE	Polyacrylamide Gel Electrophoresis
PBS	Phoshate Buffered Saline
PFA	Paraformaldehyde
PRG4	Proteoglycan 4
PTH1R	Parathyroid Hormone 1 Receptor
PTHrP	Parathyroid Hormone-Related Protein
PVDF	Polyvinyl Difluoride

RIPA	Radioimmunoprecipitation Assay
RO	Reverse Osmosis
SDS	Sodium Dodecyl Sulphate
SPP1	Secreted Phosphoprotein 1
TEMED	Tetramethylethylenediamine
TGF	Transforming Growth Factor
THBS4	Thrombospondin 4
TNC	Tenascin C
V	Volts
VAV3	Vav-3 guanine nucleotide exchange factor
VEGF	Vascular Endothelial Growth Factor
xg	x gravity

1. Introduction

1.1 Types of Cartilage

There are three main types of cartilage, hyaline, elastic and fibrocartilage (Salter, 1998, Umlauf et al., 2010). According to a review of the history of the understanding of cartilage by Benedek (2006), this tissue was first documented by Aristotle in the fourth century BC who recognised it only in the ears and nostrils. Articular cartilage was first described by Galen in 175 BC (Benedek, 2006).

1.1.1 Hyaline Cartilage

Hyaline cartilage is the most abundant out of the three types (Umlauf et al., 2010). Articular cartilage is a form of hyaline cartilage present in synovial joints (Figure 1.1) (Martel-Pelletier et al., 2008, Umlauf et al., 2010). This tissue is avascular, aneural and acts as a load-bearing material, allowing the frictionless movement of joints (Martel-Pelletier et al., 2008).

Although articular cartilage is the focus of this project, the next two sections will provide a brief overview of the features of fibrocartilage and elastic cartilage; the two other main types of cartilage.

1.1.2 Fibrocartilage

Fibrocartilage can be found in the intervertebral discs and menisci (Salter, 1998), tendons, ligaments and the temporomandibular joint (Benjamin and Ralphs, 1998, Benjamin and Ralphs, 2004, Hagandora et al., 2012, Murphy et al., 2013). Fibrocartilage is avascular and aneural and contains cells which are large, round or oval and embedded within the extracellular matrix (ECM) (Benjamin and Ralphs, 2004). Fibrocartilage contains densely packed collagen fibres and is a highly ordered tissue (Nerurkar et al., 2011); however, it is difficult to distinguish from other tissues due to it having no clear boundaries and there is some suggestion that it should be thought of as a translational tissue (Benjamin and Ralphs, 2004). A recent study aimed to see if a spectrum of cartilage tissues, from fibrous to hyaline, could be engineered using porcine chondrocytes from costal cartilage (Murphy et al., 2013). The authors clearly demonstrate a decrease in collagen type I staining with increasing length of aggregate culture; however the images for collagen type II are less clear. The quantitative data shows an increase in the overall collagen content and in collagen type II expression with increasing aggregate culture length. Collagen type I expression was too low to be detected quantitatively. Overall the results would appear to suggest that the chondrocytes begin to synthesise a more fibrous tissue before progressing to a more hyaline-like tissue (Murphy et al., 2013). A characteristic feature of fibrocartilage is that it contains both type I and type II collagen

(Benjamin and Ralphs, 2004). The ratio of these collagens can vary depending on the location of the tissue (Benjamin and Ralphs, 2004). Aggrecan and versican have been found in fibrocartilage (Benjamin and Ralphs, 2004). The former is associated with regions rich in collagen type II, whereas the latter is associated with collagen type I rich areas (Benjamin and Ralphs, 2004). Decorin and biglycan have also been found in tendon and intervertebral disc fibrocartilage (Benjamin and Ralphs, 2004).

1.1.3 Elastic Cartilage

Cartilage that contains high levels of elastic fibres is known as elastic cartilage (Hall, 2005). The elastic cartilage of the mammalian external ear, the pinna, is the most studied (Hall, 2005). Auricular chondrocytes secrete an elastin-rich matrix and contain matrix vesicles related to this purpose (Hall, 2005). Elastic cartilage is also found in the larynx (Salter, 1998). Up to 20% of the dry weight of this tissue can be elastic fibres (Salter, 1998).

Nielsen and Bytzer (1979), studied elastic cartilage of the rat epiglottis. Chondrocytes were described to be in lacunae which were surrounded by territorial matrix (Nielsen and Bytzer, 1979). This matrix was observed to be between 0.6 and 1.2µm thick and there was a clear boundary between this and the interterritorial matrix (Nielsen and Bytzer, 1979). Cox and Peacock (1977) looked at elastic cartilage from the

ears of rabbits. The authors claim that there is a difference between cartilage at the tip and base of the ear. It is also stated that features of the cartilage change with age. It is difficult to confirm these statements from the figures presented (Cox and Peacock, 1977).

Before discussing the features of articular cartilage, the overall structure of the synovial joint will be reviewed.

1.2 The Synovial Joint

Where two or more parts of the skeleton come together, this is called a joint (Benjamin, 1999). A synovial joint has a cavity that is surrounded by a fibrous capsule which links the two or more skeletal elements together (Benjamin, 1999) (Figure 1.1). These capsules are fibrous connective tissues, made of collagen fibres (Ralphs and Benjamin, 1994) and are commonly strengthened by ligaments (Benjamin, 1999). In some cases capsules are strengthened by tendons; an example of this is the extensor tendon in the finger (Benjamin, 1999). The orientation of fibres within the capsule and the thickness of the capsule itself varies depending on the level of stress it is exposed to (Ralphs and Benjamin, 1994). For example, the anterior part of the capsule in the hip joint is thicker than the posterior part because the centre of gravity passes behind the joint, which can also often hyperextend (Ralphs and Benjamin, 1994). It is important to note that not all joints in the body are synovial; there are also cartilaginous and fibrous joints

(Benjamin, 1999). Examples of such joints include those between the vertebral bodies (cartilaginous) and sutures linking bones in the skull cap (fibrous) (Benjamin, 1999). The capsule and surrounding ligaments act together to limit joint movement and hold the bones together (Benjamin, 1999). All the non-articular surfaces within the joint are lined with the synovial membrane, which secretes and absorbs synovial fluid (Benjamin, 1999). In healthy joints, this fluid is relatively acellular and pale yellow in colour (Ropes et al., 1939, Ropes et al., 1940). This fluid is responsible for lubricating the joint and delivers some of the nutrients to the articular cartilage (Benjamin, 1999). In fact, nutrients have to pass from synovial capillaries through the synovial membrane into the synovial fluid (Buckwalter and Hunziker, 1999). This fluid then has to move through the cartilage matrix in order to reach the chondrocytes (Buckwalter and Hunziker, 1999). The synovial membrane usually consists of one to three layers of synoviocytes and is supported by vascular connective tissue (Benjamin, 1999). Both the synovium and capsule are innervated, the latter more so than the former (Ralphs and Benjamin, 1994). These nerves are the same nerves which supply the muscles acting on the joint (Ralphs and Benjamin, 1994).

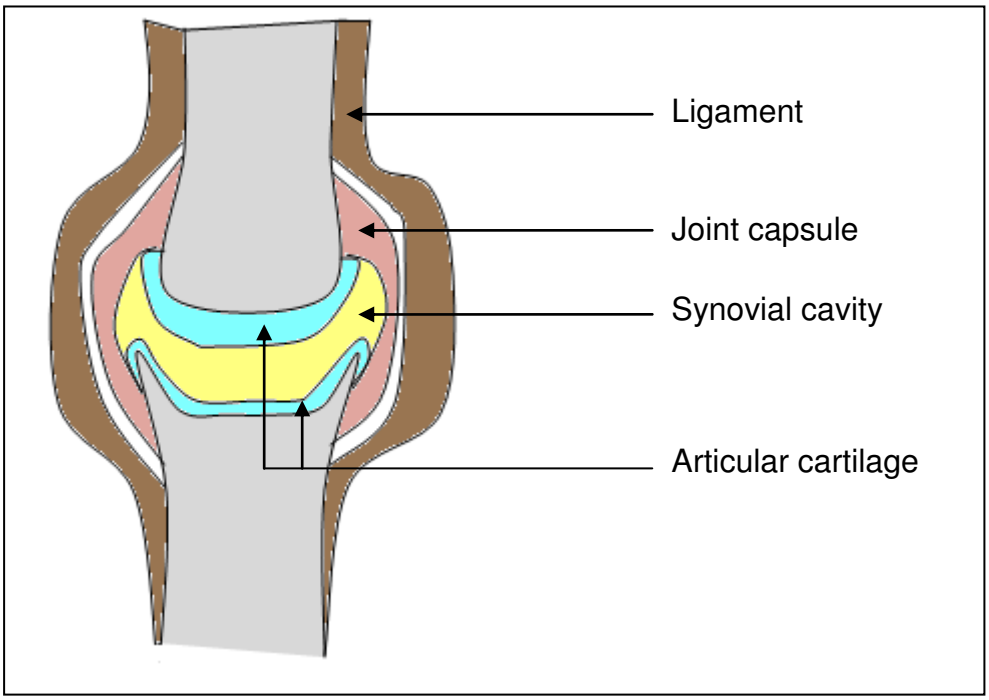


Figure 1.1: A synovial joint. This diagram showing the main features of a synovial joint was adapted from (Benjamin, 1999, Setton, 2008).

The shape and position of the articular surfaces and associated muscles and ligaments all influence the stability of the joint (Benjamin, 1999). Joints are most stable when there is maximum possible contact between the bones within the joint; this is called the close-packed position (Benjamin, 1999). When in this position, the capsule and ligaments are at their most tense (Benjamin, 1999).

Normal function of the synovial joint is dependent on the smooth, low friction gliding and load bearing surface provided by the articular cartilage (Buckwalter and Hunziker, 1999). Although articular cartilage can differ in thickness, cell density, matrix composition and mechanical

properties between species and even within an individual; all synovial joints have the same general structure and components and perform the same functions (Buckwalter and Hunziker, 1999). The structure of articular cartilage will now be reviewed in detail.

1.3 Cartilage Structure: Zones of Articular Cartilage

It is universally recognised that articular cartilage can be split into four zones. However there are slight differences in the literature in the nomenclature of these zones. Some authors divide articular cartilage into the superficial, middle, deep and calcified zones (Poole et al., 1987, Dowthwaite et al., 2004, Goldring and Goldring, 2005, Martel-Pelletier et al., 2008, Karlsson and Lindahl, 2009), whereas others refer to the superficial zone, transitional zone, middle or radial zone and calcified zone (Buckwalter and Mankin, 1998, Buckwalter and Hunziker, 1999, LeBaron and Athanasiou, 2000, Temenoff and Mikos, 2000, Goldring and Goldring, 2005, Umlauf et al., 2010) (Figure 1.2).

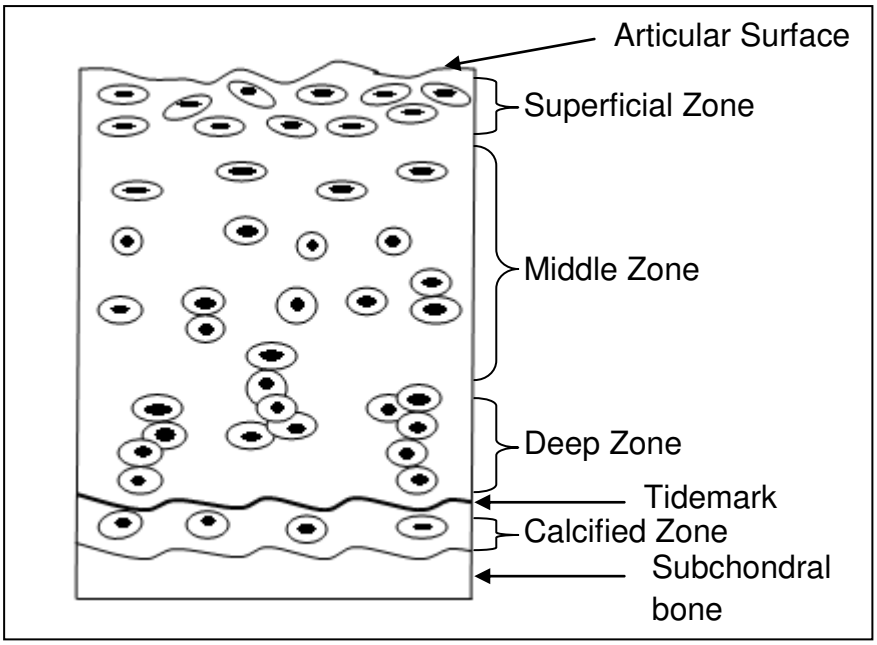


Figure 1.2: The structure of articular cartilage. There are four zones in articular cartilage but the nomenclature of the zones in articular cartilage varies in the literature (see section 1.3).

1.3.1 Superficial Zone

The first zone, the superficial zone, is the thinnest of the four (Buckwalter and Mankin, 1998, Buckwalter and Hunziker, 1999, Martel-Pelletier et al., 2008) and represents about 10% of the tissue (LeBaron and Athanasiou, 2000). This zone comprises of two layers (Buckwalter and Mankin, 1998, Buckwalter and Hunziker, 1999, LeBaron and Athanasiou, 2000, Temenoff and Mikos, 2000). The upper layer is a thin, acellular film consisting of fine fibrils with a small amount of polysaccharide called the lamina splendens (Buckwalter and Mankin, 1998, Buckwalter and Hunziker, 1999, LeBaron and Athanasiou, 2000,

Temenoff and Mikos, 2000). Underneath this layer is the cellular part of the zone. Here, the chondrocytes have a flatter, more disc-like morphology and they are aligned parallel to the articular surface (Buckwalter and Mankin, 1998, Buckwalter and Hunziker, 1999, LeBaron and Athanasiou, 2000, Temenoff and Mikos, 2000, Goldring and Goldring, 2005, Martel-Pelletier et al., 2008). Although these cells secrete surface zone proteoglycan (Dowthwaite et al., 2004), they produce a matrix that is high in collagen and low in proteoglycan content compared to deeper zones (Buckwalter and Mankin, 1998, Buckwalter and Hunziker, 1999, Martel-Pelletier et al., 2008). The collagen fibrils in this zone are relatively thin and are parallel to the articular surface (Buckwalter and Mankin, 1998, Buckwalter and Hunziker, 1999, LeBaron and Athanasiou, 2000, Temenoff and Mikos, 2000, Goldring and Goldring, 2005, Martel-Pelletier et al., 2008) (Figure 1.3). This zone has a greater tensile stiffness compared to the other zones because of the dense network of collagen fibrils parallel to the surface (Buckwalter and Mankin, 1998, Buckwalter and Hunziker, 1999, LeBaron and Athanasiou, 2000, Temenoff and Mikos, 2000). Although it is stated throughout the literature that there are four zones in articular cartilage, a recently published paper claims that there is an additional zone before the superficial zone referred to as the most superficial zone (Fujioka et al., 2013). In fact, the authors suggest that this zone is split into three layers. However they do admit that some layers were only visible using certain techniques and indicate that the second layer might be an artefact caused by sample preparation (Fujioka et al., 2013).

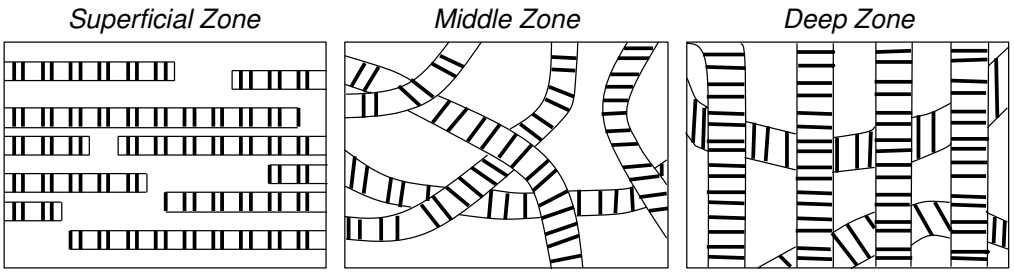


Figure 1.3: Collagen fibril orientation and thickness in articular cartilage. Fibril diameter increases with depth and orientation changes from parallel in the superficial zone to perpendicular in the deep zone. This figure was adapted from (Buckwalter and Hunziker, 1999, Buckwalter et al., 2005)

1.3.2 Middle Zone

As stated previously, the next zone is either referred to as the middle zone or the intermediate zone. This zone is the largest section of articular cartilage; it can be between 40% and 60% of the total cartilage height and weight (Goldring and Goldring, 2005, Martel-Pelletier et al., 2008). The chondrocytes in this zone are more rounded (Buckwalter and Mankin, 1998, Buckwalter and Hunziker, 1999, Martel-Pelletier et al., 2008) and secrete cartilage intermediate layer protein (Dowthwaite et al., 2004, Karlsson and Lindahl, 2009). They also have more synthetic organelles, Golgi membranes and endoplasmic reticulum than those in the superficial zone (Buckwalter and Mankin, 1998, Buckwalter and Hunziker, 1999, LeBaron and Athanasiou, 2000, Temenoff and Mikos, 2000). The matrix in this zone consists of thicker collagen fibrils (Goldring and Goldring, 2005, Martel-Pelletier et al., 2008), which are

aligned obliquely or randomly to the articular surface (Temenoff and Mikos, 2000) (Figure 1.3). Although the collagen fibrils are thicker, the overall concentration of collagen is lower in this zone compared to the superficial zone (Buckwalter and Mankin, 1998, Buckwalter and Hunziker, 1999, LeBaron and Athanasiou, 2000, Temenoff and Mikos, 2000). Water content is also lower but proteoglycan concentration is higher (Buckwalter and Mankin, 1998, Buckwalter and Hunziker, 1999, LeBaron and Athanasiou, 2000, Temenoff and Mikos, 2000).

1.3.3 Deep Zone

The third zone can be called the middle (in literature where the second zone is referred to as the transitional zone), radial or deep zone. Chondrocytes in this zone are also spheroid in shape and are organised in columns (Buckwalter and Mankin, 1998, Buckwalter and Hunziker, 1999, LeBaron and Athanasiou, 2000, Temenoff and Mikos, 2000). They secrete a matrix that contains the biggest collagen fibrils (Buckwalter and Mankin, 1998, Buckwalter and Hunziker, 1999, Martel-Pelletier et al., 2008), which are aligned perpendicular to the articular surface (Figure 1.3) and extend into the tidemark (Buckwalter and Mankin, 1998, Buckwalter and Hunziker, 1999, Temenoff and Mikos, 2000). There are also collagen fibrils in a different alignment surrounding individual chondrocytes or stacks of chondrocytes in the territorial matrix (see section 1.4.2) (Buckwalter and Mankin, 1998,

Buckwalter and Hunziker, 1999, Temenoff and Mikos, 2000, Martel-Pelletier et al., 2008). Interestingly, cell density decreases from the superficial zone to the deep zone (Goldring and Goldring, 2005) but the cell volume increases. In fact, chondrocytes in the second and third zones have twice the cell volume of the cells in the superficial zone (Goldring and Goldring, 2005).

1.3.4 Calcified Zone

The final zone is separated from the upper layers by a tidemark (Hollander et al., 2010). The main function of this calcified zone is to anchor the articular cartilage to the subchondral bone (Martel-Pelletier et al., 2008) In addition to this, it acts as a mechanical buffer between the subchondral bone and uncalcified articular cartilage (zones one to three) (Goldring and Goldring, 2005). The chondrocytes in this layer have a more hypertrophic phenotype and secrete type X collagen and alkaline phosphatase (ALP) (Martel-Pelletier et al., 2008). They are also smaller in volume than the cells in zones two and three and have small quantities of Golgi complex membranes and endoplasmic reticulum (Buckwalter and Mankin, 1998, Buckwalter and Hunziker, 1999, Temenoff and Mikos, 2000).

The structure and components of the ECM will now be discussed in greater detail, beginning with the regions of the matrix followed by its components.

1.4 Cartilage Extracellular Matrix (ECM)

The majority of articular cartilage is composed of extracellular matrix (ECM), which contains water, type II collagen and proteoglycans (Stockwell, 1978, Hollander et al., 2010). Although water represents 70% of the wet weight of unloaded cartilage, it is unevenly distributed throughout the tissue, with the highest concentration (80%) near the cartilage surface (Martel-Pelletier et al., 2008, Hollander et al., 2010).

1.4.1 Regions of ECM: Pericellular Matrix

There are three distinct areas of articular cartilage ECM; the pericellular, territorial and interterritorial regions (Poole et al., 1987, Buckwalter and Mankin, 1998, Buckwalter and Hunziker, 1999, Youn et al., 2006). Immediately surrounding the chondrocyte is the pericellular matrix (Buckwalter and Mankin, 1998, Buckwalter and Hunziker, 1999). The cell membrane appears to be attached to this matrix (Buckwalter and Mankin, 1998, Buckwalter and Hunziker, 1999). This matrix has a high concentration of proteoglycans (Poole et al., 1980, Buckwalter and

Mankin, 1998, Buckwalter and Hunziker, 1999) but also contains non-collagenous proteins such as the anchorin CII, which is a cell-membrane-associated molecule (Buckwalter and Hunziker, 1999). In a study by Poole *et al* (1980), staining for proteoglycan was more intense in the pericellular matrix surrounding chondrocytes nearer the articular surface compared to deeper zone cells. Staining was also intense around those cells close to the junction with the subchondral bone. This group also looked for link protein and found clear, defined pericellular staining in the middle zone of the cartilage (Poole et al., 1980). Link protein is a small glycoprotein which aids the stabilisation of aggrecan aggregates (Kiani et al., 2002). Poole and colleagues (1987), showed that there is a difference in the pericellular matrix between zones in adult human articular cartilage. In the middle and deep zones, the pericellular matrix appeared to be surrounded by a capsule. This capsule was absent in the superficial zone (Poole et al., 1987). The authors adopted the term 'chondron' in this study, which refers to the chondrocyte, pericellular matrix and capsule being one functional unit (Poole et al., 1987). This group also studied the morphology and structure of chondrons isolated from canine tibial cartilage (Poole et al., 1988). They showed that single chondrons appeared to have two different structures. In some chondrons there was a loosely woven tail at one pole of the chondrocyte; in others the chondron appeared ovoid in shape. These latter chondrons would often have channels at one pole. Clusters of chondrocytes were shown to have a dense capsular sheath separating individual cells but the whole

stack was surrounded by a continuous sheath (Poole et al., 1988) (Figure 1.4).

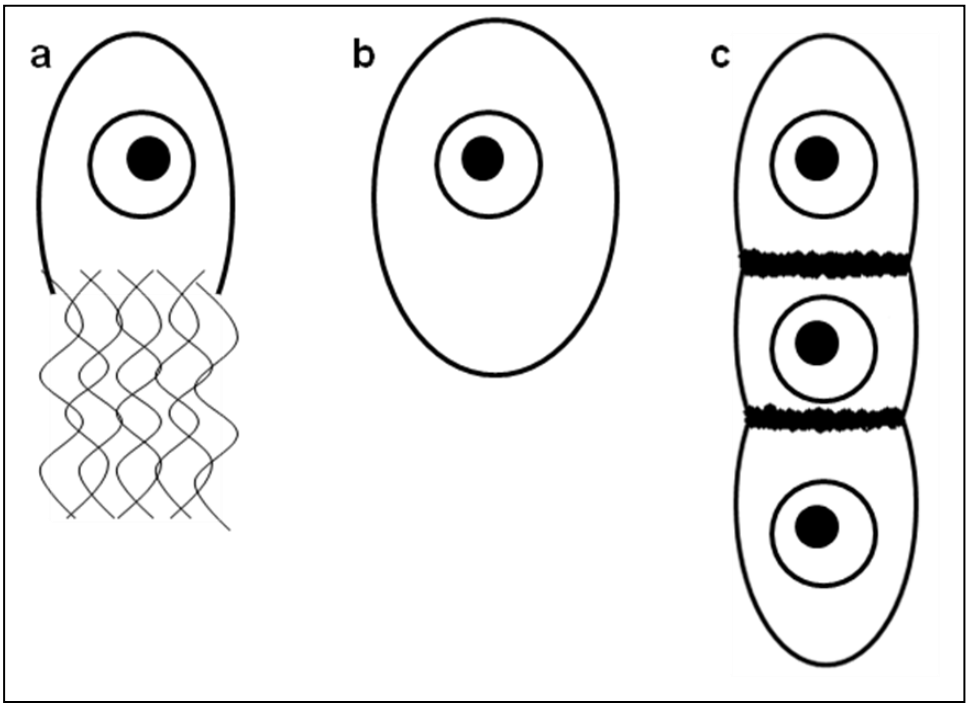


Figure 1.4: Chondron morphology. Single chondrons either have a loosely woven tail (a) or an ovoid structure (b). Columns of chondrocytes are surrounded by a single capsule but individual cells are separated by a dense sheath. This figure was devised based on the data presented in (Poole et al., 1988).

Another study by Poole *et al* (1991), aimed to identify further features of canine chondrons by immobilizing isolated chondrons in thin layers of agarose gel and subjecting them to immunohistochemical analysis. The authors observed strong staining for collagen type VI in the matrix immediately next to the cell (Poole et al., 1991). They acknowledge

that different fixatives had different effects in the sample and admit to reaching a compromise of using a fixative that preserved the ultrastructure of the chondron but reduced epitope activity (Poole et al., 1991). Single keratan sulphate chains were observed in the tail of the chondron and in the parts of the capsule between two adjacent chondrons (Poole et al., 1991). Bovine chondrons have also been shown to stain positively for collagen types VI and II as well as keratin sulphate and chondroitin-6-sulphate (Wang et al., 2008). Depending on the enzyme treatment of the chondrons, keratan sulphate epitopes associated with core protein were detected in marginal regions with weak staining around the chondrocyte itself; or staining was intense throughout the capsule including immediately surrounding the chondrocyte. Expression of epitopes of glycosaminoglycan attachment region of the core protein and hyaluronate binding region of the core protein was high throughout the chondron (Poole et al., 1991). Youn *et al* (2006), found that the volume of the chondron increased from the superficial zone to the deep zone. However, when related to the volume of the enclosed cell, the volume of the pericellular matrix remained constant throughout the zones. Interestingly, this group also found that in the deep zones, the volume of the pericellular matrix increased as the cell number per cluster increased. However the opposite was seen in the ratio of pericellular matrix volume: cell volume (Youn et al., 2006).

Knudson (1993) studied the formation of the pericellular matrix by embryonic chick tibial chondrocytes and adult rat chondrocytes. It was found that monolayer chondrocytes stripped of their pericellular matrix reformed this matrix within 30 minutes after being incubated with hyaluronan and aggrecan, whereas incubating with hyaluronan alone for two hours generated no matrix. This suggests that hyaluronan and aggrecan interact together to keep the pericellular matrix attached to the cell surface. Cells were also incubated with hyaluronan hexasaccharide (HA_6) in this study. This competes for the hyaluronan binding sites on the cell surface (Knudson, 1993). When HA_6 was added to the cells in excess, no pericellular matrix was formed. Therefore, hyaluronan must be able to bind to the receptors on the cell surface to anchor the matrix to the cell but in addition, an aggregating proteoglycan is also required to form the pericellular matrix (Knudson, 1993).

Non-fibrillar collagens such as collagen type VI are also present (Poole et al., 1991, Poole et al., 1992, Arican et al., 1996, Buckwalter and Mankin, 1998, Buckwalter and Hunziker, 1999, Soder et al., 2002). One study showed that collagen type VI was confined to the pericellular matrix in healthy canine cartilage but was present beyond the pericellular matrix in osteoarthritic cartilage (Arican et al., 1996). Chondrocytes can have cytoplasmic extensions which reach into and go through the pericellular matrix into the territorial matrix (Buckwalter

and Mankin, 1998, Buckwalter and Hunziker, 1999). The pericellular matrix is between 1 and 2µm; this thickness varies around the chondrocyte with the thickest covering at the poles of the ovoid-shaped cell (Stockwell, 1975). Interestingly, it has been shown that there is a clear distinction between the pericellular matrix and other matrix areas in older cartilage, whereas in younger cartilage these regions seem to merge together (Stockwell, 1975).

1.4.2 Regions of ECM: Territorial Matrix

The territorial matrix surrounds the pericellular matrix around the individual cells and columns of chondrocytes (Buckwalter and Mankin, 1998, Buckwalter and Hunziker, 1999). It has been described as forming a cocoon around the cells (Mittelstaedt et al., 2011). The collagen fibrils in this area are thin and adhere to the pericellular matrix (Buckwalter and Mankin, 1998, Buckwalter and Hunziker, 1999). This network possibly provides mechanical protection for the chondrocytes during loading of the tissue (Buckwalter and Mankin, 1998, Buckwalter and Hunziker, 1999). The boundary between the territorial matrix and interterritorial matrix is difficult to distinguish because there are many collagen fibrils connecting the two areas (Buckwalter and Mankin, 1998, Buckwalter and Hunziker, 1999).

Hughes and colleagues (2005), conducted a study on mouse articular cartilage and found that the territorial matrix in the middle zone was made of sheets of randomly oriented collagen fibrils. A similar sheet-like pattern was observed in the deep zone. Here the collagen fibrils appeared to mainly align parallel to each other and perpendicular to the articular surface. In addition to this, there were also collagen fibrils weaving in and out of the collagen sheets (Hughes et al., 2005). This zone was also shown to extend into the calcified zone (Hughes et al., 2005).

1.4.3 Regions of ECM: Interterritorial Matrix

The majority of the volume of articular cartilage is taken up by the interterritorial matrix (Buckwalter and Mankin, 1998, Buckwalter and Hunziker, 1999, Youn et al., 2006). This part of the matrix is mainly composed of type II collagen and aggrecan (Youn et al., 2006). The collagen fibrils here have the largest diameter and are oriented perpendicular to the articular surface (Buckwalter and Mankin, 1998, Buckwalter and Hunziker, 1999).

Having discussed the various regions of articular cartilage, the components within this tissue will be reviewed in the subsequent sections.

1.4.4 Components of ECM: Collagens

Collagens are a key component of articular cartilage ECM. Although there are several types present (VI, IX, X, XI, XII and XIV), collagen type II is the most abundant (Aigner and Stove, 2003, Goldring and Goldring, 2005, Martel-Pelletier et al., 2008).

1.4.4.1 Type II collagen

Collagen type II corresponds to about 80% of the total collagen in articular cartilage (Aigner and Stove, 2003). It is a triple helical collagen composed of three identical polypeptide chains $\alpha1[II]_3$ (Goldring and Goldring, 2005, Martel-Pelletier et al., 2008). These chains are synthesised as pro- α-chains, which may have large propeptides at the C- and N- termini (Martel-Pelletier et al., 2008). There are short telopeptide regions between the propeptides and the helix-forming sections (Martel-Pelletier et al., 2008). The propeptides are essential for formation of the triple helix, which occurs within the chondrocyte (Martel-Pelletier et al., 2008). This procollagen is then released from the cell and the propeptides are removed by a procollagen-N-protease and procollagen-C-protease from the N- and C- termini respectively (Martel-Pelletier et al., 2008, Ricard-Blum, 2011). There are two types of type II procollagen that can be formed, IIA and IIB (Martel-Pelletier et al., 2008). These two forms result from the alternative splicing of RNA

at exon two (Ryan and Sandell, 1990, Nah et al., 2001). This exon codes for a cysteine-rich globular region of the peptide (Ryan and Sandell, 1990, Nah et al., 2001). Type IIA collagen is normally found in newly formed cartilage and contains a 69 amino acid cysteine-rich domain (Bruckner and van der Rest, 1994). Expression of procollagen types IIA and IIB has been shown in the chicken limb bud; with the former predominating at days four and seven. However, by day nine type IIB was the predominant form (Nah et al., 2001). Although, type IIA expression is usually associated with chondrogenic precursor cells, it has been detected in OA cartilage (Aigner et al., 1999, Nah et al., 2001). In addition to this, one research group has suggested that type IIA procollagen could be a marker of dedifferentiated chondrocytes (Gouttenoire et al., 2010). They found that type IIA procollagen expression increased with increasing passage of embryonic chondrocytes and such expression was upregulated by TGF-β1. They also demonstrated that type IIB procollagen was upregulated by BMP-2. However, the authors only observed the chondrocytes up to P2 and refer to P1 chondrocytes as dedifferentiated. It would be useful to measure expression over a longer time period to ensure a consistent pattern of expression is seen with increasing passage. The same group have also shown that expression of type IIB procollagen by embryonic murine rib cage chondrocytes decreases from passage one to passage six (Gouttenoire et al., 2004).

Following processing (removal of the propeptide), collagen molecules are assembled into fibrils (Martel-Pelletier et al., 2008). Molecules are joined by aldimine-derived cross-links that go from the telopeptide of one molecule to the triple helix of another (Martel-Pelletier et al., 2008). These links are modified to pyridinoline cross-links which can be markers for mature collagen (Martel-Pelletier et al., 2008). Cross-links stabilize the fibril and over time other, nonaldimine-derived cross-links accumulate in the fibrils (Martel-Pelletier et al., 2008). These links help the collagen fibrils resist proteolytic degradation (Martel-Pelletier et al., 2008). The collagen triple helix is resistant to most proteases but can be cleaved by collagenases (Martel-Pelletier et al., 2008).

1.4.4.2 Type XI collagen

This is another fibrillar collagen and is also specific to cartilage (Goldring and Goldring, 2005, Martel-Pelletier et al., 2008). In adult cartilage it constitutes 3% of the total collagen content (it is about 10% in foetal cartilage) (Eyre et al., 2006, Martel-Pelletier et al., 2008). Three different α-chains make up the triple helix α1[XI]α2[XI]α3[XI] (Martel-Pelletier et al., 2008, Ricard-Blum, 2011, McAlinden et al., 2014). This collagen is synthesised in a similar way to collagen type II but only the C-propeptide is removed (Martel-Pelletier et al., 2008). Type XI collagen molecules form heterotypic molecules with type II collagen molecules (Martel-Pelletier et al., 2008). In fact, a recent

paper has shown that in homozygous knock-in mice which exclusively express type IIA procollagen, these IIA molecules covalently cross-link to type XI molecules forming a heteropolymer of type IIA and type XI collagen (McAlinden et al., 2014). Another study has shown that type XI collagen has a high affinity for the heparan sulphate and dermatan sulphate (Vaughan-Thomas et al., 2001). This group also found that binding of type XI collagen to chondrocytes was reduced in the presence of heparin or heparinase, indicating that this collagen binds to the cell surface proteoglycan heparan sulphate (Vaughan-Thomas et al., 2001).

1.4.4.3 Type IX collagen

Type IX collagen is a fibril-associated collagen with interrupted triple helix (FACIT) (Martel-Pelletier et al., 2008). It is also composed of three different α-chains, α1[IX]α2[IX]α3[IX] (Martel-Pelletier et al., 2008, Parsons et al., 2011, Ricard-Blum, 2011). Each of these chains contain three triple-helical sequence regions that are separated by non-triple-helical sections (Olsen, 1997). Collagen type IX is synthesised in two forms; the long form contains a large amino-terminal globular domain whereas the short form lacks this region (Olsen, 1997). The former is the major type in cartilage and the latter can be found in tissues including the vitreous and early limb buds (Olsen, 1997). Collagen type IX is present at the surface of type II/type XI heterotypic fibrils (Olsen,

1997, Martel-Pelletier et al., 2008) and has been shown to have a high affinity for binding to fibronectin (Parsons et al., 2011). Like type XI collagen, this molecule represents 10% of the total foetal collagen (Eyre et al., 2006, Martel-Pelletier et al., 2008). However, this is reduced to 1% in adult cartilage (Eyre et al., 2006, Martel-Pelletier et al., 2008).

1.4.4.4 Type VI collagen

This is a non-fibrillar collagen also present in but not specific to articular cartilage (Martel-Pelletier et al., 2008). It only represents around 1% of the total collagen (Eyre et al., 2006, Martel-Pelletier et al., 2008). It is composed of three alpha chains (α1[VI]α2[VI]α3[VI]) and has a central triple helix region with large non-helical terminal sections (Pullig et al., 1999, Martel-Pelletier et al., 2008).

1.4.4.5 Type X collagen

Type X collagen is a short chain collagen produced by hypertrophic and growth plate chondrocytes (Sutmuller et al., 1997, Shen, 2005) and is often used as a marker for chondrocyte hypertrophy (Kwan, 1999, Studer et al., 2012). The growth plate is vital for the longitudinal growth of bones (Mackie et al., 2011, Michigami, 2013). Growth plate chondrocytes go through several processes during bone elongation

(Mackie et al., 2011, Michigami, 2013). Firstly, they proliferate forming ordered, parallel columns (Michigami, 2013). Then they produce high levels of cartilaginous matrix before exiting the cell cycle and adopting a more hypertrophic phenotype (Mackie et al., 2011, Michigami, 2013). Once terminally differentiated, these hypertrophic cells undergo apoptosis and the cartilage matrix is mineralised (Mackie et al., 2011, Michigami, 2013).

As well as expressing collagen type X, hypertrophic chondrocytes also have an increased cell volume and express other markers including alkaline phosphatase (ALP), runt-related transcription factor 2 (RUNX2) and matrix metalloproteinase-13 (MMP-13) (Studer et al., 2012). Type X collagen is abundant in the pericellular matrix of hypertrophic chondrocytes (Chambers et al., 2002).

Collagen type X is composed of three identical α1[X] chains which contain three distinct regions (Barber and Kwan, 1996, Sutmuller et al., 1997). These are a short non-collagenous amino-terminal domain, a collagenous domain and a collagenous carboxyl-terminal domain (Sutmuller et al., 1997). The C-terminal domain is much larger than the N-terminal region and is not removed following secretion into the ECM; this is different to processing of fibrillar collagens (Barber and Kwan, 1996).

1.4.5 Components of ECM: Proteoglycans

Proteoglycans make up 5-10% of the wet weight of articular cartilage (Martel-Pelletier et al., 2008). The most abundant proteoglycan present is aggrecan (Umlauf et al., 2010), representing about 90% of the total mass of cartilage proteoglycans (Buckwalter and Mankin, 1998). Aggrecan is a large chondroitin sulphate proteoglycan (Umlauf et al., 2010) which is associated with hyaluronic acid and link protein to form large aggregates (Martel-Pelletier et al., 2008).

An aggrecan molecule has several important regions, namely the G1, Interglobular, G2, GAG attachment and G3 domains (Figure 1.5). The globular G1 domain is at the N-terminus and it is through this region that aggrecan binds non-covalently to hyaluronic acid (Valiyaveettil et al., 2005). The molecular weight of this region is about 37kDa and it is formed by an N-terminal immunoglobulin-like repeat and two proteoglycan tandem repeats (Aspberg, 2012). The interaction between link protein and the G1 domain is mediated by the immunoglobulin-like repeat and the tandem repeats form part of the interaction with hyaluronic acid (Kiani et al., 2002). This domain has been shown to be abundant in canine knee articular cartilage and was found to be more highly expressed in cartilage compared to other tissues in the canine knee joint (Valiyaveettil et al., 2005). The next region, which separates the first and second globular domains (G1 and G2 respectively) is about 150 amino acids long and is known as the

interglobular domain (Kiani et al., 2002, Valiyaveettil et al., 2005, Aspberg, 2012). This domain is unique to aggrecan and is involved in the physiological turnover of the molecule (Kiani et al., 2002).

The second globular domain is also unique to aggrecan and consists of two proteoglycan tandem repeats which are similar to those in the G1 domain (Kiani et al., 2002, Aspberg, 2012). After this domain is the GAG attachment region, which contains a keratan sulphate rich section followed by a long chondroitin sulphate region (Valiyaveettil et al., 2005). Each aggrecan molecule contains approximately 100 chondroitin sulphate chains and up to 60 keratan sulphate chains (Kiani et al., 2002). The former are usually about 20kDa, whereas the latter are normally smaller, being between 5kDa and 15kDa (Kiani et al., 2002). The final region is the third globular domain (G3), which contains four structural motifs: two epidermal growth factor-like repeats, a C-type lectin domain and a complimentary regulatory protein (Kiani et al., 2002, Aspberg, 2012). It is generated by the alternative splicing of exons during post-transcriptional processing (Kiani et al., 2002). The G3 region is not always present in mature aggrecan as it is readily lost soon after synthesis (Dudhia, 2005); in fact, the number of aggrecan molecules containing an intact G3 domain decreases with age (Dudhia, 2005).

Synthesis of aggrecan begins in the nucleus with the transcription of the gene encoding the protein core (Figure 1.5) (Mankin et al., 1994). The

resulting mRNA is then translated in the rough endoplasmic reticulum (RER) and the protein is synthesised at the ribosome (Mankin et al., 1994). This protein core is then transported to the Golgi apparatus where glycosaminoglycan chains are added (Mankin et al., 1994). Following this, the glycosylated protein is transported to the plasma membrane via secretory vesicles, where it is released into the matrix (Mankin et al., 1994). Link protein is synthesised in a similar way to aggrecan whereas the large, non-sulphated glycosaminoglycan hyaluronic acid is synthesised at the plasma membrane (Mankin et al., 1994, Roughley, 2006, Martel-Pelletier et al., 2008). Formation of the proteoglycan aggregate occurs in the pericellular matrix (Roughley, 2006, Martel-Pelletier et al., 2008).

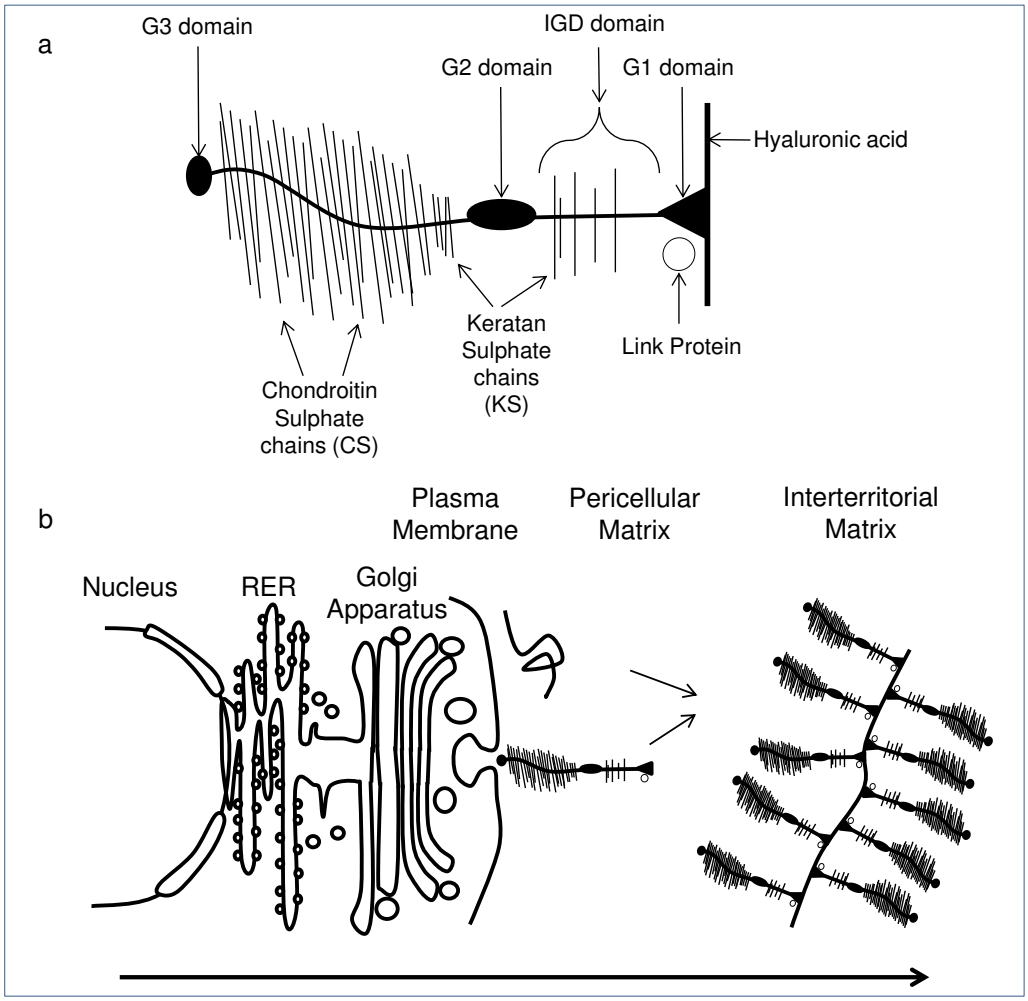


Figure 1.5: Structure and synthesis of aggrecan. (a) Aggrecan molecules possess three globular domains (G1, G2 andG3), an interglobular domain (IGD) and a GAG attachment region containing chondroitin sulphate and keratan sulphate. (b) Synthesis of aggrecan begins with transcription of genes in the nucleus, followed by translation at the Rough Endoplasmic Reticulum (RER). The glycosaminoglycan chains are then added in the Golgi Apparatus and the glycosylated protein is then transported to the plasma membrane through secretory vesicles and released into the matrix. Link protein is synthesised in a similar way but hyaluronic acid is produced at the plasma membrane. This means that the aggregate of aggrecan, link protein and hyaluronic acid can only be assembled in the pericellular matrix. Diagrams in this figure have been adapted from (Mankin et al., 1994, Kiani et al., 2002)

Each aggregate consists of a hyaluronic acid filament which can have up to 100 aggrecan molecules attached to it (Heinegard et al., 2005, Roughley, 2006). Interestingly, aggrecan is rarely completely intact in articular cartilage; it is cleaved at different sites to generate fragments of varying lengths (Valiyaveettil et al., 2005, Roughley, 2006). The link protein in these aggregates has several functions; including stabilising the aggregate and mediating a conformational change in newly formed aggrecan, enabling it to bind to the hyaluronic acid filament (Roughley, 2006, Martel-Pelletier et al., 2008).

Aggrecan is an integral part of the cartilage matrix and has been described as an osmotically active protein (Dudhia, 2005). The presence of the chondroitin sulphate and keratan sulphate chains within aggrecan, gives the molecule a high negative charge (Dudhia, 2005, Aspberg, 2012). In fact, there can be up to 9000 negative charges per aggrecan (Seror et al., 2011). This negative charge brings water into the tissue creating a high osmotic pressure (Dudhia, 2005, Seror et al., 2011, Aspberg, 2012). This occurs because the anionic groups on the GAG chains carry mobile counter ions, such as Na+ alongside them (Kiani et al., 2002). As a result, a large difference in ion concentration is generated between cartilage and the surrounding tissue and water is drawn in (Kiani et al., 2002). The incoming water causes the aggrecan abundant network to expand but the level of expansion is restricted by the stiff collagen fibres which results in the generation of a swelling

pressure (Kiani et al., 2002, Dudhia, 2005). This pressure enables cartilage to deform reversibly under mechanical load (Dudhia, 2005).

Other proteoglycans present in cartilage include decorin, biglycan, fibromodulin and lumican, which are all types of small leucine-rich repeat proteoglycans (SLRPs) (Roughley, 2006, Martel-Pelletier et al., 2008). SLRPs are part of the leucine-rich repeat superfamily (McEwan et al., 2006). The leucine-rich repeat (LRR) in the SLRP family is unique in that it is flanked by clusters of cysteines (Hocking et al., 1998). At the N-terminus there are four cysteine residues within a sequence of 20 amino acids, whereas at the C-terminus there are two cysteine residues (Hocking et al., 1998). SLRPs can be classified into groups based upon the arrangement of the cysteine residues at the N-terminus (Hocking et al., 1998, McEwan et al., 2006) (see Table 1.1). The LRR region within SLRPs varies in length; it can be between 20 and 29 amino acids long, with 24 being the most common (Hocking et al., 1998, McEwan et al., 2006). The number of repeats also varies (see Figure 1.6). Each LRR contains a characteristic motif of 11 amino acids, L-x-x-L-x-L-x-x-N-x-L, with L being leucine, N being asparagine and x representing any other amino acid. Another distinctive component of SLRPs is the “ear” repeat (Chen and Birk, 2011). This region is near the C-terminus and it is where the LRR extends laterally from the main body of the proteoglycan (McEwan et al., 2006). For decorin, biglycan, fibromodulin and lumican this is located at LRR-11 (McEwan et al.,

2006). In osteoglycin and epiphycan this “ear” repeat can be found at LRR-7 (McEwan et al., 2006).

Class of SLRP	Arrangement of cysteine residues	Proteoglycans
1	Cx_3CxCx_6C	decorin, biglycan, asporin
2	Cx_3CxCx_9C	keratocan, osteoadherin, lumican, fibromodulin
3	Cx_2CxCx_6C	opticin, epiphycan, osteoglycin

Table 1.1: Classes of small leucine-rich repeat proteoglycans (SLRPs). These proteins can be classified into groups based on the arrangement of the cysteine residues at the N-terminus. C – cysteine, x – any other amino acid. Table created based on information in (Hocking et al., 1998, McEwan et al., 2006).

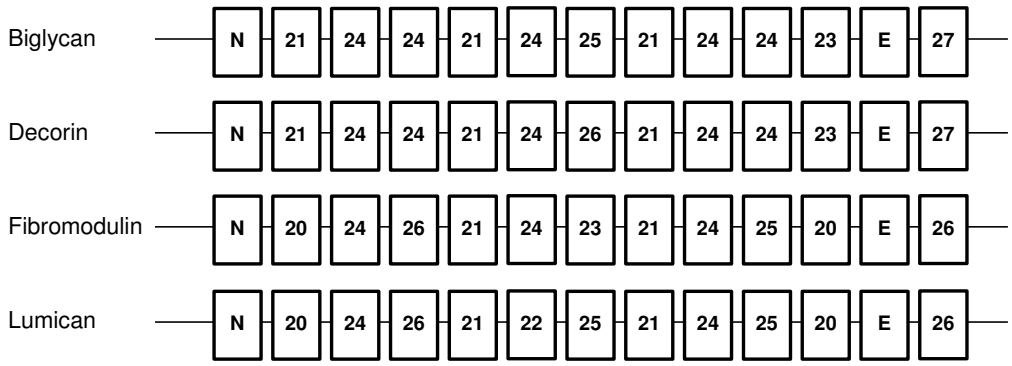


Figure 1.6: The LRRs of the SLRPs biglycan, decorin, fibromodulin and lumican. The box labelled N represent the N-terminus where the cluster of four cysteine residues reside. The boxes containing the numbers 20-27 are individual LRR regions and the figures relate to the number of amino acids within that particular repeat. The E stands for the “ear” repeat region, which lies close to the C-terminus. Figure was adapted from (McEwan et al., 2006).

Fibromodulin, lumican, decorin and biglycan have all been shown to be expressed at similar levels in canine articular cartilage (Yang et al., 2012). Expression of all of these SLRPs, except the latter has been shown in bovine articular, nasal and engineered cartilage with lumican being upregulated in the engineered cartilage (Kafienah et al., 2008). Another group assessed the effect of culture environment on the expression of aggrecan, decorin and biglycan by rabbit chondrocytes (Demoor-Fossard et al., 1998). Expression of all of these proteoglycans was shown to be higher in alginate compared to monolayer cultures. Decorin expression was significantly reduced in monolayer cultures incubated with TGF-β but addition of TGF-β to alginate cultures had little impact. Biglycan expression was only slightly increased in monolayer and alginate with TGF-β incubation. A similar pattern was also seen for aggrecan expression. IL-1β induced a reduction in expression of decorin in monolayer and alginate. Biglycan expression was also reduced in these monolayer cultures but IL-1β had little impact on expression of this SLRP in alginate cultures. Aggrecan expression was considerably reduced in monolayer in this group but a slight increase was seen in IL-1β treated alginate cultures. This suggests that physical environment and growth factors have a combined effect on the expression of proteoglycans by cultured chondrocytes. It must be noted however, that these results represent mRNA expression and may not represent protein levels within the cultures (Demoor-Fossard et al., 1998).

Decorin is the most abundant of the SLRPs in articular cartilage (Monfort et al., 2006). It contains a core protein associated with a single chain of dermatan or chondroitin sulphate (Monfort et al., 2006, Lorda-Diez et al., 2014). This proteoglycan is present throughout the pericellular and intercellular matrices (Demoor-Fossard et al., 1998, Goldring and Goldring, 2005, Roughley, 2006, Martel-Pelletier et al., 2008). Decorin obtained its name due to the fact that it 'decorates' collagen fibrils (Chen and Birk, 2011). It has high and low affinity binding sites for collagen at LRRs 4-6 and at the C-terminus respectively (Chen and Birk, 2011). This SLRP is involved in the regulation of collagen fibrillogenesis and also has a role in maintaining tissue integrity through binding with fibronectin and thrombospondin (McEwan et al., 2006, Monfort et al., 2006, Chen and Birk, 2011). It binds to collagen type II and also connects collagen type VI filaments, forming fibrils (Buchanan and Gay, 1996, Chen and Birk, 2011). Decorin can also bind to a variety of growth factors including TGF-β, IGF-1 and FGF-2 as well as interacting with tyrosine kinase receptors (Chen and Birk, 2011).

Fibromodulin and lumican are present in the interterritorial matrix and biglycan is present in the pericellular matrix (Goldring and Goldring, 2005, Roughley, 2006, Martel-Pelletier et al., 2008). Whereas decorin and biglycan can be referred to as dermatan sulphate proteoglycans, fibromodulin and lumican are keratan sulphate proteoglycans (Knudson

and Knudson, 2001, Roughley, 2006). Both fibromodulin and lumican interact with collagen fibrils and do so at the same region of the collagen molecule, however this site is separate from the site where decorin binds (Roughley, 2006). Biglycan and decorin have 55% amino acid identity, with many of the other residues being chemically similar (Hocking et al., 1998). Biglycan primarily interacts with collagen type VI but can also bind to fibronectin (Buchanan and Gay, 1996, Demoor-Fossard et al., 1998).

Now the ECM of cartilage has been reviewed, another key element of articular cartilage to be examined is the chondrocyte.

1.5 The Chondrocyte

The cells present in articular cartilage are chondrocytes and only count for 2% of the tissue (Stockwell, 1978, Goodrich and Nixon, 2006, Martel-Pelletier et al., 2008, Hollander et al., 2010, Umlauf et al., 2010). Cell density has been reported to vary between species, with rabbit articular cartilage having a cell density of $12.8x10^4$ cells/mm^3 and bovine articular cartilage having $4.7x10^4$ cells/mm^3 (Stockwell, 1978). A study looking at human articular cartilage found that cell density decreases with age (Stockwell, 1967). They reported counts of 76 and 61 cells per 0.22mm^2 from two day and six week old humans respectively (Stockwell, 1967). However, counts from humans aged

between 17 and 89 years old ranged from 7 to 16 cells per 0.22mm^2. This study also looked at the different regions of articular cartilage and found a greater number of cells in the superficial zone compared to deeper zones (Stockwell, 1967). Similar findings were observed by another research group comparing cartilage from infants (26 weeks gestation – six days postnatal), children (two – six years old) and adults (31 and 35 years old) (Gilmore and Palfrey, 1988). They found that cell density was higher in infants compared to children and adults and although counts were similar between children and adults, they were marginally higher in children samples (Gilmore and Palfrey, 1988). This group also demonstrated that cell density decreases in the deeper zones of articular cartilage (Gilmore and Palfrey, 1988).

In a more recent study, DNA content in bovine cartilage was shown to decrease with age, indicating a decrease in cell number (Erickson et al., 2011). Researchers compared cartilage from foetal (second or third trimester), juvenile (three to six months) and skeletally mature (two to three years) cows. The authors also showed that collagen content increased significantly with aging, however the exact type of collagen measured is not specified (Erickson et al., 2011). Histological images suggest that proteoglycans and GAGs increase with age but the images presented are not very clear (Erickson et al., 2011).

The next few sections will detail the development of articular cartilage, how chondrocytes are identified in culture and the methods used to attempt to maintain chondrocyte phenotype in culture.

1.5.1 Origin of the Chondrocyte (MSC Differentiation)/ Chondrogenesis

Chondrogenesis is a stage of embryogenesis which results in the formation of cartilage (Cancedda et al., 2000, Goldring et al., 2006, Pelttari et al., 2008, Mobasheri et al., 2009, Onyekwelu et al., 2009, Boeuf and Richter, 2010, Penny et al., 2012). Most of this cartilage undergoes endochondral ossification to form endochondral bone (Cancedda et al., 2000, Goldring et al., 2006, Pelttari et al., 2008, Mobasheri et al., 2009, Onyekwelu et al., 2009, Boeuf and Richter, 2010, Penny et al., 2012).

Different types of cartilage arise from mesenchymal stem cells from a range of sources. Craniofacial cartilage arises from neural crest cells, intervertebral disc, rib and sternum cartilage (referred to as the axial skeleton) originate from somites in the paraxial mesoderm and articular cartilage is derived from the lateral plate mesoderm (Goldring, 2005, Goldring and Goldring, 2005).

