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**An investigation of primary human cell sources and clinical  
scaffolds for articular cartilage repair**

Doctor of Philosophy

by

Nupur Kohli

Aston University

September 2015

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## **An investigation of primary human cell sources and clinical scaffolds for articular cartilage repair**

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### Thesis Summary

Damage to articular cartilage of the knee can be debilitating because it lacks the capacity to repair itself and can progress to degenerative disorders such as osteoarthritis. The current gold standard for treating cartilage defects is autologous chondrocyte implantation (ACI). However, one of the major limitations of ACI is the use of chondrocytes, which dedifferentiate when grown *in vitro* and lose their phenotype. It is not clear whether the dedifferentiated chondrocytes can fully redifferentiate upon *in vivo* transplantation.

Studies have suggested that undifferentiated mesenchymal stem or stromal cells (MSCs) from bone marrow (BM) and adipose tissue (AT) can undergo chondrogenic differentiation. Therefore, the main aim of this thesis was to examine BM and AT as a cell source for chondrogenesis using clinical scaffolds. Initially, freshly isolated cells were compared with culture expanded MSCs from BM and AT in Chondro-Gide®, Alpha Chondro Shield® and Hyalofast™. MSCs were shown to grow better in the three scaffolds compared to freshly isolated cells. BM MSCs in Chondro-Gide® were shown to have increased deposition of cartilage specific extracellular matrix (ECM) compared to AT MSCs.

Further, this thesis has sought to examine whether CD271 selected MSCs from AT were more chondrogenic than MSCs selected on the basis of plastic adherence (PA). It was shown that CD271<sup>+</sup>MSCs may have superior chondrogenic properties *in vitro* and *in vivo* in terms of ECM deposition. The repair tissue seen after CD271<sup>+</sup>MSC transplantation combined with Alpha Chondro Shield® was also less vascularised than that seen after transplantation with PA MSCs in the same scaffold, suggesting anti-angiogenic activity. Since articular cartilage is an avascular tissue, CD271<sup>+</sup>MSCs may be a better suited cell type compared to the PA MSCs. Hence, this study has increased the current understanding of how different cell-scaffold combinations may best be used to promote articular cartilage repair.

**Key words:** Bone marrow; adipose tissue; mesenchymal stem cells; CD271.

## *Dedication*

*I would like to dedicate this thesis to my late baby brother, Lakshay Kohli. For your memories will forever live in our hearts and your smile will never be forgotten.*

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## **List of abbreviations**

2D	Two-dimensional
3D	Three-dimensional
ACAN	Aggrecan
ACI	Autologous chondrocyte implantation
ACI-C	Autologous chondrocyte implantation – collagen membrane
ACI-P	Autologous chondrocyte implantation-periosteal flaps
ADAS	Adipose derived adult stem cells
ADAMSTS5	Disintegrin, metalloproteinase with thrombospondin motifs-5
AdMSC	Adipose mesenchymal stem cells
ADSC	Adipose derived stromal cells
AM	Acetomethyl
ANOVA	Analysis of variance
ASCs	Adipose-derived stem cells
ASCs	Adipose-derived stem/stromal cells
AT	Adipose tissue
AT MSCs	Adipose tissue-derived mesenchymal stem or stromal cells
AT SVF	Adipose tissue stromal vascular fraction
BM MNCs	Bone marrow mono-nucleated cells
BM MSCs	Bone marrow-derived mesenchymal stem or stromal cells
BM	Bone marrow
BMAC	Bone marrow aspirate concentrate
BMP	Bone morphogenetic protein
BSA	Bovine serum albumin
CAM	Cell-adhesion molecule
CCL2	Chemokine ligand 2
CFU-F	Colony forming unit-fibroblast
CM	Chondrogenic media
Col	Collagen
COMP	Cartilage oligomeric matrix protein
DAB	Diaminobenzidine
DEX	Dexamethasone
DMEM	Dulbecco's modified eagle's medium
DMMB	Dimethyl methylene blue
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonuclei acid
ECM	Extra cellular matrix
EDTA	Ethylendiamine tetra-acetic acid
EMA	European Medical Association
FBGC	Foreign body giant cell
FCS	Fetal calf serum
FGF	Fibroblast growth factor
FGFR	Fibroblast growth factor receptor
GAGs	Glycosaminoglycans
Gdf5	Growth and differentiation factor 5
H&E	Haematoxylin and eosin
HA	Hyaluronic acid
HGF	Hepatocyte growth factor
HMA	Human mitochondrial antigen
HMDS	Hexamethyldisilazane
ICRS	International cartilage repair society
IFATs	International Fat Applied Technology Society

