Sheep Fat Bingo

A Comparison of Subcutaneous Adipose-Derived Mesenchymal Stem Cells with Infrapatellar Adipose-Derived Mesenchymal Stem Cells with Regard to their Chondrogenic Ability in a Sheep Model of Osteochondral Defect Repair

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ABSTRACT

Articular cartilage is an avascular, aneural, alymphatic tissue that has a very low capability for intrinsic repair. Injuries to the tissue often lead to progressive degeneration, leading to painful joint disease. The most successful biologically-based treatments for articular cartilage lesions are cell-based therapies that introduce new cells to the lesion with the intention that they produce and replace lost extracellular matrix. Adipose-derived mesenchymal stem cells (ASC) are an attractive source of graft cells for their ease of collection, ease of expansion, and ability to differentiate into the chondrogenic phenotype and produce cartilage-specific extracellular matrix. While many studies report the inferiority of ASC with regard to their chondrogenic ability, the vast majority of these authors isolate ASC from subcutaneous (SC) fat harvested during lipoaspiration procedures. Few studies have studied the chondrogenic potential of ASC isolated from the infrapatellar (IP) fat pad and none have compared the chondrogenic potential of ASC from these two sources.

This study compared ASC isolated from IP and SC fat in an ovine model. Adipose tissue was harvested surgically from both SC and IP fat sources. ASC were isolated from each source and expanded by monolayer culture in vitro. Monolayer cultures were induced to pellet formations using a chondrogenic medium and then cultured in the mechanically-loading environment of a three-dimensional rotary cell culture system. Neocartilage pellets from each source were compared for their ability to produce the phenotypic cartilage extracellular matrix proteins collagen type II and aggrecan as well as pericellular collagen type VI. Qualitative analysis was performed with immunohistochemical evaluation of pellets by stain with antibodies against these matrix proteins. Quantitative assays for total collagen, total glycosaminoglycan (GAG), and Deoxyribonucleic Acid (DNA) content of pellets were performed to verify qualitative results. Pellets were then autologously implanted into osteochondral defects surgically created in the articular surfaces of sheep knees. After four and twelve weeks of recovery, post-mortem examination of defect repair was performed and defects removed by dissection for fixation and subsequent processing for histopathology and immunohistochemical analysis.

Qualitative comparison of SC and IP pellets revealed that IP pellets produced greater relative amounts of all extracellular matrix proteins studied than SC pellets. IP pellets were significantly larger than SC pellets and contained more total collagen and total GAG per unit of DNA than SC pellets. When implanted, none of the pellets contributed substantially to defect repair, but IP pellets remained within the defects at twelve weeks while SC pellets were absent.
In conclusion, this study shows that IP ASC have greater chondrogenic ability than SC ASC, suggesting potential for the use of IP ASC in tissue-engineered constructs for repair of articular cartilage injury.
PREFACE

bin go (interjection)

1 - used to announce an unexpected event or instantaneous result
2 - used to express endorsement of a correct assertion

The title of this thesis, “Sheep Fat Bingo” came about in a late-night moment during the writing of the discussion section of this thesis. It wasn’t quite an epiphany moment, but a moment of clarity in which all of the data seemed to pull together to lead me toward a viable explanation of the results. In those rare moments when everything seems so clear, I am given to utter (or shout) the word, “bingo”. At the time, I was indulging in optimistic visions of actually completing this work and looking for a shorter title than the 30-word behemoth that I was certain would never fit on the spine of the bound thesis. In the lead was my standard answer to the question of what the topic of my thesis was: “sheep fat and stem cells”. By combining said interjection with the lead title I arrived at “Sheep Fat Bingo” as a tongue-in-cheek play on the title of the 1965 movie, “Beach Blanket Bingo”.

In the seven-year odyssey that began upon my arrival in Dunedin in April 2004, many people have supported my effort and I would like to dedicate this work to them. First and foremost of them is my supervisor and friend Tony Poole. Tony accepted me as a student and supported my application to the University of Otago when the admissions board was less than enthusiastic about my undergraduate career. Tony believed in me, went to bat at the admissions board for me, and made this thesis possible through award of a NZ Health Research Council grant. He has shared his incredible enthusiasm for cartilage with me, supported me when necessary, pushed me when necessary, allowed me space when necessary, and I would not and could not have done this without him.

My other supervisor, Mark Walton, has also been incredibly supportive and inspirational. Early on he regaled me with tales of all of the former postgraduate students who failed to complete their theses and achieve their postgraduate degrees and the regret that was so plain in his voice prompted me to vow to myself that I was not going to be the latest of those tales. It was that vow that kept me going in my darkest hours, when quitting seemed a viable option. Mark also afforded me a great deal of faith and allowed me a free hand with the surgical procedures, making suggestions here and there that always improved my technique. I recall the hours spent in the operating theatre as the most enjoyable of these experiments and a large part of that was the fun and humour-filled atmosphere that Mark created with his dry wit and light-hearted conversation. A significant contribution that Mark made to this thesis was the fateful question asked during a post-operative debrief, “do you think that there is any
difference between subcutaneous fat and infrapatellar fat?” The asking of that question was the catalyst for the thought process that resulted in the overall experimental design. In addition to his personal contribution, the infrastructure that Mark established to perform orthopaedic procedures in sheep was critically important in enabling this work. Once the methods were discussed, it was a simple matter of scheduling the surgery and the processes that he had put in place over 20 years took over and the experiments happened. This would not have been possible without the indispensable assistance of Kay Pearson, who scheduled, organised, and assisted every procedure. In many ways the banter (bickering?) between Kay and Mark was as entertaining as a rehearsed comedy duo and belied a mutual respect and affection that neither would admit to, I am certain. Jo Draffin was also a key contributor to this thesis as it was her practiced eye that selected all of the sheep for this study. In her years as a sheep farmer’s daughter, Jo learned the art of stock selection and I am certain that her selections for this experiment reduced the inter-animal variability to a manageable degree. Jo was also an excellent operating theatre technician and her assistance during procedures is appreciated.

My first months in Tony’s lab were spent learning the techniques I would need to perform the aspects of the in vivo experiments. Kim Kennedy Parker patiently taught me every one of those techniques and in the many hours we spent together in the laboratory she was an invaluable sounding board for refinement of those methods, modification of those methods to those specific for fat and stem cells, and a valued second opinion on the results of all phases of the in vivo work. In addition, when I was working full-time during the day and unable to perform the methods that would have taken entire days or forced me to work over nights, Kim performed the processing and immunolabelling of a substantial portion of the in vivo histology slides.

Finally, and certainly not the least of my supporters, is my family. My parents, who always believed in me and encouraged me, have been a tremendous source of support and comfort over the last seven years. Although they missed me every day of my absence from them, they never once hinted at a suggestion of quitting to return to the US, even when Maxwell was born and they were unable to develop the relationship they would have liked to have with their grandson. Lucky last is my wife and partner Heidi, who never let me forget that there is more to life than sheep fat and stem cells. For tolerating my stress-induced moods, keeping me fed, financially supporting us when we needed it, and for giving me the gift of our wonderful son, Max.

Kia Ora
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LIST OF ABBREVIATIONS

3-D ............................................................... three dimensional
αMEM ...................................................... α minimal essential medium
µl ................................................................. microliter(s)
µm ............................................................... micrometer/micron
ACI ............................................................. autologous chondrocyte implantation
aDMEM ........................................................ advanced Dulbecco’s modified Eagle’s medium
AER ............................................................. apical ectodermal ridge
AMP .............................................................. adenosine monophosphate
ASC ............................................................. adipose-derived mesenchymal stem cell(s)
ATP .............................................................. adenosine triphosphate
BC .............................................................. biochemical
BMI .............................................................. body mass index
BM-MSC ..................................................... bone marrow-derived mesenchymal stem cell(s)
BMP .............................................................. bone morphogenic protein
BSA ............................................................. bovine serum albumin
C ................................................................. chondrocyte(s)
CPM ............................................................. continuous passive motion
cm ................................................................. centimetre(s)
CS .............................................................. chondroitin sulphate
DIC .............................................................. differential interference contrast
DNA ............................................................. deoxyribonucleic acid
ECM ............................................................. extracellular matrix
FABP-4 ......................................................... fatty acid binding protein - 4
FCS ............................................................. foetal calf serum
(b)FGF .......................................................... (basic) fibroblast growth factor
g ................................................................. gram(s)
GAG ............................................................. glycosaminoglycan
HA .............................................................. hyaluronic acid
HSC ............................................................. haematopoietic stem cell(s)
ICRS ............................................................ International Cartilage Repair Society
IHC .............................................................. immunohistochemistry
IL-1 ............................................................. interleukin-1
IP ................................................................................................. infrapatellar
ISCT .................................................................................. International Society for Cellular Therapy
kg ............................................................................................. kilogram(s)
KS ............................................................................................. keratan sulphate
L ................................................................................................. liter(s)
MACI ........................................................................ matrix assisted autologous chondrocyte transplant
mg ............................................................................................. milligram(s)
ml ............................................................................................. millilitre(s)
mm ............................................................................................. millimetre(s)
MMP .................................................................................. matrix metalloproteinase
MQ ............................................................................................. MilliQ
MRI ............................................................................................. magnetic resonance imaging
MSC .................................................................................. mesenchymal stem cell(s)
NBF .................................................................................. neutral buffered formalin
OA .............................................................................................. osteoarthritis
OAT ................................................................................ osteochondral autograft transfer
OCT ............................................................................................. optimal cutting temperature
PBS ................................................................................ phosphate buffered saline
PCM .................................................................................. pericellular matrix
PG ............................................................................................. proteoglycan
PSG ................................................................................ penicillin-streptomycin-glutamine
RBC ................................................................................ red blood cell(s)
RCCS .................................................................................. rotary cell culture system
RNA .......................................................................................... ribonucleic acid
RT-PCR ............................................................................... reverse transcriptase polymerase chain reaction
SC ............................................................................................. subcutaneous
SD ............................................................................................. standard deviation
SMA ................................................................................ smooth muscle actin
SVF ................................................................................ stromal vascular fraction
TC ............................................................................................. total collagen
TEM ........................................................................................ transmission electron microscopy
TGF ........................................................................................ transforming growth factor
TIMP ........................................................................................ MMP tissue inhibitor
INTRODUCTION

1. Introduction

Hyaline cartilage, the most abundant form of cartilage in the human body, derives its name from the Greek word *hyalos*, meaning “glass”. It is characterised by its translucent appearance, brought about by its low cellularity and high extracellular matrix-bound water content. Articular cartilage is the hyaline cartilage that covers the articulating ends of diarthroidal joints. It is responsible for protecting the underlying bone from the compressive, shear, and tensile forces experienced during joint loading and for greatly reducing friction during joint articulation. It is not, however, impervious to structural degeneration (despite remarkably little friction) and, due to its lack of intrinsic reparability, small injuries or defects often progressively degenerate, resulting in painful joint disease.

Non-surgical therapies for degenerative joint disease focus on the associated secondary soft tissue inflammation and associated symptomatic pain, but do nothing to arrest the progression of the disease, indeed in some instances, hastening it. In most cases the inevitable long-term treatment is an artificial joint replacement procedure. Surgical therapies to replace or repair damaged cartilage have been more successful at repairing the actual defect. However, the repair tissue is often less resilient than the original hyaline cartilage and degenerative pathology can be created at the site of graft tissue harvest. The most successful of surgical therapies now incorporate elements of tissue engineering to culture harvested cells for replacement into the articular cartilage defect, some using a scaffold within which to grow the engineered cartilage. More recently, mesenchymal stem cells (MSC) from bone marrow and adipose tissue have been engineered into cartilage. MSC-engineered cartilage has yet to be used clinically in human patients, but studies using animals have been performed with varying results.

The use of adipose-derived MSC (ASC) is an attractive option for engineering articular cartilage repair grafts because of its copious distribution, ease of harvest, and ability to differentiate into a chondrogenic phenotype. Little work has been done to compare different sources of ASC for their use in engineering articular cartilage constructs.

The work presented here compares infrapatellar fat with subcutaneous fat for the suitability of each tissue as a source of ASC for engineering hyaline cartilage graft material for use in a surgical model of articular cartilage defect repair in the sheep knee.
1.1. Structure and function of articular cartilage

1.1.1. Cartilage development

Articular cartilage is an aneural, avascular, alymphatic connective tissue that covers the articulating ends of diarthroidal joints. Morphologically, it contains a small number of cells, or chondrocytes, that are responsible for the production, organisation, and maintenance of the extracellular matrix that comprises the remainder of the tissue. The balance between hydration of matrix proteoglycans and the resistance to expansion imposed by the collagen network provides hydrodynamic load bearing properties that are critical for joint articulation and mitigation of mechanical compression borne by the joint [1]. Articular cartilage arises from a population of cells that is distinct from that of the embryonic cartilage. This population of cells occupies the surface of the newly cavitated joint and may be derived from this region of the embryonic epiphysis [2] or from cells of the interzone [3]. Most of the growth in developing cartilage is appositional, occurring at the margins of the expanding epiphyses particularly during late stages of development [2] (Figure 1). Articular cartilage does not fully complete structural differentiation until skeletal maturity as defined by closure of the epiphyseal plate [4].

![Diagrammatic representation of appositional growth of developing articular cartilage](image)

Figure 1: Diagrammatic representation of appositional growth of developing articular cartilage
Progenitor cells in the surface layer divide giving rise to two daughter cells, one being another progenitor cell and the second being a transit amplifying unit cell within the transitional zone that can then undergo further divisions and subsequent differentiation along a chondrogenic pathway. Note that maturing chondrocytes do not migrate through the matrix, but rather maintain their position relative to the original surface layer progenitor cell as the articular cartilage grows through apposition. [5]

The elements of the mammalian skeleton are laid down and differentiate in a proximodistal sequence [6]. Limb buds, in the form of ectodermal outgrowths from the flank
of the embryo, are formed during the fifth week of gestation when type I collagen first appears throughout the mesenchyme [7, 8].

Figure 2: Development of the mammalian long bone
Articular cartilage forms from original chondroblasts at each bulbous end of the long bone. Taken from Stevens and Lowe, Human Histology, p. 246, Figure 13.24 [9]

Mesenchymal cells in the progress zone beneath the apical ectodermal ridge (AER) are maintained in an undifferentiated and highly proliferative state [10] and become lineage committed based on when they leave the AER [2]. Driven by paracrine signals, such as basic fibroblast growth factor (bFGF), which is released by the AER, and tenascin [11] these cells round and condense to form a prechondrogenic core [8]. Extensive cell-cell and cell-matrix interactions, mediated by matrix glycoproteins such as tenascin [11], thrombospondins 1-4 [12], COMP [12], syndecan-3 [13], and fibronectin [14], trigger chondrogenic differentiation and patterning as chondrocyte-specific gene expression is activated. Synthesis and sequestration of specific matrix macromolecules, such as hyaluronan, aggrecan, and collagen types II and IX [8], force cells apart, terminating gap junctions and promoting further chondrogenesis. Type II collagen is originally concentrated in the ends of the epiphysis and becomes generalised in the extracellular matrix by maturity [7]. Type II collagen fibrillogenesis is moderated by procollagen proteinases with regard to supply of the molecular fibril subunits, but lateral growth control of the fibrils are mediated by the processed collagens II and IX themselves [15]. Type VI collagen, originally distributed throughout the mesenchyme of the neonatal limb bud [7], becomes concentrated in the pericellular matrix (PCM), particularly so in regions that persist to maturity and less intensely in regions that remodel during development [16]. The distribution of proteoglycans is highly dynamic during development [17], indicating a variety of functions performed during the formation of the articular cartilage matrix.

The developing articular-epiphyseal cartilage complex organizes into three transverse layers [2]. The tangential zone is characterised by a thin layer of discoid chondrocytes; the
transitional zone is characterised by small groups of spheroid chondrocytes that are randomly organised and proliferate but do not form radial columns; and the radial zone, which gives rise to adult articular cartilage, is characterised by small groups of spheroid chondrocytes that organize into radially aligned columns and do not proliferate. The radial zone is further divided into the proliferating cell or surface zone, characterised by dividing cells that form egg-shaped clusters and where the majority of growth activity in the developing cartilage (in terms of proliferation and synthesis of IGF) occurs [2]; and the hypertrophic zone with rounded, non-columnar cells; and the calcification zone in which matrix calcification encloses clusters of hypertrophic chondrocytes [8].

1.1.2. Cartilage as a tissue

1.1.2.1. Structure

Mature articular cartilage is characterised by an abundant extracellular matrix that makes up more than 95% of the tissue volume and consists mainly of sulphated proteoglycan and collagen (principally type II) [4]. Typically, tissue thickness is greatest in regions of highest physiological loads [18-20]. The remaining 5% of tissue is mostly a sparse population of chondrocytes, characterised by small size and rounded appearance. Each chondrocyte is surrounded by a complex pericellular microenvironment that is distinct from, but integrated with, the bulk of the extracellular matrix, itself divisible into a territorial matrix adjacent to the chondrocyte-pericellular complex and an interterritorial matrix between adjacent territories [4]. Articular cartilage matrix is formed under dynamic load and the combination of compressive and shear force exerted on the tissue as a result of physiological loading results in a clear division of matrix into structurally identifiable zones and concentric subdivisions. In this way it is the epitome of the “form follows function” paradigm. Articular cartilage is organised in layers from the articulating surface layer through the middle and deep layers to the calcified layer that forms the interface between cartilage and subchondral bone (Figure 3).
Figure 3: Articular cartilage structure
Histology section (left) and Illustration (right) of a vertical section of articular cartilage clearly showing surface (s), middle (m), deep (d), and calcified (c) layers, as well as the tide mark (tm) and subchondral bone (b). The illustration is Figure 293 from Gray's Anatomy and is a classic example of early understanding of the structure of articular cartilage. [21]

The surface layer is exposed to the joint cavity and covered by a surface coat of moderately dense material [22] called the lamina splendens. The surface layer represents 5-10% of the total matrix volume and is characterised by small, disc-shaped chondrocytes, low proteoglycan content, and densely packed layers of uniform diameter collagen fibres aligned tangentially to the articular surface as is the long axis of the chondrocyte [1, 4]. The packing density and tangential alignment of surface layer collagens provide articular cartilage with tension resistance (indentation during weight bearing) and characteristic hyaline opacity. Surface layer chondrocytes are aligned parallel to the dominant fibrillar orientation [4, 23] and contain a subpopulation of cells that has a limited ability to form a pericellular matrix but higher proliferative capability [24]. Dowthwaite et al. [25] further characterised this subpopulation of surface layer chondrocytes as expressing Notch 1, having an affinity for fibronectin, and an ability to form highly proliferative colony forming units in vitro that can differentiate into several tissues in the mesenchymal lineage.

The middle layer makes up 40-45% of the total matrix volume and is characterised by spherical chondrocytes, often found in pairs [23], a higher proteoglycan content than the surface layer, and more differentiated matrix comprised of an amorphous network of
obliquely oriented collagen fibres [1, 4]. Chondrocytes in the middle layer maintain their affinity for fibronectin, but lose their ability to proliferate [25].

The deep layer comprises 40-45% of the total matrix volume and is characterised by spherical chondrocytes arranged in columns, high proteoglycan content, and radially aligned collagen fibres [1, 4]. In the deep layer, chondrocyte columns are aligned parallel to what Benninghoff [26] described as “collagen arcades” that are aligned perpendicular to the articular surface and account for the most pronounced matrix differentiation in articular cartilage [4]. These collagen fibres become increasingly compacted as they approach the tidemark, which delineates the deep layer from the calcified cartilage layer, excluding proteoglycan aggregates from the area adjacent to the tidemark [4].

At the interface between articular cartilage and the subchondral bone lays the calcified cartilage layer, which accounts for the remaining 5-10% of the matrix volume [4]. The calcified cartilage is characterised by spherical chondrocytes in fluid-filled capsules, the absence of proteoglycans, a high concentration of calcium salts, and radial collagen fibres anchored in a calcified matrix [1] largely composed of type X collagen fibres [27], braced and supported by a complex interaction of calcium and other components [4]. The calcified cartilage is bounded above by the smoothly undulating tidemark, composed of calcium; phosphate; lipids; and enzymes such as alkaline phosphatase and ATPase [28], and below by the highly convoluted cement line that provides firm anchorage of the articular cartilage to the underlying subchondral bone [29] and at which point the radial collagen network abruptly terminates [1].

Chondrocytes below the surface layer of articular cartilage are surrounded by a pericellular matrix enclosed by a pericellular capsule that is itself surrounded by a territorial matrix and an interterritorial matrix between columns of territorial matrix [23] (Figure 4). The distinct ultrastructure of each of these concentric matrix zones, with regard to the presence and organisation of collagens and noncollagenous matrix proteins, gives each zone a unique ability to respond to physiological compression and suggests that they function synergistically to produce an integrated hydroelastic suspension system to withstand such physiological forces [23].
Benninghoff’s [26] articular cartilage matrix arcades that traverse radially through the deep layer from calcified cartilage to decussate through the middle layer and come to lay tightly packed in parallel with the articulating surface. Within this architecture, fine fibrils predominate in the vicinity of the chondrocyte and fibril size progressively increases with distance from the cell [22, 29].

The pericellular capsule is composed of tightly woven fine collagen fibrils oriented longitudinally, transversely, obliquely, and in a circular fashion around the pericellular matrix with a dense accumulation of proteoglycan associated with fibrils of the capsule [23]. The articular pole of each capsule is densely compacted while the basal pole has a more loosely woven and porous texture [23]. The pericellular matrix is rich in hyaluronan [30], sulphated proteoglycans [23], a range of matrix glycoproteins [31-33] and a high concentration of type VI collagen [34, 35]. Together, the chondrocyte and its pericellular microenvironment represent the chondron [36], which will be discussed in much greater detail in section 1.1.3 as it is the primary structural, functional, and metabolic unit of adult articular and other hyaline cartilages.

Surrounding each chondron is a 10-30 µm circumferential territory composed mainly of loosely organised collagen bundles with clear interdigitating spaces that are filled with proteoglycan [23, 37]. Sometimes these proteoglycan “fingers” interconnect adjacent chondrons [4], suggesting the potential for preferential diffusion paths between chondrons,
further strengthened by the presence of matrix vesicles in these spaces [4]. While hyaluronan makes up a significant portion of the total matrix [30], chondroitin sulphate is more concentrated in the territorial matrix [29] and decreases with aging [38]. By contrast, the interterritorial matrix, which fills the spaces between adjacent territorial matrices, is characterised by more tightly packed, larger diameter collagen fibres [4] arranged in a radial organisation and mixed with some random orientation punctuated by “knotted” crossover points [39, 40]. High molecular weight keratan sulphate is more concentrated in the interterritorial matrix [29] and increases with aging [38].

1.1.2. Function

Articular cartilage functions to create a dynamic, transitional interface between the stiff but compliant surface and the rigid, calcified subchondral bone. It provides a covering material that protects bone from abrasion and shear damage; it provides for joint congruity and low contact stress between apposing bones; and it provides a smooth, lubricated surface that facilitates movement and minimises friction [4]. The dynamic balance between hydration of the hydrophilic matrix proteoglycans and the resistance to expansion imposed by the collagen network allows for dissipation of the weight-bearing load over a large area by movement of the partially mobile aqueous phase. This is critical for joint articulation and the smooth transition of mechanical compression across the joint [1]. Chondrocytes play a crucial role in the synthesis, organisation, and maintenance of the extracellular matrix and are, in turn, protected by the extracellular matrix from mechanical force. The chondrocytes and extracellular matrix function synergistically to provide a hydroelastic suspension mechanism able to absorb, redistribute, and transmit physiological compressive and shearing forces to the subchondral bone [23]. The functional integrity of this system is dependent on the relatively low hydraulic permeability that controls the viscoelasticity and high swelling tendency of proteoglycans as well as entrapment of this hydrodynamic gel within the collagenous network [41, 42]. It is the permeability of the matrix that governs the aqueous flow through it, which determines the ability to deform and energy dissipation through the tissue. The success of this composite material hinges upon the very intimate bonding of collagen to proteoglycan that allows compressive loads to be efficiently transferred from tension-resistant components to compression resistant components.

At the articulating surface, the tangential arrangement of surface layer collagens provides tension resistance and load distribution [43]. The relatively low proteoglycan content of the surface layer insures little compressive deformation and efficient transfer of load to the deeper layers. The radial alignment and higher proteoglycan content of the deeper layers suggest its function as a hydroelastic shock absorber. Under load, the matrix in these layers is
deformed at constant volume and water is forced from the proteoglycans into uncompressed areas of the matrix, dissipating load/energy laterally, until a resting equilibrium is established. When load is removed, osmotic pressures exceed applied load and the proteoglycans re-imbibe water to achieve a new equilibrium between applied load and the proteoglycan swelling potential [41, 42]. Compression creates minute pressure gradients within the matrix, aided by compartmentalisation of the pericellular, territorial, and interterritorial matrices, to assist fluid flow throughout the matrix and enable it to withstand or absorb applied load without permanent deformation [23].

Chondrocytes respond to mechanical [23] and osmotic [44] challenge by varying degrees depending on which layer they are in (surface, middle, or deep), indicating the influence of the extracellular matrix. Under load, the chondrocytes in the surface layer expand laterally between the tangentially oriented sheets of collagen fibres, accentuating their flattened, discoidal shape. Loaded chondrocytes in the middle layers compact vertically and expand laterally to assume an oval shape while deep layer chondrocyte columns compact vertically with little lateral deformation [23]. Within the layers of cartilage, the concentric matrix compartments also exhibit differential responses to load. Under compression the radial collagen fibres in the territorial matrix collapse into a synchronised crimped wave form, the pericellular matrix and capsule compact vertically and deform laterally, fluid is lost from proteoglycans, matrix vesicles are pumped out into the territorial matrix, and load is distributed over the articulating surface [45]. The synchronous crimping of territorial matrix collagens is facilitated by tight packing into discrete bundles and separation of these bundles by fluid-containing proteoglycan tracts [23]. Compression in the interterritorial matrix affects individual collagen fibres, producing a random, irregular, and asynchronous crimping pattern [23]. The larger diameter, closer packing, and greater degree of stiffness due to higher concentrations of high molecular weight keratan sulphate serve to increase resistance to compressive deformation [46]. Thus, the efficacy of this integrated system is primarily dependent upon the variation in stiffness, compliance, and compressibility of collagen and proteoglycan components within different parts of the concentric matrices.

1.1.2.3. Low intrinsic reparability

In 1743, William Hunter wrote, "…from Hippocrates down to the present age, we shall find, that an ulcerated cartilage is universally allowed to be a troublesome disease;…and that, when destroyed, it is never recovered." [47]. This sentiment was echoed a century later by Sir James Paget who wrote, “there are, I believe, no instances in which a lost portion of cartilage has been restored, or a wounded portion repaired with new and well-formed permanent cartilage, in the human subject” [48]. Trauma to the articular cartilage fails to heal
spontaneously due, in part, to low mitotic activity and its avascular nature [49] and will often progress to degenerative joint disease with time [50] as the cells lose their ability to maintain the matrix that is so critically important to their survival. The immunomodulators, cytokines, and nutrients that contribute to healing of other, vascularised tissues must be carried to ailing chondrocytes by the less efficient osmotic “pump” mechanism existing in cartilage. The efficacy of this transport system is further reduced by the over hydration of defective cartilage [51]. As will be discussed in the section on articular cartilage failure (1.2.), the chondrocyte repair response bears a resemblance to immature cartilage development and primarily involves the initiation of proliferation in typically non-proliferative cells. The resultant cellular hypertrophy occurs within the restraints of the pericellular matrix capsule, which becomes stressed to the elastic limit of the collagen network. Any new matrix formed by these cell clusters is unable to withstand the strain imposed on the matrix organisation infrastructure, and therefore fails to mature and coalesce into a cartilage form that is able to handle the cyclical compressive and shear forces experienced during normal loading.

1.1.3. The chondron – the functional unit of articular cartilage

The concept of the chondron was first introduced by Benninghoff [26] who used polarized light microscopy to describe a structural unit comprised of the chondrocyte and its pericellular environment in a range of hyaline cartilages. Four decades later Szirmai [52] applied newly developed histochemical concepts and microchemical analysis to re-evaluate the chondron concept in horse nasal cartilage homogenate. He described the “micro-world” of the chondrocyte consisting of a fine collagenous “perilacunar rim” surrounding a proteoglycan-rich “lacunar space”. The fact that chondrons could be extracted by high-speed homogenization proved that the chondron was a mechanically robust anatomical unit.

Almost 20 years later, Poole [36] conclusively established the chondron as the primary structural, functional, and metabolic unit of adult articular cartilage through his work with low-speed homogenates of canine tibial cartilage. He demonstrated the viability of chondrocytes within intact chondrons isolated from his slow-speed homogenates; confirmed the presence of cartilage-specific proteoglycans, glycoproteins, and collagens; and demonstrated the metabolic function of the chondron with autoradiographic studies of aggrecan distribution and catabolism. The isolated chondron thus became a model to study the intimate relationship between the chondrocyte, its pericellular microenvironment, and the remainder of the adult articular cartilage matrix.

It is now widely accepted that the chondron is a complex microanatomical unit containing a heterogeneous mixture of matrix macromolecules differentially organised to produce a complex pericellular microenvironment intermediate between the chondrocyte and
the load-bearing territorial and interterritorial matrices. It is the pericellular matrix that provides hydrodynamic protection for the chondrocyte during physiological loading and plays a metabolic role in the pericellular sequestration, assembly, and organisation of newly synthesized macromolecules into the extracellular matrix [36, 45].

1.1.3.1. Structure

The pericellular matrix of chondrocytes initially develops as a narrow glycocalyx that consolidates over time [53] to form the chondron. High concentrations of aggrecan, link protein, and hyaluronan in the pericellular matrix are sequestered early in chondron development to form hyaluronan-aggrecan-link protein complexes. There are strong proteoglycan-collagen interactions within the pericellular matrix, as evidenced by the resistance of some proteoglycan to extraction, that account for matrix sequestration, organisation, and maintenance [1]. It has been suggested that Type VI collagen and fibronectin could act cooperatively to anchor a collagen and glycoprotein-rich calyx at the cell surface and could subsequently act as molecular scaffolds for the continued assembly and differentiation of matrix macromolecules [54].

The microenvironment of the chondron consists of two integrated parts: the pericellular glycocalyx, which is rich in aggrecan [55]; hyaluronan [30]; and fibronectin [31, 56], and the pericellular capsule, which is comprised of collagen types II, VI, IX, and XI [34, 57-59] and laminin [32]. The transparent glycocalyx completely encapsulates the chondrocyte and is in intimate contact with the cell surface through type VI collagen, fibronectin, and decorin attachment points [53]. The transparency by phase contrast and differential interference contrast (DIC) microscopy of the pericellular matrix is due to the low ratio of organic matter to water resulting from hydration of proteoglycan [60], which led to its original distinction as an empty space or “lacuna”. Removal of matrix proteoglycan causes the capsule to collapse against the cell surface, suggesting its normal anatomical position is mediated by pericellular matrix hydration [37].

Chondrocytes form intimate contacts with some regions of the capsule while other areas of the cell seem detached from the capsule but maintain contact through a series of extended microfibrils [45]. The capsular collagens form an isotropic boundary around the chondrocyte, entrapping proteoglycan within the microenvironment and limiting their expansion to create an osmotically robust chondron [44]. Each chondrocyte develops the chondron to withstand a unique set of dynamic mechanical forces and is responsible for its own pericellular microenvironment although a continuous/shared capsule in chondron columns implies cooperative interaction in the deeper layers of articular cartilage [35].
Circular, disc-shaped chondrons appear singularly in the surface layer while more spherical single chondrons are more prevalent in the middle layer of cartilage. Double chondrons prevail in the middle/deep layers, giving way to chondron columns in the deep layer [35]. This differential morphology suggests unique roles for the chondrons of each layer, from resistance to shear forces experienced in the surface layer, to transfer of mechanical load through the middle layers to the deeper layers where the bulk of resistance to compressive force would take place.

The largely unidirectional compressive force exerted upon articular cartilage and the prevailing collagenous architecture imposes a polarity on the chondron. It has been suggested that this polarity is dictated by the primary cilium, which is the extracellular component of a membrane-bound ciliary axoneme projecting out into and interacting with the pericellular matrix where it is bent or deflected against the cell surface by confinement within the chondron [61]. Intracellularly, it is continuous with a diplosomal basal body, attached distally to the cell membrane by alar sheets and has basal feet.

The primary cilia acts as a cellular cybernetic probe capable of transducing mechanical, physical, and/or osmotic changes in the cellular microenvironment and signalling those changes to secretory organelles, such as the Golgi apparatus and microtubular transport system, responsible for mediating the appropriate feedback response to applied mechanical load [61-64].

During development, the response to the directional compression would be the secretion and sequestration of matrix collagens and their integration with the surrounding matrix. In single chondrons the articular pole, facing the articular surface, is more dense and compacted than the basal pole, which tapers toward the tidemark [1]. The chondron tails are typically 10-40 microns long, but some of up to 100 microns have been reported [34]. In multiple chondron columns the tails form interconnecting segments between adjacent chondrons in a linear arrangement [1]. Proteoglycan monomer is particularly concentrated in the tail and interconnecting segments of chondron columns where it could function to protect and stabilise the chondrocyte [65]. The presence of frayed collagen fibres radiating outward from mechanically isolated chondron columns suggests a shear-resistant, structural relationship between the capsular components and the radial type II collagen network of the territorial matrix [65, 66].

1.1.3.2. Functional response of chondrons to environmental/osmotic stress

The chondrocyte is responsible for the formation and maintenance of the pericellular microenvironment and is able to remodel the chondron in response to changing environmental influences or to physicochemical and biomechanical properties of degenerating matrix [4].
The chondron response to matrix degeneration will be discussed in later sections. The geometrical changes that take place pericellularly in response to physiological loading include vertical compaction, lateral expansion, and shearing displacement with associated reduction in pericellular matrix volume [4]. In healthy articular cartilage these changes are transient and deformed chondrons almost always recover their shape and position in the matrix after removal of load.

As discussed in section 1.1.2.2., the various components of the articular cartilage matrix combine functionally to produce an integrated response to physiological load. This concept also holds true for the chondron as deformation causes loss of proteoglycan-bound water, flow-regulated by the restriction of capsular collagens, which serves to protect the chondrocyte from shear and compressive forces. However, each component of the chondron also serves other functions such as anchorage, storage of synthesized materials [4], and cell-matrix interaction mediation [45, 67]. In addition, feedback loops allow the chondrocyte and its pericellular matrix to dynamically adapt to the constantly changing environment of the loaded joint [44, 68, 69], aided by streaming potentials created by the movement of water from the chondron [23].

The pericellular capsules of chondrons in columns within the middle and deep layers of articular cartilage are mutually interconnected to form one, continuous capsule around the entire column, creating an integrated linear unit [4]. The uppermost chondron in a column has the most dense capsule, as it will bear the bulk of the compressive load, and forms a cupola-like structure over the whole column [4]. The column is stabilised by the radial collagen fibres of the territorial matrix that impinge upon, but do not penetrate, the capsule. In this way, the column can maintain its alignment perpendicular to the articular surface and thus realize the compression-resisting properties of such alignment. Displacement of the column during loading is further limited by the tail of the chondron column. The chondron tail is strongly interdigitated with the bulk of the surrounding matrix and may play an additional role in load bearing through hydroelastic cushioning provided by high sulphated proteoglycan concentrations [4].

Pericellular matrix proteoglycans and collagens, intimately linked to the chondrocyte cytoskeleton via a range of cell-specific receptors including integrins and CD44, play a major role in signalling matrix changes to the cell [44]. The stabilising mechanism of the chondron extends from the chondrocyte binding to the capsule and tail, beyond the capsule to the radial collagens, and through the radial collagens of the territorial and interterritorial matrices to the calcified cartilage, and is also involved in load bearing and cell-matrix interaction. This is a
fine example of the elegant and integrated structural economy that exists within the articular cartilage matrix.

As previously stated, the chondrocyte is able to remodel the chondron in response to changing environmental influences. With regard to proteoglycan synthesis, which in turn affects the hydrodynamic properties of the chondron and its response to mechanical and osmotic pressure, the chondrocyte response to loading depends on the type of load. The chondrocyte responds to static load by first stimulating synthesis of proteoglycans and then inhibiting proteoglycan synthesis while cyclic or intermittent loading is stimulatory [68]. The chondrocyte is able to exert influence over the matrix by export of vesicles into the surrounding matrix during mechanical compression [23, 36, 70] thus controlling macromolecular transport and strain around cells [67]. Furthermore, the composition and integrity of the pericellular matrix can influence the metabolic rate [69] and the volume regulatory response of the chondrocyte [44]. As the primary functional and metabolic unit of adult articular cartilage, the chondron therefore exerts influence over the surrounding matrix to protect the chondrocyte and is, in turn, influenced by the matrix as it responds dynamically to smoothly transfer load to the underlying bone.

1.1.4. Extracellular matrix and the role of matrix macromolecules in articular cartilage

The role of the extracellular matrix is primarily one of resistance to mechanical and osmotic stresses experienced during physiological loading with each component of the matrix performing a specific set of tasks in its integrated function, controlled by the chondrocyte. Cell synthetic activity is profoundly influenced by mechanical load [68], which implies that the cell must be able to sense applied load and respond appropriately. The chondrocyte can only direct the synthesis and sequestration of the matrix, and therefore its ability to withstand environmental stresses, by dynamically sensing its environment, which is accomplished through the primary cilium and the cell’s intimate links with the extracellular matrix. Many factors, including involvement of cyclic AMP [71]; osmotic state of proteoglycan [72]; interstitial pH [73]; the type, frequency, and amplitude of load [74]; hydrostatic pressure [75]; changes in pericellular ionic composition [76]; and deflection of primary cilia [37], have been implicated in signalling biomechanically influenced changes that the chondrocyte can respond to [4]. The signals that regulate chondrocyte function, morphology, and volume regulatory response [44] are translated through “outside-in” cell-matrix interactions that are enabled by the integration of the matrix with intracellular components including the cytoskeleton to which the primary cilium is attached [77].
The pericellular matrix interacts directly with the chondrocyte through cell-surface receptors such as anchorin CII [78], integrins [79], and CD44 [80] binding to specific matrix macromolecules such as pericellular collagens, fibronectin, and hyaluronan [81]. The presence of cell-matrix attachment sites is therefore important in stabilising the interaction between the chondrocyte, the pericellular matrix, and the extracellular matrix beyond the chondron [45] as the binding of matrix molecules to the cell surface conveys information about its physiochemical environment to the chondrocyte [82].

1.1.4.1. Type II collagen, type XI collagen, and type IX collagen

Type II collagen is the dominant collagen species in articular cartilage accounting for 90-95% of all collagens [83]. Not surprisingly, type II collagen is largely responsible for the resistance to compressive load that characterises articular cartilage. The collagen fibres are heterogeneous in diameter and composition [84], arranged generally perpendicular to the articulating surface, and crimp in either a synchronized waveform or in a random fashion, depending on zonal location, in response to compressive loading [45]. As previously discussed, this crimping serves to smoothly transmit compressive forces from the articulating surface, through the pliable middle and deep layers, to the rigid calcified cartilage and underlying subchondral bone. Straightening of the crimp requires fairly low tensile load, providing evidence of the efficiency of this energy transfer system.

The heterogeneity of type II collagen fibril diameter results from the differing composites of this collagen species found at various distances from the cell and has a profound effect on its function. In general, type II collagen fibril size increases with distance from the chondrocyte [22, 29].