Chondrogenesis is summarised in Figure 1.7 and begins with the condensation and proliferation of MSCs (Cancedda et al., 2000,

Goldring and Goldring, 2005, Goldring et al., 2006, Pelttari et al., 2008, Mobasheri et al., 2009, Boeuf and Richter, 2010, Penny et al., 2012). This condensation is initiated by TGF-β1 and in the early stages of chondrogenesis, this growth factor stimulates the production of N-cadherin and fibronectin, thereby promoting cellular interactions (Demoor et al., 2014). Another study assessing TGF-β expression during cartilage development found that TGF-β1, TGF-β2 and TGF-β3 are expressed throughout development (up to eight months) (Hayes et al., 2001). This study used *Monodelphis domestica* (grey, short-tailed opossum) as a model animal because offspring are born at early developmental stages (Hayes et al., 2001). At two months of development, the structure of articular cartilage was clearly visible and staining of all three TGF-βs was present throughout. By eight months, this staining was only present in the superficial layer (Hayes et al., 2001). BMP-2 is also involved in the condensation of MSCs (Demoor et al., 2014). Another important protein involved in chondrogenesis is the transcription factor Sox9 (Bernstein et al., 2009b, Demoor et al., 2014). Cells expressing Sox9 form aggregates and this factor also induces the expression of cartilage specific markers as well as stimulating the expression of Sox5 and Sox6 (Bernstein et al., 2009b, Demoor et al., 2014). Following condensation, chondroprogenitors proliferate, differentiate and mature; some also undergo terminal differentiation into hypertrophic chondrocytes (Goldring et al., 2006, Vinatier et al., 2009, Penny et al., 2012). A recent study determined a range a markers identifying cartilage-committed mesenchymal cells

(referred to as prechondrocytes by the authors) (Wu et al., 2013). These cells were identifiable by negative expression of CD31, CD34, CD45, CD235a and CD324 (collectively referred to as LIN), low or negative expression of CD146 and CD166, low expression of CD44 and positive expression of CD73 and BMPPR1B (Wu et al., 2013). High levels of mRNA expression for Sox9, aggrecan and collagen type II was detected in these prechondrocytes (Wu et al., 2013). Differentiated chondrocytes secrete an extracellular matrix characteristic of hyaline cartilage (Pelttari et al., 2008, Penny et al., 2012). Biomechanics also has a key role in driving chondrogenesis and this is reviewed in (Responte et al., 2012).

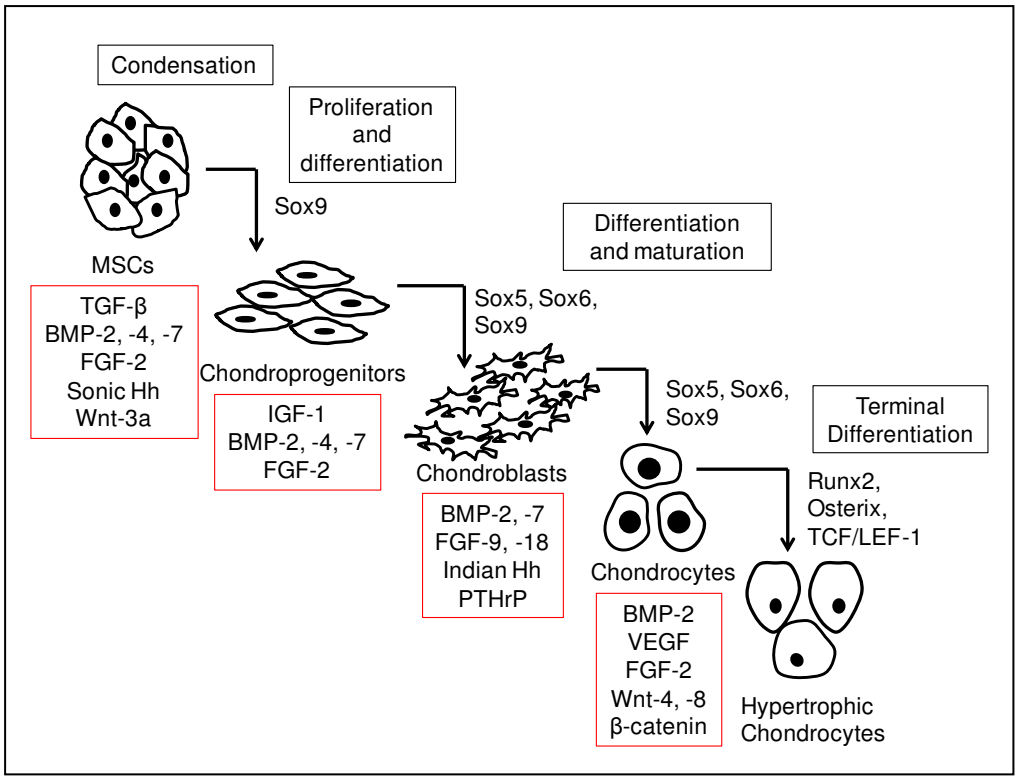


Figure 1.7: Differentiation of MSCs down the chondrogenic lineage. Black boxes indicate the stages of differentiation. The red boxes detail growth factors expressed by the cells they are underneath. Abbreviations: BMP: bone morphogenetic protein, FGF: fibroblast growth factor, Hh: hedgehog, IGF: insulin-like growth factor, PTHrP: parathyroid hormone-related protein, VEGF: vascular endothelial growth factor. Diagram is adapted from (Vinatier et al., 2009).

1.5.2 Native Chondrocyte Phenotype

When embedded in native articular cartilage, chondrocytes have two distinct morphologies. Those found in the surface zone have a disc like morphology, whereas those present in the remaining three zones have a rounded morphology (Buckwalter and Mankin, 1998, Buckwalter and Hunziker, 1999, LeBaron and Athanasiou, 2000, Temenoff and Mikos, 2000, Goldring and Goldring, 2005, Martel-Pelletier et al., 2008). One

group attempted to identify the different fractions of chondrocytes present within the entire depth of cartilage tissue (Min et al., 2002). The authors identified four distinct fractions of cells; fraction one had large cells with small nuclei, fraction two contained small cells with small nuclei, fraction three possessed large cells with large nuclei and the fourth fraction had small cells with large nuclei (Min et al., 2002). Collagen type II was found to be expressed by cells in all fractions, except fraction four. However, western blot analysis suggested weaker expression in fractions one and three. Fraction two cells appeared to have the highest expression of proteoglycans (Min et al., 2002). In a recent paper describing the phenotype of native articular chondrocytes, expression of nine specific cartilage markers and seven non-specific cartilage markers was identified (see Table 1.2) (Demoor et al., 2014). Expression of the specific markers was considerably higher than the non-specific ones, with *COL2B* and *COL2A1* being the most abundant (Demoor et al., 2014).

Specific Cartilage Markers	Non-specific cartilage markers
COL2A	Cbfa1
COL2A1	HIF-2α
COL2B	OSTCN
ACAN	COL10A1
SOX9	HTRA1
COL11A1	COL3A1
COL9A1	COL1A1
Integrin-β1	
HIF-1α	

Table 1.2: Markers of native chondrocytes. Native articular chondrocytes were found to express both specific and non-specific cartilage genes. *COL2A*, type IIA collagen; *COL2A1*, type II collagen; *COL2B*, type IIB collagen; *ACAN*, aggrecan; *SOX9*, SRY-type HMG box 9; *COL11A1* type XI collagen; *COL9A1*, type IX collagen; *HIF-1α* and *HIF-2α*, hypoxia inducible factor 1 alpha and 2 alpha; *Cbfa1*, core binding factor 1, *OSTCN*, osteocalcin; *COL10A1*, collagen type X; *HTRA1*, high temperature requirement 1; *COL3A1*, collagen type III; *COL1A1*, collagen type I. Table compiled using data presented in (Demoor et al., 2014).

Chondrocytes are an important cell source for cellular treatment of cartilage damage and as such their phenotype *in vitro* has been extensively studied. The following sections will review the markers used to identify chondrocytes *in vitro* and how the chondrocytic phenotype is lost during monolayer expansion.

1.5.3. Chondrocyte Phenotype *in vitro* and Dedifferentiation

There is considerable interest in the phenotype of chondrocytes *in vitro* due to their use in cellular treatments for cartilage damage. Articular cartilage is very poor at regenerating itself and as such cell and tissue engineering strategies are being extensively explored to repair damaged cartilage (see section 1.7). Autologous Chondrocyte Implantation (ACI) is a technique used to repair articular cartilage defects larger than $4cm^2$ (Schulze-Tanzil, 2009, Oldershaw, 2012). ACI is described in further detail in section 1.7.2. All forms of the ACI method involve isolation and expansion of chondrocytes in monolayer culture before being placed into the defect (Brittberg, 2008, Brittberg, 2010, Zeifang et al., 2010, Oldershaw, 2012). A major problem with the large cell requirements for these techniques is that the cells begin to lose their phenotype (dedifferentiate) during *in vitro* expansion.

Dedifferentiation has been described as the reversion of differentiated cells into more primitive progenitor cells (Ebeling and Fischer, 1922, Chen et al., 2005, Eguizabal et al., 2013). However in the field of chondrocyte biology, dedifferentiation usually refers to loss of the chondrocytic phenotype and adoption of a more fibroblastic phenotype. Loss of the chondrocyte phenotype whilst undergoing *in vitro* expansion has been reported in many species including, humans (Archer et al., 1990, Schnabel et al., 2002, Goessler et al., 2006, Yang et al., 2006b, Tew et al., 2008), goats (Darling and Athanasiou, 2005), cows (Brodkin

et al., 2004, Shao et al., 2013), pigs (Watt, 1988) and rabbits (Malemud and Papay, 1984, Baici et al., 1988).

In many cases, the chondrocyte phenotype is assessed by the production of specific ECM components, namely collagen type II and aggrecan. Collagen type I is also commonly used as a marker for dedifferentiation, although it has been shown that *in vivo* articular chondrocytes express this matrix protein (Kolettas et al., 1995). In fact, some researchers have found the ratio of collagen type II to collagen type I expression accurately represents the phenotype of chondrocytes in culture (Hamada et al., 2013). Although it is common to assess expression of collagen types I and II and aggrecan when looking at chondrocyte phenotype in monolayer, a wide variety of proteins have been studied (Tables 1.4 and 1.5).

Some studies have tried to identify specific markers for chondrocytes and dedifferentiated chondrocytes. Rai and colleagues (2009), looked at the expression of aggrecan, Sox9, cartilage acidic protein 1 (CRTAC1), cartilage oligomeric matrix protein (COMP) and collagen types I and II in canine chondrocytes grown in monolayer and alginate. They found that expression of all markers decreased from passage zero to passage five, except for collagen type I where the opposite was seen (Rai et al., 2009). Although expression of the latter wasn't observed until passage three (Rai et al., 2009). Aggrecan, Sox9, CRTAC1, COMP expression increased when the cells were cultured in alginate

(Rai et al., 2009). This group also aimed to generate a cell line which maintained the chondrogenic phenotype for longer; however limited data is presented for the cells transfected with human reverse transcriptase telomerase (hTERT) and redifferentiation appears to more successful in the unaltered chondrocytes (Rai et al., 2009). Having said this, the transfected cells continued to grow up to passage 20 whereas senescence was observed at passage seven with the non-transfected cells (Rai et al., 2009).

A more recent study compared the secretomes of articular chondrocytes and MSCs expanded in monolayer (Polacek et al., 2011). The categories of proteins in the secretomes of both cell types were present at similar levels (Table 1.3), with 65 specific proteins being identified in both secretomes. These proteins included, collagen alpha 2 (I), biglycan, decorin, COMP and IGF. A total of 26 proteins were exclusively detected in the chondrocyte secretome including clusterin and proteoglycan-4. The MSC secretome contained 14 proteins which were not detected in the cartilage secretome. These included the proteins collagen alpha-1 (XI chain) and BMP-1 (Polacek et al., 2011). These results give a good indication of what proteins articular chondrocytes are producing whilst in monolayer culture; however it must be noted that the authors used cryopreserved chondrocytes, so a different secretome may be observed in freshly cultured chondrocytes.

Category	Portion of articular chondrocyte secretome (%)	Portion of MSC secretome (%)
ECM components	45	43
Enzyme inhibitors and anabolic agents	7	12
Growth factors	15	9
Inflammatory regulators	14	12
Intracellular components	5	9
Metabolic enzymes	4	6
Proteases and catabolic agents	10	9

Table 1.3: The secretomes of chondrocytes and MSCs in monolayer culture. Data in this table was taken from (Polacek et al., 2011).

A few papers have assessed the change in chondrocyte markers between freshly isolated chondrocytes to cells that have undergone multiple passages (Diaz-Romero et al., 2005, Cheng et al., 2011, Hamada et al., 2013). Tables 1.4 and 1.5 summarise markers that have been found to increase (1.4) and decrease (1.5) during monolayer culture of chondrocytes. For the functions of these proteins, refer to Table 1.6.

Cheng and colleagues (2011) found nine genes were expressed at significantly higher levels in bovine cartilage compared to passage six bovine chondrocytes, whereas there were 13 markers found to be significantly upregulated in the passaged cells (Table 1.4) The authors

do acknowledge that mRNA expression was measured and that this may not result in the production of functional protein for all these markers (Cheng et al., 2011).

Increased expression with increasing culture length	
Marker	**References**
BMP-4	(Cheng et al., 2011)
Cathepsin B	(Baici et al., 1988)
Cadherin II	(Cheng et al., 2011)
CD10	(Diaz-Romero et al., 2005)
CD44	(Diaz-Romero et al., 2005, Hamada et al., 2013)
CD49c	(Diaz-Romero et al., 2005, Hamada et al., 2013)
CD49e	(Diaz-Romero et al., 2005)
CD49f	(Diaz-Romero et al., 2005)
CD51/61	(Diaz-Romero et al., 2005)
CD81	(Diaz-Romero et al., 2005)
CD90	(Cheng et al., 2011, Hamada et al., 2013)
CD99	(Diaz-Romero et al., 2005)
CD105	(Diaz-Romero et al., 2005)
CD151	(Diaz-Romero et al., 2005, Hamada et al., 2013)
CD166	(Diaz-Romero et al., 2005)
Dickkopf Homolog 3	(Cheng et al., 2011)
Fibromodulin	(Cheng et al., 2011)
Fibronectin	(Brodkin et al., 2004)
HIF-1α	(Cheng et al., 2011)
IGF binding protein	(Cheng et al., 2011)
Secreted Phosphoprotein 1	(Cheng et al., 2011)
S-100	(Cheng et al., 2011, Lehmann et al., 2013)
Tenascin C	(Cheng et al., 2011)
TGF-β3	(Cheng et al., 2011)
Twist Basic Helix-Loop-Helix Transcription Factor 1	
Type I collagen	(Archer et al., 1990, Brodkin et al., 2004, Darling and Athanasiou, 2005, Gosset et al., 2008, Tew et al., 2008, Rai et al., 2009, Munirah et al., 2010, Cheng et al., 2011, Nadzir et al., 2011, Hoshiba et al., 2012, Lehmann et al., 2013)
Type III collagen	(Schnabel et al., 2002, Cheng et al., 2011)
Versican	(Cheng et al., 2011, Ma et al., 2013)
Vitronectin	(Cheng et al., 2011, Hoshiba et al., 2012)

Table 1.4: Markers found to be upregulated during monolayer expansion of chondrocytes.

Decreased expression with increasing culture length	
Marker	**References**
Aggrecan	(Gosset et al., 2008, Rai et al., 2009, Cheng et al., 2011, Hoshiba et al., 2012)
Cartilage Oligomeric Matrix Protein	(Rai et al., 2009)
CD14	(Diaz-Romero et al., 2005, Hamada et al., 2013)
CD49a	(Diaz-Romero et al., 2005, Hamada et al., 2013)
CD54	(Diaz-Romero et al., 2005, Hamada et al., 2013)
CD106	(Diaz-Romero et al., 2005)
Clusterin	(Cheng et al., 2011)
Early Growth Response 1	(Cheng et al., 2011)
Link Protein	(Cheng et al., 2011)
Melanoma Inhibitory Activity	(Cheng et al., 2011)
Sox9	(Tew et al., 2008, Rai et al., 2009, Munirah et al., 2010, Cheng et al., 2011, Ma et al., 2013)
Superficial Zone Protein	(Darling and Athanasiou, 2005, Cheng et al., 2011)
Type II collagen	(Archer et al., 1990, Schnabel et al., 2002, Brodkin et al., 2004, Darling and Athanasiou, 2005, Yang et al., 2006b, Eleswarapu et al., 2007, Gosset et al., 2008, Tew et al., 2008, Rai et al., 2009, Munirah et al., 2010, Cheng et al., 2011, Hoshiba et al., 2012, Lehmann et al., 2013, Ma et al., 2013)
Type IX collagen	(Munirah et al., 2010, Ma et al., 2013)
Type X collagen	(Munirah et al., 2010)
Type XI collagen	(Munirah et al., 2010, Cheng et al., 2011)

Table 1.5 Markers found to be downregulated during monolayer expansion of chondrocytes.

Protein	Function*
Aggrecan	Major proteoglycan present in cartilage with a key role in compression resistance.
Cartilage Oligomeric Matrix Protein	A noncollagenous ECM protein which can facilitate the interaction between chondrocytes and their matrix as well as contributing to the structural integrity of cartilage.
Cathepsin B	Protease involved in the intracellular degradation of proteins.
Cadherin II	An adhesion protein, also known as N-cadherin, with multiple functions. It has a role in synaptogenesis in the brain and other neural tissues and also stimulates fibroblast growth factor receptor 1.[a]
CD10	Involved in the cleavage of the glycine-phenylalanine bond through the destruction of opioid peptides.
CD14	Involved in the innate response to LPS and upregulation of other cell surface molecules.
CD44	Hyaluronic acid receptor which has an involvement in cell migration and tumour growth progression.
CD49a	α1-subunit of an integrin. The integrin α1β1 is a receptor for collagen and laminin.
CD49c	α3-subunit of an integrin. The integrin α3β1 is a receptor for fibronectin, collagen, epiligrin, thrombospondin, laminin and chondroitin sulphate proteoglycan 4.
CD49e	The α5 subunit of an integrin. The integrin α5β1 is a receptor for fibrinogen and fibronectin.
CD49f	The α5 subunit of an integrin. When combined with β1, it is a receptor for laminin in platelets but when combined with β4 it is a receptor for laminin in epithelial cells.
CD51/61	Integrin α5β3, which is a receptor for vitronectin.
CD54	Has a role in leukocyte trans-endothelial migration by binding to leukocyte adhesion protein.
CD81	A member of the transmembrane 4 superfamily that forms complexes with integrins and may be involved in signal transduction.
CD90	May be involved in cellular interactions during synaptogenesis and other processes in the brain.
CD99	A cell surface glycoprotein with roles in transmembrane protein transport, T-cell adhesion and leukocyte migration.
CD105	The major glycoprotein present in vascular endothelium. It may have an important role in binding endothelial cells to integrins.
CD106	Assists the adherence of leukocytes to endothelial cells and is crucial for cell-cell recognition.
CD151	Has a major role in the assembly of the glomerular and tubular basement membranes of the kidney.
CD166	A cell adhesion molecule which binds to CD6 and has a role in neurite extension.

Protein	Function*
Clusterin	An extracellular chaperone that prevents the aggregation of non-native proteins. It is also involved in protecting cells from cytolysis by complement and apoptosis.
Dickkopf Homolog 3	Locally inhibits Wnt regulated processes so is important in vertebrate development. In adults, it has a role in bone formation and disease.
Early Growth Response 1	Regulates the transcription of genes whose products are needed for mitogenesis and differentiation.
Fibromodulin	May have a role in the assembly of the ECM. Has an impact on the rate of fibril formation.
Fibronectin	A glycoprotein that binds to numerous compounds including collagen and actin and cell surfaces. It has a role in cell adhesion, cell motility and maintenance of cell shape.
HIF-1α	α subunit of HIF-1. HIF-1 is an essential transcription factor for the regulation of cellular and systemic responses to hypoxia.
IGF Binding Protein	Extends the half-life of IGFs and can stimulate or inhibit the growth of promoting effects of IGFs on cells in culture.
Link Protein	Stabilises the binding of proteoglycan monomers to hyaluronic acid in the ECM of cartilage.
Melanoma Inhibitory Activity	Inhibits the growth of melanoma cells *in vitro* and some other neuroectodermal tumours.
S-100	Has a role in regulating cell cycle progression, differentiation and other cell processes.
Secreted Phosphoprotein 1	A key component of mineralised matrix which binds tightly to hydroxyapatite.
Sox9	Transcription factor involved in skeletal development.
Superficial Zone Protein	Is involved in the boundary lubrication within articulating joints and also prevents the deposition of protein onto articular cartilage.
Tenascin C	Protein present in the ECM and is thought to be involved in guiding migrating neurons during development. It is also a ligand for several integrins including α8β1 and α9β1.
TGF-β3	Has roles in embryogenesis and cell differentiation.
Twist Basic Helix-Loop-Helix Transcription Factor	Regulates the transcription of a variety of genes. It inhibits myogenesis and regulates cranial suture patterning and fusion.
Type I collagen	Present in many connective tissues and is particularly abundant in bone, tendon and skin. Major role is to provided tensile strength.[b]
Type II collagen	Found in cartilage and the vitreous humour of the eye. Has key roles in embryonic skeletal development and compression resistance in cartilage.

Protein	Function*
Type III collagen	Is present in most connective tissues and has roles in regulating cortical development and inhibiting neuronal migration.
Type IX collagen	Provides structural support in hyaline cartilage and the vitreous of the eye.
Type X collagen	Specifically produced by hypertrophic chondrocytes. Thought to be involved in facilitating calcification.[c]
Type XI collagen	May be an important component in fibrillogenesis by controlling the lateral growth of type II collagen fibrils.
Versican	A proteoglycan present in the ECM which has roles in many processes including cell adhesion and proliferation and tissue morphogenesis and maintenance.
Vitronectin	It interacts with GAGs and proteoglycans and encourages cell adhesion and spreading.

Table 1.6: Proteins observed when studying the phenotype of chondrocytes in monolayer culture. This table details the functions of the proteins listed in tables 1.4 and 1.5. *Details given in the function column were obtained from Genecards - http://www.genecards.org/. Some functions were not available on Genecards and were obtained from other sources; a: (Gelse et al., 2003), b:(van Roy, 2014), c (Shen, 2005).

Another paper compared human chondrocytes at passage zero and passage four (Hamada et al., 2013). They found that expression of CD44, CD49c and CD151 increased across the passages, whereas expression of CD14, CD49a and CD54 decreased (Hamada et al., 2013). Expression of CD26 fluctuated throughout culture but was higher at passage four compared to passage zero (Hamada et al., 2013). The authors then sorted cells for high CD54 expression and low CD44 expression and examined the ability of these cells to express collagen types I and II and aggrecan (Hamada et al., 2013). As expected, the expression of collagen type II and aggrecan was higher in the sorted cells compared to unsorted cells and expression of collagen type I was lower (Hamada et al., 2013). However, none of these results were statistically significantly different, this may be due to the small sample size tested and the authors acknowledge that there were difficulties in obtaining samples (Hamada et al., 2013).

Even though there are similarities between the markers found in all of these papers, it must be recognised that there are still differences between isolation and culture techniques between groups and such differences may impact on the expression of chondrocyte markers (Oseni et al., 2013). Oseni and colleagues (2013) have begun to address this issue by isolating ovine nasal cartilage chondrocytes using different pre-digest methods and varying concentrations and incubations of collagenase type II (Oseni et al., 2013). Pre-digestion appeared to provide no benefit to cell yield and had a negative effect on

cell viability and was therefore deemed unnecessary (Oseni et al., 2013). The optimum concentration and incubation of collagenase type II was 0.2% and ten hours respectively, which resulted in a cell yield of 1-1.5x10^6 cells/gram of tissue (Oseni et al., 2013).

Diaz-Romero *et al* (2005) also took this into consideration when comparing expression of markers in human chondrocytes after 24 hours and two weeks of culture. Firstly they assessed the effect of collagenase and pronase on marker expression 24 hours after isolation (24 hours plus time for enzyme treatment (collagenase incubation: overnight; pronase incubation: one hour)). CD63 and CD82 expression was not affected by either enzyme and only CD105 expression altered in response to collagenase (Diaz-Romero et al., 2005). The expression of nine markers (CD14, CD49e, CD49f, CD44, CD54, CD81, CD105, CD106 and CD151) was affected by pronase (Diaz-Romero et al., 2005). Out of the 31 markers looked at 16 markers were found to be differentially expressed in chondrocytes cultured for 24 hours and two weeks (Diaz-Romero et al., 2005). The authors do acknowledge that this expression pattern may change as chondrocytes are cultured for longer (Diaz-Romero et al., 2005). They also mention the difficulty in obtaining samples, like Hamada and colleagues. Their approach to this problem was to cryopreserve chondrocytes but the concern is that cryopreservation can further affect the phenotype of the cells. However the authors showed, in this case, the expression profiles of

cryopreserved and freshly isolated chondrocytes were very similar (Diaz-Romero et al., 2005).

Most studies involve populations of chondrocytes isolated from all cartilage zones. Grogan and colleagues (2013) questioned whether there are markers specific to chondrocytes from each zone. They found higher expression of ten genes in the superficial zone compared to the deep zone in both human and bovine cartilage (Grogan et al., 2013). Such genes included chitinase 3-like 1 (CH13L1), endothelial growth factor–containing fibulin-like extracellular matrix protein 1 (EFEMP1), THBS4, proteoglycan 4 (PRG4) and tenascin C (TNC) (Grogan et al., 2013). Only four genes were found to be more highly expressed in the superficial zone compared to the middle zone, whereas for the genes C-type lectin domain family 3, member A (CLEC3A), vav-3 guanine nucleotide exchange factor (VAV3) and leukocyte cell–derived chemotaxin 1 (LECT1), the opposite was the case (Grogan et al., 2013). The deep zone was found to have much higher levels of integrin-binding sialoprotein (IBSP) and secreted phosphoprotein 1 (SPP1) than the superficial zone (Grogan et al., 2013). Variation in chondrocyte monolayer expansion techniques including seeding densities and passage numbers are discussed in further detail in Chapter Three, section 3.1.

It is clear that some research groups are going beyond looking at the production of key matrix markers to understand the phenotype of chondrocytes both *in vivo* and *in vitro*. This thesis will examine the expression of four proteins (in addition to looking at cell morphology and GAG production). These are the matrix proteins collagen types I and II, the transmembrane protein CD44 and the transcription factor Sox9.

Collagen type I is a fibrillar collagen like collagen type II (Jensen and Host, 1997, Gelse et al., 2003), with its triple helix being a heterotrimer of two identical α1[I] chains and one α2[I] chain (Gelse et al., 2003). It is the most abundant collagen and is present in many tissues including bone, tendon, ligament, skin and cornea (Jensen and Host, 1997, Gelse et al., 2003). In fact, it represents 90% of the organic mass of bone (Gelse et al., 2003). It is not found in hyaline articular cartilage (Gelse et al., 2003) and is frequently found to be upregulated in dedifferentiating chondrocytes (Table 1.4). Collagen type II is specific to cartilage and is usually downregulated in dedifferentiating chondrocytes (Table 1.5). The structure and function of collagen type II is covered in section 1.4.4.1.

Sox9 was chosen as it is a non-collagenous protein which has been used in multiple studies to assess the chondrocyte phenotype, with it often being found to be downregulated in monolayer-expanded chondrocytes (Table 1.5). The sox family of proteins are transcription

factors which are part of high mobility group (HMG) superfamily; they are characterised by a HMG-box DNA binding domain (de Crombrugghe et al., 2001, Kiefer, 2007, Wegner, 2010). Sox9 is important for skeletal development as mutations in this protein can lead to campomelic dysplasia, a skeletal malformation syndrome (McDowall et al., 1999, de Crombrugghe et al., 2001). This transcription factor is expressed during chondrogenesis (Wright et al., 1995) and has a similar pattern of expression to collagen type II during foetal development (Ng et al., 1997). It is frequently used as a marker for chondrogenic differentiation of mesenchymal stem cells (MSCs) (Reed and Johnson, 2008, Vieira et al., 2009, Lettry et al., 2010). Although Sox9 is a major regulator of chondrogenesis there are two other Sox proteins involved; Sox5 and Sox6 (Ikeda et al., 2004, Wegner, 2010). Together these proteins are known as the SoxTrio (Ikeda et al., 2004, Wegner, 2010). This trio has been shown to induce the chondrocyte phenotype in a range of cells including pluripotent embryonic stem cells, multipotent MSCs and terminally differentiated dermal fibroblasts (Ikeda et al., 2004).

Membrane proteins are not frequently studied when assessing the phenotype of cultured chondrocytes. CD44 has appeared in the literature, with regards to chondrocyte dedifferentiation, slightly more frequently than other membrane proteins (Diaz-Romero et al., 2005, Albrecht et al., 2009, Hamada et al., 2013). It also has a key role in

pericellular matrix organisation and assembly (Knudson and Loeser, 2002, Knudson, 2003, Goldring, 2005). For these reasons, it was selected as the fourth protein of interest. The 'CD' in the names of these membrane proteins stands for cluster of differentiation (Zola et al., 2005). This nomenclature was created during Human Leukocyte Differentiation Antigen workshops (Zola et al., 2005, Zola et al., 2007). During the early 1980s, immunologists were developing a large number of monoclonal antibodies targeted against various leukocyte cell surface molecules, all with different names (Zola et al., 2005, Zola et al., 2007). The workshops aimed to standardise the nomenclature by coding antibodies and sending them to laboratories for testing against different cell types (Zola et al., 2005). Clusters of antibodies with similar binding patterns were identified, giving rise to the terminology 'cluster of differentiation' (Zola et al., 2005).

CD44 is a transmembrane protein that was originally identified on lymphocytes and was reported to be involved in cell adhesion and homing (Sneath and Mangham, 1998, Knudson and Loeser, 2002, Goldring, 2005). As well as chondrocytes, cells in many different tissues including the lung, skin, stomach and spleen express it (Sneath and Mangham, 1998, Knudson and Loeser, 2002). The major role of CD44 in articular cartilage is to act as a receptor for hyaluronan (Knudson and Loeser, 2002, Knudson, 2003). Hyaluronan is rarely present in isolation in articular cartilage; aggrecan monomers bind to a

hyaluronan filament via a link protein (Knudson and Loeser, 2002, Knudson, 2003). The number of aggrecan monomers that bind to one filament can be over 100 (Knudson, 2003). Therefore the interaction of CD44 with hyaluronan enables these large, hydrated protein aggregates to be retained in the pericellular matrix, immediately surrounding the chondrocyte (Knudson and Loeser, 2002, Knudson, 2003). It has been reported that CD44 expression is high in proliferating cells (Sneath and Mangham, 1998), suggesting that as chondrocytes have a more proliferative role in monolayer culture, the expression of CD44 may be higher in these cells than native cells.

1.5.4 Three-dimensional (3D) Culture of Chondrocytes

One way of maintaining the chondrocyte phenotype *in vitro* is to culture the cells three-dimensionally. A range of 3D culture techniques have been used to maintain or induce the chondrocytic phenotype. A common 3D culture technique used is high density pellet culture (Abbott and Holtzer, 1966, Manning and Bonner, 1967, Kato et al., 1988, Solursh, 1991, Ballock and Reddi, 1994, Tallheden et al., 2003, Malda et al., 2004, Zhang et al., 2004, Dehne et al., 2009). Abbott and Holtzer (1966), found that chondrocytes from 10-day chick embryos cultured in pellets had similar properties to those cultured in intact vertebrae. Other studies have also shown that chondrocytes cultured in high density pellets retain their spherical morphology (Manning and Bonner,

1967, Kato et al., 1988). Ballock and colleagues (1994) found that cells cultured in pellets expressed collagen type II mRNA irrespective of the culture medium used. They also found that expression of this gene increased with decreasing levels of FBS in the medium. In fact expression was highest when FBS was absent and insulin was added to the culture medium. The authors also found that gene expression of collagen type X increased with increasing culture length, with expression similar in cultures with or without serum. (Ballock and Reddi, 1994). Stewart and colleagues (2000) compared the phenotype of equine chondrocytes cultured in monolayer, pellets, aggregates and explants in addition to observing the effects of FBS and bone morphogenetic protein two (BMP-2). Procollagen type II gene expression was higher in pellet and aggregate cultures compared to monolayer and explant cultures. Expression of this gene was significantly increased in BMP-2 treated cells cultured in monolayer and aggregates. Gene expression of procollagen type II was lower in all cells incubated with FBS, regardless of culture system; however the only significant difference reported was in monolayer culture. Aggrecan expression was highest in the control group in pellet cultures; however incubation with BMP-2 enhanced expression in monolayer cells to a similar level. Collagen type I gene expression was detected in all culture systems apart from explant culture, although expression in aggregate and pellet cultures was lower compared to monolayer (Stewart et al., 2000).

Another group set out to determine the plasticity of human chondrocytes (Tallheden et al., 2003). For the chondrogenic differentiation component of the study, cells were pelleted and cultured in chondrogenic medium (DMEM-HG with ITS premix, 1mM pyruvate, 10ng/ml TGF-β, 10-7M dexamethasone and 80µM ascorbate-2-phosphate) . In fact, the pellet culture method is often used to induce chondrogenesis in mesenchymal stem cells (Johnstone et al., 1998, Csaki et al., 2007, Stewart et al., 2007, Stewart et al., 2008, Vidal et al., 2008, Lettry et al., 2010). This method is used as it mimics the condensation of MSCs during the early stages of chondrogenesis (Cancedda et al., 2000, Goldring et al., 2006, Chen and Tuan, 2008, Pelttari et al., 2008).

In another study, researchers used pellet culture to generate articular cartilage from chondrocytes isolated from day 16 chick embryos (Zhang et al., 2004). The authors chose pellet culture believing it would stabilise the phenotype of the chondrocytes in addition to having the advantage of not having additional material such as a supporting scaffold. The tissue generated after two weeks had a hyaline-like appearance and staining for collagen type II was similar to that seen in explants. Staining for aggrecan within the pellets was pericellular and less intense than that shown in the explants. Type IX collagen staining was shown to be stronger in pellets compared to explants. Both pellets and explants were negative for type X collagen. By day 14, pellets were shown to have a matrix with a similar fibrillar density to that in the

explants however matrix was more organised in the latter (Zhang et al., 2004).

Other 3D culture methods for culturing chondrocytes have involved seeding chondrocytes into a supporting scaffold material. One material commonly used is alginate (Bonaventure et al., 1994, Hauselmann et al., 1994, Hauselmann et al., 1996, Loty et al., 1998, van Osch et al., 1998, Chubinskaya et al., 2001, Domm et al., 2002, Schulze-Tanzil et al., 2002, Lee et al., 2003, Stoddart et al., 2006, Rai et al., 2009, Brand et al., 2012, Caron et al., 2012). One group used alginate as an intermediary step to produce cartilage-like implants from bovine chondrocytes (Stoddart et al., 2006). The authors of this paper claim that implants generated without the alginate culture step shrunk in the silicon mould and consisted mainly of cellular material (Stoddart et al., 2006). However, with the alginate culture process, implants looked similar to that of the transitional zone in native bovine articular cartilage (Stoddart et al., 2006). Another study investigated genes which are differentially regulated in chondrocytes cultured in monolayer and alginate (Haudenschild et al., 2001). This group discovered that all the matrix formation cDNAs were enriched in monolayer cultures, whereas matrix remodelling factors were enriched in alginate cultures. More specifically, mRNA expression for collagen types I and II, aggrecan and connective tissue growth factor was evident in both culture systems. However, expression of collagen type II and aggrecan seemed to be lower in monolayer cultures, suggesting that the 3D culture encouraged

retention of the chondrocytic phenotype (Haudenschild et al., 2001). In a more recent study, researchers looked at the effect of cell density in alginate beads on the expression of collagen types I, II and X and the transcription factor Sox9 (Bernstein et al., 2009b). High density alginate beads had an initial seeding density of $7x10^7$ cells/ml of alginate, whereas low density beads had an initial seeding density of $4x10^6$ cells/ml. The authors found Sox9 expression to be significantly higher in the low density beads but expression of both collagen types I and II were higher in the high density beads. This difference in collagen gene expression was only significant in the early stages of the culture. Collagen type X expression fluctuated throughout the cultured period but was significantly higher in the low density beads at weeks one and four (Bernstein et al., 2009b).

A more recent study used a novel surface expansion system in an attempt to prevent chondrocyte dedifferentiation (Rosenzweig et al., 2012). This research group compared bovine chondrocytes cultured on three different surfaces, high extension silicone rubber (HESR) which was continually expanded (CE cultures), standard tissue culture polystyrene (SD cultures) and polystyrene culture dishes coated with approximately 1mm of silicone rubber (SS cultures). Cell morphology and gene expression of collagen types I and II, aggrecan, COMP and Sox9 were similar in SD and SS cultures from passages one to five. The morphology of the cells in the CE cultures more closely resembled that of a native chondrocyte. Expression of collagen type I, Sox9,

COMP and aggrecan was upregulated in these cells whereas expression of collagen type I was downregulated when compared to SD cultures. Following monolayer culture, cells from each of the three conditions were cultured three dimensionally in pellets. Staining for collagen type II and GAGs was more intense in pellets containing CE cultured cells compared to those containing SD and SS cells. These findings suggest that culturing chondrocytes on a continually expanding surface helps retain the chondrocytic phenotype during *in vitro* expansion (Rosenzweig et al., 2012).

In addition to alginate, some other materials have been used to culture chondrocytes three-dimensionally in order to encourage redifferentiation (Table 1.7). This includes synthetic polymers such as polyglycolic acid (PGA) (Homicz et al., 2003), poly(DL-lactic-co-glycolic acid (PLGA) (Chen et al., 2003) and polycaprolactone (PCL) (Garcia-Giralt et al., 2008).

Scaffold Material	References
Alginate	(Bonaventure et al., 1994, Hauselmann et al., 1994, Hauselmann et al., 1996, Loty et al., 1998, van Osch et al., 1998, Chubinskaya et al., 2001, Domm et al., 2002, Schulze-Tanzil et al., 2002, Lee et al., 2003, Stoddart et al., 2006, Rai et al., 2009, Brand et al., 2012, Caron et al., 2012)
Atelocollagen Solution	Khoshfetrat 2008
Type I collagen gel	(Lee et al., 2007)
Gelatin	(Brochhausen et al., 2013)
Silk	(Das et al., 2013)
Silk-gelatin	(Das et al., 2013)
Polyglycolic Acid (PGA)	(Homicz et al., 2003)
Poly(DL-lactic-co-glycolic acid (PLGA)	(Chen et al., 2003)
Polycaprolactone (PCL)	(Garcia-Giralt et al., 2008)
Methoxypolyethyleneglycol-block-co-poly(lactide-co-glycolide) (MPEG-PLGA)	(Foldager et al., 2011)

Table 1.7: Various scaffold materials used for the 3D culture of chondrocytes.

Three-dimensional culture alone is often not enough to completely reverse or prevent chondrocyte dedifferentiation. Therefore, there are additional changes to the culture system that can be made in order to enhance redifferentiation.

Some research groups have altered monolayer expansion conditions in an effort to maintain the chondrocyte phenotype or enhance redifferentiation (de Haart et al., 1999, Lee et al., 2005), whilst others

have adjusted 3D culture conditions to maximise redifferentiation (Domm et al., 2002, Murphy and Polak, 2004, Duval et al., 2009, Babur et al., 2013). Some researchers have attempted to optimise both stages of chondrocyte culture to successfully maintain the chondrogenic phenotype (Egli et al., 2008).

Aspects of the culture system that can be altered include changing the physical conditions surrounding the 3D construct and adding soluble factors to the culture medium.

The media used to culture chondrocytes both in monolayer and in 3D varies in the literature. Mandl and colleagues (2002) expanded human chondrocytes in four different media preparations (DMEM+10% FCS, DMEM+ITS+, DMEM+ITS+5ng/ml FGF2 and DMEM+100ng/ml FGF2) before seeding the cells into alginate. Alginate cultures were again incubated in two different solutions (DMEM+10% FCS+25µg/ml ascorbic acid and DMEM+IGF-1 and TGF-β1+25µg/ml ascorbic acid). Collagen type I expression was significantly lower in the group expanded in DMEM+100ng/ml FGF2 followed by culture in DMEM+IGF-1 and TGF-β1+25µg/ml ascorbic acid compared to cells expanded in media containing serum, followed by the same conditions. Collagen type II expression was significantly higher in the beads seeded with cells cultured in media containing serum or, followed by DMEM+IGF-1 and TGF-β1+25µg/ml ascorbic acid (Mandl et al., 2002). In a more recent study, human chondrocytes were expanded in monolayer in two

different media compositions, DMEM/Ham's F12+10% FCS and DMEM/Ham's F12+10% FCS+5ng/ml FGF2+5µg/ml insulin before being seeded into collagen sponges (Claus et al., 2012). Once seeded into the sponges the cells were again cultured in two different media, DMEM/Ham's F12+10% FCS+50µg 2-phospho-L-ascorbic acid+200ng/ml BMP-2α + 5µg/ml insulin and 100nM thyroxin T3 or DMEM/Ham's F12+10% FCS+50µg 2-phospho-L-ascorbic acid. The authors found that collagen type II was only expressed in the sponges cultured in supplemented media; however the genes Sox9 and aggrecan were expressed in all groups. However Sox9 protein was not expressed by cells in sponges cultured in non-supplemented media, regardless of the monolayer expansion conditions (Claus et al., 2012).

Yang and colleagues (2006a), cultured human chondrocytes in two different media preparations in monolayer, followed by another two solutions in 3D culture. The authors also used two 3D culture systems, pellets and collagen coated filters. Interestingly, as well as having different media, different seeding densities were used. The exact culture conditions used in this study is summarised in Table 1.8. The authors showed that pellets seeded with chondrocytes expanded in basic media (BEC) were negative for collagen type II whereas those seeded with chondrocytes expanded with growth factor supplemented media (GFSEC) contained regions positive for this collagen type II. BEC filters also had regions positive for collagen type II whereas GFSEC filters were positive throughout. GAG content was found to be

significantly higher in filters compared to pellets; however the content was significantly higher in both GFSEC cultures compared to BEC cultures (Yang et al., 2006a).

Ascorbate is a common feature in these media designed to help maintain the chondrocyte phenotype in *in vitro* culture. Therefore, this vitamin will be used in the latter stages of this thesis in an attempt to enhance any redifferentiation observed in the 3D cultures. Ascorbate is discussed in greater detail in Section 5.1, Chapter Five.

Monolayer culture	
DMEM containing L-glutamine, 10% FBS, 1 x non-essential amino acids, 10mM HEPES, 0.2mM ascorbic acid, 0.4mM proline (BEC).	DMEM containing L-glutamine, 4.5mg/ml glucose, 25mM HEPES, 10% FBS. After the first three days 10ng/ml bFGF was added (GFSEC).
3D culture	
Pellet	**Collagen coated filters**
DMEM containing Glutamax, 0.1% human serum albumin, 0.2mM ascorbic acid, 1xITS+10ng/ml IGF-1 and 10ng/ml TGF-β2.	DMEM containing L-glutamine, 2% human serum albumin, 1xITS, 5ng/ml TGF-β2 and 0.4mM ascorbic acid.

Table 1.8: Different culture media used in a study by Yang *et al* (2006a) for the culture of chondrocytes.

As well as adding soluble factors to culture media, researchers have also varied the physical environment surrounding the chondrocytes to encourage and enhance redifferentiation. One group investigated how adhesion sites within agarose gels and the stiffness of the agarose itself impacted chondrocyte redifferentiation (Schuh et al., 2012). The

authors found that increasing adhesion sites and stiffness had a negative impact on chondrocyte redifferentiation. Another way of altering the *in vitro* environment is to lower the oxygen tension to closer to what is experienced in the joint. This is the second mechanism that will be used later in the thesis to enhance redifferentiation and is discussed in greater detail in Section 5.1, Chapter Five.

Up to this point, only healthy cartilage has been reviewed. Articular cartilage can be damaged through injury, osteoarthritis (OA) or spontaneously as a consequence of the condition osteochondritis dissecans (Clar et al., 2005). The following sections will deal with cartilage damage and the treatment of cartilage lesions with a particular look at cellular therapies and regenerative medicine.

1.6 Cartilage Damage

Articular cartilage can be damaged in three main ways, through osteoarthritis, a sudden accidental injury or osteochondritis dissecans (NHS, 2014). Another way in which cartilage can be damaged is through infection (NHS, 2014). It is difficult to ascertain the frequency of cartilage damage as those with mild damage may not always seek medical assistance (NHS, 2014). Although it is known that around 10000 people a year in the UK seek treatment for cartilage damage (NHS, 2014). In some cases, traumatic injuries to cartilage can lead to

the development of OA; out of 46 million people in America diagnosed with OA, 12% of cases were associated with trauma (Waters et al., 2014). It is not clear whether all cartilage injuries lead to OA or whether there is a critical size or depth of injury which results in the development of OA (Loken et al., 2010).

There are two key elements to cartilage injury resulting from acute trauma; the structure of the collagen matrix and the loading rate of the tissue (Gallo and Mosher, 2013). When a blunt injury occurs during a traffic accident, there is a high rate of loading. This energy is dispersed across the superficial layer of the articular cartilage and can cause superficial cracks in the tissue (Gallo and Mosher, 2013). Oblique surface-layer tears can occur if excessive shear force is applied to cartilage (Gallo and Mosher, 2013). In a paper studying 993 knee arthroscopies, 20% of the patients had localized lesions without degeneration (Aroen et al., 2004). Out of this 20%, 44% were partial thickness lesions, 47% were localized full thickness lesions and 9% were categorised as osteochondritis dissecans. Across the total number of patients (993), 59% reported an acute onset of symptoms whereas the remaining 41% experienced a more gradual onset. This group also reported the causes of injury in these patients; sport was the majority at 49% with the next highest being unknown at 23%. Traffic accounted for only 4% of the injuries seen. Interestingly, this group found that soccer and team handball contributed to most of the sporting injuries at 30% and 13% respectively (Aroen et al., 2004). Another

paper looking at 1000 knee arthroscopies found chondral or osteochondral lesions in 61% of the patients studied (Hjelle et al., 2002). The majority of these lesions were OA lesions (44%). The remaining lesions were categorised as follows, focal chondral lesions (28%), chondromalacia patellae (23%), osteochondritis dissecans (2%) and other (3%) (Hjelle et al., 2002). Chondromalacia patella is a condition which involves softening or breakdown of articular cartilage on the medial and odd facet of the patella and fibrillation of the patellar bone (Macmull et al., 2012, Pak et al., 2013). The exact cause of this condition remains to be confirmed and there is currently no cure for it (Macmull et al., 2012, Pak et al., 2013).

The exact pathophysiology of osteochondritis dissecans is unknown and there is some debate as to whether the term osteochondrosis would be more accurate due to the lack of inflammation observed in this condition (Edmonds and Polousky, 2013, McCoy et al., 2013). In this disease there is damage to the subchondral bone and articular cartilage (McCoy et al., 2013, Shea et al., 2013). Features of the disease can include softening, swelling, early separation, partial detachment or complete separation resulting in the formation of a loose body (Shea et al., 2013). The most common region to be affected is the femoral condyles of the knee (Eismann et al., 2014), although this disease can also affect ankle and elbow joints (Paterno et al., 2014).

1.6.1 Osteoarthritis

Osteoarthritis (OA) is an extremely common arthritic disease in humans (Chen and Tuan, 2008, Felson, 2009, Penny et al., 2012). It is progressive and degenerative and as such can also be called degenerative joint disease (Miljkovic et al., 2008, Mobasheri et al., 2009, Buhrmann et al., 2010, Richardson et al., 2010, Penny et al., 2012). There a numerous risk factors for OA, including age, sex, obesity, trauma and overuse (Chen and Tuan, 2008, Felson, 2009, Penny et al., 2012). Degradation of cartilage, inflammation and the formation of osteophytes are the major events in OA (Abramson and Attur, 2009, Felson, 2009, Mobasheri et al., 2009, Penny et al., 2012) (Figure 1.8). There are two major proteinases involved in matrix degradation in OA, matrix metalloproteinases and aggrecanases (Martel-Pelletier et al., 2008, Umlauf et al., 2010) In the early stages of OA, patients experience acute pain which later becomes chronic (Felson, 2009, Penny et al., 2012). Current treatments for OA are unable to stop progression of the disease and focus more on alleviating pain (Felson, 2009, Penny et al., 2012).

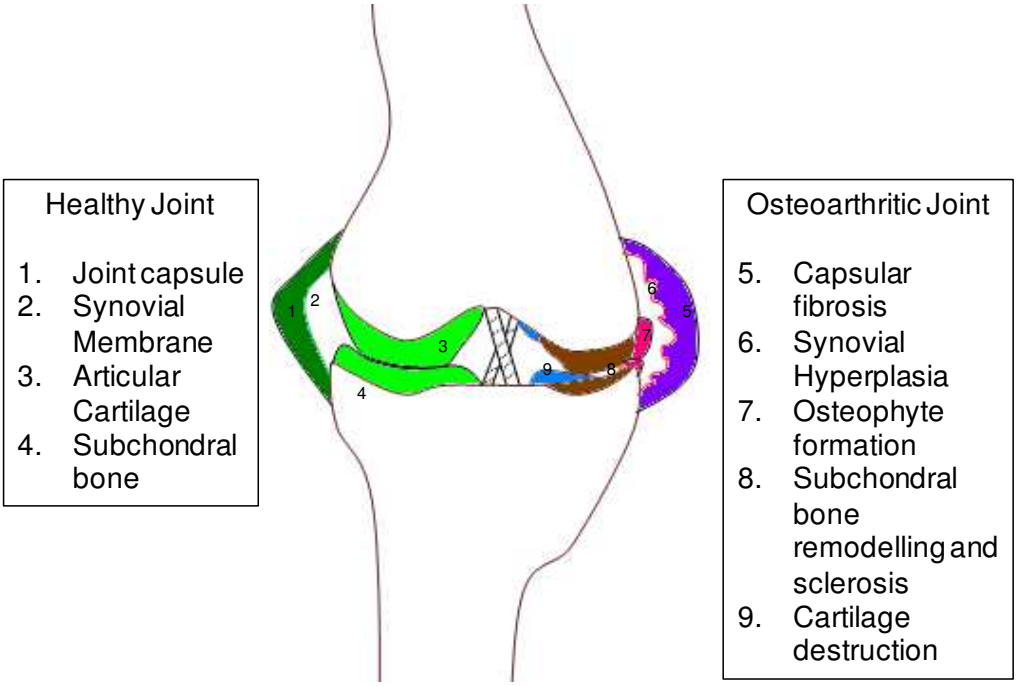


Figure 1.8: Comparison of the features of a healthy and osteoarthritic joint. The cartilage is severely worn and subchondral bone remodelling is seen in the diseased joint. Figure is adapted from (Aigner and Stove, 2003, Aigner et al., 2006).

It is not only humans that are affected by OA. Spontaneous OA is common in athletic and older horses (Goodrich and Nixon, 2006, Penny et al., 2012). Other species including dogs (Liu et al., 2003, Cook et al., 2010, Rychel, 2010), guinea pigs and mice (Bendele, 2002) also suffer from OA.

OA can be a primary or secondary complaint and in dogs it is commonly secondary to other musculoskeletal disorders (Henrotin et al., 2005). Such disorders include hip and elbow dysplasias (Clements et al., 2010, Cook et al., 2010) and osteochondrosis (Bari et al., 1989, Arican et al., 1994). All breeds of dogs can develop OA regardless of their shape

and size (Rychel, 2010); however some breeds are more predisposed to it than others. Labrador Retrievers have a much higher risk of developing OA (Liu et al., 2003, Todhunter et al., 2003, Clements et al., 2010), whereas Greyhounds are rarely affected by OA (Todhunter et al., 2003). Male dogs are also more susceptible to developing OA (Clements et al., 2010). OA is said to affect up to 20% of dogs over one year old (Clements et al., 2010) and accounts for 37% of all lameness in the dog (Arican et al., 1994).

The dog has been extensively used as a model for OA. The most common model used is transection of the anterior cruciate ligament (Altman et al., 1984, Fife, 1986, Pelletier et al., 1988, Pelletier et al., 1992, Matyas et al., 1999, Liu et al., 2003, Matyas et al., 2004, Rogachefsky et al., 2004, Kuroki et al., 2011, Gharbi et al., 2013). The dog is a very useful model because the anatomy of the knee is very similar to humans and many features including the articular cartilage, synovium and joint capsule are histologically and biochemically highly conserved between species (Cook et al., 2010).

1.7 Treatments for OA

Most of the treatments for OA focus on alleviating symptoms rather than preventing the progression of the disease. One problem is that OA is often present for a long time before a patient experiences any noticeable discomfort (Matyas et al., 2004). There is current research

which aims to identify biomarkers of early OA, with the hope that these will help identify cases of OA in the early stages of disease or identify predisposing factors. Recent progress in this area of OA research is reviewed in (Mobasheri, 2012).

1.7.1 Pharmaceuticals and Nutraceuticals

There is a hierarchy of treatment for OA in humans, which has been described as a treatment pyramid (Lohmander and Roos, 2007, Roos and Juhl, 2012). All treatment begins with educating the patient about their lifestyle and advising them about weight loss and exercise regimes (Lohmander and Roos, 2007, Roos and Juhl, 2012). The next level of treatment, which can be combined with the information and advice in the first line of treatment or can be a second line of treatment, is non-surgical intervention (Lohmander and Roos, 2007, Roos and Juhl, 2012). This can include pharmacological pain relief, physiotherapy, occupational therapy and acupuncture (Lohmander and Roos, 2007, Roos and Juhl, 2012).

Pharmacological relief includes paracetamol, non-steroidal anti-inflammatory drugs (NSAIDs) and opiods (Lohmander and Roos, 2007, Swift, 2012). Paracetamol is opted for first due to the fact that it is relatively effective at relieving pain and has fewer side effects than NSAIDs (Dougados, 2007, Swift, 2012). This is in contrast to treatment

of OA in veterinary species where NSAIDs are chosen first (Rychel, 2010). NSAIDs work by inhibiting the enzyme cyclooxygenase (COX) which is involved in the production of prostaglandins and thromboxanes (Henrotin et al., 2005). There are two isoforms of COX, COX-1 and COX-2 (Henrotin et al., 2005, Solomon and Goodson, 2007). The first is constitutively expressed whereas the latter is inducible and expressed in cells involved in inflammation in diseased tissue (Henrotin et al., 2005, Solomon and Goodson, 2007). Prostaglandin E_2 has been found in the synovial fluid of OA patients and seems to contribute to pain, inflammatory erythema and fever (Solomon and Goodson, 2007). Therefore inhibiting the COX enzymes reduces the production of such compounds and subsequently the pain and fever they cause. Carprofen, Meloxicam and aspirin are examples of NSAIDs (Henrotin et al., 2005, Solomon and Goodson, 2007). Over 40 NSAIDs have been developed but a quarter of those have since been removed from the market due to their toxicity (Solomon and Goodson, 2007). There are also NSAIDs which specifically inhibit COX-2 (Henrotin et al., 2005, Solomon and Goodson, 2007). However a few of these have been withdrawn from the market due to severe side effects (Solomon and Goodson, 2007). In addition to NSAIDs, opioids can be used to manage pain but they have many side effects including nausea, dizziness and constipation (Swift, 2012).

Another class of treatments for OA are nutracueticals. Glucosamine is one of the popular products in the UK but limited evidence is available for its affectivity (Swift, 2012). In fact, in veterinary species, glucosamine with chondroitin is a highly recommended treatment for OA (Rychel, 2010).

These treatments are effective at reducing pain and increasing mobility but they do not act against the underlying problem of damaged cartilage.

1.7.2 Surgical Treatments

The top level of the treatment pyramid is surgical intervention. Some surgery just replaces the entire joint or alleviates pain whereas other surgical options aim to repair the damaged cartilage, either by prompting spontaneous repair or inserting newly formed cartilage.

Debridement is one such surgical technique which aims to temporarily relieve OA patients of the pain they are suffering (Hunziker, 2002). Osteotomy is also used to reduce pain and improvement alignment of the joint; however it is only recommended for OA patients who also have deformities (Hunziker, 2002).

Microfracture, on the other hand is a technique used to initiate spontaneous repair of the damaged articular cartilage (Hunziker, 2002). This involves drilling small holes in the joint, throughout the cartilage lesion, down to the subchondral bone (Hunziker, 2002). This encourages the spontaneous repair response and a blot clot consisting of a fibrin matrix forms and fills the defect (Hunziker, 2002). Subsequently mesenchymal cells penetrate the matrix and the clot is converted to a vascularised scar-like tissue (Hunziker, 2002). The disadvantage with this repair is that the tissue formed is more like fibrocartilage and as such is mechanically inferior (Hunziker, 2002). It also begins to degenerate after a few weeks (Hunziker, 2002).

Autologous chondrocyte implantation (ACI) is an established treatment for cartilage lesions that are larger than $4cm^2$ (Schulze-Tanzil, 2009, Oldershaw, 2012, Steinert et al., 2012). It is also used as a secondary treatment where microfracture has failed (Steinert et al., 2012). This technique involves harvesting cartilage from a non-loading bearing surface and isolating chondrocytes from it (Richardson et al., 2010, Steinert et al., 2012). These chondrocytes are then expanded in monolayer before injecting the cells back into the patient (Brittberg, 2008, Brittberg, 2010, Zeifang et al., 2010, Oldershaw, 2012, Steinert et al., 2012). The major problems with this technique include dedifferentiation of the cells during monolayer culture, calcification and cell leakage (Steinert et al., 2012). The first version of ACI was ACI

with a periosteal flap (ACI-P) (Zeifang et al., 2010). This involved covering the defect (once filled with chondrocytes) with a periosteal flap and sealing it with fibrin glue (Zeifang et al., 2010). There were many disadvantages to this technique, including the requirement of a second surgical procedure to obtain the periosteal flap and sutures to secure it (Zeifang et al., 2010). The chondrocytes in the defect were also unequally distributed using this method (Zeifang et al., 2010). ACI-P was subsequently modified to use a flap made of porcine type I/III collagen (ACI-C) (Zeifang et al., 2010). The latest ACI modification is matrix-induced autologous chondrocyte implantation (MACI) (Zeifang et al., 2010). With this technique chondrocytes are embedded in a biological scaffold and implanted and secured into the defect with fibrin glue (Zeifang et al., 2010). Bartlett and colleagues (2005) compared the outcomes of ACI-C and MACI used to treat osteochondral defects in the knee. All patients complained of pain and limited function prior to the operation. The average defect size was 6.0cm^2 in the ACI-C group and 6.1cm^2 in the MACI group. The majority of patients had good repair tissue one year after the procedure in both treatment groups; however the most of the tissues contained fibrocartilage (Figure 1.9) (Bartlett et al., 2005).