IFN- $\gamma$	Interferon-gamma
Ig	Immunoglobulin
IHH	Indian hedgehog
IL	Interleukin
IPA	Isopropyl alcohol
ISCT	International Society for Cellular Therapy
ITS	Insulin transferrin selenium
LNGFR	Low-affinity nerve growth factor receptor
LREC	Local research ethical committee
MACI	Matrix-induced autologous chondrocyte implantation
MACS	Magnetic associated cell sorting
MMP	Matrix metalloproteinase
MSCs	Mesenchymal stem or stromal cells
Mtan1	Matrilin-1
NGFR	Nerve growth factor receptor
OA	Osteoarthritis
P/S	Penicillin/streptomycin
P75NTR	Neurotrophin receptor
PA MSCs	Plastic adherent mesenchymal stem or stromal cells
PCL	Polycaprolactone
PCM	Peri-cellular matrix
PCR	Polymerase chain reaction
PDLG	Poly-DL-lactide-co-glycolide
PGA	Polyglycolic acid
PI	Propidium iodide
PLA	Processed lipoaspirate
PLGA	Poly-DL-lactic-co-glycolic acid
PLLA	Poly-L-lactic acid
RA	Rheumatoid arthritis
RNA	Ribonucleic acid
ST MSCs	Synovial tissue-derived mesenchymal stem or stromal cells
SD	Standard deviation
SEM	Scanning electron microscopy
SEM	Standard error of the mean
Sox-9	Sex determining region-Y-box 9
SVF	Stromal vascular fraction
TGF- $\alpha$	Transforming growth factor- alpha
TGF- $\beta$	Transforming growth factor- beta
Tgfr2	Transforming growth factor- beta type II receptor
T <sub>H</sub> 1	T helper cells 1
T <sub>H</sub> 2	T helper cells 2
TIMP-1	Tissue inhibitor of metalloproteinases-1
TNF- $\alpha$	Tumor necrosis factor-alpha
TSG-6	Tumor necrosis factor-inducible gene 6
UV	Ultraviolet

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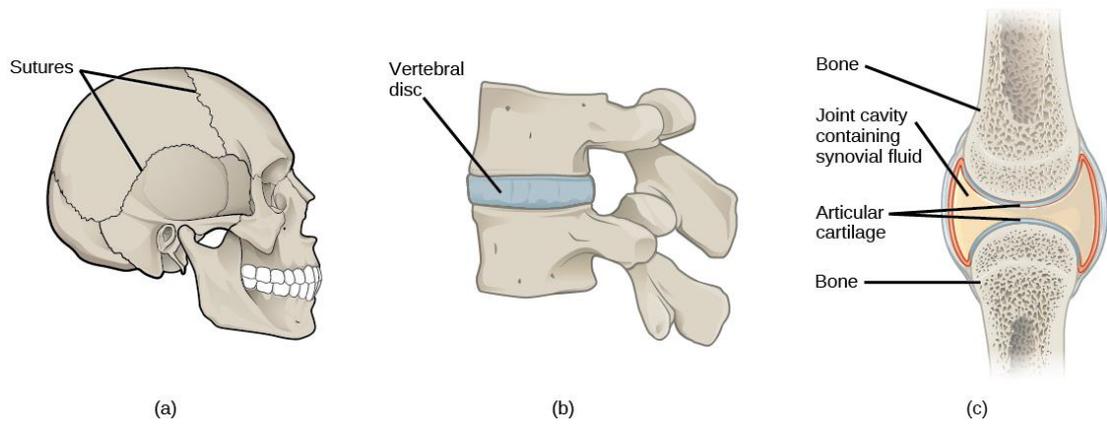
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## **Chapter 1: Introduction**