Fine fibrillar heterotypic type II collagens, wrapped around a type XI collagen core [34, 85] and decorated on its surface in a bottle-brush fashion by type IX collagen [34, 45], predominate in the chondron capsule [66] and pericellular matrix [53]. These fine, long, weakly banded fibrils of a uniform 20 nm diameter [15] are required to form the tight weave of the capsule that is responsible for containing pericellular proteoglycans and restricting the loss of water during physiological loading. In bovine epiphyseal cartilage, both the proteoglycan and non-proteoglycan forms of type IX collagen are present, but the non-proteoglycan form is preferentially incorporated into the matrix [86] to maturity, and specifically in the chondron capsule [87]. Due to its preferential localisation in the chondron it has been suggested that it functions to regulate type II collagen diameter [66], but Li and Olsen [88] showed that it functions to stabilise interactions within the fine fibrillar network or between fibrils and the extracellular matrix. This serves to maintain tissue integrity as the cationic NC4 domain of type IX collagen projects from the fibril surface and interacts with
anionic domains of other pericellular matrix molecules [53] aiding in fibril formation efficiency and stability [15].

The role of type II collagen diameter regulation is instead performed by type XI collagen [89], with a bulky N-terminal that is large enough to cover 8–10 adjacent type II collagen molecules on the fibril surface, thereby preventing further growth in diameter as long as molar ratios of collagen types II and XI are maintained up to 8:1 in immature cartilage and 10:1 in adult cartilage [15]. In the extracellular matrix the weight proportion of type II collagen exceeds 90%, lateral growth control is lost [15] and large, banded type II collagen fibrils predominate [66]. Therefore, the heterogeneous type II collagen in articular cartilage performs two differing and crucial functions. In the pericellular matrix and capsule the fine heterotypic copolymers of type II collagen, wound around a type XI core with type IX collagen projecting from the surface, weave tightly to sequester hydrated proteoglycans within the capsule and restrict flow of fluid from the chondron, providing hydrodynamic protection for the chondrocyte during physiological loading. Further from the cell, the large, banded collagen type II fibres of the extracellular matrix crimp when loaded, smoothly transferring the compressive force to the underlying bone. It stands to reason that the thicker and stronger fibrils would be located away from the chondron, where the bulk of weight would be borne, relieving the cell of most of the load. A close molecular bond between collagen and associated proteoglycan means that the hydrophilic proteoglycan is restricted in the amount of water it can imbibe. Therefore, it is the proteoglycan that restricts the water flow as it needs increased compression to release entrapped water.

1.1.4.2. Type VI collagen

Type VI collagen is a beaded filament that consists of three polypeptide chains (α1, α2, and α3) that contain a short triple helical domain (105 nm), at each end of which is a large N- and C-terminal globular domain [90], and are bundled together as a monomeric unit. The monomers assemble intracellularly into dimers and tetramers that are then secreted into the extracellular matrix and assemble into microfibrillar structures by end-to-end association [91]. The mosaic structure of type VI indicates multifunctional characteristics capable of providing not only mechanical support through its collagenous sequences but also an additional range of functions that include cell adhesion and collagen binding [92]. Type VI collagen fibres have been observed in association with the cell surface as well as major collagen fibres of the extracellular matrix, providing cohesive interaction between the chondrocyte, the pericellular matrix, and the extracellular matrix [93].

The similarity of type VI globular domains to domains present in adhesive extracellular matrix proteins such as von Willebrand factor, cartilage matrix protein, and
fibronectin [91] is a strong indicator of its central role in the sequestration and organisation of other macromolecules during chondron formation [35]. The structural colocalisation of type VI collagen and fibronectin at the surface of cultured chondrocytes and mechanically isolated chondrons [54], as well as the upregulation of both proteins during articular cartilage degeneration [24, 54], imply a combined role in cell-matrix adhesion and matrix-matrix cohesion during the development and maintenance of the pericellular matrix. Aside from fibronectin, type VI collagen interacts with a number of other matrix components (Figure 5) including decorin; fibromodulin; hyaluronan; perlecan; and heparin [24, 67], providing further evidence that it plays a critical part in the assembly and organisation of pericellular matrix architecture [1, 53].

Figure 5: Illustrative view of the chondrocyte pericellular matrix
An illustration of the intricate relationships that exist between the chondrocyte and the pericellular matrix molecules as well as between the collagens, proteoglycans (PG), glycosaminoglycans (GAG), and glycoproteins that form the pericellular matrix. Adhesion molecules such as hyaluronan receptor (CD44), integrin, and anchorin CII bind pericellular collagens that in turn bind each other, PG, GAG chains, and glycoproteins. This matrix of pericellular macromolecules serves many purposes, arguably the most important of which is protection of the chondrocyte. Courtesy of CA Poole.

Type VI collagen binds to the chondrocyte membrane at its articular pole [34] through its triple helical domain [24], mediated by cell surface receptors such as integrins; CD44; Anchorin CII; and NG-2, which also serve to provide signalling potentials between the cell and its pericellular microenvironment [34]. Given this critical relationship with the cell surface and its low affinity for type II collagen [67], it is not surprising that type VI collagen is most concentrated in the pericellular matrix and capsule [35]. It is particularly concentrated at the inner margin of the capsule, whereas at the outer margin it forms dense aggregates that create a distinct boundary with the radial type II collagen fibres of the territorial matrix [35]. It is less concentrated in the chondron tail and interconnecting segments between adjacent
chondrons in a column or cluster [35] where it integrates with the radial collagen network to stabilise the collagens, proteoglycan, and glycoproteins of the pericellular matrix [34, 54].

It has been suggested that, within normal articular cartilage, type VI collagen is found exclusively in the chondron [67], but the presence, albeit minimal, of type VI collagen has been noted in the territorial matrix, and to an even lesser degree in the interterritorial matrix, where it presents as randomly distributed punctuate deposits [34]. The role of type VI collagen in the territorial and interterritorial matrices has yet to be discerned but it is possible that it serves to stabilise the radial collagen network and assist in maintenance of matrix integrity while radial collagens crimp under load.

1.1.4.3. Proteoglycan, glycosaminoglycan, and matrix glycoproteins

A proteoglycan is a protein that has one or more attached glycosaminoglycan (GAG) chains [94]. GAG chains are large extended structures with highly charged sulphate and carboxylate groups, and they dominate the physical properties of the protein to which they are attached [82]. Within the extracellular matrix of articular cartilage, proteoglycans serve primarily to bind water and create fluid-filled compartments, critical to the compression resistant properties of the pericellular and extracellular matrices. However, the various proteoglycans in the pericellular and extracellular matrices of articular cartilage also serve a number of other functions, mainly in cell and matrix adhesion, which will be discussed in this section.

The “minor” extracellular proteoglycans and glycoproteins of the extracellular matrix include: decorin; biglycan; fibromodulin; fibronectin; link protein; laminin; and even type IX collagen, which has a proteoglycan form [31, 32, 45, 53, 82, 95]. In general, they assist in matrix organisation and stabilisation by binding to the cell surface, collagens, and other matrix macromolecules (Figure 5) [82, 96], but also play roles in collagen fibril assembly as do collagen type IX [53, 88] and decorin [53, 97], which also mediates chondrocyte metabolism by binding TGF-β [82, 98].

The vast majority of proteoglycan in adult articular cartilage is aggrecan, accounting for 80-90% of all proteoglycans [83]. In the pericellular matrix, aggrecan is found as a huge multimolecular aggregate comprised of a single hyaluronic acid (HA) chain bound to as many as 800 [60] proteoglycan monomers [99] and noncovalently bound to and stabilised by link protein, which is homologous with the N-terminal domain of aggrecan [100]. The proteoglycan monomer consists of an extended protein core with up to 150 sulphated GAG chains, consisting mainly of chondroitin sulphate (CS) and keratan sulphate (KS) [101]. The presence and location of epitopes within the GAG chains, which govern the physical properties of the protein to which they are attached, varies with the source, developmental
status, and pathological status of chondrocytes, regulated by the chondrocyte itself [102]. HA is a crucial component of articular cartilage and performs many critical functions from joint development, where it plays a central role in joint cavitation [2], to the maintenance and homeostasis of adult cartilage [103], and it is considered by some to be the scaffold upon which the articular cartilage matrix is formed [60, 81, 83]. However, as most of the HA in cartilage is associated with aggrecan [99], and other associated components are required for the formation of the extracellular matrix [104], it will only be discussed in relation to its function within the HA-aggrecan complex.

Amounts of HA-aggrecan complex components in cartilage, extracted by guanidine – HCL, are 79% CS, 16.3% KS, and 4.3% HA and are similar in chondrons and intact cartilage [56], suggesting that the HA-aggrecan complex exists mainly in association with the chondron. However, since a fraction of cartilage and chondron samples were resistant to extraction and the resistant fraction was enriched with CS (70.9%) and HA (10.5%) [56], it is assumed that the amounts of these components would be higher in intact tissue.

Aggrecan monomer is secreted into the cartilage matrix in an immature form that undergoes a time-dependent maturation from low- to high-affinity for binding HA [82]. The aggrecan-link protein-HA complex then assembles extracellularly, as HA does not pass through the rough endoplasmic reticulum and Golgi apparatus as do aggrecan and link-protein [81]. A portion of aggrecan migrates from the pericellular matrix into the territorial matrix, and from there into the interterritorial matrix where it is stored long-term [55]. Through the rapid turnover and migration of the pericellular-associated aggrecan, modulated by environmental factors such as load intensity and frequency [68], the chondrocyte controls the retention and maturation of aggrecan prior to sequestration of HA-aggrecan complexes in the functional load bearing matrices of articular cartilage.

The primary function of proteoglycan in adult articular cartilage is to bind water in the matrix. The high fixed negative charge attracts counter ions and the osmotic imbalance caused by a local high ionic concentration draws water from the surrounding areas, keeping the matrix hydrated [82]. The resultant concentration gradients may also serve to promote interactions that are concentration-dependent. The movement of aqueous solution throughout the matrix provides the chondron and extracellular matrix with much of its ability to withstand compressive forces by dissipating energy created during loading and also facilitates the movement of matrix vesicles from the chondrocyte [4], enabling the chondrocyte to exert influence over its environment at distances from the cell. However, without the restraint imposed by the fine fibrillar collagens, water, and surely proteoglycan, would rapidly and unilaterally escape the matrix during loading and the compressive resistance would be lost.
A secondary, yet also crucial, function of the HA-aggrekan complex is in pericellular matrix formation. It has been suggested that the ability of the chondrocyte to assemble a pericellular matrix requires the expression of the cell surface HA receptor CD44 and the binding of HA to those receptors and that the pericellular matrix is built on a scaffold of HA, thus bound to the cell surface [60, 81, 103]. It has further been suggested that the thickness of the pericellular matrix is a function of the average length of the HA and that the mobility and density of HA-aggrekan complexes serve to exclude large particles from the pericellular matrix [60]. There is evidence to support these theories, but they seem incomplete without any mention of the role of pericellular collagens in these functions.

Type VI collagen is known to interact with HA [34], binding the HA-aggrekan complex [54] and the cell surface as well as other matrix macromolecules, as discussed in section 1.1.4.2., thus performing an integral function in sequestration and organisation of the pericellular matrix. It is therefore far more likely that collagen type VI and aggrekan act cooperatively, rather than exclusively, to sequester matrix macromolecules and organise the pericellular matrix.

In the territorial and interterritorial matrices, collagen and proteoglycan interact to form networks of significant strength. Fingers of proteoglycan interdigitated between spaces in radial collagen bundles also connect adjacent chondrons and may serve to facilitate diffusion of fluids, nutrients, and other humoral factors [4] in addition to acting as a lubricant [83], allowing collagen fibres to slide over each other more easily, facilitating differential movement, and limiting collagen microstructural damage from shear during loading. Furthermore, matrix stiffness is directly related to the collagen:proteoglycan ratio, with higher concentrations of aggrecan reducing the number and/or strength of molecular interactions and therefore matrix stiffness [83].

1.2. Articular cartilage degeneration

Osteoarthritis (OA) is one of the most common diseases in humans, the most commonly occurring form of joint disease, and the single largest cause of locomotory disability in the adult population [12]. The term "osteoarthritis" implies an inflammatory disease, a misconception arising from episodic secondary synovitis. As the diseased cartilage itself does not become inflamed it is more accurate to use the terms "osteoarthrosis," or "degenerative joint disease," [105]. For the sake of simplicity, the condition of articular cartilage degeneration will henceforth be referred to as “OA” in this document.

OA is considered a noninflammatory form of arthritis, but there can be a significant episodic inflammatory component as a result of the synovium engulfing larger degenerative fragments with a resultant inflammatory response, causing patient discomfort. Although the
aetiology of OA remains elusive, the imbalance between the synthesis and degradation of the cartilage results in deterioration of the articular surface. Although primary, or idiopathic, is the most prevalent form of OA [106], the cause of it is unknown. In addition to lifestyle, a number of disorders including anatomic, metabolic, neurologic, traumatic, and inflammatory, are well-known to contribute to secondary OA, but the pathogenesis remains unclear. The clinical presentation of each classification is very similar and it is generally not important to distinguish between the two forms as non-operative treatment for both is limited to mitigation of discomfort caused by transient inflammation and considerable localised pain, particularly when weight bearing. As discussed in section 1.1.2.3., cartilage has a very limited ability for self-repair so the progression of articular cartilage degeneration can be variable, taking many years or only a few years from the initiation of the degenerative process to the first symptoms, but most often leads to surgical intervention, which will be discussed subsequently in section 1.3.

OA is characterised by localised increased synthesis and turnover of matrix components and activation of cell division in some previously non-dividing cells [102]. One of the earliest macroscopic changes associated with OA is increased cartilage swelling [51] caused by enzymatic disruption of the fibrillar collagen network [45], resulting in increased permeability of the pericellular matrix and overhydration of proteoglycan as it is released from the constraints of collagen association. The resultant dilution of matrix GAG causes an increase in hydraulic permeability and a loss of volume regulation, significantly altering the stress-strain characteristics and fluid environments of the chondrocytes and decreasing the compressive resistance of the pericellular matrix by 50% [107].

The degradation of proteoglycan, digested by stromelysin (MMP-3), is one of the first changes to occur in osteoarthritic cartilage [108] with associated disorganisation of residual matrix around the majority of chondrocytes [109] as the matrix macromolecules responsible for matrix adhesion and integrity deteriorate. Interleukin-1 (IL-1) is a critical mediator of cartilage catabolism during OA [110] and is responsible for the down regulation of proteoglycan and type II collagen production [111] as well as the up regulation of matrix metalloproteinases (MMP) such as stromelysin, plasminogen activator, and collagenase [108, 109, 112], with a subsequent decline in MMP tissue inhibitor (TIMP) secretion [112]. These changes, while very small, are enough to tip the balance towards degeneration over the many years it takes to develop degenerative joint disease. The initial changes in the collagen and proteoglycan distribution within the chondrons in OA are followed by chondrocyte proliferation [113] as morphological and physicochemical changes in pericellular matrix organisation promote mitosis and clonal proliferation. Chondrocyte division and expansion
within the pericellular matrix results in cluster formation with each chondrocyte in a cluster surrounded by a dense calyx rich in type VI collagen [34] with few fibrillar collagens [1]. Swelling of the chondron capsule precedes the cell division that occurs within, forcing remodelling of the pericellular microenvironment until gaps form in the chondron capsule to allow migration of chondrocytes out of the now-permeable capsule [114]. The decline in pericellular fibrillar collagens, precipitated by a loss of pericellular type IX collagen [59] and likely enabling permeation of the capsule, is accompanied by a concurrent increase in type VI collagen synthesis and sequestration with a broader distribution [34, 86, 115] in the middle and deep zones whereas the superficial zone of articular cartilage shows reduced levels of type VI collagen [67]. The type VI collagen produced is an immature form with a larger chain composition [116], which is subject to modulation as a result of the proliferative state of the cells, and may represent an elegant mechanism for adjusting the function of the type VI collagen microfibrils in articular cartilage [92]. The stimulation of type VI collagen and fibronectin [54] production may be an attempt to ensure continued cell-matrix integrity during chondron remodelling, preferentially accumulating around the cell enabled by, and benefiting from, its remarkable resistance to MMP catabolism [24]. This is supported by the expression of the NG-2 receptor on the surface of immature and proliferating chondrocytes, which interacts directly with type VI collagen [34].

Articular chondrocytes in OA appear to adopt a less differentiated phenotype. Chondrocytes in OA not only produce an immature form of type VI collagen, but also express procollagen type IIA, originally expressed at chondrogenesis during early development [117]. The alteration of GAG epitope (3B3) expression that occurs in OA is also related to developmental changes as re-initiation of a high level of chondroitin sulphate synthesis resembles what occurs in early development [102]. Similarly, an increase in tenascin levels at the onset of OA [118] suggests a similarity between degenerating cartilage attempting repair and developing cartilage. Furthermore, as the cascade of degeneration gains momentum the increased expression of the cell surface markers CD105 and CD166, normally co-expressed in mesenchymal progenitor cells, in OA indicates a cellular reversion to a less differentiated state [119]. This may activate normally quiescent chondrocytes to proliferate [120], and possibly acting to prevent terminal differentiation of chondrocytes in later stages of OA [121]. However, the proliferative response within a matrix straining for cohesion, coupled with the expression of immature matrix components, is inadequate to prevent the degradation of the extracellular matrix and the degeneration of the tissue, leading to fibrillation and eventual erosion of the articular surface.
1.3. Surgical methods of articular cartilage defect repair

There is a direct correlation between knee injuries and later development of OA [122] and patients who injure joints in their youth, develop secondary OA on average 10 years earlier than patients without such injuries [123]. There is a high unmet medical need with respect to the treatment of traumatic articular cartilage injuries in the younger patient (specifically the type of injury I am aiming towards developing a surgical therapy for) particularly considering long-term outcome goals [124]. The clinical goals of any surgical method of articular cartilage repair are to provide sustained (2 years or more) improved joint function and decreased joint symptoms [125], as well as the prevention of the need for any type of arthroplasty at an early age. To achieve these, the therapeutic focus must be on arresting degeneration and promoting regeneration of the articular cartilage. While these outcomes may be met by repair tissue other than hyaline cartilage, the regeneration of hyaline cartilage is highly valued by orthopaedic surgeons [124] and it is well known that fibrocartilage is biomechanically inferior to and less durable than hyaline cartilage [126, 127].

The need for evidence-based long-term outcomes arises from the understanding that the surgical procedure itself may affect short-term results. Irrigation and debridement during arthroscopy may improve symptoms and function by removal of torn or loose tissue and the procedure itself may have a strong placebo effect [125]. Arthrotomy has been shown to induce slight changes in the histological, biochemical (decreased GAG content), and ultrastructural (increased metabolism) properties of articular cartilage [128-130]. While both the beneficial and detrimental effects are thought to be transient, the possibility exists that the procedure itself may damage the articular cartilage while providing temporary symptomatic relief.

Current methods to repair articular cartilage defects are designed to fill the defect with endogenously- or exogenously-derived graft tissue. The current standards of care include allograft, in which intact cadaverous cartilage is transplanted to the defect; autologous chondrocyte transplantation (ACI); matrix-assisted ACI (MACI); osteochondral autograft transfer (OAT)/mosaicplasty; and microfracture, all of which will be described in detail later in this section. The size of the defect largely dictates the recommended procedure with allograft the only recommendation for defects greater than 5 cm²; MACI, ACI, mosaicplasty, and allograft for defects of less than 5 cm²; and any procedure is suitable for small defects with realignment osteotomy recommended if the lesion is in a compartment under “more than normal physiological compression” [131].

There is no agreement in the published literature on the superiority of one technique over the other and authors typically publish reports lauding their preferred method. Several
studies have claimed that mosaicplasty is better than microfracture [132-134] while another claimed that although microfracture yielded better functional scores than ACI, histologically there was no difference between the two and there was no statistical correlation between histological and clinical findings [135]. Yet another published that ACI is superior to mosaicplasty [136] and others that the results of microfracture are as good as any other available treatments [137], lavage and debridement are no better than placebo [138], MACI is better than microfracture [139], and ACI and Mosaicplasty are of equivalent efficacy [140], but debridement alone is effective in nearly 1/3 of patients, calling into question the need for additional procedures [141]. In a canine model, there were no significant reparative differences seen in microfracture (with or without type II collagen matrix) and MACI. However, differences in stiffness and streaming potential, consistent with hypertrophic-like remodelling that typically precede degenerative changes, resulted from the harvest procedure [142] while in a similar human study, MACI resulted in better clinical scores and regrowth at 2 years than microfracture [139]. Finally, one study published that ACI repair tissue is closer to normal cartilage than microfracture repair after 24 months magnetic resonance imaging (MRI) assessment, but no difference was seen after 12 months MRI [143].

The generally low methodological quality in published studies, and noted deficiencies with regard to the type of study, description of rehabilitation regime, outcome criteria, outcome assessment, and patient selection criteria [144], contributes to large variations on reported outcomes, even within each surgical procedure, and prevents an accurate assessment of the superiority of any treatment over another. Conflicts of interest that would preclude an objective assessment of the efficacy and cost-effectiveness of ACI were also found in several published studies that tout the benefits of ACI alone or as compared to other chondral-lesion therapies [145]. Furthermore, no significant differences were found between each kind of therapy in a retrospective analysis of over 60 published studies involving nearly 4000 patients that underwent microfracture, mosaicplasty, periosteal transplantation, and ACI [144]. This study found that 27 different scoring systems were used in the 60 studies, highlighting the lack of standardisation that exists with clinical and histological scoring systems, making objective comparison of studies more difficult. In addition, many published histological scoring systems were primarily designed for use in animal studies of cartilage repair [146], the published guidelines of the Histology Endpoint Committee of the International Cartilage Repair Society (ICRS) [147] being a standout exception. Even the results of opinion surveys are confounding as the majority of polled orthopaedic surgeons perform microfracture as their default articular cartilage repair procedure, but favour ACI over microfracture for outcome goals [124].
## 1.3.1. Autologous Chondrocyte Transplantation

Autologous chondrocyte transplantation (ACI) is the most commonly used cell-based therapy for the treatment of cartilage defects in young adults [148-150] and has been shown to improve quality of life [151]. It is indicated for lesions of 2-12 cm² [152, 153] and best outcomes are achieved in femoral condylar lesions in patients younger than 40 years [135, 152]. It is comprised of two procedures, the first of which is arthroscopic debridement of damaged cartilage and subsequent harvest of cartilage from a “low-weight bearing” surface, although debrided cartilage can also be used [154]. Chondrocytes are isolated and expanded in culture for 14 to 21 days before being injected into the defect beneath a flap of periosteum, which is harvested from the proximal medial tibia during the same procedure [127]. There is some question as to the source of reparative cells since the periosteum is well-known to contain a population of chondrogenic progenitor cells [155] that may contribute to the newly formed tissue. Chondrocytes cultured for ACI have low levels of apoptotic cells [156] and the age of the patient does not affect the viability or proliferative potential of the cells [157]. The resultant repair tissue is initially fibrocartilaginous but may transform with time to a more hyaline-like tissue [146, 158] and will complete maturation in the 9-12 months post-implantation period [159]. After 12 months, repair in most patients is fibrocartilage, with fewer repairs resulting in fibrohyaline cartilage, hyaline cartilage, and fibrous tissue, respectively [160]. Molecular markers for cartilage degradation in the repair tissue increase from surgery to 6 months when they drop off to preoperative levels [161] and, together with similar enzymatic activity taking place during this period [162], may indicate that remodelling is taking place. However, it is also possible that these events are in response to the injury caused by the cartilage harvest procedure, the effects of which have not been studied in detail and are poorly understood.

Proponents of ACI cite the presence of type II collagen and proteoglycan in repair tissue as an indication of its hyaline nature [162, 163], with little, if any, mention of the important collagen:proteoglycan ratio. In addition, type II collagen is also produced by some fibrocartilages such as intervertebral disc [164], and chondroitin sulphate and keratan sulphate are similarly found in fibrocartilage [165], casting some doubt on these claims. The source of repair tissue is also not definitively known. Possible sources include injected cultured chondrocytes, periosteal cells from the flap cover, adjacent cartilage, underlying bone/bone marrow, or the synovium. Labelling of injected cells has revealed that cultured chondrocytes can be retained in the defect [163, 166] but variations in the number of cells retained and the number of new cells populating the defect make it unlikely that injection is the sole source.
and histological samples clearly indicate the contribution of underlying bone marrow to repair tissue [166].

With regard to clinical scores following surgery, the most marked improvement is seen from 3 to 12 months [167]. However, it is not uncommon for patients to experience a transient deterioration in clinical scores at 3 months followed by a progressive improvement to 24 months [168]. It is possible that the initial improvement in clinical scores is due to the relief provided by debridement, after which the immaturity and inferior biomechanical properties of the fibrocartilage repair tissue fail to provide the joint with pain-free smooth articulation and adequate compressive resistance until it matures further. Interestingly, the canine model contradicts the human clinical improvement data. In one study, the benefits of ACI over periosteal or non-treated control groups were limited to a significant increase in reparative tissue filling of lesions, but this singular benefit did not persist longer than 3 to 6 months and was followed by degradation by 12-months [166]. Similar circumstances have also been reported in other models of cartilage repair [49, 169-171].

Adverse events following ACI include adhesions, arthrofibrosis, hypertrophic changes [172], delamination [173], and damage to adjacent cartilage from suturing [166], as well as periosteal extrusion (lifting and detachment of periosteal patches), which accounted for 13% of ACI-related adverse events in one study [174]. Another study cited that periosteal flap issues were the root cause of 10-39% of all complications [173] and 73% of complications in patients required to undergo reoperation less than 2 years following implantation, whereas cartilage-related problems were responsible in 70% of cases more than 2 years after operation. Furthermore, debridement of hypertrophic grafts in the latter group appeared to be detrimental [175].

Recently, the ACI procedure has been modified and marketed as matrix-assisted autologous chondrocyte implantation, or MACI. MACI is essentially the ACI procedure with cultured chondrocytes seeded onto a scaffold (commonly a bi-layer collagen type I-III membrane) and fixed in place with fibrin glue rather than under a sutured periosteal flap. MACI can be indicated for chondral defects of approximately 5 cm² [176]. It is based on the assumption that chondrogenic cells require a matrix for their attachment and containment [177]. While the requirement for chondrogenic cell containment in the defect is obviously critical, the need for a matrix to attach to is debatable since truly chondrogenic cells will readily produce their own matrix, as described in previous sections. In addition, collagen types I and III, of which the MACI scaffold are constructed, are known to be fibrogenic while collagen type II is widely accepted to be the phenotypic chondrogenic collagen.
The advantages of MACI over ACI include increasing surface area for cell attachment, allowing for regulation of implanted cell density, and facilitating arthroscopic implantation [177] as well as being suture free, requiring less operating time and surgical exposure [178]. However, the lack of suture allows for transplant detachment, albeit in a small percentage of cases [179] and it is more costly than ACI [180]. The role of the fibrin sealant in healing of chondral defects is not well understood but there is some evidence that it promotes the migration and proliferation of human articular chondrocytes [181], possibly accounting for its perceived improvement over ACI.

There is no significant difference in the repair tissue seen between ACI and MACI but MACI is associated with fewer adverse events [182], particularly hypertrophic graft/periosteal flap complications [173]. Several materials for scaffold/matrix and fixation combinations have been tested [183] but little is still known about the fixation stability of implants despite its well-known importance for successful therapy. In addition, synthetic matrices have been shown to cause surface irregularities in the tissue surrounding implants in a rabbit model [184]. Seeded cell density appears to have no effect on outcome histology in a rabbit model [185]. Biopsy at 1-year following MACI reveals hyaline-like cartilage with fibrocartilage [178], an irregular surface, and chondron clustering, all indicative of incomplete healing, which evolved to a smooth and continuous surface at the 2-year biopsy [186]. However, D’Anchise did not show images of the 2-year histology sections and cited “well-organised cell-clusters” as typical of hyaline cartilage, which it is not. Therefore, the assumption is that the clusters remain at 2-years, indicating the potential for reversion to degenerative cartilage while allowing for the possibility of sustained repair.

1.3.2. Microfracture

Microfracture is arguably the simplest and least invasive surgical method of articular cartilage repair, excluding lavage and debridement, which do not actually repair the damaged tissue but rather remove fragments of tissue that may impede smooth joint articulation. It is indicated for small to medium-sized lesions due to acute or repeated-acute injury [187], osteonecrosis [188] and mild to moderate osteoarthritic changes [189]. It is performed arthroscopically and involves the removal of all degenerative cartilage from the lesion with subsequent puncture of the subchondral bone with a specially designed awl. Through these punctures, which leave the bony plate intact, bone-marrow, and its resident population of mesenchymal stem cells, fills the lesion and creates a fibrin “super clot” that differentiates and remolds into fibrocartilage repair tissue [187] that contains type II collagen [189]. As a marrow-stimulating technique, microfracture takes advantage of the body’s own healing potential.
While significant improvement in functional scores have been noted in the first 18-months post-surgery, ICRS scores deteriorated in the next 18-month period in some studies [190], particularly in patients older than 40 years [191]. The founder of this procedure, Steadman, reported significant improvement in pain and functionality after average 11 years in patients 45 years or younger [192] and others have noted similar symptomatic improvement [193], but age [132, 191] and location of the lesion [190] are predictors of functional improvement with best results achieved in young patients with femoral condylar lesions [135, 190-192]. Other factors that may affect outcomes include body mass index (BMI), duration of preoperative symptoms, and fill grade [194].

Advantages of microfracture include minimal invasiveness, low relative cost, its single-procedure nature without the need for harvest, and an absence of heat necrosis due to drilling as exists in procedures such as mosaicplasty. Recommended rehabilitation involves weight-bearing restrictions and continuous passive motion (CPM) [195, 196] and the postoperative protocol “may be as important as the surgery itself” [197] although one study found no difference in outcomes following a post-operative regime of weight-bearing as tolerated and an absence of CPM [198].

The durability of the fibrocartilage repair tissue is a concern [138] although the procedure has been successfully used on active professional athletes [199]. However, while pain and swelling was reduced with associated functional improvement and rapid return to high-level sport, only approximately half of the athletes performed at the pre-injury level after 2-3 years [132, 200, 201] and the majority of athletes showed a decline in sport activity level after 6 years [202].

1.3.3. Osteochondral Autograft Transfer/Mosaicplasty

Mosaicplasty, or osteochondral autograft transfer (OAT), is a method to repair chondral defects of weight-bearing surfaces by transplanting cylindrical osteochondral plugs harvested from non weight-bearing sections of the knee. The resultant repair tissue is a composite of the transplanted hyaline cartilage plugs filled with fibrocartilage in between, regenerated by the recruitment of MSC from the bone marrow, which is brought to the defect through drilling of recipient holes. In this way it can be seen as a variation, or debatably, an improvement, on the microfracture technique. It can be performed arthroscopically and rehabilitation can be initiated relatively early. Recommended rehabilitation following surgery includes immediate exercise and non-weight-bearing for two to four weeks followed by a two week period of partial loading with the possible inclusion of CPM to mitigate stiffness and pain [203]. The size of the reparable defect is limited to small- to medium-sized by the number of plugs available for harvest [204-206], although it has been used in conjunction
with autologous chondrocyte transplantation in larger defects [207], and is not indicated for osteoarthritic lesions unless it can be performed in the early stages of OA and in combination with osteotomy. The best prognostic outcomes are achieved in patients aged younger than 50 years [204] with best results achieved in the lateral femoral condyle [208]. OAT can provide good restoration of joint congruity and freedom from pain, crepitation, and locking [209] with good to excellent results reported in 92% of patients treated with femoral condylar implantations in a ten-year study [210, 211].

While long-term results are positive and the graft appears durable, OAT has several disadvantages inherent in the harvest of viable osteochondral tissue and the fate of the donor site. Donor sites are usually left empty and, at best, become filled with fibrous tissue with the potential for collapse of adjacent bone and cartilage [212]. Attempts to correct this have included the harvest of bone plugs from the proximal tibia with or without periosteal cover, and have been largely unsuccessful [212]. An extensive margin (approximately 1/3) of cell death in the graft has been reported, particularly at the periphery of the graft, which is likely to compromise lateral integration and articular reconstruction [213]. Clinical adverse affects include donor site morbidity, deep infections, and intra-articular bleeding (haemarthrosis) [210]. Graft incongruity with the articular surface may have a deleterious affect with protuberance leading to fissuring of plugs, fissuring around recipient site, a “catching sensation”, and pain, whereas depression may allow overgrowth of fibrocartilage-like tissue [214]. Seating of grafts by impact loading is associated with structural damage, loss of viability of chondrocytes, and subsequent degeneration of the articular cartilage [215]. Discrepancies in graft length vs. recipient hole depth could create instability (if the hole is deeper) or require the use of excessive force to implant (if the graft is longer), leading to issues seen with impact loading [216]. In addition, stiffness discrepancies between graft and normal cartilage have been reported [217], possibly due to the thickness of the cartilage layer [218]. Histologic evaluation of post-implantation of osteochondral plugs shows chondrocyte cluster formation, fissuring, and loss of proteoglycan [219], which are all indicative of continuing cartilage degeneration.

1.4. Regenerative medicine/ tissue engineering of articular cartilage

Given the limitations that exist with the current surgical therapies for the repair of articular cartilage defects, the field of tissue engineering has risen to the challenge and has made great progress in overcoming these limitations. Autologous cartilage and osteochondral grafts are now being engineered to pre-defined sizes and shapes to provide tailored repair solutions. However, tissue engineers are also limited by their incomplete understanding of the
regulatory roles played by biological, chemical, and mechanical factors on the development of hyaline articular cartilage constructs.

Articular cartilage engineering methods typically involve the monolayer expansion of biopsy tissue followed by three-dimensional (3-D) culture in gels, scaffolds, or scaffold-free systems, many of these within bioreactor culture systems. The two cell types currently used for engineering articular cartilage constructs are chondrocytes and mesenchymal stem cells, both of which will be discussed later in more detail. Scaffolds are designed to provide for adhesion/retention of cells, homogenous distribution of cells within the defect, protection of cells from mechanical force until they develop a robust extracellular matrix, ease of handling and fixation, and even stimulation of tissue maturation as in the case of biomimetic materials [220]. Often, the scaffold or substrate with the best ease of handling or fixation characteristics does not have desirable tissue formation properties.

Variations in culture conditions and results make it difficult to know which individual factors influence the fate of in vitro engineered cartilage [221]. For example, whereas higher seeding density may yield higher rates of matrix production [222, 223], seeding method and density depends on the type of scaffold used with regards to density, porosity, and pore interconnectivity. In this way, each variable leads down a path of multiplicative variables and also multiplicative effects on the quality or hyaline-like nature of neo-cartilage produced. Therefore, many methods are developed and compared but it may be an impossible task to identify the individual culture conditions that have resulted in a desirable quality in the graft tissue.

1.4.1. Chondrocytes

1.4.1.1. Collection/isolation

The harvest of healthy cartilage for isolation and expansion of chondrocytes for the production of engineered neocartilage repair tissue is exactly the same methodology used in ACI and MACI procedures with the same associated morbidity to the harvest site. The differences between them are based in the second phase of culture in tissue engineering methods, which is typically performed in a 3-D culture system, whereas ACI and MACI procedures return the monolayer cells to the defect site following expansion. While the graft tissue resulting from tissue engineering methods may be of a more hyaline nature due to the in vitro differentiation phase, the fact remains that a new defect has been created for the purpose of cellular harvest. Cartilage biopsy tissue is typically collected by arthroscopy and often during exploratory examination during which the joint may be debrided and/or lavaged. The possibility exists that cartilage debrided from the margins of the defect may be suitable for isolation and expansion of chondrocytes, but the state of cartilage at harvest may affect the
behaviour of cultured chondrocytes [224] making the use of cells from degenerative tissue an unattractive option.

Once harvested, chondrocytes must be released from the complex extracellular matrix, which requires potent and pleiotropic enzymes such as pronase to completely digest and may affect cell viability. Supplements, such as ascorbic acid, have been shown to improve chondrocyte survival over trypsin or collagenase alone [225].

1.4.1.2. Culture

1.4.1.2.1. Monolayer/ de-differentiation

As discussed in previous sections, the natural state of chondrocytes is rounded, post-mitotic, and intent on extracellular matrix production and maintenance. To proliferate they must de-differentiate and become fibroblast-like. They alter their morphology from round or polygonal to flat, elongated cells, exhibit contractile behaviour, possibly due to the presence of α-smooth muscle actin (SMA) [226], and preferentially produce type I collagen over type II collagen [227]. Even though chondrocytes in monolayer upregulate type II collagen genes and express aggrecan proteins [228] in early culture, the degree of chondrocyte phenotypic protein expression declines over time relative to type I collagen expression. Once released from the ECM, chondrocytes re-enter the cell cycle and metabolic activity becomes focused on proliferation and migration, as evidenced by the upregulation of cell-surface proteins involved in cell-cell adhesion and others distinctive to mesenchymal stem cells [229]. Genes distinctive to mesenchymal stem cells [230] are also upregulated, strongly suggesting the reversion of differentiated chondrocytes to an undifferentiated state in monolayer. Furthermore, chondrocytes that have been expanded in monolayer have the ability to differentiate toward different tissue types within the mesenchymal lineage [231, 232].

Several growth factors have been used to enhance proliferation of monolayer chondrocytes for expansion and reduce population doubling times. However, Jakob et al. [227] found that the factors that most potently support chondrocyte proliferation also most strongly induce dedifferentiation. Furthermore, serially passaged chondrocytes do not appear able to recover their chondrogenic potential without supplementation of growth factors [233]. Therefore, the challenge for tissue engineers is to supplement chondrocyte culture medium in early passage to achieve adequate expansion for clinical use, but to use a supplementation regime that also promotes subsequent redifferentiation. FGF-2 and TGFβ together were found to additively promote proliferation rates in passage 1 but increase doubling times in passage 2 [227], suggesting that receptor expression changes in serial passage. However, Jakob et al. [227] also found TGFβ and dexamethasone to be inducers of redifferentiation once the cells were cultured in a 3-D environment, demonstrating that “the conditions of chondrocyte
expansion in monolayers modulate at the transcriptional level their commitment to redifferentiation and their ability to respond to regulatory molecules in a 3-D environment”. Whereas supplementation of monolayer cultures with a combination of TGF-β and FGF-2 increases proliferation and induces dedifferentiation [234], the presence of FGF-2 in monolayer expansion allows for chondrocytes to fully maintain their potential for redifferentiation once transferred to 3-dimensional culture systems, possibly through inhibition of thick F-actin structures [233]. The addition of FGF-2 to monolayer cells has been shown to inhibit production of SMA and type I collagen, promote proliferation, and induce responsiveness to BMP-2 during subsequent three-dimensional (3-D) cultivation [235]. Gigout et al. [236] showed that low calcium levels in culture medium promoted the chondrogenic phenotype and chondrocytic (rounded) morphology, indicating that nutrition and ionic balance may also play roles in determination of chondrocyte morphology and preservation of chondrogenic ability.

1.4.1.2.2. 3-D / re-differentiation

The common objective of the second and third phases of articular cartilage engineering is a shift from proliferation to matrix production and recapitulation of the chondron and extracellular matrix. The second phase involves the formation of a cell pellet that allows formerly flattened cells to become rounded once again and initiate cell-cell communication in a 3-D environment. This phase is followed by, and probably causes, the production of appropriate extracellular matrix molecules to re-form the chondron and secrete the extracellular matrix that will become the new tissue, forcing the cells apart as the pellet grows, mimicking the appositional growth seen in early development. As will be discussed below, this is best accomplished in a 3-D culture system and a variety of biomechanical and biochemical stimuli have been used to promote the desired production of a hyaline matrix. As discussed earlier, scaffolds have been commonly used to provide the 3-D environment required for redifferentiation. However, viable chondrocytes will readily provide their own matrix and 3-D environment given the opportunity [237] and micromass and pellet culture have been used effectively with this principle in mind. Tallheden et al. [238] showed this in an elegant gene microarray study of how chondrocytes temporally redifferentiate from days 7 to 14 of pellet culture, mimicking the formation of early limbs buds in embryonic development. More recently, Hayes et al. [239] further characterised the growth characteristics of pellet cultured chondrocytes and showed high cellular turnover and growth through appositional and interstitial mechanisms.