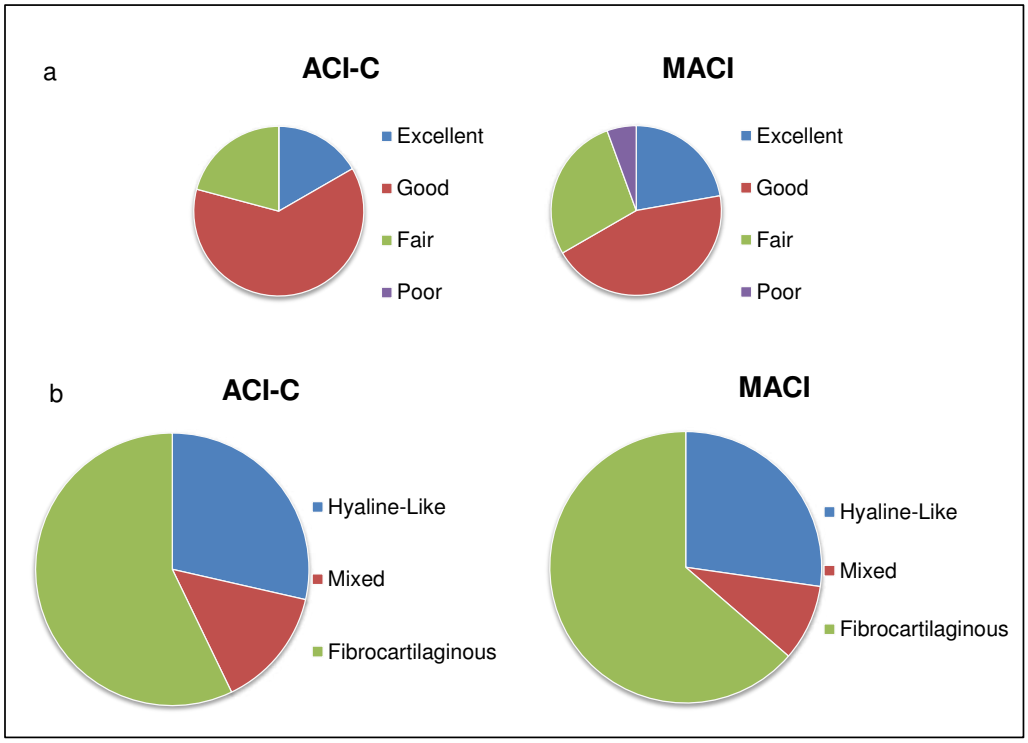


Figure 1.9: Comparison of ACI-C and MACI treatments for osteochondral defects in the knee. (a) Charts indicating the grade of the repair tissue, 24 patients and 18 patients were assessed in the ACI-C and MACI groups respectively. (b) Charts indicating the type of repair tissue, data represents 14 patients in the ACI-C group and 10 patients in the MACI group. Charts were generated based on the data given by (Bartlett et al., 2005). The reason behind differing patients for each assessment is not clear. ACI-C (autologous chondrocyte implantation with a collagen flap) and MACI (matrix-induced autologous chondrocyte implantation).

Another research group compared the outcomes of knee defects treated with ACI-P or MACI (Zeifang et al., 2010). Inclusion criteria for the study were patients aged between 16 and 50 years with defects between 2.5cm^2 and 6.0cm^2. At six months after the operation, defect repair and filling was more extensive in the MACI treated patients. However at 12 months, knee function was significantly improved in both

treatment groups and similar knee function was also seen at 24 months. Hypertrophy was observed more frequently in ACI-P treated patients but more MACI treated patients required revisions after the initial operation (Zeifang et al., 2010). These results seem to suggest that, although MACI is perceived to be an improvement on the original ACI technique, the restoration of the function of the affected joint is actually similar between the two treatments. Another paper details the histological quality of MACI repair tissue in the knee (Enea et al., 2012). For this study lesion size was 2cm^2 or greater and follow-up biopsies were taken six months after the MACI procedure. Authors report that when biopsies were taken, 14 out of 33 biopsies were taken from asymptomatic patients, whereas the remaining 19 were taken from patients experiencing a recurrence in or persistence of their original symptoms. Out of these biopsies, 30.3% were classified as normal, 51.5% as nearly normal, 12.2% as abnormal and 6.1% as severely abnormal. The authors also state that 21% of biopsies contained mostly hyaline matrix, 6% were mostly a fibrous matrix and another 6% were a mixture of the two, with fibrocartilage often on top of a layer of hyaline cartilage (Enea et al., 2012). This again indicates that although improvement was seen in some patients, MACI does not repair damaged cartilage back to its native form in many cases. Although ACI has undergone several modifications since it was introduced in 1987, repair of damaged cartilage is still a major challenge for orthopaedic surgeons (Filardo et al., 2013).

The final surgical option is joint replacement. In fact, total hip replacement is frequently used to treat end-stage OA (Mellon et al., 2013). This procedure is now very common, with 71 672 total hip replacements being undertaken in 2011 in England and Wales alone (Mellon et al., 2013). Although the survival of the replacements are very good (90% - 95% last for up to ten years and approximately 85% last for up to 20 years), they can still fail (Mellon et al., 2013). Failure can be for a variety of reasons, including infection, dislocation and aseptic loosening, with the latter being the most common (Mellon et al., 2013). Total knee replacement is also a common treatment for OA affected joints (Liddle et al., 2013). In the USA, 676 000 total knee replacements were carried out in 2009 and this figure is expected to increase to 3.5 million by 2030 (Liddle et al., 2013). This procedure is well-established and can be considered to be the gold standard treatment for knee OA but it may not be appropriate for every patient (Liddle et al., 2013). A recent study followed patients that had undergone total ankle replacement for end stage ankle OA (Rosello Anon et al., 2014). At the final follow-up (this varied between patients but averaged at 37 months after surgery), 15 patients were satisfied with the outcome of the surgery, 1 patient was satisfied with reservations and 2 patients were dissatisfied (Rosello Anon et al., 2014). In another study 114 out of 225 patients were found to be dissatisfied with their knee replacement (Ali et al., 2014). Those dissatisfied were found to have a lower range of motion and higher levels of pain, anxiety and depression (Ali et al., 2014). These studies suggest that total

replacement of a joint affected by severe cartilage damage and / or OA can be very effective for some patients but they are not the best solution for everybody.

Even current surgical treatments don't successfully restore the joint back to its native state and in some cases the repair tissue is of poor quality (Stoddart et al., 2009). Therefore current research is looking into other ways of repairing cartilage, mainly tissue engineering and regenerative medicine.

1.7.3 Tissue Engineering and Regenerative Medicine

Tissue engineering essentially involves the reconstruction of mammalian tissues which have structural and functional properties which closely mimic those of the native tissue (Hunziker, 2002). This process can either be done exclusively *in vitro* or it can be started *in vitro* and then finished off *in vivo* (Hunziker, 2002). There are three major elements to a tissue engineered construct; a matrix scaffold, cells and signalling molecules (Hunziker, 2002). Cartilage tissue engineering has been intensively researched for the past two decades; however there are still only a few treatment options which have reached the clinic (Johnstone et al., 2013).

During the past two decades many different cells (the optimum source is yet to be identified) and scaffolds have been evaluated for the regeneration of articular cartilage each with their own benefits and disadvantages (Stoddart et al., 2009, Johnstone et al., 2013, Bhardwaj et al., 2014). Successful regenerative medicine depends on cell biologists working together with tissue engineers and surgeons to repair or regenerate damaged tissues and organs (Krampera et al., 2006, Helder et al., 2007, Penny et al., 2012).

The first cell option for cartilage repair and regeneration is autologous chondrocytes and these are already approved for clinical use (Johnstone et al., 2013, Wright et al., 2013, Kwak et al., 2014). A recent paper studied patients following autologous chondrocyte implantation for osteochondral lesions of the talus (Kwak et al., 2014). Before the operation, 26 patients rated their ankles as poor and three rated them as fair. After the procedure, nine ankles were given an excellent rating, 14 good, five fair and one poor. Although most patients observed an improvement, one procedure failed and out of the 23 patients actively involved in sport before the procedure only ten returned to the same level of activity after the operation (Kwak et al., 2014). This suggests that ACI is not the best solution to treat cartilage damage for every patient. Another study aimed to characterise the cells contained in repair tissue following ACI (Wright et al., 2013). When compared with adjacent macroscopically normal cartilage, ACI repair

tissue was found to contain fewer GAGs and less collagen type II. This group isolated cells from the repair tissue, chondrocytes from normal cartilage and MSCs from subchondral bone. They found all cells to be positive for CD73, CD90 and CD105 and negative for CD19, CD31, CD34, CD45 and HLA-DR. Expression of CD14 in repair tissue cells and chondrocytes varied, whereas MSCs were negative for CD14 (Wright et al., 2013). This would suggest that repair cells are similar to chondrocytes but are not as effective as native cells in producing cartilage ECM.

Chondrocytes have the advantage of a low risk of immunological reaction and they possess the native phenotype (Johnstone et al., 2013, Roelofs et al., 2013). However large numbers of chondrocytes are needed for larger cartilage defects and a consequence of expansion is a loss of phenotype (see Section 1.5.3). Another research group have investigated the potential of chondrons (a chondron consists of the chondrocyte itself and its surrounding pericellular matrix) for regenerating articular cartilage (Vonk et al., 2010, Vonk et al., 2014). In their 2010 paper they demonstrated that chondrons encapsulated in alginate beads expressed greater levels of aggrecan and collagen type II than those containing chondrocytes alone (Vonk et al., 2010). Beads containing chondrons were also shown to contain more GAGs (Vonk et al., 2010). In a more recent paper using damaged cartilage, the same group showed similar results with respect to chondrons exhibiting a

more chondrocyte-like phenotype *in vitro* compared to chondrocytes alone (Vonk et al., 2014). Allogeneic chondrocytes could be another option offering a potential off-the-shelf solution (Johnstone et al., 2013). Almqvist and colleagues (2009) investigated whether implanting alginate beads containing human mature allogenic chondrocytes were a safe and feasible option for the treatment of symptomatic cartilage lesions in the knee. Patients had an average defect size of 2.6cm^2. Twelve patients had suffered from a traumatic injury and nine had non-traumatic injuries (e.g. focal OA). Alginate beads containing 20x10^6 cells/ml were cultured for two weeks prior to implantation. Patients follow-up was carried out for a maximum of 24 months after the operation. The level of pain experienced by patients significantly decreased post-operatively and decreased over time. There were no adverse effects from the implantation. Of those patients who consented to 12 month biopsy, two contained hyaline-like repair tissue, six contained a mixture of hyaline-like and fibrocartilaginous tissue, four consisted of fibrocartilaginous tissue. In addition to this, there was one biopsy that was categorised as fibrous vascularised tissue. In terms of actually filling the defect, two were completely filled, four were hypertrophic and seven were underfilled (Almqvist et al., 2009). This data would seem to suggest that the success of particular cellular treatment will vary greatly depending on the patient and the size and cause of the defect.

Over the past few years interest in the use of mesenchymal stem cells (MSCs) in regenerative medicine has grown substantially. MSCs are multipotent rather than totipotent because they can differentiate into a restricted range of cells (Smith and Webbon, 2005, Taylor et al., 2007). These cells have been isolated from a range of species including dogs, humans, horses, rodents and rabbits (Javazon et al., 2004, Penny et al., 2012). They are also extracted from a number of different tissues, including bone marrow, adipose tissue, umbilical cord (blood and matrix) and placental chorionic villi (Penny et al., 2012). The two major advantages of these cells is that they can be expanded *in vitro* without worrying about a loss of phenotype and there are multiple sources available (Johnstone et al., 2013). However, the major problem with this cell type is the lack of consistency with isolation procedures and their specific phenotypic identity. The International Society for Cellular Therapy have tried to address this by publishing criteria that should be used to characterise human mesenchymal stromal cells (Dominici et al., 2006). There are three components to this criteria; firstly the cells should be adherent to plastic (Dominici et al., 2006). Secondly 95% of cells should express the surface markers CD73, CD90 and CD105 and 2% or less should express CD11b, CD19 CD34, CD45 and HLA-DR (Dominici et al., 2006). Finally, the cells must be multipotent and have the ability to undergo chondrogenic, adipogenic and osteogenic differentiation (Dominici et al., 2006).

Despite the fact that isolation, characterisation and chondrogenic differentiation techniques of MSCs vary in the literature and controversy surrounds their exact identity (Johnstone et al., 2013); one research group is hopefully that a one-step surgical procedure involving MSCs will one day be available (Helder et al., 2007). Whilst the surgeon operates on the patient a tissue engineer isolates MSCs from adipose tissue and seeds them onto a graft (Helder et al., 2007). When the graft is ready the surgeon implants it all in the same operation (Helder et al., 2007). The authors state that the whole process would take around two and a half hours (Helder et al., 2007).

Although these cells hold much promise for use in treating cartilage lesions, another problem with inducing chondrogenesis in MSCs is premature hypertrophic differentiation (Chen and Tuan, 2008). This generates cartilage similar to the growth plate rather than articular cartilage (Chen and Tuan, 2008).

Another cell type that can be used is the induced pluripotent stem cell (iPS). These cells would create a large source of patient specific cells capable of regenerating articular cartilage, which can be generated from a starting cell population that is harvested via a minimally invasive technique (Diekman et al., 2012, Johnstone et al., 2013). A skin sample can be harvested from a patient with an articular defect and fibroblasts can be isolated via enzymatic digestion (Tsumaki et al., 2014). These cells can then be reprogrammed into iPS cells using

transfection (Tsumaki et al., 2014). The resultant iPS cells can either be differentiated into chondrocytes and then implanted into a defect or they can be used generate cartilage *in vitro* (Tsumaki et al., 2014). It is important to note that iPS derived chondrocytes will dedifferentiate in culture like chondrocytes isolated directly from articular cartilage (Tsumaki et al., 2014). Therefore, it is imperative that expansion of iPS cells is carried out before differentiation. One research group aimed to generate tissue engineered cartilage from murine iPS cells from a starting population predifferentiated down the chondrogenic lineage (Diekman et al., 2012). Fibroblasts were isolated from the tails of eight to ten week old mice and were shown to express the stem cell markers Nanog, Oct4 and SSEA1. These undifferentiated iPS cells were also shown to form teratomas when injected into murine kidneys and testes. The authors cultured these iPS cells in chondrogenic media and sorted for cells expressing collagen type II. This sorted population expressed significantly higher levels of collagen type II and aggrecan than the unsorted cells. They also produced more GAGs but also expressed higher levels of collagen type X. When implanted into an *in vitro* defect, both cell populations generated cartilage like matrix, however tissue generated by the sorted population had a significantly higher shear strength (Diekman et al., 2012). In another paper, iPS cells from different sources were shown to have different effects (Uto et al., 2013). Nanog green fluorescent protein (GFP) iPS cells (the GFP protein was knocked in to the Nanog locus) were shown to produce teratomas. Green mouse iPS cells developed from the bone marrow of EGFP-

transgenic mice, when embedded into a collagen hydrogel, filled a cartilage defect with cartilage-like tissue in the patellar groove of the left femur in eight week old mice (Uto et al., 2013). Research into these cells is still in the early stages and issues surrounding efficiency, reproducibility and control of differentiation need to be refined before they are introduced into the clinic (Johnstone et al., 2013).

The final cell option available is embryonic stem cells (Johnstone et al., 2013). These cells have the capability to differentiate into any cell type within the body and could offer and off-the-shelf treatment option (Johnstone et al., 2013). One paper has shown a three step method of differentiating human embryonic stem cells (hESCs) into chondrocytes (Oldershaw et al., 2010). The first stage is differentiating the hESCs into the primitive streak mesendoderm. At the end of this stage, the cells have a large, prominent nuclei and express *NANOG*, *OCT4*, *CDH1*, *GSC2*, *T* and *GATA4*. The second stage involves differentiation to the mesoderm. Cells at the end of this stage form densely packed clusters in culture and express *MIXL1*, *KDR*, *SOX9* and *ACAN*. The final stage is chondrogenic differentiation. These cells form 3D aggregates in culture and express *PDFGRB*, *COL2A1*, *SOX9*, *SOX6*, *CD44* and *ACAN* (Oldershaw et al., 2010). For the functions of the proteins these genes encode, see Table 1.9.

Gene	Function[a]
ACAN	Encodes for the protein aggrecan. Aggrecan is an essential component of cartilage ECM, contributing to compression resistance.
CD44	Encodes for a cell surface glycoprotein which has roles in cell adhesion, migration and cell-cell interactions.
CDH1	Encodes for the calcium dependent cell-cell adhesion glycoprotein, cadherin 1.
COL2A1	Encodes the alpha-1 chain of collagen type II. Collagen type two is a major component of cartilage ECM and has a crucial role in providing compressive resistance in this tissue.
GATA4	Encodes a zinc-finger transcription factor which is thought to be involved in the regulation of genes with roles in embryogenesis and myocardial function and differentiation.
GSC2	Encodes a protein thought to have a role in development.
KDR	Encodes a tyrosine-protein kinase receptor. It is a cell surface receptor for vascular endothelial growth factors A, C and D.
MIXL1	Encodes a transcription factor which has multiple roles during development. These include cellular differentiation from the primitive streak to blood and morphogenesis of the heart and gut during embryogenesis.
NANOG	Encodes a transcription regulator involved in the proliferation and self-renewal of embryonic stem cells and the inner cell mass.
OCT4	Encodes a transcription factor that has major roles in embryonic development and stem cell pluripotency.
PDGFRB	Encodes a cell surface tyrosine kinase receptor for platelet derived growth factor homodimers (B and D) and heterodimers (A and B). It is involved in many processes including cell proliferation, differentiation and survival.
SOX6	Encodes a transcription factor which has roles in many developmental processes including formation of the skeleton and neurogenesis.
SOX9	Encodes a transcription factor involved in skeletal development. It is important in chondrogenesis.
T	Encodes an embryonic nuclear transcription factor which is important in the formation and differentiation of mesoderm.

Table 1.9: Genes expressed by human embryonic stem cells during a three step directed differentiation protocol. [a] Functional information for each gene was obtained from Genecards - http://www.genecards.org/

In another study sheep embryonic stem-like cells were transplanted into cartilage defects and compared with empty and glued-only defects (Dattena et al., 2009). Defects treated with the embryonic stem-like cells were shown to be filled with hyaline cartilage and joints appeared to be macroscopically healthy (Dattena et al., 2009). In a more recent paper, researchers looked at the ability of chondroprogenitors derived from hESCs to repair damaged cartilage (Cheng et al., 2014). During chondrogenic differentiation, hESCs were shown to express typical chondrogenic gene markers, Sox5, Sox6, Sox9, Collagen type II and Aggrecan. Following differentiation into chondrocytes, cells were encapsulated into fibrin gels and implanted into osteochondral defects (2mm wide and 2mm deep) in the trochlear grooves of the distal femurs of athymic rats. At four weeks after implantation, the defects treated with fibrin containing cells were partially repaired, mostly with flat, shiny and translucent tissue. Defects treated with fibrin alone contained minimal, rough and irregular repair tissue. The same difference was seen 12 weeks after implantation; fibrin only treated defects were poorly repaired, whereas joints treated with cells appeared to be fully repaired. The cells derived from hESCs formed hyaline-like repair tissue and there were still some of these cells remaining in the tissue at 12 weeks, suggesting that these cells contributed to the formation of the cartilage-like repair tissue (Cheng et al., 2014).

Although these cells are a promising alternative to chondrocytes, they do have their own disadvantages. The major problem with these cells

is the ethical considerations and regulations. Also, like with iPS cells there is a risk of teratoma formation (Johnstone et al., 2013).

The other important component of a tissue engineered construct is the matrix or scaffold that surrounds the cells (Hunziker, 2002, Kolacna et al., 2007, Stoddart et al., 2009, Johnstone et al., 2013). The ideal scaffold must possess several qualities (Stoddart et al., 2009, Johnstone et al., 2013). Firstly the scaffold must be made of a substance that will not evoke an immune reaction in the patient (Stoddart et al., 2009, Johnstone et al., 2013). Secondly it should be biodegradable and any fragments created during degradation should also not cause an immune response (Stoddart et al., 2009, Johnstone et al., 2013). In addition to this, the material should be engineered in a way that optimises cell-material interactions (Stoddart et al., 2009). The scaffold should be large enough to fit inside the defect it is designed for (Stoddart et al., 2009). Tissue engineered scaffolds must also be able to withstand high levels of mechanical loading (Stoddart et al., 2009). Finally an ideal scaffold or matrix should be porous to enable seeded cells to migrate or native cells to infiltrate and growth factors and signalling molecules to filter through (Hunziker, 2002).

Many different scaffolds have been tested, including protein- and carbohydrate-based polymers and artificial polymers (Table 1.10) (Hunziker, 2002). However only a few biodegradable scaffolds have been commercialised for the use of cartilage repair (Stoddart et al.,

2009). One such product is CaRes, which is a collagen type I gel (Stoddart et al., 2009). Chondrocytes are directly seeded into the gel without prior monolayer expansion but are further cultured in the scaffold prior to implantation (Stoddart et al., 2009). Scaffolds are also discussed in Chapter 6.

Protein-based scaffolds	**Carbohydrate-based scaffolds**	**Artificial polymer scaffolds**
Fibrin	Polylactic acid	Dacron
Type I collagen	Polyglycolic acid	Teflon
Type II collagen	Hyaluronan	Carbon fibres
Gelatine	Agarose	Polyesterurethane
Silk	Alginate	Polybuturic acid
	Chitosan	Polymethylmethacrylate
		Hydroxyapatite
		Polyethylene glycol

Table 1.10: Different scaffolds investigated for cartilage tissue engineering.

Adapted from (Hunziker, 2002).

1.8 Summary, Hypotheses and Aims/Objectives of the Project

Articular cartilage can be damaged through injury or OA. OA is a debilitating and painful disease in humans and other species including dogs and horses. Most of the current treatment focuses on alleviating pain and there is no treatment which can stop the progression of the disease. Articular cartilage is extremely poor at regenerating itself and there is currently no completely successful treatment which repairs

cartilage back to its native state. Therefore, extensive research into regenerating cartilage either entirely *in vitro* or partially *in vivo* is currently being undertaken. Cells are an integral part of cartilage repair and although chondrocytes are used for ACI, their major disadvantage is their limited availability. The consequence of this is the requirement for *in vitro* expansion, which ultimately leads to a loss in the chondrocyte phenotype. Other cell types including MSCs are also being investigated for their regenerative capacity, however as of yet no optimum cell source has been identified. Other research into cellular strategies for cartilage repair have involved a variety of scaffolds but again no ideal scaffold and cell combination has been identified.

The overall aim of this thesis was to develop a greater understanding of chondrocyte dedifferentiation and redifferentiation *in vitro* using canine chondrocytes. Not only do dogs suffer with OA, but they have also been used as models for this disease. This thesis contains three experimental chapters, each with individual aims and objectives. These are as follows:

<u>Chapter 3: Chondrocyte Dedifferentiation in Monolayer Culture</u>

Aim: To examine the phenotype of canine articular chondrocytes expanded to P5 in monolayer and confirm dedifferentiation.

Hypothesis One: Canine chondrocytes will alter in morphology with increasing passage, adopting a more fibroblastic phenotype.

Hypothesis Two: Expression of collagen type II and Sox9 will decrease with increasing passage whereas collagen type I and CD44 will increase.

Chapter 4: Establishment of Chondrocyte 3D culture

Aim: To establish a 3D culture of canine chondrocytes and assess the phenotype of monolayer-expanded cells within this system.

Hypothesis One: Chondrocytes previously expanded in monolayer will begin to adopt a more native phenotype (redifferentiate) in 3D culture. Expression of collagen type II and Sox9 will increase whereas expression of collagen type I and CD44 will decrease. Synthesis of GAGs should also be detected in the 3D culture system.

Chapter 5: Enhancement of Chondrocyte Redifferentiation in 3D culture

Aim: To identify factors that will enhance chondrocyte redifferentiation in 3D culture.

Hypothesis One: Addition of ascorbic acid (25µg/ml) to the cell culture medium will enhance the redifferentiation of canine chondrocytes cultured three dimensionally following expansion in monolayer culture.

Hypothesis Two: Culturing chondrocytes in a 3D system in an oxygen concentration of 2.4% will enhance the redifferentiation of monolayer expanded canine chondrocytes.

Hypothesis Three: Addition of IL-1β (10ng/ml) to the cell culture medium will inhibit the redifferentiation of canine chondrocytes cultured three dimensionally following expansion in monolayer.

2. Materials and Methods

2.1 Tissue and Cell Sources

Cartilage tissue and positive control tissues were obtained from animals euthanized for reasons other than research, where possible the history of the animals were obtained to check for joint problems. As the tissue was taken from dogs euthanized for unrelated clinical reasons, this project does not fall under the Animals (Scientific Procedures) Act 1986 or the Veterinary Surgeons Act 1966. Approval for the use of clinical material was obtained from the local Ethical Review Committee. Various dog breeds were used and animals were aged between 2 years and 11 years old. For more information on the animal material used for the work detailed in this thesis, see Appendix A.

2.2 Antibodies

All the antibodies used in this project are detailed in tables 2.1 (primary antibodies) and 2.2 (secondary antibodies). All antibodies from Aviva Systems Biology came as a lyophilized powder. All vials were centrifuged at 12 000xg for 20 seconds. Powder was resuspended in

phosphate buffered saline (PBS) (Invitrogen, Paisley, UK) containing 0.02% sodium azide (Sigma). Vials were vortexed briefly to distribute the liquid before being centrifuged as before. Antibodies were aliquotted and stored at -20^{o}C. All antibodies from the Developmental Studies Hybridoma Bank (DSHB) were aliquotted on arrival and stored at 4^{o}C. All other antibodies were either kept at 4^{o}C or -20^{o}C according to manufacturer's instructions.

Antibody	Supplier	Catalogue Number	Specificity	Application
COL1A1	Aviva Systems Biology	ARP59998	Rabbit polyclonal against C terminal region of collagen type I	IF, WB
COL1A1	Aviva Systems Biology	ARP59999	Rabbit polyclonal against C terminal region of collagen type I	IF, WB
CD44	Aviva Systems Biology	ARP61023	Rabbit polyclonal against C terminal region of CD44	IF, WB
SOX9	Aviva Systems Biology	ARP37986	Rabbit polyclonal against C terminal region of Sox9	IF, WB
SOX9	Aviva Systems Biology	P100797	Rabbit polyclonal against N terminal of Sox9	IF, WB
COL1A2	Aviva Systems Biology	ARP58441	Rabbit polyclonal against middle region of collagen type I	IF, IHC, WB
Hermes-1	DSHB	N/A	Rat anti-CD44	WB
H4C4	DSHB	N/A	Mouse anti-CD44	WB
P1G12	DSHB	N/A	Mouse anti-CD44	WB
CIIC1	DSHB	N/A	Mouse anti-collagen type II	WB
II-II6B3	DSHB	N/A	Mouse anti-collagen type II	WB
M-38	DSHB	N/A	Mouse anti-collagen type I	WB
Anti-Collagen II	Abcam	ab34712	Rabbit polyclonal against collagen type II	IHC, WB
Rabbit polyclonal IgG	Abcam	ab27478	Isotype control	IHC, WB
Mouse IgG isotype control	Vector Labs	I-2000	Isotype control	IHC
β-actin	Sigma	A5136	Mouse anti-human β-actin	WB

Table 2.1: Primary antibodies used in this project. For each antibody the supplier, specificity and application are given and where appropriate the catalogue number is also provided.

Antibody	Supplier	Catalogue Number	Specificity	Application
Goat pAb to Rb IgG (DyLight® 488) Excitation: 493nm, Emission: 518nm	Abcam	ab98462	Fluorescent conjugated secondary antibody	IF
Fluorescein anti-mouse IgG (H&L) Excitation: 490-500nm, Emission: 510-520nm	Vector Laboratories	FI-2000	Fluorescent conjugated secondary antibody	IF
Anti-rabbit IgG, HRP-linked Antibody	Cell signalling technology	#7074	HRP conjugated secondary antibody	WB
Polyclonal Rabbit Anti-Rat Immunoglobulins/ HRP	DAKO	P0450	HRP conjugated secondary antibody	WB
Biotinylated universal (anti-mouse IgG / rabbit IgG) antibody	Vector Laboratories	PK6200	Biotinylated secondary antibody	IHC

Table 2.2 Secondary antibodies used in this project. For each antibody the supplier, catalogue number, specificity and application are given.

2.3 Cartilage Harvest and Chondrocyte Isolation

2.3.1 Canine Forelimb and Hindlimb Dissection and Cartilage Harvest

Articular cartilage was harvested either from the canine elbow joint in the forelimb or the stifle joint in the hindlimb (Figure 2.1). All limbs were shaved before being washed with cold water and washing up liquid. These were then scrubbed in cold water and 10% trigene. The limbs were then left to soak in 2% bleach for 30 minutes. Once taken out of the bleach each limb was sprayed with 70% ethanol (Fisher Scientific, Loughborough, UK) before the skin was removed. The cartilage was checked to see if it appeared macroscopically healthy (Figure 2.2). Healthy cartilage should be smooth and white as shown in by Marijnissen and colleagues (2002). If deemed healthy, the opened joints were flooded with sterile PBS. Cartilage was harvested using a sterile scalpel (Swann Morton, Sheffield, England) and forceps.

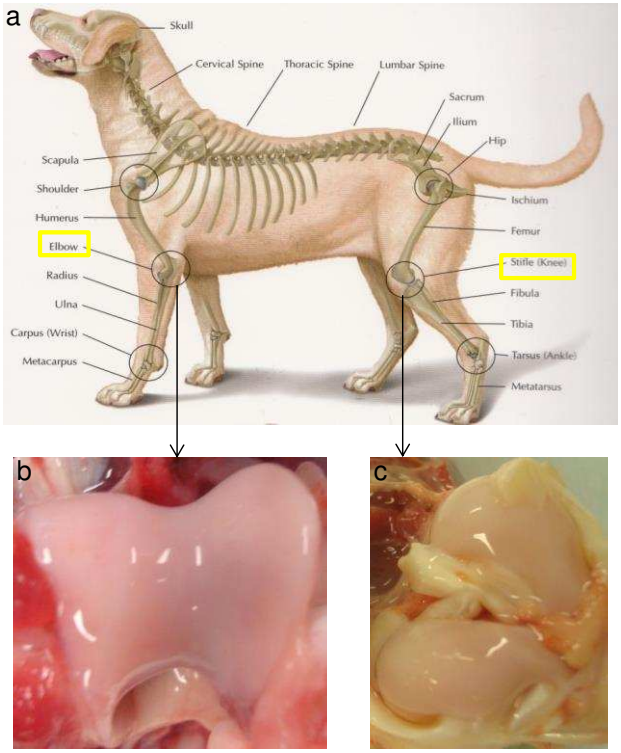


Figure 2.1: Location of the canine elbow and stifle joints. (a) Location of the elbow and stifle joints in a dog (image taken from (Johnstone and Biery, 2003)) (b) a dissected and washed elbow joint (c) a dissected and washed stifle joint. The photographs in (b) and (c) were taken immediately prior to cartilage harvest. Photographs were taken using a Sony Cyber-shot DSC-W30 digital camera.

Thin shavings were taken in order to increase the efficiency of the tissue matrix digestion and therefore cell release. These shavings were placed into a falcon tube (Greiner Bio-One, Gloucestershire, UK) containing collection media (Low Glucose Dulbecco's Modified Eagle Medium (DMEM) containing 4mM L-glutamine, 1000mg/L glucose and 110mg/L sodium pyruvate (Fisher Scientific) supplemented with 2% antibiotics (10,000Units/ml penicillin and 10,000µg/ml streptomycin) (Invitrogen)).

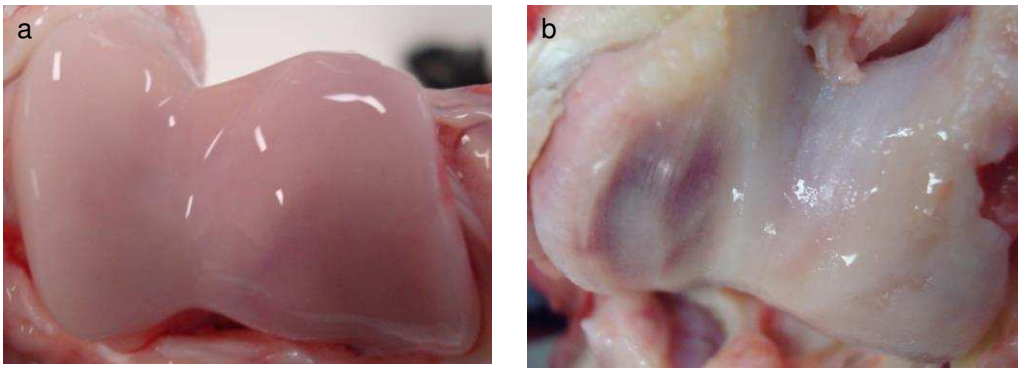


Figure 2.2: Macroscopically healthy and degenerate articular cartilage. Throughout this study healthy cartilage was harvested to isolate chondrocytes. Healthy cartilage is smooth and shiny as shown in (a). The cartilage in (b) is not smooth or shiny and is significantly worn. No cartilage was harvested from the joint in (b). Photographs were taken using a Sony Cyber-shot DSC-W30 digital camera.

2.3.2 Chondrocyte Isolation from Articular Cartilage

In order to isolate chondrocytes from cartilage shavings, the cartilage tissue was digested using the enzyme collagenase. Firstly, the shavings were tipped into a sterile petri dish (Thermo Scientific, Roskilde, Denmark) with a small volume of the collection media and cut into smaller chips using a sterile scalpel. This was done to increase the surface area available for the enzyme to digest. These chips were then placed in a falcon tube containing sterile PBS supplemented with 10% antibiotics on a roller for 20 minutes. The PBS was removed and the sterile filtered 0.1% Collagenase Type I (Invitrogen) solution (Collagenase Type I dissolved in DMEM supplemented with 2%

antibiotics) was added. The cartilage shavings and collagenase solution were then poured into a T25 flask (Thermo Scientific, Germany) and placed in a 37°C, 5% CO_2 incubator overnight for 16-18 hours. Subsequently, the flasks were placed on a shaker for two hours at room temperature. Once the cartilage had been digested, the cellular solution was sterile filtered, using a 70µm cell strainer (BD Biosciences, Erembodegem, Belgium) into a falcon tube. This solution was then centrifuged at 200xg for ten minutes at 20°C. The cell pellet was washed with sterile PBS using the same centrifuge conditions as before. After the second wash, collection media was added to the cell pellet and a cell count was performed using the Trypan Blue dye exclusion assay (see section 2.5.1). Once counted, the cells were washed for a final time in PBS and centrifuged as before. After the final wash the pellet was resuspended in culture medium (DMEM supplemented with 2% antibiotics and 10% foetal calf serum (FCS) (Invitrogen)) and the cellular suspension was split into three flasks (where possible, see Chapter Three, section 3.2.1), already containing culture medium. These flasks were kept in a 37°C, 5% CO_2 incubator with media being changed at least twice a week.

2.4 Expansion of Chondrocytes in Monolayer

Once seeded into T25 flasks the cells were monitored under the inverted light microscope, Leica DM IL and images were captured using the DFC490 camera and Leica Application Suite (Leica, Milton Keynes, UK) and media was changed every two to three days. When the cells were 90-100% confluent, they were passaged. The culture medium was removed and the cells were washed with sterile PBS. The cells were then incubated with 1ml 1x TrypLE express (Invitrogen) (5ml for T75 flasks and 10ml for T175 flasks) at 37^{o}C and 5% CO_2. Flasks were observed under a light microscope at five-minute intervals for cell detachment. Once a suitable level of cell detachment had occurred, the cell suspension was dispensed into a sterile falcon tube. The surface of each flask was washed with a small volume of 1x TrypLE express (1ml for T25 flasks, 2ml for T75 flasks and 3ml for T175 flasks) to maximise the cell removal. This cell solution was added to the sterile falcon tube. Sterile PBS was added and the whole solution was centrifuged at 200xg for ten minutes at 20^{o}C. The wash with sterile PBS and centrifugation was repeated once, before the cell pellet was resuspended in collection medium and the cells were counted using the Trypan Blue dye exclusion assay (section 2.5.1). After the cell count, the cells were washed with sterile PBS and centrifuged as before. The cell pellet was resuspended in cell culture medium and the resulting cell suspension was split into three flasks already containing culture medium. This

whole process was repeated for every passage. Depending on the downstream applications, cells were split into T25s, T75s or T175s (Thermo Scientific).

2.5. Cell Viability Assays

2.5.1 Trypan Blue

Cell pellets were resuspended in a volume of media (DMEM with 2% antibiotics) appropriate for the size of the particular cell pellet. An aliquot of the cell suspension was removed and dispensed into a sterile eppendorf to which Trypan Blue (Fisher Scientific) was then added. 10µl of this solution was then dispensed into each side of a haemocytometer (Sigma, St. Lois, USA), which was viewed under a light microscope at x10 objective. The number of viable cells (appear as bright cells) and non-viable cells (stained blue) were counted and the concentration and percentage of viable cells were calculated (Figure 2.3). Five squares on each side of the haemocytometer (ten squares in total) were counted and an average count was taken. To get the concentration of cells/ml, the average number of cells per square was multiplied by the dilution factor and then by 10000.

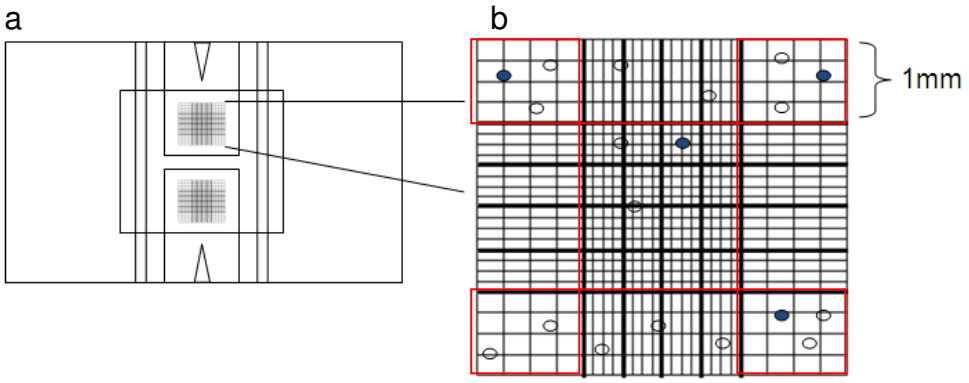


Figure 2.3: Cell counting using a haemocytometer. (a) Diagram of a haemocytometer. (b) The grid used to count the cells. Only cells in the four corner squares and central squares were counted (indicated by red squares). Dead cells stained blue whereas live cells appeared bright.

2.5.2 Cyquant

The Cyquant NF assay (Invitrogen) uses a fluorescent dye to measure cellular DNA content within a sample. This will indicate the cell number in the sample as DNA content within cells is highly regulated, making the values closely proportional. Cells were seeded into 96 well plates (Corning black plates with clear bottoms, supplied by Fisher Scientific) and left to adhere for a minimum of four hours. Plates were kept at 37^{o}C, 5% CO_2 during this time. When appropriate, cells were seeded at a known density; to determine the cell density Trypan Blue was used. After the cells had adhered, culture medium was aspirated and 100µl of Cyquant NF dye reagent diluted in Hanks Balanced Salts Solution (HBSS) (Invitrogen) buffer was added to each well. Following

optimisation (Figure 2.4) cells were incubated with the dye for 10mins, at 37°C, 5% CO_2. This optimisation also identified the maximum cell density that could be used for this assay is 10000 cells/well. The fluorescence was measured at room temperature in fluorescence microplate reader (Fluostar Optima, BMG Labtech GmbH, Ortenberg, Germany) with the excitation filter set at 485nm and the emission filter set at 520nm.

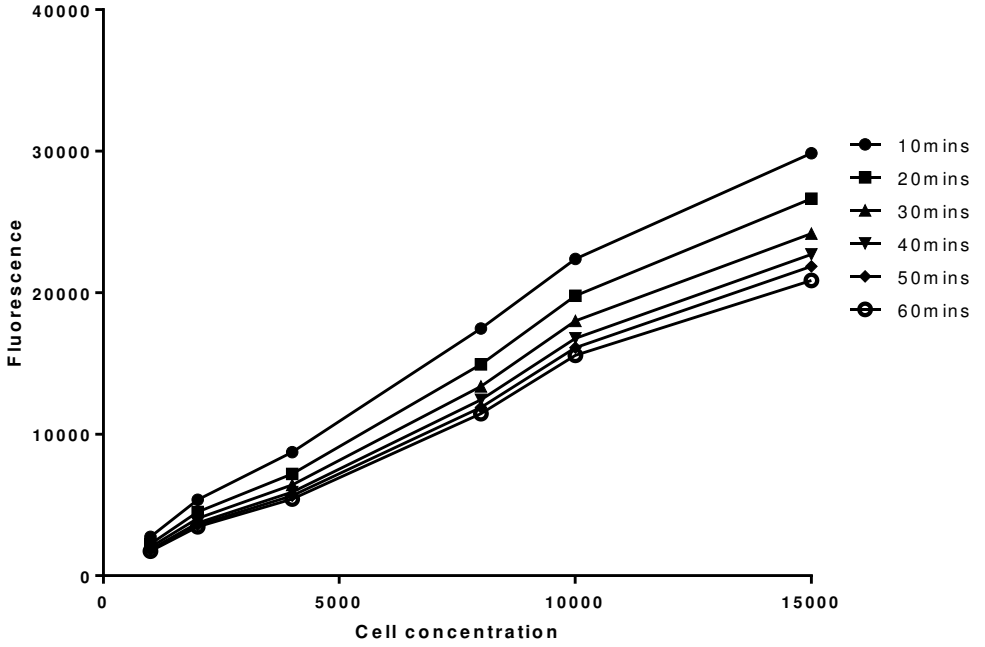


Figure 2.4: Cyquant Assay optimisation. Cells were seeded into a 96 well plate at different densities and were incubated with the Cyquant NF dye for different lengths of time. The fluorescence started to plateau after a density of 10000 cells/well and the level of fluorescence detected began to dip after 10 minutes particularly at higher densities. Therefore a 10 minute incubation was selected for all further assays and the maximum number of cells seeded per well was 10000.

2.5.3 Alamar Blue

The Alamar Blue assay measures the metabolic activity of cells through a oxidation-reduction (REDOX) reaction. Alamar Blue has been used to assess the viability of cells *in vitro* since the early nineties (O'Brien et al., 2000). O'Brien and colleagues (2000) identified that Alamar Blue and resazurin are in fact the same compound. One of the first uses of the resazurin reduction test was to assess the level of bacteria in milk (John, 1939, Nixon and Lamb, 1945). Whilst the cells are growing, the surrounding environment is chemically reduced and that reduction continues during cell growth. If cell growth is inhibited the environment becomes more oxidised. In a reduced environment, the Alamar blue dye changes from non-fluorescent blue to fluorescent red.

Chondrocytes were counted using Trypan Blue and seeded into 24 well plates (Thermo Scientific) at known densities to generate a standard curve. This assay was primarily used to assess the viability of chondrocytes seeded into alginate beads. When this assay was performed on alginate beads, individual beads were placed into a well in a 24 well plate. Cells were left to adhere to the plastic overnight (incubated at 37^{o}C, 5% CO_2). Media was aspirated and all wells were washed three times with sterile PBS. The Alamar Blue stock solution (AbD Serotec, Oxford, UK) was diluted 1:10 with HBSS. A volume of

1ml of this solution was added to each well; plates were wrapped in aluminium foil (SLS, Nottingham, UK) and incubated at 37°C, 5% CO_2 for 90mins. Alamar blue solution (100µl) was transferred to a 96-well plate; this was performed in triplicate for each well of the 24-well plate. This 96-well plate was wrapped in foil during transport to the fluorescent plate reader (Fluostar Optima). The fluorescence was measured at room temperature with the excitation filter set at 560nm and the emission filter set at 590nm.

2.6 Cryopreservation and Revival of Canine Chondrocytes

Culture medium was removed from the flasks. The cells were briefly rinsed with TrypLE express before being incubated at 37°C, 5% CO_2 with TrypLE express. The cells were checked for detachment every five minutes. When the cells had lifted, the solution was drawn up and dispensed into a sterile falcon tube. An equal volume of DMEM supplemented with 2% antibiotics was added to inactivate the TrypLE express. This solution was then centrifuged at 1000xg for five minutes at 20°C. After the supernatant was removed the cells were resuspended in 1ml of freeze medium (90% 50:50 DMEM supplemented with 2% antibiotics: FCS and 10% Dimethyl Sulphoxide (DMSO) (Fisher Scientific). A cell count was then performed, using the Trypan Blue Dye exclusion assay (section 2.5.1). The required volume

of freeze media was added in order to obtain a cell concentration of 1-2x10^6 cells/ml. Aliquots of 1ml of this solution were dispensed into cryoprotective vials, which were then placed in a Nalgene Mr Frosty (a passive freezer). The Mr Frosty was kept in a -20^{o}C freezer for one hour before being transferred to a -80^{o}C freezer overnight. The vials were then removed from the Mr Frosty and placed into liquid nitrogen storage.

Cell revival has to be carried out quickly in order to maximise cell survival. After the vial was removed from liquid nitrogen storage the cell solution was rapidly thawed in a 37^{o}C water bath. Once thawed, the contents of the vial were transferred to a falcon tube and 10ml of culture medium was added (drop-wise at first). The cells were then centrifuged at 200xg for five minutes at 20^{o}C and the supernatant discarded. The pellet was resuspended in culture medium and the whole cell solution was pipetted into a T25 flask. The flask was placed in a 37^{o}C, 5% CO_2 incubator and left for two days for the cells to settle. When the cells were between 90% and 100% confluent, they were passaged using the method previously described.

2.7. 3D Culture of Canine Articular Chondrocytes

2.7.1 High Density Pellet Culture

Articular cartilage was harvested and chondrocytes were isolated and expanded as described in sections 2.3.1 and 2.3.2. When the cells were 90-100% confluent, pellet culture was attempted. Firstly, the culture medium was removed and the cells were washed with sterile PBS. The cells were then incubated with TrypLE express at 37^{o}C and 5% CO_2. Flasks were checked at five-minute intervals for cell detachment. The lifted cells were dispensed into a sterile falcon tube and an equal volume of DMEM supplemented with 2% antibiotics was added. The cell solution was centrifuged at 1000xg for five minutes at 20^{o}C. After the supernatant was removed the cell pellet was resuspended in 1ml of culture medium. A small aliquot of this cell suspension was taken for cell counting as described earlier. The required volume of culture medium was then added to obtain a cell concentration between $1x10^6$ and $2x10^6$ cells/ml. Aliquots (1ml) of the cell suspension were then pipetted into 1.5ml sterile eppendorfs. These eppendorfs had holes in the lid, made using a hypodermic needle, to assist with gaseous exchange. The cells were then centrifuged at 400xg for five minutes at 20^{o}C. All pellets were incubated at 37^{o}C and

5% CO_2 for 21 and 35 days. The media was changed every two to three days.

2.7.2 Alginate Bead Culture

The method used to seed the cells into alginate was adapted from (Liebman and Goldberg, 2001). When the cells were between 90% and 100% confluent, culture medium was removed and the cells were washed with sterile PBS. TrypLE was then added to the flask, which was placed in back in the 37°C, 5% CO_2 incubator and checked every five minutes for cell detachment. Once a significant proportion of the cells had lifted, the whole solution was drawn up and dispensed into a sterile falcon tube. A further volume of TrypLE express was added to the flask to further enhance the number of cells lifted from the flask. An equal volume of DMEM supplemented with 2% antibiotics was added to this solution which was then centrifuged at 1000xg for five minutes at 20°C. The supernatant was discarded and the cell pellet was resuspended in 1ml of DMEM with 2% antibiotics. A cell count was then performed as previously described. The cells were centrifuged for a second time at 250xg for ten minutes at 20°C. Whilst centrifuging, 102mM $CaCl_2$ (Fisher Scientific) was added to each well of a six well plate (Thermo Scientific). Once the supernatant had been removed, the cells were resuspended in 1.2% (low viscosity) alginate to get a cell

concentration of $2x10^6$ cells/ml. The 1.2% alginate solution was made by dissolving 1.2g of alginic acid from brown algae (Sigma) in 100ml of 0.155M NaCl. This solution was then autoclaved prior to its use in cell culture. 1.2% alginate has been used for chondrocyte culture by many research groups (Hauselmann et al., 1994, Beekman et al., 1997, van Osch et al., 1998, Chubinskaya et al., 2001, Domm et al., 2002, Stoddart et al., 2006, Brand et al., 2012, Caron et al., 2012) The cells were mixed thoroughly with the alginate using a sterile pipette. This cellular suspension was then drawn up through a 18G needle (Terumo Europe, Leuven, Belgium) into a sterile 10ml syringe (Terumo Europe). The suspended chondrocytes were expressed dropwise through the 18G needle into the $CaCl_2$ in the six well plate. Each well contained 20 drops. The instantly formed beads were left to polymerize for a further ten minutes in the $CaCl_2$ solution. The beads were then washed three times with 0.155M NaCl (Fisher Scientific). After the final wash, culture medium was added to each well and the plate was placed in a 37^oC, 5% CO_2 incubator. Media was changed every two to three days.

The size of the needle used to generate the beads and the chondrocyte seeding density were selected based on experimental work which is discussed in Chapter Four, section 4.2.2.1.

2.7.3 Varying the Culture Conditions of the Alginate Beads

Beads were set up as before and were left to settle in culture medium (DMEM supplemented with 2% antibiotics and 10% FCS) for four days at 37^{o}C and 5% CO_2. On the fourth day media was removed and treatments were added or beads were placed in different environmental conditions (Figure 2.5). Media was changed every three days and treated media was made fresh immediately before it was changed. Cultures were terminated after 35 days. The exact changes in culture conditions are detailed in the following two sections.

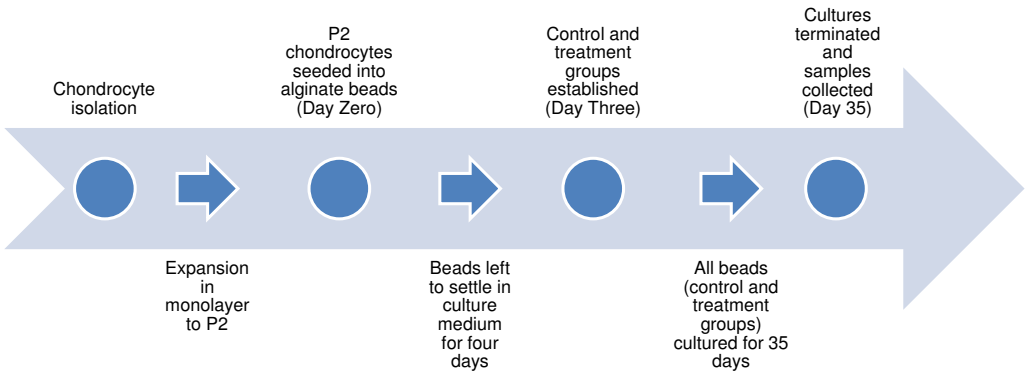


Figure 2.5: Timeline of alginate bead experiments involving changes in culture conditions. Canine articular chondrocytes were isolated and expanded in monolayer for two passages before being seeded into alginate beads. The beads were cultured in normal culture conditions (DMEM+10%FBS+2% antibiotics, 21% oxygen, 5% CO_2 and 37°C) to allow the cells to settle into the new culture environment. On the third day, a selection of beads was kept in normal culture conditions and two treatment groups were created. Some beads had ascorbate added to the culture medium whereas another selection of beads were incubated in reduced oxygen conditions. P2: passage two.

2.7.3.1 Ascorbate

A stock of 1mg/ml ascorbic acid (Sigma) was made in nanopure water and aliquots were stored at -20°C. Aliquots were thawed on ice prior to media change. Ascorbic acid was added to culture medium to a final concentration of 25µg/ml. Media was removed from the wells and

media containing ascorbate was added. Beads were cultured as before and media was changed every three days.

2.7.3.2 Environmental Conditions

Culture media was aliquotted into a sterile falcon tube and placed into an incubator set at 2.4% oxygen overnight. This enabled the media to adjust to the reduced oxygen conditions. Plates were briefly taken out of the incubator, along with the prepared media and media was changed. Plates were immediately placed back in the reduced oxygen incubator after media change. All other conditions (37^{o}C and 5% CO_2) remained the same.

2.7.3.3 Inflammatory Mediator

The inflammatory mediator IL-1β (Recombinant Equine IL-1β/IL-IF2, R&D Systems, Minneapolis, USA) was reconstituted in PBS at a concentration of 10µg/ml. This stock was aliquotted and stored at -20^{o}C. Aliquots were thawed on ice prior to media change. IL-1β was added to culture media to a final concentration of 10ng/ml. Beads were cultured as before and media was changed every three days.

2.8 Histological Staining

Fixation and processing of high density pellets and alginate beads had to be altered in order to maintain the integrity of the samples. This is discussed in detail in sections 4.2.1 (high density pellets) and 4.2.2.2 (alginate beads) in Chapter Four.

2.8.1 Haematoxylin and Eosin

Slides were placed in Histoclear (National Diagnostics, Hull, UK) for ten minutes before being placed in graded alcohol baths (100%, 95% and 70%) (Ethanol Absolute, Fisher Scientific) for two minutes each. After being washed with tap water the slides were left in Haematoxylin (BDH, VWR International, Poole, UK) for three minutes. The slides were washed again before being dipped in 1% acidic industrial methylated spirit (IMS) (IMS (Fisher Scientific) with Hydrochloric Acid (Acros Organics, Geel, Belgium) for two to four seconds. After another wash, the slides were dipped in ammoniated water (ammonia solution (35%): Fisher Scientific). The slides were then left in Eosin (MERCK, Darmstadt, Germany) for five minutes after being washed. The slides were washed for a final time before being dipped in graded alcohol solutions (70%, 95% and 100%). Before being transferred to xylene (Fisher Scientific) for five minutes, the slides were left in Histoclear for

five minutes. The slides were dipped in a second xylene bath before they were mounted with DPX mounting medium (BDH). The slides were left in the fume cupboard at room temperature for 24 hours for the mountant to set. Slides were observed under a light microscope, DM5000B fitted with digital camera DCF350FX (Leica) at x5 x10 and x20 objectives.

2.8.2 Alcian Blue

Slides were placed in Histoclear for ten minutes followed by graded alcohol baths (100%, 95% and 70%) for two minutes each. The slides were then washed under running water for two minutes before being placed in Alcian Blue (pH2.5) (Alcian Blue 8GX (Sigma) in 3% glacial acetic acid (Fisher Scientific)). Following incubation in the stain, slides were washed in water and dipped in graded alcohol baths (70%, 95% and 100%). Various concentrations and incubation times of Alcian Blue and washing times were trialled to achieve optimal staining. This work is discussed in detail in Chapter Four, section 4.2.2.3. The slides were then placed in Histoclear for five minutes and then xylene for five minutes before being mounted with DPX mounting medium. The slides were left in the fume cupboard at room temperature for 24 hours for the mountant to set. The slides were observed under a light microscope

DM5000B fitted with digital camera DCF350FX (Leica) at x5, x10 and x20 objectives.

2.8.3 Safranin O

The following method was obtained from Professor Susan Chubinskaya's group based at Rush University in Chicago. This method had been adapted specifically for alginate from (Rosenberg, 1971). Slides were placed in Xylene for 15 minutes (three separate baths for five minutes each) followed by a series of alcohol baths, 100% ethanol for six minutes (three baths; two minutes each), 95% ethanol for four minutes (two baths; two minutes each), 70% ethanol for two minutes and 50% ethanol for two minutes. The slides were then washed in RO water for 15 minutes. Following deparaffinization, the slides were placed in 0.1% fast green (Raymond A Lamb Ltd, Eastbourne, UK) for three minutes. After two dips in 1% acetic acid water, the slides were left in 0.1% Safranin O (Raymond A Lamb Ltd) for four minutes. After staining, the slides were dehydrated by placing them in graded alcohol baths (70% ethanol, one dip; 95%, three dips and 100% ethanol (two baths, two minutes each)). The slides were then placed into 50:50 ethanol : xylene for two minutes before being put in xylene for nine minutes (three separate baths, three minutes each). The slides were mounted with DPX mounting medium and left in the

fume cupboard at room temperature for 24 hours for the mountant to set. The slides were observed under a light microscope DM5000B fitted with digital camera DCF350FX (Leica) at x5, x10, x20 and x40 objectives.

2.9 1,9-dimethylmethylene blue (DMMB) Assay

2.9.1. Papain Digestion

In order to evaluate glycosaminoglycan (GAG) production in a test sample, that sample must first be digested with the enzyme papain. Papain is a cysteine protease found in papaya that cleaves peptide bonds in basic amino acids (histidine, lysine and arginine), leucine and glycine.

Alginate beads were removed from culture, placed in a six well plate and washed with PBS. Individual beads were placed into pre-weighed eppendorfs, which were re-weighed in order to calculate the wet weight of the bead. The eppendorfs were left open and parafilm (Fisher Scientific) was used to cover the top. A few holes were pierced into the parafilm and the beads were snap frozen in liquid nitrogen. The eppendorfs were then placed in the freeze drier (Alpha 1-2 LD plus,

SLS) overnight. Then the eppendorfs were re-weighed to obtain the dry weights of the beads. These samples were then stored at -80^{o}C until required.