## **1.1 Definition, function and types of joints**

A joint is the location where two bones meet. Joints are the essential organs of the locomotion system in that they provide mechanical support and stability to body segments. Structurally, the joints are classified into three different types based on the materials binding the bones together and the presence or absence of a cavity. These are: fibrous, cartilaginous and synovial joints (Figure 1.1). Functionally, the joints are classified into synarthroses, amphiarthroses and diarthroses joints, based on the extent of movement. Fibrous joints are held together by collagen fibres and are mostly synarthrotic, i.e. where the bones have very limited or no movement (the term “synarthroses” comes from Greek for fixed or immovable joints). An example of synarthroses or the fibrous joints are the junctions of bones in between the skull. Cartilaginous joints are the joints in which bones are connected by cartilage. Cartilaginous joints are amphiarthrotic as they allow greater movement between the bony surfaces than fibrous joints, e.g. those found between the two pubic bones or joints between two vertebral bodies of the spine. Both fibrous and cartilaginous joints lack a joint cavity. The synovial joints are diarthrodial joints, simply meaning “freely movable joints” and are very different from fibrous and cartilaginous joints in that they permit extensive motion of the opposing bones. Along with the knee, other examples of diarthrodial joints are those of the shoulder, hip, elbow and the ankle. These are the only joints that contain a fluid filled joint cavity (Mow and Huiskes 2004). There are many different types of synovial joints depending on the shape of the articulating surfaces and the range of motion; these are ball-socket joints, condyloid joints, hinge joints, saddle joints, gliding joints and pivot joints (Ateshian and Eckstein 2004). In the following sections of this thesis, the knee synovial hinge joint will be described in more details.



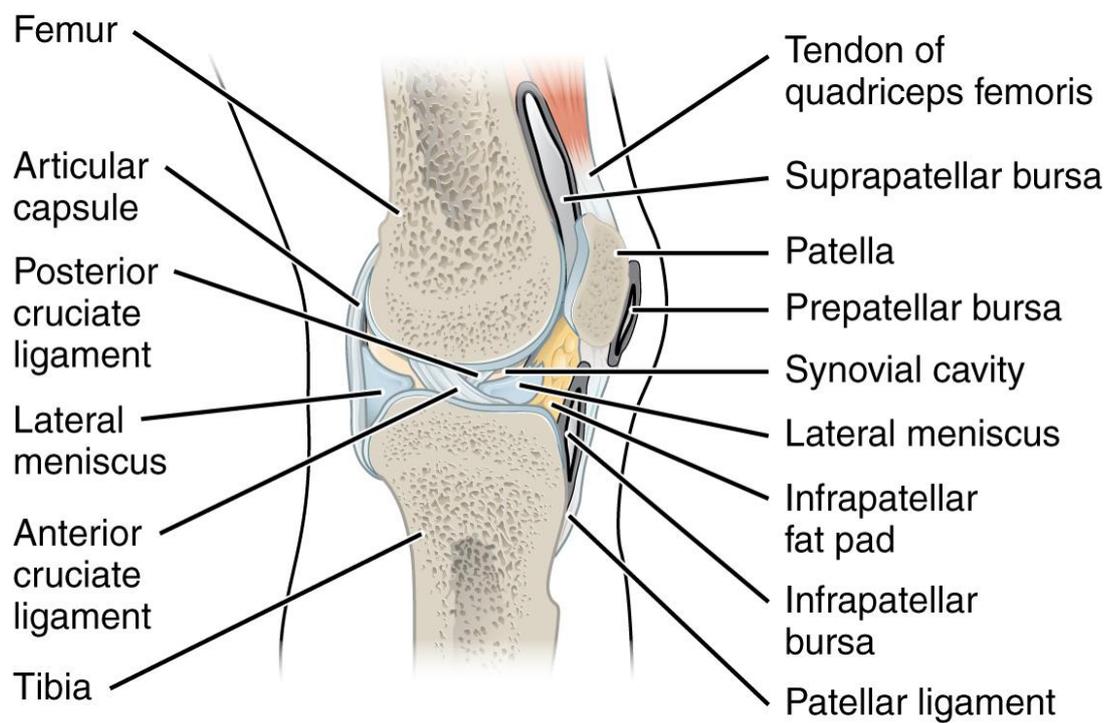
**Figure 1.1. Structural classification of joints.** (a) A fibrous joint between the junction of bones in the skull, (b) a cartilaginous joint between two vertebral bodies of the spine, (c) a synovial joint of the knee. Image derived from [cnx.org](https://cnx.org)<sup>1</sup>