It is well-known that the appropriate biomechanical environment is a strong promoter of chondrocyte redifferentiation and subsequent chondrogenesis [240]. Static culture has been
shown to yield small and fragile constructs, while turbulent flow yields constructs with fibrous outer capsules [233], both of which had poor mechanical properties. However, cultures that were freely suspended in dynamic laminar flow in rotating vessels yielded large constructs with high collagen and proteoglycan content and superior mechanical properties that reached or exceeded freshly explanted native cartilage and persisted for 7 months [241]. Similarly, perfusion bioreactors have been shown to support elevated GAG synthesis and retention [242, 243] resulting in greater uniformity of cells and matrix [244].

The majority of the chondrogenic promoting effect of 3-D culture is based in the exposure of chondrocytes to biomechanical forces in a low-shear and high mass-transfer environment. The manipulation of that environment has also been shown to produce chondrogenic matrix-promoting results. Dynamic deformational loading of constructs within a bioreactor yielded superior mechanical properties and higher GAG and hydroxyproline content [245] most likely through stimulation of metabolism [246-249] although it must be considered that development of structural organisation within the extracellular matrix may change the required mechanical conditioning regimen. Reduced oxygen culture conditions and intermittent hydrostatic pressure have also increased type II collagen production and phenotype stability [250, 251].

In addition to the biomechanical environment, the biochemical signals to which the cultured chondrocytes are exposed, during both phases of culture, have been shown to have a profound effect on the ability to redifferentiate and develop chondrogenic pericellular and extracellular matrices. As noted earlier in Jakob’s work, dexamethasone has long been known to be an inducer of chondrogenesis in de-differentiated chondrocytes and MSC alike [252-254] increasing chondrogenic nodule formation [253] and amplifying protein synthesis although it also suppressed proteoglycan synthesis and aggregation in the presence of TGF-β1 and foetal calf serum [255]. TGF-β is a key factor in the regulation of proliferation and differentiation, especially during chondrogenesis [256]. Members of the TGF-β superfamily have been shown to promote chondrogenesis in embryonic limb mesenchymal cells [257, 258] as well as mesenchymal stem cells isolated from foetal rat muscle explants [259] and periosteum-derived cells [260]. However, the effects of TGF-β on articular chondrocyte proliferation can be either inhibitory or stimulatory, depending on culture conditions, timing of supplementation, and state of cellular differentiation [261, 262]. Addition of chondroitin sulphate to type I collagen scaffolds increased proliferation, which does not seem advantageous during the redifferentiation phase, but also appeared to preserve the chondrocytic phenotype [263]. While it may seem plausible that the addition of articular cartilage matrix proteins to chondrocytes in chondrogenic culture would provide the building
blocks of extracellular matrix and potentiate their formation and organisation, the opposite is
ture as a feedback mechanism involving the hyaluronan receptor CD44 results in decreased
proteoglycan synthesis when extracellular proteoglycan concentration is high [264]. Similarly,
the addition of high molecular weight hyaluronan markedly decreases the mRNA expression
of both aggrecan and type II collagen [265], emphasizing that the cellular microenvironment,
including the composition of the extracellular matrix and concentrations of soluble mediators
secreted by adjacent cells, has a profound effect on the metabolic processes of chondrocytes.

Computational modeling has reduced the amount of guesswork involved in selecting
the appropriate culture environment from the myriad of possible biomechanical and
biochemical factors. Algorithms have now been developed to analyse the dynamic
composition of engineered cartilage [266] and the effect of nutrition [267-269] and fluid
mechanics [270-272] on growth kinetics within 3-dimensional cultures. Using these models
along with experimental data it is now possible to devise methods of expansion and
subsequent redifferentiation of cultured chondrocytes through the judicious application of
growth factors and biomechanical stimuli.

1.4.2. Mesenchymal Stem Cells

Stem cells are defined as cells that have the capacity for self-renewal and have the
potential to differentiate into specialised tissue types. Mesenchymal stem cells (MSC) are
primordial cells of mesodermal origin, found within connective tissues such as muscle [273,
274]; bone [252, 253, 275]; adipose [276]; skin [277]; vascular tissue [278]; cartilage [25];
and in rare instances, circulating blood [279], that have the potential to differentiate into other
tissues in the mesenchymal lineage. Each MSC divides, sometimes asymmetrically to produce
a daughter cell and a lineage-specific progenitor cell [280], to produce additional MSC and
fulfil their capacity for self-renewal. It should be noted that clonal populations of cultured
MSC are heterogeneous with regard to their differentiation potential as different fractions will
have tri-lineage, bi-lineage, and uni-lineage potential [281]. MSC have also been found with
transdifferentiation potential [282, 283], or the potential to differentiate into lineages other
than mesenchymal, such as neuronal cells [284, 285] and hepatocytes [286, 287]. These cells
provide connective tissues with their reparability as they leave their niche environment and
migrate to the site of need, thought to be mediated by intracellular surface antigens and cell
adhesion molecules, growth factors, and proteolytic enzymes [288-290] released during tissue
damage and remodelling. Once there, they initiate repair by differentiating to replace lost
cells, fusing with endogenous cells, initiating trophic effects [291], and transferring functional
mitochondrial DNA [292].
MSC have been called by many names and the International Society for Cellular Therapy (ISCT) issued a statement in 2005 to suggest standard nomenclature for MSC to mean “multipotent mesenchymal stromal cells” [293]. The varied tissue sources and methods of isolation used amongst researchers have also added to the ambiguity of MSC characterisation and have made comparisons of published results difficult. To address this, the Mesenchymal and Tissue Stem Cell Committee of the ISCT proposed a set of minimal criteria to define human MSC for both academic and industrial research investigations [294]. Therefore, MSC are currently defined as cells that are adherent to plastic; are positive (≥ 95%) for the surface antigens CD105, CD73, and CD90; are negative (≤ 2%) for the haematopoietic surface antigens CD45, CD34, CD14 or CD11b, CD79α or CD19, and HLA-DR; and have the potential to differentiate into osteoblasts, adipocytes, and chondroblasts in standard in vitro differentiation culture conditions. It is recognized that the surface antigen expression of non-human species is not universally well characterised [295] and a lack of antibody cross-reactivity may not allow for surface antigen specificity to be used for criteria in animal models.

MSC are a very attractive option as a cell source for tissue engineering of articular cartilage for several reasons, not the least of which is that their autologous harvest does not create a new lesion in the very tissue that is to be repaired. In addition, because monolayer expanded chondrocytes have been found to have plasticity [231, 232] and several similarities exist between dedifferentiated chondrocytes and MSC [25, 281, 296, 297], it is likely that the dedifferentiation of chondrocytes during monolayer expansion renders them to a MSC-like state. Therefore, it is reasonable to consider the use of MSC for the purposes of engineering neocartilage constructs as the concepts and many methods of chondrocyte redifferentiation and 3D culture discussed in the previous section would apply.

The two sources of MSC that will be discussed in this section are bone marrow and adipose tissue. Although MSC from both sources have been exhaustively compared for their chondrogenic ability [298-305] there is neither agreement nor conclusive evidence of the superiority of one over the other in the literature. It is possible that the ideal culture conditions for MSC differs with the cell source, making an objective, controlled comparison difficult to accomplish.

1.4.2.1. Bone marrow-derived MSC

Bone marrow contains two distinct populations of stem cells. The best characterised is the haematopoietic stem cell (HSC) population that gives rise to the cellular components of blood. The other resident population of stem cells derives from the marrow stroma, which provides the haematopoietic system with structural support for developing blood components.
MSC derived from bone marrow stroma (BM-MSC) were discovered in the 1960’s [306] with osteogenic potential and since have been found to have the potential to differentiate into other connective tissue cell types in addition to bone, including cartilage, fat, tendon, muscle, and marrow stroma [308, 309] as well as the ability to revert to undifferentiated states in the absence of stimulus [310]. MSC are typically isolated from bone marrow and separated from the HSC by gradient centrifugation followed by removal of non-adherent cells in subsequent monolayer culture, although fluorescence activated cell sorting (FACS) technology based on MSC-specific cell-surface antigens have allowed for more simple and efficient methods of MSC isolation [311, 312]. The expansion of bone marrow-derived MSC is very similar to that of chondrocytes. Release from the stroma creates a mitogenic response in which the MSC are transformed into highly proliferative fibroblast-like colony forming units (CFU) [313-315]. Subsequent monolayer culture in medium supplemented with appropriate growth factors and cytokines preserve the MSC morphology and prevent differentiation commitment to the fibroblast phenotype.

The bone marrow has been considered the in vivo residence for the uncommitted MSC and, once entering the blood system to home into sites of regeneration, provides connective tissues with continual replenishment during homeostatic and reparative activity [308, 316]. For this reason, BM-MSC are considered an ideal source of cells for engineering of articular cartilage graft tissue. However, a major disadvantage of bone marrow as a source of MSC is the difficult nature of tissue harvest. Bone marrow is usually aspirated from the iliac crest or tibia and the procedure is not only very painful, but also carries a serious risk of infection.

1.4.2.2. Adipose-derived MSC

Another source of MSC that has attracted attention in recent years is adipose tissue [276, 317]. Like bone marrow, adipose tissue is derived from the embryonic mesoderm and can be classified as a connective tissue. Adipose-derived mesenchymal stem cells (ASC) are easily harvested in large quantities in a minimally invasive procedure, typically during liposuction, under local anaesthesia and with little discomfort. ASC possess the same mesodermal and ectodermal differentiation capacity as MSC from other sources such as bone marrow and express the requisite cell surface antigen profile [288] as discussed in section 1.4.2. It has recently been suggested that ASC may not elicit a response from T cells and can possibly suppress an immune response [318, 319], introducing the possibility of their use in allogenic transplantation. Since the harvest of bone marrow, like cartilage, is quantity-limited, the numbers of isolated MSC cells may also be quite low [309], requiring extensive in vitro expansion. Aside from the time and expense involved in BM-MSC expansion, the possibility of culture contamination is increased with each passage. Therefore, the ease of harvest, along
with robust proliferative capacity [299] and clonogenic potential [107], would seem to make adipose tissue an excellent source of MSC for tissue engineering of neocartilage constructs.

The isolation of ASC is slightly different than that of BM-MSC as the nature of the tissue requires. Lipoaspirate or tissue from lipectomy is physically minced and then subjected to proteolytic enzyme digestion, much like the isolation of chondrocytes from cartilage. Serial centrifugation then separates the fat from the cellular fraction, often called the stromal vascular fraction (SVF), which is then selectively cultured in monolayer for plastic adherent cells [276, 320]. Bone marrow aspirate of 30 ml yields approximately $1 \times 10^5$ cells, while a liposuction (subcutaneous [SC] fat) aspirate of 100 cm$^3$ yields approximately $3.3 \times 10^6$ cells [321]. Because of the volumes of tissue and cells available, the primary cultures can be used therapeutically or differentiated without the need for passage expansion, although expansion is sometimes performed for selection of a more homogeneous cell population [318] or transfection with viral vectors [322]. Chondrogenic differentiation of ASC is no different than that of monolayer expanded chondrocytes or BM-MSC, involving the same biomechanical and biochemical stimuli [323].

An alternative source of adipose tissue for harvest of ASC is the infrapatellar (IP) fat pad. IP fat is easily harvested arthroscopically and is often resected during such procedures to afford the surgeon a better view of the articular surface. It is sometimes removed during arthroplasty to prevent infrapatellar contraction syndrome, in which cells within the fat pad assume a fibroblastic phenotype [324]. Clinically, the harvest of IP fat during exploratory arthroscopy would provide a ready source of ASC for future return during defect repair. It has been shown to have chondrogenic potential comparable to BM-MSC and ASC from SC fat [325] and cell yield appears to be higher than either bone marrow- or SC-derived MSC [326]. Furthermore, the IP fat pad is significantly different from the product of liposuction and a large proportion of it is composed of dense collagenous tissue [325]. TGF-β has been detected in IP fat following injury to the anterior cruciate ligament [327] and the collagen content of the IP fat pad increases after reconstruction of the anterior cruciate ligament [328], indicating a possible role for the IP fat pad in repair of knee injuries. Given this, and the reversion of a population of cells within the fat pad to a fibroblastic phenotype in response to injury, it is tempting to postulate that ASC from the IP fat pad “home in” to the source of injury, which would make IP fat-derived ASC (IP-ASC) an ideal cell source for the engineering of articular cartilage constructs.

1.4.2.3. The stem cell niche

Recently, the microenvironment in which stem cells reside, or niche, has received a great deal of attention. This is particularly true for MSC as the niche will have certain
characteristics that allow it to function ideally as an organ system. However, the majority of that attention has been focused on how the environments into which MSC migrate, or are transplanted, influence the differentiation of MSC.

The primary function of the MSC niche (of origin) appears to be support of the stem cell phenotype and maintenance of an undifferentiated state [329] although the role of the niche in determination of MSC function and potential is not well understood. It has been shown that cell-cell and cell-extracellular matrix interactions can induce differentiation of uncommitted cells [330] and that the extracellular matrix can modulate the local concentrations of secreted differentiation factors [331], but only once they have egressed the niche. Embryonic stem cells have been shown to respond with different differentiation profiles when cultured on tissue-specific extracellular matrices [332], proving the importance of the extracellular matrix in driving plasticity. In addition to the soluble mediators found within the extracellular matrix that direct MSC differentiation, it is now coming to light that the physical properties of the microenvironment can also regulate MSC fate [333, 334], but still very little is known about how the niche influences the potential of MSC to differentiate into particular cell lineages, mostly due to an inability to study stem cells in vivo. Recent studies have attempted to recreate the MSC niche in an ex vivo environment [335] to achieve a better understanding of the behaviour of MSC in vivo as well as define the role of signalling molecules in regulating MSC function [336-338]. Transmission electron microscopy (TEM) studies of epithelium have revealed that differentiated cells create anatomically distinct environments for neighbouring stem cells to reside within [339]. These cells can influence the expression of differentiation mediators in adjacent MSC and, thus, their differentiation path [340]. Other studies utilising TEM have shown that MSC from different sources may bear the same phenotypic characteristics but differ ultrastructurally [341], possibly providing insight into the microenvironment from which they originate. However, it is still unclear how these microenvironments, or niches, from which MSC originate determine their ability to differentiate into a particular tissue type. It seems likely that if MSC can be so profoundly influenced by their microenvironment to differentiate solely based on something like the elasticity of the matrix [334], then the characteristics of their niche of origin would ‘pre-program’ them to a certain degree toward tissue types with similar characteristics.

1.5. Animal Models of articular cartilage defects/repair

All currently used animal models of articular cartilage repair have some value and specific advantages, as well as limitations associated with their use. It has been suggested that animal models are best suited to testing aspects of the key principles that govern reparative processes rather than trying to scale up procedures developed for relatively small animals with
intrinsically different articular cartilage structure [342]. The key to animal model selection would therefore be to match the advantages and limitations of the model to the hypothesis or question(s) being posed.

Important issues to consider when using animal models of articular defect repair include the type of articular defect(s) studied, the age of the animal, differences in articular cartilage, joint motion, and contact stresses among species [125, 342]. The goal in using an animal model in developing therapies for human benefit is to mimic the human condition as closely as possible, providing relevant information for the design of human clinical studies. When studies of articular cartilage repair are performed in humans, the goal is the restoration of pain-free function of a joint. Therefore, much of the repair outcome is assessed through clinical scores and assessment of pain and ability to resume normal activities. Evaluating pain in animals is difficult and is usually achieved by addressing changes in gait or posture. Therefore, the objectives of animal studies of articular cartilage repair relate to the restoration of a joint surface that is anatomically, mechanically, and histologically and has considerable in vivo longevity similar to the original joint surface [343].

The most commonly used animal models of articular cartilage repair are rabbit and dog, mostly due to the availability of background research data, relatively low cost, and ease of handling [344]. However, larger animals such as sheep and goats are rapidly growing in popularity as their background database becomes more complete, particularly as models of surgical device implantation and surgical repair methods. In addition to the higher vertebrate level, larger joint size [345, 346], and non-companion animal status of the sheep as compared to rabbits and dogs, the degree of flexion of the sheep stifle joint is less than in rabbits and dogs [344, 347] and the cartilage thickness to body weight ratio is more similar to humans [348], suggesting a closer analogy to human knee joints and making the sheep an attractive option for translational research [349]. Furthermore, rabbit cartilage, aside from being very thin, maintains some intrinsic reparability at maturity [170, 350, 351], possibly confounding objective evaluation of cartilage repair methods in that animal model.

While the ovine model of articular cartilage defect repair is used more frequently in recent years, the model is still not well described. However, common attributes of results in studies using sheep provide some insight into characteristics of the ovine model. Absent from discussions, but apparent from reported standard deviation and error, interanimal variability appears as a common trait in studies using sheep models [352, 353]. This is possibly inherent in the nature of outbred domestic animals such as sheep. A lack of spontaneous healing is also commonly reported in ovine cartilage repair models. Since the sheep knee has approximately 60% of the contact area of a human knee, critical defect size has been estimated at 7 mm for
the sheep [354], although defects as small as 4 mm in diameter have failed to heal spontaneously [352]. Thus the use of 6mm defects in this study is consistent with the estimated value of 7mm. The ability of sheep to load operated limbs within the week following surgery has also been discussed as integral to the development of functional repair cartilage with both loading and contact time returning to pre-operative values after eight weeks of post-operative recovery [355]. On the contrary, the immediate loading after surgery has also been described as a departure from the clinical scenario [355].

It is well known and accepted that there are fundamental differences between human articular cartilage and even the most similar animal model, particularly regarding cellular density and intrinsic architecture [356]. The selection of an appropriate animal model of articular cartilage repair hinges on the understanding of the differences that are relevant to the study design. For example, human articular cartilage permeability and Poisson’s ratio are known to be significantly different than most other species [125] so any study including analysis of compressive and elastic strain or hydrodynamics in an animal model would have to account for and address this variable. Also, in the canine model of ACI, degeneration following delamination of the graft occurs after 3-6 months [166, 177] whereas in humans, durable integration between graft and host tissue is typically seen, forming more hyaline-like cartilage with time [162]. Similarly, understanding the differences that exist between species is critical to extrapolating results between similar experiments with differing animal models. Dorotka et al. [357] noted significant differences between their results in an ovine model and similar studies in dogs [358] and rabbits [359]. Overall, the use of animal models is an excellent way to test in vitro cartilage repair results in an in vivo system, but the appropriate selection of species and joint, as well as consistency of these two variables between in vitro and in vivo studies, is critical [360].
THESIS STATEMENT AND EXPERIMENTAL DESIGN

2. Thesis Statement and Experimental Design

Many researchers have studied the chondrogenic potential of ASC but the vast majority have used SC fat as the source of cells [255, 323, 361-364]. The availability of large volumes of processed lipoaspirate as a waste product of liposuction has enabled the field of ASC research to progress; however, the differences that exist between ASC from different sources of adipose tissue are not well understood. Adipose tissue is a ubiquitous tissue found in contact with virtually every organ system in the body. Given that “cytokines, growth factors, adhesion molecules, and extracellular matrix components in the stem cell microenvironment play important roles in stem cell fate determination” [297], it would stand to reason these factors would also play a role in determination of the differentiation potential of ASC. It is likely that ASC surrounding different organ systems would possess differing potential germane to the support of that organ system. Specifically, the fact that the IP fat pad is exposed to compression and shear, the same biochemical stimuli as articular cartilage in the knee, as well as the integration of the synovium with the IP fat pad, may uniquely enable the chondrogenic differentiation potential of ASC residing within.

2.1. The chondrogenic ability of ASC from infrapatellar fat is greater than that of ASC from subcutaneous fat.

This thesis was designed to determine whether ASC from IP fat have greater potential for chondrogenesis than ASC from SC fat as assessed by their ability to produce hyaline-like neocartilage constructs. Chondrogenic potential was determined by the ability of ASC from each source to differentiate into rounded cells that form chondrons and secrete the phenotypic extracellular matrix molecules of hyaline cartilage: collagen type II, collagen type VI, and aggrecan. In addition, the repair potential of the neocartilage constructs from each ASC source was then studied by implantation into a full-thickness osteochondral defect model in sheep.

2.2. Overview of experimental design

2.2.1. Justification

2.2.1.1. Model

As discussed in section 1.5, sheep were used as a model in these experiments for several reasons, including the lack of spontaneous healing, the ability to load joints soon after surgery, and the similarity of sheep and human knees, particularly with regard to the size of the joint and articular surface. This latter point was critically important to these experiments
as they were designed for translation to human therapeutic use. One disadvantage of using large animal models is the limitation on the number of animals that can be used for ethical, economic and practical reasons. As this \textit{in vivo} experiment was intended as a proof-of-concept, the focus was on qualitative comparison rather than statistical analysis. It is recognised that a larger study involving groups sizes powered for statistical significance will be required to fully assess the procedure. A substantial benefit of the use of the sheep model in these experiments is their availability in Otago and the pre-existing supply chain within the Section of Orthopaedic Surgery at the University of Otago, School of Medicine. This supply chain is a direct result, and facility, of the many years of experience with ovine models of knee injury and repair that Dr. Mark Walton has amassed. That experience, coupled with my own similar if less extensive experience, in ovine surgical models made sheep a natural choice for these experiments. In addition, the animal housing and surgical facilities at the University are uniquely adapted to work with sheep, making them quite simple to manage.

\textbf{2.2.1.2. Endpoints}

Collagen type II was chosen as a phenotypic marker of chondrogenesis because it is the most prevalent type of collagen species in the cartilage extracellular matrix. While it is found in other connective tissues, its organisation around the pericellular, territorial, and intraterritorial matrices is unique to cartilage. It is also the most widely accepted marker of chondrogenesis in the literature.

Collagen type VI is somewhat less accepted as a phenotypic marker of chondrogenesis. However, its role in the formation of the chondron and sequestration of extracellular matrix molecules makes it an ideal choice for evaluation of chondron development, particularly in early culture. The presence of type VI collagen in the pericellular microenvironment is critical to chondron development and therefore must be included in any assessment of hyaline-like cartilage formation.

The presence of aggregcan in cultured neocartilage pellets indicates that the matrix is capable of holding fluid, providing the cell within the chondron with much of its compressive resistance. For the cultured constructs to bear load without damage to the cell, the hydrodynamic infrastructure of the chondron, of which aggregcan is an integral part, must be intact.

\textbf{2.2.1.3. Methods of evaluation}

Immunohistochemical (IHC) staining is an excellent way to verify the presence of a particular matrix protein, such as collagen or proteoglycan. It allows for visualisation of the protein’s location in relation to the cell and its organisation around the cell and within the tissue or object of interest. The relative intensity of staining can provide a qualitative
comparison of samples from different groups, but its limitation is inherent in the inability to quantify the relative amounts of protein in each sample without the use of image analysis software. Even with the use of such software, great care would have to be taken to insure that photomicrographs were taken with the same settings to objectively compare samples. In addition, the inspection of series of sections through the entire object of interest would be necessary to comprehensively and objectively assess the amounts of total proteins present throughout the pellet. An excellent method for the confirmation of comparative assessment of IHC results is through biochemical analysis of sample digests for the same proteins that were viewed by staining. Amounts of protein present in pellet digests can be analysed for total protein and compared definitively per unit measure. In this way, a complete picture of the type, organisation, and amount of extracellular matrix proteins can be compared across groups.

2.2.2. Study design

2.2.2.1. In vitro

In vitro comparisons of neocartilage pellets made from IP ASC, SC ASC, and chondrocytes (C) were made after one, two, and three weeks of chondrogenic pellet culture. SC- and IP-derived ASC pellets made from the in vivo experiments ASC 5 and ASC 7, respectively, were compared with each other and with chondrocyte-derived pellets from the same experiments by biochemical assays (total collagen, total GAG, and DNA content) and immunohistochemical stains (collagen type II, collagen type VI, and aggrecan epitope 5D4) after one and three weeks of culture. As this experiment was primarily to compare surgical outcomes, it was necessary to compare SC pellets made from four sheep with IP pellets made from four different sheep. As chondrocyte pellets were made from all eight sheep, comparisons of IP and SC pellets with pooled chondrocyte pellet data from all sheep as well as same-animal comparisons were possible.

At the two-week point, IP pellets were compared with SC pellets made from the same animals in experiment ASC 6 and chondrocyte pellets from experiments ASC 5 and ASC 7 were compared to both (Table 1). In experiment ASC 6, fat was harvested from the IP fat pad and SC fat of three female sheep. ASC were isolated from each harvest and primary cultures immediately split to assess the impact of selective enrichment by adhesion to plastic culture flasks for either up to two hours or at three days. Comparison of selectively-enriched (two hours of adhesion) monolayer cultures to non-selectively cultured cells (allowed to adhere for three days) was performed as a subset of all ASC 6 assessments. Following two passages, these twelve cultures were subjected to chondrogenic differentiation methods to form neocartilage pellets. Pellets were harvested at the conclusion of the second week for analysis.
of extracellular matrix formation by the same IHC and biochemical (BC) methods used to compare pellets from ASC 5 and ASC 7. These pellets were also compared with two week-old chondrocyte pellets from ASC 5 and ASC 7. Since this latter comparison was inherently of differing animal sets, IP and SC pellets were compared with pooled chondrocyte pellets.

Table 1: Study design of in vitro analysis

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Number of Animals</th>
<th>Cell Source</th>
<th>Enrichment</th>
<th>Analysis</th>
<th>Time Point (Weeks)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ASC 6</td>
<td>3</td>
<td>IP</td>
<td>Adhesion</td>
<td>IHC, BC</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>SC</td>
<td>Adhesion</td>
<td>IHC, BC</td>
<td>2</td>
</tr>
<tr>
<td>ASC 5</td>
<td>4</td>
<td>C</td>
<td>None</td>
<td>IHC, BC</td>
<td>1, 2, &amp; 3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>SC</td>
<td>None</td>
<td>IHC, BC</td>
<td>1 &amp; 3</td>
</tr>
<tr>
<td>ASC 7</td>
<td>4</td>
<td>C</td>
<td>None</td>
<td>IHC, BC</td>
<td>1, 2, &amp; 3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>IP</td>
<td>None</td>
<td>IHC, BC</td>
<td>1 &amp; 3</td>
</tr>
</tbody>
</table>

Adipose tissue from two sources (IP and SC) was collected from each animal, cultured with or without enrichment by adhesion for ASC, and evaluated for their chondrogenic ability after each of three weeks of pellet culture with immunohistochemical (IHC) staining for type II collagen, type VI collagen, and aggrecan, and biochemical (BC) analysis for total collagen, GAG, and DNA content. These pellets were compared with each other and chondrocyte pellets from experiments.

2.2.2.2. *In vivo*

These experiments were referred to as ASC 5 (SC fat) and ASC 7 (IP fat). Adipose tissue from two groups of four female sheep each was harvested from either the lumbar area (SC) or the infrapatellar fat pad (IP) of the left knee. ASC were isolated from each tissue sample and cultured for three weeks under chondrogenic conditions to form neocartilage pellets. At the end of the third week of culture, pellets were autologously implanted into subchondral defects in the femoral condyle of the right knee created during the same procedure and just prior to implantation. A control defect was created in the same knee and adjacent to the test defect in each animal and filled with the fibrin sealant used to affix the
neocartilage implants. At 4 and 12 weeks following implantation, 2 animals from each group were euthanized and defects collected for histological and immunohistochemical evaluation (Table 2 and Figure 6).

![Flowchart of study procedures](image)

**Figure 6:** Process flow of *in vivo* study procedures in experiments ASC 5 and ASC 7.

**Table 2: Study design of in vivo (ASC 5 [SC] and ASC 7 [IP]) analysis**

<table>
<thead>
<tr>
<th>Group</th>
<th>Number of Animals</th>
<th>Source of ASC</th>
<th>SxH</th>
<th>SxI</th>
<th>Lesions</th>
<th>Euthanasia</th>
<th>Analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>ASC 5</td>
<td>2</td>
<td>SC</td>
<td>Week 0</td>
<td>Week 3</td>
<td>Test, Control</td>
<td>Week 7</td>
<td>Histology, IHC</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Week 15</td>
<td></td>
</tr>
<tr>
<td>ASC 7</td>
<td>2</td>
<td>IP</td>
<td>Week 0</td>
<td>Week 3</td>
<td>Test, Control</td>
<td>Week 7</td>
<td>Histology, IHC</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Week 15</td>
<td></td>
</tr>
</tbody>
</table>

Two groups of four animals each were autologously implanted with ASC-derived neocartilage pellets cultured from either infrapatellar IP or SC fat. Fat was harvested from sheep during the first procedure (SxH) and ASC isolated from the fat harvest. Isolated ASC were cultured for three weeks to form neocartilage pellets that were then implanted (SxI) into subchondral lesions formed during the implantation procedure. Following 4 and 12 weeks of postoperative rehabilitation, animals were euthanized and lesions collected for histological and immunohistochemical (IHC) analysis.
METHODS

3. Methods

With the exception of histopathology processing, all methods, including surgical, cell and tissue culture, immunohistochemistry, biochemistry, and histopathologic assessment of repair were performed by the candidate.

3.1. Cell culture

3.1.1. Monolayer

3.1.1.1. Isolation of cells

3.1.1.1.1. Adipose-derived stem cells

Adipose tissue was surgically collected as described in section 3.4.2. Aseptically excised adipose tissue was transferred to a Class II biosafety cabinet from the surgical theatre in warm Advanced Dulbecco’s Modified Eagle’s Medium (aDMEM) with 1% Penicillin-Streptomycin-Glutamine 100x added (aDMEM/PSG) (Invitrogen). The tissue was minced with scissors until the pieces were approximately 1mm² and washed twice with sterile phosphate buffered saline (PBS). 1-2 g of tissue was then transferred to a sterile 25 ml glass universal jar containing 10 ml of 0.05% Collagenase Type II (0.5 mg/ml) (Invitrogen) in aDMEM/PSG and mixed overnight on a rotating tube rack at 37°C. The entire contents of the digest were transferred into a 50 ml polypropylene centrifuge tube (Corning) and the universal jar was rinsed into the same tube with 3 ml of aDMEM. Tubes were then centrifuged at 300 x g for 5 minutes. The liquid fraction/cell suspension was carefully aspirated and transferred to a 15 ml polypropylene centrifuge tube (Corning), leaving the remaining fat behind in the 50 ml tube, and centrifuged at 600 x g for 5 minutes to form cell pellets. The supernatant was poured off and the pellet was resuspended in 100 µl aDMEM/PSG, diluted to 5 ml with aDMEM/PSG and centrifuged at 600 x g for 5 minutes. The supernatant was removed, the cell pellet resuspended in 100 µl aDMEM/PSG, and then diluted to 500 µl with aDMEM/PSG. 20 µl was removed and added to 20 µl of Trypan Blue (Gibco). 20 µl of this mixture was used for counting on a haemocytometer (La Fontaine). The cell suspension was diluted to 10 ml with aDMEM and transferred to a 75 cm² polystyrene cell culture flask (Corning). The 15 ml centrifuge tube was washed with 10 ml aDMEM/PSG and added to the culture flask for a final volume of 20 ml of cell suspension. Cultures were incubated at 37°C, 5% CO₂ for either 1-2 hours or 3 days, and then washed with 10 ml of warm sterile PBS to remove any non-adherent cells. The purpose of limiting the time from cell plating to first wash was to select for cells that would most readily adhere to plastic and thus enrich the cell
population for ASC. In the development of this method I found that cells would begin to adhere to plastic within the first 2 hours of plating, so this time period was chosen. I theorised that a more homogenous population, enriched with ASC, could be achieved through selection by limiting the adhesion period. Three days is the most that medium should be left without changing as nutrients become depleted and carbon dioxide released by aerobically metabolizing cells lowers the pH of the medium. Following adherence of cells, culture medium was replaced with 20 ml warm aDMEM/PSG + 1% Foetal Bovine (Calf) Serum (FCS) of New Zealand Origin (Invitrogen) for monolayer expansion. Medium was changed every 3 days. The doubling rate was calculated by determining the number of doublings in culture divided by the number of days in culture (i.e., doublings/day = (ln(final cell number/starting cell number)/ln(2))/number of days in culture)).

3.1.1.1.2. Chondrocytes

Aseptically collected cartilage was placed onto a 130 mm watch glass with a small amount of aDMEM/PSG. Cartilage was finely chopped to 1mm² pieces using 2 scalpels (#22). Minced cartilage was transferred to a 90mm petri dish with 20 ml aDMEM/PSG + 2% FCS + 1.2 mg/ml of 40x ascorbate-phosphate (25 µl/ml) and incubated overnight at 37°C and 5% CO₂. The following day cartilage was rinsed twice with aDMEM/PSG and then approximately 0.5 g transferred to each 20 ml glass universal jar containing 20 ml of aDMEM/PSG + 5% FCS and 26.4 mg/ml pronase I (Roche) and incubated on a roller (14 rpm) at 37°C for 90 minutes. Pronase solution was carefully removed and cartilage was rinsed twice with warmed aDMEM/PSG. 20 ml of aDMEM/PSG + 5% FCS + 1.2 mg/ml collagenase II (Gibco) was then added to the jars and incubated on a roller (14 rpm) at 37°C for 3 hours or until cartilage chips were no longer visible. Once digestion was complete, the digest solution was transferred to a 50 ml centrifuge tube and diluted to 50 ml with aDMEM/PSG. The contents of each 50 ml tube was then transferred to 4 x 15 ml centrifuge tubes and centrifuged at 600 x g for 10 minutes. Supernatant was discarded and the cell pellet was resuspended in 100 µl of aDMEM/PSG. The cell suspension was diluted to 10 ml with aDMEM/PSG and centrifuged at 600 x g for 10 minutes. The supernatant was discarded, the cell pellet was resuspended in 100 µl of aDMEM, and all cell suspensions from the same animal were pooled into a 50 ml centrifuge tube and diluted to 40 ml with aDMEM/PSG. The cell suspension was filtered through a 70 µm strainer into 15ml conical tubes and centrifuged at 600 x g for 10 minutes. Cell pellets were resuspended with 100 µl of aDMEM/PSG each, pooled in a 15 ml centrifuge tube and diluted to 2 ml with aDMEM/PSG. 20 µl was removed and added to 20 µl of Trypan Blue. 20 µl of this mixture was used for counting on a haemocytometer and the remaining cell suspension diluted to 10 ml with aDMEM/PSG. 100
µl of a 5 mg/ml solution of 100X DNAase (Roche) was added and the mixture incubated for at least 15 minutes at 37°C and 5% CO₂. The cell suspension was centrifuged at 600 x g for 10 minutes. The cell pellet was resuspended with aDMEM/PSG to a final concentration of 1 x 10⁶ cells/ml and 75 cm² culture flasks were seeded with 2 x 10⁵ cells. 19.8 ml of aDMEM/PSG + 2% FCS, 5ng/ml FGF-2 (CytoLab), 1ng/ml TGF-β1 (CytoLab) was added to give a total of 20 ml in each flask. Cultures were incubated at 37°C and 5% CO₂ and medium was changed every 3 days.

3.1.1.2. Confirmation of plasticity (MSC differentiation assay)

Adipose tissue was surgically collected from the infrapatellar fat pad of a live, anaesthetised female sheep and ASC were isolated and cultured per the method in section 3.1.1.1. Following addition to the culture flask, cells were allowed to adhere for 3 days prior to the first wash and medium was subsequently changed every 3 days. The monolayer culture was passaged twice and Passage 2 cells were harvested with trypsin EDTA. A Human Mesenchymal Stem Cell Functional Identification Kit (the Kit) (R&D Systems, Minneapolis, MN, USA) was used to confirm the tri-lineage potential of isolated cells. All differentiation supplement solutions, stains, and antibodies were supplied in a pre-formulated state with the Kit. All differentiation supplement solutions were warmed to 37°C before addition to basal medium and fresh differentiation medium was prepared for each medium change.

3.1.1.2.1. Confirmation of adipogenic potential

3.1.1.2.1.1. Adipogenic differentiation culture

To prepare the culture plate, 12mm round coverslips were sterilized with absolute ethanol and placed into each well of a 24-well culture plate (Corning). 0.5 ml of sterile PBS was added to each well and the culture plate was stored in a 37°C, 5% CO₂ incubator until needed. A suspension of 3.7 x 10⁵ cells in 5 ml of αMEM (α Minimal Essential Medium, Gibco) was prepared, PBS was removed from wells, and 0.5 ml of the cell suspension was added to each well for a final density of 3.7 x 10⁴ cells/well (2.1 x 10⁴ cells/cm²). Cultures were incubated at 37°C, 5%CO₂. After 2 days of incubation, medium was removed and replaced with 0.5 ml of adipogenic differentiation medium, which was prepared by the addition of 50 µl of a 100X concentrated solution containing hydrocortisone, isobutylxanthine, and indomethacin to 5 ml of αMEM (solution supplied pre-formulated with the Kit). Medium was changed every 3 days.

3.1.1.2.1.2. Immunocytochemistry of adipocytes

2 coverslips were collected with forceps on days 8, 15, 21, and 28, washed twice with 1 ml of PBS and fixed with 4% paraformaldehyde for 20 minutes at room temperature. Day 8 cultures were stored in PBS at 4°C until processed on Day 15. All other cultures were
processed on the day of collection as follows. After fixation, cultures were washed three times with 0.5 ml of 1% bovine serum albumin (BSA) in PBS for 5 minutes. Cells were then permeabilized and blocked with 0.5 ml of 0.3% Triton X-100, 1% BSA, and 10% normal donkey serum in PBS for 45 minutes at room temperature, as recommended in the assay instructions provided by the manufacturer. Goat anti-mouse fatty acid binding protein-4 (FABP-4) antibody was diluted in PBS containing 1% BSA and 10% normal donkey serum to a final concentration of 10 µg/ml and a negative control prepared with PBS containing 1% BSA and 10% normal donkey serum with no antibody. After blocking, cells were incubated with 300 µL/well of either diluted goat anti-mouse FABP-4 antibody or control without antibody overnight at 4°C. The next morning the cells were washed 3 times with 0.5 ml of 1% BSA in PBS for 5 minutes. Cy3-conjugated donkey anti-goat IgG (Jackson Laboratories) was reconstituted with distilled water and then diluted with 1% BSA in PBS to a final concentration of 2.5 µg/ml. Cells were incubated with 300 µL/well of secondary antibody in the dark for 1 hour at room temperature. Cells were washed 3 times with 0.5 ml of 1% BSA in PBS for 5 minutes. 1% BSA was replaced with distilled water and coverslips removed from wells and mounted cell-side down onto cleaned glass slides with 10 µl of Vectashield.

3.1.1.2.1.3. Histology of adipocytes

On Day 28, 2 cultures were removed, washed 3 times in PBS for 5 minutes, fixed in 4% paraformaldehyde for 20 minutes at room temperature and washed 3 more times with PBS for 5 minutes. 0.5 mg of oil red-O was dissolved in 5 ml of 70% ethanol and filtered with a 20 µm filter. Cells were incubated with oil red-o working solution for 15 minutes at room temperature. Coverslips were washed 3 times with PBS and mounted cell-side down onto cleaned glass slides with Vectashield (Vector Laboratories, Burlingame CA, USA).