Preparing individual beads resulted in very low quantities of sample. After a further literature search the preparation of beads for the GAG assay was altered. The following method was adapted from (Uitterlinden et al., 2007). After washing with PBS, five beads were placed in sterile eppendorfs and 1ml of 1x dissolving buffer (55mM sodium citrate (Sigma), 150mM NaCl and 30mM EDTA (BDH)) was added. Samples were vortexed for one minute. After vortexing 1ml of papain solution (papain from papaya latex, Sigma) was added and tubes were placed in a waterbath set at 60^{o}C overnight. In order to prevent evaporation, lids were closed tightly and wrapped in parafilm. Any solution not used immediately was stored at -80^{o}C. Stocks of papain solution were made in advance, aliquotted and kept at -80^{o}C. To make the papain solution (concentration: 1.06mg/ml), 0.0264g of papain was dissolved in 25ml of papain buffer (0.1M dibasic sodium phosphate (Sigma)), 0.005M cysteine hydrochloride (Sigma) and 0.005M EDTA (Sigma), pH6.5). For papain buffer preparation see Appendix B.

2.9.2 DMMB Assay

Firstly the DMMB solution (pH1.5) was prepared (see Appendix B for recipe). This solution was stored at room temperature in a foil-wrapped bottle. Then the standards were prepared. A stock solution of chondroitin-4-sulphate (chondroitin sulphate sodium salt from shark cartilage, Sigma) was prepared by dissolving 100mg in RO water to give a concentration of 1mg/ml. This was then diluted 1:10 with blank papain solution to create a working solution with a concentration of 100µg/ml. The working solution was further diluted with blank papain solution to generate a range of concentrations for the standard curve (0, 5, 10, 15, 20, 25, 30, 40, 50 and 75 µg/ml). Blank papain solution was added to a 96 well plate, followed by the standards and then the test samples (the volume of all solutions added to the 96 well plate was 40µl). All solutions were loaded in triplicate. DMMB solution was added to all wells (200µl) and the plate was read in a LT-4000 Microplate Reader at 540nm within ten minutes of adding the DMMB solution.

2.10 Immunofluorescence

Cells were seeded into 96 well plates (Corning, supplied by Fisher Scientific) at a density of 10000 cells/well and were left to adhere

overnight in a 37°C / 5% CO_2 incubator. Once adhered, media was removed from the wells and each well was rinsed with PBS. Cells were then fixed with ice-cold methanol (Fisher Scientific) for ten minutes. In the initial run, 4% paraformaldehyde (Sigma) fixation was compared with methanol fixation. Following this experiment, methanol was used for fixation as it enabled more defined staining (see Figure 2.6). After fixation cells were washed twice with PBS. After the final wash, fresh PBS was added to each well and plates were stored in the fridge (at 4°C) until required. Plates were sealed with parafilm to help prevent them drying out.

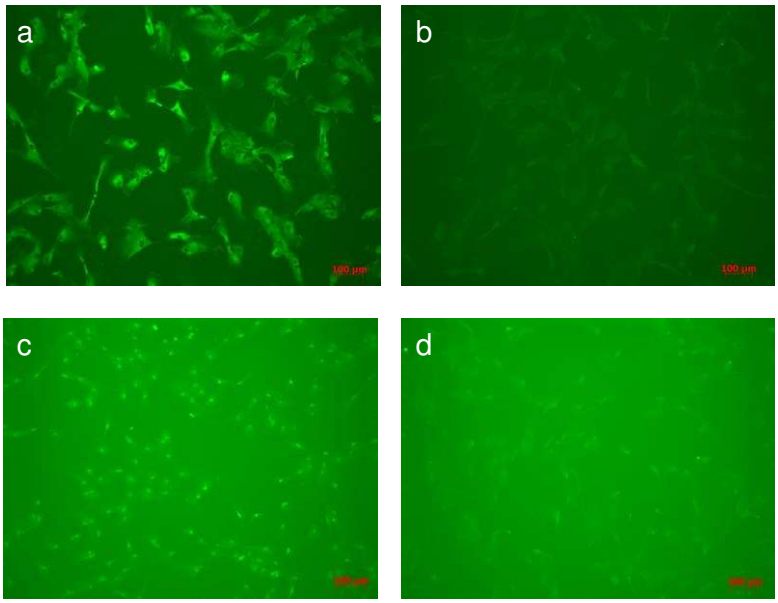


Figure 2.6: Comparison of methanol and paraformaldehyde fixation. Chondrocytes were seeded into 96 well plates and after being left to adhere were fixed with ice-cold methanol (a) and (b) or 4% paraformaldehyde (c) and (d) for ten minutes. (a) and (c) Cells were evaluated for the expression of collagen type I using the Aviva antibody COL1A2 at a concentration of 1µg/ml. (b) and (d) are the corresponding isotype controls. The staining appears to be masked in the cells fixed with paraformaldehyde. Magnification: x100, scale bar 100µm.

When ready for staining, plates were taken out the fridge and the PBS was removed. Cells were then incubated with 0.1% Triton X-100 (Sigma) in PBS for ten minutes at room temperature, which permeabilizes the cells. The cells were then washed three times with PBS (five minutes each time). In order to block non-specific binding of the primary antibody, cells were incubated with 3% bovine serum albumin (BSA) (Fisher Scientific) for one hour at room temperature. Following this, the blocking solution was removed and the appropriate primary antibody (diluted in 3% BSA) was added to each well. Cells

were incubated with the primary antibody overnight at 4°C. The primary antibody was removed and cells were washed three times with PBS (five minutes each time). Cells were then incubated with the secondary antibody (also diluted in 3% BSA) for one hour at room temperature, in the dark. The secondary antibody was removed and cells were washed three times with PBS (five minutes each time). Plates were wrapped in aluminium foil and placed in the fridge until imaging. Plates were imaged using the Axiovert 200 Inverted Fluorescence Microscope System at the Waltham Centre for Pet Nutrition.

2.11 Immunohistochemistry

2.11.1 Preparation of Positive Control Tissues and Test Samples

Canine articular cartilage was used as a positive control tissue for the antigen collagen type II as it is a major component of the extracellular matrix in this tissue. It was also used as a positive control for CD44. Articular cartilage shavings were harvested as described in section 2.3.1 and placed immediately in 10% neutral buffered formalin (NBF) (Neutral Buffered Formaldehyde Concentrate, Surgipath Ltd, Peterborough, UK) for 24 hours at room temperature. Following fixation, the shavings were stored in 70% ethanol until processing. Shavings

were then placed in cassettes in the tissue processor and were processed for 17 hours (see Appendix C).

After the tissue was processed, it was embedded in paraffin wax. The embedded tissue was kept at room temperature until sectioning. The tissue was cut into 5µm sections using a microtome. Sections were straightened using 30% ethanol followed by floating in a water bath heated to 42^{o}C. Sections were then placed on polysine slides (Fisher Scientific) which were left to dry on a hotplate for one hour at 40^{o}C. To fully bake the sections onto the slides, slides were left in an oven set to 50^{o}C overnight. Slides were stored at room temperature until staining.

Canine tendon tissue was used as a positive control for collagen type I detection as it is an abundant extracellular matrix protein in this tissue. Canine forelimbs were prepared for cartilage harvest as described previously. Before cutting into the joint, tendons were removed and placed in collection media. Following this, tendons were washed with sterile PBS. Tendons were cut into small sections and placed in 1.5ml eppendorf tubes. Tubes containing tendon tissue were immersed into liquid nitrogen to snap freeze the tissue. Snap frozen tissue was stored at -80^{o}C until required.

In order to prepare the tissue for sectioning, an eppendorf containing tendon segments was placed on the cryobar of the cryotome (set to -60^{o}C). Plastic moulds were coated with Vaseline before being lined with a small volume of OCT mounting medium (Raymond A Lamb Ltd). Moulds were placed in the cryotome and as the OCT began to set tendon sections were inserted vertically into the mounting medium. The moulds were left in the cryotome until the OCT had completely frozen. Following this, the remaining exposed tendon was covered with OCT and blocks were left in the cryotome until the OCT had completely frozen. The blocks were removed from the plastic mould and stored at -20^{o}C until sectioning. The tissue was cut into 5µm sections using a cryotome and placed on polysine slides. Slides were stored at -20^{o}C until staining.

The processing of the alginate bead test samples had to be optimised; this optimisation is discussed in Chapter Four, section 4.2.2.2. Briefly, beads were processed manually before being embedded in paraffin wax. Bead samples were sectioned as described for the cartilage tissue sections.

2.11.2 Detecting Collagen Types I and II and CD44 Expression

For samples embedded in paraffin wax, the slides need to be dewaxed and rehydrated before any staining protocol can be applied. Slides were placed in xylene twice for five minutes each before being placed in graded alcohol baths (100%, 95% and 70%) for five minutes each. Slides were washed in PBS for five minutes, following a five minute wash in RO water.

Tissue embedded in OCT requires fixation after sectioning. Slides were placed in ice cold methanol for ten minutes before being washed twice with PBS for five minutes each time. These slides do not require dewaxing and rehydration, so after the final PBS wash the same staining protocol as used for the wax embedded sections was followed.

Slides were blotted dry and a circle was drawn around each tissue or bead section with a paraffin pen (liquid blocker super PAP pen, Sigma). This helps retain any solutions on the slide and also limits the total volume of each solution used as only enough to cover the section is required. It is at this point that the slides are transferred to a humidity chamber. This helps to keep the slides moist and prevent them from drying out. Slides were then incubated for ten minutes at room

temperature with 3% H_2O_2 (Fisher Scientific) to block endogenous peroxidase activity. After being washed with PBS twice (five minutes each time) sections were incubated with avidin solution (Avidin and Biotin Blocking Kit, Vector Laboratories, California, USA) for 15 minutes at room temperature. All washing steps were performed in troughs as opposed to pipetting PBS over the slides in the humidity chamber. This was done to further reduce any potential background staining. Slides were briefly washed with PBS before being incubated with biotin solution (Avidin and Biotin Blocking Kit, Vector Laboratories) for 15 minutes at room temperature. The avidin and biotin solutions are added to block any endogenous biotin, biotin receptors and avidin binding sites present in the tissue. Following another brief wash with PBS slides were incubated with 2.5% normal horse serum (diluted in PBS) (Vectastain ABC Kit Universal, Vector Laboratories, Peterborough, UK) for 20 minutes at room temperature. The serum is used to block non-specific binding of the primary antibody. Excess serum was blotted off the slide and the appropriate primary antibody, diluted in 2.5% horse serum, was added to each section. Slides were incubated with the primary antibody inside the humidity chamber at 4^{o}C overnight.

Following the overnight incubation, slides were washed three times (ten minutes each time) with PBS containing 0.1% Tween20 (Tween20, Fisher Scientific). After the final wash slides were placed back in the humidity chamber and incubated with biotinylated universal (anti-mouse

IgG / rabbit IgG) secondary antibody (Vectastain ABC Kit Universal, Vector Laboratories) for 30 minutes at room temperature. Whilst incubating, the AB complex (Vectastain ABC Kit Standard, Vector Laboratories, California, USA) was prepared as it has to be left at least 30 minutes before use. After incubating with the secondary antibody, the slides were washed for ten minutes with PBS containing 0.1% Tween20 and for a further ten minutes in PBS alone. The AB complex was then added to the sections and slides were incubated with this solution for 30 minutes at room temperature inside the humidity chamber. Slides were then washed two times with PBS (five minutes each time). After washing, diaminobenzidine (DAB) substrate (Peroxidase Substrate Kit, Vector Laboratories, California, USA) was added to the sections. With each different sample DAB was added to the slide and the change in colour was monitored under a light microscope. The incubation times varied from three minutes to ten minutes for different samples. Incubation times for negative controls were matched to the time required for the corresponding test section. Slides were then washed in running tap water for five minutes, before being counterstained with haematoxylin. Slides were placed in haematoxylin for 15-30 seconds before being rinsed in tap water until the water ran clear. Slides were then dipped twice in 1% acidified IMS, rinsed in water, dipped twice in ammoniated water and rinsed again in water.

The standard protocol following this final rinse in water is to dip the slides in graded alcohol baths (70%, 95% and 100%) before placing them in two xylene baths (five minutes each). This prepares the slides for mounting with DPX mountant. However, this process caused sections to lift off of the slides. An alternative method for preparing slides for mounting is to let them dry out completely. Therefore, after the final rinsing step, slides were placed in an oven set to 30^{o}C for a minimum period of one day to allow the slides to dry out. Once completely dry, slides were mounted with DPX mountant. Before observing the slides under the microscope they were left overnight (in the fume hood at room temperature) to allow the mountant to set. Slides were observed under a light microscope Nikon 50i at x10, x20 and x40 objectives and images were captured using the DS-2M/5M/Fi1 camera with DS-U2/L2 controller and the EclipseNet software. For each tissue and sample three sections were tested. Multiple images were captured at several objectives for each tissue and sample and representative images are presented in this thesis.

2.12 Western Blotting

2.12.1 Selection and Preparation of Positive Controls

For the antigens collagen type II and I the positive control tissues selected were canine articular cartilage and canine tendon respectively. These tissues were harvested as previously described. After harvest, cartilage shavings were washed in PBS and weighed. Pre-weighed cartilage was placed in a mortar which was pre-cooled using liquid nitrogen and crushed using a pre-cooled pestle. Crushed cartilage was placed in an eppendorf and sample buffer containing protease inhibitors (Protease Inhibitor Cocktail, Sigma-Aldrich) was added (111mg cartilage, 100µl sample buffer and 4µl protease inhibitors per eppendorf). The recipe for sample buffer is given in Appendix B. This solution was boiled for five minutes before being centrifuged at 10000xg for ten minutes. The supernatant was split into aliquots which were subsequently stored at -80^{o}C.

Tendon tissue was harvested and washed as previously described. After washing tendons were cut into chunks and weighed (110mg of tendon tissue was placed in each eppendorf). Tendon sections were placed in eppendorfs and snap frozen in liquid nitrogen. Pre-weighed tendon tissue was placed in a mortar which was pre-cooled using liquid

nitrogen and crushed using a pre-cooled pestle. Crushed tendon was placed in an eppendorf containing sample buffer (1ml) and protease inhibitors (40µl). Samples were heated to 95^{o}C for five minutes before being centrifuged at 10 000xg for ten minutes. The supernatant was aliquotted into eppendorfs and stored at -80^{o}C.

For the antigens CD44 and Sox9, canine spleen and canine testis tissue lysates were used respectively. Canine spleen and testis tissue was removed from a cadaver during post-mortem and placed in a -80^{o}C freezer until collection. Tissue was collected from the Veterinary Laboratories Agency, Sutton Bonington, transported on ice and placed immediately in the -80^{o}C freezer until processing.

To generate tissue lysates, testis and spleen tissue were homogenised in a TissueLyser II (Qiagen GmbH, Hilden, Germany). For the homogeniser to work effectively, a maximum of 25mg of tissue should be placed in an eppendorf along with a ball bearing. This is reduced to 10mg for spleen tissue due to its high cell density. Each tissue was weighed before being placed in an eppendorf containing a ball bearing. RIPA buffer (see Appendix B for recipe) containing protease inhibitors was also added to the tube (625µl RIPA buffer + 25µl protease inhibitors per 25mg of testis and 250µl RIPA buffer + 10µl protease inhibitors per 10mg of spleen tissue). Samples were placed in the

homogeniser for two runs (three minutes each) before being put on ice for 30 minutes. Lysates were then centrifuged at 10000xg at 4°C until all the tissue fragments had sedimented into the bottom of the tube. The supernatant was split into sterile eppendorfs and stored at -80°C.

2.12.2 Preparation of Cellular Samples

For cell monolayer lysates, all media was removed from the flasks and flasks were rinsed three times with sterile PBS. Flasks were washed thoroughly to remove as many serum proteins as possible. RIPA buffer containing protease inhibitors was added to the flasks (0.5ml RIPA buffer + 20µl protease inhibitors for a T25, 1ml RIPA buffer + 40µl protease inhibitors for a T75 and 2ml RIPA buffer + 80µl protease inhibitors for a T175), which were incubated with this solution, on ice, for 30 minutes. All further procedures except centrifugation were performed on ice. Cells were scraped off of the flask and the cell suspension was pipetted into a dounce glass homogeniser. Cells were manually sheared 30 times using this homogeniser. The solution containing the lysed cells was then transferred to eppendorfs and centrifuged at 10000xg at 4°C. The supernatant was removed, placed in a sterile eppendorf and stored at -80°C. Samples were aliquotted after the protein concentration was determined (see section 2.12.3). Each tube contained a volume equating to 25µg of protein. This was

done to minimise the number of freeze-thawing steps prior to using the sample on a western blot.

Alginate beads were removed from six well plates and placed in a sterile petri dish. Beads were washed briefly with sterile PBS to remove any remaining media. Five beads were placed in each eppendorf containing dissolving buffer (55mM sodium citrate, 150mM NaCl and 30mM EDTA). Samples were vortexed for one minute. All aliquots were then placed in a falcon tube and centrifuged at 200xg for ten minutes. The supernatant was pipetted into a fresh falcon tube and stored at -80^{o}C. The cell pellet was resuspended in 0.5ml RIPA buffer and 20µl protease inhibitors and incubated on ice for 30 minutes. The cell solution was then transferred to a dounce homogeniser and cells were manually sheared, on ice, 30 times. The solution containing the lysed cells was then transferred to an eppendorf and centrifuged at 10000xg at 4^{o}C. The supernatant was removed, placed in a sterile eppendorf and stored at -80^{o}C. Samples were aliquotted following determination of the protein concentration; each aliquot represented 25µg of protein.

2.12.3 Determining Protein Concentration

The Pierce bicinchoninic acid (BCA) protein assay kit (Thermo Scientific) was used to determine the protein concentration of all samples, except the cartilage and tendon lysates, which were loaded onto gels volumetrically. These latter two samples were prepared in sample buffer which would interfere with the protein assay.

This assay works by detecting the reduction of Cu^{2+} to Cu^{1+} by protein. In particular, the macromolecular structure of the protein, the amino acids, cysteine, tryptophan and tyrosine and the number of peptide bonds are said to be involved in the reaction being detected by this assay. The change in colour from green to purple is caused by BCA molecules binding to cuprous cations (Cu^{1+}) at a ratio of 2:1.

For the standard curve, BSA was diluted in RO water at a range of concentrations, 1500µg/ml, 1000µg/ml, 750µg/ml, 500µg/ml and 250µg/ml. Protein assays were performed in 96 well plates (Thermo Scientific) and each sample was loaded in triplicate. Blank wells containing RO water only and RIPA buffer only were loaded first, followed by the BSA standards and then each sample. 10µl of blanks, standards and test samples were loaded. 200µl of working solution was then added to each well. Working solution was prepared by mixing 50

parts of Reagent A (contains sodium carbonate, sodium bicarbonate, BCA and sodium tartrate in 0.1M sodium hydroxide) to 1 part Reagent B (contains 4% cupric sulphate). Plates were then wrapped in aluminium foil and placed in a 37^{o}C incubator for 30 minutes. Absorbance was measured at 540nm and protein concentration was calculated using the Multiskan Ascent V1.24 Plate reader and software.

Occasionally the original sample was either too concentrated or dilute to be measured. If the sample was too concentrated, it was further diluted in RO water and retested as above. Samples that were too dilute were placed back the -80^{o}C freezer to re-freeze. Once frozen, samples were placed in a freeze drier (Alpha 1-2 LD plus, SLS) until completely dry (this varied depending on the starting volume of the sample). The protein lysate was then resuspended in a smaller volume of RO water and the concentration was measured again following the same method as before.

2.12.4 Preparation of SDS-PAGE Gels

Glass plates were wiped with weak HCl before being sprayed with 70% IMS to clean them. Spacers were also cleaned with 70% IMS prior to use. Pairs of plates and spacers were slid into the plate holder and screwed into place. The bottoms of the plates were then wiped with

Vaseline, in order to prevent leakage. The sealed plates were clipped into the gel casting stand and water was pipetted between the plates to check for leakage. The water was removed and resolving gel (10%) was added (see table 2.3 for the components of the gel). 50% isopropanol was then pipetted on to the top of the gel to straighten it. Gels were left for 15 minutes to set at room temperature. Once set, the isopropanol was removed and the top of the gel was rinsed with RO water. Stacking gel (4%) (see table 2.3 for gel components) was then pipetted on top of the resolving gel and a comb inserted. This was left to set at room temperature for 45 minutes. Once the gel had set, the plate holder was unclipped from the gel casting stand and clicked into the gel cassette, which was then placed in the tank. The tank was then filled with running buffer (for the recipe, see Appendix B). If gels were made in advance, the tank containing the gels were wrapped in cling film and kept in the fridge for a maximum of 48 hours.

Resolving Gel	Stacking Gel	Supplier
Tris pH8.8 1.5M	Tris pH6.8 0.625M	Tris Base – Fisher Scientific
30% Acrylamide	30% Acrylamide	Geneflow, Staffordshire, UK
Sodium Dodecyl Sulphate	Sodium Dodecyl Sulphate	Sigma
RO water	RO water	N/A
TEMED	TEMED	Sigma
Ammonium Persulphate	Ammonium Persulphate	Sigma

Table 2.3: The components of an SDS-PAGE gel. Where appropriate the supplier information has been given. Exact quantities of each chemical are given in Appendix B.

2.12.5 Gel Electrophoresis

Samples were removed from the -80^{o}C freezer and thawed on ice. Gels were taken out of the fridge (if made in advance) and the combs were removed. Sample buffer (for recipe see Appendix B) was prepared as a 5x stock and aliquots were kept at -20^{o}C. Sample buffer was thawed at room temperature. Once samples had thawed, aliquots corresponding to 25µg of protein were pipetted into sterile eppendorfs and sample buffer was added (the final concentration of sample buffer in each tube was 1x). Volumetrically loaded samples (already containing sample buffer) were aliquotted into sterile eppendorfs. All

samples were heated to 95°C for five minutes in a heat block before being centrifuged at 295xg for two minutes. This centrifugation aimed to draw all of the sample back down into the bottom of the tube. Before loading samples onto the gel 3µl of a molecular weight marker (Precision Plus Protein Standards Kaleidoscope, Bio-Rad) was loaded into the first lane. This was followed by the positive control and then the test samples. The gel was routinely run for 1 hour at 150V, however the molecular weight marker was monitored during this time and on some occasions the gel need slightly longer to run.

2.12.6 Blotting

Whilst gels were running, the PVDF membrane (Bio-Rad, Hertfordshire, UK) and filter paper (Fisher Scientific) were cut to size and the membrane activated. In order for the proteins on the gel to bind to the membrane during transfer, the membrane needs to be activated. Membranes were activated by being placed in 100% methanol on a shaker for ten minutes at room temperature. Following activation, membranes were washed in RO water and then placed in transfer buffer until needed. Once cut to size, filter paper was soaked in transfer buffer; sponges in the transfer cassette were also soaked in transfer buffer.

Once the gels had stopped running, running buffer was removed and the tank containing the gels was rinsed twice with RO water. Each gel was left in the cassette until the transfer cassette was prepared. Firstly, the transfer cassette was placed with the black plate at the bottom. A soaked sponge was placed on top, followed by three sheets of soaked filter paper. The gel was then removed from the cassette and the stacking gel was cut off. The remaining gel was rinsed in transfer buffer before being reversed and placed on the filter paper. It is important to keep everything in the transfer cassette moist, so transfer buffer was added when necessary. The activated membrane was then placed on top of the gel, followed by three sheets of pre-soaked filter paper. Finally, a second piece of soaked sponge was placed on top. Any air bubbles were rolled out using a pipette. Cassettes were then clipped shut and placed inside the transfer tank. Once all cassettes were prepared (maximum of four in one tank), the tank was filled with transfer buffer. Transfer was set to 100V for two hours at room temperature.

After transfer, membranes were placed in 5% milk (Marvel, Dublin, Ireland) in TBS-Tween (the recipe for TBS-Tween is in the Appendix B) for two hours at room temperature on a shaker. This was to block non-specific binding of the primary antibodies.

2.12.7 Coomassie Blue and Ponceau S

After transfer, gels were placed in Coomassie Blue stain (see Appendix B for full recipe) on a rocker overnight. Gels were then placed in destaining buffer (see Appendix B for recipe) overnight. Following destaining, gels were rinsed with RO water and then stored in RO water at room temperature until imaging. Gels were scanned using the ImageQuant 300 machine. This was to check the transfer of the proteins from the gel to the membrane. Some gels were also stained prior to transfer, (see figure 2.7 to see the difference before and after transfer).

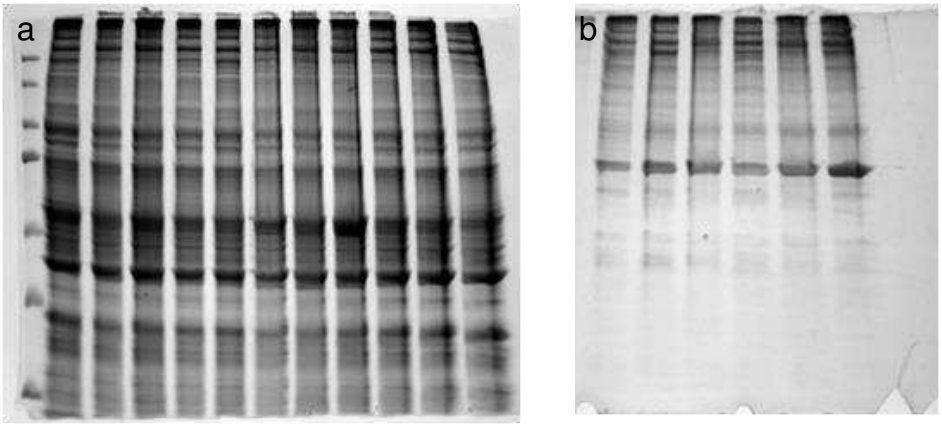


Figure 2.7: SDS-PAGE gels stained with Coomassie Blue before and after transfer. (a) Before transfer (b) After transfer. Some of the higher molecular weight proteins and more abundant proteins are left on in the gel after transfer.

Another way to assess transfer is to stain the membrane with Ponceau S. Membranes were incubated with Ponceau S stain (0.1% Ponceau S (Sigma) in 5% acetic acid) for ten minutes at room temperature on a shaker. After staining, membranes were washed in RO water until the background was clear. Membranes were photographed using a Sony Cyber-shot DSC-W30 digital camera.

2.12.8 Primary and Secondary Antibody Incubation

Following incubation with block, membranes were incubated with primary antibody, diluted in 5% milk in TBS-Tween, overnight at 4^{o}C on a roller. Membranes were then washed five times with TBS-Tween (five minutes each) before being incubated with the secondary antibody (diluted in 5% milk in TBS-Tween) for one hour at room temperature on a roller. After this incubation, membranes were washed as stated before.

2.12.9 Detection

Following the final wash, membranes were incubated with chemiluminescence solution (Biological Industries, Haemek, Israel) for five minutes at room temperature. Excess chemiluminescence solution

was removed and the membranes were placed in a plastic wallet in an x-ray cassette. The plastic sheets were wiped to further remove any excess chemiluminescence solution. All further steps were performed in the dark. X-ray films (CL-Xposure Film, Thermo-Scientific and Amersham Hyperfilm ECL, SLS) were placed on top of the membranes and the cassette was closed. Membranes were exposed to films between one second and ten minutes. After the appropriate exposure time had elapsed, films were removed from the cassette and placed into the X2 auto-red film developer. Films were scanned using the ImageQuant 300 machine. Films to which densitometric analysis was applied were scanned using a GS-800 Calibrated Densitometer. Densitometric analysis was performed using ImageJ and proteins of interest were normalised to expression of the housekeeping gene β-actin.

2.13 Statistical Analysis

Statistical analysis was performed using the software GraphPad Prism 5. One way ANOVA with Tukey's multiple comparison post-test was applied to data with a p value of <0.05 being significant.

3. Chondrocyte Dedifferentiation in Monolayer Culture

3.1 Background

In chondrocyte biology, dedifferentiation usually refers to loss of the chondrocytic phenotype and adoption of a more fibroblastic phenotype. An early study into the loss of the chondrocyte phenotype *in vitro* was conducted by Holtzer and colleagues (Holtzer et al., 1960). The authors sought to determine whether the differentiated state of a cell would be retained after several divisions *in vitro* (Holtzer et al., 1960). The authors claim that cells isolated from ten-day chick embryos had a reduced ability to retain the differentiated phenotype in pellet culture with increased monolayer culture (Holtzer et al., 1960).

Articular cartilage is very poor at repairing itself and as such cell and tissue engineering strategies are being extensively explored to repair damaged cartilage. Autologous Chondrocyte Implantation (ACI) is a technique favoured forrepairing articular cartilage defects larger than $4cm^2$ (Schulze-Tanzil, 2009, Oldershaw, 2012). ACI has undergone several modifications since its inception in 1987 (Ringe and Sittinger, 2009, Schulze-Tanzil, 2009, Brittberg, 2010, Zeifang et al., 2010,

Oldershaw, 2012), but all forms of the ACI method involve isolation and expansion of chondrocytes in monolayer culture before being placed into the defect (Brittberg, 2008, Brittberg, 2010, Zeifang et al., 2010, Oldershaw, 2012). A major problem with the large cell requirements for these techniques is that the cells begin to lose their phenotype during *in vitro* expansion. Despite the advances in ACI technology, treatment of cartilage lesions are still a major challenge for orthopaedic surgeons, particularly when there are added complications, such as osteoarthritis (Filardo et al., 2013).

Loss of the chondrocyte phenotype whilst undergoing *in vitro* expansion has been reported in many species including, humans (Archer et al., 1990, Schnabel et al., 2002, Goessler et al., 2006, Yang et al., 2006b, Tew et al., 2008), goats (Darling and Athanasiou, 2005), cows (Brodkin et al., 2004, Shao et al., 2013), pigs (Watt, 1988) and rabbits (Malemud and Papay, 1984, Baici et al., 1988). However, all these studies have differed in their approach to chondrocyte expansion, their assessment of dedifferentiation and how this is information is reported.

One difference is the age of the animals used in the studies. In the human studies for example, the average age ranges from 29.4 years (Hamada et al., 2013) to 84 years (Schnabel et al., 2002). Both of these papers aimed to study the phenotypes of chondrocytes in culture

with the latter looking to identify markers to distinguish dedifferentiated cells. Some studies have investigated differences in chondrocytes with age. Guerne *et al* (1995) demonstrated that DNA synthesis in human chondrocytes decrease with age (samples taken from humans aged between late twenties and early eighties; the exact ages are not given). A more recent study investigated how ageing affects human chondrocyte yield, proliferation, chondrogenic capacity following expansion and response to growth factors during culture (Barbero et al., 2004). The authors claim that cell viability was unaffected by age but the cell yield did decrease with age. Proliferation of chondrocytes was similar across all age groups (twenties, thirties, forties, fifties, sixties, over seventy). Proliferation was enhanced in all groups cultured with growth factors (TGFβ, FGF-2 and PDGF-BB). GAG synthesis was similar across all ages although a reduction with age was seen in cultures with growth factors (Barbero et al., 2004). Another research group states that the population doublings of rabbit chondrocytes decrease with age, quoting 11-12 doublings for four week old rabbits, 8-10 for six month old rabbits and 3-4 doublings for three and a half year old rabbits (Evans and Georgescu, 1983). This group also made the observation that canine chondrocytes are smaller than those from human and rabbit cartilage and that they maintain their morphology in culture longer than rabbit chondrocytes (Evans and Georgescu, 1983). A different group showed that the number of cells present in cartilage was higher in younger rabbits (1-2 months) compared to older rabbits (four years) (Adolphe et al., 1983). They also found that chondrocytes

in the latter group had a larger volume. Cell population growth was shown to be faster with younger chondrocytes (Adolphe et al., 1983). A more recent paper looked at the transcriptome of equine chondrocytes from young (four years) and old (>15 years) horses (Peffers et al., 2013). A variety of genes were shown to be differentially expressed in the two groups with collagen type II alpha 1, biglycan and hyaluronan synthase 3 expression lower in older chondrocytes (Peffers et al., 2013). In some literature, the ages of the animals from which the cartilage is harvested is not given (Watt, 1988, Goessler et al., 2006, Tew et al., 2008, Shao et al., 2013). Although it must be acknowledged that in some cases, particularly when working with non-human species, it is not possible to obtain accurate background information about samples. For example, the pig tissue used by Watt and colleagues (1988) was obtained from a butcher. Taking all this into consideration, there may be differences in chondrocyte phenotype which are attributable to age of the animal from which tissue was harvested; however it is not always possible for accurate ages to be obtained. This was sometimes the case with the canine material used in this thesis (see Appendix A for animal details).

In addition to this, the information provided in terms of seeding chondrocytes into a culture vessel differs considerably between publications. Some papers don't list any cell numbers (Malemud and Papay, 1984, Hamada et al., 2013, Shao et al., 2013), some give cell

densities (Schnabel et al., 2002, Darling and Athanasiou, 2005, Yang et al., 2006b, Tew et al., 2008, Nadzir et al., 2011) and others state the total number of cells per vessel (Baici et al., 1988, Watt, 1988, Archer et al., 1990). The exact cell seeding information given in a range of papers, covering a range of species is given in Table 3.1. One of the papers listed in Table 3.1 gives two cell densities (Watt, 1988). This is because the purpose of the work was to assess the affect of cell density on the phenotype of the cultured chondrocytes (Watt, 1988). Collagen type I and II expression was detected in cultures at both densities but staining was stronger and more widespread in high density cultures particularly with collagen type II (Watt, 1988). This would suggest that seeding density has an impact on chondrocyte phenotype and therefore should be a consideration when expanding chondrocytes in monolayer culture. Another paper detailing a protocol for culturing immature murine articular chondrocytes provides recommended seeding densities for various tissue culture plates in order to limit dedifferentiation (Gosset et al., 2008). This again suggests seeding density plays an important role in the phenotype of chondrocytes in culture and should therefore be documented.

Another aspect of the chondrocyte expansion process that differs in the literature is passaging. In some publications, culture time is only reported in terms of the numbers of days or hours with no indication of the numbers of passages undertaken during that time. One research

group refer to low, medium and high density cultures but this only refers to the number of days, rather than cell or passage number (Malemud and Papay, 1984). When passage details are given, there is a considerable difference between the number of passages observed. Some groups have expanded the cells to passage two (P2) (Schnabel et al., 2002, Yang et al., 2006b) or passage three (P3) (Munirah et al., 2010); others have left the culture for slightly longer up to passage four (P4) (Baici et al., 1988, Darling and Athanasiou, 2005, Hamada et al., 2013) and passage five (P5) (Shao et al., 2013). One group looked at human chondrocytes expanded in monolayer up to passage 17 (P17) (Tew et al., 2008). The authors of this paper state that confluence took ten days but provide no further information on the exact level of confluence at each passage. Other researchers do give more detailed information in terms of confluence at passage; two papers quote 80% confluence at passage for rabbit (Nadzir et al., 2011) and human (Hamada et al., 2013) chondrocytes, whereas in another paper the cell density prior to passage is given ($5x10^4$ cells/cm^2) (Darling and Athanasiou, 2005). It should also be acknowledged that passage is a subjective term and that some researchers believe population doublings are a more accurate way of monitoring cells in culture (Greenwood et al., 2004). One group aiming to identify a measurable parameter for chondrogenic capacity of monolayer-expanded chondrocytes provided an initial seeding density (20000 cells/cm^2), subsequent densities (10000 cells/cm^2), confluence at passage (80-90%) and population doublings (Giovannini et al., 2010). Another research group assessed

population doubling limit of human chondrocytes cultured in 5% and 21% oxygen (Moussavi-Harami et al., 2004). They showed that the limit was enhanced in the lower oxygen group (60-70 doublings) compared to the 21% oxygen group (30-40) (Moussavi-Harami et al., 2004). Considering chondrocytes *in vivo* are exposed to relatively low oxygen tensions and do not proliferate, it is perhaps surprising that a higher population doubling limit was observed at the lower oxygen tension.

Species	**Seeding Information**	**Reference**
Human	$7.9x10^4$ cells/cm^2	(Archer et al., 1990)
	$1.5x10^4$ cells/cm^2	(Schnabel et al., 2002)
	$5.0x10^3$ cells/cm^2	(Yang et al., 2006b)
	$2.0x10^4$ cells/cm^2	(Tew et al., 2008)
Goat	$1.6x10^4$ cells/cm^2	(Darling and Athanasiou, 2005)
Pig	$1.04x10^4$ cells/cm^2*	(Watt, 1988)
	$1.04x10^2$ cells/cm^2**	
Rabbit	$5.24x10^4$ cells/cm^2	(Baici et al., 1988)
	$1.0x10^4$ cells/cm^2	(Nadzir et al., 2011)

Table 3.1: Seeding details given in different studies for chondrocytes from various species. How chondrocyte seeding is described varies between papers and the numbers of cells also differs between studies. * and ** classified as low density and high density respectively in (Watt, 1988).

In addition to differences in culture technique, there are also differences in the proteins studied to determine the phenotype of chondrocytes cultured *in vitro*. Tables 1.4 and 1.5, Chapter 1, show various markers which have been shown to be upregulated (1.4) and downregulated (1.5) in monolayer-expanded chondrocytes.

Out of the proteins listed in the Tables 1.4 and 1.5, four (collagen types I and II, Sox9 and CD44) were chosen to assess the phenotype of canine chondrocytes expanded in monolayer. The reasons behind the selection of these proteins are given in section 1.5.3, Chapter 1.

Although it is known that chondrocytes dedifferentiate during monolayer expansion, the nature of how this change in phenotype is observed varies considerably in the literature. The aim and subsequent hypotheses of this chapter were as follows:

Aim: To examine the phenotype of canine articular chondrocytes expanded to P5 in monolayer and confirm dedifferentiation.

Hypothesis One: Canine chondrocytes will alter in morphology with increasing passage, adopting a more fibroblastic phenotype.

Hypothesis Two: Expression of collagen type II and Sox9 will decrease with increasing passage whereas collagen type I and CD44 will increase.

In addition to this, due to the limited number of antibodies available that are already known to cross-react with canine antigens, antibody evaluation and validation was also expected to be conducted.

3.2 Results

3.2.1 Change in Chondrocyte Morphology with Increasing Passage

To assess the change in morphology of freshly isolated chondrocytes, the cells were expanded in monolayer until P5. The original plan was to seed cells into flasks at a known density of 8,000 cells/cm^2 (200,000 cells in total per flask) for P0 and 20,000 cells/cm^2 (500,000 cells in total per flask) for later passages (Figure 3.1). These figures were based on previous cell counts obtained. From two previous canine samples 600,000 and 604,000 chondrocytes were freshly isolated from cartilage shavings. On both occasions, these cells were split into three T25 flasks, at densities of 8,000 cells/cm^2 and 8,053 cells/cm^2 respectively.

For these same cells, flasks were pooled at P1 to be seeded into larger (T75) flasks. The three T25 flasks seeded at a density of 8,000 cells/cm^2 generated a total of 13.95x10^6 cells and two flasks seeded at a density of 8,053 cells/cm^2 generated a total of 3.54x10^6 cells. Taking into consideration the difference in cell numbers obtained and the smaller flask size in the plan a seeding density of 20,000 cells/cm^2 was deemed appropriate.

Another part of the monolayer expansion plan was to keep the time of passage consistent, some groups have controlled passage by time point (cells were passaged every seven days (Lee et al., 2003)). However it was deemed more appropriate to assess the level of confluence prior to passaging. It was decided that cells should be passaged once they had reached 90-95% confluence; this would generate a relatively high cell yield without the cells becoming over-confluent.

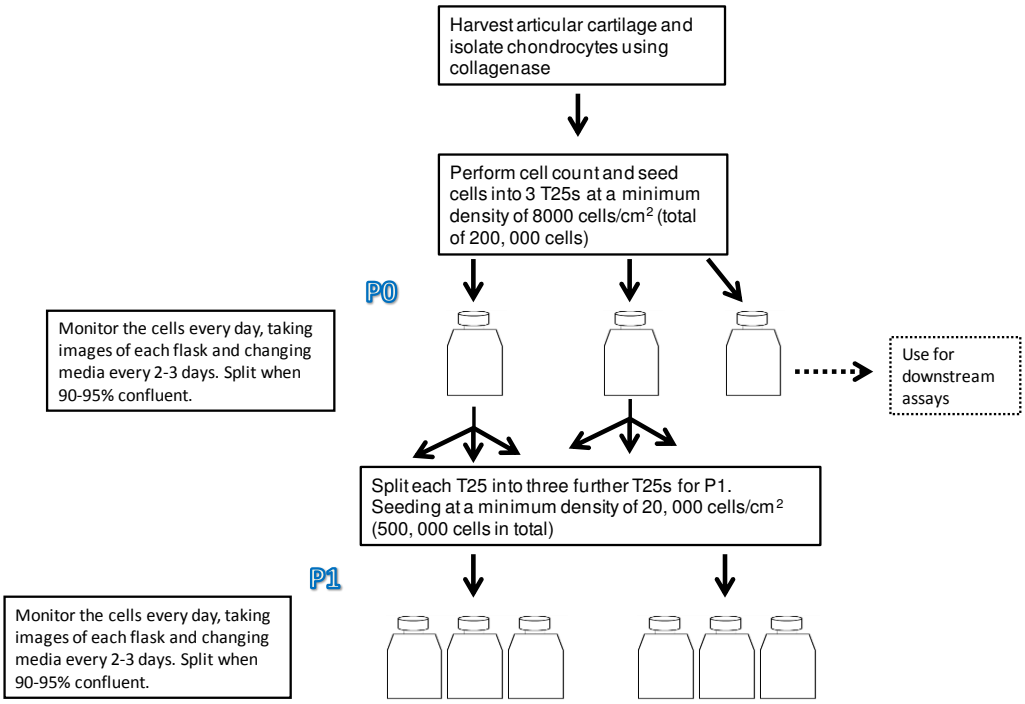


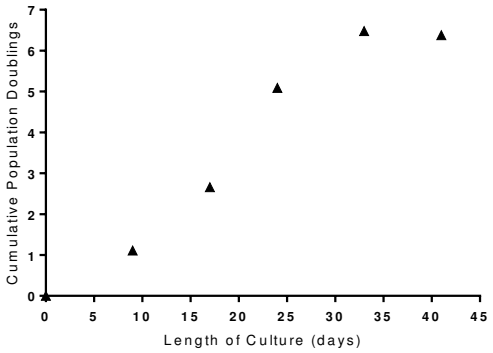
Figure 3.1 Monolayer cell culture plan. Chondrocytes were closely monitored throughout the culture process and passaged once they had reached 90-95% confluence. Diagram is representative of all passages, with the cell density used at P1 planned to be used at all subsequent passages. The dashed arrow indicates a flask that was used to generate samples for western blotting. In subsequent passages three flasks from each passage were used.

Elements of the cell culture plan had to be adapted based on the tissue available. For example, the first tissue to become available after the cell culture plan was devised was cartilage shavings sent from Dr Rebecca Lewis, University of Liverpool. The initial yield of chondrocytes isolated from this tissue was too low to be counted accurately and the entire population was seeded into one T25 flask. The cell culture plan was followed as closely as possible with these

cells however, cell densities were far lower (between 7,000-8,500 cells/cm^2) at each passage. At P5 the seeding density was just under 3,400 cells/cm^2). Due to the lack of an initial cell count and low seeding densities, the phenotypic analysis of these cells is not shown, as it would not truly represent typical canine chondrocyte culture.

The next tissue to become available was cartilage was harvested from the forelimbs of a four year old Rottweiler. After enzyme treatment there were enough cells to seed into three T25 flasks at a density of 13,200 cells/cm^2 (330,000 cells in total per flask). Cells were expanded in monolayer up to P5. In another change to the plan, it was decided that the cell density should be kept as consistent as possible throughout all passages, including P0. The seeding densities at each passage are listed in Figure 3.2b. Each passage took between eight and nine days to reach 90-95% confluence, except passage five which took eleven days. At each passage the viability of the cells was assessed. It ranged from 98.0% to 95.0%. The cumulative population doublings were also calculated during the monolayer expansion of these cells. Cell doubling was relatively slow during initial expansion and then was in exponential phase until day 25. After this point cell growth slowed down and after 34 days began to decline.

a



b

Passage	Seeding density (cells/T25 flask)	Length of culture (days)
0	330000	9
1	356000	8
2	361875	8
3	335714	9
4	371428	8
5	307333	11

Figure 3.2: Monolayer expansion of canine chondrocytes. (a) Cumulative population doublings of chondrocytes expanded in monolayer (b) passage, seeding density and length of culture for the same cells as in (a). Cells were isolated from cartilage harvested from a four year old Rottweiler. Population doublings were calculated using the formula: PD = [log(N/Xo)] / log(2), where PD = population doubling, N = final cell count, Xo = starting (baseline) cell count.

The morphology of the cells was closely monitored throughout the culture process. Before attaching to the cell culture plastic, freshly isolated chondrocytes appeared spherical in shape (Figure 3.3a). Once attached, chondrocytes were flatter and more ovoid in shape (Figure 3.3b). At P0 at 90-95% confluence, the majority of the cells had an ovoid morphology but a few had already begun to stretch out and adopt a more fibroblastic morphology (Figure 3.3c). The stretching and flattening out became more pronounced from P0 to P5 (Figure 3.3). Cells also increased in size with increasing passage and by passage five, cells began to grow in clusters rather than a continuous monolayer (Figure 3.3).

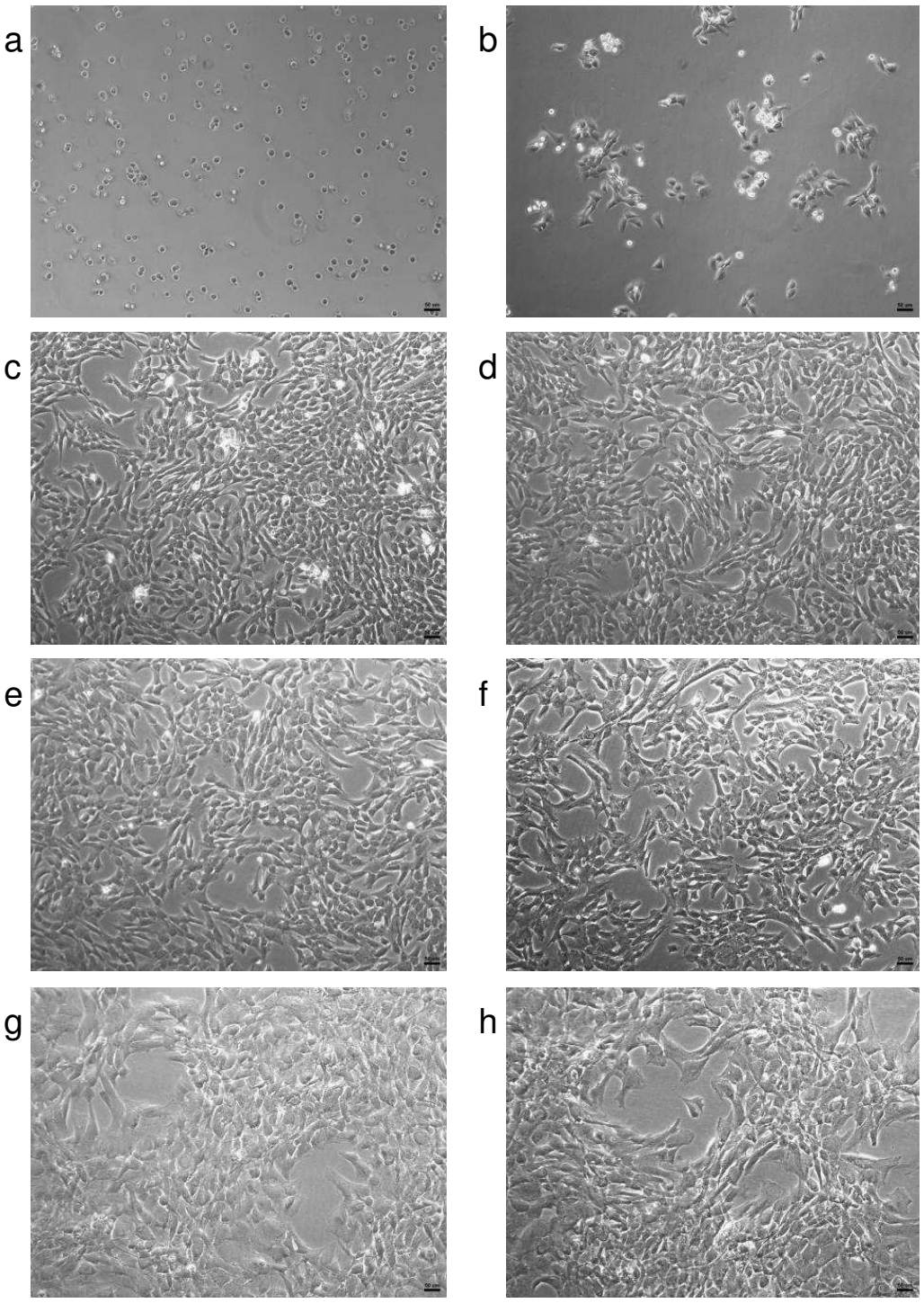

a
b
c
d
e
f
g
h

Figure 3.3: Morphology of canine chondrocytes expanded in monolayer. Primary canine chondrocytes (isolated from cartilage from a four year old Rottweiler) at various stages of monolayer culture. (a) one day after seeding, (b) four days after seeding (c) P0 at 90-95% confluence, (d) P1 at 90-95% confluence, (e) P2 at 90-95% confluence, (f) P3 at 90-95% confluence, (g) P4 at 90-95% confluence, (h) P5 at 90-95% confluence. Magnification: x100, scale bar: 50µm. Cells appear spherical before attachment. Once attached the cells adopt a polygonal morphology and become increasingly elongated with increasing passage.

3.2.2 Antibody Evaluation and Western Blotting Optimisation

To assess the dedifferentiation and redifferentiation of canine chondrocytes when expanded in monolayer and cultured three-dimensionally, the expression of collagen types I and II, CD44 and Sox9 was going to be assessed. In order to do this, the cross-reactivity of antibodies for these antigens in canine tissue was evaluated.

First, a panel of antibodies from Aviva Systems Biology was applied to canine chondrocytes fixed in 96-well plates. All of the antibodies were selected based upon the broad cross-reactivity stated in the datasheets. Three of the antibodies (ARP37986, ARP61023 and P100797) listed dog as one of the species they react with. Each antibody was tested at a range of concentrations (5µg/ml, 2µg/ml, 1µg/ml and 0.5µg/ml). Out

of the two antibodies targeted against the C-terminus of collagen type I, the second antibody tested (ARP59999), appeared to have a more intense and specific staining (Figure 3.4 a-d). One of the SOX9 antibodies (ARP37986) and a collagen type I antibody targeted against the N-terminus had strong and specific staining when compared to the isotype control (Figure 3.4 g-h and k-l). The other antibody targeted against Sox9 (P100797) and the antibody targeted against CD44 appeared to have the same pattern of staining as the isotype control, indicating non-specific binding (Figure 3.4 e-f and i-j).

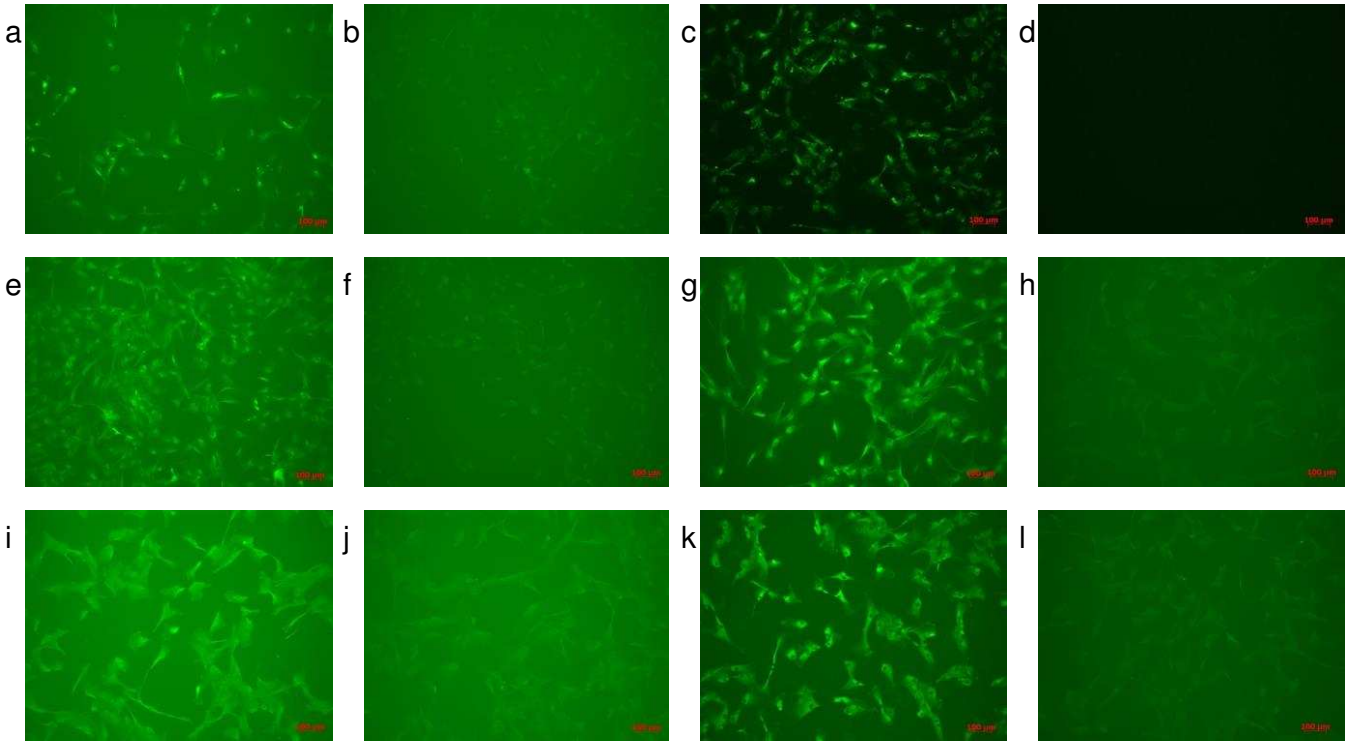


Figure 3.4: Evaluation of Aviva antibodies for cross-reactivity with canine antigens. Canine chondrocytes were seeded into 96-well plates and fixed using ice-cold methanol. (a) COL1A1 (ARP59998), (c) COL1A1 (ARP59999), (e) CD44 (ARP61023), (g) SOX9 (ARP37986), (i) SOX (P100797), (k) COL1A2 (ARP58441); (b, d, f, h, j and l) Isotype control (rabbit polyclonal IgG-ChIP grade, ab27478). Concentration of all antibodies: 1µg/ml. Secondary antibody: goat polyclonal to rabbit IgG DyLight 488 (ab98462): 1:300 dilution. Magnification: x100, scale bar: 100µm. The antibodies used in (c), (g) and (k) appear to stain specifically whereas the staining in (a), (e) and (i) looks to be non-specific.

Alongside the Aviva antibodies, a DSHB antibody targeted against collagen type II was tested for its cross reactivity with canine antigens. The CIIC1 antibody had a range of species listed under cross-reactivity including human, bovine, ovine and mouse and was selected for this reason. This antibody was applied to cells that had been grown and fixed on sterile coverslips. It was tested at a range of dilutions from undiluted to 1:100 (Figure 3.5). There was no difference in staining between any of the dilutions indicating non-specific binding. However it should be noted that an isotype control was not included with this experiment and therefore limited conclusions can be drawn. Therefore, this test was repeated (along with an isotype control) using cells seeded and fixed in 96-well plates and all concentrations were completely negative.