## **1.2 Structure and function of the synovial knee joint**

The knee is one of the largest joints in the human body formed between three bones, the thigh bone or femur, the shin bone or tibia and the patella. Another bone called the fibula sits just behind the tibia and only contacts this bone. The distal end of the femur looks like two convex processes known as condyles which meet the concave processes on the proximal end of the tibia. This joint is also called the tibiofemoral joint as it's the joint formed between the tibia and the femur. Anterior to the tibiofemoral joint is the patella where its smooth posterior surface faces the femur and forms the patellofemoral joint. The ends of the bones that come together to form the synovial joint are covered by a layer of connective tissue called articular or hyaline cartilage (Khan *et al.*, 2007). The term hyaline, meaning "glass-like" represents the smooth appearance of the articular cartilage that helps in reducing friction during joint movement. In between the femur and tibia is a thick pad of fibrocartilage called the meniscus that acts as a shock absorber and helps to provide stability during joint movement. Like with all synovial joints, the knee joint is also encapsulated within a joint capsule consisting of a fibrous outer layer and a thin layer of synovial membrane that lines the joint capsule. The synovial membrane produces synovial fluid that fills the space in the joint cavity and acts as a lubricant to reduce friction. The synovial fluid, articular cartilage and the supporting bones form the diarthrodial joints that function together to provide smooth gliding almost friction free movements (Archer *et al.*, 2003). In addition, the knee joint is encapsulated by collateral and cruciate ligaments. Collateral ligaments are extracapsular and restrict the sideways motion of the knee, whereas cruciate ligaments are intracapsular as they connect the femur and the tibia at the centre within the joint capsule and limit rotation and forward and backward motion of the tibia. Along with the collateral and cruciate ligaments, several other important structures provide stability to the knee and help cushion and protect the joint during movement. These include small pockets of synovial fluid, known as bursae, that surround the joint to reduce friction from the movement of tendons present in the joint, as well as the infrapatellar fat pads

that act as shock absorbers and help cushioning the knee from external impact (Figure 1.2) (Mow *et al.*, 2004).

The knee is a hinge joint and therefore it functions to provide the flexion and extension of the lower leg relative to the thigh. The anatomy of the bones and ligaments and the alignment of different structures of the knee joint limit the knee's range of motion; however, the joint does allow for approximately 120 degrees of flexion. The knee hinge joint also allows for limited medial and lateral rotation when the knee is flexed, which distinguishes knee hinge joints from other hinge joints found in the body. The movements at the knee joint are vital for many day-to-day activities like walking, running, sitting and standing. The joint also ensures, during these movements, that the loads and bending moments acting along the long bones remain within acceptable physiological limits. Although these joints undergo an enormous range of loading conditions, the cartilage layer undergoes little wear and tear under normal situations. The knee joint functions effectively under the very high loads and for up to seven or eight decades. This movement and loading must demand effective lubrication to minimize friction and breakdown of articular cartilage. Wear of articular cartilage of the knee by biochemical or biomechanical means can lead to arthritis. Therefore, articular cartilage is one of the essential components in the effective functioning of human knee joint. (Mow *et al.*, 2004).