3.1.1.2.2. Confirmation of osteogenic potential

3.1.1.2.2.1. Osteogenic differentiation culture

To prepare the culture plate, 12mm round coverslips were sterilized with absolute ethanol and placed into each well of a 24-well culture plate. 0.5 ml of sterile PBS was added to each well and culture plate was stored in a 37°C, 5% CO₂ incubator until needed. PBS was removed from wells and cells in αMEM were seeded into each well at a density of 7.4 x 10³ cells/well (4.2 x 10³ cells/cm²). Cultures were incubated at 37°C, 5%CO₂. After 2 days of incubation, medium was removed and replaced with 0.5 ml of osteogenic differentiation medium, which was prepared by the addition of 250 µl of a 20X concentrated solution containing dexamethasone, ascorbate-phosphate, and β-glycerolphosphate to 5 ml of αMEM (solution supplied pre-formulated with the Kit). Medium was changed every 3 days.
3.1.1.2.2. Immunocytochemistry of osteocytes

2 coverslips were collected with forceps on days 15, 21, and 28, washed twice with 1 ml of PBS and fixed with 4% paraformaldehyde for 20 minutes at room temperature. After fixation, cultures were washed three times with 0.5 ml of 1% bovine serum albumin (BSA) in PBS for 5 minutes. Cells were then permeabilized and blocked with 0.5 ml of 0.3% Triton X-100, 1% BSA, and 10% normal donkey serum in PBS for 45 minutes at room temperature. Mouse anti-human osteocalcin antibody was diluted in PBS containing 1% BSA and 10% normal donkey serum to a final concentration of 10 µg/ml and a negative control prepared with PBS containing 1% BSA and 10% normal donkey serum with no antibody. After blocking, cells were incubated with 300 µL/well of either diluted mouse anti-human antibody or control without antibody overnight at 4°C. The next morning the cells were washed 3 times with 0.5 ml of 1% BSA in PBS for 5 minutes. AlexaFluor 546-conjugated goat anti-mouse IgG (Molecular Probes) was diluted with 1% BSA in PBS to a final concentration of 4 µg/ml on Day 15 and 21, and 10 µg/ml on Day 28. Cells were incubated with 300 µL/well of secondary antibody in the dark for 1 hour at room temperature. Cells were washed 3 times with 0.5 ml of 1% BSA in PBS for 5 minutes. 1% BSA was replaced with distilled water and coverslips removed from wells and mounted cell-side down onto cleaned glass slides with 10 µl of Vectashield.

3.1.1.2.2.3. Histology of osteocytes

On Day 28, 4 cultures were removed, washed 3 times in PBS for 5 minutes and fixed in ice-cold 70% ethanol for 1 hour. Alizarin red-S stain for calcium was diluted to 20 mg/ml in distilled water and buffered with NH₃OH to pH 4.2. Cells were washed with PBS for 5 minutes and incubated with alizarin red-S working solution for 10 minutes at room temperature. A differentiation solution of 95% ethanol and concentrated HCl was added to the cells for 15 seconds and washed 3 times with PBS for 5 minutes. Coverslips were washed 3 times with PBS and mounted cell-side down onto cleaned glass slides.

3.1.1.2.3. Confirmation of chondrogenic potential

3.1.1.2.3.1. Chondrogenic differentiation culture

2.5 x 10⁵ cells in αMEM were transferred to a 15 ml centrifuge tube and centrifuged at 200 x g for 5 minutes. The medium was removed and the pellet resuspended in 1.0 ml of DMEM/F-12 basal medium. The cells were centrifuged again at 200 x g for 5 minutes, after which the medium was aspirated and discarded. The cells in each tube were resuspended in chondrogenic medium, which was prepared by the addition of 25 µl of a 100X concentrated solution containing dexamethasone, ascorbate-phosphate, proline, pyruvate, TGF-β3, insulin, transferrin, selenious acid, BSA, and linoleic acid to 2.5 ml of DMEM/F-12 basal medium.
Cells were centrifuged at 200 x g for 5 minutes. The caps on each tube were loosened to allow gas exchange and cultures were incubated at 37°C and 5% CO₂ for up to 28 days. On day 2 and every 3 days afterward medium was changed with chondrogenic supplement in DMEM/F-12 in 5 of the 6 tubes and with chondrogenic medium containing 40x ascorbate-phosphate (25 µl/ml)(Sigma Aldrich); TGF-β1 (2 pg/ml)(Peprotech); 10⁻⁷ M dexamethasone (10 µl/ml)(Sigma Aldrich); and linoleic acid (5 ng/ml)(Sigma Aldrich) in advanced Dulbecco’s Modified Eagle’s Medium (aDMEM)(Invitrogen) in the remaining tube. It was believed that the chondrogenic medium developed by our group was superior to the kit-supplied chondrogenic medium so the sixth pellet was used, grown in our group’s medium, to assure that at least one pellet would be cultured in what was considered to be the best conditions for differentiation.

3.1.1.2.3.2. Immunocytochemistry of chondrocytes

On days 14, 21, and 28, 2 pellets were collected, transferred to 24-well culture plates and washed twice with 1.0 ml of PBS for 5 minutes. Pellets were fixed with 4% paraformaldehyde for 20 minutes at room temperature and then washed two more times with PBS for 5 minutes each time. Pellets were placed into a 10 mm cryomould (Sakura Finetek) and covered with optimal cutting temperature (O.C.T.) compound (Sakura Finetek). Moulds were then frozen in liquid nitrogen and stored at -80°C until cut into 10 µm sections. Sections were mounted onto Superfrost + slides and encircled with a wax pen. Mounted sections were permeabilized and blocked with 0.5 ml of 0.3% Triton X-100, 1% BSA, and 10% normal donkey serum in PBS for 45 minutes at room temperature. Goat anti-human aggrecan antibody was diluted in PBS containing 1% BSA and 10% normal donkey serum to a final concentration of 10 µg/ml and a negative control prepared with PBS containing 1% BSA and 10% normal donkey serum with no antibody. After blocking, sections were incubated with 300 µl of either diluted antibody or control without antibody overnight at 4°C in a covered container. The next morning the sections were washed 3 times with 0.3 ml of 1% BSA in PBS for 5 minutes. Cy3-conjugated donkey anti-goat IgG (Jackson Laboratories) was reconstituted with distilled water and then diluted with 1% BSA in PBS to a final concentration of 2.5 µg/ml. Sections were incubated with 300 µl of secondary antibody in the dark for 1 hour at room temperature. Cells were washed 3 times with 0.3 ml of 1% BSA in PBS for 5 minutes. 1% BSA was replaced with distilled water and excess water was subsequently removed. 10 µl of Vectashield was placed on each section and a coverslip added.
3.1.2. 3-Dimensional Culture

3.1.2.1. Chondrogenic medium

Chondrogenic differentiation medium was prepared by the addition of 100x penicillin-streptomycin-glutamine (10 µl/ml)(Invitrogen); 40x ascorbate-phosphate (25 µl/ml)(Merck); TGF-ß1 (2 pg/ml)(Peprotech); 10^{-7} M dexamethasone (10 µl/ml)(Sigma Aldrich); and linoleic acid (5 ng/ml)(Sigma Aldrich) to Advanced Dulbecco’s Modified Eagle’s Medium. This chondrogenic medium was developed by our group, based on R. P. Jakob’s method [365], to provide the cells with the appropriate hormones, growth factors, and differentiation mediators for chondrogenesis as discussed in section 1.4.1.2.2.

3.1.2.2. Pellet formation

Medium was aspirated from the culture flask and cells were washed with 10 ml of warm PBS to remove residual foetal calf serum. 5 ml of 0.25%trypsin/ 1mM EDTA (Gibco) was added to cells and incubated for approximately 3 minutes or until virtually all cells were detached from the flask. 7 ml of aDMEM/PSG was added to the flask and the cell suspension was transferred to a 15 ml centrifuge tube and centrifuged at 600 x g for 5 minutes. The cell pellet was resuspended in 100 µL and diluted to 1 ml with aDMEM/PSG. 100 µl of DNAse and 8.9 ml of aDMEM/PSG was added to the tube, mixed, and incubated at 37°C and 5% CO₂ for at least 15 minutes. Following incubation, cells were centrifuged at 600 x g for 5 minutes. The cell pellet was resuspended in 100 µl and diluted to 2 ml with chondrogenic medium, prepared as described in section 3.1.2.1. 20 µl was removed and added to 20 µl of Trypan Blue. 20 µl of this mixture was used for counting on a haemocytometer. 3 x 10⁵ cells were placed into each LidBac culture tube (Eppendorf), diluted to 500 µl with chondrogenic medium and centrifuged at 600 x g for 5 minutes. Each lid was opened and replaced with a sterile membrane lid before incubating at 37°C, 5% CO₂ on an orbital shaker set at 40 rpm for 2 days. After 2 days, cell pellets were transferred to either 24-well culture plates (experiment ASC 6) or a rotary cell culture system (RCCS) as described in the next section, 3.1.2.3 (ASC 5 and ASC 7). In method development with the RCCS it was noted that pellets sometimes formed clumps within the culture vessel. For the purposes of surgical implantation (experiments ASC5 and ASC7), pellet clumping was determined to be acceptable and less important than providing the optimal mechanical environment to pellets in preparation for implantation into the joint. As experiment ASC6 was a direct comparison of pellets from two sources grown under identical conditions, it was determined that the maintenance of pellet separation within culture wells was more important than optimisation of pellet growth. Therefore, pellets in experiment ASC6 were cultured in 24 well culture plates. The 24 well
culture plates were placed on an orbital shaker in the incubator at 37°C and 5% CO₂ and medium changed every 3 days.

3.1.2.3. Rotary cell culture system

The Rotary Cell Culture System™ (RCCS-4, Synthecon) base unit (Figure 7) was cleaned with 70% ethanol and placed on to the bottom shelf of a humidified incubator at 37°C and 5% CO₂. A 50 ml disposable culture vessel (Synthecon) was filled with αDMEM and fitted to the RCCS base unit. The next day, the vessel was removed from the base unit and relocated in the Class II biosafety cabinet. The sample port plug and syringe port lid were removed and stored inside a foil packet with an isopropanol swab. The αDMEM was removed from the vessel through the syringe port with a 20 ml syringe (Terumo). After 2 days of culture in LidBac tubes each cell pellet was dislodged from the bottom of the tube by gentle flicking. Using a transfer pipette with the tip cut to allow for a larger opening, each pellet was aspirated from the tube and placed into the RCCS vessel through the sample port. Once all pellets were thus transferred, all medium remaining in the RCCS vessel was aspirated with a transfer pipette through the sample port. 45 ml of warmed chondrogenic medium, as prepared in section 3.1.2.1., was transferred into the vessel with a Powerpette (Jenkens) and a 25 ml serological pipette (Corning). The sample port plug was replaced and wiped with an isopropanol swab. The remaining capacity of the vessel was filled with additional warmed chondrogenic medium through the syringe port using a 10 ml syringe (Terumo) with care being taken to remove all air bubbles from the vessel. The syringe port was closed off while pressure was being applied to the syringe to create positive pressure within the vessel. The syringe port lid was replaced and the entire vessel was wiped with an isopropanol swab. The vessel was screwed on to the RCCS base unit in the incubator at 37°C, 5% CO₂ and the unit set to 10 rpm.

Every other day medium was changed through the syringe port. Both syringe port lids were removed and stored in a foil packet with an isopropanol swab. A 20 ml syringe was
affixed to one syringe port and the port opened to equalize the pressure within the vessel. The other syringe port was then opened to allow air to enter the vessel and the existing medium was removed with care being taken to avoid aspiration of any cell pellets. New, warmed, chondrogenic medium was injected slowly into the vessel with a new 20 ml syringe through a syringe port and the syringe port closed while pressure was applied to the syringe to create positive pressure within the vessel. The lids were replaced on both syringe ports and the entire vessel was wiped clean with an isopropanol swab. The RCCS control unit was switched off, the vessel was replaced on to the base unit and the unit switched back on.

Neo-cartilage pellet samples were collected through the sample port during medium changes. Once medium had been removed from the vessel, the sample port plug was removed and placed into a foil packet with an isopropanol swab. Using a transfer pipette with the tip cut to allow for a larger opening, samples were aspirated from the vessel and stored appropriately for later processing. The medium was replaced and the vessel restored to the base unit as previously described.

3.1.2.4. Pellet diameter measurement

Pellets were measured using an optical dissecting microscope. X- and Y-axis diameters were measured at right angles and the arithmetic mean of the two measurements was recorded as the pellet diameter.

3.2. Immunohistochemistry

3.2.1. Pellet

3.2.1.1. Collagen type II

10 µm sections mounted on Superfrost + slides were encircled with a wax pen and rehydrated with (pH 7.2-7.4) for 10 minutes at room temperature in a covered plastic staining tray lined with a moist paper towel. Sections were washed with 0.1% BSA in PBS for 10 minutes and then 3 more times with tris-HCl (pH 5.5) for 10 minutes each. Sections were incubated with testicular hyaluronidase (2mg/ml in tris-HCl, pH 5.5) for 60 minutes at 37°C to remove aggrecan and expose the target epitopes and then washed with PBS for 10 minutes at room temperature. Sections were washed twice for 10 minutes with 0.1% BSA in PBS and then blocked with normal goat serum (20 µl/ml in PBS/0.1%BSA) (Jackson ImmunoResearch) for 2 hours at room temperature. Serum was removed from sections and replaced with rabbit anti-collagen type II (1.0 mg/ml diluted to 1:100 in 0.1%BSA/PBS) (Rockland) and incubated overnight at 4°C. The following day sections were washed thrice for 15 minutes with 0.1% BSA in PBS. 50 µl of Alexa Fluor® 488 goat anti-rabbit IgG (2 mg/ml diluted to 1:1000 in 0.1% BSA/PBS) (Molecular Probes) was added to each section and incubated for 3 hours at room temperature in the dark. Following incubation with
secondary antibody, sections were washed thrice with PBS for 10 minutes at room temperature. 10 µl of Vectashield was added to each section and then the slide was covered with an ethanol-cleaned coverslip and sealed with clear nail polish. Slides were kept in the dark at 4°C.

3.2.1.2. Collagen type VI

10 µm sections mounted on Superfrost + slides were stained for collagen type VI as described in Section 3.2.1.1 with the following exceptions:

After blocking, serum was removed from sections and replaced with rabbit anti-collagen type VI (1.0 mg/ml diluted to 1:1000 in 0.1% BSA/PBS) (Rockland) and incubated overnight at 4°C. The following day sections were washed thrice for 15 minutes with 0.1% BSA in PBS. 50 µl of Alexa Fluor® 488-conjugated goat anti-rabbit IgG (2 mg/ml diluted to 1:1000 in 0.1% BSA/PBS) (Molecular Probes) was added to each section and incubated for 3 hours at room temperature in the dark.

3.2.1.3. Aggrecan

10 µm sections mounted on Superfrost + slides were encircled with a wax pen and rehydrated with tris-acetate buffer (pH 8.0), prepared by mixing equal amounts of 1M tris(hydroxymethyl)methylamine (BDH) and 1M sodium acetate (Merck), for 10 minutes at room temperature in a covered plastic staining tray lined with a moist paper towel. Chondroitinase ABC (0.4 u/ml in tris-acetate buffer) (Sigma) was added to sections and incubated for 1 hour at 37°C in a humidified hybridization oven to expose the target epitopes of aggrecan. Sections were then washed twice with 0.1% tween 20 (Sigma) in PBS (tween/PBS) for 5 minutes each wash and blocked with 5% BSA in tween/PBS, for 1-2 hours at room temperature. After blocking, sections were washed thrice with tween/PBS for 5 minutes each and mouse anti-keratan sulfate, 5D4 (1 mg/ml used at 1:100 in tween/PBS) (Seikagaku) was added to sections and incubated overnight at 4°C. Sections were washed with tween/PBS thrice for 5 minutes and then 50 µl of Alexa Fluor® 488-conjugated goat anti-mouse IgG (2 mg/ml diluted to 1:1000 in tween/PBS) (Molecular Probes) was added and incubated in the dark for at least 1 hour at room temperature. Following incubation with secondary antibody, sections were washed thrice with tween/PBS for 5 minutes at room temperature. 10 µl of Vectashield was added to each section and then the slide was covered with an ethanol-cleaned coverslip and sealed with clear nail polish. Slides were kept in the dark at 4°C.
3.2.2. Implantation site

3.2.2.1. Collagen type II

10 µm sections were de-mineralized and de-waxed as described in section 3.5.1, mounted on Superfrost + slides, and stained for collagen type II as described in section 3.2.1.1.

3.2.2.2. Collagen type VI

10 µm sections were de-mineralized and de-waxed as described in section 3.5.1, mounted on Superfrost + slides, and stained for collagen type VI as described in section 3.2.1.2.

3.2.2.3. Aggrecan

10 µm sections were de-mineralized and de-waxed as described in section 3.5.1, mounted on Superfrost + slides, and stained for aggrecan as described in section 3.2.1.3.

3.3. Biochemical assays

Samples for biochemical analysis of collagen, GAG, and DNA content of beads were prepared by digestion in papain. Five PBS tablets (Sigma P4417) were added to each litre of milli-Q (MQ) water and stirred until completely dissolved to prepare PBS solution. The resulting solution yielded 0.01 M phosphate buffer, 0.0027 M potassium chloride and 0.137 M sodium chloride at pH 7.4. Papain buffer was prepared by combination of 5mM cysteine (DL cysteine, Sigma, C9768)/L PBS with 5mM sodium EDTA/L PBS and stored at 4°C until used. Neocartilage beads were pooled into vials per animal per time point with 4.46 µl of papain in 1 ml of papain buffer. All vials were incubated at 60°C for 12 hours. Pellet-papain digests were frozen at -20°C until use. All biochemical analyses were performed on these papain digests.

3.3.1. Total collagen

Total collagen content in pellet samples was assessed via the measure of hydroxyproline, found primarily in collagenous amino acid sequences and is therefore used as an indicator of collagen content.

Papain digests were hydrolyzed by the addition of 500 µl of sample to 500 µl of concentrated hydrochloric acid (HCl) in a 15 ml centrifuge tube and incubation at 100°C in a heat block overnight. Hydrolyzed samples were concentrated in Vivaspin2 columns (VivaScience #VS0211) per manufacturer's instructions and then freeze-dried for 6 to 8 hours to remove the acid. Samples were then transferred to 500 µl tubes, sealed with paraffin and stored at 4°C for eight days until used.

500 ml of stock buffer was prepared by combining 28.5 g sodium acetate tri-hydrate, 18.75 g tri-sodium citrate di-hydrate, 2.75 g citric acid, and 200 ml propan-2-ol. Solids were
first dissolved in 250 ml of water before the addition of propan-2-ol. Water was then added to a final volume of 500 ml. 150 ml of diluent was prepared by the combination of 100 ml propan-2-ol and 50 ml water. Oxidant was prepared by the addition of 0.7 g chloramine-T to 10 ml water and 50 ml stock buffer. Colour reagent was prepared by the combination of 7.5 g dimethylamino benzaldehyde, 11.25 ml perchloric acid (60%), and 62.5 ml propan-2-ol. Hydroxyproline standards were prepared from 0 to 5 µg/ml in water.

Hydrolysates were reconstituted in double-distilled water and then spun in a microcentrifuge at maximum speed for 10 minutes to remove any insoluble, particulate matter. The supernatant was collected, taking care not to include any insoluble residue. To each well of a 96 well plate was added 30 µl of sample or standard, 70 µl of diluent and 50 µl of oxidant. 125 µl of colour reagent was then added, mixed well on a plate shaker and incubated at 70°C for 10 to 20 minutes. Once samples turned a peach colour they were immediately read on a plate reader at an absorbance wavelength of 540 nm.

3.3.2. GAG

DMMB solution was prepared by dissolution of 32 mg 1,9-DMMB (Serva # 20335) in 20 ml absolute ethanol overnight with orbital rotation and stirring at room temperature. In a 2 L measuring cylinder 1.5 L MQ water was added to 59 ml of 1M NaOH and 7 ml of 98% formic acid. The dissolved DMMB was added to this solution, and topped up to 2 L with MQ water, stirring for an additional two hours. The absorbance of the DMMB solution was checked against MQ water as a blank at 525 nm and 592 nm and stored in a foil- wrapped bottle until used.

Standard curve solution was prepared with chondroitin sulphate C (sodium salt from shark cartilage; Sigma C-4384). A stock solution (0.5 mg/ml in water, stored at -20°C) was made and then diluted to get five standards at 0, 10, 20, 30 40, and 50 µg/ml in papain buffer. 40 µl of each standard was added by pipette into triplicate wells of a 96-well flat bottom EIA microtitre plate (ICN cat# 76-381-04). 40 µl of each papain-digested sample, at appropriate dilution in papain buffer, was added into duplicate wells. 200 µl of DMMB solution was added to all wells with a multi-channel pipette and the plate was immediately read at 525 nm. The concentration of GAG in the sample (µg/µL) was calculated from the standard curve with consideration given to dilution of samples.

3.3.3. DNA

As all neocartilage pellets were formed from the same number of cells, the DNA content of pellets was used not only to normalize total collagen and GAG content to assess chondrogenic matrix production on a cellular basis, but also as a measure of cell viability.
following induction of chondrogenesis. A higher DNA content indicates a greater number of cells.

DNA content of samples was assessed using the Quant-it dsDNA HS Assay Kit (Invitrogen). Papain digests were hydrolyzed as described in the collagen assay. All other buffers and reagents were supplied with the assay kit. Standards used were: blank; 0; 0.5; 1; 2; 4; 6; 8; and 10 ng/µL. DNA buffer/reagent solution was prepared by the mixture of 200 µl of reagent and 40 ml of buffer in a 50 ml conical tube (Falcon).

10 µl of standard, diluted with 10 µl papain buffer, and 20 µl of sample were added in triplicate to each respective well containing 200 µl of DNA buffer/reagent mixture. 20 µl of papain buffer was added to each of three wells as a blank. The plates were read on a POLARstar OPTIMA multi-detection microplate reader using the PicoGreen® method (excitation at 485 nm, emission at 520 nm, gain of 1000).

3.4. Surgery/animal model

All procedures using animals were approved under University of Otago Animal Ethics Committee Approval 74/04 and adhered to strict aseptic technique. Eight female, non-pregnant, crossbred Romney sheep over two years of age and weighing between 75 Kg and 85 Kg were used for the in vivo study. Animals were selected on the basis of general health, similarity of size, and age by a qualified technician. Animals found to be pregnant were excluded from the study. Age was determined by dental development and all care was taken to select animals that were young adult and of a similar age.

3.4.1. Pre-operative preparation and maintenance of anaesthesia

The area over the jugular vein was shaved to the skin and cleansed with Hibicet before 10 ml of thiopentone (50 mg/ml) was injected into the vein to induce anaesthesia. Once the animal showed signs of jaw tone slackening, an additional 10 ml bolus of thiopentone was administered, also via the jugular vein. The sheep was intubated with a size 9.0 mm endotracheal tube and the cuff filled with air to occlusion. A gastric drain tube was inserted into the oesophagus and allowed to drain into a bucket. Anaesthesia was maintained by 3% Halothane carried on 2 L/minute O₂. During the limiter placement procedure, in which a device to limit the range of motion of operated legs and therefore retain ASC grafts in situ, the animal was also maintained on 0.5 L/minute NO₂. The area over the saphenous vein contralateral to the operated leg was shaved to the skin and cleansed with Hibicet before introduction of a 20 gauge indwelling catheter, through which a drip set was attached from a bag of 500 ml of 0.9% NaCl, with 1 ml/10 kg of Alamycin added, was initiated and maintained until wound closure. The area over the dorsal lumbar spine was shaved to the skin for placement of the cautery earth-ground pad. The operating site was shaved to the skin and
prepared for surgery with 3 alternating swabs of Hibicet and 70% ethanol. Following limiter placement (section 3.4.3), the operating site was re-swabbed with Hibicet and 70% ethanol alternatively. While the sheep was anesthetized, heart rate, respiration rate, O₂ saturation, expired CO₂, capillary refill time, mucous membrane colour, and reflexes (eye-touch response and jaw tension) were monitored approximately every 15 minutes and recorded. Breathing was assisted with a ventilator set to a tidal volume of approximately 500 ml. During wound closure, 5 ml of Marcaine (bupivicaine) was administered intra-articulary for analgesia.

3.4.2. Collection of tissue

3.4.2.1. Subcutaneous fat

The sheep was prepared for surgery as described in section 3.4.1. and laid in lateral recumbence. A 40-50 mm crescent-shaped incision in the skin approximately 80 mm medial to the iliac crest was opened with cautery and the subcutaneous adipose tissue was exposed. The adipose tissue was collected into a pre-weighed sterile vial containing 4 ml of warm aDMEM + 1% PSG and stored in an incubator at 37°C until processed for MSC isolation. The wound was closed in 2 layers with 2.0 Dexon (Davis and Geck).

3.4.2.2. Infrapatellar fat

The sheep was prepared for surgery as described in section 3.4.1. and laid in left lateral recumbence to allow access to the medial aspect of the left knee. The right leg was tied in extension to remove it from the surgical field. A 50-60 mm incision in the skin overlying the medial aspect of the left knee, parallel and ~5 mm medial to the patellar tendon and ~10 mm distal to the femoral condyle, was opened by cautery. The tissue overlaying the joint capsule was dissected bluntly to the fascia. The fascia was pierced with a stab incision and either cut or torn along the fascia plane with scissors to expose the capsule. Approximately 1 cm³ of the underlying infrapatellar fat was excised with either cautery or scissors from the central portion of the fat pad without exposing the articular surface. The adipose tissue was collected into a pre-weighed sterile vial containing 4 ml of warm aDMEM + 1% PSG and stored in an incubator at 37°C until processed for MSC isolation. Wounds were closed in 3 layers with 2.0 Dexon (Davis and Geck).

3.4.2.3. Cartilage

During the defect creation procedure described in section 3.4.4, the majority of cartilage was removed from within the lesion margins with a 6 mm curette (Figure 8) and remaining cartilage scraped down to subchondral bone with a #11 scalpel blade. The lesion margins were cleaned to original delineation with a #11 scalpel blade. All removed cartilage was collected into a pre-weighed sterile vial containing 4 ml of aDMEM/PSG and transferred to the laboratory on ice until isolation of cells was performed.
3.4.3. Placement of external limiter

External limiters were placed on the legs of sheep in a procedure that spanned the duration of the defect creation/implantation procedure. Schanz pins were placed immediately following induction of anaesthesia at the start of the procedure and limiting rods were placed after implantation while animals were being weaned off inhalant anaesthesia. Limiter placement is described below in its entirety with references made to the timing of each step.

The sheep was anaesthetized and the surgical site(s) prepared per the procedures in section 3.4.1. The animal was transferred to the x-ray table in the radiography suite and laid in lateral recumbence with the left leg tied to the table in full extension to remove it from the radiographic field of view. The right leg was aseptically draped and the drape cut to allow access to the femur and tibia. The right greater trochanter was palpated before a stab incision to the bone was made parallel to the femur and along the distal end of the trochanter as it slopes down toward the shaft. A 5.0 mm Schanz pin, loaded into the chuck of a hand drill, was inserted into the incision and positioned on the crown of the bone. Slight force was applied to the pin to create a purchase mark and the pin then drilled into the femur until it penetrated through both cortices. Using fluoroscopy, the distal end of the femur was briefly visualized and the tip of a scalpel blade (#11) was positioned at the mid-point of the distal end of the femur, approximately half-way along the patellar groove so as to avoid any structure such as the lateral collateral ligament. A stab incision to the bone was then made at that point. A 5.0 mm Schanz pin, loaded into a hand drill, was inserted into the incision and the desired
positioning verified briefly with fluoroscopy. The pin was marked with slight pressure and carefully drilled into the femur until firmly penetrating both cortices. Using fluoroscopy, the proximal end of the tibia was briefly visualized and a scalpel blade (#11) positioned slightly posterior to the mid-point of the bone, approximately 20 mm distal to the tibial plateau and a stab incision to the bone was made at that point. A 5.0 mm Schanz pin, loaded into a hand drill, was inserted into the incision and carefully drilled into the tibia until firmly seated in both cortices. The tibia was palpated approximately 50 mm from the distal end until the location of the saphenous blood vessel running over the bone at that point was located. A stab incision was made with a scalpel blade (#11) on to the centre of the bone and a safe distance from the blood vessel. A 2.5 mm drill bit loaded into a hand drill was inserted into the incision, positioned on the crown of the bone and its position verified briefly with fluoroscopy. The drill was carefully advanced in half-turns so that it seated without moving from the original mark. The mark was then carefully deepened with full turns and the drill bit passed fully through both cortices, perpendicular to the crown. A 5.0 mm Schanz pin, loaded into a hand drill, was inserted into the incision and seated into the 2.5 mm pilot hole. The Schanz pin was advanced into the tibia with great care taken to keep it perpendicular to the crown so that it would properly seat in the pilot hole drilled into the medial cortex. The pin was drilled into the tibia until the tip was barely palpable through the skin on the medial aspect of the tibia. The animal was then prepared for pellet placement surgery per the procedures in section 3.4.1. and transferred to the operating theatre.
The fixed angle directly over the knee is arranged to allow the sheep to stand comfortably. The transverse bar is hinged at the distal end with a slide at the proximal end that has limited movement due to the locking collar affixed at the same end, thus limiting the range of knee joint motion.

Following placement of pellets and Tisseel into the lesions, the sheep was rolled into lateral recumbence on its left side and the surgical drape moved to expose the Schanz pins but keep the wound site covered and sterile. The adjustable rod was placed onto the proximal femoral pin and the distal tibial pin so that the limiting stopper and couple were positioned at the femoral end. The limiting couple was positioned on the adjustable rod and affixed so that the leg was extended sufficiently to fully cover both lesions with the meniscus, but at an angle that would allow for natural and comfortable standing. The femoral pins were then joined by an 8.0 mm diameter steel rod (clean, but not sterile), which was affixed to the pins over the adjustable bar with non-sterile Ostron Blue, a polymethylmethacrylate cement (GC Corporation), so that the steel rod and cement would prevent the removal of the adjustable rod from the proximal femoral pin. The tibial pins were similarly joined by a second 8.0 mm steel rod so that the distal end of the rod would prevent removal of the distal end of the adjustable rod from the distal tibial pin (Figure 9). Once the cement had fully cured to hardness, the
animal was returned to dorsal recumbence and the surgical drapes replaced to allow for wound closure. Following wound closure, swabs soaked in nitrofuracin were wrapped around the base of each pin where they entered the skin. Swabs were changed at least once weekly.

3.4.4. Defect creation

The sheep was prepared for surgery as described in section 3.4.1. and laid in right lateral recumbence to allow access to the medial aspect of the right knee. The left leg was tied in extension to remove it from the surgical field. A 50-60 mm incision in the skin overlying the medial aspect of the right knee, parallel and ~20 mm medial to the patellar tendon, was opened by cautery. The tissue overlaying the joint capsule was dissected bluntly to the fascia. The fascia was pierced with a stab incision and either cut or torn along the fascia plane with scissors to expose the capsule. The incision was extended proximally to the muscle boundary and distally to the meniscus to provide the best possible exposure of the condyle. The joint was flexed maximally to expose the broadest part of the condyle normally covered by the meniscus. Using a 6 mm biopsy punch two lesions were marked on the broadest area of the exposed condyle, one on either side of the crown of the condyle, approximately 2 mm from the meniscus and approximately 5 mm apart. The cartilage within the lesion boundaries was removed and collected per the method in section 3.4.3.3. A pilot hole was drilled into the centre of each lesion with a 1 mm drill bit and a hand drill. Using a 6 mm drill bit and either a hand drill or auger handle, the defect was reamed to a depth of approximately 3-4 mm below the articular surface until welling blood was evident (Figure 10). Bone fragments were removed from the lesions and the wound was flushed profusely with saline irrigation.

![Figure 10: Defect creation](image)

Defects were reamed through to subchondral bone with a 6 mm drill bit until welling of blood was evident. Once drilling was complete and edges of each defect were cleaned with a scalpel blade, the defects were ready for implantation with neocartilage pellets.

3.4.5. Implantation

Pellets were transferred from the culture laboratory to the surgical theatre in a 70 µm nylon cell strainer (BD Falcon) sitting inside a sterile 6-well polystyrene culture plate.
(Corning) with warm aDMEM/PSG. The cell strainer was removed from the culture well with forceps (Figure 11) and pellets removed from the strainer with a spatula.

Figure 11: A cell strainer containing neocartilage pellets ready for implantation

The test lesion (lateral) was swabbed with gauze and pellets placed into the defect until they were raised slightly above the articular surface so that they would be exposed to compression and shear. Tisseel (Baxter Biosurgery) was carefully injected over the top of the pellets and into the control lesion (medial) to minimize the amount of Tisseel in the wound and given a minute to set firmly (Figure 12). Excess Tisseel was cut from around the lesions with a scalpel blade (#11) to minimise adhesion with any other tissues. A spatula was used to guide the meniscus over the lesions as the joint was carefully extended. The leg was held in extension as the limiter was fixed in place according to the method in section 3.4.3. Following limiter fixation the wound was closed in 3 layers with 2.0 Dexon.
Figure 12: Placement of neocartilage pellets into the defect
Neocartilage pellets in place within the defect, prior to addition of Tisseel (left) and once Tisseel had been added to both test and control lesions (right). The profile of the pellets was raised slightly above the cartilage surface exposing pellets to compressive load and shear and providing mechanical stimulus to continue matrix production \textit{in situ}.

3.4.6. Rehabilitation

Following wound closure the drapes were removed and the animal, still on the operating table, retired to the recovery area where it was monitored for signs of awakening. Once a swallowing and chewing response was evident the oesophageal and endotracheal tubes were removed. Prior to full recovery from anaesthesia, the sheep was transferred from the operating table into a canvas sling suspended from an overhead support so that fully recovered sheep could stand comfortably on a metal grate but its weight would be borne by the sling when resting. The animal was fed hay \textit{ad libitum} and grain was provided several times per day. Water was provided \textit{ad libitum}. The incision site and schanz pin tracks were inspected daily for signs of infection and schanz pin swabs were changed at least once weekly. Clinical observations, including site observations and general health, were performed and recorded at least once daily while the animal was in the sling. After 3 weeks, the area over the jugular vein was shaved and 10 ml of thiopentone (50 mg/ml) was injected intravenously to effect, followed by an additional 10 ml of thiopentone. Once the anaesthetic was fully effective, the schanz pins were removed and pin tracks were cleaned and flushed with a betadine solution if necessary. After overnight recovery the sheep was released to deep litter for one week, during which gait, weight bearing, and general health were assessed and recorded. At four weeks post-surgery, the sheep was either euthanized for evaluation of tissues or transferred to a holding farm where it was allowed free-range for an additional 8 weeks until returned to the facility for euthanasia and evaluation of tissues.

3.4.7. Euthanasia and necropsy

The sheep was euthanized by captive bolt pistol followed by exsanguination. The right hip was disarticulated and the muscles over the femur removed. The femur was placed in a table vice so that the condyle was up and the tibia and fibula were facing outward. The patellar tendon was cut and the patella released to expose the articular surface. The
surrounding ligaments and tendons were severed to fully expose the condyle and lesions. A fine hacksaw blade was used to cut the lesions out of the condyle by first cutting around and 5-10 mm away from the lesions and the cutting underneath the lesions to release them from the condyle. The test and control lesions were separated by a duridium style single edged razor blade (Gem Scientific) and then each lesion was halved with the same blade. Each half of each lesion was placed into a pot of 10% neutral buffered formalin (NBF) and labelled for the appropriate demineralization method as described in section 3.5.1.

3.5. Histopathology

3.5.1. Demineralization

Tissue samples were removed from NBF and placed into either 10% formic acid or 7.5% EDTA (buffered to pH 7.0 with HCL). Both methods of demineralization were used to increase the probability of successful demineralization in the shortest time while maintaining the antigenicity required for subsequent immunohistochemical staining. Formic acid is widely used and known for rapid and complete demineralization, but has been shown to destroy antigenic reactivity [366]. In contrast, EDTA demineralisation, while possibly slower and less efficient, has been shown to preserve antigenic reactivity [366]. For EDTA demineralization, tissue samples were assessed for residual calcium by x-ray set to 0.125 exposure and positioned 5 cm from the specimen and the solution renewed every 2-3 days. Once no residual calcium was detected, the tissue specimen was washed for several hours in distilled water and then placed into 70% ethanol until processed. For formic acid demineralization, the solution was sampled by ammonium oxalate testing and renewed every 2-3 days until testing revealed no detectable calcium. Ammonium oxalate testing was performed by first shaking the specimen container to suspend any precipitated mineral. 5 ml of solution was then removed from the centre of the specimen container and placed into a glass vial. The sample was buffered to pH 9-10 with concentrated ammonium hydroxide. The contents of the vial were mixed and if a white precipitate formed it was recorded as positive for calcium ions and the formic acid solution was renewed. If the sample remained clear after agitation, 5 ml of saturated ammonium oxalate was added and left for 30 minutes at room temperature. If, after 30 minutes had passed, a precipitate formed, it was recorded as positive for calcium ions and the formic acid solution was renewed. However, if the solution remained clear after 30 minutes then the mixture was recorded as negative for calcium ions and the specimen was washed for several hours in distilled water and placed into 70% ethanol until processed.
3.5.2. Slide processing

3.5.2.1. Haematoxylin & eosin stain

Mounted paraffin sections were first hydrated by submersion in ethanol of successively decreasing concentration to water. Following hydration, slides were bathed in Harris haematoxylin for 6-8 minutes and then washed well in running water. Slides were dipped into 0.2% HCL in 70% alcohol for a few seconds to differentiate and then dipped into ammonia water to “blue”. Slides were washed well in running water and then dehydrated sequentially with 70%, 95%, and absolute ethanol. Sections were stained in alcoholic eosin (0.1% eosin Y) for 3 minutes and then dehydration was completed with 3 more changes of absolute ethanol for 1 minute each. Sections were then cleared with xylene and mounted with DPX.

3.5.2.2. Mallory’s trichrome stain

Mounted paraffin sections were first hydrated by submersion in ethanol of successively decreasing concentration to water. Nuclei were stained with Harris haematoxylin for at least 10 minutes and then washed well in running water. Slides were dipped into 0.2% HCL in 70% alcohol for a few seconds to differentiate and then dipped into ammonia water to “blue”, leaving nuclei over stained. Sections were stained in 1% acid fuschin and washed well in running water until collagen was almost colourless. Slides were stained in Mallory’s Aniline Blue-Orange G (0.5 g aniline blue, 2.0 g orange G, and 1.0 g phosphotungstic acid in 100 ml of distilled water) for 20-30 minutes and then washed in running water until the aniline blue no longer came away. Slides were dehydrated from water through to absolute ethanol and cleared in two changes of xylene before being mounted with DPX.

3.5.3. Histopathology assessment

The degree to which pellets from each ASC cell source repaired the lesions was assessed via Mallory’s trichrome staining as well as haematoxylin and eosin staining. Mallory’s trichrome was used to highlight the extracellular matrix collagen and mineralization within the defect while haematoxylin and eosin were used to stain cells. Slides of defects from each cell source group were compared with each other as well as control lesions from the same animal. Test sections from each animal were examined for the presence of neocartilage pellets within the lesion, and test and control lesions from each animal were examined for: new matrix formation; new matrix organisation; the integration of newly formed tissue with host tissue; and surface congruence. For this assessment, an Olympus AX-70 light microscope (Olympus Optical, Japan) was used in brightfield mode. A UPlanFl x4 magnification objective with a numerical aperture of 0.13 (Olympus Optical, Japan) was used to observe and capture large areas of histology sections. A UPlanFl x20 magnification objective with a
numerical aperture of 0.50 and Phase setting of 1 (Olympus Optical, Japan) was used to observe and capture detail of points of interest within sections. Digital images were captured using a Spot RT Colour digital camera (Diagnostic Instruments, USA) (Software version 3.5.0). Adobe Photoshop CS Version 9.0.2 (Adobe) Photomerge was used to compile all x4 magnification images into a single composite image of the entire defect. No other editing of images was performed.

The fate of neocartilage pellets following implantation into the lesions was determined by the use of antibody stains against the phenotypic cartilage matrix proteins collagen type II and aggrecan epitope 5D4, as well as collagen type VI, which is a marker of pericellular matrix organisation (section 1.1.4.2). Matrix production and organisation were evaluated as an assessment of whether the pellets continued to differentiate and form hyaline-like cartilage in vivo. For this assessment, an Olympus AX-70 light microscope (Olympus Optical, Japan) was used in epifluorescence mode equipped with a fluorescence filter cube (Olympus Optical, Japan) with the following specifications: green excite bandpass at 520-550nm, dichroic mirror at 565nm, and long pass filter at 580nm. A UPlanFl x4 magnification objective with a numerical aperture of 0.13 (Olympus Optical, Japan) was used to observe and capture large areas of histology sections. A UPlanFl x20 magnification objective with a numerical aperture of 0.50 (Olympus Optical, Japan) was used to observe and capture detail of points of interest within sections. Digital images were captured using a Spot RT Colour digital camera (Diagnostic Instruments, USA) (Software version 3.5.0). Gain was adjusted within the Spot software to most accurately and faithfully reproduce the section as observed through the objective lens and avoid the pixel saturation point, thereby establishing an upper limit for intensity. Adobe Photoshop CS Version 9.0.2 (Adobe) Photomerge was used to compile all x4 magnification images into a single composite image of the entire defect. No other editing of images was performed.