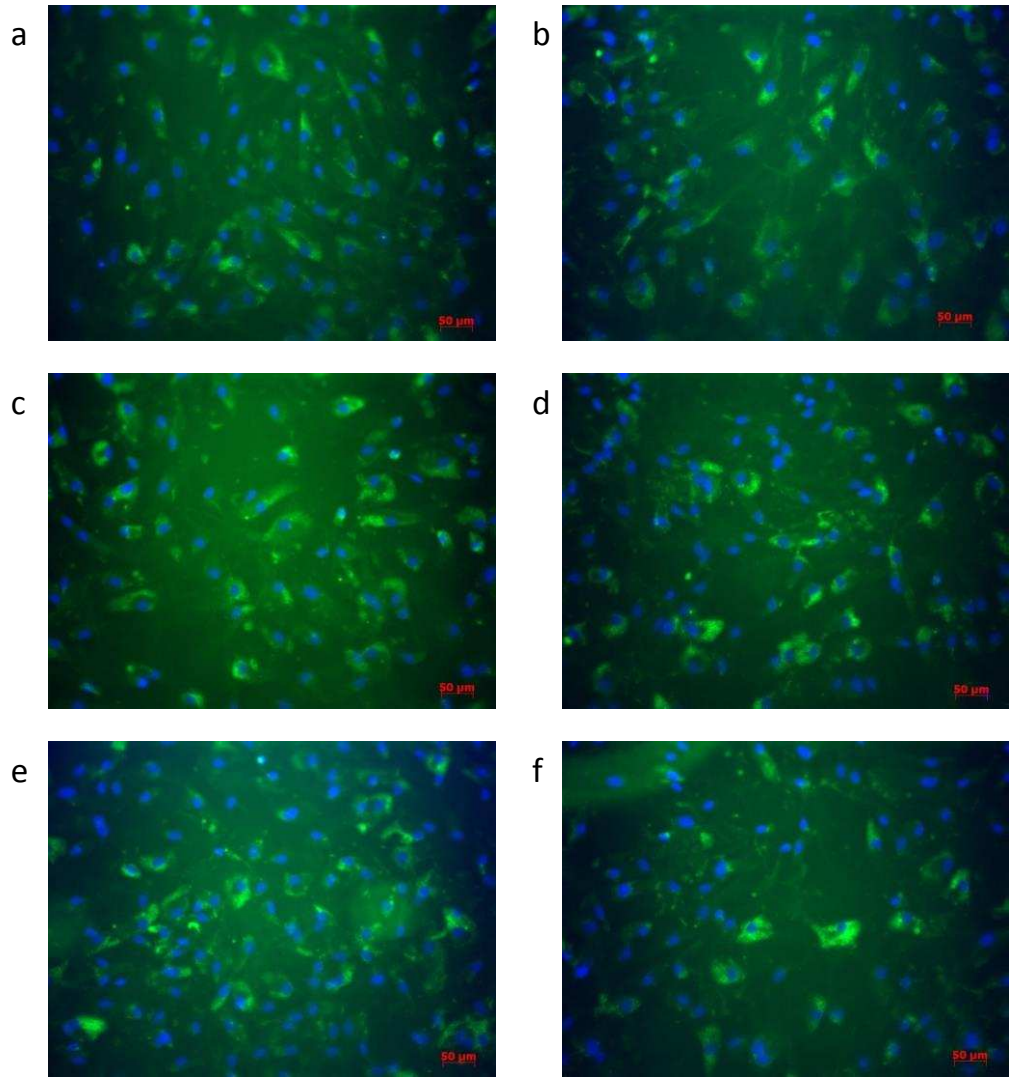


Figure 3.5: Evaluation of the DSHB antibody CIIC1 for cross-reactivity with canine antigens. Cells were seeded onto coverslips and fixed with ice-cold methanol. The primary antibody was tested at the following dilutions (a) neat, (b) 1:1, (c) 1:5, (d) 1:10, (e) 1:50 and (f) 1:100. Secondary antibody: Fluorescein anti-mouse IgG (H+L) (FI-2000): 1:100 dilution. Magnifcation: x100, scale bar: 50µm. Staining was similar at all concentrations, indicating non-specific binding.

To confirm the cross-reactivity with canine antigens, western blotting was carried out. Throughout the antibody validation process the

western blotting protocol was deconstructed to identify the optimum method for each step. This optimisation is described in section 3.2.2.1 before moving on to the validation of specific antibodies for individual antigens; collagen type I (section 3.2.2.2), collagen type II (section 3.2.2.3), Sox9 (section 3.2.2.4) and CD44 (section 3.2.2.5).

3.2.2.1 Western Blotting Protocol Optimisation

First, the protein profiles of all samples were checked. Samples were loaded onto and ran on an SDS-PAGE gel. Gels were then immediately placed in Coomassie Blue rather than proceeding to transfer (Figure 3.6). This generated a reference for all samples which could be checked against if problems were encountered further downstream. It also enabled the assessment of appropriate loading for each sample, particularly for the canine positive control lysates. For canine cartilage, spleen and testis lysates a clearer banding pattern was observed with the higher load (Figure 3.6a). The equine cartilage lane (lysate kindly given by Adam Williams, University of Nottingham) was completely blank. This may have been due to the sample being too dilute. For the canine testis lysate, 10µg seemed an optimal volume for loading as the gel began to become overloaded when 20µg was loaded. For the dedifferentiation samples (canine chondrocyte lysate samples from P0 to P5), the protein banding was similar across all

samples, with bands throughout the length of the gel (Figure 3.6b and c). In the second gel containing canine chondrocyte lysates, the lanes spread a little, this may be due to there being spaces between the lanes (Figure 3.6c).

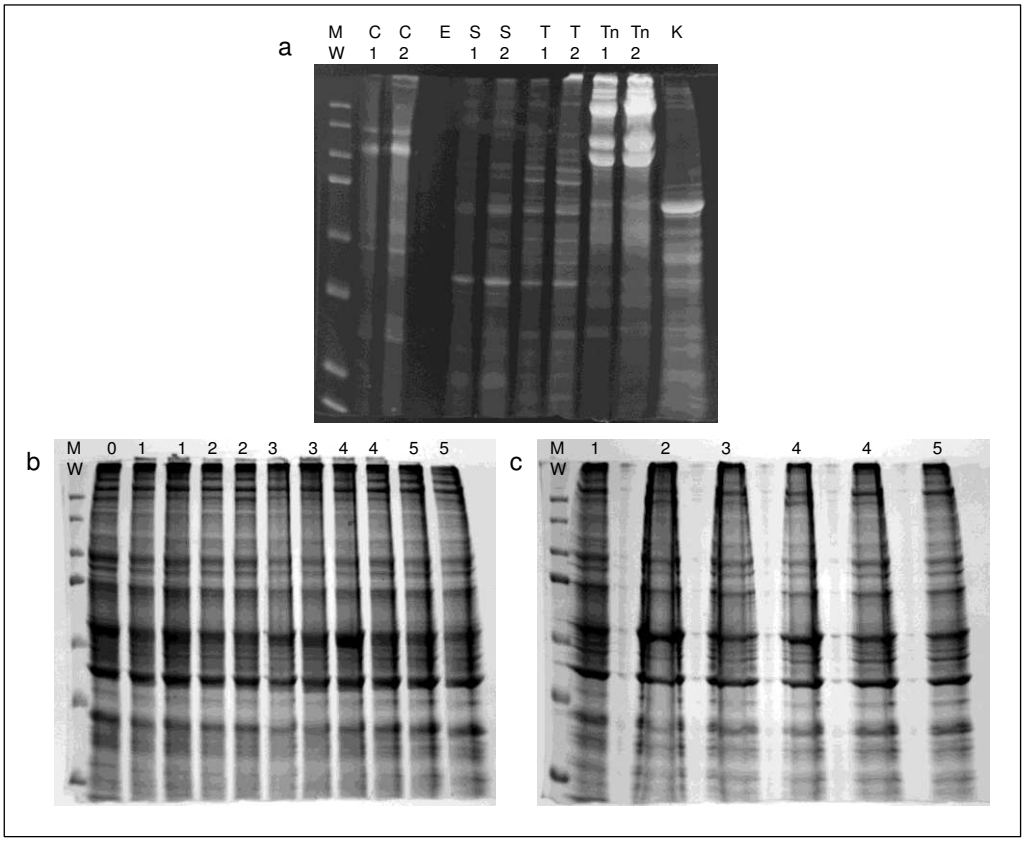


Figure 3.6: SDS-PAGE gels of positive control tissues and chondrocyte cell lysates stained with Coomassie Blue. This was done to generate a protein profile reference for all samples. (a) gel containing canine and equine positive control tissue lysates. Gel was scanned in reverse to show the bands more clearly (b) and (c) gels containing canine chondrocyte lysates. MW: molecular weight marker, C1: canine cartilage lysate (2µl), C2: canine chondrocyte lysate (10µl), E: equine chondrocyte lysate (20µl), S1: canine spleen lysate (10µg), S2: canine spleen lysate (20µg), T1: canine testis lysate (5µg), T2: canine testis lysate (10µg), Tn1: canine tendon lysate (10µl), Tn2: canine tendon lysate (20µl), K: canine kidney lysate (25µg). The numbers in (b) and (c) refer to passage number.

The next stage of the western blotting process to be optimised was the transfer time and conditions. Canine and equine samples were tested alongside porcine diaphragm cell lysate (kindly provided by Dr Zoe Redshaw, University of Nottingham). Two identical gels were either transferred at 100V for two hours at room temperature (short-term) or at 30V overnight at 4^{o}C. The blots were probed with an antibody targeted against the housekeeping gene histone H3. An aliquot of this rabbit polyclonal anti-histone H3 antibody (ab1791) was kindly given by Dr Zoe Redshaw, University of Nottingham. This antibody had previously been shown to work in the porcine sample, consistently detecting a single band at 20kDa; therefore this was included as a positive control. The predicted molecular weight of this antigen is 15kDa but the datasheet advises that the antibody will detect a band at 17kDa. There was a band at 15kDa in the porcine sample and at around 17kDa in the canine cartilage, spleen and testis samples (Figure 3.7c and d). No band was detected at the predicted molecular weight in the equine sample (equine chondrocyte lysate kindly provided by Ismail Hdud, University of Nottingham). In terms of transfer time, there seemed to be little difference in the bands detected on the blot between the short and long transfer; however the gels stained with Coomassie Blue post-transfer indicate that the short-term transfer was more efficient (Figure 3.7a and b). Therefore short-term transfer was routinely adopted as part of the protocol.

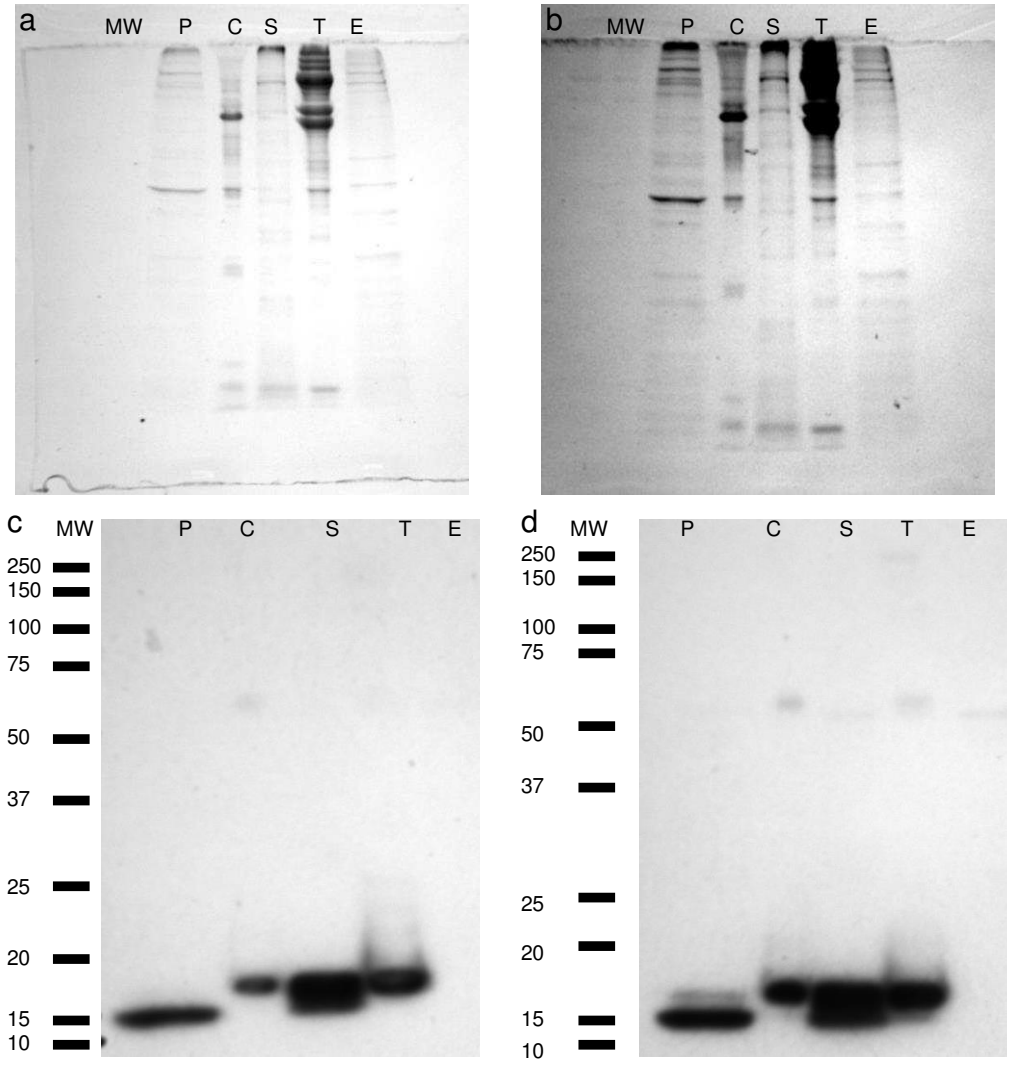


Figure 3.7: Detection of histone H3 in porcine, canine and equine samples, comparing short-term and long-term transfer. (a) and (b) SDS-PAGE gels stained with Coomassie Blue after two hours of transfer at 100V at room temperature (a) and overnight transfer at 30V at 4°C (b). (c) and (d) western blots for histone H3, exposure: one second. (c) short-term transfer and (d) long-term transfer. MW: molecular weight marker, P: porcine diaphragm satellite cell lysate (5µg), C: canine cartilage lysate (10µl), S: canine spleen lysate (20µg), T: canine tendon lysate (10µl), E: equine chondrocyte lysate (25µg). Transfer time had little effect on the protein bands detected on the blot but the stained gels indicate the shorter transfer was more efficient.

A frequent problem encountered during antibody validation was the detection of multiple non-specific bands. Therefore, the next part of the western blotting process to be optimised was blocking times and solutions. Blocking with 5% milk in TBS-Tween for two hours at room temperature was compared with using 3% BSA in TBS-Tween for two hours at room temperature. It was also decided to test whether diluting the primary antibody in TBS-Tween alone or in blocking solution made a difference to the bands detected. H4C4, a DSHB antibody targeted against CD44, was selected for this test and three different concentrations of the antibody were used for each condition. The conditions are summarised in Table 3.2. The highest concentration used was 0.5µg/ml, which was the same as was used previously. As there were non-specific bands detected by this antibody in a previous blot, the other two concentrations were lower to try and further reduce non-specific binding. Secondary antibody conditions were matched to that of the primary.

Antibody Dilution (Concentration in µg/ml)	Blocking and Primary Antibody Conditions				
	Milk Block	BSA block	1°Ab (TBS)	1°Ab (TBS+Milk)	1°Ab (TBS+BSA)
1:1144 (0.5)	✓		✓		
	✓			✓	
		✓	✓		
		✓			✓
1:1500 (0.38)	✓		✓		
	✓			✓	
		✓	✓		
		✓			✓
1:2000 (0.29)	✓		✓		
	✓			✓	
		✓	✓		
		✓			✓

Table 3.2: Optimisation of blocking and primary antibody conditions using H4C4. H4C4 was used at three different concentrations. Milk block: 5% milk in TBS-Tween, BSA: 3% BSA in TBS-Tween, TBS: TBS-Tween only. When the primary antibody contained blocking solution, a stock solution of the block was made and part of this was used to dilute the primary antibody. Secondary antibody conditions were matched to that of the primary.

Three bands were detected in the majority of the blots, even in the negative controls where the primary antibody was omitted (Figure 3.8). The multiple bands could indicate non-specific binding of the primary antibody; however as some of these bands were also present in the negative control, this may suggest cross-reaction with the secondary antibody. Interestingly, the milk block appeared to be more effective than BSA at the higher antibody concentrations; however, the opposite was the case at the lowest antibody concentration. Out of all the combinations, blocking with 5% milk and diluting the primary antibody in

milk appeared to be the most efficient at reducing non-specific binding. Even after ten minutes exposure, most of the bands were quite faint. Therefore, four blots (two blocked with milk and two blocked with BSA) were selected to be re-probed with more concentrated primary antibody. Two concentrations were tested, 1.1µg/ml (1:500) and 2.9µg/ml (1:200). There were no clear bands detected on any of the four blots; only background staining was observed. The background staining was weaker on the blots blocked with milk, further indicating milk to be the optimum blocking solution. To confirm whether milk was more efficient at blocking non-specific binding, new gels were run with different samples and a different antibody was selected for testing. This time the Aviva anti-Sox9 antibody (ARP37986) was chosen and used at two different concentrations, 1.44µg/ml (1:700) and 1.0µg/ml (1:1000). There was a considerable difference between the two blocking solutions; there were multiple bands in all samples in the blots blocked with 3% BSA (Figure 3.9). The binding appeared to be much more specific in the blots blocked with 5% milk (Figure 3.9a and b). However a band at the predicted molecular weight of 56kDa only appears in the blots blocked with 3% BSA (Figure 3.9c and d).

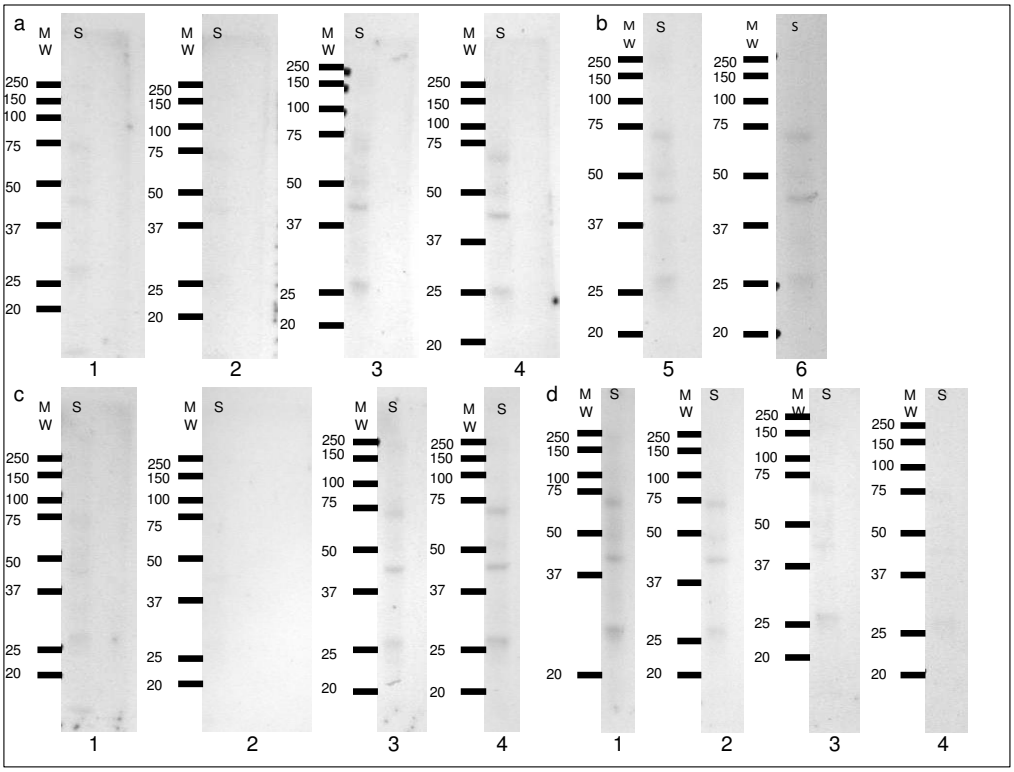


Figure 3.8: Optimisation of blocking for western blotting using H4C4. The DSHB anti-CD44 antibody, H4C4, was selected to assess the affect of blocking material on the bands detected. H4C4 was tested at three different concentrations: 0.5µg/ml (a), 0.38µg/ml (c) and 0.29µg/ml (d). (b) Blots incubated with secondary antibody only. 1: Block: 5% Milk; Primary Antibody: TBS-Tween only, 2: Block: 5% Milk; Primary Antibody: 5% Milk, 3: Block: 3% BSA; Primary Antibody: TBS-Tween only, 4: Block: 3% BSA; Primary Antibody: 3% BSA. For each of these conditions, the secondary antibody conditions matched the primary. 5: Block: 5% Milk, Secondary Antibody: TBS-Tween only, 6: Block: 5% Milk, Secondary Antibody: 5% Milk. Milk and BSA were diluted in TBS-Tween and the secondary antibody was used at 1:5000. MW: molecular weight marker, S: canine spleen lysate (10µg). Exposure: ten minutes. 5% Milk was more effective at blocking than non-specific binding that 3% BSA, except at the lowest concentration where 3% BSA appeared to be more effective.

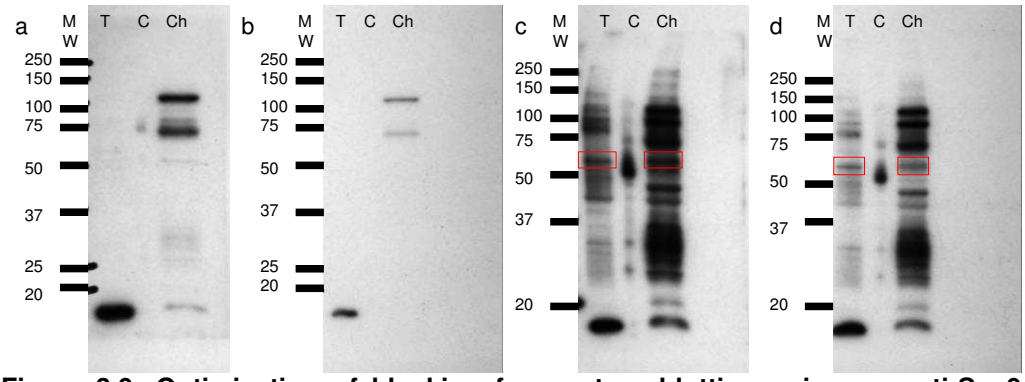


Figure 3.9: Optimisation of blocking for western blotting using an anti-Sox9 antibody (ARP37986). The primary antibody was tested at two concentrations, 1.44µg/ml (a) and (c) and 1.0µg/ml (b) and (d). Blots were either blocked with 5% milk (a) and (b) or 3% BSA (c) and (d). Exposure: 30 seconds. T: canine testis lysate (10 µg), C: canine cartilage lysate (5µl), Ch: P2 canine chondrocyte lysate (25µg). 5% milk was clearly more effective at blocking non-specific binding; however a band at the predicted molecular weight of 56kDa was only detected in the blot blocked with 3% BSA. The red box indicates the band of interest.

After the general optimisation of the western blotting process, the following steps were incorporated into the standard protocol, which was used for all test samples. Firstly, all gels were transferred for two hours at 100V at room temperature (short-term transfer). Secondly, membranes were blocked in 5% milk in TBS-Tween for two hours at room temperature and finally, all antibodies were prepared in 5% milk in TBS-Tween.

The following sections detail the processes involved in identifying a suitable primary antibody for the target canine antigens, collagen types I and II, Sox9 and CD44.

3.2.2.2 Antibody Evaluation: Collagen Type I

The first step to assess cross reactivity with canine tissue was to test the antibody on canine tissue lysates where the antigen of interest is abundant. For collagen type I this was canine tendon lysate. Three antibodies targeted against collagen type I from Aviva Systems Biology were tested for their cross reactivity with canine antigens. Two of these antibodies, ARP59998 and ARP59999, were targeted against the C-terminus of collagen type I and have a predicted molecular weight of 137kDa. The other collagen type I antibody (ARP58441) was targeted against the middle region and had a predicted molecular weight of 150kDa. The banding pattern observed in the positive control lanes was different for each of the three antibodies (Figure 3.10). For two of the antibodies (ARP59999 and ARP58441), there was a strong band detected at around 250kDa (Figure 3.10b and c). The latter antibody detected a second strong band at around 110kDa (Figure 3.10c). The other antibody targeted against collagen type I (ARP59998) detected multiple bands in the tendon lysate, in a different pattern to the other two antibodies (Figure 3.10a). This antibody also detected multiple non-specific bands in a canine chondrocyte sample.

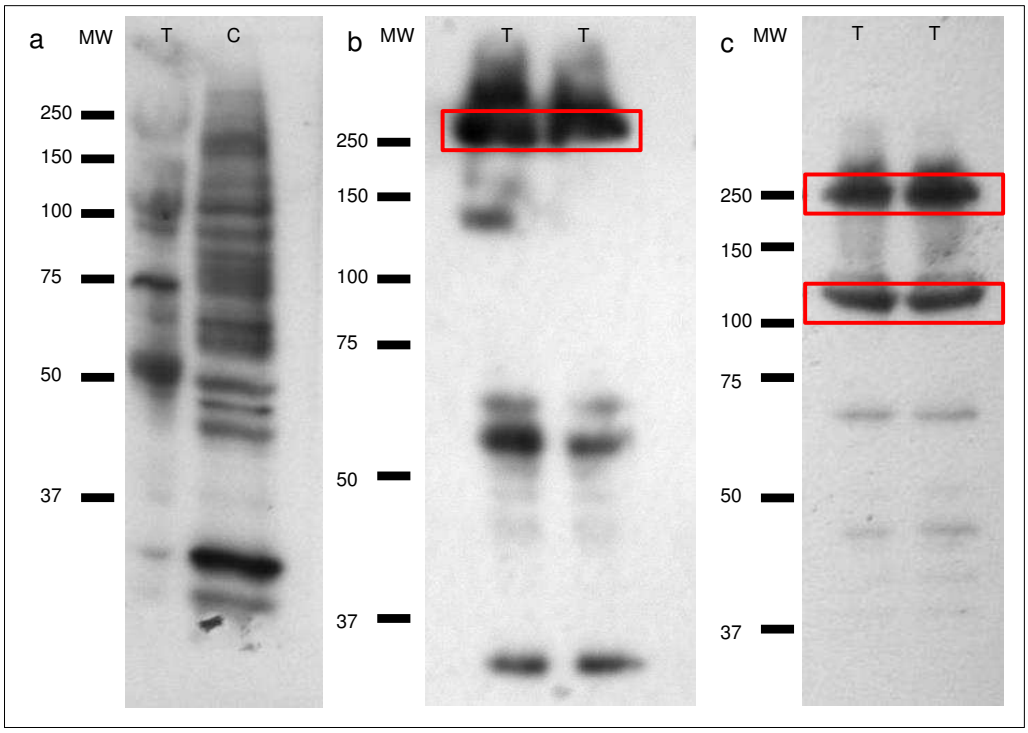


Figure 3.10 Detection of collagen type I in canine samples. (a) ARP59998 at 1.0µg/ml, 30 seconds exposure, (b) ARP59999 T 0.2µg/ml, 15 seconds exposure, (c) ARP58441 at 1.0µg/ml, 40 seconds exposure. MW: molecular weight marker, T: canine tendon lysate (20µl in (a) and 10µl in (b) and (c)) and C: P4 canine chondrocyte lysate (25µg). The predicted molecular weights were 137kDa for ARP59998 and ARP59999 and 150kDa for ARP38441. Red boxes indicate potential bands of interest.

Following this ARP59998 was discontinued due to the lack of defined bands detected in either of the canine samples. Both ARP59999 and ARP58441 detected specific bands, although the band at 250kDa was much higher than the predicted molecular weight. This may be due to insufficient denaturing of the collagen protein. The second intense band detected by ARP58441 may represent one of the α chains. The

bands detected below 75kDa may be degradation products. In subsequent tests ARP58441 consistently detected bands at 250kDa and 110kDa so this was selected for use on test samples and ARP59999 was discontinued.

3.2.2.3 Antibody Evaluation: Collagen Type II

Like with collagen type I, the first stage of the antibody validation process was to test antibodies targeted against collagen type II on a canine positive control tissue. In this case it was canine cartilage lysate. Two DSHB antibodies targeted against collagen type II were tested for their cross-reactivity with canine antigens. CIIC1 was tested at three dilutions, 1:25, 1:50 and 1:100 and II-II6B3 was tested at 1:25 and 1:50 on canine cartilage lysates. Like CIIC1, II-II6B3 was stated to have a broad cross-reactivity. All blots were completely negative. To check that the negative result was not caused by problem with the transfer, all membranes were washed and probed for the housekeeping protein β-actin. β-actin was detected across all blots in all samples. This suggests that the problem was due to the primary antibody itself; either a loss of activity due to prolonged storage or lack of cross-reactivity with canine antigens. Fresh aliquots of these two anti-collagen type II antibodies were ordered to confirm whether the negative result was due to loss of reactivity or a lack of cross-reactivity. The fresh batch of

CIIC1 and II-II6B3 were tested at a concentration of 0.5µg/ml on canine cartilage lysate and equine cartilage lysate. There were no bands detected in any sample for either of the two antibodies. This again suggests that these antibodies do not work on canine and equine samples. To confirm whether this was the case, the membranes were washed and reprobed with a more concentrated secondary antibody and exposure was increased to ten minutes. This only generated light background staining with no bands being detected.

These negative results led to a search for a commercial antibody either known or predicted to work in dog. The Abcam anti-collagen type II antibody (ab37412) was selected and tested on different volumes of canine cartilage lysate (Figure 3.11). In all samples a strong band was seen at about 150kDa (predicted molecular weight: 142kDa). There was also a band detected between 50kDa and 60kDa which could be a degradation product. Three different dilutions of the antibody were tested, 1:1000, 1:5000 and 1:10000 with 1:5000 providing optimal detection.

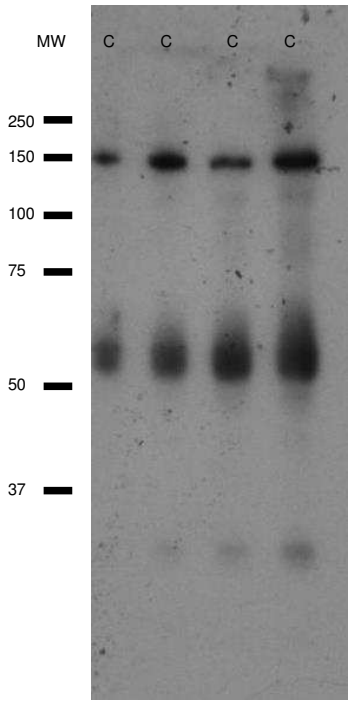


Figure 3.11: Evaluation of the anti-collagen type II antibody (ab37412) for cross-reactivity with canine antigens. MW: molecular weight marker and C: canine cartilage lysate. Canine cartilage lysate was loaded volumetrically, from left to right: 2µl, 5µl, 7µl and 10µl were loaded. Antibody dilution: 1:5000; exposure: ten seconds. Predicted molecular weight: 142kDa.

The two DSHB antibodies targeted against collagen type II (CIIC1 and II-II6B3) were discontinued due to a lack of cross-reactivity. The anti-collagen type II antibody was carried forward for use on test samples due to the specific banding pattern observed in the canine positive control.

3.2.2.4 Antibody Evaluation: Sox9

The positive control tissue sample used to assess the cross-reactivity of the two anti-Sox9 antibodies from Aviva Systems Biology against canine antigens was canine testis lysate. One Sox9 antibody (ARP37986) was targeted against the C-terminus, whereas the other antibody was targeted against the N-terminus (P100797). Both antibodies are predicted to recognise a protein of 56kDa. When these antibodies were applied to canine testis lysate and P0 canine chondrocyte lysate, slightly different banding patterns were observed (Figure 3.12). The first antibody (ARP37986) detected bands at the predicted molecular weight of 56kDa in both samples (Figure 3.12a). However there were also strong bands detected further up the blot in both samples. This could be due to glycosylation or dimers and trimers being present. The second Sox9 antibody (P100797) detected a faint band at 56kDa in the positive control tissue, however there was a strong band detected at the same molecular weight in the canine chondrocyte lysate (Figure 3.12b). Unlike the first antibody, the bands detected further up the blot in the chondrocyte lysate were much fainter than the band at 56kDa (Figure 3.12b). The negative control blot, where the primary antibody was omitted, contained no bands (Figure 3.12c).

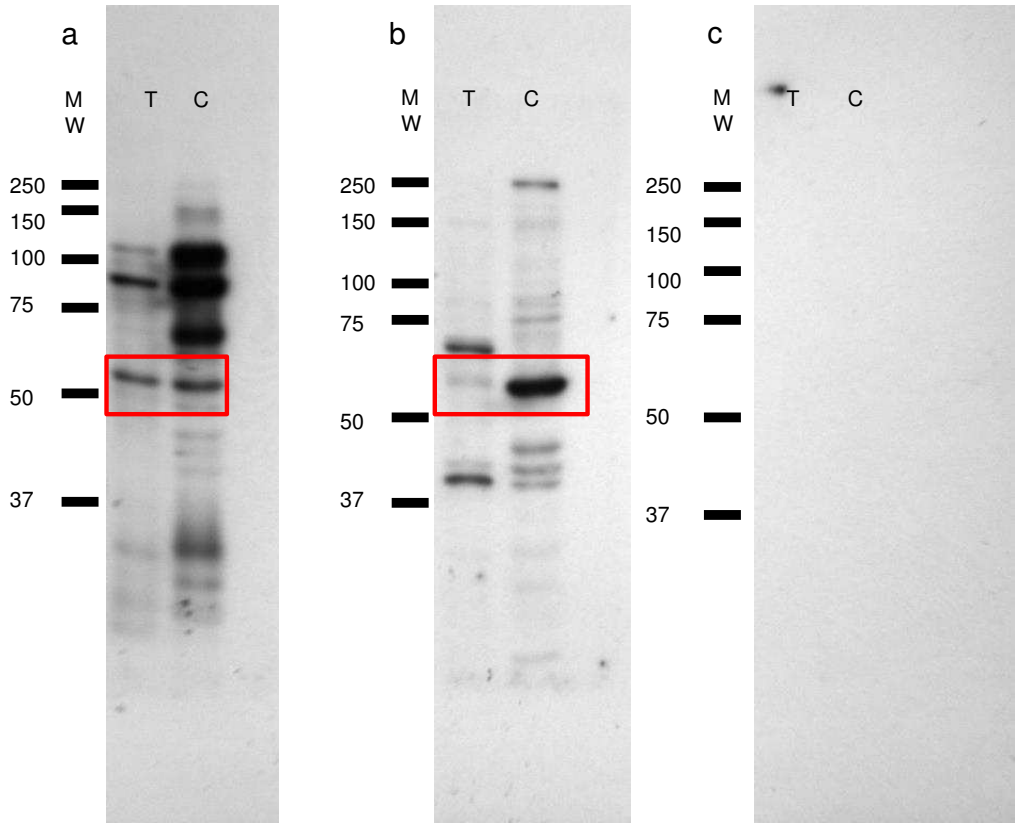


Figure 3.12: Detection of Sox9 in canine samples. (a) Sox9 antibody ARP37986 at 1.44µg/ml (b) Sox9 antibody P100797 at 1.44µg/ml (c) Negative control (primary antibody omitted). MW: Molecular weight marker, T: canine testis lysate (10µg) and C: P0 canine chondrocyte cell lysate (19.74µg). Exposure: 30 seconds. Predicted molecular weight: 56kDa. Red boxes indicate bands of interest.

The antibody targeted against the C-terminus (ARP37986) was carried forward for further testing as it detected a band at the predicted molecular weight in the positive control sample and the canine chondrocyte lysate. However, in further tests this band was absent. In fact when tested on a human sample known to contain Sox9 (K562, Human erythromyeloblastoid leukemia cell line, kindly given by Dr Freya Shephard, University of Nottingham) alongside the canine positive control, the only bands detected were below the predicted molecular weight of 56kDa. Three bands were detected in the K562 cell lysate and only one band was detected in the canine sample at around 17kDa (Figure 3.13). Due to the inconsistency in the banding pattern observed, the use of this antibody was discontinued.

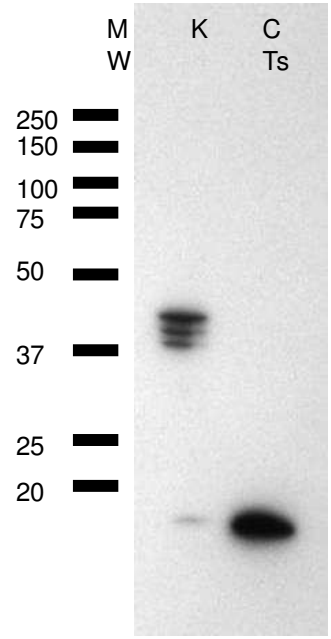


Figure 3.13: Detection of Sox9 in human and canine positive control samples. Membranes were incubated with the anti-Sox9 antibody (ARP37986) at 1.44µg/ml. MW: molecular weight marker, K: K562 cell lysate (2µl), CTs: canine testis lysate (10µg). Exposure: two minutes 30 seconds. Predicted molecular weight: 56kDa.

3.2.2.5 Antibody Evaluation: CD44

As with all the other antibodies, the first stage of validation was to test the antibodies on a canine positive control tissue, which for CD44 is the spleen. The first antibody to be tested was from Aviva Systems Biology (ARP61023), which was predicted to detect a band at 40kDa. However the molecular weight of CD44 can be increased to 80-100kDa due to the attachment of glycosaminoglycan side chains (Sneath and Mangham, 1998). This antibody detected various bands in different

samples (Figure 3.14). In the positive control tissue, the spleen, a single band at 50kDa was detected. In the canine chondrocyte sample, multiple bands were detected with the strongest being at around 110kDa. The banding pattern was different again in freshly isolated equine chondrocytes (equine chondrocyte lysate kindly provided by Ismail Hdud, University of Nottingham) with two distinct bands at around 60kDa and 120kDa. This difference in molecular weights could be due to the fact that there are many different isoforms of CD44 as well as the different levels of glycosaminoglycan attachment. The UniProt database only has limited information for canine CD44 and that given is for a fragment of the protein. However the entries for murine and human CD44 are much more developed with 13 and 19 isoforms being listed respectively.

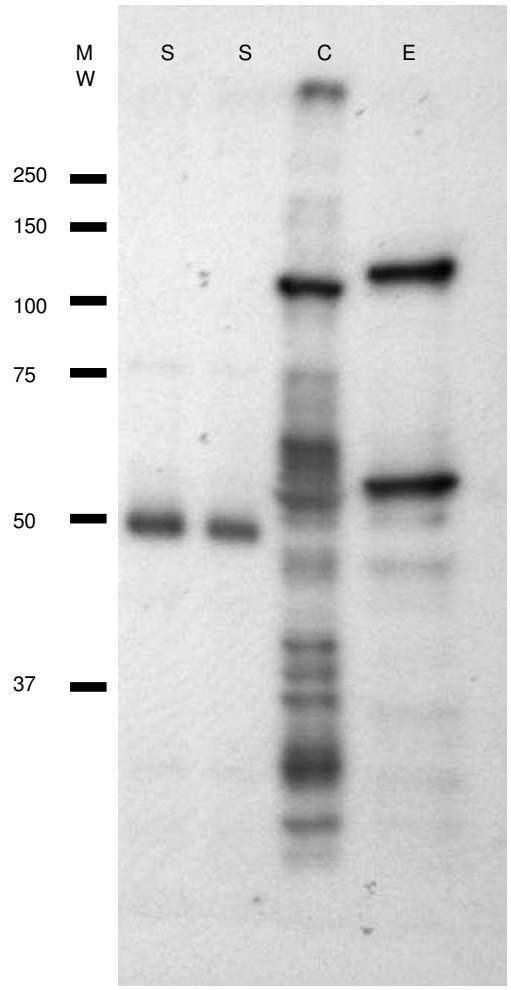


Figure 3.14 Detection of CD44 in canine and equine samples. CD44 (ARP61023) was tested for cross-reactivity with canine and equine samples at a concentration of 1.44µg/ml. MW: Molecular weight marker, S: canine spleen lysate (20µg), C: P0 canine chondrocyte cell lysate (37µg) and E: freshly isolated equine chondrocyte cell lysate (20µg). The blots was exposed to film for 30 seconds. The predicted molecular weight according to the antibody datasheet was 40kDa. The strong bands may appear at different molecular weights in different samples because of the many isoforms of CD44 and different glycosaminoglycan attachments.

Due to the difference in banding patterns seen in the different samples tested for CD44, it was decide to test another panel of antibodies

against this antigen. The aim of this was to try and identify a specific band for CD44 in the samples of interest. Three antibodies targeted against CD44 were selected from the DSHB catalogue (Hermes-1, H4C4, and P1G12). All of the antibodies were stated to cross-react with human samples. Hermes-1 was listed as reacting with other species and P1G12 was stated to work in the macaque. All three CD44 antibodies (Hermes-1, H4C4, and P1G12) were tested on canine spleen lysates at two different concentrations (0.5µg/ml and 1µg/ml). Blots using H4C4 and P1G12 were completely blank, whereas the blots probed with Hermes-1 had multiple bands (Figure 3.15).

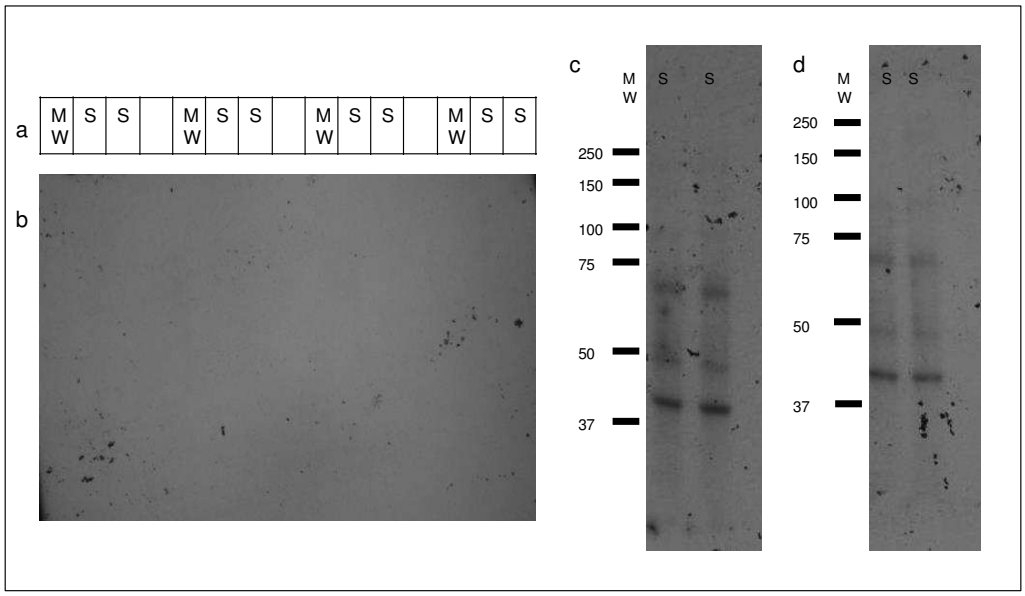


Figure 3.15: Evaluation of three DSHB antibodies targeted against CD44 for their cross-reactivity with canine antigens. (a) Layout of the lanes in the gel that corresponds to the blank blot in (b), (b) Blot for CD44 using the antibodies H4C4 and P1G12 at 1µg/ml and 0.5µg/ml; exposure: two minutes, (c) and (d) Blot for CD44 using Hermes-1 at 1µg/ml (c) and 0.5µg/ml (d); exposure: 15 seconds. M: molecular weight marker, S: canine spleen lysate (10µg). No bands were detected by H4C4 and P1G12, whereas multiple bands were detected by Hermes-1.

H4C4 and P1G12 were raised in mouse whereas Hermes-1 was raised in rat; therefore a different secondary antibody was applied to the two blots with the mouse primary. This means that the lack of bands detected in the blots probed with H4C4 and P1G12 could have been due to the secondary antibody. Another problem could have been that the exposure time was too short. Therefore, the blot probed with H4C4 and P1G12 was washed and re-probed with a more concentrated secondary antibody and the exposure was increased to ten minutes. This resulted in only a light background staining across all blots and no bands were detected (Figure 3.16).

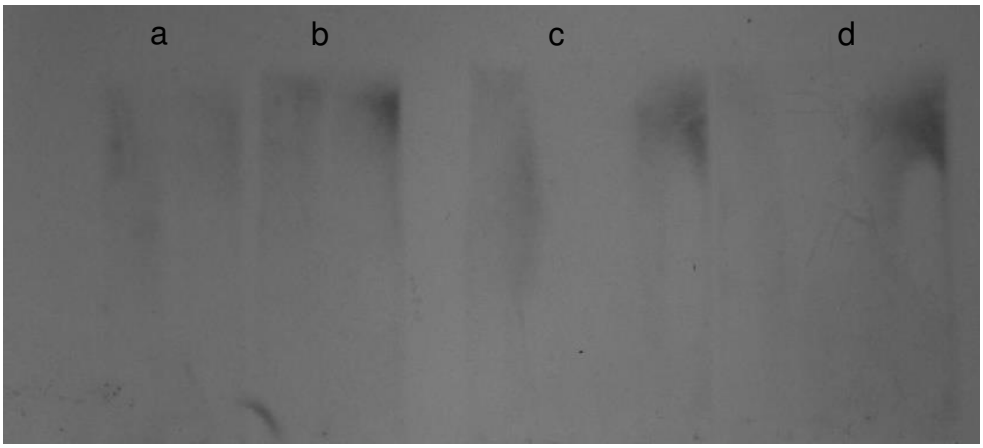


Figure 3.16: Adjustment of secondary antibody conditions for the DSHB primary antibodies. Primary antibodies were as follows: (a) and (b) H4C4 and (c) and (d) P1G12. Concentrations of the primary antibodies were 1µg/ml in (a) and (c) and 0.5µg/ml in (b) and (d). The secondary antibody used was a DAKO anti-mouse / anti-rabbit antibody and was diluted 1:20000. Exposure: ten minutes. Even after the addition of a more concentrated secondary antibody and a prolonged exposure, only background staining was observed.

In a final attempt to identify an antibody that detects canine CD44, H4C4 was tested again using a different secondary antibody. Canine chondrocyte lysate was added to the gel alongside the positive control and Hermes-1 was also re-tested. Hermes-1 was raised in rat so the secondary antibody was not changed from previous testing. A strong band was detected in the canine chondrocyte lysate at around 80kDa by the Hermes-1 antibody (Figure 3.17b). No such band was present in the negative control (Figure 3.17c), suggesting that this is a specific band for CD44. However, there were no bands present in the positive control tissue lysate (Figure 3.17b). This may be explained by the Ponceau Red staining of the membrane post-transfer (Figure 3.17a). There appears to be very few bands in the spleen lysate lanes and there is no band in the predicted molecular weight range for CD44. This could be rectified by loading more of the positive control tissue. Only one band was detected in the blot probed with H4C4 and this was at around 12kDa (Figure 3.17d). This band was also present in the negative control, indicating non-specific binding. As there was a band detected by Hermes-1 within the predicted molecular weight range of 80-95kDa, this antibody was carried forward for use on experimental samples.

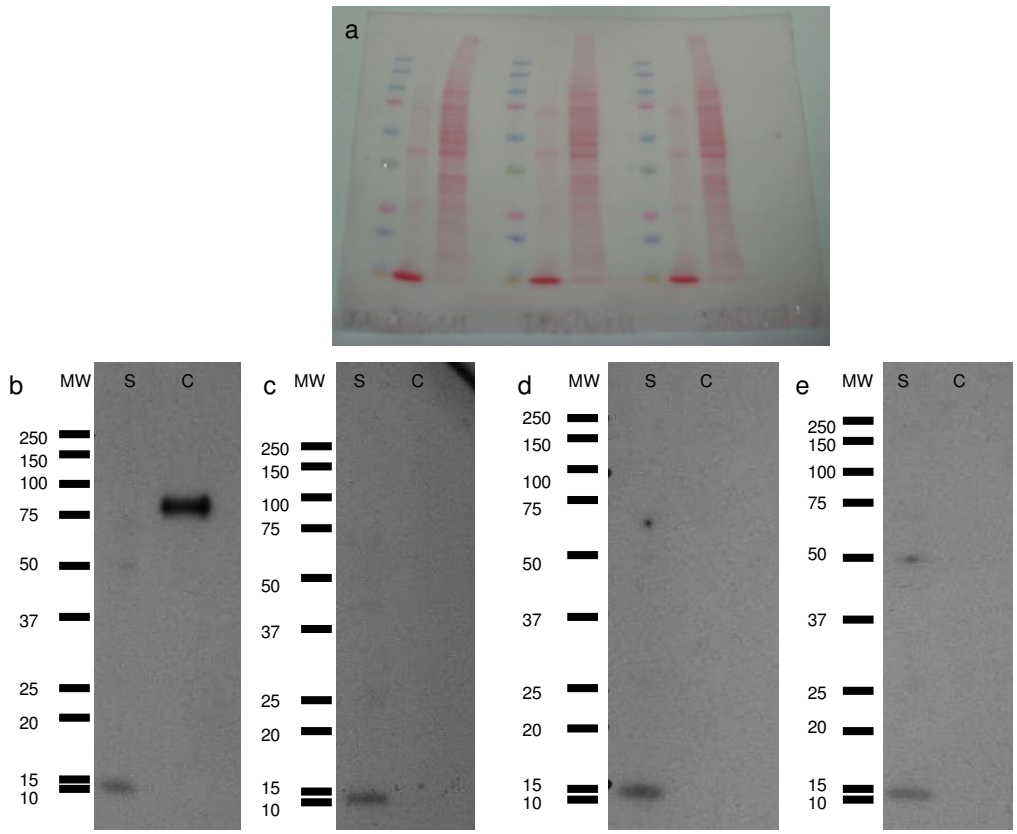


Figure 3.17: Detection of CD44 in canine samples using Hermes-1 and H4C4. (a) In order to check the transfer, membranes were stained with Ponceau Red prior to blocking. (b) western blot for CD44 using the Hermes-1 antibody at 0.34µg/ml (1:1000), (c) negative control (primary antibody omitted), (d) western blot for CD44 using the antibody H4C4 at 0.29µg/ml (1:2000), (e) negative control (primary antibody omitted). MW: molecular weight marker, S: canine spleen lysate (10µg), C: canine chondrocyte lysate (25µg). Exposure: ten minutes. A specific band within the expected molecular weight range was only detected by Hermes-1 and only in the canine chondrocyte lysate sample. Lack of bands in the positive control lysate could be explained by the fact that the bands appear faint on the membrane stained with Ponceau Red, suggesting that more positive control lysate should have been loaded.

For a summary of all the antibodies evaluated and the reasons behind discontinuing or continuing them see Table 3.3.

Antibody	Company	Status	Reason
COL1A1 (ARP59998)	Aviva Systems Biology	Discontinued	No specific banding pattern observed in canine samples.
COL1A1 (ARP59999)	Aviva Systems Biology	Discontinued	Although a specific band was detected at 250kDa, ARP58441 was observed to be more consistent in detecting two distinct bands.
COL1A2 (ARP58441)	Aviva Systems Biology	Continued	Specific bands consistently detected at 250kDa and 110kDa in canine positive control samples.
C11C1	DSHB	Discontinued	Lack of cross-reactivity with canine antigens.
II-II6B3	DSHB	Discontinued	Lack of cross reactivity with canine antigens.
Collagen type II (ab37412)	Abcam	Continued	Specific band detected in canine positive control samples at 150kDa.
Sox9 (ARP37986)	Aviva Systems Biology	Discontinued	Lack of consistency in banding pattern, multiple bands detected.
Sox9 (P100797)	Aviva Systems Biology	Discontinued	Multiple non-specific bands detected.
CD44 (ARP61023)	Aviva Systems Biology	Discontinued	Multiple non-specifc bands detected.
Hermes-1	DSHB	Continued	Specific band detected within the predicted molecular weight range.
H4C4	DSHB	Discontinued	Inconsistency in banding pattern ranging from non-specific bands to a complete lack of bands.
P1G12	DSHB	Discontinued	Lack of cross-reactivity with canine antigens.

Table 3.3: Evaluation of antibodies targeted against collagen types I and II, Sox9 and CD44. The names, catalogue numbers (where appropriate) and companies of the antibodies are given. Reasons for the continuation or termination of antibodies are given. The rows highlighted in yellow represent the antibodies used on the test samples.

3.2.3 Change in Expression of Collagen Types I and II and CD44

Collagen type I expression was detected in all passages (band at around 150kDa); although bands were barely visible in the P0 and P1 sample (Figure 3.18b). There was a general trend of an increase in collagen type I expression with increasing passage; however there was a decrease in expression at P4 (Figure 3.18a). Despite this general trend none of the results were statistically significant when compared to P0 data. In the collagen type II blots, two distinct bands were detected at 250kDa and 150kDa (Figure 3.18f). Expression of the larger isoform decreases with increasing passage, however there was a small increase in expression from P2 to P3. Expression at every passage was significantly lower than that at P0 (all passages $P = < 0.001$, except P4 where $P = < 0.0001$) (Figure 3.18d). Expression of the smaller isoform was lower in P1, P2 and P4 and higher in P3 and P5 compared to P0 (Figure 3.18e). However none of these differences in expression were statistically significant. CD44 expression increased from P0 to P5, although expression remained relatively low in early passages (P1 to P3) (Figure 3.18h-i). Expression at P5 was the only result to be statistically significant when compared to the expression at P0 ($P= < 0.05$).

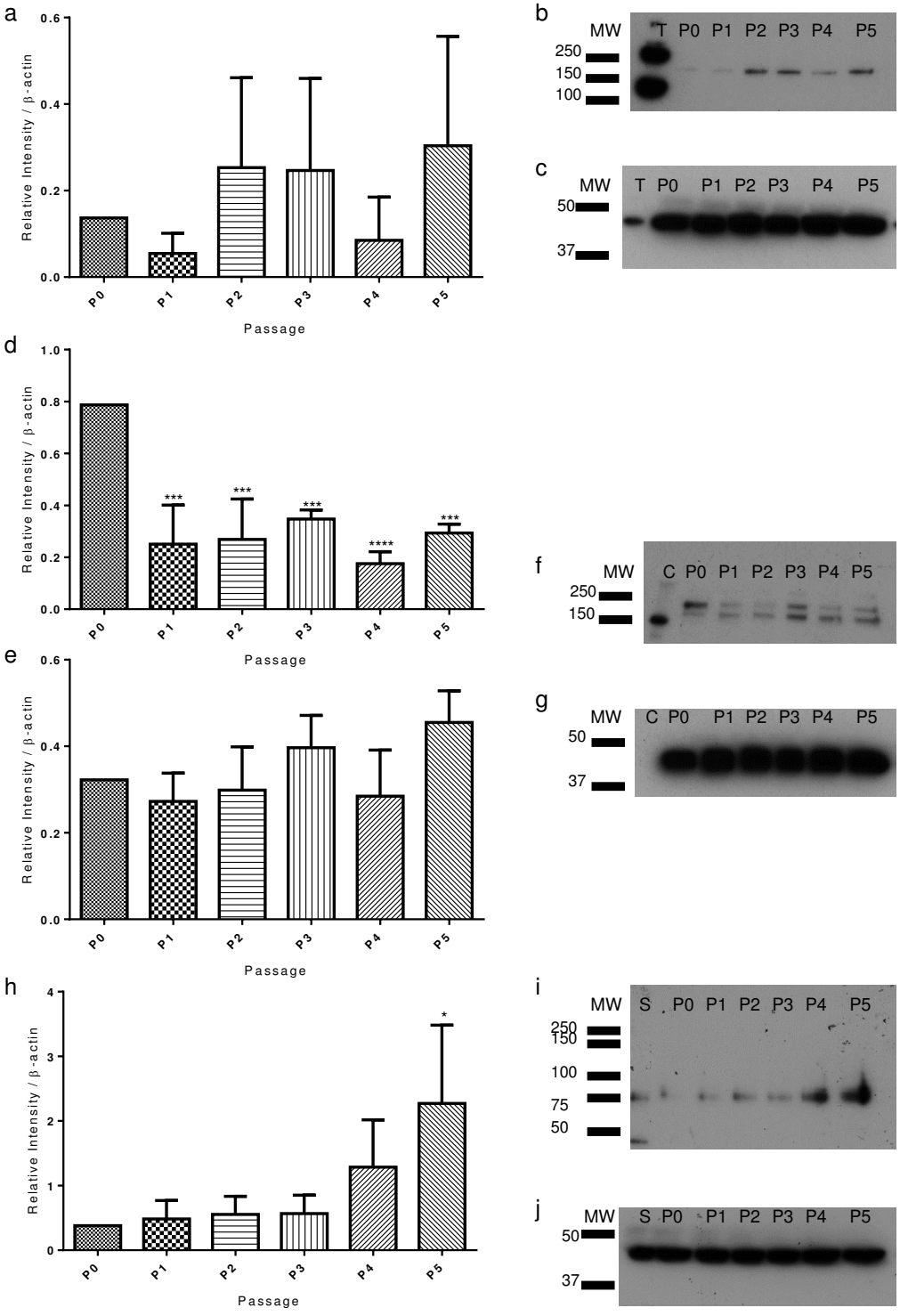

a
Relative Intensity / β-actin
0.0
0.2
0.4
0.6
P0
P1
P2
P3
P4
P5
Passage
b
MW
T P0 P1 P2 P3 P4 P5
250
150
100
c
MW
T P0 P1 P2 P3 P4 P5
50
37
d
Relative Intensity / β-actin
0.0
0.2
0.4
0.6
0.8
1.0
***
***
***
****
***
P0
P1
P2
P3
P4
P5
Passage
f
MW
C P0 P1 P2 P3 P4 P5
250
150
e
Relative Intensity / β-actin
0.0
0.2
0.4
0.6
P0
P1
P2
P3
P4
P5
Passage
g
MW
C P0 P1 P2 P3 P4 P5
50
37
h
Relative Intensity / β-actin
0
1
2
3
4
*
P0
P1
P2
P3
P4
P5
Passage
i
MW
S P0 P1 P2 P3 P4 P5
250
150
100
75
50
j
MW
S P0 P1 P2 P3 P4 P5
50
37

Figure 3.18: Expression of collagen types I and II and CD44 in canine chondrocytes expanded in monolayer up to P5. (a) Densitometry for collagen type I blots (n=3), (b) representative western blot for collagen type I, (c) representative western blot for beta actin; (d) and (e) densitometry for collagen type II blots (n=3), (d) is for the top band and (e) is for the bottom band, (f) representative western blot for collagen type II, (g) representative western blot for beta actin; (h) densitometry for CD44 blots (n=3), (i) representative western blot for CD44, (j) representative western blot for beta actin. MW: molecular weight marker, T: canine tendon lysate, C: canine cartilage lysate, S: canine spleen lysate, P: passage, P0-P5: canine chondrocyte cell lysates. Exposures: (b) and (f) two minutes, (i) five minutes, (c), (g) and (j) one second. * P = <0.05, *** P = <0.001, **** P = <0.0001. Collagen type I expression generally increased with increasing passage however none of the results were significantly different from P0. Two distinct bands were detected for collagen type II; the higher molecular weight form significantly decreased with increasing passage, whereas the lower molecular weight form fluctuated throughout the culture period. CD44 expression increased with increasing culture but the only significant difference in expression occurred at P5.