**Figure 1.2. Sagittal section of synovial knee joint.** Anatomical structures of the synovial hinge joint formed between the two long bones of the knee and the accessory structures including bursa, fat pad and the ligaments. Image taken from [cnx.org](http://cnx.org)<sup>2</sup>

### **1.3 Cartilage**

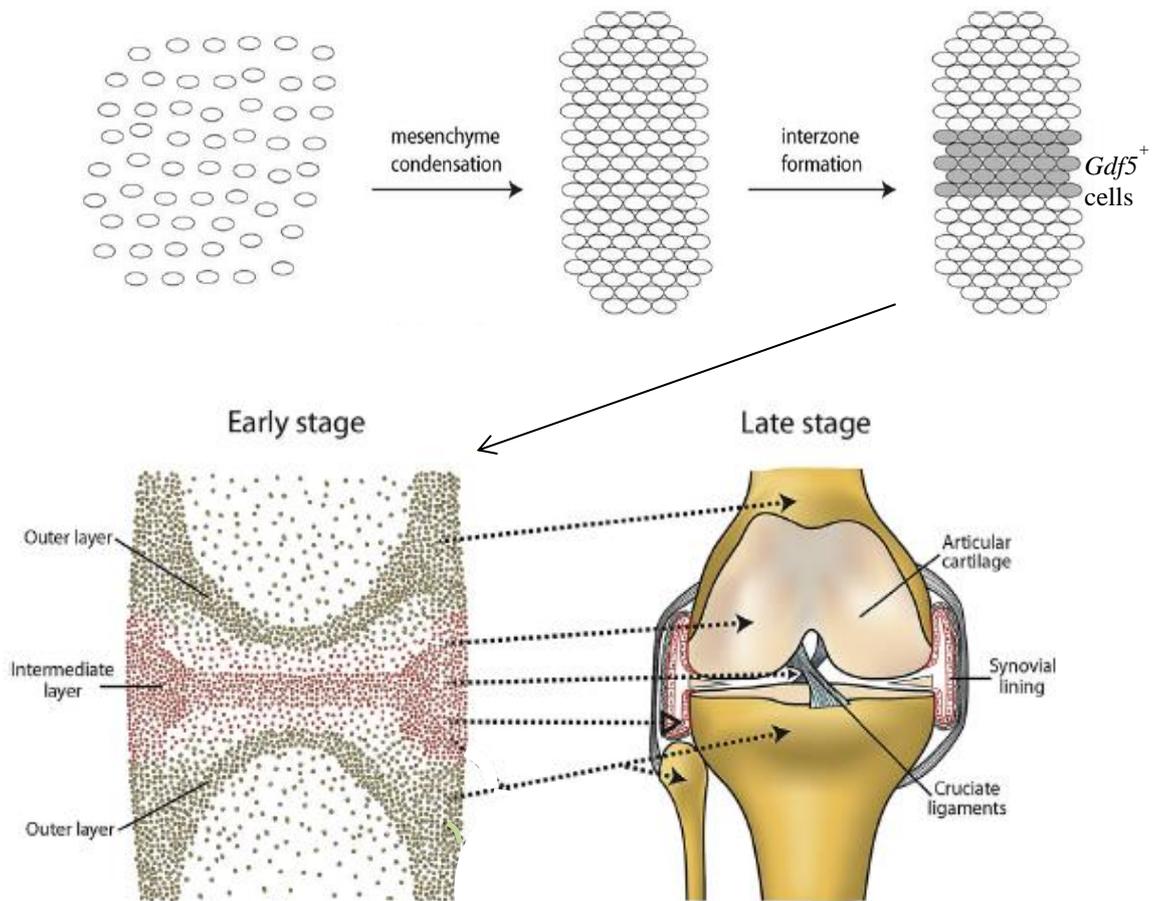
Cartilage is a highly specialized connective tissue that is composed of a single cell type called a chondrocyte which occupies only 5% of the tissue volume (Freyria & Mallein-Gerin 2012). The sparsely populated chondrocytes are embedded within a dense hydrated extracellular matrix (ECM). Depending on the biochemical composition and the molecular integrity of the ECM, there are three major type of cartilaginous tissues i.e., hyaline cartilage, elastic cartilage and fibrocartilage. The surface of the synovial joints is covered with a special type of hyaline cartilage called the articular cartilage (Mow *et al.*, 1992; Poole *et al.*, 2001; Becerra *et al.*, 2010). Apart from the synovial joints, hyaline cartilage is also found in the larynx, the trachea, the nasal septum and the sternal end of the rib cage. The cartilage found in the ears, epiglottis and the eustachian tube is more flexible due the presence of elastic fibres and hence referred to as elastic cartilage. It generally appears pale white in colour and opaque in texture. Fibrocartilage on the other hand, contains layers of thick collagen fibres in its ECM and therefore, appears to be fibrous and relatively rough. They are found in two major sites in the human body, which are the annulus fibrosus in the intervertebral discs of the spine and the meniscus of the knee (Mow *et al.*, 2004).