3.6. Statistical Analysis

The in vitro results were expressed as a mean ± standard deviation (SD). The significance of the results was determined using the Student t-Test within Microsoft Excel. The test was run as paired when sample numbers was the same in both arrays and when samples were missing from a set then the test was run as two samples with equal variance. A two-tailed distribution was used. A p value of < 0.05 was considered as significant.
RESULTS

4. Results

4.1. In vitro

4.1.1. Cell culture

4.1.1.1. Yield

Yield of ASC from each harvest of adipose tissue is presented here.

ASC 5: The cellular yield of subcutaneous fat from four animals is summarised in Table 3 below. Isolated cells were allowed to adhere to culture flasks for 4.5 hours or until all cultures displayed adherent cells with varying degrees of fibroblast-like morphology and tendril formation. All ASC were ringed by red blood cells with a clearly defined buffer zone around each, which was devoid of red blood cells and most likely consists of a hyaluronan coat [60]. At this point all cultures were washed and expansion medium added.

### Table 3: Cellular yield from subcutaneous fat harvested from sheep in experiment ASC 5

<table>
<thead>
<tr>
<th>Sheep #</th>
<th>Total Fat (g)</th>
<th>Total Cells (x10⁵)</th>
<th>Yield (cells x10⁵/g tissue)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SC1</td>
<td>0.881</td>
<td>0.50</td>
<td>0.57</td>
</tr>
<tr>
<td>SC2</td>
<td>0.833</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>SC3</td>
<td>1.707</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>SC4</td>
<td>1.337</td>
<td>0.30</td>
<td>0.22</td>
</tr>
</tbody>
</table>

No cells were visible in the counting field of samples from animals SC 2 and SC 3.

ASC 6: Infrapatellar (IP) and subcutaneous (SC) fat was collected from each of three sheep and processed per the method for isolation of ASC. In each of the three animals the IP fat was far less vascular and adhered to containers and tools less than SC fat. Overnight digestion of fat also differed between IP and SC groups. The IP fat seemed to have digested more completely, forming a particulate suspension with a thin layer of fat on the surface of the orange-coloured medium, indicating that the medium had become acidic. The SC fat adhered to the lid of the digestion vial in a large mass and the medium remained very pink and clear. After centrifugation at 300 x g, the SC fat rose to the surface leaving clear medium that was easily aspirated. In contrast, the IP fat sample had debris at the bottom of the tube after spinning and a fine layer of fat on the surface that was easily resuspended, transferring a
substantial amount of lipid and debris into the next step. After spinning at 600 x g, the IP suspension formed a large cell pellet with some red blood cells included and a thin layer of lipid at the surface, whereas the SC suspension formed a very small cell pellet, also with red blood cells included.

Cellular yields from each of the sheep in ASC 6 are summarised in Table 4 below. The total yield from each tissue source from each animal was split equally over 4 x T75 (75 cm²) culture flasks to the following densities:

<table>
<thead>
<tr>
<th>Sheep #</th>
<th>ASC Source</th>
<th>Total Fat (g)</th>
<th>Total Cells (x10⁵)</th>
<th>Yield (x10⁵ cells/g fat)</th>
<th>Plating Density (x10³ cells/cm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>IP</td>
<td>0.941</td>
<td>20.0</td>
<td>21.3</td>
<td>6.67</td>
</tr>
<tr>
<td></td>
<td>SC</td>
<td>0.774</td>
<td>0.10</td>
<td>0.129</td>
<td>0.033</td>
</tr>
<tr>
<td>2</td>
<td>IP</td>
<td>1.059</td>
<td>7.10</td>
<td>6.70</td>
<td>2.23</td>
</tr>
<tr>
<td></td>
<td>SC</td>
<td>1.028</td>
<td>0.40</td>
<td>0.389</td>
<td>0.133</td>
</tr>
<tr>
<td>3</td>
<td>IP</td>
<td>1.050</td>
<td>7.14</td>
<td>7.14</td>
<td>2.50</td>
</tr>
<tr>
<td></td>
<td>SC</td>
<td>1.034</td>
<td>0.50</td>
<td>0.480</td>
<td>0.167</td>
</tr>
</tbody>
</table>

Yield from IP fat is approximately an order of magnitude greater than yield from SC fat harvested at the same time from each animal.

**ASC 7:** Adipose tissue was collected from the infrapatellar fat pad of four sheep as follows: IP1= 0.366 g; IP2= 0.453 g; IP3= 0.693 g; and IP4= 0.376 g. Following digestion, there were no ASC visible in the haemocytometer counting field but many red blood cells were evident. All cultures were transferred to 75 cm² culture flasks with expansion medium.

4.1.1.2. Expansion

The data is presented the same as in section 4.1.1.1 in order to aid correlation of the two sets.

**ASC 5:** Single cells and cell clusters within primary cultures formed actively dividing colonies of various sizes. Cultures grew at different rates and were harvested at approximately 90% confluence (at day 6 for cultures SC1 and SC4 and at day 9 for cultures SC2 and SC3). Expansion results for ASC 5 are found below in Table 5. Culture SC1 yielded 5.02 x 10⁶ cells (1.11 population doublings/day) that were then passaged to 9 new flasks of 5.57 x 10⁵ cells per flask (0.74 x 10⁴ cells/cm²). Culture SC2 yielded 2.08 x 10⁶ cells that were then passaged to 9 flasks of 2.5 x 10⁵ cells per flask (0.33 x 10⁴ cells/cm²). Culture SC3 yielded 1.08 x 10⁶ cells that were then passaged to 9 flasks of 1.3 x 10⁵ cells per flask (0.17 x 10⁴ cells/cm²). Culture SC4 yielded 1.82 x 10⁶ cells (0.99 population doublings/day) that were then passaged...
to 9 flasks of 2.03 x 10^5 cells per flask (0.27 x 10^6 cells/cm^2). Culture SC1 passage 1 (P1-1) reached confluence after 6 days and yielded an average of 5.6 x 10^6 cells per flask (0.55 population doublings/day). Culture SC2 passage 1 (P1-2) was confluent after 5 days and yielded an average of 5.2 x 10^6 cells per flask (0.88 population doublings/day). Culture SC3 passage 1 (P1-3) reached confluence at day 7 and yielded an average of 9.1 x 10^6 cells per flask (0.88 population doublings/day). Culture SC4 passage 1 (P1-4) reached confluence first after 4 days of expansion and yielded an average of 5.0 x 10^6 cells per flask (1.16 population doublings/day).

**Table 5: Summary of expansion results for experiment ASC 5**

<table>
<thead>
<tr>
<th>Culture</th>
<th>Passage</th>
<th>Density (10^4 cells/cm^2)</th>
<th>Days to Confluence</th>
<th>Yield (10^6 cells/flask)</th>
<th>Doubling Rate (population doublings/day)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SC1</td>
<td>0</td>
<td>n/a</td>
<td>6</td>
<td>5.02</td>
<td>1.11</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>0.74</td>
<td>6</td>
<td>5.60</td>
<td>0.55</td>
</tr>
<tr>
<td>SC2</td>
<td>0</td>
<td>n/a</td>
<td>9</td>
<td>2.08</td>
<td>n/a^a</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>0.33</td>
<td>5</td>
<td>5.20</td>
<td>0.88</td>
</tr>
<tr>
<td>SC3</td>
<td>0</td>
<td>n/a</td>
<td>9</td>
<td>1.08</td>
<td>n/a^a</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>0.17</td>
<td>7</td>
<td>9.10</td>
<td>0.88</td>
</tr>
<tr>
<td>SC4</td>
<td>0</td>
<td>n/a</td>
<td>6</td>
<td>1.82</td>
<td>0.99</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>0.27</td>
<td>4</td>
<td>5.0</td>
<td>1.16</td>
</tr>
</tbody>
</table>

^aNo cells were visible in the counting field of primary cultures of SC 2 and SC 3 so it was not possible to calculate population doubling rate.

**ASC 6:** After 3 days of primary culture, IP samples were generally more confluent and proliferative than primary culture SC samples. This was also true of those not subjected to selective-enrichment for adherent cells (sections 2.2.2.1 and 3.1.1.1.1), even accounting for variability between animals. The IP non-enriched cultures were most proliferative and reached confluence earliest followed by the IP selectively-enriched, SC non-enriched and finally, SC selectively-enriched cultures (Figure 13). This was also evident in the yields and population doubling rates, as shown in Table 5.
Figure 13: ASC 6 primary monolayer cultures after three days. IP cultures are more proliferative with a greater degree of confluence than SC cultures and non-enriched cultures (3-day) are more established and confluent than selectively-enriched cultures (2-hour). Magnification 10X.

A summary of expansion results for experiment ASC 6 is found below in Table 6. "n" denotes “non-selective” cultures allowed to adhere for three days, whereas “s” denotes “selectively enriched” cultures washed after two hours. Animal 1 IP fat non-enrichment (IP1n) and animal 2 IP fat non-enrichment (IP2n) cultures were harvested on day 4 to yield 13.8 x 10^6 cells (0.70 population doublings/day) and 7.4 x 10^6 cells (0.85 population doublings/day), respectively. IP1s and IP3n cultures were harvested on day 5 and yielded 12.1 x 10^6 cells (0.52 population doublings/day) and 13.5 x 10^6 cells (0.55 population doublings/day), respectively. IP2s and IP3s cultures were harvested on day 6 to yield 5.85 x 10^6 cells (0.51 population doublings/day) and 7.35 x 10^6 cells (0.55 population doublings/day), respectively. On day 9, SC2n, SC2s, SC3n, and SC3s cultures were harvested to yield 6.05 x 10^6 cells (0.80 population doublings/day), 4.15 x 10^6 cells (0.74 population doublings/day), 7.05 x 10^6 cells (0.79 population doublings/day), and 5.0 x 10^6 cells (0.74 population doublings/day), respectively. On day 12, SC1n and SC1s cultures were harvested to yield 9.04 x 10^6 cells (0.54 population doublings per day) and 3.51 x 10^6 cells (0.43 population doublings/day), respectively.
Table 6: Culture expansion results for experiment ASC 6

<table>
<thead>
<tr>
<th>Culture</th>
<th>Days to Confluence</th>
<th>Starting Density (10^6 cells/flask)</th>
<th>Yield (10^6 cells/flask)</th>
<th>Doubling Rate (population doublings/day)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IP1n</td>
<td>4</td>
<td>2.00</td>
<td>13.8</td>
<td>0.70</td>
</tr>
<tr>
<td>SC1n</td>
<td>12</td>
<td>0.10</td>
<td>9.0</td>
<td>0.54</td>
</tr>
<tr>
<td>IP1s</td>
<td>5</td>
<td>2.00</td>
<td>12.1</td>
<td>0.52</td>
</tr>
<tr>
<td>SC1s</td>
<td>12</td>
<td>0.10</td>
<td>3.5</td>
<td>0.43</td>
</tr>
<tr>
<td>IP2n</td>
<td>4</td>
<td>0.71</td>
<td>7.4</td>
<td>0.85</td>
</tr>
<tr>
<td>SC2n</td>
<td>9</td>
<td>0.04</td>
<td>6.1</td>
<td>0.80</td>
</tr>
<tr>
<td>IP2s</td>
<td>6</td>
<td>0.71</td>
<td>5.9</td>
<td>0.51</td>
</tr>
<tr>
<td>SC2s</td>
<td>9</td>
<td>0.04</td>
<td>4.2</td>
<td>0.74</td>
</tr>
<tr>
<td>IP3n</td>
<td>5</td>
<td>0.75</td>
<td>13.5</td>
<td>0.83</td>
</tr>
<tr>
<td>SC3n</td>
<td>9</td>
<td>0.05</td>
<td>7.1</td>
<td>0.79</td>
</tr>
<tr>
<td>IP3s</td>
<td>6</td>
<td>0.75</td>
<td>7.4</td>
<td>0.55</td>
</tr>
<tr>
<td>SC3s</td>
<td>9</td>
<td>0.05</td>
<td>5.0</td>
<td>0.74</td>
</tr>
</tbody>
</table>

**ASC 7**: A summary of culture expansion results from ASC 7 is found below in Table 7. On day 4, primary culture of cells from animal 4 reached confluence, was devoid of red blood cells, and was harvested to yield 9.36 x 10^6 cells, which was then split over 9 new flasks of 1.04 x 10^6 cells each (1.38 x 10^4 cells/cm^2) as passage 1 (P1 - 4). On day 6, all remaining primary cultures were devoid of red blood cells and harvested to yield 1.13 x 10^6 cells (animal 1); 4.28 x 10^6 cells (animal 2); and 5.49 x 10^6 cells (animal 3). Each harvest was split over 9 new flasks of (P1 - 1) 1.25 x 10^5 (0.167 x 10^4 cells/cm^2); (P1 - 2) 4.75 x 10^5 (0.633 x 10^4 cells/cm^2); and (P1 - 3) 6.1 x 10^5 cells (0.813 x 10^4 cells/cm^2) each. After 5 days, cultures P1 - 2, P1 - 3, and P1- 4 were confluent and harvested to yield 3.2 x 10^6 (0.55 population doublings/day), 2.73 x 10^6 (0.43 population doublings/day), and 10.7 x 10^6 (0.67 population doublings/day) cells per flask, respectively. Culture P1 - 1 was confluent on day 7 and harvested to yield 1.05 x 10^6 cells per flask (0.44 population doublings/day).
### Table 7: Culture expansion results for experiment ASC 7

<table>
<thead>
<tr>
<th>Culture</th>
<th>Passage</th>
<th>Density (10^4 cells/cm²)</th>
<th>Days to Confluence</th>
<th>Yield (10^6 cells/flask)</th>
<th>Doubling Rate (population doublings/day)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IP1</td>
<td>0</td>
<td>n/a</td>
<td>6</td>
<td>1.13</td>
<td>n/a&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>0.167</td>
<td>7</td>
<td>1.05</td>
<td>0.44</td>
</tr>
<tr>
<td>IP2</td>
<td>0</td>
<td>n/a</td>
<td>6</td>
<td>4.28</td>
<td>n/a&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>0.633</td>
<td>5</td>
<td>3.2</td>
<td>0.55</td>
</tr>
<tr>
<td>IP3</td>
<td>0</td>
<td>n/a</td>
<td>6</td>
<td>5.49</td>
<td>n/a&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>0.813</td>
<td>5</td>
<td>2.73</td>
<td>0.43</td>
</tr>
<tr>
<td>IP4</td>
<td>0</td>
<td>n/a</td>
<td>4</td>
<td>9.36</td>
<td>n/a&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>1.38</td>
<td>5</td>
<td>10.7</td>
<td>0.67</td>
</tr>
</tbody>
</table>

“n/a” indicates “not applicable”. *No cells were visible in the counting field of primary cultures for any animal, making calculation of population doubling rates impossible.

4.1.1.3. Characterisation of monolayer population

Freshly plated cells isolated from both fat types were mostly small, round; enucleate, non-proliferative cells, most likely red blood cells (RBC) given their morphology, although no confirmatory analysis was performed. The other cell type looked much like a fibroblast, with spindle-shaped cell membranes, and often formed clumps. As the culture progressed, the RBC were displaced by the fibroblast-like cells, and formed a perimeter around the larger, highly proliferative fibroblast-like cells until gradually being excluded from the culture. The RBC were increasingly washed away with each medium change as they did not maintain adherence to the culture flasks.

4.1.1.3.1. Differentiation assay results

The differentiation assay kit (section 3.1.1.2, page 48) results confirmed that the protocol used to isolate mesenchymal stem cells from adipose tissue resulted in a population of cells with tri-lineage potential. Cells of the same culture, derived from macerated IP adipose isolate, were able to differentiate into adipogenic, osteogenic, and chondrogenic cells.

#### 4.1.1.3.1.1. Adipogenic

4.1.1.3.1.1.1. Monolayer culture

On day 2 of monolayer culture, adipogenic differentiation medium was added and the culture became fully confluent after 5 days. Lipid droplets were first seen after six days in adipogenic culture medium, were clearly visible after 8 days, and were abundantly visible after 11 days (Figure 14). After 14 days in adipogenic culture, cells detached easily from the coverslip.
**Figure 14**: Phase contrast images of adipogenic monolayer culture after 14 days. Lipid droplets are clearly visible and abundant. Magnifications: A – 10X; B – 20X.

4.1.1.3.1.1.2. **Fatty Acid Binding Protein - 4**

Clear increases in staining intensity and number of cells stained with an antibody against Fatty Acid Binding Protein - 4 (FABP-4) were seen between days 7, 14, 21, and 28 of adipogenic culture (Figure 15). Negative control slides were devoid of any staining at all time points.

**Figure 15**: Adipogenic monolayer cultures stained with antibody against FABP-4 at 7 days, 14 days, 21 days, and 28 days of culture. There is a clear and progressive increase in intensity and number of cells stained, indicating an increase in expression of FABP-4 in the cells and therefore differentiation toward an adipogenic phenotype. Magnifications 10X.
4.1.1.3.1.3. Oil red-O.

Similar to the progression of FABP-4 staining, clear increases in the number of lipid droplets visible by oil red-O staining were seen between all time points from day 7 through 28 days of adipogenic culture (Figure 16).

![Figure 16: Adipogenic monolayer cells after 28 days of culture Lipid droplets stained with oil red – O. Magnification 10X.](image)

4.1.1.3.1.2. Osteogenic

4.1.1.3.1.2.1. Monolayer culture

On day 2 of monolayer culture, osteogenic differentiation medium was added and the culture became fully confluent after 5 days. After nine days in osteogenic culture the cellular phenotype changed to smaller elongated cells that appeared to stack upon one another in a lattice-like fashion (Figure 17).

![Figure 17: Osteogenic monolayer culture after 14 days A dense lattice-like cellular organisation predominates with smaller more tightly packed cells stacked upon one another. Magnification (A) 10X and (B) 20X.](image)

4.1.1.3.1.2.2. Osteocalcin

Osteogenic monolayer cultures stained positively for osteocalcin after 14, 21, and 28 days in differentiation medium. Negative controls were devoid of staining at all time points. The number of cells stained and intensity of staining increased from days 14 to 28 (Figure 18). Staining first appeared at the periphery of each coverslip culture and progressed to uniformly stain all of the culture by day 28.
Figure 18: Osteogenic monolayer cultures after 14 days, 21 days, and 28 days stained with antibody for Osteocalcin. Cells at all time points were positive for osteocalcin expression. Magnifications 20X.

4.1.1.3.1.2.3. Alizarin red-s

After 28 days in osteogenic differentiation medium, osteogenic monolayer cultures stained positively for calcium with alizarin red-s (Figure 19). In contrast to osteocalcin staining, alizarin red-s stain was most intense at the centre of each coverslip, fading in intensity toward the periphery.

Figure 19: Osteogenic monolayer culture after 21 days in osteogenic differentiation medium stained with alizarin red-s for calcium deposition. Cultures stained most intensely in the centre of each coverslip, presumably where cells were most tightly packed with the greatest degree of cell stacking. Magnification 10X.
4.1.3.1.3. Chondrogenic

After 2 days in chondrogenic medium, pellets had formed and by day 5 they had grown larger and were clearly visible. By day 5 the control pellet, cultured in chondrogenic medium developed by our laboratory, was larger than the tests pellets cultured in chondrogenic medium supplied with the kit. By day 8 the control pellet was approximately twice as large as the tests pellets and approximately 3 times larger than tests pellets by day 11. After 19 days of pellet culture the test pellets appeared to stop growing, whereas the control pellet continued to grow until collection and fixation on day 21.

4.1.3.1.3.1. Aggrecan

Aggrecan staining in test pellets after 14 days in chondrogenic culture was positive and grew more intense by day 21. Staining at day 28 was also positive with no visible increase in intensity. Staining of the pellet grown in control medium, developed within our laboratory, was positive at day 21, and aggrecan appeared more widely distributed and well organised (Figure 20).

Figure 20: Test (t) and control (c) chondrogenic pellets at 14, 21, and 28 days of culture, stained with antibody against aggrecan
Staining of test pellets increased in intensity from day 14 (d14t) to day 21 (d21t), but do not appear to become more intense by day 28 (d28t). Pellet growth was also marginal between days 14 and 21, and similarly so between day 21 and 28. The control pellet (d21c), grown in chondrogenic medium developed within our lab, appears more organised, with more uniform aggrecan distribution, and several times larger than test pellets at any time point. Magnification 10X.
4.1.1.4. Enrichment (ASC 6: 2-hour vs. 3-day)

To test whether ASC populations in primary cultures could be enriched by selecting for the cells that preferentially adhered to the flasks, cultures were washed after two hours of being added to culture plates or three days after addition to culture plates. Furthermore, the International Society for Cellular Therapy specifies a preferential adherence to plastic as one of the characteristics of MSC [294], supporting the hypothesis that this method would minimise the heterogeneity of the culture resulting from my ASC isolation protocol.

Pellets grown from populations of ASC enriched by selective adhesion (2-hour) were compared with those grown from cultures using standard procedures not including enrichment (3-day).

4.1.1.4.1. Monolayer culture

The effect of enrichment on the doubling rate of ASC cultures for individual animals is presented in Figure 21. Actual values have been presented in Table 6 in section 4.1.1.2. In a per-animal comparison, washing primary culture flasks after two hours of seeding appears to have diminished the doubling rate of the resident cells in each sheep. The largest number of population doublings per day was seen in cultures of both tissue types (IP and SC fat) that were allowed to adhere to culture flasks for three days, when the first medium change was performed (Figure 21).

![Figure 21: Cell doubling rate was reduced by enrichment for ASC by selective adhesion to plastic ASC were preferentially selected for by allowing them to adhere to plastic culture flasks for either 2 hours or 3 days. Cultures allowed to adhere for 3 days doubled more times per day than cultures allowed to adhere for only 2 hours.](image)

When cultures of each type (2-hour and 3-day) were compared as a group, 3-day IP cultures were significantly more proliferative than 2-hour IP cultures (p = 0.03). 3-day SC
cultures were more proliferative than 2-hour SC cultures, but not significantly so (p = 0.055) (See Figure 22).

Figure 22: Effect of selective enrichment on proliferative ability of ASC
The enrichment of ASC by selective adhesion to culture flasks significantly reduced the population doubling rate of IP cultures. While the doubling rate of SC cultures was reduced by selective adhesion, the difference was not significant.

4.1.1.4.2. Pellet size

Enrichment for ASC by selective adhesion had no significant effect on pellet size. SC fat derived pellets ranged from 0.6 mm to 0.9 mm in diameter. The mean diameter of enriched SC derived pellets was 0.83 mm while the mean diameter of non-enriched, SC derived pellets was 0.72 mm, but the differences between the two groups of pellets did not reach statistical significance. IP fat derived pellets ranged from 1.18 mm to 1.5 mm in diameter. The mean diameter of enriched IP derived pellets was 1.27 mm while the mean diameter of non-enriched IP derived pellets was 1.31 mm and the differences between these two groups of pellets likewise did not reach statistical significance (Figure 23).
Effect of selective enrichment on pellet size

Enrichment for ASC by selective adhesion to plastic had no significant effect on pellet size. Although pellets made from IP fat were generally larger than those derived from SC fat, no significant (NS) difference in size existed between pellets derived from enriched (2-hour) and non-enriched cultures (3-day).

4.1.1.4.3. Immunochemistry

In order to compare whether enrichment for ASC had any effect on the resultant population's ability to form neocartilage pellets, monoclonal antibodies directed against chondrogenic matrix proteins were used. Pellets grown in 24 well culture plates on an orbital shaker were collected and fixed in paraformaldehyde after 14 days of chondrogenic culture. Immunostaining for the extracellular matrix components specific to the chondrogenic phenotype (type II collagen and aggrecan) as well as the pericellular differentiation marker, type VI collagen, allowed me to assess the patterns of matrix secretion and organisation discussed in Chapter 1. Pellets from each group were thus evaluated for their ability to produce a pericellular and territorial matrix rich in type II collagen, type VI collagen, and aggrecan.

4.1.1.4.3.1. Type II collagen

Qualitative analysis of immunohistochemical sections revealed patterns of staining for extracellular type II collagen that were indistinguishable between groups of enriched and non-enriched cells from both fat sources. All subsets clearly showed type II collagen, stained with a fluorescent marker conjugated to an antibody against it, filling the spaces between unstained cells (Figure 24).
4.1.1.4.3.2. Type VI collagen

Immunohistochemical staining of day 14 pellets for type VI collagen clearly displayed the pericellular localization of this collagen species in all groups. While obvious differences in matrix organisation revealed by this stain existed between sources of fat, these will be presented in section 4.1.2. No differences were seen in type VI collagen staining between enriched and non-enriched groups (Figure 25).
Figure 25: Comparison of ASC neocartilage pellets stained for type VI collagen
Staining of pellets derived from cultures enriched for ASC is indistinguishable from that of non-enriched cultures. Chondrocytes appear as unstained ovals or circles within pericellular microenvironments stained for type VI collagen at various intensities. The pattern of type VI collagen organisation is similar between enriched and non-enriched sections within each fat source subset. Magnification 40X.

4.1.1.4.3.3. Aggrecan

Aggrecan distribution in ASC-derived neocartilage pellets appeared unaffected by enrichment for ASC (Figure 26) and immunohistochemical staining for the 5D4 epitope was weak in all groups at day 14 and so weak in SC groups that photomicrographs were not possible. In positive sections, staining appeared more generalized throughout the extracellular matrix, filling the spaces not occupied by cells and collagens type II and VI (Figure 27).

Figure 26: Comparison of aggrecan distribution in day 14 pellets derived from IP fat
In both pellets, aggrecan is concentrated at the periphery of the pellet with less staining toward the centre. Distribution, organisation, and degree of staining appear similar in both groups indicating that enrichment for ASC did not have a substantial effect on the expression or distribution of aggrecan in IP-derived pellets. Magnification 10X.
Figure 27: 5D4 staining of a day 14 pellet derived from IP fat
Aggrecan distribution is generalised throughout the extracellular matrix, filling areas between adjacent cells and matrix collagens, sometimes concentrated in the pericellular microenvironment. Magnification 40X.

4.1.1.4.4. Biochemistry

Biochemical assay data for total collagen, GAG and DNA content of day 14 pellets were used to quantitatively confirm the results from immunohistochemical analysis.

4.1.1.4.4.1. Total collagen

After 14 days of chondrogenic pellet culture mean total collagen of selectively enriched (2-hour) pellets was 1.38 ± 0.30 ng/ng of DNA and of non-enriched (3-day) pellets was 1.24 ± 0.60 ng/ng of DNA (Table 8). Total collagen content of SC pellets was below the limits of detection of the assay for all samples so a statistical comparison is not possible. However, it can be argued that enrichment by adhesive selection did not result in a population of cells capable of forming pellets with enough collagen to be detectable in this assay. A student's t-test indicated that enrichment for ASC had no statistically significant effect on the total collagen content of IP pellets at day 14 (p = 0.60).
Table 8: Total collagen content of enriched (2-hour) and non-enriched (3-day) neo-cartilage pellets after 14 days of chondrogenic culture

<table>
<thead>
<tr>
<th>Group</th>
<th>Mean Collagen Content (ng/ng DNA)</th>
<th>Standard Deviation (±)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IP 2-hour</td>
<td>1.38</td>
<td>0.30</td>
</tr>
<tr>
<td>IP 3-day</td>
<td>1.24</td>
<td>0.60</td>
</tr>
<tr>
<td>SC 2-hour</td>
<td>0.00*</td>
<td>0.00</td>
</tr>
<tr>
<td>SC 3-day</td>
<td>0.00*</td>
<td>0.00</td>
</tr>
</tbody>
</table>

*Total collagen contents of all SC pellets were below the limit of detection of the assay.

4.1.1.4.4.2. GAG content

After 14 days of chondrogenic pellet culture, mean GAG content of enriched IP pellets was 28.24 ± 7.06 ng/ng DNA and mean GAG content of non-enriched IP pellets was 22.13 ± 7.92 ng/ng DNA. A student's t-test indicated that enrichment had no significant effect on GAG content of IP pellets (p = 0.47). Mean GAG content of enriched SC pellets was 6.38 ± 4.91 ng/ng DNA and mean GAG content of non-enriched SC pellets was 6.85 ± 2.65 µg/ng DNA. A student's t-test indicated that enrichment made no significant difference to the GAG content of SC pellets (p = 0.56) (Figure 28).

![Figure 28: Total GAG content of enriched (2-hour) and non-enriched (3-day) neo-cartilage pellets after 14 days of chondrogenic culture](image)

4.1.1.4.4.3. DNA content

As discussed in section 3.3.3, DNA content of pellets was used to determine cell viability following chondrogenic induction. To accomplish this, the DNA concentration in
ng/µl of pellet lysate was normalized against the number of pellets in each lysate to yield the total DNA content in each pellet, and thus, the resulting cellularity per pellet.

After 14 days of chondrogenic culture, mean DNA content of enriched IP pellets was 4.66 ± 0.96 ng/pellet and mean DNA content of non-enriched IP pellets was 8.82 ± 5.22 ng/pellet. A student's t-test indicated that enrichment had no significant effect on cellularity of IP pellets following chondrogenic induction (p = 0.40). Mean DNA content of enriched SC pellets was 1.09 ± 0.332 ng/pellet and mean DNA content of non-enriched SC pellets was 1.85 ± 0.67 ng/pellet. A student’s t-test indicated that enrichment for ASC had no significant effect on cellularity of SC pellets following chondrogenic induction (p = 0.23) (Figure 29).

![Figure 29: Cellularity of neocartilage pellets following chondrogenic induction, as assessed by DNA content of pellets, was unaffected by enrichment for ASC](image)

4.1.2. Comparison of IP ASC, SC ASC, and chondrocyte pellets

Using the same criteria by which enriched and non-enriched ASC populations were compared, IP- and SC- derived ASC were compared with each other and with chondrocytes for their ability to produce the phenotypic chondrogenic matrix molecules, collagen type II and aggrecan, as well as the pericellular matrix marker collagen type VI, in pellet culture. Two sets of data over 3 weeks of differentiation were evaluated. First, data from ASC 6 (section 4.1.1.4.) were pooled by source of fat rather than enrichment protocol and were used to evaluate chondrogenesis at two weeks of differentiation. Despite the smaller sample size, this dataset is particularly interesting because samples from different sources of fat were compared within the same animals, thus reducing inter-animal variability. Pellets at two weeks were not compared with chondrocyte derived pellets from the same animals as cartilage was not available from these animals, but pellets made from other animals were used
for comparison at the week two time point. The second data set (week one and week three samples) were analyzed as a subset of the *in vivo* study and were therefore comparisons from two distinct sets of animals by necessity. Since articular cartilage was removed from animals during defect creation, pellets were made from the chondrocytes isolated from this tissue. So, at one and three weeks, comparison of ASC-derived with chondrocyte-derived pellets from the same animals was possible.

4.1.2.1. **Pellet morphology (size)**

The size of IP and SC pellets was compared using the pellet diameter measurements presented in section 4.1.1.4.2. Pellet diameter measurements were not taken from chondrocyte-derived pellets as these measurements were taken only in experiment ASC 6. IP pellets were significantly larger than SC pellets (*p* < 0.0001) after 14 days in chondrogenic culture (Figure 30). Mean IP pellet diameter (1.30 ± 0.07 mm) was nearly twice that of mean SC pellet diameter (0.75 ± 0.09 mm).

![Mean Diameter of IP and SC pellets](image.png)

**Figure 30:** Mean diameter of IP- and SC-derived neocartilage pellets. IP pellets were nearly twice as large as SC pellets (*p* < 0.0001).

4.1.2.2. **Immunohistochemistry**

4.1.2.2.1. **Collagen type II**

Collagen Type II distribution and organisation in IP pellets was very different from that of SC pellets at all time points. IP pellets appeared to stratify into three layers or zones (Figure 31). The outer layer was typified by circumferentially aligned matrix surrounding cells made clearly visible by pericellular capsules brightly stained for collagen type II. The central zone of the pellet was typified by a diffuse matrix staining of lesser intensity than the
pericellular capsules stained in the outer layer. Between these two areas was a transition zone of circumferentially arranged fibrillar matrix stained less intensely than the outer layer matrix.

Figure 31: Day 14 IP pellet stained for collagen type II
Day 14 IP pellet stained for collagen type II at 10X magnification showing three distinct zones of organisation as highlighted in the inset at 40X magnification. The central zone (c) is comprised of densely packed rounded cells. Transition layer (t) cells appear flattened and elongated, more like fibroblasts than chondrocytes. Outer layer (o) cells become visibly rounded again, indicative of a return to the chondrocyte phenotype.

Chondrocyte pellets after two weeks of chondrogenic culture showed the highest degree of pericellular sequestration of collagen type II (Figure 32). The central zone displayed the least intense staining, mainly focused in the pericellular microenvironment. The transitional zone in chondrocyte pellets was less distinct by collagen type II staining, but appeared as a further darkening at the outer rim of the central zone. The outer layer of chondrocyte pellets stained most intensely for collagen type II, with moderate staining filling the area between rounded chondrocytes encircled by brightly stained capsules.

Collagen type II staining IP pellets at week 2 was broadly distributed throughout the pellets and was most intense in the outer layer, particularly in the pericellular region (Figure 32). The central zone of IP pellets stained uniformly for collagen type II, completely filling the spaces between rounded, unstained chondrocytes, but did not brightly stain the pericellular region. Staining in the transitional zone of these pellets was limited to circumferential fibres that appeared to stream around the centre of each pellet.

In SC pellets at week 2, very little matrix organisation was evident and collagen type II staining was weak (Figure 32). The central zone appeared devoid of collagen type II, and although collagen distribution increased toward the outer margins of the pellets, the matrix remained disorganised with only moderate staining.
Figure 32: Week two chondrocyte (C), IP, and SC pellets stained for collagen type II
A comparison of week two neocartilage pellets derived from chondrocytes (C), IP, and SC fat at 20X and 40X magnification (inset area) stained for collagen type II. Distribution of collagen is broader in C and IP pellets than SC pellets, particularly in central regions where SC pellets display limited collagen type II. While the three layers are apparent in all pellet types, collagen type II distribution in SC pellets is generally sparse and disorganised in comparison with IP pellets and chondrocyte pellets are even more organised with regard to matrix deposition and organisation. Chondrocyte pellets show the greatest degree of pericellular location and the least difference in organisation of collagen type II between zones.

4.1.2.2. Collagen type VI

Collagen type VI staining in neocartilage pellets revealed stratification of pellets into layers or zones, similar to those visualized by collagen type II staining. While the specific staining patterns of pellets differed from those of collagen type II, the organisation of the stained collagen was similar, with dense packing predominant in the central zone, giving way to circumferentially arranged collagens in the transitional zone surrounded by an outer layer of well-defined cells enclosed by brightly stained pericellular capsules (Figure 33).

After 2 weeks of culture, collagen type VI staining in chondrocyte (C) and IP pellets revealed a broad distribution and a high degree of organisation, with chondrocyte pellets showing the greatest degree of matrix organisation. Collagen type VI staining in the central zone of chondrocyte pellets was restricted to the pericellular capsules, clearly evident as
bright round rings surrounding each negatively contrasted chondrocyte. In the central zone of IP pellets, collagen type VI was more broadly distributed and appeared more fibrillar than pericellular. Staining in the transitional zones of chondrocyte and IP pellets was similarly weak, but still visible between elongated cells arranged circumferentially, the outer parts of both chondrocyte and IP pellets showing clear pericellular sequestration. By contrast, collagen type VI staining in SC pellets, although more abundant than collagen type II, showed a similar state of matrix disorganisation. While the three layers of the pellet matrix could still be seen, the distribution of collagen type VI was more fibrillar than pericellular with virtually no cellular encapsulation evident (Figure 33).
While the general organisation of the pellet into outer (o), transitional (t), and central (c) zones/layers was evident in each cell source group, the differences in distribution and organisation of collagen type VI were distinct and clearly visible. The degree of organisation and pericellular distribution of collagen type VI was greatest in chondrocyte pellets, moderate in IP pellets, and least in SC pellets, which lacked any real organisation or regular distribution of collagen type VI. 4X Magnification (C); 10X Magnification (IP and SC); 20X Magnification insets.

4.1.2.2.3. Aggrecan

Aggrecan distribution and organisation in neocartilage pellets after 2 weeks of chondrogenesis, as assessed by staining for the 5D4 epitope, varied between pellet types (Figure 34). Chondrocyte derived pellets had the broadest distribution of aggrecan, organised to uniformly fill the spaces between chondrocytes. Although the outer transitional and central zones were still visible by the orientation and morphology of the resident cells, there was only a slight difference in staining between zones. The brightest staining was seen around the pericellular capsule of some cells. Aggrecan distribution in week two IP pellets was
concentrated mostly in the outer layer with less staining evident in the central and transitional zones, which were still recognizable by the morphology and orientation of resident cells. Organisation of aggrecan in all layers of IP pellets appeared less complete than chondrocyte pellets to form an integrated meshwork around the cells. Distribution of aggrecan in SC pellets was very sparse at two weeks with the brightest staining in the central region. There was little organisation of matrix in these pellets but differences in collagen organisation made the different layers or zones visible (Figure 34).

Figure 34: Week two chondrocyte (C), IP, and SC pellets stained for aggrecan epitope 5D4
After two weeks of chondrogenic culture, the central (c), transitional (t), and outer (o) zones are visible but less distinct than pellets stained for collagen type II and collagen type VI. Chondrocyte pellets showed the broadest distribution of aggrecan, followed by IP pellets. SC pellets showed very little aggrecan distribution and even less matrix organisation. Aggrecan distribution in chondrocyte pellets was generally uniform and well organised to fill the spaces between negatively contrasted chondrocytes, with slightly greater intensity immediately pericellularly. Aggrecan distribution in IP pellets was most intense in the outer layer and organised into a meshwork that appeared to flow between cells.
4.1.2.3. Biochemistry

Biochemical assays were used to quantitatively compare the extracellular matrix production capability of each cell type (Table 9). At weeks one and three, total collagen, GAG, and DNA were measured to compare IP, SC, and chondrocyte pellets from the *in vivo* experiments, ASC 5 and ASC 7. Therefore, data from four animals in the IP group were compared with data from four different animals in the SC group, and each of these were compared with data from pellets derived from chondrocytes taken from all eight animals as well as chondrocyte pellets from within the same experiments. At the two-week time point, total collagen, GAG, and DNA data from IP pellets were compared with data from SC pellets made from the same three animals in the *in vitro* experiment (ASC 6). Total collagen, GAG, and DNA content were also evaluated over time within each cell source group.

Table 9: Experimental design of biochemistry assessment of chondrocyte (C), IP, and SC pellets

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Number of Animals</th>
<th>Cell Source</th>
<th>Analysis</th>
<th>Time Point (Weeks)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ASC 6</td>
<td>3</td>
<td>IP</td>
<td>TC, GAG, &amp; DNA</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>SC</td>
<td>TC, GAG, &amp; DNA</td>
<td>2</td>
</tr>
<tr>
<td>ASC 5</td>
<td>4</td>
<td>C</td>
<td>TC, GAG, &amp; DNA</td>
<td>1 &amp; 3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>SC</td>
<td>TC, GAG, &amp; DNA</td>
<td>1 &amp; 3</td>
</tr>
<tr>
<td>ASC 7</td>
<td>4</td>
<td>C</td>
<td>TC, GAG, &amp; DNA</td>
<td>1 &amp; 3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>IP</td>
<td>TC, GAG, &amp; DNA</td>
<td>1 &amp; 3</td>
</tr>
</tbody>
</table>

Total collagen (TC), GAG, and DNA content of pellets were analyzed after one, two, and three weeks of chondrogenic culture as explained in the text.
4.1.2.3.1. Total collagen

At week one, mean total collagen content of chondrocyte pellets pooled from all animals in experiments ASC 5 and ASC 7 was significantly greater (p < 0.05) than the mean total collagen content of IP pellets (Figure 35). Within experiment ASC 7, week one chondrocyte pellets had significantly more total collagen than IP pellets from the same animals (p = 0.05).