3.3 Discussion

The first issue addressed in the chondrocyte monolayer expansion process was the seeding density. This varied considerably in the literature, so a cell culture plan was designed with seeding densities incorporated, which were based on previous counts obtained. However, this plan had to be adjusted depending on the availability of samples. Tissue availability is not just a limitation with canine samples; other groups have also reported difficulties in obtaining samples (Diaz-Romero et al., 2005, Hamada et al., 2013).

Freshly isolated chondrocytes took between nine and ten days to become between 90-95% confluent. This is similar to the rate observed for human chondrocytes seeded at a density of 20 000 cells/cm^2 which took ten days to become confluent (Tew et al., 2008). However another paper analyses P2 human chondrocytes at 42 days (Schnabel et al., 2002). The canine chondrocytes used in this study reached 90-95% confluence at P2 after a total of between 23 and 25 days in culture. The difference in values between this study and the work done by Schnabel *et al.* (2002) could be attributed to a difference in the size of culture vessel used. This study used T25 flasks whereas the human chondrocytes were seeded into T75 flasks.

In terms of morphology, freshly isolated chondrocytes appeared spherical before attaching to the tissue culture plastic. Once attached the cells adopted a more polygonal shape and became increasingly elongated with increasing passage. This change in morphology was also observed in ten-day embryonic chick chondrocytes (Abbott and Holtzer, 1966). One hour after seeding 100% of the cells had a rounded morphology whereas after 48 hours this was reduced to 2% with 98% having a stellate morphology (Abbott and Holtzer, 1966). Archer and colleagues (1990) observed the morphology of human surface zone chondrocytes and deep zone cells separately. They showed that the surface zone cells spread in a more bipolar fashion compared to the deep zone cells which adopted a more polygonal morphology (Archer et al., 1990). Although the authors do state that there were some deep zone cells that also adopted this morphology (Archer et al., 1990). Both cell types were observed to be highly fibroblastic after only 14 days of culture (Archer et al., 1990). This is in contrast to the canine chondrocytes in this study, which didn't undergo extensive elongation even after 53 days in monolayer culture. In another paper the morphology of human chondrocytes expanded in monolayer in three different media types (F12:DMEM + 10%FBS + growth factors, F12:DMEM + 2%FBS + growth factors and F12:DMEM + 10%FBS) was observed (Munirah et al., 2010). The images shown in the paper are for P0 (taken at seven days) and P3 (Munirah et al., 2010). No time point is given for the latter passage as the authors acknowledge confluence rates for each group were different (Munirah et

al., 2010). Cells exhibited a polygonal morphology in all groups at day seven; however this morphology was only retained at P3 by those chondrocytes expanded in media containing growth factors (Munirah et al., 2010). The chondrocytes grown in media without growth factors had adopted a more fibroblastic morphology by P3 (Munirah et al., 2010).

In order to determine the phenotype of the canine chondrocytes expanded in monolayer, four proteins were chosen for analysis of expression. Once chosen, the next step was to identify suitable antibodies for these target antigens and optimise immunohistology and western blotting techniques as appropriate. As there are limited antibodies available for these antigens which are known to cross-react with canine samples, various antibodies were screened to evaluate their cross reactivity. It has been noted in a recent review that it is not always easy to identify the specific materials used in a paper in order to exactly replicate the results shown (Vasilevsky et al., 2013). This paper studied 238 manuscripts from 84 journals and looked at the reporting of antibodies, model organisms, knockdown reagents, constructs and cell lines (Vasilevsky et al., 2013). The authors found that only 46% of the commercial antibodies and 43% of the non-commercial antibodies used were identifiable (Vasilevsky et al., 2013). The authors recognise that commercial antibodies do come with datasheets; however they acknowledge that the quality of the information provided can vary

considerably (Vasilevsky et al., 2013). They also recognize that actually 63% of researchers use journal articles to select appropriate antibodies (Vasilevsky et al., 2013).

After an initial screening of a panel of antibodies targeted against the chosen antigens using immunofluorescence, it was decided that western blotting would provide a clearer picture in terms of antibody cross-reactivity. In fact, some researchers are of the opinion that antibody specificity must be determined by western blotting prior to use for immunohistochemistry applications (Kurien et al., 2011). The western blotting process undertaken in this chapter required extensive optimisation and as there are many steps involved in western blotting which can affect the end-result, each step had to be evaluated. The various levels of optimisation that can be undertaken for western blotting are reviewed extensively in (Kurien and Scofield, 2003, Kurien and Scofield, 2006, Alegria-Schaffer et al., 2009).

Following the optimisation of western blotting and antibody evaluation, three antibodies were selected, one targeted against collagen type I, one against collagen type II and one against CD44, for testing on canine chondrocyte lysates from multiple passages (P0 to P5). The fourth marker had to be withdrawn from the study due to the lack of an effective primary antibody. Overall, collagen type I expression

increased from P0 to P5, however there were decreases in expression at P1 and P4 and none of the results were significantly different from the expression observed at P0. This general trend of an increase with increasing passage or culture is in agreement with results seen in published literature (Brodkin et al., 2004, Darling and Athanasiou, 2005, Yang et al., 2006b, Munirah et al., 2010, Nadzir et al., 2011, Hamada et al., 2013). It must be noted that many of these groups have looked at mRNA expression rather than protein expression. To get a true sense of what the cells are producing, protein expression should also be assessed. One group who studied chondrocytes expanded up to passage 17 found that collagen type I gene expression increased from P0 to P3 but remained relatively constant after this passage (Tew et al., 2008).

The opposite pattern, in part, was observed for collagen type II. Two distinct bands were detected in all chondrocyte lysates, compared to only one band in the cartilage lysate. This may be due to the presence of different forms of collagen type II. There are two types of procollagen, types IIA and IIB (Ryan and Sandell, 1990, Bruckner and van der Rest, 1994, Nah et al., 2001, Martel-Pelletier et al., 2008). Although type IIA collagen is normally associated with newly formed cartilage (Bruckner and van der Rest, 1994) it has been suggested to be a marker of dedifferentiated chondrocytes (Gouttenoire et al., 2010). This group found that type IIA procollagen expression increased with increasing

passage of embryonic chondrocytes (Gouttenoire et al., 2010). Another possible explanation for the multiple bands is the detection of collagen at various stage of processing. When collagen is first synthesised, it contains propeptides at the N- and C- termini which are essential for the formation of the triple helix (Martel-Pelletier et al., 2008, Ricard-Blum, 2011). The band at the higher molecular weight may represent collagen prior to the cleavage of the propeptides. Also, as the primary antibody used was polyclonal; it has the potential to detect multiple forms of the target protein. The expression of the larger molecular weight form of collagen type II significantly decreased with increasing passage with all values being significantly lower than that at P0. This decrease with increasing culture length is also demonstrated in the literature but like with the collagen type I studies, many groups look at gene expression rather than protein expression (Brodkin et al., 2004, Darling and Athanasiou, 2005, Yang et al., 2006b, Eleswarapu et al., 2007, Tew et al., 2008, Munirah et al., 2010, Hamada et al., 2013).

CD44 expression was shown to increase from P0 to P5 with only the expression at P5 being significantly higher than P0. Diaz-Romero (2005) and colleagues looked at the expression of a range of cell surface markers by flow cytometry of monolayer chondrocytes at zero hours and 24 hours. They found that the level of CD44 increased in this time, indicating that an increase in CD44 expression occurs early-on in the monolayer expansion process. Another group also looked at CD44

expression using flow cytometry, this time studying cells from P0 to P4 (Hamada et al., 2013). The authors of this paper found that there was a sharp increase in expression from P0 to P2; there was a slight increase from P2-P3 but a minimal decrease from P3 to P4 (Hamada et al., 2013). This is in contrast to the data presented in this chapter in which the greatest increase in expression is demonstrated at the latter passages. This difference could be due to the different isoforms of CD44 present. Another paper studying the gene expression of CD44 in monolayer chondrocytes expanded for four weeks showed that expression of the standard and long tail forms of CD44 fluctuated throughout the culture period (Albrecht et al., 2009). However expression of the short-tail form was higher in the latter stages of the culture (from day 18 onwards) (Albrecht et al., 2009).

It must be noted that results in this chapter have been described in relation to passage, which is subjective term and may mean different things to different researchers. Therefore, it may be useful to also take population doublings into consideration as this may affect the phenotype of the cells. For example, those chondrocytes analysed during the lag phase of expansion may more closely resemble native chondrocytes compared to those in the exponential phase. One research group observed that the number of round cells decreased with increasing population doublings (Nadzir et al., 2011). This was also observed by another group culturing rabbit chondrocytes on collagen

substrate and in collagen gel (Kino-Oka et al., 2009). Nadzir and colleagues (2011) also demonstrated that collagen type I expression increased with increasing population doublings. Collagen type II expression was slightly more complex with expression being low at low and high population doublings but there was also a considerable peak at 5.1 population doublings (termed middle age by the authors) (Nadzir et al., 2011). This could indicate variation in phenotype depending on the stage of culture. Another research group described chondrocytes as being dedifferentiated at two, five and eight population doublings (Rackwitz et al., 2014). They also claimed that collagen type I expression increased and collagen type II expression decreased with increasing population doublings but the data is not shown (Rackwitz et al., 2014).

3.4 Conclusion

The results in this chapter clearly show that canine chondrocytes dedifferentiate in monolayer, although the change in morphology appears to be less dramatic than that reported for human chondrocytes. This could be due to the differences in initial seeding densities and cell culture technique. Expression of collagen type I and CD44 increased with increasing passage, although the only significant difference in expression was at P5 for CD44. Collagen type II significantly

decreased in expression from P0 to P5, however there was a lower molecular weight isoform detected that fluctuated in expression across the passages.

Also highlighted in this chapter was the difficulty in obtaining a regular supply of tissue and the problems involved with identifying suitable antibodies which specifically detect the antigens of interest.

The next chapter will deal with encouraging the chondrocytes to redifferentiate into the chondrocytic phenotype through establishing a three dimensional cell culture system.

4. Establishment of Chondrocyte 3D Culture

4.1 Background

Various 3D culture techniques have been used to maintain or induce the chondrocytic phenotype however the one method that is most frequently used is high density pellet culture (Abbott and Holtzer, 1966, Manning and Bonner, 1967, Kato et al., 1988, Solursh, 1991, Ballock and Reddi, 1994, Stewart et al., 2000, Tallheden et al., 2003, Malda et al., 2004, Zhang et al., 2004, Dehne et al., 2009). Multiple research groups have shown that chondrocytes cultured in high density pellets retain their spherical morphology (Manning and Bonner, 1967, Kato et al., 1988). Expression of collagen type II or its precursor procollagen type II has also been shown to expressed by chondrocytes cultured in high density pellets (Ballock and Reddi, 1994, Stewart et al., 2000). However, evidence of hypertrophy through collagen type X expression has been shown in pellet culture (Ballock and Reddi, 1994). Pellet culture has also shown to be ineffective at completely preventing chondrocyte dedifferentiation in in vitro culture as collagen type I expression has also been detected (Stewart et al., 2000). Pellet culture has also been used as a mechanism to induce chondrogenesis in mesenchymal stem cells (Johnstone et al., 1998, Csaki et al., 2007, Stewart et al., 2007, Stewart et al., 2008, Vidal et al., 2008, Lettry et al., 2010), as it mimics the condensation of MSCs during the early stages of

chondrogenesis (Cancedda et al., 2000, Goldring et al., 2006, Chen and Tuan, 2008, Pelttari et al., 2008).

Another way to culture chondrocytes three dimensionally is to seed them into a supporting scaffold material. One such material is alginate (Bonaventure et al., 1994, Hauselmann et al., 1994, Hauselmann et al., 1996, Loty et al., 1998, van Osch et al., 1998, Chubinskaya et al., 2001, Domm et al., 2002, Schulze-Tanzil et al., 2002, Lee et al., 2003, Stoddart et al., 2006, Rai et al., 2009, Brand et al., 2012, Caron et al., 2012). Some groups have also used synthetic polymers such as polyglycolic acid (PGA) (Homicz et al., 2003), poly(DL-lactic-co-glycolic acid (PLGA) (Chen et al., 2003) and polycaprolactone (PCL) (Garcia-Giralt et al., 2008) for chondrocyte redifferentiation in 3D culture.

As high density pellet culture is commonly used to help maintain the chondrocyte phenotype *in vitro* and has the advantage of no additional materials being required, this method will be applied to the canine chondrocytes in this study. The aim and hypothesis of this chapter were as follows:

Aim: To establish a 3D culture of canine chondrocytes and assess the phenotype of monolayer-expanded cells within this system.

Hypothesis One: Chondrocytes previously expanded in monolayer will begin to adopt a more native phenotype (redifferentiate) in 3D culture. Expression of collagen type II will increase whereas expression of collagen type I and CD44 will decrease. Synthesis of GAGs should also be detected in the 3D culture system.

4.2 Results

4.2.1 High Density Pellet Culture

Due to limited tissue availability, the initial work with high density pellet cultures was carried out with cryopreserved canine chondrocytes (cells were at P1 when frozen). These cells were revived and expanded in monolayer culture until 100% confluence was reached. The chondrocytes were lifted off tissue culture plastic and dispensed into 15ml falcon tubes. Cells were centrifuged to form pellets with an initial density between $1.75x10^5$ and $1.94x10^5$ cells. The initial cell density used for pellet culture ranges in the literature (Table 4.1). Due to the limited availability of cartilage and chondrocytes, relatively low densities were trialled first. After 21 days in culture at 37°C, 5% CO_2 the cells had formed a very small pellet (Figure 4.1a). Four of the pellets were removed from the media at this stage for processing, embedding and sectioning. Due to the small size of the pellets, three were left for a

further two weeks to see if a longer culture period would generate significantly bigger pellets (Figure 4.1b and 4.1c). Little change in size was observed after 14 extra days in culture.

Species	Initial Cell Density	Reference
Bovine	$2.5x10^5$	(Kawanishi et al., 2007)
	$1.5x10^6$	(Bernstein et al., 2009a)
Human	$2x10^5$	(Tallheden et al., 2003)
	$5x10^5$	(Scotti et al., 2012)
Rabbit	$8x10^4$	(Kato et al., 1988)
Rat	$1.6x10^5$	(Ballock and Reddi, 1994)
Equine	$2x10^5$	(Stewart et al., 2000)
Chick	$1\text{-}5x10^6$	(Abbott and Holtzer, 1966)

Table 4.1: Different cell densities used for pellet culture of chondrocytes.

The small size of the pellets made processing using the automated programs on the tissue processor very difficult. Therefore this was adapted slightly by embedding the pellets in 1% agarose (Fisher Bioreagents) after fixation in 10% neutral buffered formalin (NBF) and prior to processing. The pellets were then processed using the tissue processor before being embedded in paraffin wax. It proved difficult to obtain sections of these pellets due to the fact that the wax shattered when trying to section through the agarose gel. In addition, their small size made it difficult to visualise them within the wax. As the pellets were no bigger after 35 days, culture and processing was terminated

and the pellet culture was attempted again, aiming to generate larger pellets.

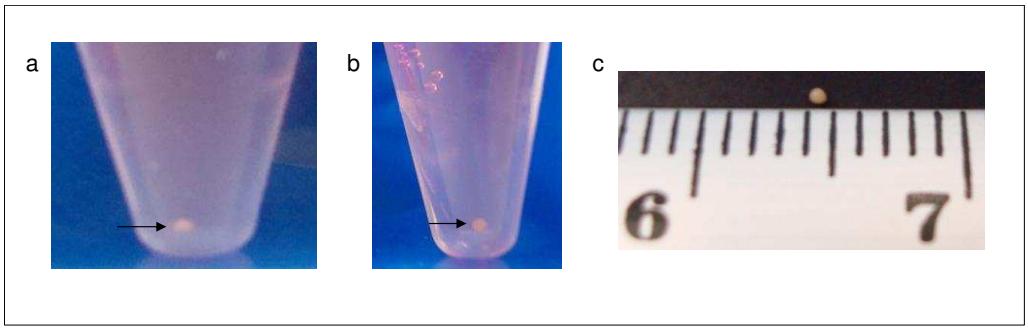


Figure 4.1: Canine chondrocyte high density pellet culture. (a) pellet cultured for 21 days (b) and (c) pellet cultured for 35 days. Pellets at both time points were very small and difficult to manipulate out of culture. The size of the pellets did not appear to change from day 21 to day 35. Pellets were photographed using a Sony Cyber-Shot DSC-W30 digital camera.

In order to generate a cell pellet large enough for embedding and sectioning, high density pellet culture was attempted for a second time with higher initial density. Cryopreserved canine chondrocytes (P1 when frozen) were revived and expanded in monolayer (passaged once) before being pelleted. Another potential reason for the lack of growth seen in the previous pellets could have been inefficient gaseous exchange. Therefore, the system was adapted to improve this and cells were pelleted in sterile eppendorfs with holes in the lids. Two types of holes were made (Figure 4.2a and 4.2b). All pellets had a starting cell density of between $1x10^6$ and $2x10^6$ cells. After 21 days, some of the pellets were removed for fixing, embedding and sectioning (Figure 4.2c).

The pellets were very delicate and one broke up during the fixation period (Figure 4.2d). As the pellets were still very small and the agarose technique used previously didn't work, snap freezing the pellets in OCT after fixation in 10% NBF was attempted (Figure 4.2e). The sections were then stained with Haematoxylin and Eosin (H&E) (Figure 4.2f and 4.2g). A considerable amount of the tissue had been lost. There are two possible reasons for this; the fixation method used was not appropriate for the embedding technique opted for and the staining procedure washed away some of the material from the slide. Culture of the rest of the pellets was stopped after 35 days. At this point they were fixed in 10% NBF and stored in 70% ethanol until processing. These pellets were considerably larger than those generated after the first attempt of high density pellet culture (Figure 4.2h). The size of the pellet was unaffected by the type of hole made in the eppendorf, therefore, as the holes made using the hypodermic needle were consistently the same size, only these eppendorfs were used for further high density pellet culture studies.

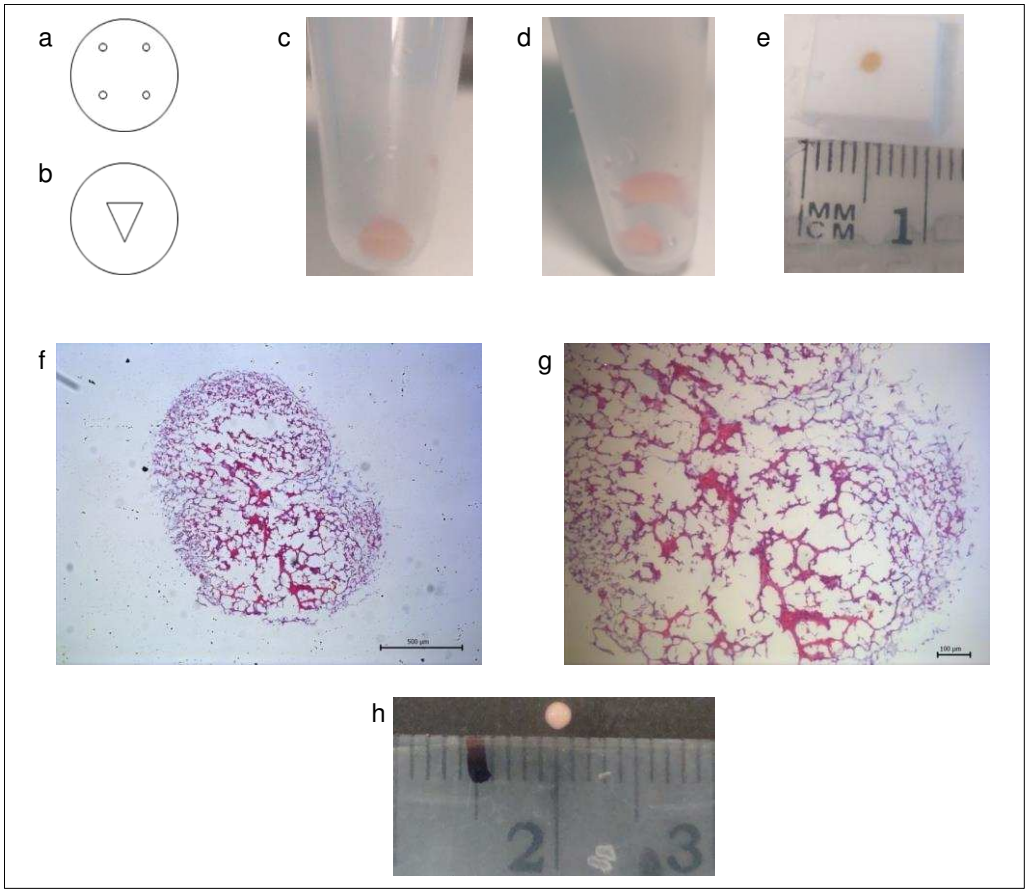


Figure 4.2: Adjustment of high density pellet culture conditions and processing. (a) Diagram representing the lid of an eppendorf containing holes made using a hypodermic needle. (b) Diagram representing the lid of an eppendorf containing a hole made using a sterile scalpel. (c) Chondrocyte pellet after 21 days in culture in an eppendorf containing hypodermic needle holes in the lid (starting cell density: $1.21x10^6$ cells). (d) A broken up cell pellet, which was cultured for 21 days in an eppendorf with a scalpel hole in the lid (starting cell density: $1.21x10^6$ cells). (e) Chondrocyte pellet embedded in OCT. (f) Haematoxylin and Eosin (H&E) stained pellet (5µm section, magnification: x50, scale bar: 500 µm). (g) The same section as in (f) but viewed at a magnification of x100 (scale bar: 100 µm). (h) Chondrocyte pellet after 35 days in culture. Cell pellets were considerably larger than the previous attempt although some were very fragile and difficult to process. Substantial sample loss was observed through histological staining which was probably due to incompatible fixation and processing.

The 35-day pellets were still very delicate and one broke-up whilst being transferred from the eppendorf to the tissue processing cassette. The two remaining pellets, which were both cultured in eppendorfs containing scalpel holes in the lids, were successfully transferred to the cassettes (Figure 4.3a and 4.3b). Only one of these pellets was not destroyed in the tissue processor and was subsequently embedded in paraffin wax. This pellet (starting density of around $2x10^6$ cells) was analysed histologically using Alcian blue (Figure 4.3c) and H&E (Figure 4.3d) staining. This time the tissue was not lost and positive Alcian blue staining indicated glycosaminoglycan (GAG) production.

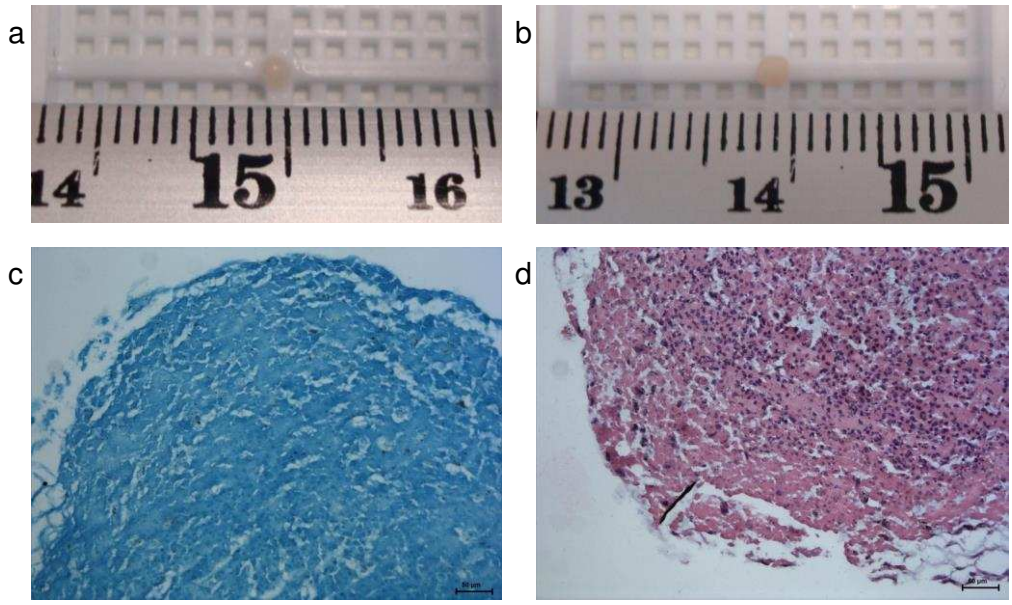


Figure 4.3: Canine chondrocyte high density pellets cultured for 35 days. (a) 35-day pellet; starting cell density: $1.18x10^6$ cells. (b) 35-day pellet; starting cell density: $2.27x10^6$ cells. (c) The pellet in (b) stained with Alcian blue. (d) The pellet in (b) stained with H&E. The pellet in (a) was destroyed in the tissue processor. (c) and (d) magnification: x200, scale bar: 50 µm., sections: 5µm.

Due to the instability of the pellet cultures observed during culture and during processing for downstream applications it was decided that the cells would need a support mechanism for 3D culture. The supporting material chosen was alginate. Alginate was chosen because it has been used to culture chondrocytes from a variety of species including humans (Hauselmann et al., 1996, Chubinskaya et al., 2001), cows (Hauselmann et al., 1994, van Osch et al., 1998, Lee et al., 2003), rabbits (De Ceuninck et al., 2004), rats (Loty et al., 1998) and dogs (Rai et al., 2009).

4.2.2 Alginate Bead Culture

4.2.2.1 Size, Cell Density and Cell Viability

The first stage of establishing the alginate bead culture was to identify the appropriate size of the bead and the initial cell density. The size of each alginate bead is governed by the size of the needle used to dispense the alginate solution into the calcium chloride solution. The calcium, which is a divalent cation, interacts with the α-guluronic acid monomer in the alginate, forming ionic bonds between the copolymers (Martinsen et al., 1989, Paige et al., 1996, Shakibaei and De Souza, 1997, Mierisch et al., 2002, Drury et al., 2004, Wilson et al., 2013).

An array of needle sizes has been used in the literature to generate alginate beads. They range from 20 gauge (20G) to 23G (Bonaventure et al., 1994, Hauselmann et al., 1994, Hauselmann et al., 1996, van Osch et al., 1998, Chubinskaya et al., 2001, Domm et al., 2002, Lee et al., 2003, De Ceuninck et al., 2004, Stoddart et al., 2006, Bernstein et al., 2009a, Brand et al., 2012, Caron et al., 2012). Therefore, a range of needle sizes was assessed for the size of beads they produced (Figure 4.4a). Two pipette sizes and the syringe itself was also assessed for bead generation. Beads generated with an 18 gauge needle gave large, regular sized beads (Figure 4.4a1). 1ml pipettes also generated large, regular sized beads (Figure 4.4a8), however the dispensing of the alginate was more difficult to control with the pipette.

Like with the 1ml pipette, dispensing of alginate from a 5ml pipette was also difficult to control. The two largest needle sizes (16G and 14G) generated beads that had a flattened edge, as opposed to the more spherical beads generated with the smaller gauge needles (Figure 4.4a5 and 6). 18G needles were selected to be used for all alginate bead work, as they produced large, regular sized beads. To confirm that beads were a regular size, alginate solution was dispensed through a 18G needle and 20 beads were selected at random and placed along a ruler (Figure 4.4b). In addition, four beads were randomly selected and weighed. The average weight of the beads without chondrocytes was 11.1mg (range: 9.3mg-12.2mg).

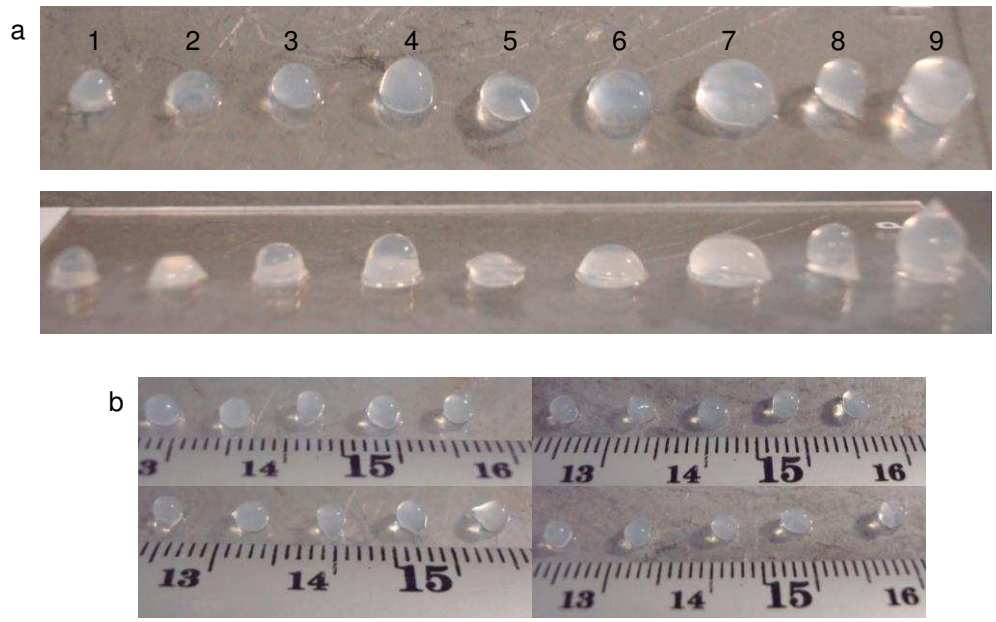


Figure 4.4: Size evaluation of alginate beads. (a) Different sized needles and pipettes were used to determine the optimum size for alginate bead culture, 1: 21 gauge (G) needle, 2: 20G needle, 3: 19G needle, 4: 18G needle, 5: 16G needle, 6: 14G needle, 7: 10ml syringe, 8: 1ml pipette, 9: 5ml pipette. (b) Beads generated with an 18G needle; 20 beads were randomly selected and assessed for regularity in size. Beads were photographed using a Sony Cyber-Shot DSC-W30 digital camera.

The next step of the alginate encapsulation process to be assessed was the seeding density of the chondrocytes. This varies in the literature from $5x10^5$ cells/ml of alginate using canine chondrocytes (Rai et al., 2009) to $1x10^7$ cells/ml of alginate used in a bovine chondrocyte study (Lee et al., 2003). In order to maximise the number of beads generated from the cells available, a cell density close to that used by Rai and colleagues (2009) was aimed for. Due to limited availability of fresh tissue, cryopreserved canine chondrocytes (P1 when frozen) were revived and expanded in monolayer (until 100% confluence was

reached) before being encapsulated into alginate beads. The initial cell density used of $4x10^5$ cells/ml of alginate was shown to be too low, through light microscopy (Figure 4.5b) and H&E staining (Figure 4.5c). The next time the beads were established, two different seeding densities were used. The cells used for this were cryopreserved canine chondrocytes (P1 when frozen) expanded in monolayer to P4. Some beads were seeded with $1x10^6$ cells/ml of alginate whereas others were seeded with $2x10^6$ cells/ml of alginate. These two densities were chosen based upon the range of densities used in the literature (Table 4.2). Also taken into consideration was the quantity of tissue available and the potential number of beads that could be generated. Beads were observed under the light microscope DM IL microscope and DCF490 camera (Leica) during culture (Figure 4.5e and f) and once culture had been terminated beads were fixed, embedded, sectioned and stained with H&E (Figure 4.5h-k). Cells were in much closer contact with each other at the higher densities and it was decided that all future beads would need to contain a minimum of $2x10^6$ cells/ml of alginate.

Species	Cell Density (cells/ml of alginate)	Reference
Human	3-4x10^6	(Bonaventure et al., 1994)
	4x10^6	*(Hauselmann et al., 1996)
	8x10^6	
	4x10^6	(Chubinskaya et al., 2001)
	2x10^6	(Schulze-Tanzil et al., 2002)
	8x10^5	(Brand et al., 2012)
Bovine	4x10^6	(Hauselmann et al., 1994)
	8x10^6	(van Osch et al., 1998)
	1x10^7	(Lee et al., 2003)
	4x10^6	(Stoddart et al., 2006)
Rat	1x10^6	(Loty et al., 1998)
Rabbit	2x10^6	(De Ceuninck et al., 2004)
Canine	5x10^5	(Rai et al., 2009)
Porcine	4x10^6	(Bernstein et al., 2009a)

Table 4.2: Chondrocyte seeding densities within alginate beads in different species. *Hauselmann and colleagues (1996) used two different densities depending on the downstream applications, the lower density being for metabolic studies; the higher density used for morphometric and biochemical analysis.

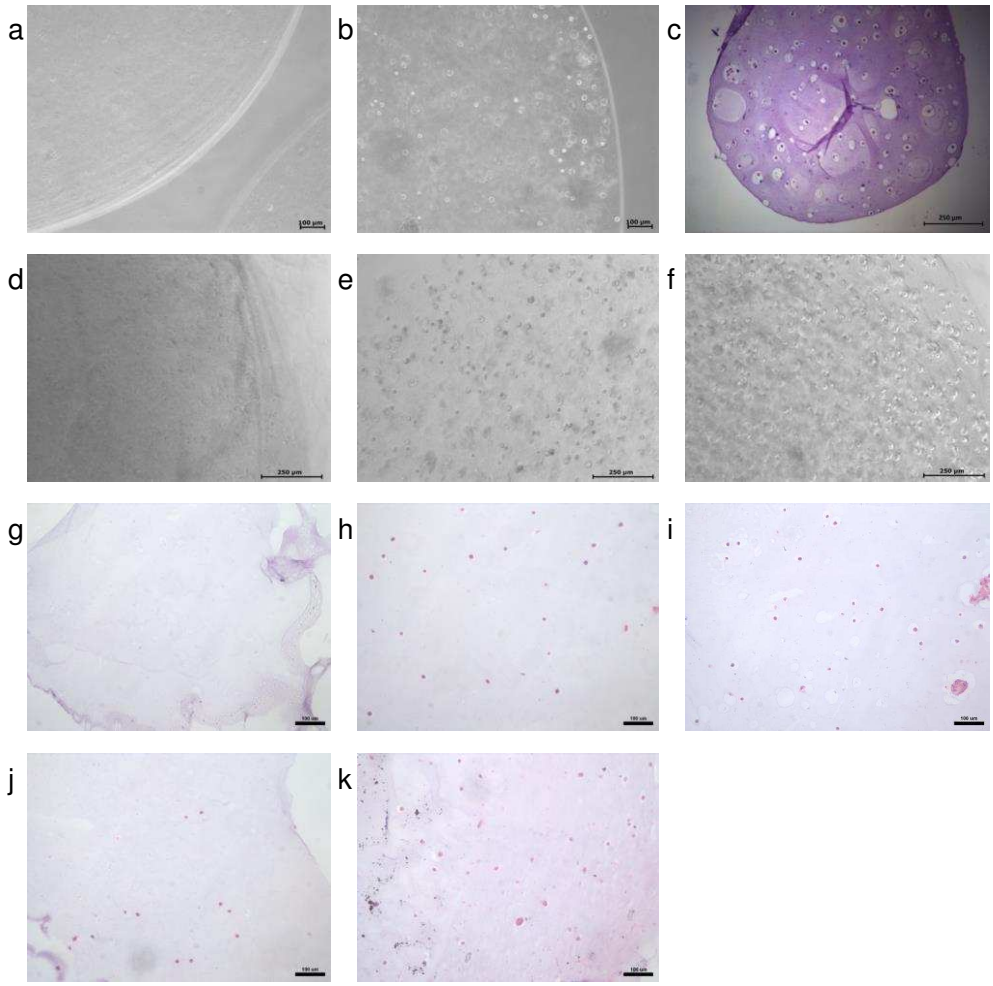


Figure 4.5: Evaluation of cell density within alginate beads. Cryopreserved canine chondrocytes were revived and expanded in monolayer before being seeded into alginate beads. The first cell density trialled, $4x10^5$ cells/ml of alginate (b) and (c), was too low. Therefore in the next culture higher densities were trialled. Specifically beads were seeded with $1x10^6$ cells/ml of alginate (e), (h) and (j) and $2x10^6$ cells/ml of alginate (f), (i) and (k). (a), (d) and (g) are alginate beads without any chondrocytes seeded. Cell density within the beads was monitored using light microscopy during culture (a)-(b) and (d)-(f) and H&E staining (c) and (g)-(k). The images in (a) and (b) were taken two days after the beads were established and (d)-(e) were taken one day after the beads were set-up. The bead in (c) was removed and fixed on day 23 of culture (day zero is when the beads were created). The beads in (g)-(i) were removed and fixed on day three and the beads in (j) and (k) on day seven. Images (a)-(f): magnification: x100, scale bars 100µm (a) and (b) and 250µm (c)-(f); (g)-(k) magnification: x200, scale bar 250µm. Sections in (c) and (g)-(k): 5µm.

In addition to observing the density of cells within the beads, the viability of the chondrocytes was also assessed. As well as determining cell viability prior to seeding into alginate using trypan blue, some cells were seeded into wells of a 96-well plate and the Cyquant NF assay was perfomed. Fluorescence was shown to increase with increasing number of seeded cells (Figure 4.6a). To assess the viability of chondrocytes encapsulated within the beads, the Alamar Blue assay was used. The Alamar Blue assay measures metabolic activity of the cells within the beads with metabolically active cells maintaining a reduced environment. As non-viable cells would not be metabolically active, Alamar Blue data can be interpreted as giving an indication of cell viability within the construct. Beads containing revived canine chondrocytes (initial seeding densities of $1x10^6$ cells/ml of alginate and $2x10^6$ cells/ml of alginate) were cultured in normal culture medium (DMEM+10%FBS+2% antibiotics) at 37^oC, 5% CO_2 until day seven. On day seven media was changed to contain various concentrations of sodium nitroprusside (SNP) (0.5µM, 1µM, 0.5mM and 1mM). SNP was added to some cultures to induce cell death in order to check that the process of alginate culture did not have an adverse effect on the chondrocytes in terms of cell viability. Control media had no SNP added. SNP ($Na_2Fe^{++}(CN)_5NO.2H_2O$) is a nitric oxide donor and has been used in previous studies to induce and study cell death in a range of cell types including chondrocytes (Yamada et al., 1996, Notoya et al., 2000, Rabkin and Kong, 2000, Del Carlo and Loeser, 2002, Kuhn and Lotz, 2003, Yoshioka et al., 2003, Kim et al., 2005). Beads were

incubated with the fresh media overnight under the same conditions. In the beads with the lower starting density the lower concentrations of SNP (0.5µM and 1µM) appeared to improve the viability of the cells within the beads (Figure 4.6c). The same affect was seen in the higher density beads incubated with 1µM SNP (Figure 4.6d). In both groups the two highest concentrations of SNP (0.5mM and 1mM) appeared to decrease the viability of the chondrocytes within the beads. Due to a loss of some beads during the culture and analysis only two beads were analysed for each concentration. Therefore, another batch of beads were incubated with SNP overnight. In this experiment the beads were seeded with P2 canine chondrocytes with an initial starting density of $2x10^6$ cells/ml of alginate. Two additional lower concentrations (0.5nM and 1nM) were added to see if they also had beneficial effects on cell viability. Three beads were tested at each concentration. 0.5nM had little impact on cell viability and 1nM caused a slight decrease in cell viability. 0.5µM, 1µM and 0.5mM increased viability, with it peaking in the beads incubated with 1µM. The highest concentration decreased cell viability, however none of the results were statistically significant.

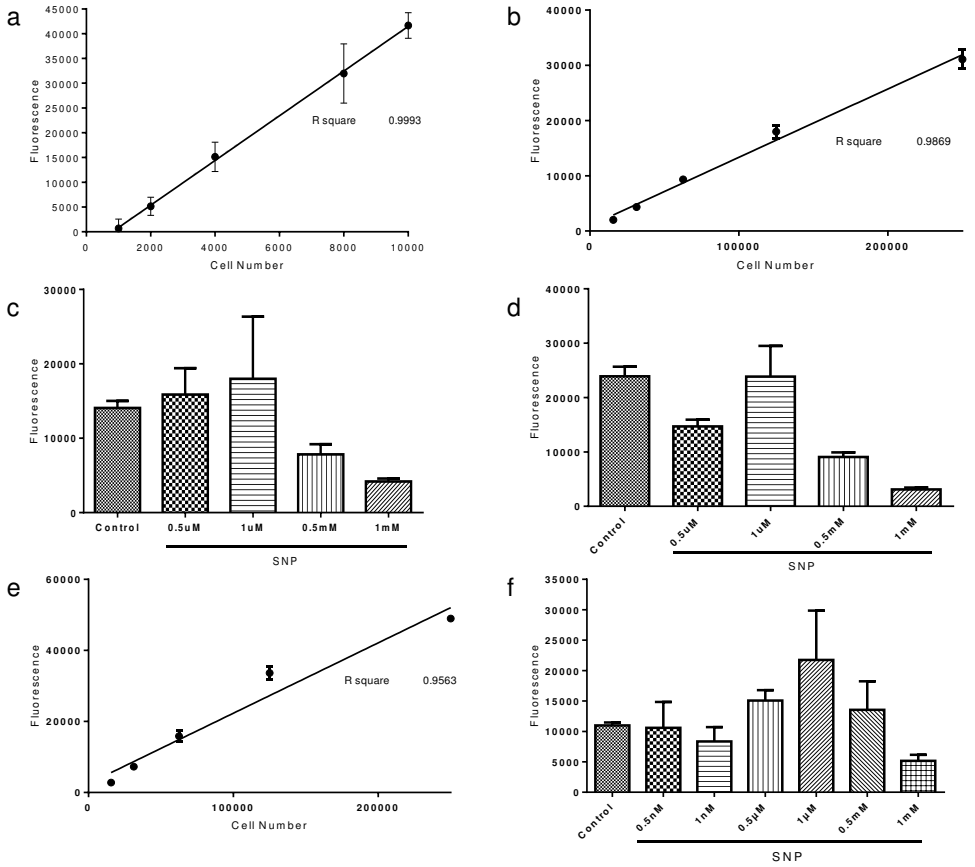


Figure 4.6: Cell viability prior to seeding and within alginate beads. Revived canine chondrocytes were expanded in monolayer up to P4. Some of these P4 cells were seeded at known densities into wells of a 96-well plate for the Cyquant NF assay (a); another aliquot of cells were seeded into wells of a 24-well plate at known densities to generate the standard curve for the Alamar blue assay (b). The remaining cells were seeded into alginate at two different densities, $1x10^6$ and $2x10^6$ cells/ml of alginate. Beads were cultured for seven days before being incubated with various concentrations of SNP (0.5µM, 1µM, 0.5mM and 1mM) overnight. (c) $1x10^6$ beads and (d) $2x10^6$ beads post SNP incubation (N=2 for each SNP concentration). A second batch of beads seeded with P2 canine chondrocytes at an initial density of $2x10^6$ cells/ml of alginate were incubated with the same concentrations of SNP along with two other concentrations; 0.5nM and 1nM. (e) Standard curve, (f) cell viability after incubation with SNP (N=3 for each concentration). (c)-(f) Alamar Blue Assay data. No concentration of SNP significantly affected the health of the cells within the beads.

4.2.2.2 Processing Alginate Beads for Histological Analysis

Different research groups have used various methods for fixing and alginate beads for histological analysis. Hauselmann and colleagues (1994) incubated alginate beads with 2.5% glutaraldehyde for two hours, whereas Stoddart *et al* (2006) embedded beads in OCT prior to fixation. Another group incubated beads in 4% PFA (in 29mM $NaH_2PO_4x1H_2O$, 28mM NaHPO4, 53mM saccharose and 10mM $CaCl_2$) for 30 minutes (Domm et al., 2002), whereas Chubinskaya and colleagues (2001) fixed beads in 4% PFA, 0.1M cacodylate buffer (pH7.4) containing 10mM $CaCl_2$ for four hours at 20^{o}C. Bernstein *et al* (2009a) used two different methods of fixation for histology and immunohistology. For the former, the authors fixed the beads in acetone for five minutes at -20^{o}C followed by methanol for five minutes at -20^{o}C (Bernstein et al., 2009a). For the latter the authors state 4% PFA was used but the incubation time is not provided (Bernstein et al., 2009a). In a more recent study beads were fixed in 70% ethanol overnight (Brand et al., 2012). In order to generate sections of beads for histological staining, a suitable fixation and embedding process had to be defined.

Taking into consideration the differences in fixatives used in the literature, the first fixative to be trialled was 4% PFA for 1.5 hours at 4^{o}C. These beads were extremely fragile and could not be manipulated for processing and embedding. This fragility could have been caused by

calcium leaching out of the beads, weakening the ionic bonds between the polymers in the alginate. For the next batch of alginate beads different fixatives, containing different ratios of 4% PFA and 102mM $CaCl_2$ (PFA only, 90:10, 70:30, 50:50, 30:70 and 10:90) were tested. The incubation time was kept the same as before. These conditions were first tested on alginate-only beads. The beads in 4% PFA only dissolved, whereas all the other beads were still intact and were successfully removed from the six-well plate into tissue processing cassettes. Fixatives containing ratios of 4% PFA and 102mM $CaCl_2$ were then applied to alginate beads containing P1 canine chondrocytes and another set of alginate only beads. Five beads were incubated in each of the five fixation solutions as before. All beads from all fixatives were successfully transferred to tissue processing cassettes post fixation. As these beads were stable enough to transfer to the tissue processing cassettes post fixation, the processing protocol was continued. Cassettes were placed in the tissue processor in which the beads were gradually dehydrated before being prepared for embedding. The exact solutions and times used are summarised in Table 4.3. None of the beads were still intact after being in the tissue processor. There are two possible reasons for this: (1) the PFA was diluted to less than 4%, so the beads may not have been fixed for long enough (2) the beads may have melted in the wax, as the wax has to be heated to 55^{o}C to be in liquid form. Therefore, in the next batch of fixation solutions a final concentration of 4% PFA was ensured by starting with a more concentrated stock solution. The three solutions with the

highest PFA content were selected for the next test (PFA: $CaCl_2$ ratios: 90:10, 70:30 and 50:50).

Container	Solution	Time (mins)	Process
1	70% Ethanol	30	Gradual dehydration of the tissue
2	80% Ethanol	30	
3	95% Ethanol	30	
4	100% Ethanol	15	
5	100% Ethanol	15	
6	100% Ethanol	15	
7	100%Ethanol	15	
8	Histoclear	30	Preparation for the wax
9	Histoclear	30	
10	Histoclear	30	
11	Paraffin	30	Preparation for embedding
12	Paraffin	45	

Table 4.3: Solutions and times used in the tissue processor to prepare samples for embedding in wax. The tissue processor has a variety of automated programs where the times in the solutions vary.

In addition to this, the processing protocol was reduced and performed manually. Although it was suspected that the beads may have melted in the wax, they could have been destroyed earlier on in the process. Due to the small size of the beads, incubation in each solution was severely reduced. The manual protocol up to the last Histoclear incubation is summarised in Table 4.4. To assess the survival of the

beads within the paraffin wax, beads were observed in the molten wax and timed. Molten wax was placed into a metal mould, which was placed on a heated stirrer (Heidolph MR 3001) with a thermometer attached. The wax was heated to 55^{o}C, keeping the conditions the same as in the tissue processor. The beads fixed in the 90:10 solution only lasted 90 seconds in the wax. The 70:30 beads were still intact even after seven minutes in the wax and the 50:50 beads were still intact after five minutes. It was decided that the 90:10 solution would be discarded as a potential fixative and that five minutes would be the optimum incubation in molten wax.

Solution	Time (mins)
70% Ethanol	5
80% Ethanol	5
95% Ethanol	5
100% Ethanol	5
100% Ethanol	5
Histoclear	5
Histoclear	5

Table 4.4: Solutions and times used for manually preparing alginate beads for embedding in wax. The solutions are the same as those used in the automated process but the timings have been severely reduced.

Alginate beads seeded with canine chondrocytes were fixed using the 70:30 or 50:50 fixation solutions. These beads were then processed manually using the solutions and timings in Table 4.3. The final stage of processing before embedding was a five-minute incubation in molten wax. These beads were sectioned and stained with H&E and alcian blue (Figure 4.7). Little difference in staining was observed between the beads fixed with the different solutions, however the structure of the beads and the cells within them appeared to be marginally better preserved by the 50:50 solution (Figure 4.7b and d) compared to the 70:30 solution (Figure 4.7a and c). Therefore the 50:50 solution was used for all subsequent fixation of alginate beads. A basic overview of the processes involved in successfully fixing and processing the beads is provided in Figure 4.8.

One problem highlighted during this test was the intense background staining of the alginate with the Alcian Blue. This may be because alginate is an anionic polymer (Lee and Mooney, 2012) and alcian blue contains cationic isothiouronium groups which are thought to interact with polyanionic molecules within tissues via electrostatic bonds (Myers et al., 2008). Thus the Alcian Blue staining protocol had to be evaluated to determine a method which would differentiate between staining of the alginate and staining of GAGs produced by the chondrocytes within the beads.

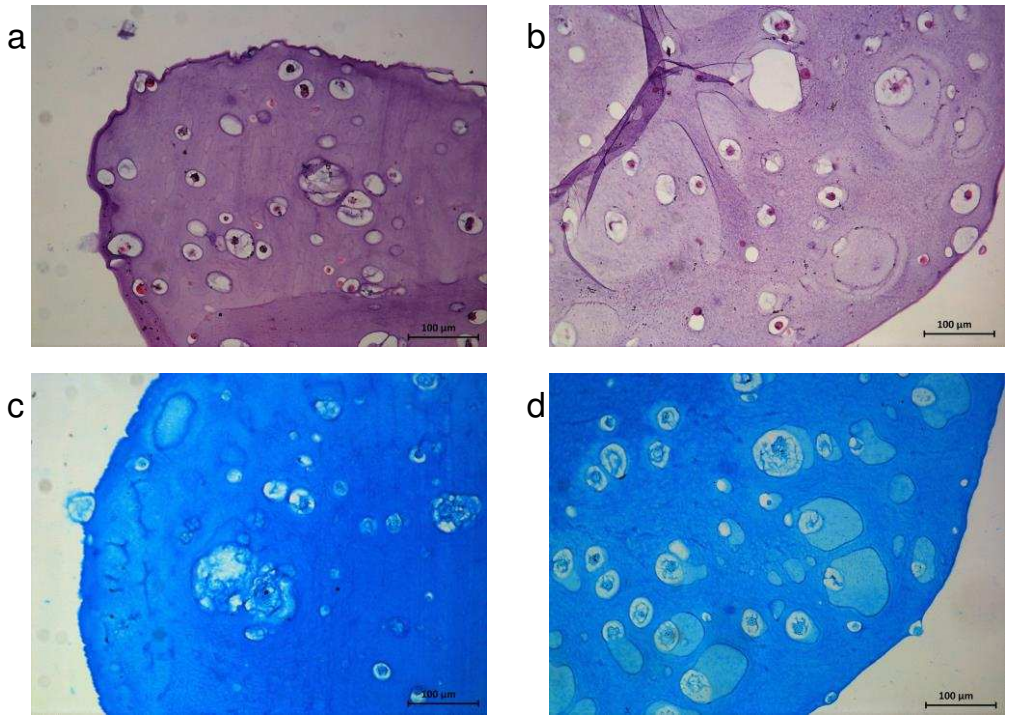


Figure 4.7: Optimisation of fixation and processing of alginate beads seeded with canine chondrocytes. Alginate beads were fixed with solutions containing different ratios of 4% PFA and 102mM $CaCl_2$; either 70:30 (a) and (c) or 50:50 (b) and (d). Beads were manually processed, embedded in wax and sectioned. 5µm sections were stained with H&E (a) and (b) or alcian blue (c) and (d). There was minimal difference between the two fixation solutions; the 50:50 solution seemed marginally better at preserving the cells within the beads, so this was selected for all subsequent fixation of alginate beads. All images were taken with a magnification of x200, scale bars: 100µm.

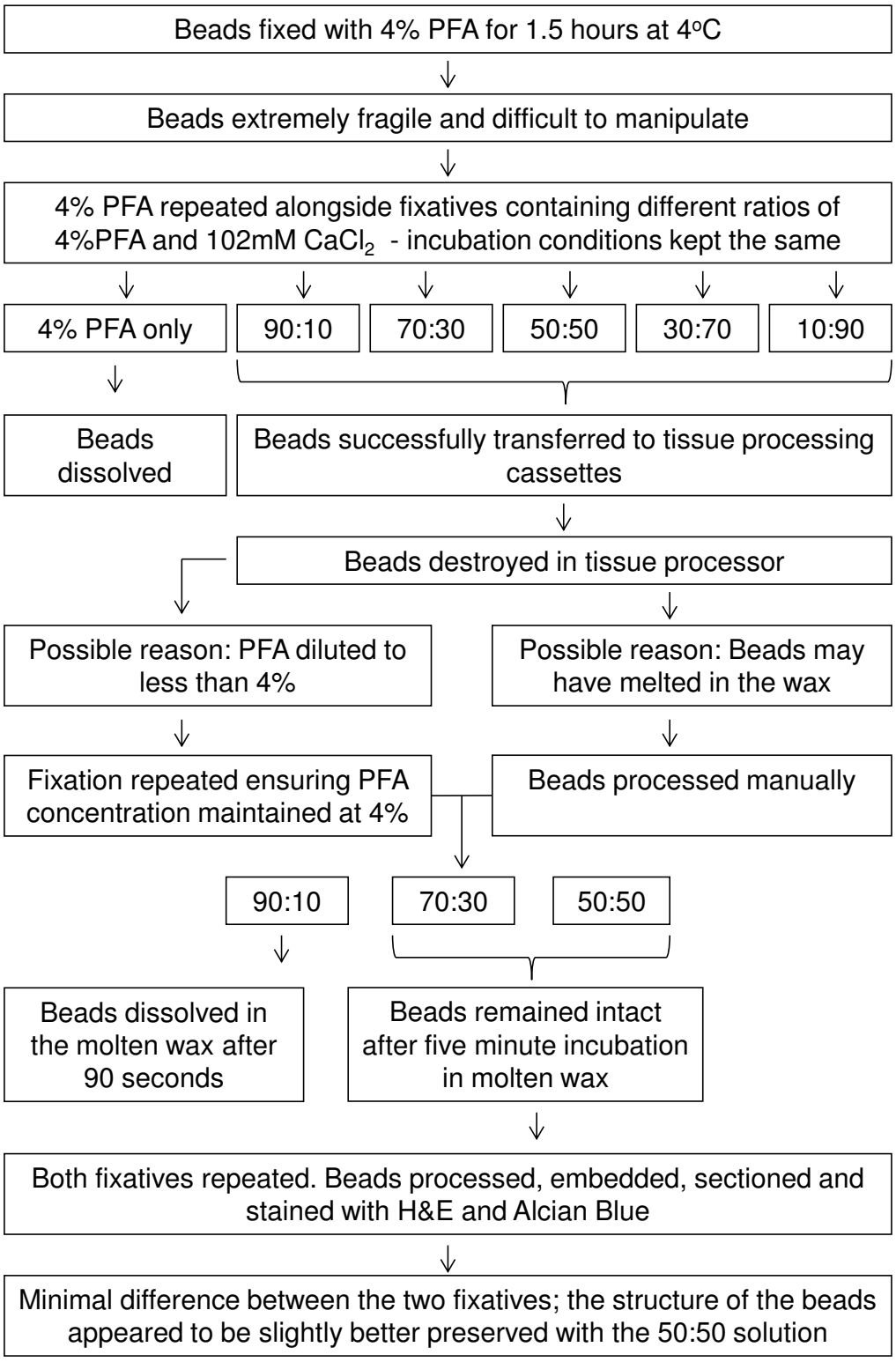


Figure 4.8: Overview of the processes involved in successfully fixing and processing alginate beads for histological analysis.

4.2.2.3 Alcian Blue Optimisation

The steps taken to reduce the background staining of the alginate with alcian blue are summarised in Figure 4.9. Briefly, various conditions were changed throughout the optimisation process, beginning with increasing the length of the washing step. The problem with intense background staining was initially observed in beads seeded with canine chondrocytes. The optimisation of staining was performed on alginate-only beads. This was to ensure that the stain would specifically indentify GAGs produced by the chondrocytes within the beads as opposed to staining the alginate. Reducing the incubation times and altering the washing times still resulted in a uniform, strong staining of the alginate. The most effective condition for reducing staining of the alginate was having the alcian blue at a concentration of 0.02%, dipping the slides in this solution three times and then washing the slides for ten minutes (Figure 4.9). The pH of the alcian blue solution was kept constant at 2.5 throughout the optimisation process.

As the background staining of the alginate had been reduced to acceptable level, which should enable the differentiation between staining of alginate and GAGs produced by the chondrocytes; day three beads seeded with canine chondrocytes (initial density of $2x10^6$ cells/ml of alginate) were stained using the 0.02% alcian blue (three dips, ten minute wash). Staining of the alginate was very weak; however, there was no specific staining indicating GAG production (Figure 4.10a and b).