#### **1.3.1 Articular cartilage development**

The developmental stages of cartilage and other synovial joint structures are tightly synchronized (Archer *et al.*, 2003; Khan *et al.*, 2007). The cartilaginous skeleton arises from mesenchyme and is first recognised at about five weeks of gestational age (Glenister 1976). In the primitive mesenchyme, cells appear stellate, however during cartilage and joint development they condense and become rounded and their cytoplasm to nuclear ratio decreases. This condensation process is controlled by various factors including cell-adhesion molecules (CAMs) e.g., cadherins and N-CAMs and membrane receptors, such as CD44 (Archer *et al.*, 2003). This cell-condensation process results in the formation of pre-cartilage blastemata in which the blastema cells start secreting matrix molecules. As long as this

intercellular space/ matrix remains sufficiently pliable, the blastema continues to expand by the interstitial growth of cellular divisions. The cells at the periphery condense to form a bilaminar perichondrium whose outer layer forms a fibrous tissue while the inner layer contains the chondroblasts that are responsible for the appositional growth of the developing cartilage anlage (Glenister 1976; Koyama *et al.*, 2008). The chondroblasts undergo further divisions and form chondrocytes that secrete ECM molecules intrinsic to the cartilage type and express cartilage specific genes. Then, in a remodelling phase, chondrocytes undergo hypertrophy and express angiogenic factors that encourage vascularisation. Here, the mid-shaft or the diaphysis of the long bones develops in a process called endochondral ossification at the primary ossification centre. The cartilaginous epiphysis appears at the ends of diaphysis just before or after birth. Postnatally, chondrocytes in the epiphysis undergo hypertrophy and the matrix next to the hypertrophic chondrocytes gets invaded by vessels of the cartilage canals and forms the secondary ossification centre. With the further growth of the secondary ossification centre, the surrounding epiphyseal cartilage gets thinner (Blumer *et al.*, 2008; Caldwell & Wang 2015). Traditionally, it was believed that articular cartilage represents a remnant of epiphyseal cartilage that does not undergo endochondral ossification (Archer *et al.*, 1994). However, that idea was constantly challenged and studies now suggest that a special cell population called interzone cells may be responsible for the formation of different joint structures (Archer *et al.*, 2003; De Bari *et al.*, 2010). In the developing cartilage anlagen of the long bones, at the prospective joint site, the cells lose their rounded shapes and become elongated and express growth and differentiation factor 5 or *Gdf5*. This region of elongated cells sandwiched by cartilage anlagen is called interzone which separates into two outer layers and an inner intermediate layer (Archer *et al.*, 2003; Khan *et al.*, 2007; Iwamoto *et al.*, 2013). The cells flanking the interzone dorsally and ventrally activate the expression of TGF $\beta$  type II receptor (*Tgfb2*), while the anlagen-bound chondrocytes in the intermediate layer start expressing Matrilin-1 (*Matn1*) (Hyde *et al.*, 2007; Decker *et al.*, 2014). The chondrocytes in the flanking outer interzone region lose collagen type II and up-regulate the expression of hypertrophic markers such as collagen type X and initiate the expression of matrix