At week three, mean total collagen content of chondrocyte pellets pooled from all animals in experiments ASC 5 and ASC 7 was significantly greater (p < 0.001) than the mean total collagen content of IP pellets (Figure 35). Within experiment ASC 7, week three chondrocyte pellets had significantly more total collagen than IP pellets from the same animals (p < 0.05). Total collagen from SC pellets was below the lower limit of detection of the assay at both weeks one and three.

![Figure 35: Mean total collagen content of IP, and chondrocyte pellets at weeks one and three](image)

Chondrocyte pellets pooled from experiments ASC 5 and ASC 7 contained more collagen than IP (ASC 7) pellets at both time points, the difference being significant at week one (p < 0.05) and very significant at week three (p < 0.001).

In ASC 6, the mean total collagen content of IP pellets at week two was 1.31 ± 0.53 ng/ng DNA and the total collagen content of SC pellets was below the lower limit of detection of the assay.

The mean total collagen content of pooled week three chondrocyte pellets was significantly greater than the mean total collagen content of pooled chondrocyte pellets from week one (p < 0.001). Collagen content of chondrocyte pellets from sheep in experiment ASC 5 increased significantly from week 1 to week 3 (p < 0.01), and an increase in collagen
content of pellets from ASC 7 sheep was not significant from week one to week three (Table 10).

Table 10: Mean total collagen content of chondrocyte pellets

<table>
<thead>
<tr>
<th></th>
<th>Mean Total Collagen Content of Chondrocyte Pellets (ng/ng DNA)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>WEEK ONE</td>
</tr>
<tr>
<td>ASC 5</td>
<td>1.98 ± 0.43</td>
</tr>
<tr>
<td>ASC 7</td>
<td>2.50 ± 1.29</td>
</tr>
<tr>
<td>ASC 5 + ASC 7</td>
<td>2.24 ± 1.00</td>
</tr>
</tbody>
</table>

Displayed as pellets from each experiment as well as pooled from both experiments at weeks one and three

IP pellets contained more total collagen at week three than IP pellets from the same animals at week one, but the difference was not significant (p = 0.29). IP pellets from different animals (experiment ASC 6) contained more total collagen than either the week one or week three pellets, highlighting the issue of inter-animal variation (Table 11).

Table 11: Mean total collagen content of IP pellets from weeks one through three

<table>
<thead>
<tr>
<th>Week 1 (ASC 7)</th>
<th>Week 2 (ASC 6)</th>
<th>Week 3 (ASC 7)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.12 ± 0.12*</td>
<td>1.31 ± 0.53</td>
<td>0.31 ± 0.31*</td>
</tr>
</tbody>
</table>

(*p = 0.29)

4.1.2.3.2. GAG content

As shown in Table 12, at week one mean GAG content of chondrocyte pellets pooled from experiments ASC 5 and ASC 7 was significantly greater than the mean GAG content of IP pellets (p = 0.01) and of SC pellets (p < 0.01). When compared within the same animals, chondrocyte pellets contained more GAG than IP pellets, but this was not significant (p = 0.32), while chondrocyte pellets from experiment ASC 5 contained significantly more GAG than SC pellets from the same animals (p < 0.01). IP pellets contained significantly more GAG than SC pellets (p < 0.05) at week one (see also Figure 37).
### Table 12: Mean total GAG content of chondrocyte (C), IP, and SC pellets at week one

<table>
<thead>
<tr>
<th>Source</th>
<th>ASC5 Total GAG (ng/ng DNA)</th>
<th>ASC7 Total GAG (ng/ng DNA)</th>
<th>ASC5 + ASC7 Total GAG (ng/ng DNA)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td>107.46 ± 19.99 †</td>
<td>34.88 ± 34.88 †</td>
<td>71.17 ± 49.85 † ‡</td>
</tr>
<tr>
<td>IP</td>
<td>NA</td>
<td>10.23 ± 2.40 ‡</td>
<td>NA</td>
</tr>
<tr>
<td>SC</td>
<td>4.61 ± 2.12 †</td>
<td>NA</td>
<td>NA</td>
</tr>
</tbody>
</table>

(*p = 0.01; †p < 0.01; ‡p = 0.32; ‡p < 0.01; ‡p < 0.05)

At week two, IP pellets (25.19 ± 8.87 ng/ng DNA) contained significantly more GAG than SC pellets of the same animals in experiment ASC6 (6.61 ± 4.33 ng/ng DNA, p < 0.01) (Table 15).

As shown in Table 13, at week three the mean GAG content of chondrocyte pellets pooled from experiments ASC5 and ASC7 was significantly greater than the mean GAG content of (ASC7) IP pellets (p < 0.05) and (ASC5) SC pellets (p < 0.05). The mean GAG content of chondrocyte pellets from experiment ASC7 was significantly greater than that of IP pellets from the same animals (p < 0.05). The mean GAG content of chondrocyte pellets from experiment ASC5 was significantly greater than that of SC pellets from the same animals (p < 0.01). While IP pellets from sheep in ASC7 at week three contained more than six times the GAG of week-three SC pellets from sheep in ASC5 the difference was not significant (see also Figure 37).

### Table 13: Mean total GAG content of chondrocyte (C), IP, and SC pellets at week three

<table>
<thead>
<tr>
<th>Source</th>
<th>ASC5 Total GAG (ng/ng DNA)</th>
<th>ASC7 Total GAG (ng/ng DNA)</th>
<th>ASC5 + ASC7 Total GAG (ng/ng DNA)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td>349.55 ± 102.32 †</td>
<td>56.69 ± 2.12 †</td>
<td>203.12 ± 174.62 ‡</td>
</tr>
<tr>
<td>IP</td>
<td>NA</td>
<td>19.98 ± 19.55 ‡</td>
<td>NA</td>
</tr>
<tr>
<td>SC</td>
<td>3.27 ± 2.11 †</td>
<td>NA</td>
<td>NA</td>
</tr>
</tbody>
</table>

(*p < 0.05; †p < 0.05; ‡p < 0.05; ‡p < 0.01; ‡p = 0.17)
As shown in Table 14, the mean GAG content of chondrocyte pellets pooled from experiments ASC5 and ASC7 at week three was significantly greater than the mean GAG content of pooled chondrocyte pellets from week one (p < 0.05), as was the difference between weeks one and three in experiment ASC5 (p < 0.05). While GAG content of chondrocyte pellets in ASC7 increased from week one to week three, the difference was not significant (p = 0.37).

Table 14: Mean total GAG content of chondrocyte pellets

<table>
<thead>
<tr>
<th>Mean Total GAG Content of Chondrocyte Pellets (ng/ng DNA)</th>
<th>WEEK 1</th>
<th>WEEK 3</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>ASC5</td>
<td>107.46 ± 19.99</td>
<td>349.55 ± 102.32</td>
<td>&lt; 0.05</td>
</tr>
<tr>
<td>ASC7</td>
<td>34.88 ± 34.88</td>
<td>56.69 ± 2.12</td>
<td>= 0.37</td>
</tr>
<tr>
<td>ASC5 + ASC7</td>
<td>71.17 ± 49.85</td>
<td>203.12 ± 174.62</td>
<td>&lt; 0.05</td>
</tr>
</tbody>
</table>

Mean total GAG content of chondrocyte pellets from ASC7, ASC5, and pooled from both experiments at weeks one and three. GAG content increased significantly in pooled samples and in ASC5, but not significantly in ASC7.

While week-three IP pellets contained nearly twice the GAG of week one IP pellets from the same animals and slightly less than the GAG content of week-two IP pellets from sheep in ASC6, neither of these differences were significant. However, week two IP pellets from ASC6 sheep contained significantly more GAG than week one IP pellets from ASC7 sheep (p < 0.01), again highlighting the issue of inter-animal variation (Table 15).

Table 15: Mean total GAG content of IP pellets from weeks one through three

<table>
<thead>
<tr>
<th>Mean Total GAG Content of IP Pellets (ng/ng DNA)</th>
<th>Week 1 (ASC7)</th>
<th>Week 2 (ASC6)</th>
<th>Week 3 (ASC7)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>10.23 ± 2.40*</td>
<td>25.19 ± 8.87#</td>
<td>19.98 ± 19.55#</td>
</tr>
</tbody>
</table>

( * p = 0.34; # p = 0.68; $ p < 0.01)

The mean GAG content of SC pellets at week three was less than that of SC pellets from the same animals at week one and approximately half the mean GAG content of SC pellets from different animals (ASC6 sheep) at week two, although neither of these differences was significant. While the GAG content of week two pellets from ASC6 sheep was greater than that of week one pellets from ASC5 sheep, the difference was not significant (Table 16).
Table 16: Mean total GAG content of SC pellets from weeks one through three

<table>
<thead>
<tr>
<th>Week 1 (ASC5)</th>
<th>Week 2 (ASC6)</th>
<th>Week 3 (ASC5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.61 ± 2.12$^*$</td>
<td>6.61 ± 4.33$^*$</td>
<td>3.27 ± 2.11$^*$#</td>
</tr>
</tbody>
</table>

($p = 0.38; ^*p = 0.20; ^# p = 0.38$)

Figure 36: Mean GAG content of IP and SC pellets from the same animals at week two (day 14). IP pellets contain significantly more GAG than SC pellets from the same animals ($p < 0.01$).

Figure 37: Mean GAG content of chondrocyte, IP, and SC pellets from different animals at weeks one and three. Chondrocyte derived pellets pooled from experiments ASC 5 and ASC 7 contain significantly more GAG than either IP (ASC 7) or SC pellets (ASC 5) at both time points ($p \leq 0.01$). IP pellets contain more GAG than SC pellets at both time points, but was only significant at week one ($p < 0.05$).
4.1.2.3.3. DNA content

As seen in Table 17 and Figure 38, after one week in chondrogenic culture, pellets from all sources contained approximately the same number of cells, with the exception of the week one SC sample from animal 4. Table 18 illustrates that IP pellets contained significantly more DNA than SC pellets from the same sheep at week two. As seen in Table 19 and Figure 38, the DNA content of pellets from experiment ASC 7 were not significantly different and beset by a similar variability as the other experiments.

Table 17: DNA content of C and SC pellets from experiment ASC 5 at weeks one and three

<table>
<thead>
<tr>
<th>ASC 5</th>
<th>ng DNA/ Pellet</th>
<th>ng DNA/ Pellet</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Week 1</td>
<td>Week 3</td>
</tr>
<tr>
<td>Sheep #</td>
<td>SC</td>
<td>C</td>
</tr>
<tr>
<td>1</td>
<td>3.98</td>
<td>3.31</td>
</tr>
<tr>
<td>2</td>
<td>1.98</td>
<td>3.33</td>
</tr>
<tr>
<td>3</td>
<td>4.68</td>
<td>2.86</td>
</tr>
<tr>
<td>4</td>
<td>14.03</td>
<td>3.89</td>
</tr>
<tr>
<td>MEAN</td>
<td>6.16 ± 5.37</td>
<td>3.35 ± 0.42</td>
</tr>
</tbody>
</table>

NA = No SC pellet sample was available from animal 2 at the three-week time point. The result of the week one SC pellet sample from animal 4 appears to be out of the range of the other sample data and increases both the mean and the standard deviation for that group.
Figure 38: DNA content of pellets at weeks one and three
Cellularity of chondrocyte (C), IP, and SC pellets at weeks 1 and 3 of chondrogenic culture, as measured by the DNA content. No significant differences between groups existed at either time point, although SC and C pellets trended downward at week 3 while IP pellets maintained their DNA content, although above-range outlier data at week 1 for C and SC pellets may account for this. Chondrocyte values were pooled from ASC 5 and ASC 7 pellets.

Table 18: DNA content of IP and SC pellets from experiment ASC 6 at week two

<table>
<thead>
<tr>
<th>ASC 6</th>
<th>Week 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sheep #</td>
<td>IP</td>
</tr>
<tr>
<td>1</td>
<td>4.58</td>
</tr>
<tr>
<td>2</td>
<td>10.31*</td>
</tr>
<tr>
<td>3</td>
<td>5.33</td>
</tr>
<tr>
<td>MEAN</td>
<td>6.74 ± 3.12*</td>
</tr>
</tbody>
</table>

*The IP sample from sheep 2 appears to be out of the range of other IP samples from the other two animals and accounts for an increase in both the group mean and the standard deviation. *IP pellets contain significantly more cells than SC pellets (p < 0.05).
Table 19: DNA content of C and IP pellets from experiment ASC 7 at weeks one and three

<table>
<thead>
<tr>
<th>ASC 7</th>
<th>ng DNA/ Pellet Week 1</th>
<th>ng DNA/ Pellet Week 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sheep #</td>
<td>IP</td>
<td>C</td>
</tr>
<tr>
<td>1</td>
<td>7.05</td>
<td>2.66</td>
</tr>
<tr>
<td>2</td>
<td>3.20</td>
<td>2.81</td>
</tr>
<tr>
<td>3</td>
<td>12.05</td>
<td>24.17</td>
</tr>
<tr>
<td>4</td>
<td>4.60</td>
<td>4.41</td>
</tr>
<tr>
<td>MEAN</td>
<td>6.73 ± 3.89</td>
<td>8.51 ± 10.47</td>
</tr>
</tbody>
</table>

The result of the week one C pellet sample from animal 3 appears to be out of range from the other sample data and increases both the mean and the standard deviation for that group. The week one result of the IP pellet from animal 3 is similarly higher than the other animals, and contributes to the increase in mean and variability of that group, but not to the same degree as the C sample.

4.2. *In vivo* Testing

When referring to the subject animals in this section, animal number from 1 through 4 will be preceded by either an "SC" to designate SC group animals from experiment ASC 5 or an "IP" to designate IP group animals from experiment ASC 7. Therefore, animal 1 from experiment ASC 5 will be referred to as animal "SC 1".

4.2.1. Observations

4.2.1.1. Surgical

Unless otherwise noted, all surgical procedures were performed as per the method presented in section 3.4. No complications arose pre- or post-operatively.

4.2.1.1.1. Fat harvest

Fat harvest data are presented below in

**Sheep SC 1** -- 0.88 g of subcutaneous fat was excised from a layer just below the skin. The harvested adipose tissue was very vascular.

**Sheep SC 2** -- 0.83 g of subcutaneous fat was excised from a layer just below the skin. The harvested adipose tissue was very vascular.

**Sheep SC 3** -- 1.71 g of subcutaneous fat was excised from a deeper layer of fat than animals SC 1 and SC 2. The harvested adipose tissue was less vascular than the tissue harvested from animals SC 1 and SC 2.

**Sheep SC 4** -- 1.34 g of subcutaneous fat was excised from a deeper layer of fat than animals SC 1 and SC 2, similar to sheep SC 3. The harvested adipose tissue was less vascular than the tissue harvested from animal SC 1 and SC 2, also similar to sheep SC 3.
Sheep IP 1 -- 0.37 g of infrapatellar fat was excised from the central portion of the fat pad, without exposing the articular surface. Adipose tissue was excised in one large piece and included the overlying membrane.

Sheep IP 2 -- 0.45 g of infrapatellar fat was excised from the central portion of the fat pad without exposing the articular surface. The adipose tissue was excised in one large piece and included the overlying membrane.

Sheep IP 3 -- 0.69 g of infrapatellar fat was excised from the central portion of the fat pad without exposing the articular surface. The adipose tissue was excised in one large piece and included the overlying membrane (Figure 39).

Sheep IP 4 -- 0.38 g of infrapatellar fat was excised from the central portion of the fat pad without exposing the articular surface. The fat pad of sheep IP 4 was less substantial than the other animals and was excised in small pieces.
4.2.1.1.2. Defect creation/ implantation

Cartilage from 8mm diameter full-thickness defects weighed between 44 mg and 70 mg.

Sheep SC 1 -- Two defects were created on the thickest part of the exposed condyle, each equidistant from the crown. The drill bit slipped out of the lateral defect during reaming and created a shallow furrow, approximately 6 mm long and 3 mm wide, lateral to the defect (Figure 40). The neocartilage constructs available for implantation consisted of pellet clumps of various sizes (Figure 41). These clumps, when placed into the medial defect, filled the lesion to approximately 70% full with the remainder of the lesion filled with Tisseel. The control lesion (lateral defect) was completely filled with Tisseel. The 27gauge needle used to apply the Tisseel was too fine to allow the viscous fraction of the Tisseel to flow as readily and equally with the liquid fraction. As a result, the liquid fraction flowed faster from the syringe than the viscous fraction, filling the defect with Tisseel that appeared too thin and took longer than normal to set.

Figure 40: Surgically created defects in sheep SC 1
Test (medial) and control (lateral) defects in the articular cartilage of sheep SC 1. The furrow created by the drill during reaming is visible radiating down and left from the control defect (arrow).

Figure 41: Neocartilage pellets implanted into animal SC 1.
Neocartilage constructs available for implantation into animal SC 1 consisted of several pellet clumps of various sizes.
Sheep SC 2 -- Two defects were created on the thickest part of the exposed condyle, each equidistant from the crown. The neocartilage constructs available for implantation consisted of 2 similar-sized pellet clumps that, when implanted side-by-side within the medial defect, filled approximately half of the lesion (Figure 42). The remainder of the test lesion, and the entire control lesion (lateral defect), were filled with Tisseel administered through a 21 gauge needle that produced good mixing of the two fractions that resulted in a properly viscous material that set appropriately and formed a good seal. Upon leg extension the Tisseel came out of the control lesion and had to be replaced. The replacement Tisseel stayed within the control defect upon extension the second time.

Figure 42: Test lesion in animal SC 2
Test (medial) lesion in animal SC 2 filled with neocartilage implants before (left) and after (right) administration of Tisseel. Constructs available for implantation into this sheep's defect consisted of 2 similar sized and shaped pellet clumps that filled approximately half of the lesion when implanted.
Sheep SC 3 -- Two defects were created on the thickest part of the exposed condyle, each equidistant from the crown (Figure 43). The cartilage over the lateral lesion felt and sounded thicker when delineating margins with the biopsy punch. The edges of the lateral lesion were less clean than those of the medial lesion and needed to be trimmed with a scalpel. The neocartilage constructs available for implantation consisted of one very large pellet clump that nearly filled the entire defect by itself and a second, smaller, pellet clump that completed filling of the defect once implanted. The implants were sealed over with Tisseel administered through a 21 gauge needle that mixed appropriately and set properly to form a good seal over the lesion. Tisseel was also used to completely fill the control lesion, but dislodged during joint extension. Tisseel was replaced into the control lesion and remained in place during joint extension the second time.

Figure 43: Defects in animal SC 3
Test (medial) and control (lateral) defects before (left) and after (right) implantation with neocartilage pellets.
Sheep SC 4 -- When entering the joint capsule a great deal of fat obscured the view of the condyle and a fair amount was removed to improve visualisation of the articular surface. Two defects were created on the thickest part of the exposed condyle, the test (medial) defect directly over the crown and the control defect approximately 5 mm lateral to that, and appeared to be seated in equal thicknesses of cartilage. The pellets to be implanted were not clumped and filled the medial defect entirely (Figure 44). A 27 gauge needle was also used to administer Tisseel into the test and control lesions of this animal, and the result was the same as in sheep SC 1, namely a thin very liquid Tisseel that took longer than normal to set and did not cure appropriately. The Tisseel was removed with minimal loss of pellets, and replaced to satisfaction.

Figure 44: Defects created in the articular cartilage of animal SC 4
Neocartilage pellets available for implantation completely filled the test (medial) defect. Tisseel was thin and less viscous than was appropriate, requiring re-administration.
Sheep IP 1 -- The condyle was exposed and the joint flexed to reveal the broadest surface of the condyle normally hidden by the meniscus. Two defects were created on the surface approximately 2 mm proximal to the meniscus and approximately 5 mm apart. Neocartilage pellets available for implantation were not clumped and were placed into the lateral lesion and Tisseel was added to both lesions with a 21 gauge needle, mixing appropriately and setting properly (Figure 45). The meniscus was replaced over the lesions as the joint was extended and a few pellets squeezed out from under the Tisseel.

Figure 45: Defects created in the articular cartilage of sheep IP 1 Test (lateral) and control (medial) lesions before (left photo) and after (right photo) implantation with neocartilage pellets. Both defects have been sealed over with Tisseel prior to closure.
Sheep IP 2 -- The condyle was exposed and the joint flexed to reveal the broadest surface of the condyle normally hidden by the meniscus. Two defects were created on the surface approximately 2 mm proximal to the meniscus and approximately 5 mm apart. Neocartilage pellets available for implantation were not clumped and were placed into the medial lesion and Tisseel was added to both lesions with a 21 gauge needle, mixing appropriately and setting properly (Figure 46). All implanted pellets stayed in place as the meniscus was replaced over the lesions and the joint was extended.

Figure 46: Defects in the articular cartilage of sheep IP 2
Test (left) and control (right) lesions before (upper left photo), during (upper right photo), and after (bottom left photo) implantation with neocartilage pellets. Both lesions were sealed with Tisseel prior to leg extension.
Sheep IP 3 -- upon exposure of the capsule there was an inordinate amount of fat obscuring the articular surface and it was realised that the incision was made far too laterally. A second subcuticular approach was opened 30 mm medial to the original incision and the resulting exposure was very good, providing clear visualisation of the broadest surface of the condyle normally hidden by the meniscus. The two lesions were located 5 mm to either side of the crown of the condyle. While removing cartilage from the medial lesion the curette slipped across the surface and scored the condyle distal to the lesion (Figure 47). Neocartilage pellets available for implantation were not clumped and were placed into the lateral lesion and Tisseel was added to both lesions with a 21 gauge needle, mixing appropriately and setting properly. All implanted pellets stayed in place as the meniscus was replaced over the lesions and the joint was extended.

Figure 47: Implantation of neocartilage pellets into defects in animal IP 3
Neocartilage pellets (Upper left photo) were implanted into the lateral (left) defect in sheep IP 3. A cut, caused by the cartilage removal curette, is visible in the articular cartilage between defects (arrows). Lesions before (lower left photo) and after (lower right photo) implantation and before administration of Tisseel.
Sheep IP 4 -- The exposure of the condyle was good, but the broadest part of the condyle surface was difficult to visualize because of joint tightness. Locations for the lesions were selected with the control lesion on top of the most distal section of the trochlear ridge and the test lesion 5 mm medial to that. As the joint remained flexed, laxity improved until better exposure of the target surface was achieved. During cartilage removal with a curette it was noted that the cartilage layer over the control lesion was very thin. During lesion reaming it was noted that the bone beneath the test lesion was harder and bled less than the control or previous animals’ lesions. Neocartilage pellets available for implantation were not clumped and were placed into the test lesion and Tisseel was added to both lesions with a 21gauge needle, mixing appropriately and setting properly (Figure 48). All implanted pellets stayed in place as the meniscus was replaced over the lesions and the joint was extended.

In general, animals recovered well from surgery without complications. All sheep resumed pre-surgery appetite levels within the first post-operative week. Acclimation to slings and external limiters was accomplished smoothly with all sheep appearing relaxed and displaying no signs of stress. When animals were placed in deep litter pens, prior to return to
the holding farm, gait and posture were assessed. In all cases, sheep were fully weight bearing before the end of the week in deep-litter holding pens with no observed alteration of gait.
4.2.1.2. Gross necropsy

4.2.1.2.1. Four-week recovery

Sheep SC 1 -- Upon exposure the joint was found in very good condition. There was some soft tissue scaring below the incision, as expected, but no arthrofibrosis. The joint surfaces were in very good condition except for the furrow created beside the control (medial) lesion during the defect creation procedure, which was clearly evident. Neocartilage implants were still within the defects after 4 weeks of healing (Figure 49). The implants do not appear to have changed over the recovery period, neither in size nor appearance.

Figure 49: Gross necropsy of Sheep SC 1 at four weeks
Test (t) and control (c) lesions (left photo) at four weeks post-implantation. Little healing is evident and pellets are still visible in the test lesion (right photo) as seen in cross section.
Sheep SC 4 -- The exposed joint looked in very good condition. There was little to no neovascularisation or arthrofibrosis and there was ample volume of synovial fluid that was clear with good string. Both lesions were concave with red centres, but filled completely. Neocartilage implants were still within the test defect after 4 weeks of recovery (Figure 50). The implants do not appear to have changed over the recovery period, neither in size nor appearance.

Figure 50: Gross necropsy of sheep SC 4 at four weeks
Little difference was seen between test (t) and control (c) lesions and little healing is evident in the defects (top photo). Lesions remained mostly filled with a translucent material. Pellets were still visible within the test defect in cross-
Sheep IP 2 -- Grossly, the joint looked healthy with no apparent neovascularisation except for a small amount in the infrapatellar fat pad directly over the lesion. Synovial fluid stream was good and the colour was clear. The test lesion was moderately concave and blood tinged with obvious margins. Upon dissection of the lesion, pellet implants were distinct and clearly visible in the lesion, and did not appear to have grown significantly in situ or integrate well with host tissue (Figure 51). The control lesion was less concave than the test lesion, coloured similarly to surrounding cartilage and uniform in appearance. The access wound looked good and healing appeared complete. No sign of infection or any other pathology was present.

Figure 51: Gross necropsy of sheep IP 2 at 4 weeks
After four weeks of recovery, defects were mostly filled but little healing was evident. Pellets were visible within the test (t) (medial) defect on gross examination.
Sheep IP 3 -- Upon exposure of the joint there was a fair amount of blood-tinged synovial fluid visible. The synovial fluid, while bloody, had good string. The joint looked generally healthy with no apparent neovascularisation apart from in the fat pad directly over the lesions. The test lesion was extremely concave and dark with blood. The lesion margins were distinct and sharp. The implanted pellets were not visible until the lesion was dissected. Dissection of the lesion revealed large, distinct pellets melding with the base of the defect, giving way to more amorphous, fully melded beads at the lesion surface. The control lesion was adjacent to the lateral margin of the condyle with the lateral margin of the lesion extending beyond the margin of the articular surface. The control lesion was completely filled with a uniformly neutral coloured material, and the lesion surface was slightly concave (Figure 52). The access wound looked completely healed with no obvious pathology.

Figure 52: Gross necropsy of animal IP 3
After four weeks of recovery, the control (c) (right) defect was mostly filled with a translucent material while the test (t) defect was mostly filled with a red-tinged material (top photo). Pellets were still visible in the test lesion in cross section (bottom photo).
4.2.1.2.2. Twelve-week recovery

**Sheep SC 2** -- The joint was in good general condition with good volume of synovial fluid that was clear with good string. There appeared to be very little difference between test and control lesions. Both were irregularly shaped with red centres and white translucent margins. New tissue appears to have grown inward from the lesion margins, softening them over the recovery period (Figure 53). Neocartilage implants did not appear to be in the test lesion and a small white nodule was removed from the fat overlaying the lesion.

![Image of sheep joint](image)

**Figure 53:** Gross necropsy of sheep SC 2 after 12 weeks of recovery
There is little difference between test (t) and control (c) lesions as both are partially healed and mostly filled with a red-tinged translucent material.
**Sheep SC 3** -- Upon exposure the joint appeared in good general condition with clean articular surfaces and ample volume of synovial fluid with good string. The test lesion looked very good. The repair tissue was uniformly coloured with good integration between the margins and surrounding tissue, forming a congruent surface. The implant plug appeared to extend down well into subchondral bone. The control lesion had a deep red centre with white translucent margins. Repair tissue appeared to have grown inward from the margins (Figure 54).

![Gross necropsy of sheep SC 3 after twelve weeks of recovery](image)

**Figure 54**: Gross necropsy of sheep SC 3 after twelve weeks of recovery
The test (t) (medial) lesion has healed well and filled consistently, integrating well with host tissue at the wound margins. The test lesion contains a concave and red centre, but otherwise healed fairly well with repair tissue appearing to have grown in from the margins.
Sheep IP 1 -- Joint condition was excellent, containing ample synovial fluid with good string. A secondary lesion, immediately medial to the control (medial) lesion, was evident and appeared to have been formed by the patella running over that part of the condyle. Upon inspection of the patella, a fibrous/bony ridge on the medial aspect was observed. The test lesion appeared as a deep red cavity with no evidence of implants or repair tissue grossly or in bisect following dissection. By contrast, the control lesion had healed to a nearly perfect congruent surface that was completely covered with repair tissue well integrated with surrounding cartilage (Figure 55). Upon dissection of the lesions, the bisect view of the control lesion showed an organised cartilage layer overlying the reformed bone that had integrated ideally with existing bone (Figure 56). The soft tissue of the surgical access wound looked completely healed with no obvious pathology.
Figure 56: Bisect view of test and control lesions from sheep IP 1 after 12 weeks of recovery
The test (left) lesion is unhealed and appears as a large red cavity. By contrast, the control (right) lesion is completely healed with an organised cartilage layer over newly formed bone integrated well with underlying host tissue.

**Sheep IP 4** -- A fibrous protrusion was observed upon external palpation of the medial aspect of the knee that, after dissection, appeared localized to the soft tissue overlying the medial condyle and just below the skin. While the knee flexed and extended fully, it did so at an externally rotated angle. The joint looked generally healthy and contained a large volume of synovial fluid with good string. A lesion was observed on the proximal patella that, due to the abnormal external rotation, corresponded with a longitudinal fissure-type lesion in the control defect that appeared to contain some infectious-looking material (Figure 57). Otherwise, the control lesion was repaired well at the margins. The test lesion was unhealed with little or no repair tissue evident. When viewed in bisect, pellet remnants were still visible within the depth of test defect. The bisected control lesion appeared to be mostly healed at the margins but an abscess at the centre of the lesion appeared to have formed (Figure 58). The soft tissue of the surgical access wound looked completely healed with no obvious pathology.

Figure 57: Gross necropsy of sheep IP 4 after 12 weeks of recovery
Apart from a secondary lesion (arrow) caused by abnormal tracking of the patella across the lateral surface, the control lesion (c) (lateral) appears partially healed, albeit with a deep red fissure in the centre of the lesion. By contrast the test lesion (t) appears unhealed.
Figure 58: Bisected test and control lesions of sheep IP 4
Pellets (arrows) are still visible within the test (left) lesion, which is unhealed. The control (right) lesion appears to be unhealed around an abscess at the centre of the defect. The margins are healed well and integrated nicely with surrounding cartilage.

4.2.2. Histopathology

4.2.2.1. Four-week recovery

Sheep SC 1 – Four weeks following surgery, the control defect of sheep SC 1 was characterised by the absence of replacement of the full cartilage thickness. Chondron clusters formed within the native cartilage at the defect margins, indicative of the repair process. Mallory's trichome staining revealed that the repair matrix contained a combination of stromal cells, erythrocytes, acellular Tisseel fragments, and thin crimped bands of collagen (Figure 59). Collagen type II was most concentrated at the defect margins and at the articulating surface of the repair (Figure 60). Collagen type VI was more generally distributed throughout the repair matrix, most concentrated at the interface between the defect and host matrix (Figure 61). There was a weak, generalised distribution of aggrecan throughout the repair tissue with no apparent organisation (Figure 62).

Similar to the control defect, the test defect lacked congruency of the articulating surface with a failure to re-create the hyaline structure of the native cartilage. Mallory's trichrome stain revealed a repair matrix composed of stromal cells, erythrocytes, Tisseel fragments, and the remains of implanted pellets (Figure 63). Stress fractures were evident in the calcified cartilage, which indicate the point at which the native cartilage collapsed into the defect. Pellets within the defect maintained pre-implantation amounts of collagen type II (Figure 64) and collagen type VI (Figure 65). The pellets also contained lipid droplets, indicating a reversion to the adipogenic phenotype (Figure 63). Apart from that contained in the pellets, little collagen exists within the defect. Some collagen type II appears in thin crimped bands nearest the articulating surface, particularly at the defect margins (Figure 64). Collagen type VI was most concentrated at the interface between repair and host matrices (Figure 65). The only aggrecan that appeared within the defect was found only at the defect margin and likely originated from the host tissue (Figure 66).
Figure 59: Control defect of Sheep SC 1 stained with Mallory’s Trichrome
At the base of the defect, stromal cells migrate into the defect and the repair matrix contains little collagen (A). At the left margin of the defect, the host cartilage collapses into the defect to be integrated into the repair matrix (B). In the centre of the defect acellular Tisseel is surrounded by repair matrix concentrated with stromal cells and erythrocytes (C). At the right margin, cells migrate out of chondron clusters in the host cartilage to contribute to and integrate with the repair matrix (D). Central from the right surface margin, the repair matrix contains a high cellular concentration, bands of collagen, and blood vessels (E). Below the right surface margin, at the interface with the subchondral bone, marrow spaces that communicate with the defect are likely the source of invading stromal cells (F).
Figure 60: Control defect of Sheep SC 1 stained for collagen type II

Chondrocytes within the host cartilage at the left margin display the pericellular concentration of collagen type II typical of hyaline articular cartilage (A). Collagen type II within the repair matrix is limited to the margins of the defect (A, B and D) and a small “island” of host tissue (C) possibly separated from the cartilage at the right margin by deterioration of the articular surface. At the right margin, chondron clusters form in the native cartilage from which cells migrate to secrete collagen type II into the repair matrix (D).
Figure 61: Control defect of Sheep SC 1 stained for collagen type VI
Apart from the unstained mass of Tisseel in the centre of the defect (A), the repair tissue has a high concentration of generally distributed collagen type VI. At the base of the defect, stromal cells migrate toward the surface from underlying marrow spaces to populate the defect and secrete new matrix (B). At the defect margin the generalised distribution of collagen type VI in the repair matrix contrasts with the highly organised concentration of collagen type VI in the pericellular microenvironment of chondrons within the native cartilage (C).
Figure 62: Control defect of Sheep SC 1 stained for aggrecan epitope 5D4
There appears to be a generalised distribution of aggrecan throughout sections of the repair tissue with no real organisation. At the surface, the margins of the defect display a similar degree of aggrecan concentration, albeit with less structure than the adjacent host tissue (A and D). At the surface of the defect, there appears to be little aggrecan at the centre, although the Tisseel appears stained (B). Aggrecan is present in the matrix at the base of the defect where it communicates with underlying marrow spaces (C).
Figure 63: Test defect of Sheep SC 1 stained with Mallory’s Trichrome
Incomplete filling of the defect has taken place, with cavities evident within the defect. Pellets remain in the defect, containing collagen, stromal cells and lipid droplets, indicating a reversion to an adipogenic phenotype (B and C). Below the native cartilage at the right margin of the defect, stress fractures in the calcified layer appear where the cartilage has collapsed into the defect, as indicated by the arrow (A). At the right defect margin, chondron clusters form in the host cartilage from which cells migrate to secrete collagen in thick bands adjacent to a pellet that contains lipid droplets (B). In the centre of the defect, a pellet containing a combination of ASC, collagen, erythrocytes, and lipid droplets, is surrounded by other pellets, Tisseel, and erythrocytes (C).
There is little evidence of collagen type II in the repair matrix, in contrast to the collagen type II distribution typical of hyaline cartilage seen in the host cartilage outside the defect margins (A). There is good integration between repair and host matrices at both defect margins (A and F). At the left margin, chondron clusters in the host cartilage release cells into the repair, which secrete collagen type II in thin bands (A). At the right margin, the chondron clusters are not evident, although the distribution of collagen type II in the repair matrix is similar (F). The collagen type II content in the repair matrix decreases with distance from the margin (B). At the centre of the defect, a pellet bordering an empty space has a high collagen type II content (C). A pellet on the surface has become congruent with the articulating surface of the defect and still contains some collagen type II, although it is largely composed of lipid droplets (D). At the interface of subchondral bone and the defect, weakly-stained bands form an interface between the bone and the disorganised repair matrix (E).
Figure 65: Test defect of Sheep SC 1 stained for collagen type VI
At both margins at the surface of the defect hyaline cartilage with bright pericellular staining meets banded repair tissue that also contains collagen type VI, but in a more generalised matrix than focused pericellular distribution (A and F). The centre of the defect is composed of a mixture of empty space, unstained Tisseel, and a large pellet with a moderate amount of collagen type VI at the upper pole (B and C). At the defect interface with the subchondral bone, stromal cells and kinked bands containing collagen type VI stream between fragments of bone (D). At the articulating surface of the defect a pellet containing lipid droplets contains a moderate amount of collagen type VI (E).
Figure 66: Test defect of Sheep SC 1 stained for aggrecan epitope 5D4
Apart from the host tissue bordering the defect margins (A and D) there is very little evidence of aggrecan in the defect repair matrix. The pellet in the centre of the defect lacks aggrecan (B), even at the uppermost pole. Some aggrecan staining appears below the surface at the right margin of the defect where the host cartilage has collapsed into the defect (C).
**Sheep SC 4** – Four weeks after surgery, the control defect of sheep SC 4 is filled with a highly cellular repair tissue that has good integration with the host cartilage at one margin and is marred by detachment of the repair matrix from the native cartilage at the opposite margin (Figure 67). Within the native cartilage at the margins, chondron clusters have formed in an attempt at repair (Figure 67). At one margin, delamination of the native matrix in the calcified layer has occurred where the host cartilage has collapsed into the defect (Figure 67). The repair matrix steadily loses collagen with distance from the margins toward the centre where collagen type II (Figure 68) and collagen type VI (Figure 69) appear as thin tendrils aligned parallel to the articulating surface and mixed with stromal cells and erythrocytes. The matrix at the base of the defect, where it communicates with marrow spaces and anchors to the underlying bone, is dense with collagen (Figure 67), but not collagen type II (Figure 68) and little collagen type VI (Figure 69). Similarly, a thin band of collagen appears at the articulating surface of the defect matrix but this matrix also contains little collagen type VI (Figure 69) and less collagen type II (Figure 68). Aggrecan appears sparsely within the defect, concentrated at the defect margins and in a very thin layer across the articulating surface (Figure 70).