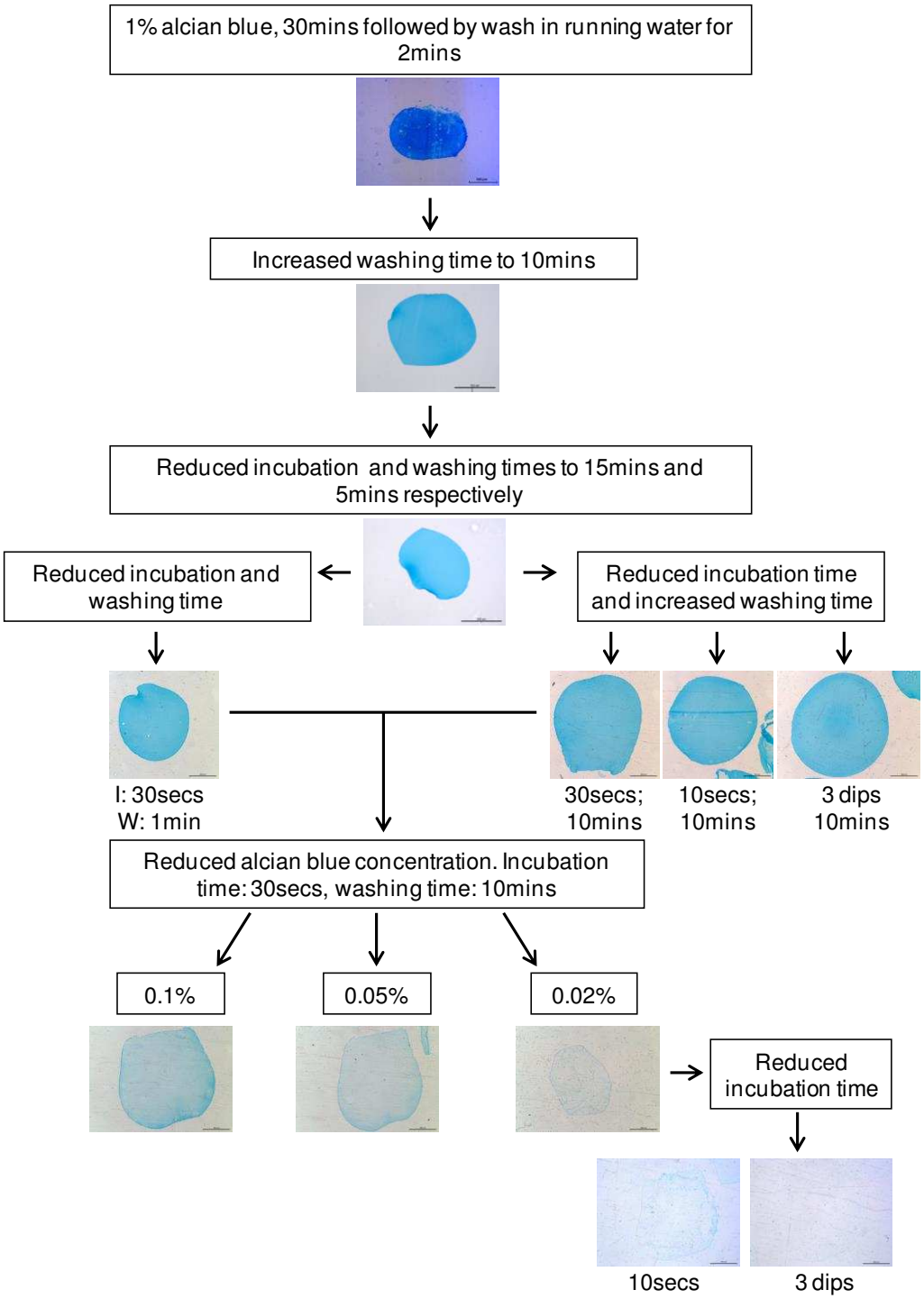

1% alcian blue, 30mins followed by wash in running water for 2mins
Increased washing time to 10mins
Reduced incubation and washing times to 15mins and 5mins respectively
Reduced incubation and washing time
Reduced incubation time and increased washing time
I: 30secs
W: 1min
30secs; 10mins
10secs; 10mins
3 dips 10mins
Reduced alcian blue concentration. Incubation time: 30secs, washing time: 10mins
0.1%
0.05%
0.02%
Reduced incubation time
10secs
3 dips

Figure 4.9: Optimisation of Alcian Blue staining of alginate beads. Various factors in the alcian blue staining process were altered in order to reduce to background staining of the alginate. A combination of reducing the incubation time with and concentration of alcian blue as well as increasing the washing time was found to significantly reduce the background staining. The lowest background was observed with 0.02% alcian blue, three dips and 10mins washing. The pH of the alginate was kept at 2.5 throughout. Images: magnification: x50, scale bars: 500µm.

It was decided to increase the incubation time to ten and twenty seconds (Figure 4.10c-f). Again only generic background staining was observed. There are two possible reasons for this; 0.02% is too weak to stain for GAGs or there was insufficient GAG production after three days in culture to be detected by the histological stain Alcian Blue. In addition to optimising the Alcian Blue staining conditions, it was thought, due to the small size of the beads, that the fixation time could be reduced. Some beads were fixed for one hour at 4^{o}C and another group were fixed for 10mins at room temperature. It was difficult to determine whether there was any difference between the two fixing periods from the Alcian Blue images, so some sections were stained with H&E (Figure 4.10g and h). The H&E images show little difference between the two fixation times. As the beads appeared to be sufficiently fixed after ten minutes, this shorter incubation was adopted in all future work.

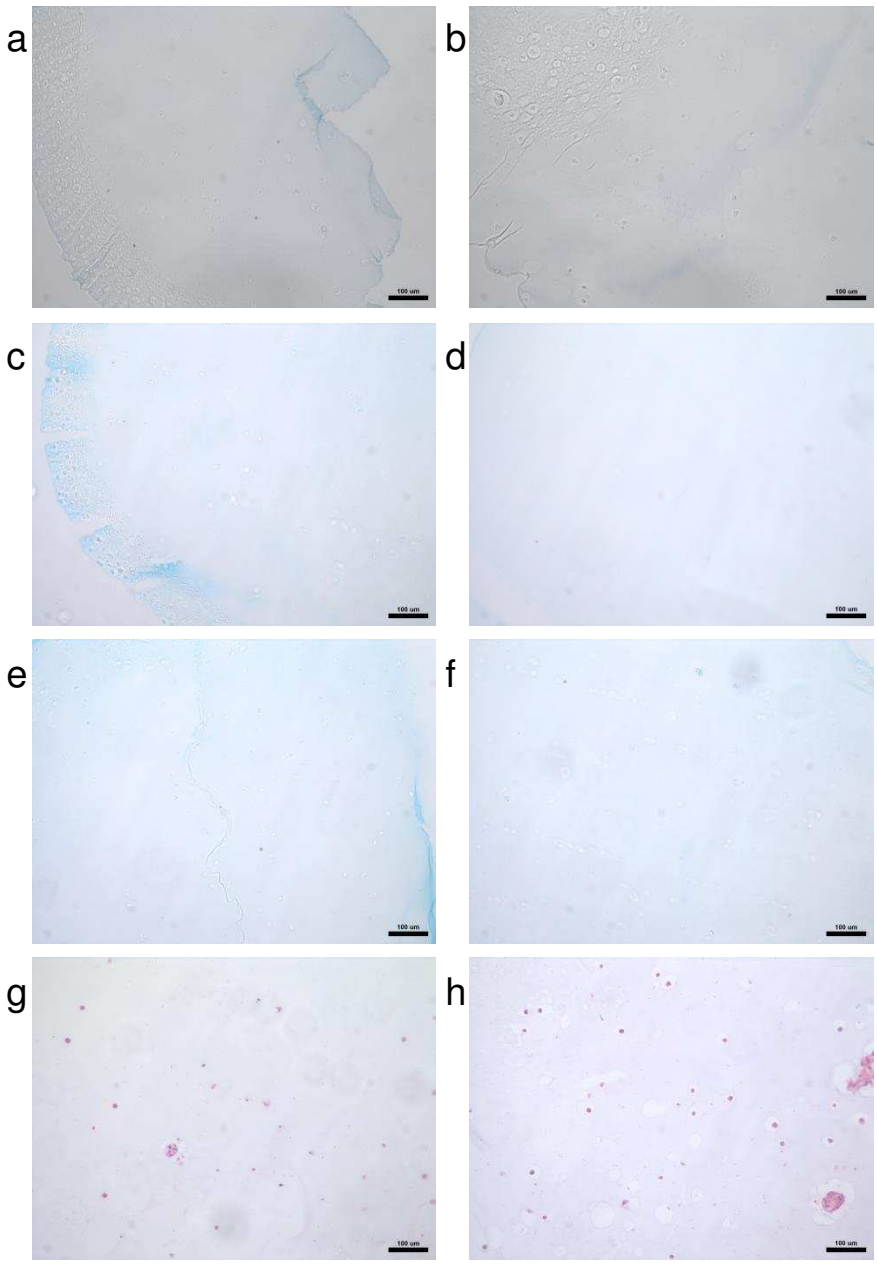


Figure 4.10: Alcian Blue and H&E staining of alginate beads seeded with canine chondrocytes. Initial seeding density for alginate beads: $2x10^6$ cells/ml. Beads were either fixed for one hour at 4°C (a, c, e and g) or ten minutes at room temperature (b, d, f and h). Slides were dipped three times in Alcian Blue (a and b) or incubated with the stain for ten (c and d) or twenty (e and f) seconds. Sections in (g) and (h) were stained with H&E. Only generic background staining was seen in all the sections stained with alcian blue. There appeared to be no difference between samples fixed for different lengths of time. All images taken with a magnification of x200, scale bars: 100µm.

4.2.2.4 Safranin O

Due to the lack of specific staining observed with Alcian Blue, a different stain was tested. Safranin O has been used in the literature to stain for GAGs produced by chondrocytes in alginate beads (Chubinskaya et al., 2001, Chubinskaya et al., 2007, Chubinskaya et al., 2008). Safranin O is a positively charged dye that binds to polyanions (molecules or chemical complexes containing multiple negatively charged sites) (Rosenberg, 1971), therefore it will bind to the negatively charged chondroitin 6-sulphate or keratan sulphate (Rosenberg, 1971). Professor Susan Chubinskaya's group at Rush University routinely stain chondrocyte seeded alginate beads with Safranin O and their method was followed for the staining of alginate beads in this study. Their protocol was adapted from that given in (Rosenberg, 1971). Some slides were sent to Professor Chubinskaya's group for staining. Although the alginate stained intensely, like with the Alcian Blue, the staining for GAGs could be differentiated from this background staining (Figure 4.11).

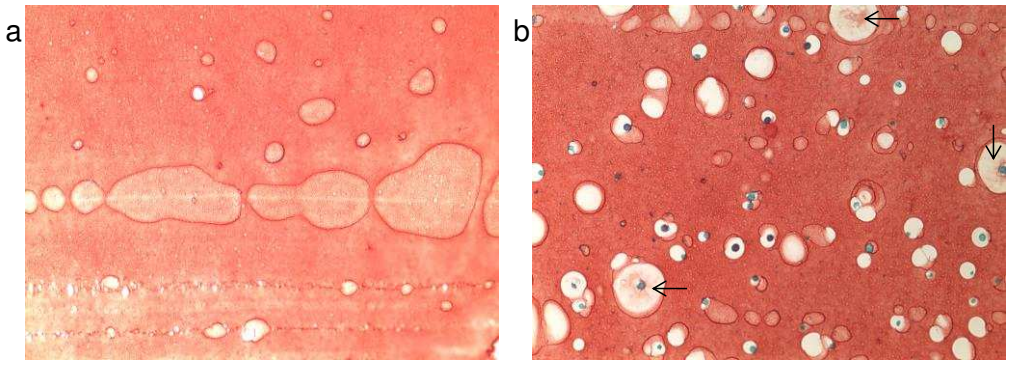


Figure 4.11: Safranin O staining of alginate beads. (a) Alginate-only bead (b) chondrocyte seeded bead (arrows indicate staining for GAGs). Images: magnification: x200. Sections: 5µm. No scale bars were provided by Professor Chubinskaya's group.

As staining of alginate and GAGs could be differentiated with Safranin O, this was used to stain test samples.

4.2.2.5 Phenotype of Canine Chondrocytes of Different Passages Cultured within Alginate Beads

Having established the 3D culture system of encapsulating chondrocytes within alginate beads, it was decided to test different passages within this environment to see if their differentiation capabilities differed depending on the length of time spent in monolayer culture. It had been reported previously that chondrocytes lose their ability to redifferentiate after prolonged monolayer culture (Benya and Shaffer, 1982). Canine chondrocytes were expanded in monolayer and encapsulated within alginate beads at passages two, three and four. The cells were seeded into alginate beads at an initial density of $2x10^6$ cells/ml of alginate. Beads were culture in culture medium

(DMEM+10%FBS+2% antibiotics) at 37°C, 5% CO_2 for 28 days. GAG production within the beads was assessed by Safranin O staining (Figure 4.12). Staining was observed in beads seeded with chondrocytes from all three passages (Figure 4.12b-d). There was no clear difference in the staining between the different beads.

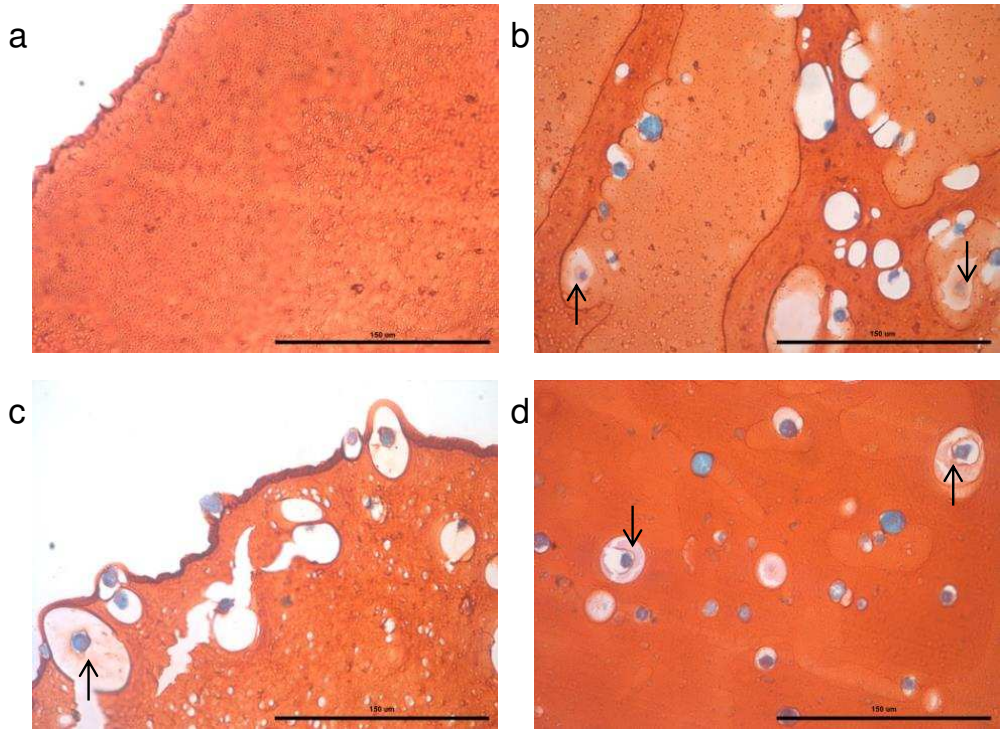


Figure 4.12 Alginate beads seeded with canine chondrocytes from different passages stained for GAG production using Safranin O. (a) Alginate-only bead, (b) bead seeded with P2 cells, (c) bead seeded with P3 cells, (d) bead seeded with P4 cells. Arrows indicate staining of GAGs; staining appeared to be similar in all beads, regardless of the passage of the cells seeded. Sections: 5µm. Images taken with a magnification of x400, scale bar: 150µm.

The chondrogenic phenotype of the cells encapsulated within the beads was assessed by detecting for collagen types I and II and CD44 by

immunohistochemistry and western blotting. The staining for collagen type I in the beads seeded with P2 and P3 canine chondrocytes looked similar to that observed in the isotype control (Figure 4.13b-f) with intense staining immediately surrounding the cells. Some intense staining was also seen in the same area in the beads seeded with P4 cells (Figure 4.13g); however in some sections there was some lighter, marginally more widespread staining.

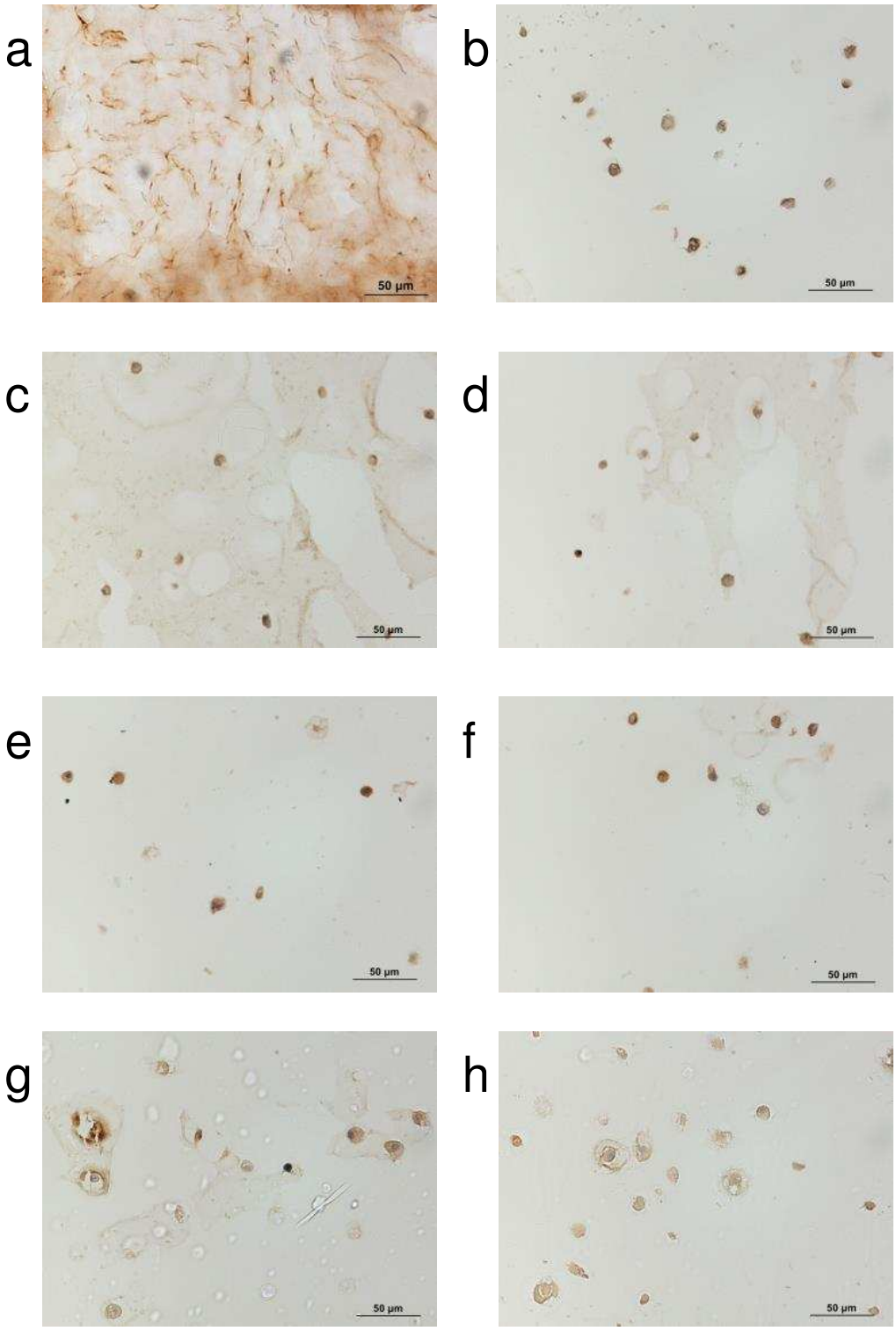

a
b
c
d
e
f
g
h
50 µm

Figure 4.13: Expression of collagen type I in alginate beads seeded with P2, P3 and P4 canine chondrocytes. (a) Canine tendon tissue stained for collagen type I (positive control), (b) alginate bead seeded with P2 canine chondrocytes incubated with an isotype control primary antibody (c-h) alginate beads seeded with chondrocytes from different passages stained for collagen type I; P2 (c-d), P3 (e-f) and P4 (g-h). Staining in the P2 and P3 beads appears similar to the isotype control, indicating non-specific staining. There are areas similar to the isotype control in the P4 beads but there are also areas of more diffuse staining. Sections: 5µm. Images taken with a magnification of x400, scale bars: 50µm.

Staining for collagen type II within the P2-seeded beads had the same distribution as the isotype control (immediately surrounding the cells) but was more intense (Figure 4.14b-d). Beads seeded with P3 chondrocytes also stained intensely for collagen type II in the area immediately surrounding the cells (Figure 4.14e-f). Staining was strongest where there was a cluster of cells present. Collagen type II expression appeared to be the strongest in P4-seeded beads (Figure 4.14g-h). Intense staining was observed around the cells as well as a lighter staining throughout bead.

CD44 staining was observed immediately surrounding the cells (Figure 4.15d-e). Staining appeared to be more intense and specific in all samples compared to the isotype control; the latter showed light staining of the alginate and slighter darker pockets of staining around the cells (Figure 4.15c). The strongest detection of CD44 was observed in beads seeded with P4 chondrocytes.

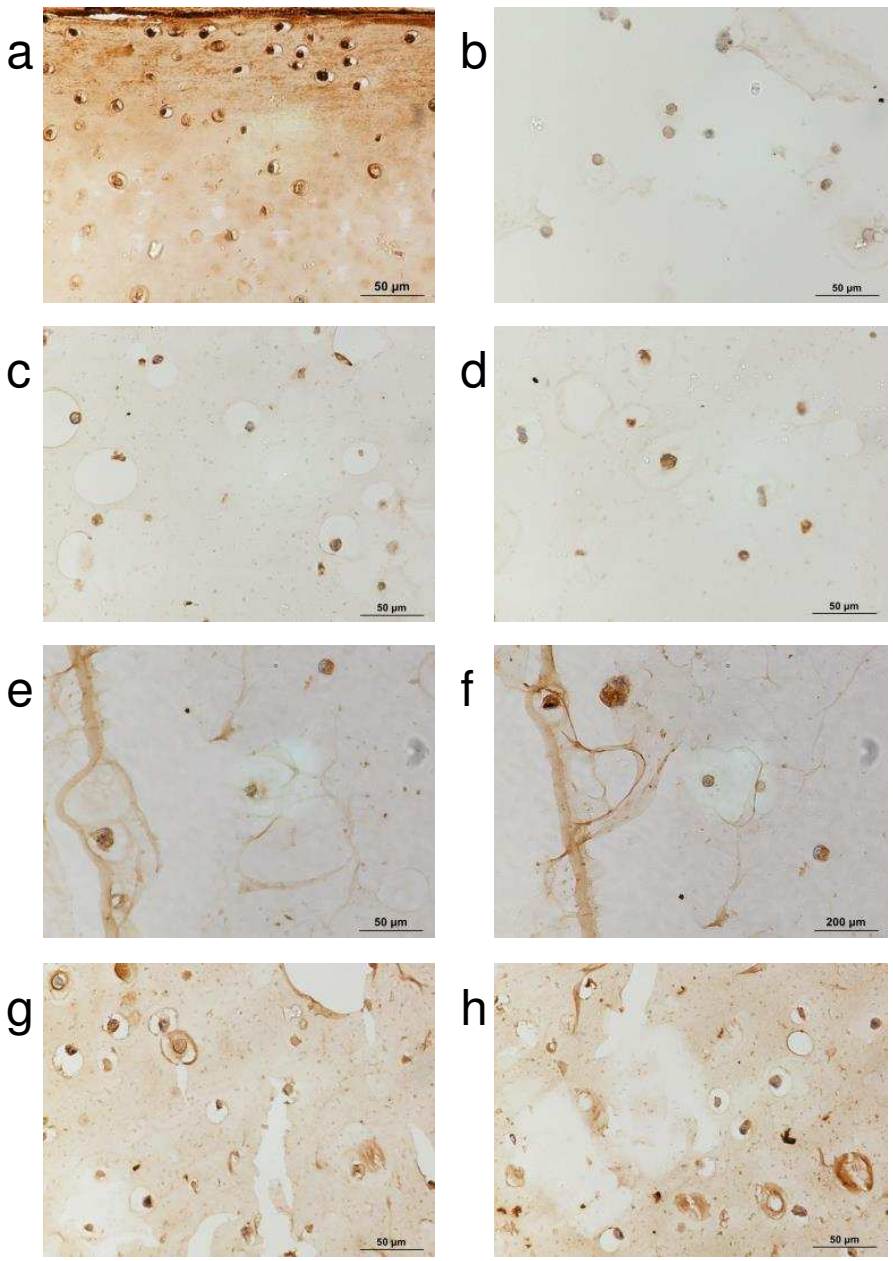


Figure 4.14: Expression of collagen type II in alginate beads seeded with P2, P3 and P4 canine chondrocytes. (a) Canine cartilage tissue stained for collagen type II (positive control), (b) alginate bead seeded with P2 canine chondrocytes incubated with an isotype control primary antibody (c-h) alginate beads seeded with chondrocytes from different passages stained for collagen type II; P2 (c-d), P3 (e-f) and P4 (g-h). The strongest staining was seen in beads seeded with P4 canine chondrocytes and was dispersed throughout the bead. Sections: 5µm. Images taken with a magnification of x400, scale bars: 50µm.

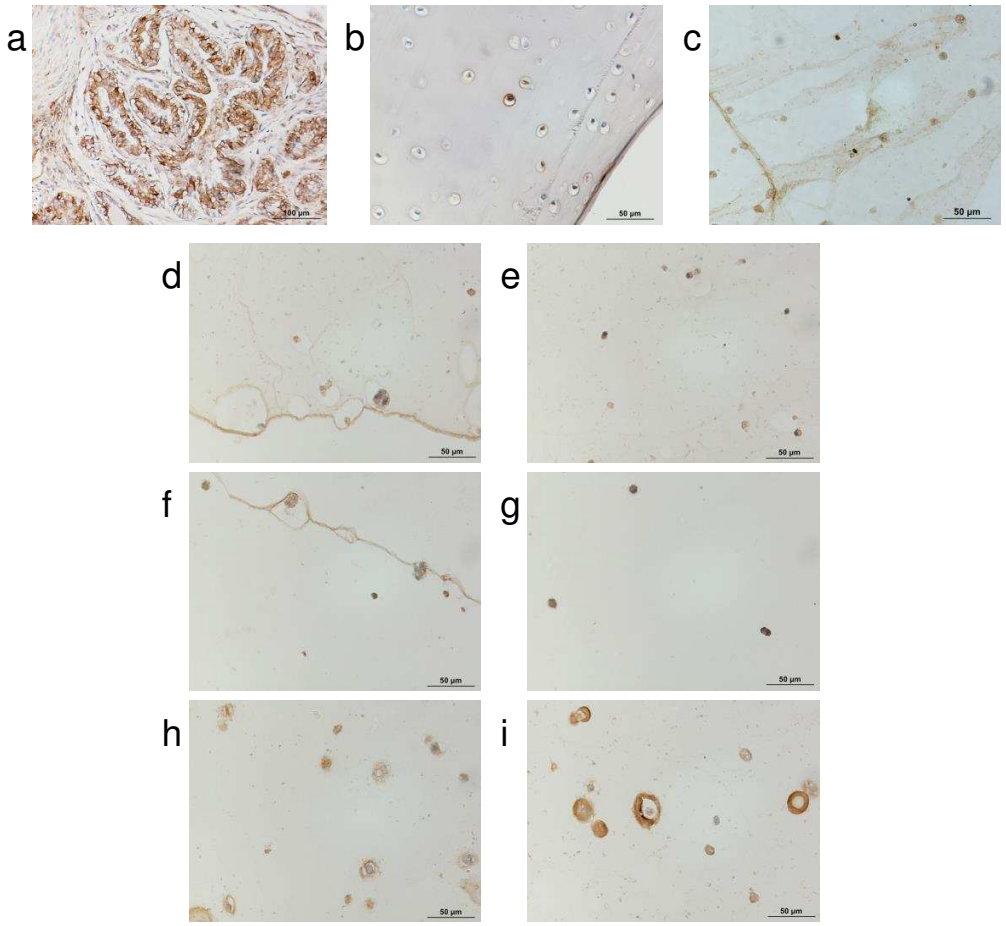
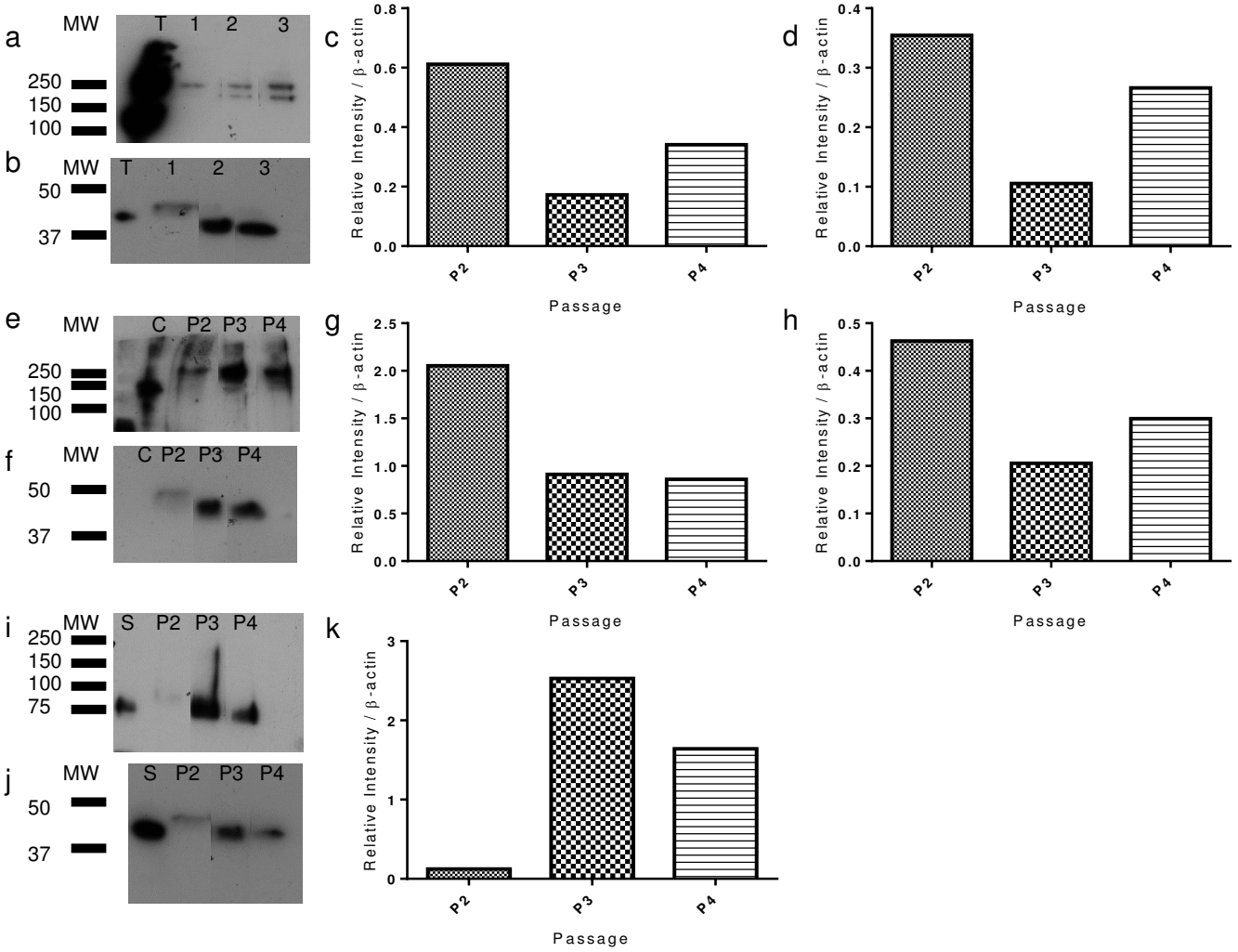


Figure 4.15: Expression of CD44 in alginate beads seeded with P2, P3 and P4 canine chondrocytes. (a) Canine mammary tumour tissue stained for CD44 (positive control) (unstained tissue section and anti-canine CD44 antibody (MAB5449, R&D Systems) kindly provided by Dr Cinzia Allegrucci, University of Nottingham), (b) Canine cartilage tissue stained for CD44 (positive control), (b) alginate bead seeded with P2 canine chondrocytes incubated with an isotype control primary antibody (c-h) alginate beads seeded with chondrocytes from different passages stained for CD44; P2 (c-d), P3 (e-f) and P4 (g-h). Sections: 5µm. Images taken with a magnification of x200 (a), scale bar: 100µm and a magnification of x400, scale bars: 50µm (b-i).

To further investigate the expression of collagen types I and II and CD44 by the cells encapsulated within the beads, beads were

dissociated and a cell lysate was generated for use in western blotting. For the sample (P2), 10 beads and 20 beads were both dissociated to generate a cell lysate for western blotting. The protein concentration in both samples was too low to obtain an accurate value. Therefore, in subsequent samples the number of beads used to generate cell lysates was increased to 50 beads. The P2 samples were freeze-dried to concentrate the protein within the sample. The larger sample (20 beads) produced a high enough protein concentration to load 25µg on to the SDS-PAGE gel. 25µg of all samples were loaded onto the SDS-PAGE gel.

In the blots for collagen type I, two bands were detected in the samples from the P3 and P4 beads (Figure 4.16a). The lower band is not visible in the blot for P2 beads; the densitometry result for this sample may have been influenced by the shadowing from the positive control band (Figure 4.16d). The densitometry for the higher molecular weight band indicates that collagen type I expression is highest in the beads seeded with P2 chondrocytes (4.16b). Collagen type II expression was also highest in the P2 seeded beads (Figure 4.16e-h). CD44 expression was lowest in the beads seeded with P2 chondrocytes (i-k). There was a peak in CD44 expression in P3 seeded beads.


a
MW
T 1 2 3
250
150
100
b
MW
T 1 2 3
50
37
c
Relative Intensity / β-actin
0.8
0.6
0.4
0.2
0.0
P2 P3 P4
Passage
d
Relative Intensity / β-actin
0.4
0.3
0.2
0.1
0.0
P2 P3 P4
Passage
e
MW
C P2 P3 P4
250
150
100
f
MW
C P2 P3 P4
50
37
g
Relative Intensity / β-actin
2.5
2.0
1.5
1.0
0.5
0.0
P2 P3 P4
Passage
h
Relative Intensity / β-actin
0.5
0.4
0.3
0.2
0.1
0.0
P2 P3 P4
Passage
i
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250
150
100
75
j
MW
S P2 P3 P4
50
37
k
Relative Intensity / β-actin
3
2
1
0
P2 P3 P4
Passage

Figure 4.16: Expression of collagen types I and II and CD44 by chondrocytes cultured within alginate beads. Cells were expanded in monolayer and seeded into alginate at P2, P3 and P4. Chondrocytes were cultured in alginate beads for 28 days. (a) western blot for collagen type I (b) corresponding beta actin blot (c) densitometry for the upper band on the blot in (a), (d) densitometry for the lower band on the blot in (a); (e) western blot for collagen type II, (f) corresponding beta actin blot, (g) densitometry for the upper band on the blot in (e), (h) densitometry for the lower band on the blot in (e); (i) western blot for CD44, (j) corresponding beta actin blot, (k) densitometry for the blot in (i). 25µg of each bead lysate was loaded onto the gel. All lysates were generated from 50 beads except P2 which was generated from 20 beads. Exposures: (a) and (e): one minute 30 seconds, (i): three minutes, (b), (f) and (j): one second. The P2 chondrocytes had the highest expression of both collagen types and the lowest expression of CD44. T: canine tendon lysate (10µl), C: canine cartilage lysate (7µl), S: canine spleen lysate (20µg). For original western blot images see Appendix D.

4.3 Discussion

Many research groups have used various methods of 3D culture to maintain the chondrocyte phenotype, induce chondrogenic differentiation and generate articular cartilage *in vitro*. One of the most common methods used is high density pellet culture and as such this technique was initially adopted for this study. However, pellets were very unstable during culture and processing. Therefore, it was decided that the chondrocytes would need a supporting material for 3D culture. Although it must be acknowledged that some research groups use an alternative method for generating high density pellets. Instead of

centrifugation, some researchers have seeded chondrocytes at high densities into 48 (Ibold et al., 2009) and 96 (Lubke et al., 2005) well plates. This could be considered as an alternative to providing a supporting structure.

Alginate was chosen because it has been used to culture chondrocytes from a variety of species including humans (Hauselmann et al., 1996, Chubinskaya et al., 2001), cows (Hauselmann et al., 1994, van Osch et al., 1998, Lee et al., 2003), rabbits (De Ceuninck et al., 2004), rats (Loty et al., 1998) and dogs (Rai et al., 2009). Alginate is a naturally occurring polymer found in the intercellular spaces and cell walls of brown algae (Nussinovitch and Nussinovitch, 2011, Lee and Mooney, 2012).

Seeding the cells into alginate beads created a more stable 3D culture, which could be manipulated and processed for downstream analysis. When the viability of the cells within the beads was assessed using different concentrations of SNP, it was found that 1µM, 0.5µM and 1nM increased cell viability and 0.5nM and 0.1nM only slightly decreased cell viability. Results for 0.5mM SNP were variable and 1mM SNP consistently reduced cell viability although none of this data was statistically significant. Chondrocyte cell death induced by 1mM SNP incubation has also been reported in the literature (Notoya et al., 2000, Kim et al., 2005, Cherng et al., 2008). Another group reported significant cell death of chondrocytes after incubation with 1.6mM SNP

(Kuhn and Lotz, 2003). Two papers found 0.5mM SNP decreased chondrocyte viability (Notoya et al., 2000, Cherng et al., 2008), however only Cherng and colleagues (2008) found this to be significant.

There are differences in the literature in terms of how alginate beads are processed for downstream applications, fixation for histological techniques being one example. Considerable work was involved in developing a technique for the successful fixation, processing, embedding and sectioning of alginate beads.

It had been previously reported that chondrocytes lose their ability to redifferentiate after prolonged monolayer culture (Benya and Shaffer, 1982). In addition to this, different research groups have seeded chondrocytes into alginate at different passages including P0 (Bonaventure et al., 1994, Hauselmann et al., 1994, Hauselmann et al., 1996, Chubinskaya et al., 2001), P2 (Bonaventure et al., 1994), P3 (Rai et al., 2009), P4 (Bernstein et al., 2009a) and P5 (Caron et al., 2012). One group recommend that human chondrocytes passaged no more than three or four times should be used for alginate encapsulation (Brand et al., 2012). Therefore, once the alginate bead culture had been established, chondrocytes from different passages were seeded into the beads to see if passage number had an effect on redifferentiation capacity. Canine chondrocytes were expanded in monolayer up to P4 and cells from P2, P3 and P4 were seeded into alginate beads at a density of $2x10^6$ cells/ml of alginate. Beads

containing cells from each passage were assessed for the presence of collagen types I and II and CD44 using immunohistochemistry. Staining for collagen type I was non-specific as the staining observed was similar to that in the isotype control. Sections stained for collagen type II exhibited a similar pattern to that of the negative control apart from beads seeded with P4 chondrocytes which had positive staining throughout the beads. Staining for CD44 also appeared to be strongest in beads seeded with P4 chondrocytes. To provide a clearer insight into the proteins expressed by the chondrocytes within the beads, western blotting was performed.

Collagen type I expression was highest in beads seeded with P2 chondrocytes, followed by P4-seeded beads and P3-seeded beads respectively. A similar pattern was observed for collagen type II expression; although for one of the bands, intensity was slightly lower in for P4 beads compared to P3. CD44 expression was considerably lower in P2-seeded beads compared to those seeded with P3 and P4 chondrocytes. Expression peaked in beads seeded with P3 chondrocytes. The majority of studies involving chondrocytes seeded into alginate beads use cells from one passage. One group did compare mRNA expression of collagen type X and aggrecan by P0 and P2 cells encapsulated in alginate (Bonaventure et al., 1994). They found that expression of both genes were higher in P0 cells (Bonaventure et al., 1994). The authors also show that collagen type II mRNA was expressed in P2 cells but no data is shown for the P0 cells

(Bonaventure et al., 1994). This would seem to suggest that the P2 chondrocytes have retained at least some of their native phenotype by still expressing collagen type II. Lee and colleagues (Lee et al., 2003) cultured bovine chondrocytes in monolayer up to P4 and subsequently seeded cells in alginate and cultured them for seven days. Alongside this, they also seeded freshly isolated cells into alginate and passaged cells into new batches of alginate, again up to P4 (Lee et al., 2003). The immunohistochemistry data presented was just for P4 (Lee et al., 2003). The images show that cells previously expanded in monolayer prior to alginate culture were positive for collagen type I but were negative for both collagen type II and chondroitin sulphate (Lee et al., 2003). However, for cells passaged in alginate the opposite was the case (Lee et al., 2003). Part of this data contrasts with the data presented in this chapter as collagen type II expression was clearly identified the P4 group through western blotting. Another group assessed the redifferentiation of P5 human chondrocytes (Caron et al., 2012). The authors compared redifferentiation in monolayer, alginate and pellet culture. All cells were cultured in the same chondrogenic medium (DMEM/F12, 1% antibiotic/antimycotic and 1% NEAA, supplemented with 1% insulin-transferrin-sodium selenite medium supplement (ITS), 1% L-ascorbic acid-2-phosphate and 10ng/ml TGF-β3) (Caron et al., 2012). Interestingly, mRNA expression of collagen type II was higher in alginate beads and pellets, however the highest protein expression was seen in monolayer cultures (Caron et al., 2012). This would seem to suggest that there is a difference seen in mRNA

and protein expression in different culture conditions and could be an interesting avenue to explore further with the work in this thesis. Although collagen type II was detected in alginate beads seeded with P4 canine chondrocytes, levels were lower than P3 and P2, suggesting a reduced ability to synthesise collagen type II with increasing passage. Collagen type II expression was also observed in monolayer culture at all passages (P1-P5) (see Chapter 3, Figure 3.18), although there was a decrease with increasing passage. This would appear to contrast with Caron's group who observed high levels of collagen type II in monolayer culture at P5. Collagen type I mRNA and protein expression was highest in alginate beads (Caron et al., 2012). This would suggest that alginate culture does not completely redifferentiate monolayer expanded chondrocytes. This is in agreement with the findings in this thesis as dedifferentiation markers CD44 and collagen type I were expressed in all alginate cultures.

4.4 Conclusion

The majority of the work presented in this chapter focused on establishing a 3D culture system for canine articular chondrocytes, which could be manipulated for downstream applications and analysis. After initial problems with the high density pellet culture method, a supporting material was chosen. Alginate beads were successfully established and after adjusting the preparation of samples, the

phenotype of the cells encapsulated within the beads could be assessed using immunohistochemistry and western blotting.

The final part of this chapter was to see if passage number of the monolayer cultures had an impact on chondrocyte redifferentiation. In terms of collagen type II and CD44 expression, P2-seeded alginate beads appeared to contain cells with a more chondrocyte-like phenotype. However, collagen type I expression was still high in these beads. These initial results suggest that P2 chondrocytes have a greater capacity for redifferentiation; however 3D culture alone is not enough to promote complete redifferentiation. The next chapter focuses on ways of enhancing the redifferentiation process.

5. Enhancement of Chondrocyte Redifferentiation in 3D Culture

5.1 Background

The *in vitro* environment in which chondrocytes are cultured in varies considerably from their native, *in vivo* environment. Therefore, there are many factors which can have an impact on chondrocyte dedifferentiation and subsequent redifferentiation *in vitro*. Altering the culture environment from 2D to 3D alone is not enough to reverse or prevent the dedifferentiation of chondrocytes *in vitro*. Therefore additional changes must be made to the culture system to enhance chondrocyte redifferentiation. Broadly speaking, these adjustments can be grouped into adding soluble factors to culture media or changing the physical conditions surrounding the 3D constructs.

Although the exact compositions of media used to maintain the chondrocyte phenotype *in vitro* vary, ascorbate is a common feature. Ascorbate (Vitamin C) is crucial for collagen synthesis as it acts as a cofactor for the hydroxylation of prolines and lysines, which form the intermolecular bonds in the collagen triple helix (Barnes, 1975, Pinnell, 1985, Stabler and Kraus, 2003). Schiltz and colleagues (1977) found that ascorbate affected the hydroxyproline content of collagen in tooth

germs. In another paper studying human fibroblasts, incubation with L-ascorbic acid and D-isoascorbic acid was found to increase collagen expression (Murad et al., 1981). In the same study, enzymes involved in the hydroxylation of lysine and proline (lysyl hydroxylase and prolyl hydroxylase respectively), were found to be upregulated in ascorbate treated cultures. However, the latter enzyme was only upregulated by treatment with L-ascorbic acid for 24 hours (Murad et al., 1981). In a later study, the same group also showed that collagen production by human fibroblasts was increased by various forms of ascorbate (L-ascorbate, D-isoascorbate, D-ascorbate and L-dehydroascorbate) (Murad et al., 1983). In fact, it was shown that L-ascorbate induced large increases at much lower concentrations compared to the other forms of ascorbate. Lysyl hydroxylase activity was also increased by all forms of ascorbate and again L-ascorbate had the biggest impact (Murad et al., 1983).

In addition to this, ascorbate is often included in chondrogenic media used to direct MSCs to differentiate down the chondrogenic lineage (Ma et al., 2003, Koerner et al., 2006, Richardson et al., 2006, Csaki et al., 2007, Giovannini et al., 2008, Stewart et al., 2008, Vidal et al., 2008, Colleoni et al., 2009, da Silva Meirelles et al., 2009, Vieira et al., 2009). It has also been shown that gene expression of collagen type II, Sox9 and aggrecan increased in mouse embryonal carcinoma-derived cell line (ATDC5) cells following incubation with ascorbate (Altaf et al., 2006). However it must also be noted that gene expression of collagen

type X was also enhanced in these cells following ascorbate incubation (Altaf et al., 2006), indicating hypertrophic differentiation.

In addition to adding soluble factors to culture media, it is thought that mimicking the *in vivo* environment as closely as possible will enhance chondrocyte redifferentiation. There are many elements to the *in vitro* culture of chondrocytes which differ greatly from the natural chondrocyte environment. One such condition is oxygen tension. Traditionally, *in vitro* cell culture is conducted at atmospheric conditions, which means the oxygen tension chondrocytes are exposed to is around 21% (160mmHG). The oxygen tension within the joint is considerably lower than this. Lund and colleagues (1970) measured oxygen tension within 103 human knee joints. They found that oxygen tension ranged from 26.53mmHG (3.5%) in patients suffering with rheumatoid arthritis to 42.92mmHG (5.6%) in osteoarthrosis joints. Another group measured oxygen tensions within the knee joints of naïve, pre-arthritic and arthritic mice, finding tensions of 29mmHG (3.8%), 25mmHG (3.3%) and 15mmHG (2.0%) respectively (Etherington et al., 2002). Zhou *et al* (2004) reviewed the oxygen tensions within rabbit knee, dog knee, bovine ankle and knee and human knee cartilage using mathematical modelling. They found that the lowest oxygen tensions ranged from ~5% in the rabbit knee to ~1.5% in the human knee (Zhou et al., 2004). It has been said that hypoxia (a term used to describe low oxygen conditions) alongside mechanical loading, are two factors that have a major impact on adult

cartilage (Lafont, 2010). One study has shown that human chondrocytes cultured in monolayer downregulated 101 genes which were subsequently upregulated following incubation in hypoxic conditions (Lafont et al., 2008). Gene expression of aggrecan and collagen type II was found to be significantly upregulated following culture under reduced oxygen conditions (Lafont et al., 2008). Hypoxia-inducible factors -1α (HIF-1α) and -2α (HIF-2α) are vital for chondrocyte survival in low oxygen conditions (Schipani, 2005, Lafont et al., 2007, Lafont, 2010). In oxygen tensions above 5%, these proteins are rapidly degraded (Lafont, 2010). In hypoxic conditions, HIF- 1α will bind to HIF-1β, which is constitutively expressed by the cell (Lafont, 2010). This heterodimer will then translocate to the nucleus to activate gene expression (Lafont, 2010). In one paper, it is suggested that HIF-2α up-regulates cartilage matrix gene expression through induction of Sox9 (Lafont et al., 2007).

Taking into consideration the various and complex conditions used to help maintain or induce the chondrocytic phenotype *in vitro*, it was decided that a soluble factor and a change in physical condition should be tested in the first instance for their impact on redifferentiation. Due to the fact that ascorbate appears frequently in chondrogenic media and is a key cofactor in collagen synthesis, this was chosen as the soluble factor. In terms of changing the physical environment surrounding the alginate beads, it was decided to lower the oxygen tension. This condition was chosen as reducing the oxygen tension would be one

way of trying to more closely mimic *in vivo* conditions. Specifically, an oxygen tension of 2.4% was used for the experiments detailed in this chapter. Oxygen tension levels within the joint, quoted in the literature, range from 1.5% to 5% (Lund-Olesen, 1970, Etherington et al., 2002, Zhou et al., 2004), therefore it was decided to use the lowest tension possible within this range. Due to equipment constraints, this level was set at 2.4%. It was decided to test these factors in isolation initially to assess their individual contribution to chondrocyte redifferentiation.

It was also thought to be important to include a factor that would inhibit chondrocyte redifferentiation, in order to confirm that the process of 3D culture encourages redifferentiation before additional changes to the culture conditions were made. This would then help to illustrate that any enhancement seen by the addition of ascorbate or incubation under reduced oxygen conditions were caused by these changes in culture conditions. For this part of the study, the inflammatory cytokine, IL-1β was chosen. IL-1β is mainly produced by monocytes and induces production of neutral metalloproteinases and serine proteinases in chondrocytes (Goldring et al., 1988). It is also has a major involvement in the pathogenesis of OA (Wojdasiewicz et al., 2014), so the effect of IL-1β on the phenotype of cultured cells may have implications on cellular treatments for degenerative joint disease. It has also previously been shown to reduce matrix production (Cook et al., 2000) and expression of collagen type II in canine chondrocytes (Kuroki et al., 2004). In addition to this, IL-1β has been described as a factor that can

be used to accelerate the dedifferentiation of chondrocytes in monolayer culture (Goldring et al., 1994). Goldring and colleagues (1994) have shown that expression of collagen type II by immortalised human chondrocytes decreased with increasing culture time in the presence of IL-1β. A more recent paper by a different group also showed a reduction in expression of collagen type II with IL-1β by immortalised human chondrocytes in monolayer (Robbins et al., 2000). Il-1β incubation had the opposite effect on collagen type I expression. This group also demonstrated similar findings with chondrocytes cultured in alginate (Robbins et al., 2000). Another research group investigate the effects of short exposure to IL-1β (one, three and six hours) on equine chondrocytes (Takafuji et al., 2005). A significant decrease in collagen type II expression was only observed after six hours of exposure (Takafuji et al., 2005). This would suggest that short term exposure has little effect on the dedifferentiation of chondrocytes in culture.

Therefore the aim and subsequent hypotheses for this chapter were as follows:

Aim: To identify factors that will enhance chondrocyte redifferentiation in 3D culture.

Hypothesis One: Addition of ascorbic acid (25µg/ml) to the cell culture medium will enhance the redifferentiation of canine chondrocytes cultured three dimensionally following expansion in monolayer culture.

Hypothesis Two: Culturing chondrocytes in a 3D system in an oxygen concentration of 2.4% will enhance the redifferentiation of monolayer expanded canine chondrocytes.

Hypothesis Three: Addition of IL-1β (10ng/ml) to the cell culture medium will inhibit the redifferentiation of canine chondrocytes cultured three dimensionally following expansion in monolayer.

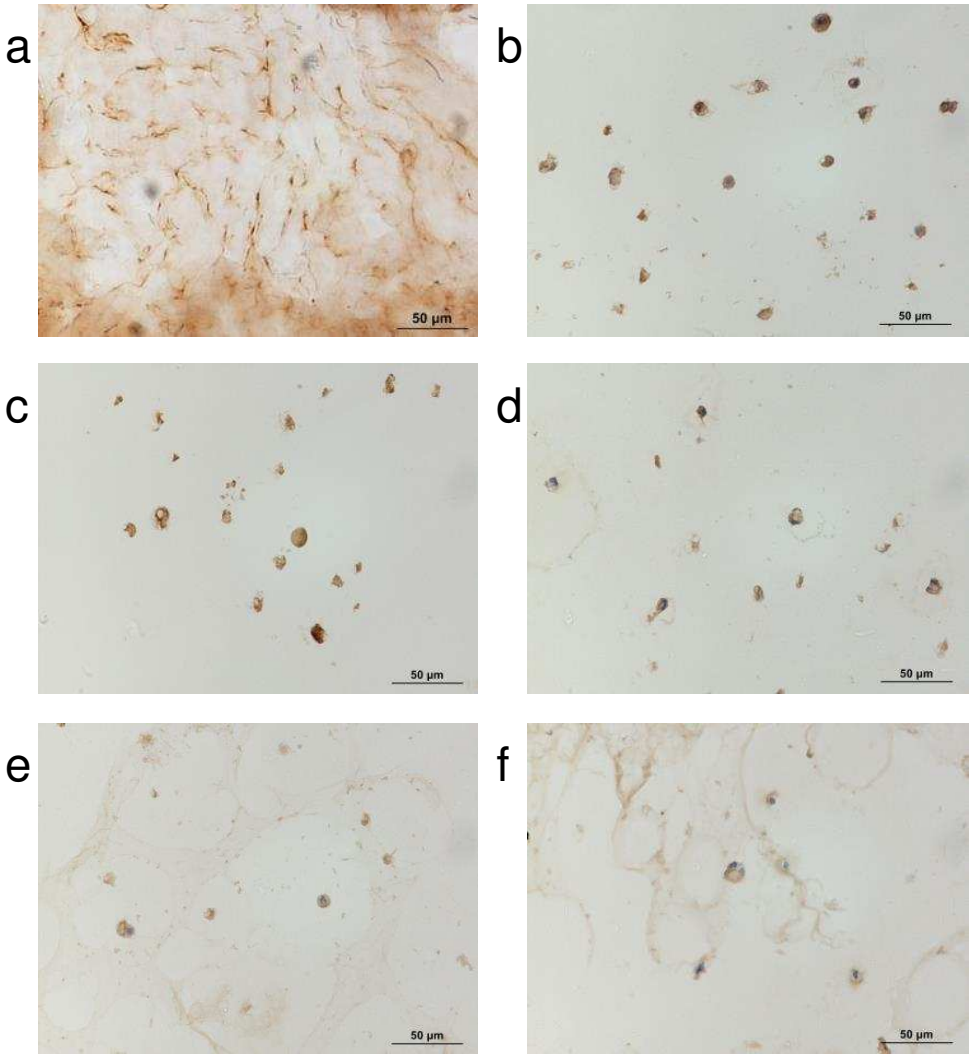
5.2 Results

5.2.1 Expression of Collagen Types I and II and CD44 by Chondrocytes Cultured within Alginate Beads in Response to Ascorbate, Reduced Oxygen Tension and IL-1β

Alginate beads were seeded with P2 canine chondrocytes and were cultured for 35 days. Beads were placed in culture medium

(DMEM+10%FBS+2% antibiotics) at 37°C, 5% CO_2, 20% oxygen for four days, in order to allow the cells to settle into the beads. On day four (day zero being the day cells were seeded into alginate) the beads were grouped into different treatments. Control beads remained in the same conditions. The IL-1β group was cultured in media containing 10ng/ml IL-1β. Beads treated with ascorbate were cultured in media containing 25µg/ml ascorbate. The beads in the reduced oxygen tension group were cultured in the same medium as the control group but were incubated in 2.4% oxygen, 5% CO_2. Beads were created from chondrocytes isolated from two different animals. Beads from each animal were selected and pooled for downstream assays.

Expression of collagen type I was initially assessed using immunohistochemistry. Dark areas of staining surrounding the cells was seen in the isotype control (Figure 5.1b). This staining was also seen in the control and IL-1β beads (Figure 5.1c and d). Staining appeared different to the isotype control in beads incubated with ascorbate or in reduced oxygen conditions (Figure 5.1e and f). However in all sections the staining surrounding the cells appeared to be non-specific.


a
b
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f
50 µm

Figure 5.1: Alginate beads seeded with P2 canine chondrocytes incubated in various conditions for 35 days, stained for collagen type I. (a) canine tendon stained with collagen type I (positive control), (b) representative isotype control (day 14 control bead incubated with an isotype control primary antibody), (c) control bead (culture medium, 37°C, 5% CO_2, 20% oxygen), (d) IL-1β bead (culture medium supplemented with 10ng/ml, 37°C, 5% CO_2, 20% oxygen), (e) ascorbate bead (culture medium supplemented with 25µg/ml ascorbate, 37°C, 5% CO_2, 20% oxygen) and (f) reduced oxygen bead (culture medium, 37°C, 5% CO_2, 2.4% oxygen). The majority of the staining appeared to be non-specific as it was similar in the test samples and the isotype control. There was more disperse, lighter staining in (e) and (f) which could indicate higher expression of collagen type I in these groups as this staining wasn't present in the isotype control. Images taken with a magnification of x400, scale bars: 50µm. Sections: 5µm.

There was strong staining for collagen type II immediately surrounding the cells in all of the treatment groups and the control group (Figure 5.2c-d); however there was also evidence of similar staining in the isotype control (Figure 5.2b). There appeared to be stronger staining and more disperse staining in the ascorbate treated beads (Figure 5.2e), which could indicate higher collagen type II production in this group. Although the staining of the reduced oxygen beads appeared to be stronger than the isotype control (Figure 5.2f), it was similar to control group beads, suggesting that collagen type II synthesis was not enhanced in this group.