degradative enzymes. Subsequently, this region turns into bone marrow cavity with the supply of blood vessels and bone forming osteoblasts (Decker *et al.*, 2014). Cells from the intermediate layer of the interzone appear later in the cartilage epiphysis. The *Gdf5* positive, *Matn1* positive cells give rise to non-articular chondrocytes e.g., growth plate chondrocytes while the *Gdf5* positive, *Matn1* negative cells adjacent to the cartilage anlagen differentiate into articular chondrocytes (Hyde *et al.*, 2007; Decker *et al.*, 2014). A schematic representation of this process is provided in Figure 1.3.



**Figure 1.3. Development of cartilage.** A schematic representation of the stages of development of cartilage. Following mesenchymal condensation, an interzone is formed, which consists of *Gdf5* positive cells. The interzone then divides into three layers, two outer layers and an inner intermediate layer during early stages of embryogenesis. Cells within the intermediate layer that are *Gdf5*,*Matn1* negative form articular cartilage and cells that are *Gdf5*,*Matn1* positive form other joint structures such as synovial lining and ligaments. Cells in the outer layer undergo hypertrophy and form bone. Abbreviations: *Gdf5*, growth and differentiation factor 5; *Matn1*, Matrilin-1. Image adapted from De Bari *et al.*, 2010 and Caldwell *et al.*, 2015.

### **1.3.2 Articular cartilage composition and structure**

The articular cartilage lines the end of the long bones of the knee. It is a tissue with low friction and high capacity to bear load and therefore, is essential to permitting smooth movement of one bone against another (Athanasίου *et al.*, 2010). It is composed of chondrocytes that are embedded within an extracellular matrix which is aneural, avascular and alymphatic in nature. Chondrocytes receive their nutrition by diffusion through the matrix and are highly specialised in synthesizing and maintaining the ECM infrastructure (Buckwalter & Mankin 1997; Becerra *et al.*, 2010).

#### *Composition of the articular cartilage*

The articular cartilage is composed of highly specialised macromolecules and proteins that allow the tissue to functionally fulfil the mechanical demands of the articulating joints. The composition of cartilage changes as the tissue develops; however, a mature cartilage is primarily composed of a fluid phase of water and electrolytes and a solid phase of collagens, proteoglycans, non-collagenous proteins and the chondrocytes. Water constitutes about 68-85% of the wet weight of the cartilage. Water allows for a low friction gliding surface and acts as a medium for lubrication in addition to permitting load-dependent deformation. Collagens account for 10-20% of the wet weight of the tissue and proteoglycans account for 5-20% of the wet weight of cartilage (Buckwalter & Mankin 1997; Pearle *et al.*, 2005; Becerra *et al.*, 2010). The unique mechanical properties of the cartilage are attributed to the interactions of collagen and proteoglycans within a charged fluid environment (Athanasίου *et al.*, 2010). This section of the thesis will describe the biochemical composition of the articular cartilage and the importance that the structural integrity of this tissue has in its function as a mechanical surface.

### ***Water***

Approximately 30% of the water is found in the intrafibrillar space of collagen and its amounts are modulated by the diameters of the collagen fibres and the swelling pressure generated by the surrounding proteoglycans. A small percentage resides in the intracellular space and remainder is contained in the molecular pore space of the ECM and acts as the primary carrier for transporting nutrients and waste with the tissue. Inorganic ions including sodium, calcium, potassium and chloride are present in the extracellular tissue fluid (Linn and Sokoloff 1965; O'Hara *et al.*, 1990; Maroudas *et al.*, 1991). Water permeating the tissue also plays an important mechanical role. Through interactions with the proteoglycans present, water provides articular cartilage with tremendous compressive resistance. The interstitial fluid can be extruded from the tissue by applying a pressure gradient across the tissue or by the constant compressive loads the articular cartilage is exposed to. As the fluid flows through the small pores of the ECM, it causes high frictional resistance and it is this frictional resistance coupled with the pressurization of water within the ECM that gives the articular cartilage the ability to withstand high compressive loads (Williams *et al.*, 2007).