Four weeks following implantation surgery, the pellets are evident in the test defect of sheep SC 4. Some pellets were invaded by stromal cells and erythrocytes and were in the process of being integrated into the surrounding repair matrix (Figure 71). Other pellets appear encapsulated and remain separate from the surrounding matrix. All pellets have a similar matrix protein composition, containing some collagen type II (Figure 72), some aggrecan (Figure 74) and slightly more collagen type VI (Figure 73). The host cartilage has formed chondron clusters at the margins and loses collagen as it collapses into the defect, creating stress fractures in the calcified cartilage (Figure 71). The stromal cells within the repair have produced an extracellular matrix that contains collagen type II (Figure 72), collagen type VI (Figure 73), and aggrecan (Figure 74), all aligned parallel to the articulating surface and growing over the native cartilage that has collapsed into the defect (Figure 71).
Figure 67: Control defect from Sheep SC 4 stained with Mallory’s Trichrome
In the host cartilage at the defect margins, chondron clusters form from which cells migrate through a matrix that has delaminated from the underlying calcified cartilage (A). Central to the margin, the collagen concentration decreases and the cellular concentration increases (B). Areas of high cell density appear in the defect repair matrix just below the surface (C) and at the base of the defect (D). At the base of the defect, the collagen concentration appears greatest where it anchors the new matrix to the underlying bone (F). At the right surface margin, the host cartilage has detached from the repair at a tenuous interface (E), possibly during histological processing. The likely connection point is noted with the double-sided arrow.
Collagen type II is absent from the majority of repair tissue within the defect. It does not appear at all at depth (A), at the surface (B), or at the margin interface (C). The host cartilage stains weakly for collagen type II where chondron clusters have formed (D).
Collagen type VI appears weakly throughout the defect, most concentrated at the interface between host and repair tissue. At the margin of the defect, collagen type VI is concentrated in the repair matrix that is formed by cells that migrate out of chondron clusters within the host cartilage (A). This concentration is maintained across the surface where gaps in the matrix appear (B). In the centre of the defect, it surrounds autofluorescent erythrocytes (C) to extend in a thin layer over the native cartilage at the opposite defect margin (D).
Figure 70: Control defect of Sheep SC 4 stained for aggrecan epitope 5D4
Aggrecan distribution in the repair matrix within the defect is focused at the articulating surface (A) and at the base, where it is anchored to the bone (B). The greatest distribution of aggrecan within the repair tissue is found where the new matrix has broken away from the host cartilage (C).
Stress fractures in the calcified layer of native cartilage on both sides of the defect indicate where the host matrix has collapsed into the defect (A and H) forming chondron clusters and losing collagen (B) to be integrated within the repair matrix (C). Below the surface pellets remain with varying collagen and cellular content, and display different stages of integration with the repair matrix. Clusters of adjacent pellets share collagen-rich outer layers (D), but remain isolated from the rest of the repair matrix by Tisseel at the upper margin and newly-forming stroma below. Other larger, rounded pellets are in the process of being invaded by stromal cells from below that reorganise the collagens into the surrounding matrix as they work their way toward the surface of the defect (G). At the centre of the defect surface, a mixture of Tisseel, erythrocytes, and kinked ribbons of collagen predominate (E), extending toward the right margin where Tisseel and erythrocytes are replaced by a more collagenous matrix that integrates with the native cartilage at a well-defined interface (F).
Figure 72: Test defect of Sheep SC 4 stained for collagen type II
Collagen type II appears within the defect as banded ribbons. At the defect margin, brightly-stained chondron clusters form within the host cartilage, in contrast with adjacent pellets, which lack staining (A). While the pellet itself contains no collagen type II, the surrounding matrix contains limited collagen type II (B). At the articulating surface of the defect, a Tisseel fragment sits atop a matrix containing thin strands of collagen type II (C). At the right surface margin, the host cartilage matrix contains brightly-stained chondron clusters and integrates with a repair matrix containing only thin, kinked collagen type II fibres (D).
While ubiquitously distributed, collagen type VI appears concentrated in regions of matrix reorganisation within the repair tissue. At the defect margin, it is concentrated in the chondron clusters formed in the host cartilage, becoming more generally distributed in the repair matrix beyond the interface and less concentrated in the adjacent pellet (A). Small strands of collagen type VI penetrate the Tisseel that separates a pair of adjacent pellets from the matrix at the articulating surface (B). The collagen type VI in the intersection of these pellets and a third, less organised, pellet contributes to the binding of these three pellets (C). At the right margin, where the host cartilage has collapsed into the defect, the interface is clearly defined and the general distribution of collagen type VI within the repair matrix is in stark contrast to the pericellular sequestration of type VI collagen seen in the native cartilage (D).
The pellets remaining within the defect four weeks after implantation display varying degrees of aggrecan content and distribution. Aggrecan is generally distributed throughout the matrix surrounding the pellets with the most concentrated distribution at the articulating surface (A). While aggrecan pervades the matrix surrounding the pellets, very little aggrecan is actually retained within the pellets. In the domain between adjacent pellets, aggrecan has penetrated even the narrowest margins and no merging of pellets is evident (A, B, and C). In all cases there is a clear distinction between the surface of the pellet and the surrounding matrix (D).
Sheep IP 2 – Four weeks after surgery, the defect of Sheep IP 2 has almost completely filled with a dense, collagenous matrix with good integration of host and repair tissue (Figure 75). The bulk of the repair matrix is highly cellular, containing both stromal cells and erythrocytes (Figure 75). Immunohistochemical staining reveals that collagen type II is arranged parallel to the articulating surface in a thick band that extends across the defect, whereas below the surface it is equally concentrated but less organised (Figure 76). Collagen type VI appears concentrated at the interface between the repair and host matrices, particularly where the repair matrix anchors to the underlying bone (Figure 77). Collagen type VI is also concentrated around blood vessels that appear throughout the defect (Figure 77). Some aggrecan appears within the repair matrix but in a lesser concentration than in the bordering host cartilage (Figure 78).

The majority of the test defect of sheep IP 2 is filled with pellets that have survived in situ for the four weeks since implantation. The repair matrix surrounding the pellets contains little collagen, except at the interface with subchondral bone at the base of the defect (Figure 79). The host cartilage has retained its integrity, resisting collapse into the defect, and chondron clusters appear at the margins, indicative of the repair process (Figure 79). The pellets appear at various depths throughout the defect and at varying degrees of integration with the surrounding matrix and each other (Figure 79). Matrix protein composition and organisation within the pellets has not changed in the four weeks since implantation, indicating that the resident cells have maintained their state of differentiation. Immunohistochemical staining for collagen type II (Figure 80), collagen type VI (Figure 81), and aggrecan epitope 5D4 (Figure 82) reveals a similar distribution and organisation of these matrix proteins to that of pellets studied in vitro (see section 4.1.2.2), in contrast to the surrounding matrix that is generally lacking collagen (Figure 80 and Figure 81) and aggrecan (Figure 82).
Figure 75: Control defect from Sheep IP 2 stained with Mallory’s Trichrome

The defect surface at the left margin is congruent with the host cartilage and good integration between native and repair matrix extends the full thickness of the cartilage (A). The repair matrix at the surface of the defect has a thin layer dense with cells and collagens, aligned parallel to the articulating surface (B), becoming less collagenous with distance from the surface until it reaches the underlying bone and marrow spaces, where the collagen concentration decreases (C). The repair matrix below the surface is less collagen-dense but also highly cellular. Stromal cells can be seen entering the defect from the surrounding marrow spaces where they communicate with the repair matrix (D). In contrast to the left margin, the native cartilage at the right margin appears to have collapsed into the defect and become overgrown with cells and collagen from the repair (E). Stromal cells have entered the defect from communicating marrow spaces and filled the space under the collapsed cartilage to embed it within the repair matrix (F).
Figure 76: Control defect of Sheep IP 2 stained for collagen type II
At the left margin, the host cartilage displays a collagen type II distribution typical of hyaline cartilage (A). Chondron clusters appear below the surface and within the repair matrix, staining brightly for collagen type II within the pericellular microenvironment (B). Collagen type II distribution throughout the repair matrix is diffuse and weak, with bands at the surface parallel to the plane of articulation and a more random distribution below (C). In the host cartilage at the right margin, the brightly stained chondron capsules are absent where the native cartilage has collapsed into the defect (D).
Figure 77: Control defect of Sheep IP 2 stained for collagen type VI

The distribution of collagen type VI within the defect is generalised throughout the matrix and concentrated at the interface between the native and repair tissue. At the surface margins (A and F), inherent chondrons of the native cartilage stain with similar intensity to the adjacent repair tissue undergoing reorganisation. Central to the margins at the articulating surface of the repair, collagen type VI concentration diminishes slightly in the matrix, concentrating around blood vessels (B and C). Just below the surface at the right margin, a pocket of chondron clusters stain brightly for collagen type VI (E). At the base of the defect, collagen type VI concentrates at the interface between underlying bone and repair matrix (D).
Unlike the even distribution of aggrecan in the native cartilage (A), aggrecan within the repair matrix is generally distributed and largely unorganized. It appears to form a thin layer across the articulating surface (B, C and D), decreasing in concentration in the repair matrix with distance from the surface. Within the defect at the right margin, a fragment of cartilage that has collapsed into the defect retains most of its aggrecan (E). The native cartilage at the same margin is highly concentrated with aggrecan at the interface with the underlying bone (F).
Figure 79: Test defect of Sheep IP 2 stained with Mallory’s Trichrome
At least eleven pellets of various morphology and collagen content are visible within the defect. Despite the large number of pellets remaining, the surface of the defect remains unfilled. Within the defect some pellets retain cellularity and seem to integrate with the surrounding matrix (A). In contrast, other pellets are less cellular, more collagen dense, and maintain a strict barrier between them and the surrounding matrix (B). Pellets at the surface are covered with a thin layer that is collagen dense and more cellular than central regions of the pellet (C). Adjacent pellets sometimes merge to form a cohesive matrix with cells and collagen that fill the spaces between (D), but as often as not are separated by a thin layer of cells and do not share matrix. At the margin of the defect, the host cartilage is populated with chondron clusters (F), and abruptly terminates at the full cartilage thickness, collapsing into the defect to be integrated into the repair tissue of stromal cells, collagen, erythrocytes and Tisseel fragments (E).
While the defect is characterised by a large concavity, the pellets remaining within the defect retain a substantial amount of their collagen type II in situ. The host cartilage at both margins (A and G) displays collagen type II distribution typical of hyaline cartilage and ends abruptly at the defect. The pellet closest to the articulating surface of the defect (B) displays the highest concentration of collagen type II when compared with pellets further below the surface (E). Some adjacent pellets remain separated by Tisseel fragments (C) while others merge at shared borders (D). The little collagen type II that exists within the repair matrix appears at the articulating surface, particularly at the defect margins (A and F).
Figure 81: Test defect of Sheep IP 2 stained for collagen type VI

The pellets remaining within the defect retain a substantial amount of collagen type VI and stand out from the surrounding matrix, which contains type VI only at the interface between repair and host matrix. At the left surface margin, the native cartilage has separated from the adjacent repair tissue, which stains with similar intensity to the native chondrons (A). The collagen type VI-rich repair at this interface appears to be an extension of the nearest pellet, which is separated from a thin, brightly stained surface layer by a matrix largely devoid of collagen type VI (B). The common border of two adjacent pellets, one with clear pericellular differentiation evident in the centre of the pellet (arrow), at the centre of the defect is separated by a thin collagen type VI-poor domain (C). This pattern is repeated at the interface between three other adjacent pellets that are also separated by a collagen type VI-poor matrix containing erythrocytes (D). Despite the high collagen type VI concentration within one of these pellets, the centre of it appears hollow (E). Unlike the interface at the opposite margin, the repair matrix at the right margin contains substantially less collagen type VI than the chondrons in the adjacent native cartilage (F).
Figure 82: Test defect of Sheep IP 2 stained for aggrecan epitope 5D4

The aggrecan concentration and distribution seen in the native cartilage (A), typical of hyaline cartilage, is in stark contrast to the general lack of aggrecan existing within the repair matrix. The pellets remaining within the defect contain a moderate amount of aggrecan, also in contrast to the inter-pellet matrix that lacks staining for 5D4 (B). The largest concentration of aggrecan within the pellets exists in the pole of the pellet closest to the articulating surface (C) and the pellets with the greatest aggrecan content are at the articulating surface itself (F). In between two adjacent pellets, a bright band of aggrecan appears (D) while a similar interface between three other pellets is dominated by autofluorescent erythrocytes (E).
Sheep IP 3 – Four weeks after surgery, the repair matrix within the control defect of Sheep IP 3 generally lacks collagen despite good integration with native repair tissue at the margins (Figure 83). The collagen that does appear within the defect is likely to have originated from the host cartilage, which contains chondron clusters at the margins that have released cells and matrix proteins into the surrounding repair (Figure 83). Immunohistochemical staining for collagen type II and collagen type VI reveals that the collagen concentrated at the defect margins contains little collagen type II (Figure 84) and predominantly collagen type VI (Figure 85). Collagen type VI also appears at the base of the defect where it is anchored to the underlying bone (Figure 85). No aggrecan was evident in the repair matrix (Figure 86).

Four-weeks following implantation surgery, several large pellets are evident within the repair matrix of the sheep IP 3 test defect (Figure 87). These pellets display differing collagen concentrations as well as states of integration with the surrounding matrix (Figure 87). The matrix surrounding the pellets also varies in collagen content and concentration. Although some collagen is present, particularly at the defect margins, the amount is insubstantial in comparison with the collagen content of the pellets. At the articulating surface of the defect, a relatively dense band of collagen is evident and immunohistochemical staining confirms the presence of a small amount of collagen type II (Figure 88) and a greater amount of collagen type VI (Figure 89). Aggrecan appears both in the pellets and in the repair matrix at the margins of the defect (Figure 90). The aggrecan in the repair matrix has likely originated from the native cartilage that has formed clusters and released cells and matrix proteins into the defect (Figure 90).
Figure 83: Control defect of Sheep IP 3 stained with Mallory’s Trichrome

The repair matrix within this defect is characterised by a general lack of collagen despite the fair integration between native and repair tissue at the margins (A). Where the base of the repair meets the underlying bone, cells enter the defect from communicating marrow spaces (B) and secrete a layer of collagen that anchors the repair (D). The only collagen that exists within the central portions of the repair matrix are found as weakly stained patches of thin wavy bands (C). At the defect margin, the native cartilage is populated with chondron clusters from which cells migrate and secrete new collagenous matrix (F), which dilutes with distance from the margin (E).
Figure 84: Control defect of Sheep IP 3 stained for collagen type II
Apart from the interface between host and repair tissue at the defect margins, the matrix within
the defect contains little to no collagen type II. At the left margin, the native cartilage has formed
chondron clusters that release cells and collagen type II into the defect (A). The pattern at the
right margin is similar except that the native cartilage has collapsed into the defect and the
clusters, concentrated with collagen type II, are trapped against the subchondral bone (B). The
rest of the defect matrix remains unstained in the absence of collagen type II (C).
Collagen type VI distribution within the repair matrix appears concentrated in areas of interface between healing native tissue and newly formed repair. At the surface of the left defect margin it appears concentrated around the blood vessels (A). Just below that it is highly concentrated where the repair matrix integrates with underlying bone (B), continuing in a thin band all along the base of the defect and filling the marrow spaces where they communicate with the defect (D). This bond then widens once more at the surface where the repair matrix meets the native cartilage (E and F). The surface regions of the defect contain substantially less collagen type VI, except around blood vessels, containing autofluorescent erythrocytes (C).
Figure 86: Control defect of Sheep IP 3 stained for aggrecan epitope 5D4
The brightly stained native matrix (F), indicating the high aggrecan content typical of hyaline cartilage, contrasts sharply with the repair matrix that contains very little aggrecan. Unlike the surface layers of the repair matrix, which remain unstained and evidently do not contain any aggrecan (A, D and E), parts of the deeper zones, where the defect integrates with the underlying bone, contain small amounts of aggrecan (B and C).
Figure 87: Test defect of Sheep IP 3 stained with Mallory’s Trichrome

Several large pellets remain in the defect with varying collagen content. The fringes of some pellets merge with the surrounding matrix (A) while other pellets maintain a strict separation between their border and the surrounding matrix (D). The pellet deepest within the defect is an extreme case of the former, with sections of the pellet being resorbed into the underlying bone (C) as the defect is repaired from below. At the surface, a mass comprised of collagen, cells, and Tisseel (B) is tethered to a band of collagen that runs beneath the articulating surface and spans from the host cartilage of one side to the other (E). Integration of the host and repair matrices at the defect margins is affected through release of cells from chondron clusters that secrete matrix as they migrate into the defect (F).
Figure 88: Test defect of Sheep IP 3 stained for collagen type II
Several pellets are evident within the defect and stain marginally for the presence of collagen type II. The pattern of collagen type II distribution from pellet to pellet differs, some consistently dense, some sparse in the centre with a dense corona (A), and some with the same dense corona but containing empty spaces at the centre (B). At the surface, a dense layer of repair matrix contains a small amount of collagen type II with variable areas of concentration and distribution (C and D). At the defect margin, the native cartilage contains chondron clusters concentrated with collagen type II and integrates with repair tissue that has a higher collagen type II concentration than further toward the centre of the defect (E). In the middle layer of the host cartilage, chondron columns bend as the native matrix collapses into the defect (F).
Apart from the collagen type VI sequestration within the pellets remaining in the defect, diffuse yet distinct layers of collagen type VI are also found within the repair matrix. A base layer of collagen type VI at the interface of the repair matrix and the underlying bone is evident, as is a thinner band near the articulating surface (B). The tissue growing over the native cartilage at the left margin contains a small amount of collagen type VI in comparison to the brightly stained chondrons and chondron clusters within the host tissue (A). The opposite margin matrix is characterised by much greater concentrations of both cells and extracellular collagen type VI (C). The distribution of collagen type VI within the pellets has not changed in the four weeks since implantation, remaining concentrated in the central zones and less so in the surface zones (D, E, F and G) (See Section 4.1.2.2.2).
Figure 90: Test defect of Sheep IP 3 stained for aggrecan epitope 5D4
Aggrecan is evident throughout this section, appearing at the surface in the native matrix as expected of hyaline cartilage, in the repair tissue that has overgrown the surface of the native cartilage (A), across the articulating surface of the defect, and also within pellets to varying degrees (B, C, D and E). Aggrecan within the pellets is mostly concentrated in the circumference and lacking at the centre of the pellets (B, C and D). At the right margin the native cartilage is hyper cellular and contains a substantial amount of aggrecan (F), even in the matrix that has collapsed into the defect (G).
4.2.2.2. Twelve-week recovery

Sheep SC 2 – After 12 weeks of recovery, the control defect from sheep SC 2 had formed incomplete repair with a noncohesive matrix, riddled with gaps and empty spaces. The repair formed was highly cellular and contained some collagen, as indicated by Mallory’s trichrome staining (Figure 91). Immunohistochemical staining revealed that this collagenous matrix did not contain collagen type II (Figure 92), but that collagen type VI was concentrated at the defect margins and the site where the repair matrix encounters open space that communicates with the joint cavity (Figure 93). Antibody staining for aggrecan epitope 5D4 indicates no aggrecan content within the repair matrix except for a patch deep within the centre of the defect that is bordered by a Tisseel fragment above and an empty space that communicates with the joint below (Figure 94). The native cartilage at the margins of the defect is degenerative, populated by chondron clusters and characterised by a loss of collagen. Stress fractures in the calcified cartilage indicate the point at which the host cartilage collapsed into the defect (Figure 91).

Twelve weeks following implantation surgery, the test defect of sheep SC2 has filled in with a repair matrix comprised of bone and Tisseel fragments surrounded by a high concentration of stromal cells and some collagen (Figure 95). There was no evidence that pellets remain in the defect. The collagen content of the repair matrix does not include collagen type II (Figure 96), and collagen type VI can only be seen at the defect margins, more concentrated where the defect anchors to the underlying bone than at the surface (Figure 97). The lack of aggrecan within the defect makes it possible to see where the host cartilage has collapsed into the defect and has been driven below the surface to be overgrown by, and integrated with, the repair matrix (Figure 98). The telltale stress fractures in the calcified cartilage below the point of collapse are further evidence of this event (Figure 98). The host cartilage at the margins of the defect that remains at the articulating surface is characterised by chondron clusters and a loss of collagen in the extracellular matrix (Figure 95).
Incomplete repair of the lesion has left the defect largely devoid of new cartilage formation. At the left margin of the defect the host cartilage is fissured and highly cellular (A) with stress-fracturing of the calcified cartilage (D) where the articular cartilage has collapsed into the defect. Further in toward the centre of the defect surface can be found the greatest degree of new matrix formation with a dense concentration of stromal cells and blue-stained collagen (B). Of the many cavities existing within the depths of the defect, possibly the rudiments of marrow spaces, most are irregularly-shaped with a few forming ovoid “holes” (C).
Figure 92: Control defect from Sheep SC 2 stained for collagen type II
Practically no collagen type II exists within the defect, which has a large cavity between the remains of Tisseel and underlying bone that communicates with the surface. The left margin of the defect is not well integrated with the host cartilage at the surface (A) and the matrix below is thick with stromal cells and segmented by vessels containing autofluorescent erythrocytes (B). Toward the centre of the lesion surface, matrix lacking collagen type II covers what remains of the unstained, acellular Tisseel. At the right margin of the defect an eruption of stromal cells spills out across the articulating surface and supports a cartilage fragment populated by chondron clusters that stain faintly for collagen type II (D). In the depths of the defect where it communicates with the underlying marrow space, the likely source of the stromal cell eruption is a dense and highly cellular matrix surrounding the formation of a new marrow space (E).
Figure 93: Control defect from Sheep SC 2 stained for collagen type VI
Aside from the cavity at the centre of the defect, the remaining repair tissue stains consistently for collagen type VI except for a thin layer at the articular surface (B, C and D). The host cartilage at the left surface margin appears to have delaminated at the subchondral bone to be partially covered by tissue with mixed collagen type VI and erythrocytes (A). In the depths of the defect, new marrow spaces are forming within a matrix stained for collagen type VI (E) where stromal cells stream into the cavity at what looks like a river delta (F). The greatest degree of matrix re-organisation, as determined by collagen type VI concentration, occurs at the trough of the defect cavity (E and F).
Figure 94: Control defect from Sheep SC 2 stained for aggrecan epitope 5D4
Apart from a small section of matrix in direct contact with a nodule of Tisseel (D) and tissue adjacent to a section of bone deep within the defect (F), the repair tissue within the defect is lacking aggrecan, as evidenced by an absence of staining. Aggrecan distribution within the host cartilage is typical of hyaline cartilage, but the densely cellular tissue adjacent to it is remarkably different in its lack of aggrecan (A). At the articulating surface of the defect, cell-dense tissue lacking aggrecan supports a fragment of the host cartilage populated by chondron clusters (B). At the surface of the right margin of the defect, a severe boundary where the host tissue appears to have cleaved off (C) opposes a matrix infused with autofluorescent erythrocytes across the gap. At the base of the cavity within the defect (E) the cells and matrix appear to be shedding into the empty space.
Apart from the incongruence of the articular surface, the lesion has generally filled with a collagenous matrix, with the red-stained remains of Tisseel filling the central depths of the defect and no evidence of pellets remain. The host cartilage response to the injury can be seen clearly through the formation of chondron clusters (A) and the adjacent repair tissue is a band of highly cellular tissue, gradually becoming more matrix-based toward the centre of the defect surface (B). Invaginations in the surface (B and C) layer appear to be lacking collagen as it extends over the surface of the host cartilage at the right defect margin (D). In the deep layers of the defect, bands of collagen line the base (E) just above newly-forming marrow spaces, some densely populated with stromal cells (F).
In general, the repair matrix of the defect is lacking collagen type II and no pellet remains are evident. The only collagen type II visible by staining is in the native cartilage outside the margins of the defect. In particular, a chondrogenic zone in the left margin below the surface contains a matrix of highly proliferative cells, both in chondron clusters and individual cells that stain for collagen type II in the pericellular microenvironments (A). Toward the surface from this area, surface layer chondrons orientate into the defect as the host cartilage collapses and is covered with a layer of stromal cells lacking any collagen type II (B) that extends down into (C) and across the surface of the defect (D) to cover the host tissue at the right margin (E). In the depths of the lesion, a nodule of unstained, acellular Tisseel is encapsulated (F).
Figure 97: Test defect from Sheep SC 2 stained for collagen type VI
The focus of collagen type VI at the margins of the defect, both surface and at depth, indicate that organisation of repair matrix has occurred from the host matrix inward toward the defect. At the left surface margin, chondron clusters form, with pericellular microenvironments stained intensely for collagen type VI (A). As the host cartilage collapses into the defect, the clusters release cells that retain their pericellular collagen type VI into the defect to affect chondrogenesis (B). This phenomenon is duplicated at the opposite margin where matrix dense with cells (D) merges with chondron clusters within the host cartilage (E). In the centre of the defect, the cellular matrix layer spans across the surface that lacks a great deal of organisation or collagen type VI (C). At depth, the remains of Tisseel are enveloped by repair matrix that also lacks collagen type VI (F).
Figure 98: Test defect of Sheep SC 2 stained for aggrecan epitope 5D4
Nearly all of the aggrecan within the defect appears to originate from the host cartilage that has collapsed into the defect, as indicated by the presence of stress fractures in the calcified cartilage (arrow). At the left surface margin, the host cartilage contains chondron clusters that contain aggrecan in the pericellular microenvironments (A). At the right margin, chondron clusters have released cells and aggrecan into the repair (B), extending from the osseo-chondral junction (D) downward into the deeper layers of the defect (E). Further into the native cartilage at the right of the defect, a surface incongruity can be seen with a higher cell density and a slight loss of aggrecan in the repair (C). At the centre of the defect, matrix lacking aggrecan surrounds remains of Tisseel (F).
**Sheep SC 3** – After twelve weeks of recovery, the control defect of Sheep SC 3 is nearly filled, mostly with new matrix, but partly with Tisseel that has been lightly invaded by stromal cells and encapsulated within a discrete space (Figure 99). The greatest degree of new formation and organisation of repair matrix is in the depth of the defect, which has formed from the margin-in and from the base-up (Figure 99), indicative of the pathway through chondrogenesis that newly remodelled bone takes. The host cartilage at the margins is more cellular, with less organisation, than healthy hyaline cartilage and a loss of collagen with distance from the native tissue (Figure 99). The repair matrix is lacking in collagen type II (Figure 100), but contains a substantial amount of collagen type VI, particularly at the defect interface with the host tissue (Figure 101). Aggrecan appears at the articulating surface of the defect and diminishes with distance from the surface (Figure 102).

Twelve weeks after implantation, no evidence of pellets remains within the defect (Figure 103). Filling of the defect is good, with good integration of the defect margins with host tissue, which has collapsed into the defect as indicated by stress fractures in the calcified cartilage (Figure 103). Hypertrophic chondron clusters appear in the native cartilage at the margins with an associated loss of collagen from the native matrix (Figure 103). The base of the defect is lined by a thick band of highly chondrogenic cells that are rounded with collagenous pericellular capsules within a collagen-rich extracellular matrix. At the interface of this matrix with the underlying bone, mineralisation is taking place as intra-chondral ossification advances toward the articulating surface (Figure 103). The repair matrix that covers the surface of the lesion contains kinked collagen ribbons and has a low cellularity, except at the articulating surface, which has a thin but dense layer of cells, aligned parallel to the articulating plane (Figure 103). Round lipid droplets appear at the articulating surface, indicative of an apparent reversion of cells and matrix to an adipogenic phenotype (Figure 103). Little collagen type II exists within the repair matrix, appearing in limited amounts at the defect margins (Figure 104). In contrast, type VI collagen appears generally distributed throughout the repair matrix and highly concentrated at the articulating surface and at the defect margins (Figure 105). Aggrecan also appears generally distributed throughout the repair matrix and particularly concentrated in a thick band across the articulating surface of the defect (Figure 106).
Figure 99: Control defect from Sheep SC 3 stained with Mallory’s Trichrome
Incomplete filling of the defect is seen, with an incongruent surface lacking collagen at the centre. Non-resorbed fragments of bone remain from surgery and have been encapsulated (D). At the left defect margin (A), the host matrix is more cellular, with less organisation, than hyaline cartilage with a loss of collagen staining away from the native tissue. At the right margin, two step changes can be seen with three distinct zones as the healthy native cartilage increases in cell and collagen density at the defect interface (C) that loses both cells and collagen from the matrix in the central defect surface (B). At the base of the defect, densely packed collagen sheets line the interface of repairing bone (E).
Figure 100: Control defect of Sheep SC 3 stained for collagen type II
Practically no collagen type II exists within the defect and the only substantial staining occurs in
the host tissue matrix at the defect margins. The first “step” at the left defect margin contains cells
with capsules that lack collagen type II (A) whereas cells as well as matrix in the second “step”
stain positively for the presence of collagen type II. Central to this stained zone, the repair tissue
lacks any collagen type II (B), as does the encapsulated Tisseel in the depths of the defect (C). At
the right margin of the lesion, a bright band of collagen type II marks the interface where cell
capsules and general matrix both stain with similar intensity (D). At the base of the defect, cell
capsules stain intensely and thin bands of collagen are shown to consist of type II, suggesting
differentiated chondrocytes, chondron formation, and pericellular localisation of collagen type II
(E).
Figure 101: Control defect from Sheep SC 3 stained for collagen type VI
Apart from the gaping cavity at the centre of the defect occupied by bone fragments (D), the remaining repair tissue stains consistently for collagen type VI. The host matrix at the left margin of the defect displays collagen type VI staining typical of hyaline cartilage (A), but the difference between pericellular focus and general distribution of collagen type VI is clearly seen at the interface further from the native tissue (B). In the centre of the defect surface the repair tissue contains the increased and generalised distribution of collagen type VI that would be expected of tissue undergoing massive organisation of matrix, and cells are no longer visible by their brightly stained pericellular microenvironments (C). At the deepest region of the defect, pericellular staining is evident with a mix of rounded cells and clusters as well as cells that appear to be undergoing osteogenesis (E).
Figure 102: Control defect from Sheep SC 3 stained for aggrecan epitope 5D4
There appears to be a generalised distribution of aggrecan in the articulating surface zone of the
defect, extending from the left interface with the host cartilage (A), across the centre (B) where
staining is more uniform than at either margin, to the right margin of the defect (C). The
concentration of aggrecan within the matrix appears to diminish with distance from the surface
with very little staining evident at the upper edge (D) and base (E) of a large empty space within
the defect.
Figure 103: Test defect from Sheep SC 3 stained with Mallory’s Trichrome
Filling of the defect is good, with good integration of the defect margins with host tissue (A), although no evidence of pellets is present. Stress fractures in the calcified cartilage (B) indicate where the host tissue has collapsed into the defect. The repair tissue at the articulating surface stains positively for collagen and displays a crimped, aligned morphology in layers with rounded cells in residence (C). Deeper into the defect a mass of acellular Tisseel is surrounded by rounded cells that have formed a pericellular capsule (D). At the right margin of the defect, the host cartilage meets densely cellular collagenous matrix (E) that decreases in both cell and collagen concentration with distance from the host tissue. At the surface of this same margin, chondron clusters form at the interface matrix, which is clearly delineated in both the arrangement and density of the cells within (F).
Figure 104: Test defect from Sheep SC 3 stained for collagen type II
Collagen type II is lacking from the bulk of the repair tissue and is only found at the circumference of the defect. At the surface, host cartilage contains chondrons with brightly-stained capsules that become clusters at the interface with repair matrix (A), which loses all collagen type II content in the centre (B). A similar pattern of collagen type II loss is evident at the opposite surface margin (C). Below the surface, the repair tissue seems to sit in a crucible of collagen type II matrix, secreted by a host of rounded, chondrogenic cells within brightly-stained pericellular capsules, supporting the repair and binding it to the underlying bone (D, E, and F).
The ubiquitous distribution of collagen type VI in the repair tissue is evidence of the massive matrix organisation taking place. The native cartilage displays the typical pericellular sequestration of collagen type VI with chondron clusters appearing at the defect margin, signalling the attempt at repair (A). Remodelling of this matrix appears to occur from the outside-in as rounded, chondrogenic cells with brightly stained pericellular capsules advance inward from surrounding areas (B). The interface between host and repair matrix is evident by a sudden increase in both cellularity and a more general distribution of collagen type VI (C), organised as kinked, layered bands that extend across the articulating surface (D). At the surface, rounded lipid droplets indicate a reversion to an adipogenic phenotype (D).
Figure 106: Test defect of Sheep SC 3 stained for aggrecan epitope 5D4
Staining intensity appears to increase from the host cartilage at the defect margins in a clear
delineation of the interface between native and repair tissue (A). The matrix maintains that
concentration of aggrecan until the centre of the defect surface (B) where three “fronts” of repair
meet, and a loss of aggrecan from the matrix takes place. At the right margin, the aggrecan
concentration increases again and the repair matrix integrates with the chondron cluster-filled
host cartilage (C). At the base of the defect, rounded, chondrogenic cells with pericellular
concentrations of aggrecan advance inward from the surrounding bone and marrow spaces into
the repair where the distribution of aggrecan becomes more generalised throughout the matrix
(D).
**Sheep IP 1** – After twelve weeks of recovery, the control defect of Sheep IP 1 contains a highly formed, highly organised, and completely filled matrix. The new repair has formed into distinctly separate cartilage and subchondral bone layers that are difficult to distinguish from the native tissue (Figure 107). Cartilage layers within the defect are in the process of forming and chondrons are not radially aligned into the columns typical of middle-layer hyaline cartilage, but surface layer cells are parallel to the articulating surface and deep layer chondrons are beginning to align (Figure 107). Defect margins are evident by the presence of chondron clusters, an increase in cellularity from host to repair matrix, the absence of a well-defined tide mark, and a slight decrease in the uniformity of collagen distribution in the repair (Figure 107). The subchondral bone has reorganised completely, with formation of marrow spaces similar to those in the adjacent native bone (Figure 107). Immunohistochemical analysis reveals a uniform distribution of collagen type II across the repair. The surface layer contains chondrons arranged parallel to the articulating surface with brightly-stained pericellular capsules (Figure 108). The major differences between native cartilage and repair matrix, with regard to collagen type II distribution, are that chondrons in the repair matrix contain less pericellular collagen type II and lack the collagen type II tails typical of hyaline cartilage (Figure 108). There is a greater disparity in the collagen type VI distribution of native and repair matrices, the repair matrix having a more generalised distribution than the host cartilage, which has sequestered collagen type VI within the pericellular microenvironment of the chondron (Figure 109). The distribution of aggrecan within the repair matrix is similar to that of the native cartilage, concentrating at the articular surface and diminishing a depth (Figure 110). The lack of uniformity in the repair matrix is in contrast to the even distribution of aggrecan within the host cartilage (Figure 110).

Twelve weeks after implantation surgery, many erythrocytes exist in the test lesion, consistent with the red-tinge to the centre of the defect seen at gross necropsy (Figure 111). The centre of the defect is concave with evidence of the remnants of pellets, which appear filled with erythrocytes. These pellet vestiges are circularly-organised collagen whirls with a high degree of cellularity (Figure 111). Immunohistochemical analysis shows the collagen composition of the pellet remains to be mainly collagen type VI (Figure 113) and lacking collagen type II (Figure 112). Characteristic fissures in the calcified cartilage are evident directly below the original margins of the lesion, where the native cartilage has collapsed into the defect (Figure 111). There is a substantial amount of new growth on one margin of the defect where chondrocyte clusters transition into a highly cellular, fibrous repair matrix above newly-forming bone (Figure 111). Areas within the defect concavity that were not subjected
to loading are characterised by a lesser degree of collagen deposition and lack of organisation of what little collagen there is (Figure 111). Collagen type II is lacking throughout the repair matrix (Figure 112) and collagen type VI is found concentrated at the articular surface and at the defect margins (Figure 113). Aggrecan distribution within the defect is most concentrated at the defect margins nearest the articulating surface and diminishes in the walls of the defect toward the trough, which contains little aggrecan (Figure 114)
Figure 107: Control defect from Sheep IP 1 stained with Mallory's Trichrome
The repair matrix within the defect is hardly distinguishable from the hyaline cartilage of the host tissue at first glance. The differences between host and repair cartilage are limited to an increase in the cellular concentration, incomplete organization of the chondrons into the columns of typical middle layer cartilage (A), a slight lack of uniformity in collagen distribution, and the absence of a well-defined tidemark. The subchondral bone has reorganized completely with formation of marrow spaces similar to those in the native bone. The deep layer chondrons appear to be the first to have organized into columns (B). The defect margin is identified by a small surface incongruity and the formation of chondron clusters in the host cartilage (C), indicative of the ongoing repair response.
Figure 108: Control defect from Sheep IP 1 stained for collagen type II
The distribution of collagen type II is uniformly typical of hyaline cartilage across the repair. The surface layer contains chondrons arranged parallel to the articulating surface with brightly stained pericellular capsules (A), while the calcified cartilage contains less collagen type II and fewer cells (B). The repair tissue within the defect is characterized by higher cell density with less pericellular collagen type II (C) than the native tissue, with radially aligned chondron columns in the deep and middle layers separated by a matrix rich in collagen type II (D).
In contrast to the organization of collagen type VI within the native matrix, which is mainly sequestered in the pericellular microenvironment of chondrons, the distribution of collagen type VI within the repair matrix is more ubiquitous. This can be partially attributed to the higher cellular concentration within the repair matrix (C and D). One of the few indicators of the defect margin is chondron clusters, which stain intensely indicating a high pericellular collagen type VI content (A). In the very centre of the defect surface, two fronts of migrating repair tissue meet and collagen type VI is concentrated at the interface (B).
Figure 110: Control defect from Sheep IP 1 stained for aggrecan epitope 5D4
The distribution of aggrecan within the repair matrix is similar to that of the native cartilage, concentrating at the articular surface and diminishing at depth. The lack of uniformity in the repair matrix (A) is in contrast to the even distribution within the host cartilage. Cells streaming into the defect from the underlying marrow spaces exhibit pericellular sequestration of aggrecan and begin to secrete it into the matrix as they move toward the surface (B, C and D).
Figure 111: Test defect from Sheep IP 1 stained with Mallory’s Trichrome
The small remains of pellets are visible in the centre of the defect and are impregnated with what appear to be a mixture of multi-nucleated cells, erythrocytes, and possibly Tisseel (A). The collagen of the pellets is denser than surrounding matrix and is circumferentially organised. The host cartilage at both margins has collapsed into the defect with delamination of the collagen at the left margin. At the right margin, a substantial growth of new tissue is characterised by an increase in cellularity and a decrease in collagen concentration within the matrix (B). In the calcified cartilage below this margin, stress fractures indicate the collapse of the native cartilage (C).
Figure 112: Test defect of Sheep IP 1 stained for collagen type II

The disparity between collagen type II content of native matrix and repair matrix clearly defines the defect margin. The stress fractures in the calcified layer of the host cartilage indicate the point at which the native cartilage collapsed into the defect (A and F). The matrix of the repair tissue that grows over the collapsed cartilage contains less collagen type II than the overgrown matrix (B). The central portions of the defect contain even less collagen type II than the defect margins and the majority of it is concentrated at the surface (C and D). The right defect margin is clearly defined between the collagen type II-poor repair and the collagen type II-rich native cartilage (E).
Unlike the concentration within the pericellular microenvironment of the host cartilage (A), collagen type VI is more generally distributed throughout the repair tissue. The capsules of chondron columns stain brightly as the columns bend, indicating a collapse of the native tissue into the defect (A). At the articulating surface, chondron columns reorganise to remain perpendicular to the surface (B). Further along the surface toward the defect, repair tissue, concentrated with generally distributed collagen type VI, integrates with host cartilage (C). In the very centre of the defect, where small pellets remain, collagen type VI is concentrated at the periphery of the pellet (D). Where the repair tissue has integrated with the native cartilage at the right margin, layers of collagen type VI (E) extend to the interface between repair and host matrix where chondron clusters appear (F).
Figure 114: Test defect from Sheep IP 1 stained for aggrecan epitope 5D4

A disparity between the concentrations of aggrecan in native tissue and repair tissue is clearly evident while the greater concentration of aggrecan at the articulating surface relative to the deeper layers within the native cartilage matrix is more subtle. Fractures in the calcified cartilage stain brightly, indicating a greater concentration than the surrounding matrix (A). The articulating surface, where the defect margins collapse toward the centre, appears to have a high aggrecan concentration (B and F), diminishing in the walls of the defect (C and E) toward the trough, which contains little aggrecan (D).
Sheep IP 4 – After twelve weeks of recovery, the control defect of sheep IP 4 is only partially filled with new matrix lacking any substantial collagen content, comprised of stromal cells and containing blood vessels filled with erythrocytes (Figure 115). The native cartilage at the defect margins is characterised by a general loss of collagen and the presence of chondron clusters (Figure 115). The angle of radially-aligned chondron columns provide evidence of the collapse of the native cartilage into the defect to be overgrown by a thin layer of repair tissue containing stromal cells (Figure 115). The stark contrast between collagen type II-rich native cartilage and collagen type II-poor repair matrix clearly identifies the defect margin (Figure 116). Collagen type VI appears concentrated at the surface of the repair matrix and in a thin layer where the defect is anchored to the underlying bone (Figure 117). Aggrecan appears in a thin layer at the very surface of the repair matrix and also at the interface between the repair matrix and the underlying bone (Figure 118).