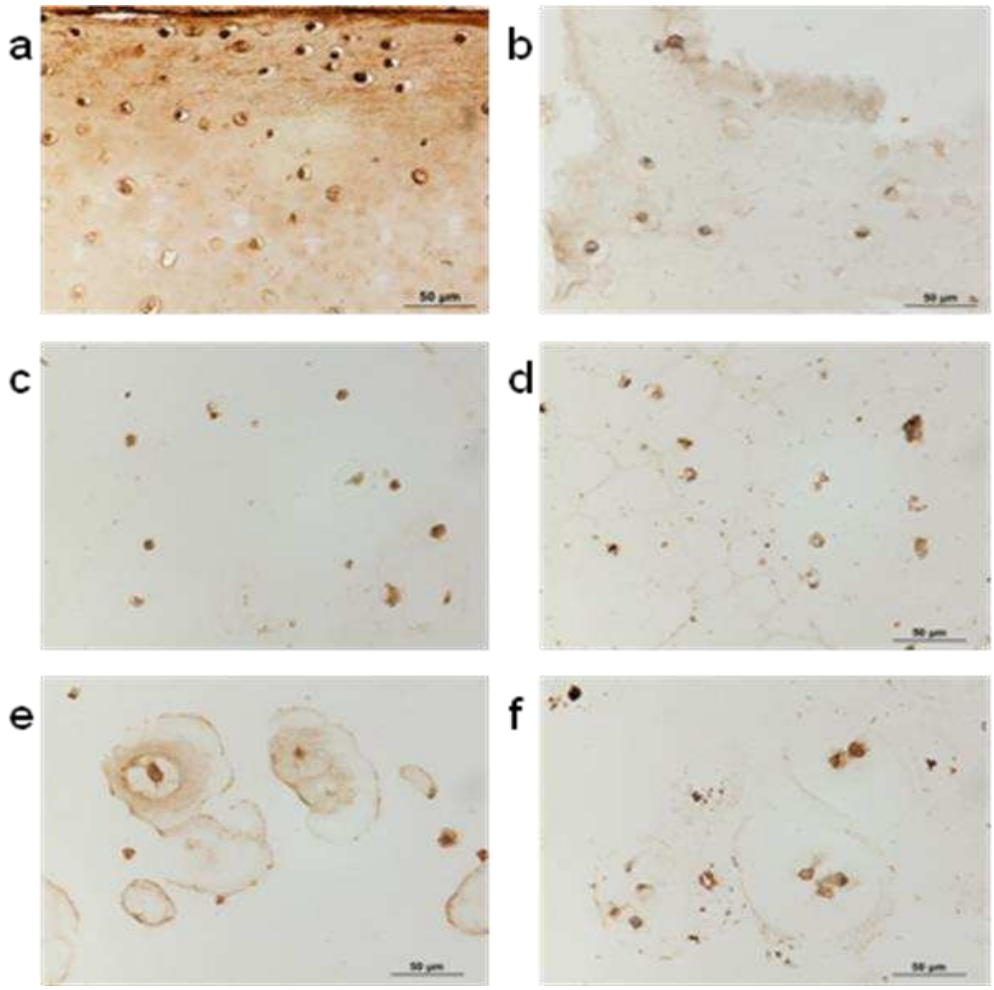


Figure 5.2: Alginate beads seeded with P2 canine chondrocytes incubated in various conditions for 35 days, stained for collagen type II. (a) canine cartilage stained with collagen type II (positive control), (b) representative isotype control (day 14 control bead incubated with an isotype control primary antibody), (c) control bead (culture medium, 37°C, 5% CO_2, 20% oxygen), (d) IL-1β bead (culture medium supplemented with 10ng/ml, 37°C, 5% CO_2, 20% oxygen), (e) ascorbate bead (culture medium supplemented with 25µg/ml ascorbate, 37°C, 5% CO_2, 20% oxygen) and (f) reduced oxygen bead (culture medium, 37°C, 5% CO_2, 2.4% oxygen). The greatest staining was observed in the ascorbate treated alginate beads. Staining surrounding the cells was present in all samples however a similar pattern of staining was observed in the isotype control. Images taken with a magnification of x400, scale bars: 50µm. Sections: 5µm.

CD44 staining appeared to be non-specific in the control, IL-1β and ascorbate groups as the pattern of staining seen surrounding the cells was similar in the isotype control (Figure 5.3b-e). However the light background staining was not seen in any of the test samples. Beads incubated in reduced oxygen conditions were negative for CD44 (Figure 5.3f).

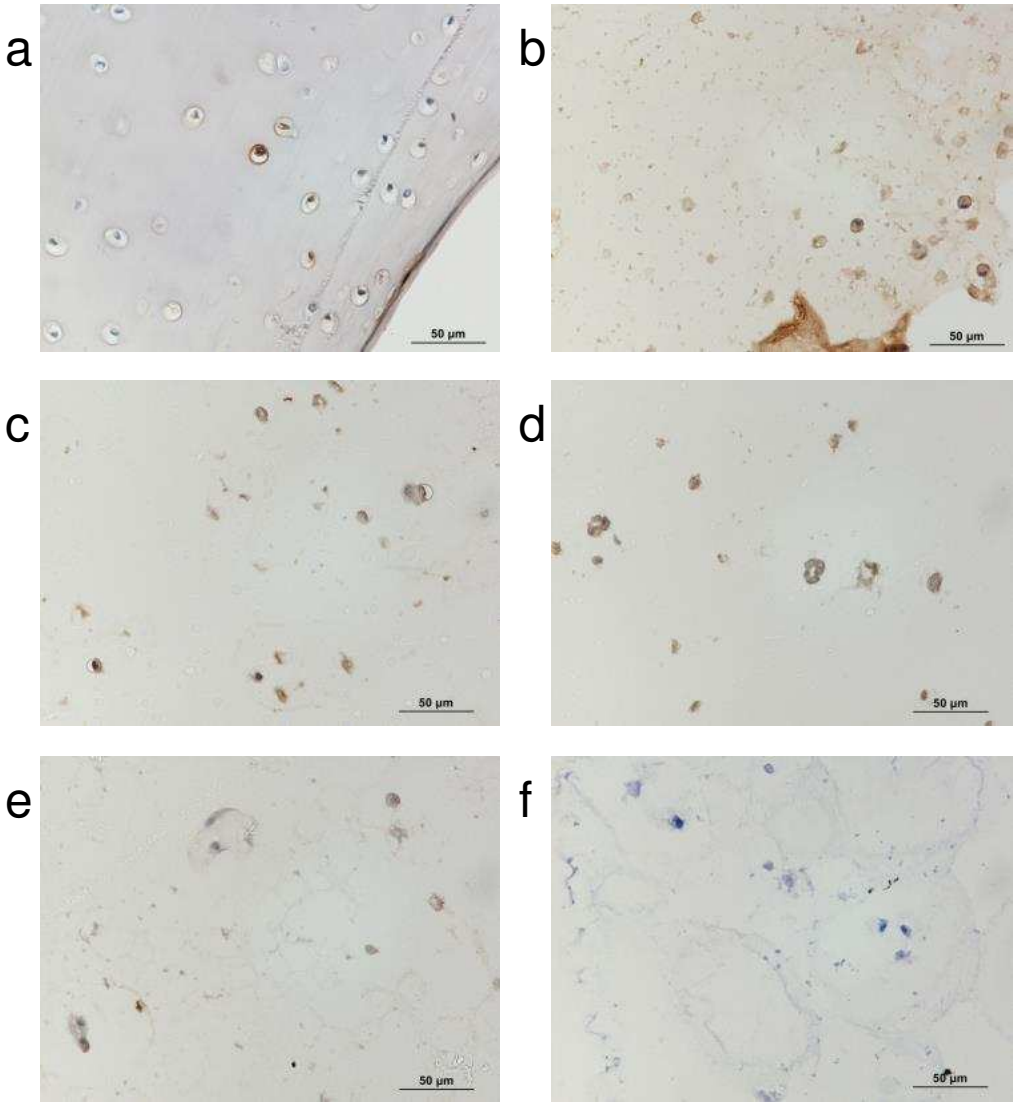


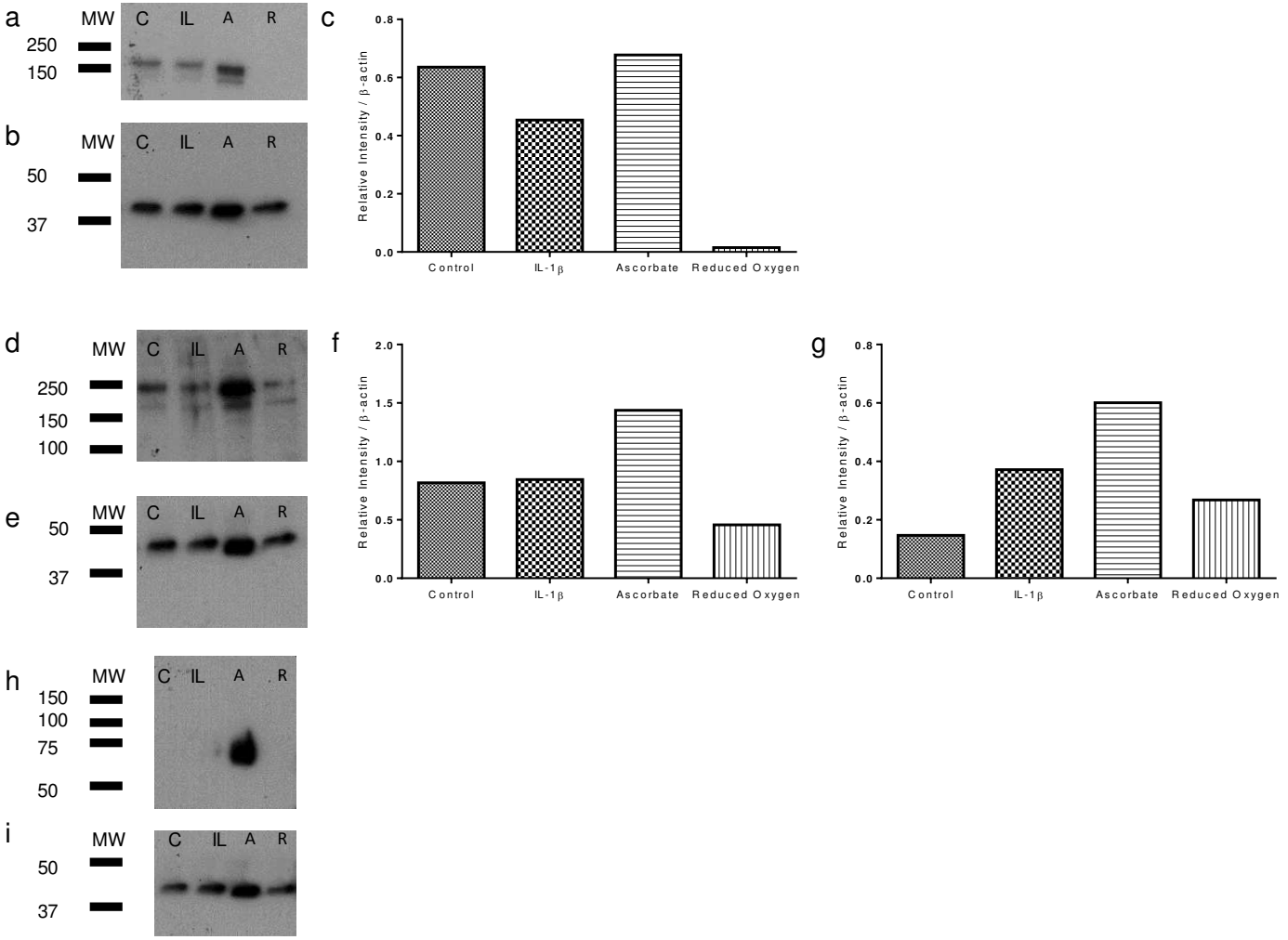
Figure 5.3: Alginate beads seeded with P2 canine chondrocytes incubated in various conditions for 35 days, stained for CD44. (a) canine cartilage stained with CD44 (positive control), (b) representative isotype control (day 14 control bead incubated with an isotype control primary antibody), (c) control bead (culture medium, 37°C, 5% CO_2, 20% oxygen), (d) IL-1β bead (culture medium supplemented with 10ng/ml, 37°C, 5% CO_2, 20% oxygen), (e) ascorbate bead (culture medium supplemented with 25µg/ml ascorbate, 37°C, 5% CO_2, 20% oxygen) and (f) reduced oxygen bead (culture medium, 37°C, 5% CO_2, 2.4% oxygen). Staining surrounding the cells was present in all samples except those incubated in reduced oxygen conditions; however a similar pattern of staining was observed in the isotype control. Beads incubated in reduced oxygen conditions appeared to be negative for CD44. Images taken with a magnification of x400, scale bars: 50µm. Sections: 5µm.

In many cases, the staining for all three antigens appeared similar to the isotype controls, indicating non-specific staining. Therefore in order to get a clearer idea of the expression of collagen types I and II and CD44 by the cells within these beads, western blotting was used. Protein lysates were generated by lysing 50 beads (25 beads were selected from each animal). Collagen type I expression was lower in the IL-1β treated group compared to the control and considerably lower in the reduced oxygen group (Figure 5.4a-c).

Two bands were detected in the collagen type II blot (Figure 5.4d). For the higher molecular weight band, expression was slightly higher in the IL-1β group compared to the control (Figure 5.4d and f). Expression was considerably higher in the ascorbate group and lower in the reduced oxygen group when compared to the control sample.

Expression of the lower molecular weight form was higher in all treatment groups compared to the control group, with expression peaking in the ascorbate group (Figure 5.4g).

CD44 was not detected in any sample except the ascorbate treated group (Figure 5.4h)


a
MW
C
IL
A
R
250
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b
MW
C
IL
A
R
50
37
c
Relative Intensity / β-actin
0.8
0.6
0.4
0.2
0.0
Control
IL-1β
Ascorbate
Reduced Oxygen
d
MW
C
IL
A
R
250
150
100
e
MW
C
IL
A
R
50
37
f
Relative Intensity / β-actin
2.0
1.5
1.0
0.5
0.0
Control
IL-1β
Ascorbate
Reduced Oxygen
g
Relative Intensity / β-actin
0.8
0.6
0.4
0.2
0.0
Control
IL-1β
Ascorbate
Reduced Oxygen
h
MW
C
IL
A
R
150
100
75
50
i
MW
C
IL
A
R
50
37

Figure 5.4: Expression of collagen types I and II and CD44 in alginate beads seeded with P2 canine chondrocytes cultured in various conditions for 35 days. (a) western blot for collagen type I (b) corresponding beta actin blot (c) densitometry for the bands in the blot in (a); (d) western blot for collagen type II (e) corresponding beta actin blot, (f) densitometry on the upper band in the blot in (d), (g) densitometry on the lower band in the blot in (d); (h) western blot for CD44, (i) corresponding beta actin blot. C: control (culture medium, 37°C, 5% CO_2, 20% oxygen), IL: IL-1β (culture medium supplemented with 10ng/ml, 37°C, 5% CO_2, 20% oxygen), A: ascorbate (culture medium supplemented with 25µg/ml ascorbate, 37°C, 5% CO_2, 20% oxygen) and R: reduced oxygen (culture medium, 37°C, 5% CO_2, 2.4% oxygen). Each protein sample was generated from 50 beads. Exposures: (a) three minutes, (d) 30 seconds, (h) five minutes, (b, e and i) one second. Expression of both collagen types was highest in the ascorbate treated group; although collagen type I expression was only marginally higher than the control. CD44 was only detected in the ascorbate treated group.

5.2.2 Change in GAG Production in Response to Ascorbate, Reduced Oxygen Tension and IL-1β

Staining for GAGs was observed in all groups (Figure 5.5b-e). Staining appeared to be more prevalent in the ascorbate treated group, however this may be due to the presence of clusters of cells; although the staining was weaker in the cell cluster in the IL-1β treated bead. In the reduced oxygen group, staining appeared to be intense immediately surrounding the cells but there was also some weaker staining further away from the cells. To get a better indication of the GAG production in the different groups, the DMMB assay was performed (Figure 5.5f).

Beads incubated with IL-1β contained significantly less GAGs than the control beads ($p=<0.001$), whereas cells within the beads incubated with ascorbate and reduced oxygen produced significantly more GAGs ($p=<0.05$ and <0.001 respectively) (Figure 5.5f). However, to get a true sense of the significance of this data, the DNA content of the beads should have been analysed and GAG data normalised to this information.

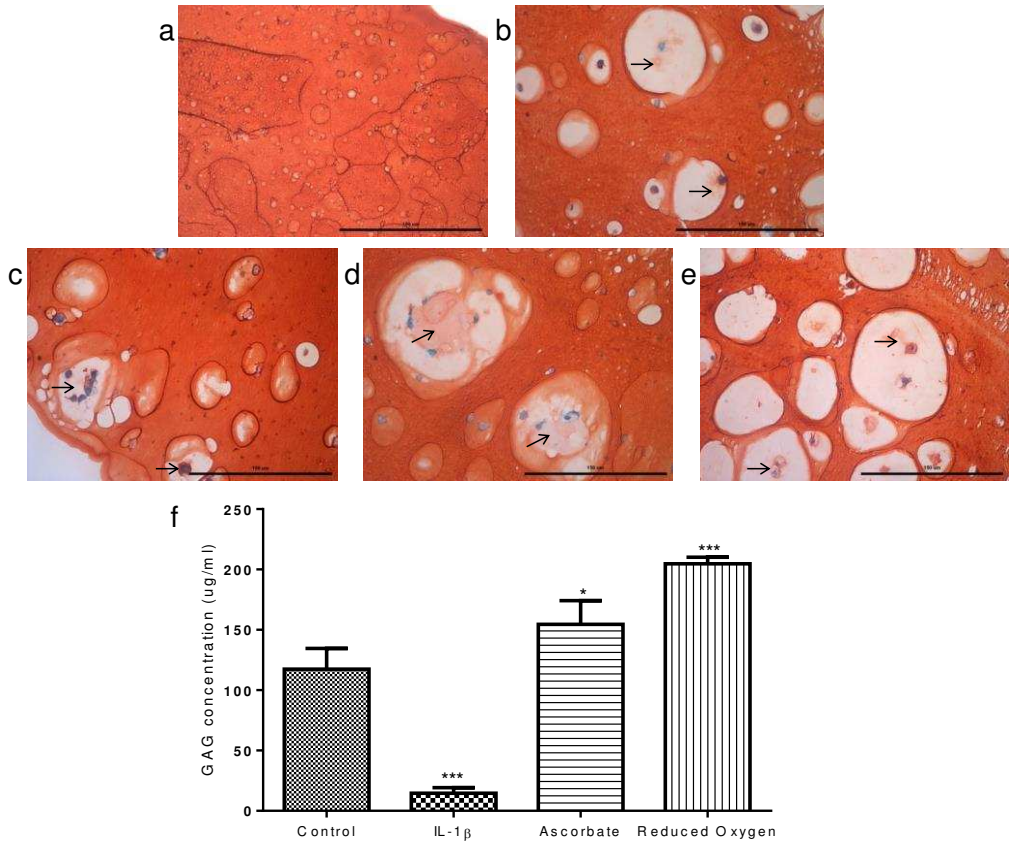

Figure 5.5: GAG staining and production in alginate beads seeded with P2 canine chondrocytes incubated in various conditions for 35 days. (a)-(e) beads stained with Safranin O, (a) alginate only bead, (b) control (culture medium, 37°C, 5% CO_2, 20% oxygen) (c) IL-1β (culture medium supplemented with 10ng/ml, 37°C, 5% CO_2, 20% oxygen), (d) ascorbate (culture medium supplemented with 25µg/ml ascorbate, 37°C, 5% CO_2, 20% oxygen) (e) reduced oxygen (culture medium, 37°C, 5% CO_2, 2.4% oxygen); Magnification: x400, scale bar: 150µm. (f) DMMB assay for GAG production. Three replicates of five beads were used for each treatment. Beads were selected and pooled from the two different animals. * p=<0.05 and ***p=<0.001

5.3 Discussion

There are many different factors used in the literature to maintain the chondrocyte phenotype both in monolayer and 3D cultures. Two factors were chosen to enhance redifferentiation, reduced oxygen tension and ascorbate. Treatment of beads with IL-1β was included as a catabolic factor which inhibits chondrocyte redifferentiation.

Assessment of collagen types I and II and CD44 expression was initially assessed by immunohistochemistry. However, the majority of the staining seen in the bead sections was non-specific. Therefore, to get a clearer indication of the phenotype of the cells within these beads, western blotting was carried out. Alongside the immunohistochemistry and the western blotting, beads were assessed for GAG production through Safranin O staining and the DMMB assay.

Collagen type I expression was considerably lower in the reduced oxygen group compared to all other groups. Two bands were detected for collagen type II, with expression of the larger form being less than the control and the smaller being greater than the control in the reduced oxygen group. CD44 expression was absent in the reduced oxygen treated group. GAG production was significantly higher in reduced oxygen beads compared to the control group, although it must be noted that this data was not normalised to the DNA content of the beads, which may have an impact on the significance of the data. Duval and

colleagues (2009), cultured P2 human chondrocytes in alginate beads at a density of $1x10^7$cells/ml at 5% oxygen (termed hypoxia) and 21% oxygen (termed normoxia). They found that when compared to P2 monolayer cells, beads from both groups had lower expression of collagen types I and III. However, expression was marginally higher in the hypoxic beads. This contrasts with the western blotting data presented in this chapter as a band for collagen type I was not detected in the reduced oxygen sample. However it must be noted that there may be differences seen between protein and mRNA expression. Collagen type II gene expression was significantly higher in both bead groups but was considerably higher in the hypoxia group compared to those incubated under normoxia. This is different to the data presented in this chapter where there was little enhancement of collagen type II synthesis in the reduced oxygen group. However it must be noted that this experiment was only conducted once and different results may be seen with an increased N number. Aggrecan gene expression was also higher in the beads but this difference was only significant in the beads incubated in hypoxic conditions (Duval et al., 2009).

Another group incubated bovine chondrocytes in reduced oxygen conditions in monolayer and 3D pellet culture. Egli *et al* (2008) incubated chondrocytes growing in monolayer in 1.5% oxygen and 21% oxygen. Proliferation was significantly slower in the 1.5% oxygen group. Pellets were incubated at three oxygen tensions, 1.5%, 5% and 21%. Gene expression results were only shown for the two monolayer

conditions and the 21% oxygen pellet group. Collagen type I expression was upregulated in all groups, whereas collagen type II was downregulated compared to fresh chondrocytes. Aggrecan was marginally upregulated in all groups whereas Sox9 was downregulated in the pellets (Sox9 expression was not detectable in the monolayer groups). Safranin O staining was found to be strongest in pellets cultured in 21% oxygen regardless of the oxygen concentration used for monolayer expansion. The authors also found that there was no difference between the 21% oxygen pellets seeded with cells incubated at 1.5% oxygen and 21% oxygen in terms of immuno-staining for collagen type II (Egli et al., 2008). This would suggest that oxygen conditions for monolayer expansion do not have an impact on the phenotype of cells cultured three dimensionally. Monolayer chondrocytes in this study were incubated at 21% oxygen and although it appeared to have little impact on the end phenotype it would be an interesting consideration for future work in this project. Babur and colleagues (2013) used slightly different oxygen tensions to represent hypoxia (2%) and normoxia (20%). This group looked at macro- and micropellets. Macropellets were formed by centrifuging $2x10^5$ cells whereas micropellets were formed using microwell inserts and contained 166 cells per pellet. Total sulphated GAG synthesis by both pellets was significantly higher in hypoxic cultures. This supports the data shown in this chapter with respect to reduced oxygen conditions enhancing GAG production. However the authors did observe differences between GAG content within the pellets and in the media

between the two pellet sizes. This could be a consideration for future work; to analyse what is being released into the medium by the chondrocytes within the alginate beads. In terms of gene expression, these authors observed similar patterns to Egli's group, however there were differences between the macro and micro pellets. For example, aggrecan expression was significantly higher in micropellets incubated in 2% oxygen when compared with all other pellets. However, expression in the 2% macropellet was found to be lower than that incubated in 20% oxygen. Collagen type II gene expression was higher in both 2% pellets but only significantly higher in the micropellets. Collagen type I expression was also significantly higher in the 2% oxygen micropellet compared to the 20% and 2% oxygen macropellets. Collagen type X expression showed a similar pattern except the expression in the 2% oxygen micropellets was significantly higher compared to all other pellet groups. Sox9 expression was significantly lower in the 2% macropellet group compared to all other pellet groups. This would seem to suggest that pellet size may have an impact on gene expression. All alginate beads generated for the work in this thesis were the same size but varying the size could provide an interesting comparison. Immunofluorescence staining indicated that pellets cultured in 20% oxygen had a more hypertrophic phenotype, staining strongly for collagen type X. These pellets also stained strongly for collagen type I. Pellets cultured in 2% oxygen stained weakly for collagen type X but stained strongly for collagen type II. Staining for the latter was weak in the 20% oxygen group (Babur et al.,

2013). This is in contrast to the immunohistochemistry data presented in this chapter where staining for collagen type II appeared to be similar in beads incubated in 21% and 2.4% oxygen. In another recent paper researchers looked at the effect of oxygen concentration on healthy and osteoarthritic human chondrocytes in pellet culture (Markway et al., 2013). For both healthy and OA chondrocytes, collagen type II and aggrecan gene expression was higher in 2% oxygen cultures compared to 20% cultures. The opposite pattern was the case for collagen types I and X, with expression being higher in the 20% oxygen cultures. Although a clear reduction in collagen type I expression was observed in the study in this thesis, in the reduced oxygen group, only slight changes in collagen type II expression was evident. Markway and colleagues (2013) also showed that intense staining of collagen type II in all groups, although it was visibly less in the 20% oxygen, healthy chondrocytes group. Collagen type I staining was shown in all groups but staining was minimal in the 20% oxygen, healthy chondrocytes pellet. Sulphated GAG was also higher in pellets cultured in 2% oxygen, for both cell types (Markway et al., 2013).

Murphy and Polak (2004) assessed the effect of different oxygen concentrations on P3 human chondrocytes seeded into alginate beads. Collagen type I gene expression was considerably lower in the beads compared to P3 monolayer chondrocytes. Statistical significance was only reported for the difference between the different oxygen tensions

(5% and 20%). Although collagen type I expression was higher in 5% oxygen beads, the difference was not significant. This is in contrast to the western blotting data presented in this chapter as collagen type I was clearly reduced in the reduced oxygen group. However statistical analysis could not be applied to this data due to only one experiment being carried out. Gene expression of collagen type II, aggrecan and Sox9 was higher in beads compared to monolayer cells. However, the difference between the two oxygen conditions became significant at different stages of culture (5% oxygen beads expressed significantly higher quantities of collagen type II after 28 days, aggrecan after 14 days and Sox9 after 7 days). Beads incubated in 5% oxygen contained significantly more GAG than beads incubated at 20% oxygen (Murphy and Polak, 2004), supporting the data presented in this chapter. Domm and colleagues (2002) also showed that chondrocytes seeded in alginate beads cultured in 5% oxygen exhibit a more chondrogenic phenotype compared to the cells cultured at 21% via immunocytochemistry. Staining for collagen types II and IX was stronger in the lower oxygen group, whereas staining for collagen type I was much stronger in the 21% oxygen group. This group also demonstrated, via western blotting that collagen type II was upregulated in alginate beads containing freshly isolated chondrocytes incubated at 5% oxygen. They also showed that collagen type II expression was absent in beads containing dedifferentiated chondrocytes incubated at 21% oxygen whereas a band for collagen type II was detected in

alginate beads containing dedifferentiated chondrocytes incubated at 5% oxygen (Domm et al., 2002).

IL-1β treated beads had a lower expression of collagen type I compared to control beads. IL-1β was used as an inhibitor to redifferentiation, in order to emphasise the redifferentiation induced by 3D culture alone and then subsequently the enhancement of this process with the altered cultured conditions. IL-1β was chosen as it had previously been shown to reduce matrix production by chondrocytes (Cook et al., 2000) and it has a major involvement in the pathogenesis of OA (Wojdasiewicz et al., 2014). Expression of collagen type II was higher than the control for both forms detected, although the difference for the higher molecular weight form was only slight. As with the reduced oxygen treated group, CD44 expression was absent in the IL-1β treated group. Sections of beads treated with IL-1β stained positively for GAGs but quantitative data showed that GAG production within these beads was significantly lower compared to the control group. Sandell and colleagues (2008) examined the effect of IL-1β on the gene expression of human articular chondrocytes. They showed that incubation with 10ng/ml IL-1β for 24 hours reduced collagen type II and aggrecan expression. The result for collagen type II is in contrast to the data presented in this chapter. They also looked at the expression of many chemokines, CCL20, CCL2, CX3CL1, CXCL6 and CXCL1 expression increased in IL-1β treated chondrocytes. This group also tested a range of IL-1β concentrations

on the human chondrocytes (0.01, 0.1 and 1ng/ml). All concentrations decreased expression of aggrecan and collagen type II and the reduction in expression increased with increasing IL-1β concentration. When assessing the effect of 0.1ng/ml IL-1β incubation at different timepoints (0 hours, 1 hour, 4 hours, 8 hours, 12 hours and 24 hours) it was shown that aggrecan and collagen type II expression was only reduced in the 24 hour incubation group. This group also found that IL-1β decreased aggrecan and collagen type II expression in cartilage explants (Sandell et al., 2008).

Ley and colleagues (2011) looked at the effect of 5ng/ml IL-1β on equine chondrocytes cultured in 3D pellets. Compared to untreated pellets, Safranin O staining for GAGs was less intense in IL-1β treated pellets. Gene expression of collagen types I and II and aggrecan was significantly lower in the IL-1β treated pellets (Ley et al., 2011). Although collagen type I expression was clearly reduced in the IL-1β group in this study, collagen type II expression only varied slightly between beads incubated at 2.4% and 21% oxygen. It must also be acknowledged that experiment in this chapter needs to be repeated so that statistical analysis can be applied.

One research group has examined the effect of a combination of oxygen tension and IL-1β on human articular and nasal chondrocytes

grown in pellets (Scotti et al., 2012). Pellets were cultured at 19% or 5% oxygen and treated with 50pg/ml IL-1β. Some pellets were incubated for a further seven days post IL-1β treatment to enable recovery. In addition to this, the authors tested immature and mature cultures; immature pellets were cultured for seven days prior to IL-1β treatment, whereas mature pellets were cultured for 14 days prior to treatment. In immature pellets seeded with articular chondrocytes, GAG staining was strongest in the recovery group (pellets incubated without IL-1β for a week following treatment) for both oxygen conditions, suggesting that the effects of IL-1β can be reversed. GAG staining was more intense in the mature pellets but the weakest staining was observed in the IL-1β treated group. This would suggest that IL-1β still has an effect on the mature pellets but the longer culture period prior to treatment allowed for greater GAG synthesis. A similar pattern was observed in the pellets seeded with nasal chondrocytes. Pellets seeded with articular chondrocytes were negative for collagen type II when cultured in 19% oxygen, except in the recovery group. This contrasts with the data presented in this chapter as collagen type II expression in the IL-1β group was detected at similar levels to the control group. All pellets cultured in 5% oxygen stained positively for collagen type II but such staining was weaker in the IL-1β group. This would suggest that oxygen conditions have an impact on the effect of IL-1β. Pellets containing nasal chondrocytes stained positively for collagen type II in all groups (staining was shown to be marginally stronger in the 5% oxygen groups). IL-1β treated beads exhibited

weaker staining for collagen type II compared to the control and recovery groups (Scotti et al., 2012).

Ascorbate treated beads showed a slightly higher expression of collagen type I compared to the control group. The highest level of collagen type II expression, for both forms detected, was seen in the ascorbate group. Beads cultured in medium supplemented with ascorbate stained positively for GAGs and produced significantly more GAGs than control beads. Benya *et al* (1984) showed that proteoglycan synthesis in rabbit articular cartilage discs increased with the addition of 25µg/ml ascorbate to the culture media. Another research group examined the effect of 50µg/ml ascorbate on bovine chondrocytes expanded in monolayer (Daniel et al., 1984). The authors found that cultures incubated with ascorbate had a more developed ECM containing dense fibrillar collagen, compared to the sparse and loose collagen network seen in control cultures. However when analysing the specific collagen types, collagen type II expression was found to be similar in the ascorbate group compared to controls, however, collagen type I expression appeared to be greater in the ascorbate group (Daniel et al., 1984), This would suggest that addition of ascorbate in monolayer encourages the production of matrix but does not prevent chondrocyte dedifferentiation in terms of collagen type I synthesis. Collagen type I expression in the study in this chapter was also marginally increased in the presence of ascorbate.

Two research groups have looked at different types of ascorbate (Clark et al., 2002, Ibold et al., 2009). Clark and colleagues (2002), incubated guinea pig cartilage explants with, L-ascorbic acid, D-isoascorbic acid, sodium L-ascorbate, sodium D-isoascorbate or Mg ascorbyl-2-phosphate. All types were tested at three concentrations designated as low (189μM), medium (378μM) and high (756μM). Out of the results shown, only the high dose of sodium L-ascorbate enhanced aggrecan expression compared to the control (untreated) group. Collagen synthesis was enhanced in all treatment groups but the increase was only significant in the high and low dose sodium L-ascorbate group and the high sodium D-isoascorbate or Mg ascorbyl-2-phosphate groups (Clark et al., 2002). In a later study, Ibold *et al* (2009), incubated high density pellets containing bovine chondrocytes with L-ascorbate, sodium ascorbate or L-ascorbate-2-phosphate. These authors also tested three concentrations, 100μM, 200μM and 400μM. The authors report that although there was no difference in pellet thickness or cell density between treatments, control pellets didn't form a stable cohesive matrix. This differs from Daniel and colleagues who observed considerable matrix formation in monolayer with the addition of ascorbate (Daniel et al., 1984). Staining for collagen types I and II appeared to be similar between treatments. Gene expression of collagen type I was downregulated in all ascorbate groups except in pellets incubated with the highest concentration of L-ascorbate. Again this differs from Daniel's research group who found collagen type I expression to be greater in ascorbate treated cultures. The 3D culture

may explain this difference to a certain extent. The combination of 3D high density pellet culture and ascorbate may be more effective at maintaining the chondrocyte phenotype and thereby reducing collagen type I expression. The alginate cultures in this thesis were incubated with 25µg/ml ascorbate which equates to 142µM ascorbate. Collagen type I expression was marginally upregulated in these cultures which contrasts with Ibold's work. All treatments enhanced collagen type II gene expression (Ibold et al., 2009). This is in agreement with the results shown in this chapter. Although Ibold and colleagues used various forms of ascorbate, on the whole, there appears to be little difference between the different types suggesting that all forms of ascorbate have similar effects on chondrocyte phenotype.

5.4 Conclusion

It is important to note that, the data presented in this chapter represents initial work into the enhancement of chondrocyte redifferentiation. Alginate beads would need to be incubated under the conditions discussed in this chapter at least two more times to confirm the effects reported and generate data to which statistical analysis could be applied. This was attempted; however both batches of beads became contaminated during the culture process. In addition, in future work the DNA content of the beads should be analysed so GAG data can be normalised to this information.

Despite this, the initial results suggest that incubation with ascorbate or under reduced oxygen conditions enhance GAG synthesis whereas IL-1β appears to reduce GAG production. In terms of expression of collagen types I and II and CD44, the immunohistochemistry data was not very conclusive. The western blotting data showed that ascorbate enhanced expression of both collagen types. Collagen type I expression was considerably downregulated in the reduced oxygen group. For collagen type II, two bands were detected; one showed increased expression in the reduced oxygen group but the other showed a decrease compared to the control group. CD44 expression was only detected in the ascorbate group.

As well as repeating the experiment detailed in this chapter, future work could include combining the conditions tested in this chapter as well as adding additional factors. As discussed in the introduction, ascorbate is a common feature of chondrocyte culture media and there are some studies that have investigated oxygen tension with media containing ascorbate (Domm et al., 2002, Malda et al., 2004). In the earlier paper, the authors show that immunocytochemical staining for collagen type I was strong in beads at 21% oxygen but absent at 5% oxygen. The opposite was the case for collagen type II. They also showed by western blotting that collagen type II expression was higher in alginate cultures containing freshly isolated chondrocytes, incubated at 5% oxygen. In addition, they showed that monolayer expanded

chondrocytes subsequently cultured in alginate did not express collagen type II when cultured at 21% oxygen. The opposite was the case at 5% oxygen (Domm et al., 2002). In the later paper, GAG content within high density pellets increased with decreasing oxygen conditions. Staining was the strongest in pellets incubated in 1% oxygen. Interestingly staining for collagen type II appeared stronger at 5% oxygen than 1% and staining for collagen type I was weakest at 5% (Malda et al., 2004). Investigating the combination of ascorbate and reduced oxygen conditions may result in enhanced collagen synthesis (caused by the ascorbate) with a reduction in the synthesis of collagen type I (a consequence of the reduced oxygen conditions).

The analytical techniques used in this chapter could also be reviewed. Perhaps techniques less heavily reliant on antibodies could be considered. Many of the papers reviewed in this discussion have studied gene expression; however changes in mRNA expression don't always translate to changes in protein expression. The potential next stages of this work, including reviewing analytical techniques, are discussed in greater detail in the next chapter.

6. Discussion

Articular cartilage can be damaged directly through injury or OA (Clar et al., 2005). It can also be damaged spontaneously as part of the condition osteochondritis dissecans (Clar et al., 2005).

Osteoarthritis (OA) is a debilitating disease which affects a range of species. It is estimated worldwide that 9.6% of men and 18.0% of women aged over 60 have symptomatic arthritis (World Health Organization, 2013). Out of those suffering with OA, 80% will experience restricted movement and a quarter will be prevented from carrying out daily tasks (World Health Organization, 2013). Dogs are also affected by OA, with up to 20% of dogs over one year old suffering with the condition (Clements et al., 2010). The dog has also been used as model for OA, with most studies inducing OA via transection of the anterior cruciate ligament (Altman et al., 1984, Fife, 1986, Pelletier et al., 1988, Pelletier et al., 1992, Matyas et al., 1999, Liu et al., 2003, Matyas et al., 2004, Rogachefsky et al., 2004, Kuroki et al., 2011, Gharbi et al., 2013). The dog is a very useful model because the canine knee is anatomically very similar to that of a human and many features including the articular cartilage, synovium and joint capsule are highly conserved between species (Cook et al., 2010).

Articular cartilage is very poor at repairing itself for two major reasons; the first being that it is an avascular tissue and therefore has limited access to mediators of repair (Newman, 1998, Lewis et al., 2006). The second reason is chondrocytes do not divide and are surrounded by their own matrix, so they cannot migrate to the injured section of the cartilage (Newman, 1998, Lewis et al., 2006). There is currently no completely successful treatment for damaged cartilage with surgical treatments often producing poor quality repair tissue (Stoddart et al., 2009). Autologous Chondrocyte Implantation (ACI), first introduced in 1987 (Brittberg, 2010), has been used to repair cartilage defects over $4cm^2$ (Schulze-Tanzil, 2009, Oldershaw, 2012). A major disadvantage of ACI is the requirement to expand chondrocytes in monolayer, in order to generate enough cells to repair the defect. ACI has been modified in an attempt to combat the problem of chondrocyte dedifferentiation (Brittberg, 2010). However successful repair of cartilage lesions is still a major challenge (Filardo et al., 2013).

In a bid to successfully repair cartilage tissue, research into tissue engineering and regenerative medicine has been extremely active over the past two decades (Johnstone et al., 2013). However as of yet, no ideal scaffold and cell combination has been identified which successfully restores articular cartilage to its native form.

The first aim of this thesis was to examine the phenotype of canine chondrocytes expanded in monolayer and confirm dedifferentiation. Canine chondrocytes expanded up to P5 were shown dedifferentiate through the decreased expression of collagen type II and increased expression of collagen type I and CD44. An increase in collagen type I expression with increasing passage has also been observed in the literature (Brodkin et al., 2004, Darling and Athanasiou, 2005, Yang et al., 2006b, Munirah et al., 2010, Nadzir et al., 2011, Hamada et al., 2013). In terms of CD44 expression, one group found an increase in expression in chondrocytes after 24 hours (Diaz-Romero et al., 2005) whereas another group reported an increase in expression from P0-P3 and a minimal decrease in expression from P3 to P4 (Hamada et al., 2013). A decrease in collagen type II expression with increasing passage has also been observed by others (Brodkin et al., 2004, Darling and Athanasiou, 2005, Yang et al., 2006b, Eleswarapu et al., 2007, Tew et al., 2008, Munirah et al., 2010, Hamada et al., 2013).

Two drawbacks with working with canine tissue were identified during Chapter Three. The first, which is echoed throughout this project, is the limited and sporadic availability of tissue. This is not a problem confined to sourcing canine tissue, others have reported difficulties in obtaining human samples (Diaz-Romero et al., 2005, Hamada et al., 2013). The other issue encountered was identifying antibodies that cross-react with canine tissue. Although dogs are important companion

animals and are a useful model for OA, there are a limited number of commercial antibodies available known to cross-react with canine tissue, particularly for the antigens studied in this work. Initially, the plan was to assess the expression of collagen types I and II, CD44 and Sox9. However, after screening several antibodies, a suitable antibody for Sox9 was not identified and was therefore removed from the study. Sopp and colleagues (2007) evaluated the cross-reactivity of monoclonal antibodies to human CD antigens to bovine antigens. They found that out of 52 antibodies, only 26 reacted with a bovine homologue to the human antigen. A further seven were found to recognise different forms of the antigen in bovine samples. When 16 of these antibodies were selected for use in western blotting, only two detected bands on the blots. However, the authors do acknowledge that this may not be a lack of cross-reactivity issue but that the antibodies may not recognise the denatured forms of the proteins (Sopp et al., 2007). A recent review has also demonstrated that there are difficulties in identifying the exact materials used in a paper (Vasilevsky et al., 2013). Vasilevsky *et al* (2013) found that only 46% of commercial and 43% of non-commercial antibodies were identifiable.

Having shown the canine chondrocytes dedifferentiated in monolayer culture, the next aim of this thesis was to redifferentiate the cells through three-dimensional culture. The stiff two dimensional monolayer culture environment causes cellular spreading and actin stress fibre

formation (Schuh et al., 2012). Culture on tissue plastic forces the cells to have a apical-basal polarity (Baker and Chen, 2012). This polarity isn't unusual for some cells (like epithelial cells) but is unnatural for chondrocytes (Baker and Chen, 2012). It is also important to note that chondrocytes are surrounded by their own matrix in their native environment. This extracellular matrix has a key role in regulating the spatial distribution of growth factors, cytokines, oxygen and nutrients (Baker and Chen, 2012). In 2D monolayer expansion, chondrocytes are not able to become completely surrounded in their own matrix, which no doubt contributes to the dedifferentiation process.

A common approach used to culture chondrocytes three-dimensionally is to generate high density pellets (Stewart et al., 2000, Tallheden et al., 2003, Zhang et al., 2004). Pellet culture is also often used to differentiate mesenchymal stem cells down the chondrogenic lineage (Johnstone et al., 1998, Csaki et al., 2007, Stewart et al., 2007, Stewart et al., 2008, Vidal et al., 2008, Lettry et al., 2010). Therefore initial attempts at 3D culture used a high density pellet system. However, all pellets were extremely fragile and in some cases, the cells did not form a cohesive pellet. This has been experienced by other researchers; Ibold and colleagues reported that their control pellets did not form a stable and cohesive matrix (Ibold et al., 2009). A possible reason for the cells not forming cohesive pellets is the low levels of matrix being produced by the dedifferentiating cells. Another research group looking

into the chondrogenic differentiation of human adipose tissue derived stem cells also commented that their control pellets were loose and fragile (Pilgaard et al., 2009). The possible explanation they give for this is having a high cell to matrix ratio within the pellets (Pilgaard et al., 2009). Therefore, it was decided that the cells would need a supporting scaffold for 3D culture. Many different scaffolds have been used in the literature to culture chondrocytes three-dimensionally (Table 6.1). Alginate was chosen as the supporting scaffold as it has frequently been used for the 3D culture of chondrocytes (Table 6.1). It is also a naturally occurring polymer (Nussinovitch and Nussinovitch, 2011, Lee and Mooney, 2012), which if used for cell transplantation is biocompatible with the host and the encapsulated cells (Ghidoni et al., 2008).

After establishing the alginate bead culture system and adjusting downstream assays, beads were seeded with different passages to see if the length of monolayer expansion had an impact on redifferentiation. A limited number of studies have assessed the redifferentiation capabilities of chondrocytes of different passages. The work in this thesis found that beads seeded with P2 canine chondrocytes appeared to contain cells with a more chondrocyte-like phenotype compared to P3 and P4 chondrocytes. However, these cells still expressed relatively high levels of collagen type I, suggesting that 3D culture alone is not enough to induce complete redifferentiation.

Therefore, the final aim of this thesis was to enhance the redifferentiation of monolayer-expanded canine chondrocytes through altering the culture environment. As discussed in previously, different researchers have used a variety of methods to enhance chondrocyte redifferentiation. Two conditions were chosen for this part of the study; adding ascorbate to the media and culturing the beads under reduced oxygen tension. The results from this work suggest that culturing beads in reduced oxygen conditions enhanced chondrocyte redifferentiation as collagen type I expression was lower than in control beads and CD44 expression was absent. However, collagen type II expression was variable. Ascorbate enhanced collagen type II expression but it also increased collagen type I expression. In addition, CD44 was only detected in the ascorbate treated beads. As discussed in Chapter Five, these results are initial data and would need to be repeated to confirm the findings and apply statistical analysis.

Now the bead culture has been established, there are many possibilities for testing additional factors and conditions, to identify optimal conditions for maintaining the chondrocyte phenotype. In the first instance, future work could include combining the culture conditions of ascorbate and reduced oxygen. Other future work could include adding other factors to the culture system. For example, bone morphogenetic protein-2 (BMP-2) and TGF-β1 have been shown to increase collagen type II expression in P1 embryonic mouse chondrocytes (Gouttenoire et

al., 2010). Another growth factor to consider could be IGF-1 as Pei and colleagues (2002) showed that chondrocytes seeded into PGA scaffolds and cultured in the presence of IGF-1 expressed high levels of collagen type II and relatively low levels of collagen type I. In another study, it was shown that high density pellets containing human chondrocytes incubated with IGF-1 during monolayer culture expressed higher levels of collagen type II and cartilage-specific proteoglycan compared to controls (Shakibaei et al., 2006). This study also showed that monolayer cells incubated with IGF-1 consistently expressed Sox9 up to P10, whereas expression was almost completely lost by P9 in control cultures (Shakibaei et al., 2006). It has also been found that a combination of TGF-β1 and IGF-1 enhanced chondrogenesis in equine bone marrow derived MSCs (Worster et al., 2001). Another potential factor to explore is the delivery of these growth factors. Traditionally, these factors are added to the culture medium and therefore have to diffuse through the scaffold to the cells. However, they could be delivered in the form of microparticles. Microparticles have been developed for the slow release of BMP-2 (White et al., 2013) and TGF-β1 (Lu et al., 2000).

Finally, another aspect of this study and the potential future work to be considered is the approach used to assess the phenotype of the redifferentiated chondrocytes. Due to the initial problems identifying suitable antibodies which cross-react effectively with canine tissue and

the inconclusive immunohistochemistry data, it may be beneficial to look at alternative forms of analysis which do not rely on antibodies. One potential alternative technique could be to measure mRNA levels of chondrocyte de- and re- differentiation markers using quantitative PCR (qPCR) (VanGuilder et al., 2008). However, an increase in mRNA expression may not always translate to an increase in protein expression. Another alternative would be to use proteomic analysis, which involves separating proteins via SDS-PAGE and identifying the bands through mass spectrometry. The use of proteomics for cartilage proteins is reviewed in (Wilson et al., 2008).

Overall, this study has confirmed the dedifferentiation of canine chondrocytes in monolayer culture in line with other chondrocyte studies. The redifferentiation of these expanded cells cultured in alginate beads has also been shown. However culture in a 3D environment was not enough to induce complete redifferentiation. Reduced oxygen tension and addition of ascorbate to the culture media appeared to enhance certain aspects of the redifferentiation process. The alginate culture system is now at a stage to be altered to identify optimal chondrocyte redifferentiation conditions, which could ultimately lead to more successful regeneration of articular cartilage.

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Appendix A: Sources and Uses of Canine Material

Breed	Sex	Age (years)	Reason for Euthanasia or Cause of Death (if known)	Usage
Rottweiler	Male	Two	Aggressive	Cell culture (freshly isolated counts referred to)
Staffordshire Bull Terrier Cross	Female	Unknown	Parvovirus	Cell culture (freshly isolated counts referred to)
Staffordshire Bull Terrier	Male	Unknown	Parvovirus	Western Blotting positive control tissues
Staffordshire Bull Terrier	Male	Three	Unknown	Sample sent by Dr Rebecca Lewis
Rottweiler	Female	Four	Severe haem diarrhoea	Multiple passage study
Staffordshire Bull Terrier	Unknown	Unknown	Unknown (suspected to be behavioural)	Immunofluorescence (antibody screening)
Tibetan Spaniel	Male	Eight	Back legs paralysed	Immunofluorescence (antibody screening)
St Bernard	Male	Eight or Nine (suspected)	Unknown cause of death	Initial Cyquant work; Cell density and SNP alginate work
German Shepherd	Male	Unknown	Behaviour	Cryopreserved chondrocytes
English Springer Spaniel	Male	Eleven	Congestive heart failure	Passage Two SNP alginate
Labrador	Male	Five or Six	History of blood in urine	Multiple passage beads
Labrador	Female	Three	Regurgitation and weight loss	Beads cultured in various conditions
Rottweiler	Male	Two	Aggressive	Beads cultured in various conditions

Appendix B: Recipes

Calcium Chloride ($CaCl_2$) (102mM)

- 11.322g $CaCl_2$ in 1L of nanopure H_2O

NB: For sterile $CaCl_2$, the solution was autoclaved

Coomassie Blue

- Dissolve 450mg Coomassie Blue in 112.5ml methanol, then add 45ml acetic acid
- Add RO H_2O to 450ml

Destaining Buffer

- 50% Methanol
- 40% nanopure H_2O
- 10% acetic acid

1,9-dimethylmethylene blue (DMMB) solution (pH1.5)

- Dissolve the following in 1L of RO H_2O
    - 0.016g DMMB
    - 2g sodium formate
    - 5ml 100% ethanol
    - 2ml formic acid
- Adjust pH to 1.5
- Store in the dark for up to three months

Papain Buffer (pH6.5)

- Dissolve the following in 90ml of RO H_2O
    - 1.42g dibasic sodium phosphate
    - 0.079g cysteine hydrochloride
    - 0.186g EDTA
- Adjust the pH to 6.5 using 1M HCL
- Add further RO H_2O to make a total volume of 100ml
- Store at 4^{o}C for up to three months

Paraformaldehyde (8%)

- Heat 800ml of RO H_2O to 55^{o}C
- Add 80g of paraformaldehyde and stir continuously
- Add 1M NaOH drop by drop until the solution clears
- Filter using filter paper
- Make up to 1L using RO H_2O
- Store at 4^{o}C for up to one month or aliquot and freeze at -80^{o}C

NB: Preparation of PFA should be undertaken in a fume cupboard

Phosphate Buffered Saline (PBS)

- 1 PBS tablet in 500ml of RO H_2O

NB: For sterile PBS, the solution was autoclaved

RIPA buffer

- 105mM NaCl
- 50mM Tris.HCl (pH 7.5)
- 5mM EGTA
- 1% Triton X-100
- 0.5% Sodium Deoxycholate
- 0.1% Sodium Dodecyl Sulphate (SDS)

Resolving Gel (10%)

- 2.5ml 1.5M Tris pH8.8
- 3.3ml 30% Acrylamide
- 4.1ml RO H_2O
- 50µl 20% SDS
- 75µl ammonium persulphate
- 9µl TEMED

Running Buffer (10x)

- 30.3g TrisBase
- 144.1g Glycine
- 5g SDS
- Mix in RO water to 1L

Sample Buffer (5x)

- 3.12ml 2M Tris-HCl pH6.8
- 1.88ml milli-Q water
- 10ml Glycerol

- 2.0g SDS
- 5ml 2-mercaptoethanol
- Small quantity of Bromophenol Blue

NB: This makes 20ml, so can be aliquotted and stored at -20^{o}C

Stacking Gel (4%)

- 2.5ml 0.625M Tris pH6.8
- 1.3ml 30% Acrylamide
- 6.1ml RO H_2O
- 50µl 20% SDS
- 75µl ammonium persulphate
- 9µl TEMED

Sodium Chloride (NaCl) (0.155M)

- 9.058g NaCl in 1L of nanopure water

NB: For sterile NaCl, the solution was autoclaved

Tris Buffered Saline (TBS) (10x)

- 24.4g TrisBase
- 175.3g NaCl
- Mix in 500ml RO H_2O and adjust pH to 8.0
- Make up to 2L with RO H_2O following setting pH

TBS-Tween

- To 1L of 1xTBS add 1ml Tween20

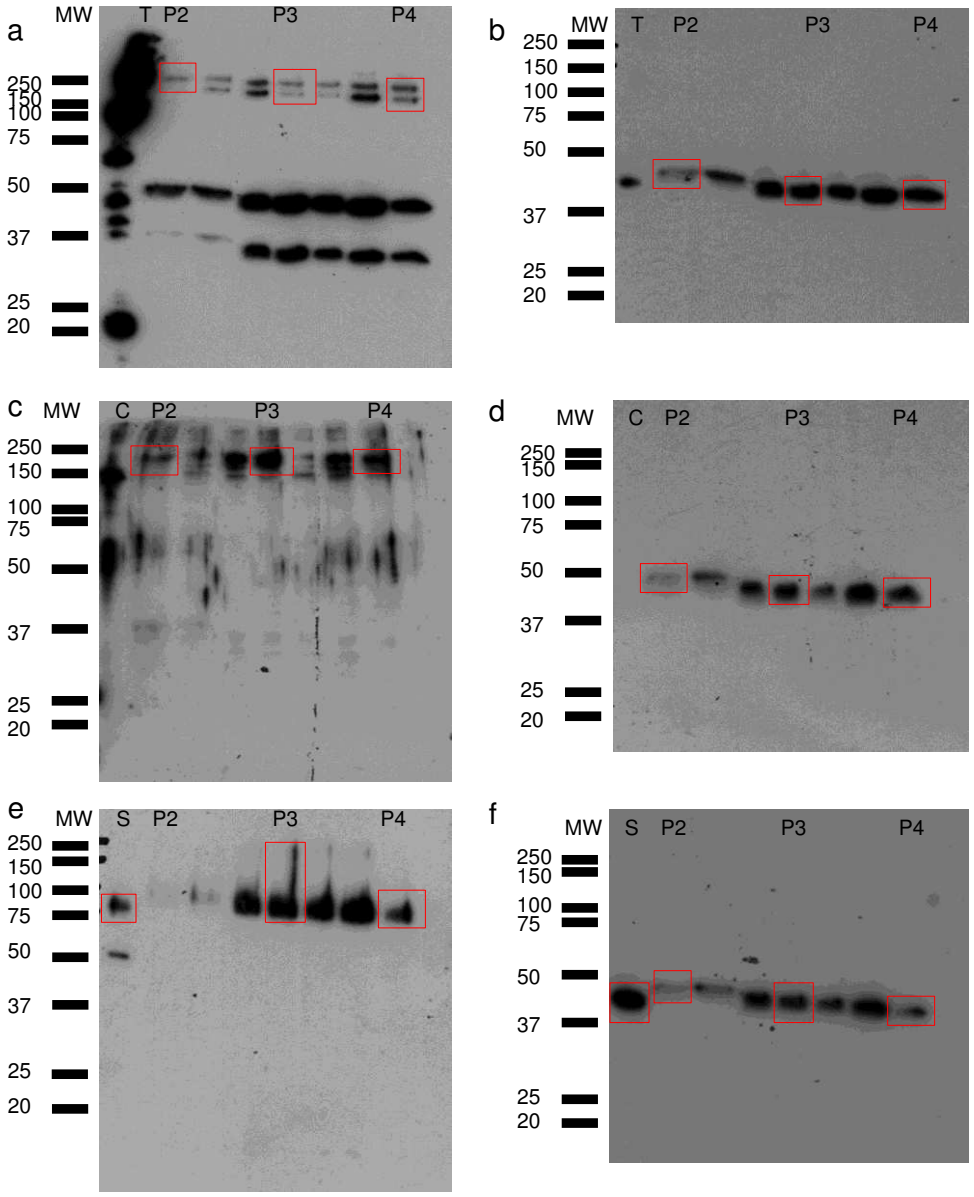
Transfer Buffer

- 7.2g Glycine
- 1.5g TrisBase
- 200ml Methanol
- 800ml RO water

Appendix C: Tissue Processing Programme Used for Articular Cartilage

Solution	Time (hours)
70% Ethanol	1.0
80% Ethanol	1.5
95% Ethanol	1.5
100% Ethanol	1.0
100% Ethanol	1.0
100% Ethanol	1.5
100% Ethanol	1.5
Histoclear	1.0
Histoclear	1.5
Histoclear	0.5
Paraffin	2.0
Paraffin	3.0

Appendix D: Western Blotting Raw Data



Original western blot images of those shown in Figure 4.16. (a) western blot for collagen type I (b) corresponding beta actin blot; (c) western blot for collagen type II, (d) corresponding beta actin blot; (e) western blot for CD44, (f) corresponding beta actin blot. 25µg of each bead lysate was loaded onto the gel. All lysates were generated from 50 beads except P2 which was generated from 20 beads. Exposures: (a) and (c): one minute 30 seconds, (e): three minutes, (b), (d) and (f): one second. T: canine tendon lysate (10µl), C: canine cartilage lysate (7µl), S: canine spleen lysate (20µg). Red boxes indicate bands of interest and the lanes that were shown in Figure 4.16