Twelve weeks after implantation, several pellets remain in the test defect of sheep IP 4. While the pellets are of a similar large-size, they display various states of reorganisation and invasion by surrounding stromal cells and erythrocytes (Figure 119). Immunohistochemical analysis confirms the retention of collagen type II (Figure 120), collagen type VI (Figure 121), and aggrecan (Figure 122) by the pellets in situ. The majority of the repair matrix appears comprised of stromal cells and little collagen except at the base of the defect where it anchors to the underlying bone (Figure 119). Immunohistochemical analysis confirms the lack of collagen type II (Figure 120) and collagen type VI (Figure 121) within the repair matrix, which also lacks aggrecan (Figure 122). The native cartilage at the defect margins displays signs of reorganisation with chondron clusters forming at the articulating surface and giving way to a collagen-poor repair matrix below (Figure 119). Telltale fractures in the calcified cartilage indicate where the native cartilage has collapsed into the defect (Figure 119).
Figure 115: Control defect of Sheep IP 4 stained with Mallory’s Trichrome
The host cartilage at the left margin of this defect attempts repair, with chondron clusters and a
general loss of collagen, well before the actual defect boundary (A). A substantial portion of the
native cartilage at the right margin has collapsed into the defect as evidenced by the angles of
radially aligned chondron columns and chondron clusters appearing in the deep layer cartilage at
the point of collapse (B). A thin layer of repair tissue containing stromal cells has overgrown the
native cartilage that has collapsed into the defect (C). Space within the defect is partially filled
with a matrix lacking any substantial collagen content, populated with stromal cells, and
containing blood vessels (D). At the base of the defect lies a large open space bounded by stromal
cells, bone, and Tisseel (E), possibly an abscess due to a localised infection.
Figure 116: Control defect of Sheep IP 4 stained for collagen type II
The native cartilage outside the defect contains collagen type II but the distribution appears more general throughout the matrix than the pericellular concentration expected of hyaline cartilage, particularly closer to the surface (A). The interfaces between repair and native matrix at both margins are characterised by distinct zones of collagen type II-rich and collagen type II-poor matrices (B and C). While filled with tissue consisting of Tisseel, stromal cells, erythrocytes and extracellular proteins, the repair matrix lacks collagen type II (D, E and F).
Figure 117: Control defect of Sheep IP 4 stained for collagen type VI

Collagen type VI distribution within this defect appears restricted to the upper half of the matrix, with the exception of a thin layer at the interface between the defect and the underlying bone (D). At the surface of the defect, collagen type VI appears particularly concentrated extending into the defect from native matrix characterized by the brightly stained pericellular capsules of chondrons clusters (A). Below the surface, collagen type VI concentrates around vessel-like structures containing autofluorescent erythrocytes (B). The right margin of the defect is especially concentrated with collagen type VI surrounding the pericellular microenvironment of masses of chondrons (C).
At the surface, aggrecan is highly concentrated within the native cartilage at the margins of the defect (A and F). Within these areas, aggrecan is found both in the pericellular microenvironment and the extracellular matrix. Further into the defect from the left margin, an area below but still exposed to the surface stains brightly for 5D4 (B). At the bottom of this chasm-like structure, only a thin layer of aggrecan remains at the very surface (C). A thin layer of aggrecan also appears at the interface between the repair matrix and the underlying bone (D). Below the surface of the right margin, pericellular capsules retain aggrecan while the extracellular matrix gradually loses it with distance from the surface (E).
Figure 119: Test defect of Sheep IP 4 stained with Mallory’s Trichrome
Several pellets remain within the defect in various states of reorganisation. The form of the deepest pellet has become nebulous as it has been invaded by stromal cells and erythrocytes that have broken the matrix apart to release the proteins into the repair, integrating with invading repair tissue (A). An adjacent pellet has retained its shape but part of its circumference has softened to allow the invasion of surrounding stromal cells. Another pellet closer to the surface appears to have maintained its original form, with rounded cells in its core, and resisted invasion from the surrounding matrix (B). The majority of the repair matrix appears comprised of stromal cells and little collagen except at the base of the defect where it anchors to the underlying bone. At the surface, the native cartilage is in a state of degeneration with chondron clusters forming at the articulating surface and giving way to a collagen-poor repair matrix below (C). Further away from the defect, fissures in the calcified cartilage mark where the host cartilage has begun to collapse into the defect (D).
Figure 120: Test defect of Sheep IP 4 stained for collagen type II

The repair matrix appears to lack collagen type II. The host cartilage outside the defect margins stains positively for the presence of collagen type II (A, B, E and F). Weak staining for collagen type II exists at the articulating surface of the repair matrix adjacent to the native cartilage at one margin (D), but this is not true of the repair interface at the opposite margin (C), which lacks collagen type II.
Figure 121: Test defect of Sheep IP 4 stained for collagen type VI
The collagen type VI distribution in the repair appears to be limited to the defect margins and what remains within pellets. At the surface of the left defect margin, the collagen type VI existing in the native cartilage extends to the repair interface (A) and spans across the defect surface in a thin layer to connect to the opposite defect margin (C), where it integrates with the collagen type VI within one of the pellets closest to the surface (D). While the border of some pellets blur, sharing collagen type VI with the extra-pellet matrix (F), some pellets remain separated from the surrounding matrix and show a layered collagen type VI pattern similar to the original pellets (see Figure 31), retaining collagen type VI within their circumference (B and E).
Figure 122: Test defect of Sheep IP 4 stained for aggrecan epitope 5D4
Aggrecan appears variably within the section. At the left defect margin it appears in the native cartilage and the extracellular matrix as well as in the pericellular microenvironment (A). At the right defect margin it appears only in the capsules of cells that populate that matrix (F). At the surface of the defect aggrecan appears in a thin layer that spans the entire articulating surface (D). Within the pellets aggrecan is more ubiquitous, displaying the same distribution that pellets did prior to implantation (B and E). At the junction between two adjacent pellets in the centre of the defect, a piece of tissue appears highly concentrated with aggrecan epitope 5D4 (C).
DISCUSSION

5. Discussion

The majority of the comparisons made of ASC with other sources of MSC cite lipoaspirate as the source of ASC [367-369]. In most cases, ASC fall short when chondrogenic potential is the comparator. This is not surprising given that most cell-based therapies for articular cartilage repair utilise a cell source that originates from within the joint, as in the case of ACI, which uses chondrocytes, but also benefits from stem cells resident in the synovium. Similarly, microfracture uses MSC enticed to the repair from bone marrow. These procedures are successful in producing repair cartilage that contains elements of hyaline cartilage, even if the structure is not truly hyaline. The reason for this is that the cells are capable of producing the correct matrix molecules in some semblance of the correct organisation. Why then, if ASC from lipoaspirate also have the capability of producing cartilage following chondrogenic induction, do MSC from bone marrow and synovium seem better suited for the task? None of the studies that compare non-adipose derived MSC with ASC ask that question. Part of the answer surely lies in the most obvious point of difference: the cells originate from completely different environments, exposed to different mechanical forces and chemical signals within the niche of origin.

If mechanical force can drive MSC toward a particular lineage [370], it can be inferred that the mechanical forces of a particular niche would push the resident MSC toward a particular lineage. Differentiation media are designed to contain optimal combinations of cytokines and growth factors for a specific target cell type. Co-culture experiments describe the way in which cells of a particular niche secrete these factors to influence neighbouring cells [371] and identify the specific soluble factors that guide chondrogenic fate [372]. So, it would seem logical that the soluble factors found in a particular niche would also push resident cells toward the predominant lineage. A recent study found that the presence of collagen type II in the matrix enhanced chondrogenesis of ASC by affecting cell shape [373]. Another study found that the geometric shape of the culture environment created changes in the contractility of the cellular cytoskeleton that induced differentiation of MSC [374]. These results clearly implicate the niche as the dominant factor in determination of lineage differentiation.

The heterogeneity of primary ASC cultures from the same source has been studied [375] and the differing chondrogenic potential of the subpopulations, also from the same source of fat, recently shown [376]. Another paper described the differences between different
sources of fat with regard to differentiation potential and cytokine/chemokine expression, but for the purpose of identifying the most appropriate cell source for investigating the pathophysiology of metabolic disorders such as obesity [377] rather than chondrogenic potential.

What has been shown in these experiments is that ASC isolated from IP adipose tissue have greater chondrogenic potential than ASC from SC adipose tissue. It is put forth that the mechanical and chemical environment of the IP niche is such that ASC from the IP fat pad are prepared for chondrogenic differentiation in a way that SC fat is not. This has been shown in an animal-matched in vitro comparison of neocartilage pellets made from ASC from each of the two sources (section 4.1.2). To investigate the potential clinical application of these results, the ability of pellets made from each of the two ASC sources to repair full-thickness articular cartilage defects (section 4.2) were compared. The sheep model enabled simulation of a human clinical scenario and implantation of full-size neocartilage pellets into surgically-created defects. Histopathologic study of the defect repair revealed the unexpected inability of the implanted pellets of either source to form hyaline cartilage, or even continue to differentiate in situ (section 4.2.2). Given this, the IP pellets were more resistant to extrinsic cellular invasion over the recovery periods than SC pellets and retained a greater semblance of pre-implantation morphology. The literature was reviewed to find reasons for the failure of the model, and theories are presented for consideration and future study.

5.1. IP ADMSC vs. SC ADMSC

5.1.1. Harvest and isolation

The differences in harvest of adipose tissue from the two sources of fat are a clinically relevant point to consider. If the joint capsule were not already open for the defect creation procedure in these experiments, access to IP fat would have been a very different endeavour, making SC collection the easier of the two by far. However, considering that arthroscopic viewing of damaged cartilage (as occurs in nearly all cases requiring cell-based therapy) allows access to the IP fat pad without the need for an open arthrotomy, the difficulty of access to IP fat is negated. The vast majority of publications cite lipoaspirate as a source of adipose tissue, but in these experiments surgical excision was determined to be an excellent way of obtaining a clean sample of relatively homogenous tissue that facilitated the subsequent isolation of ASC. Samples did not have to be washed excessively or subjected to erythrocyte lysis protocols as when using lipoaspirate, saving a great deal of time and effort.

The isolation of ASC from SC fat was very different from that of IP fat. The SC fat contained more vascular tissue than IP fat; the SC fat did not digest as completely as IP fat, and the SC fat adhered to every container and tool used during the procedure (section 4.1.1.1,
Experiment ASC 6). Given the enzymes used for digestion, it seems that IP fat contains greater collagen content in the extracellular matrix, although this was not studied. Mochizuki et al reported that IP fat had a higher specific gravity than SC fat, stating a higher fibrous content to IP fat as the reason [378]. In addition, they found that IP fat contained a greater number of nucleated cells per weight of tissue than SC fat and that these cells had a higher expression of chondrogenic genes such as COL2A1, Aggrecan, and SOX9 than SC fat [378]. While the completeness of digestion may account for the greater cellular yield from IP fat, the isolation procedure may have to be optimized for each source of fat for a true comparison of the cellular yield to be made. However, as the isolation protocol used in both studies was adapted from published methods that were developed to isolate ASC from lipoaspirate, it is likely that IP fat contains a greater number of ASC per unit of tissue. This is surprising in light of the current view is that ASC are localized in the perivascular niche and are likely pericytes [379-382], which suggests the tissue with the greatest degree of vascularity would contain a higher concentration of ASC.

5.1.2. Monolayer culture

Because the yield of cells from IP fat was greater than the yield from SC fat, IP primary cultures were seeded at a higher density and reached confluence in fewer days. Given this, the population doubling rates of both groups were similar, indicating consistent proliferative rates of cells from each source. Since one of the criteria of a MSC is its ability to adhere to plastic, it was theorized that a more homogenous cell population enriched for ASC could be achieved by limiting the time that newly-plated cells had to adhere to plastic culture flasks. As the doubling rates of the selectively-enriched and non-enriched subgroups were similar, this assessment did not support the theory (section 4.1.1.4.1). SC cultures seemed to grow slowly from small colonies or single cells, but proliferation rapidly increased with cell density to grow from 50% to confluence in a single day.

5.1.2.1. Characterisation of cell population

The characterisation of the cell population was problematic. The position statement from the International Society for Cellular Therapy gives the minimal criteria for defining MSC as being plastic adherent, expressing certain surface markers while lacking expression of others, and having tri-lineage differentiation potential [294]. Following the protocol for isolation of human ASC published by Patricia Zuk [276], and adapting it for use on sheep adipose tissue, a primary culture of cells that adhered to the plastic culture flasks was obtained. Attempts were made to enrich the population of ASC by performing an early wash to remove all but the most adherent cells. The selective adhesion-enriched cultures performed no better when compared with sheep-matched non-enriched cultures that were washed after
three days of plating (section 4.1.1.4). A recent study compared human ASC isolated following three-days of adherence, a wash one hour after plating, and selection by immunomagnetic isolation using CD49a, CD90, CD105, and CD271. While these investigators found that the washing step reduced heterogeneity, the paper noted no significant differences in adipogenic and osteogenic differentiation capacity [383], confirming the observations of this thesis. The tri-lineage differentiation potential of the isolated cells (section 4.1.1.3.1) were also confirmed. However, it was not possible to test expression of the defined surface markers. Antibodies for STRO-1 and CD105 were purchased as positive MSC markers, and CD34 as a negative marker. Dilution series were performed for determination of the optimal staining conditions for these antibodies in sheep cells. Despite several attempts, a lack of cross reactivity between the human antibodies and the sheep ASC was observed. Ultimately, the experiments progressed in confidence having met two of the three criteria for MSC classification after following the standard protocol for isolation of ASC from adipose tissue. It was only when this work was presented at the International Fat-Applied Technology Society (as it was known then) in 2006 that it became clear that this lack of cross-reactivity was a problem for many researchers working with non-human models of ASC tissue engineering.

5.1.2.2. Monolayer expansion and pellet formation

It is known that monolayer expansion conditions play a critical role in the chondrogenic potential of ASC in 3-D culture and ASC can be primed toward chondrogenesis during expansion [384]. Basic fibroblast growth factor (FGF-2) is one of the commonly used additives to monolayer expansion media for both ASC and chondrocytes. In chondrocytes it is known to limit dedifferentiation and maintain the cells’ capacity for redifferentiation [233, 385], even playing a role in in vivo proliferation of chondrocytes following injury [386]. In ASC, its role is similar [387] although a recent study claims it antagonises other chondrogenic factors such as BMP-6 [388].

Early attempts to make pellets were unsuccessful. Upon reviewing the literature at the time (2004), it was noted that few methods of ASC expansion included growth factors in the media, whereas the methods used in these experiments included FGF-2 and TGF-β. The inclusion of growth factors in the original proliferation medium stemmed from its use in a chondrocyte monolayer expansion method. The purpose was to encourage just enough dedifferentiation of chondrocytes toward a more fibroblast-like state, allowing for proliferation, while maintaining the capability for redifferentiation. As the ASC cells were already in a more plastic state it was theorized that the ASC method did not require these specific growth factors, and a higher percentage of foetal calf serum (FCS) was instead
chosen to stimulate growth. Furthermore, other attempts within our lab to form beads from harvested cartilage were similarly unsuccessful and the expiration dates of certain medium additives were questioned. Therefore, the standard proliferation medium was changed to exclude FGF-2 and TGF-β and increased the percentage of FCS to 10%. Also, new expiration dates for medium additives were put into place. It is unclear which of these changes was most influential, but the very next attempt and every subsequent attempt to form beads were successful. In hindsight, the literature published since then is clear on the benefits of FGF-2 in ASC expansion medium [387, 389], but since both test groups (IP and SC) were exposed to the same medium lacking added growth factors, the comparison remained valid.

5.2. IP pellets vs. SC pellets

5.2.1. Pellet morphology

When comparing pellet morphology through mean diameter, the highly significant difference between IP and SC pellet size (p<0.0001) (section 4.1.2.1) could have been due to: 1) a greater degree of matrix production per cell; 2) a greater cellularity; or 3) a combination of matrix production and cellularity. Given the degree of difference in DNA content between IP and SC pellets at all time points and particularly in the animal-matched comparison (p<0.05) (Table 18), it is most likely a combination of matrix production and cellularity, weighted to cellularity, which reflects directly upon the greater ability of IP ASC to survive chondrogenesis and produce chondrogenic matrix proteins.

Growth and matrix deposition characteristics of pellets can be surmised from the morphological differences of cellular organisation in the three zones or layers within each pellet (Figure 31). The appositional growth patterns of developing cartilage were discussed in section 1.1.1 and this has some relevance to how the ASC pellets grew, deposited, and organised matrix in the in vitro constructs. Bruce Caterson’s group described the appositional and interstitial growth of scaffold-free chondrocyte constructs as similar to that of articular cartilage growth and organisation in vivo [239] and observations of pellet development and morphology described in these experiments agreed.

It is likely that the central zone of the pellet, populated by densely packed rounded cells, is the remnant of initial cell pellet formation during the first twenty four hours of culture. As the surface layer cells of the newly formed pellet were exposed to mechanical forces existing in the RCCS vessel, they would begin to produce matrix in order to protect themselves. Now provided with a protective layer of separation from the circumferential tensile forces experienced during RCCS, the outer layers of central zone cells would dedifferentiate to a more elongated fibroblast-like cell type, proliferate, and migrate outward to form new outer layer cells, rounding and producing chondrogenic matrix in the process.
This growth pattern was evident in each ASC pellet with the three distinct layers, as central zone cells appear to stream and flow into the transitional zone where they flatten and produce fibrillar collagen species. Outer transitional zone cells in turn stream and flow into outer layers to become rounded and form pericellular capsules. At the very surface, cells flatten again to align circumferentially to withstand circumferential tensile forces within the rotary culture flasks. The chondrocyte pellets differed in this regard as they did not flatten in the transitional zone, but rather changed orientation to become more radially aligned. The single layer of cells at the surface of chondrocyte pellets did, however, flatten to align circumferentially, as in ASC pellets (Figure 32).

IP pellets showed a much greater capacity for formation of distinct organisational layers within the pellets than SC pellets. This organisational capacity was generally associated with increased matrix production. SC pellets generally lacked cohesiveness and where layers were present, development was inconsistent.

5.2.2. Development of extracellular matrix

In general, extracellular matrix development was greatest in the chondrocyte pellets, less so in IP pellets and least developed in SC pellets, which were typically collagen- and aggrecan-poor. Immunohistochemical analysis of pellets first revealed this qualitatively and then biochemical analysis quantitatively confirmed the results of the antibody stains. In most cases, the collagen content of the SC pellets was below the lower limit of detection of the assay.

Unilaterally, within the pellets, matrix proteins were most concentrated in the outer layers. The central zone contained a lesser concentration of collagens and GAG, and the transitional zone of ASC pellets contained the least matrix production. If the theory about ASC pellet development, as stated in the previous section is correct, then the transitional cells flatten to proliferate, losing the ability to produce matrix, then become rounded to again secrete matrix to protect themselves in the outer zone, where cellular turnover would also require proliferation for maintenance. In this way, pellets grow interstitially in the transitional zone, and appositionally at the very surface, with strong matrix production in the outer and central zones.

5.3. Femoral articular cartilage defect repair

5.3.1. The ovine model

The use of sheep in models of knee repair is a good choice because the size of the joint allows as direct a translation to the human knee as is possible in readily accessible animals, particularly in New Zealand. This is not to say that the sheep model is not without its difficulties. While they are very placid creatures, they are also very skittish, raising the
possibility of self-injury when they are handled or approached. The risk of self-injury was greatly reduced in these experiments due to the extensive experience that the surgical team had with sheep models of joint repair. Another benefit of the experience with sheep was that methods of restraint, limitation of joint movement, and even constant passive motion capability had been developed over the years. Some of these methods will be discussed in relation to the post-operative recovery of the animals in section 5.3.3.

The greatest limitation of the sheep model was the lack of cross-reactivity of ovine proteins with available antibodies. Because the sheep is not a common model of ASC differentiation studies, there is little demand for antibodies to be developed against sheep antigens. It is unlikely that the study is invalidated for the lack of full characterisation of the population of cells isolated from each source of fat, but it would have added gravitas to the comparison to know the similarities and differences between the two groups of cells. All other aspects of the isolation, culture, and differentiation of ASC were similar to those of human cells. There was also a degree of interanimal variability that, while acceptable in a proof-of-concept study such as this, would necessitate a substantial increase in animal numbers to achieve statistically significant results.

5.3.2. The surgical procedure

5.3.2.1. Defect creation

The surgical model used in the in vivo experiments was of an osteochondral defect. In addition to the complexity and variability of the addition of bone marrow-derived MSC into a composite of microfracture and ASC tissue engineering, this experiment tested the ability of neocartilage pellets to differentiate in situ in a biphasic manner. The intention was that the portion of the pellets embedded within the subchondral bone would follow a similar terminal differentiation pathway to the growth plate cartilage and begin to mineralise to form new bone. Dragoo, et al. accomplished osteochondral differentiation of ASC from human IP fat when implanted into the flanks of SCID mice [326]. In that study, cells themselves were suspended in Tisseel and implanted, each group of cells was induced with factors specific to the target lineage, and biphasic differentiation was not attempted. While this was an unlikely outcome in this experiment, the primary objective remained to produce new hyaline articular cartilage above the subchondral bone and all of the assessments in these experiments were made against that goal.

5.3.2.2. Implantation

As discussed in the previous section, the defects extended below the articular cartilage into the subchondral bone. As the SC pellets were much smaller than the chondrocyte pellets used in method development and fewer in number, many having clumped together and
merged within the RCCS vessel, there were not enough pellets left over from in vitro assessments to fill the entire defect. On the other hand, there were often too many IP pellets for the defect, and they were of a similar size to the chondrocyte pellets so attempts were made to press fit as many within the lesion as possible. While all care was taken to replace the meniscus and patella without dislodging the implant, and the joint was subsequently fixed to prevent repeated exposure of the implants, the possibility exists that parts of implants or even the entire implant may have been dislodged in animals that did not have pellets still visible in the defects at necropsy.

5.3.3. Recovery/rehabilitation

During development of rehabilitation and restraint methods, the decision was made to entrap the implant within the defect by limiting joint movement such that the meniscus always covered the defect. An external fixation system was commonly in use by the surgical group and this was used with great success. While originally concerned about the insertion of the required pins into tibia and femur, no bone instability was noted, nor was any infection introduced. The fixators allowed enough joint movement for the patella to glide over the intracondylar notch and the tibial plateau to oppose the condyle and transfer the requisite shear and compressive forces to the lesions.

5.3.4. Quality of defect repair

5.3.4.1. Gross observations

In general, the repair of control lesions was better than the repair of test lesions. At both time points, exsanguination at necropsy seemed to negatively pressurise the marrow spaces beneath the defects, forcing the lesion surfaces to concavity. Some of this negative pressure was released during dissection, sometimes observed dynamically. As the negative pressure only affected the portions of repair that remained fluid, a greater disparity between well-formed matrix and poor filling of the lesion was seen in some cases.

While the test lesions were incompletely healed after four weeks of recovery (section 4.2.1.2.1), the presence of the pellets in situ was encouraging, particularly in light of the inability to affix pellets within the lesion during method development procedures. The hope was that the pellets would continue to produce matrix in vivo, expand, and merge to fill the defect. There was no observable difference between SC and IP pellets at this time point. At four weeks, the control lesions were typically filled uniformly with translucent, sometimes blood-tinged repair material. There was little difference in healing of test and control lesions at this time point.
It was after twelve weeks of recovery that clear differences between test and control groups were seen. At least one control defect healed almost completely, making the lesion difficult to see by naked eye. In contrast, none of the test lesions healed completely and what repair existed appeared to have come from the surrounding native cartilage rather than from pellets. Surrounding the existing pellets was often the same translucent red-tinged material seen at four weeks. It was also at twelve weeks that differences between IP and SC pellets were seen, or more appropriately not seen as there was no evidence of SC pellets in either of the twelve-week test defects. Pellets remained in both of the IP test lesions and in gross examination appeared similar to those seen at four weeks, implying a lack of continued differentiation in situ.

5.3.4.2. Histopathology: tissue repair response

Examination of histology slides confirmed the gross observations and further elucidated the extent to which defects were repaired (section 4.2.2). Healing of all defects, whether test or control, appeared to originate from outside the margins of the defects. At the surface, the articular cartilage at the margins often collapsed into the defect to become engulfed within the repair matrix. The chondrocytes at the margins proliferated in response to the injury and formed clusters. Cells migrated from these clusters into the defect in a dedifferentiated state, and began to secrete a new, fibro-cartilaginous matrix. This was evident by the high relative concentration of collagen type II at the defect margins that decreased across the surface of the defect with distance from the native cartilage. The new matrix was largely devoid of collagen type II, which was usually seen as an aligned fibrous connective tissue lying parallel to the articulating surface and stretching between areas of collagen type II-poor matrix. Collagen type VI and aggrecan content also decreased in the repair matrix with distance from the native cartilage, supporting the theory that these matrix components originated from the host tissue rather than being produced by cells within the defect. When stromal cells reached the surface of the defect they flowed over the collapsed native cartilage and formed a layer over the cartilage that extended beyond the defect margins in some cases. Mallory’s trichrome stain revealed a collagenous composition to this surface layer that was weakly stained for collagen type II. Given the fibrous nature of this repair tissue, it is likely that the matrix was predominantly composed of collagen type I. Poor lateral integration of repair with host cartilage was seen in most test cases, and in several controls. These results appear to be consistent with the majority of cell-derived surgical therapies for articular cartilage defects and are likely the result of implanted cell death, dedifferentiation, poor matrix
network formation and, in the case of SC pellets, the source of cells for tissue engineering [390, 391].

Below the surface, marrow stromal cells migrated into the defect in great numbers wherever a marrow space communicated with the lesion. The migration of these cells through the lesion affected reorganisation of the matrix in its wake. When pellets were present within the defects, stromal cells surrounded and began to disintegrate the pellets, recycling liberated matrix proteins and reorganising them into the repair tissue. Stromal cells also migrated into Tisseel, dispersing it into fragments that grew smaller over time. It has been shown that fibrin sealant supports the proliferation and migration of human chondrocytes, [181]. After comparing the superior repair of Tisseel-filled control lesions (particularly Sheep IP 1, Figure 107) with the inferior repair of test lesions, the conclusion that Tisseel similarly encouraged MSC to migrate, proliferate, and form the cohesive repair matrix that test lesions lacked was made. At sub-surface margins within defects, areas of intense matrix reorganisation were evident in sections stained for collagen type VI. In these sections, collagen type VI concentrated at the interface between the defect and the subchondral bone, signalling the formation of matrix scaffolding, comprised of collagen type VI, into which new matrix components would be attracted and sequestered. The focus of collagen type VI at the margins of the defect, both at the surface and at depth, indicate that organisation of repair matrix has occurred from the host matrix inward toward the defect, but not yet reaching the central portion of the lesion. The balance of collagenous matrix deep within the defect, as revealed by Mallory’s trichrome, not stained positively for collagens type VI or type II, is most likely collagen type X which is involved in forming calcified matrix and new bone.

5.3.4.3. Histopathology: pellet response

While some space was taken by pellets remaining within the defect, and pellet matrix components were recycled into the repair by invading MSC, it is clear that the ASC within pellets did not substantially contribute to production of any new chondrogenic matrix. MSC invaded the defect like a cellular juggernaut, disintegrating anything in their path and remodelling the matrix in their wake. They swarmed around pellets, forming new matrix punctuated by new marrow spaces, sometimes penetrating the pellets and impregnating them with erythrocytes. Dragoo et al. described the ability of implanted IP ASC nodules to differentiate osteogenically and organise to not only deposit mineral, but form marrow spaces when provided with BMP-2 [326]. None of the pellets in this thesis experiment can be said to have formed marrow cavities, but the IP pellets remaining in the deepest region of the defect at twelve weeks were the first to be broken apart and fill with
erythrocytes (Figure 119). It is possible that MSC invaded these pellets first, due to their depth within the subchondral bone, and sought to remodel the matrix into the appropriate form. It is also possible that the ASC within those pellets were influenced by surrounding cells and mechanical forces to differentiate osteogenically and begin to produce a niche attractive to erythrocytes. It is likely that a combination of these events occurred, MSC invading and influencing the ASC within the pellet to change their matrix production profile as mechanical forces worked toward the same end. In addition, the formation of blood vessels within the repair tissue would enable the availability of additional chemical mediators of matrix production to further influence the process.

While in rotary cell culture, pellets showed an affinity for each other and often formed clumps of several adhesive pellets. Thus, it was expected that pellets within the defects would behave similarly and grow together to form a cohesive graft that would continue to produce matrix. Why that did not occur is the subject of great speculation with many possibilities that would have to be explored in future studies. One possibility raised by a cartilage-specific gene microarray study performed on ASC pellets by our laboratory (data not presented) suggests that pellets may undergo terminal differentiation with time, similar to the hypertrophic zone during bone growth. A recent paper showed that cartilage engineered from MSC was indistinguishable from foetal cartilage samples, suggesting that MSC-engineered cartilage repair may mimic foetal development [392]. When implanted into SCID mice, ASC pellets have been shown to lose collagen type II, exhibit fibrous dedifferentiation, or completely degenerate [393]. Given the results of these recent papers, our own preliminary results from microarray, and the histopathologic results in section 4.2.2, it is likely that implanted pellets followed the foetal cartilage development pathway, but were unable to stop at the desired stage of differentiation required to reproduce the hyaline articular cartilage phenotype sought. What is clear is that ASC pellets did not continue to produce hyaline cartilage-specific extracellular matrix when implanted. In most cases, pellets remained as they were on the day of implantation, until they were remodelled by invading stromal cells. Some pellets merged along a common border, while others maintained a strict separation. Reindel et al. reported that the adhesive strength of cartilage explants in in vitro culture depended on the viability of cells [394]. It is possible that merging pellets maintained a higher degree of cell viability after implantation into defects, but this was not examined in this study and could be determined in future work with cell fate mapping.

In comparison with IP pellets, which remained in situ at the twelve-week time point, the SC pellets did not fare as well following implantation. After four weeks, some SC
pellets showed signs of dedifferentiation to an adipogenic lineage containing what appeared to be lipid droplets within them (Figure 63). None of the SC pellets were evident in twelve-week histology sections. This could have been because 1) pellets were readily broken apart by stromal cells due to the lack of extracellular matrix to protect the ASC; 2) the SC ASC could not survive the environmental stresses experienced in the joint; or 3) SC ASC underwent terminal differentiation more quickly than IP ASC. In contrast, IP pellets were usually seen remaining in defects at both four and twelve week sections, suggesting that they were able to maintain their differentiated pellet phenotype. In addition, they were often intact and resisted disintegration, even after twelve weeks in vivo.

5.4. The role of the niche in determination of graft cell source

“...the properties of a stem cell depend as much on the niche in which it resides as on the inherent ‘stemness’ of the cell.” -Darwin Prockop [395]

Recently, MSC were found to be vascular pericytes and reside in the perivascular microenvironment [380]. It is now generally accepted that MSC function as pericytes on vessels surrounding every tissue in the body, facilitating their ability to readily migrate to areas of injury, secrete soluble repair mediators, and establish a regenerative microenvironment [396]. Given that cartilage is an avascular tissue, it is no surprise that cartilage has a limited ability for intrinsic repair. Cartilage is, however, neighbour to other vascular tissues, the infrapatellar fat pad being one of the closest and one that is exposed to the mechanical forces that make diarthroidal joints such a harsh environment.

Recent studies have sought to capitalise on the migratory capacity of MSC to home into areas of repair. Experiments involving repair of myocardial infarction and ischemic damage have been particularly successful but have also revealed that repair is made without long-term engraftment of the MSC that migrated to the site [397]. Indeed, a new model of MSC repair is emerging in which functional improvement of injured tissues is mediated by MSC without lasting engraftment or differentiation of MSC but rather secretion of soluble factors and cell-to-cell contact [395]. This model paints MSC in a new light and defines the role of the MSC as “project managers” of repair. In business, a project manager is one who guides the actions of others who carry out the work. They advise, organise, encourage, stimulate, and provide necessary resources and cues. To be effective in this role, they must know the requirements and accountabilities of each person involved in the project, knowing how to perform the tasks but not doing so themselves. If a project manager gets involved in the performance of tasks, then organisation and direction fail and the project loses focus. To relate this to MSC, just because they are able to differentiate, does not mean that they do, or
even should. The MSC that do differentiate likely lose their ability to trophically influence repair.

It has been shown that co-culture of cells can influence and direct differentiation through cell-to-cell contact [340]. The influence of mechanical forces on differentiation of ASC has also been previously discussed. It is therefore logical to theorise that the tissues in close proximity to the perivascular niche would prime the resident MSC to differentiate toward that specialised tissue type. The MSC would be similarly influenced by the mechanical environment that they share with the tissue. For this reason, it is theorised that MSC harvested from tissues in closest proximity to the tissue of interest would make the best candidates for cellular-based repair strategies for injury to that tissue. In the case of these thesis experiments, the greatest difference in the cells used to engineer neocartilage constructs for repair of osteochondral defects was the origin of those cells. It has therefore been shown that is why ASC from infrapatellar fat were far more suited to chondrogenesis than ASC from subcutaneous fat.

5.5. Limitations of this study

5.5.1. Size of the study

As a pilot study for development of a possible therapeutic method, the numbers of animals were ample to evaluate the overall merit of the methods used, but clearly not sufficient for any quantitative or statistical significance to be determined. The NZ Health Research Council grant that enabled this work was written as a proof of concept study, was funded as such, and the ethical use of animals application was similarly written and approved. Therefore, the ability to quantitatively test the procedure was not possible, but never intended.

5.5.2. Heterogeneity of results

Given that the model chosen for these experiments was a species of crossbred domestic animal, inter-animal variability was expected, and observed. Animal subjects were selected to be of a similar size, age and weight as much as was possible from a single flock offered to the University for sale and all animals were purchased at the same time. To control for this variability within the study, each animal acted as its own control and adjacent lesions on the same joint surface were compared for repair response.

5.5.3. Surgical model

As discussed previously, the model used was one of an osteochondral defect. This was necessary due to the size of the implanted pellets as the profile of the pellets rose well above the cartilage thickness height. It is acknowledged that an ideal model for repair of chronic degenerative cartilage would have been a full-thickness defect that did not break the surface of the subchondral bone. The addition of MSC from underlying marrow spaces added a
substantial variable element that was not originally intended, but could not be avoided in the assessment of the suitability of the neocartilage pellets to affect repair. As will be discussed in section 6.2, future work would involve the implantation of cells impregnated within a gel matrix that will not be limited by the cartilage thickness. This new approach would also obviate the need to limit range of motion of the operated limbs to avoid dislodging of the implanted pellets. Therefore, there would be no limiters to be placed and animals would be returned to full weight bearing and free-range holding as soon as incision wounds were healed. As has been discussed, mechanical force is a significant factor in chondrogenic differentiation and the secretion and organisation of hyaline cartilage matrix, so it would be best for animals to subject operated joints to normal load bearing as soon as is feasible. Although the limiters allowed sheep to bear load on operated joints, it is likely that motion limitation inhibited chondrogenic differentiation and hyaline cartilage matrix secretion and organisation.
CONCLUSION

6. Conclusion

6.1. Hypothesis

This thesis was designed to determine whether ASC from IP fat have greater potential for chondrogenesis than ASC from SC fat as assessed by their ability to produce hyaline-like neocartilage constructs. Chondrogenic potential was determined by the ability of ASC from each source to differentiate into rounded cells that form chondrons and secrete the phenotypic extracellular matrix molecules of hyaline cartilage: collagen type II, collagen type VI, and aggrecan. In addition, the repair potential of the neocartilage constructs from each ASC source was then studied by implantation into a full-thickness osteochondral defect model in sheep.

Results showed that IP fat contains more ASC than SC fat by weight. Visually, IP pellets were significantly larger, proving a greater survival rate and matrix production rate over SC pellets. This was qualitatively shown in immunohistochemical study of pellets from both groups and confirmed quantitatively through biochemical analysis. When implanted in vivo, IP pellets remained within defects for twelve weeks while SC pellets were absent at the same time point.

Given all of this evidence I can conclusively state that IP ASC have greater chondrogenic potential than SC ASC.

6.2. Future work

Future work would focus on developing a viable method of producing hyaline cartilage from IP ASC engineered grafts. The cell culture method should be changed to maintain more of the ASC inherent ability to affect trophic repair. I would also like to test this method in a model of full-thickness articular cartilage repair, rather than an osteochondral defect. If the size of the pellets makes this unlikely, then the ability to affect biphasic repair would be tested. Additional pilot work would be necessary to further develop the model, after which a study powered with sufficient animal subjects to obtain statistically significant quantitative data should be performed. The pellets would also be delivered in fibrin-sealant suspension rather than adding sealant over the top of pellets. I would also seek to prevent cell death at the margins of the defect in an attempt to enhance graft-host lateral integration. In addition, gene expression studies of pellets would be carried out on pellets from the original experiments to confirm the hypothesis of terminal differentiation as well as pellets and tissue explants from the new studies to characterise the defect repair expression profile. Finally, fate
mapping of implanted cells would identify the cells that do or do not contribute to repair and answer the question of what happens to pellets once they have been disassembled.

6.2.1. Chondrogenic induction period

Pellets cultured for twenty one days posed a few problems. Primarily, the size of pellets forced the model from full-thickness cartilage defect to osteochondral defect, introducing the variable of the interaction of bone marrow with ASC. The size of the pellet also created more space between pellets than was ideal. Furthermore, it is possible that three weeks in pellet culture was enough to assure the terminal differentiation of chondrogenic ASC. While other studies have described the importance of chondrogenic induction prior to implantation [398, 399], I think that pellets should be ready for implantation within one week of induction. This theory is supported by microarray studies performed by Tallheden et al. [238] and our own laboratory (unpublished) that indicate all chondrogenic genes are expressed within one week of chondrogenic induction, but decline after week one. Pilot work would be required to identify the optimal chondrogenic induction time prior to implantation.

6.2.2. Biphasic repair

If one-week pellets are still too large for fixation within a full-thickness chondral defect, I would like to explore the possibility that implanted pellets can form a biphasic repair. At the outset of my experiments, I theorised that biphasic differentiation should be possible, and I think that three weeks of chondrogenic culture may have pushed the ASC beyond their ability to differentiate to the conditions in situ. Recent studies have shown that ASC have the ability to spontaneously differentiate in situ and affect biphasic repair [400, 401], proving the possibility.

6.2.3. Revise surgical procedure

Several times during the implantation I thought that the fixation of pellets into the defect would have been far simpler if the pellets were suspended within the less viscous fraction of the Tisseel. Furthermore, the superior results of the control lesions into which Tisseel was injected and MSC from underlying bone allowed to migrate convinced me that fibrin sealant is an ideal medium for cell-based orthopaedic therapies. Recent studies have supported that concept and shown its veracity [398, 402, 403].

The histopathology sections revealed collapse and degenerative changes of the host cartilage at the margins. Regardless of the quality of the repair tissue, collapsed and degenerative host tissue will not integrate well and restoration of the articulating surface will be handicapped. A recent paper by Gilbert et al. advocates treatment of the lesions with necrosis- and apoptotic-inhibitory agents [390] to prevent GAG loss and improve integration and adhesion of repair to host margins. I think that Z-VAD-FMK can be introduced into the
lesion in one of the fibrin sealant fractions, hopefully providing extended protection against chondrocyte death and improving outcomes.

Based on the histopathology results at twelve weeks after surgery, it is clear that repair processes are still very active at the end of this period. For this reason, further studies must also include time points at six- and twelve-months post-implantation.

6.2.4. Molecular Biology (RT-PCR, Microarray)

ASC pellets from my experiments were preserved and stored in RNA later in a -80°C freezer. I would like to analyse these pellets for markers of terminal differentiation to confirm or disprove that theory. In addition, cartilage-specific gene expression studies on new pellets and sections of defect repair would further elucidate the mechanism of ASC-mediated repair in articular cartilage.

6.2.5. Fate Determination of Implanted Cells

Histopathology of defects suggests that implanted pellets do not contribute to the repair of defects. This conclusion is somewhat confounded by the surgical model in that the influx of bone marrow-derived MSC may have been primarily responsible for any observed repair. Furthermore, an antagonistic interaction between MSC and ASC is also possible. In order to obtain conclusive answers to the questions surrounding the role of ASC in repair of defects, it would be important to know the fate of implanted ASC. For this reason, future work should include fate mapping of ASC implanted as pellets or impregnated within scaffolds.
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