### ***Collagens***

Collagen is the most abundant protein in humans. Its critical role is to maintain structural integrity and to provide mechanical stability to various tissues. More than 20 different collagens have been identified so far consisting of at least 38 genetically distinct polypeptide chains (Thomas *et al.*, 1994; Ricard-Blum 2011). Structurally, collagens form right-handed triple helices comprising of three polypeptide  $\alpha$  chains (Ramachandran and Kartha 1954). The nomenclature for the collagens includes Roman numerals to indicate the genetic type with the  $\alpha$ -chain composition. The three  $\alpha$  chains can be homotrimers (identical) or heterotrimers (non-identical). An example of a homotrimeric chain is seen in collagen type II which makes 90-95% of the collagens found in articular cartilage, in addition to the small amounts of heterotrimeric chains of collagen type V, VI, IX and XI (Ricard-Blum 2011). The  $\alpha$  chains of collagens contain amino acid repeat motifs of Glycine-X-Y where X is often proline and Y is

often hydroxyproline. The number of repeats varies among different collagen types and this has been the reason that they have been categorized into different families. The majority of collagens in articular cartilage belong to the class of fibril-forming collagens, which include type II, type V and type XI collagen. Fibrillar collagens contain one major triple-helical domain, whereas collagen type IX, which belongs to a group called FACIT (Fibril-Associated Collagens with Interrupted Triple helices), contains several triple helical domains (Mow and Huijskes 2004). The collagen fibrils of one particular collagen subtype are frequently cross-linked with other collagens in cartilage. For e.g., collagen type II is the main collagen, however, it is frequently cross-linked to collagen IX and XI. Collagen type XI is found mostly within the fibrils and covalently cross-linked to collagen type II. Collagen type IX also extends into the matrix from the surface of type II fibrils and may interact with type IX molecules. These interactions may help in stabilising the collagen fibril meshwork (Eyre 2002). In articular cartilage, the primary function of collagens is to provide tensile strength and stiffness to the tissue. Collagen networks in the ECM of cartilage also functions to entrap large proteoglycans and consequently restrain their swelling pressure and provide compression stiffness (Mow and Huijskes 2004).

### *Proteoglycans*

Proteoglycans represent the second largest group of macromolecules in the ECM of articular cartilage. They are produced by chondrocytes and secreted into the ECM. Proteoglycans are large complex molecules composed of a core protein with one or more covalently attached glycosaminoglycan side chains (GAGs). The GAG chains are made up of negatively charged sulphate and carboxyl groups and extend out from the protein core while they remain separated from each other due to charge repulsion. The density of the fixed negatively charged sulphate and carboxyl groups is called the fixed charge density. These negatively charged groups attract cations and consequently result in a build of an osmotic gradient that draws water into the matrix and supports tissue hydration (Roughley 2006). The swelling pressure resulting from osmotic gradient created by the fixed charged density is called the Donnan osmotic pressure (Donnan 1924).

One of the major proteoglycans in articular cartilage is the large aggregating proteoglycan, aggrecan (Knudson & Knudson 2001). It constitutes as much as 80% to 90% of the proteoglycans present in articular cartilage. In addition to aggrecan, versican is another proteoglycan that belongs to the large aggregating proteoglycan family because of their ability to form large aggregates. Aggrecan is composed of a core protein to which attaches sulphated GAG chains of chondroitin sulphate and keratan sulphate. As many as 50 keratan sulphates and 100 chondroitin sulphates can attach to the core protein forming 90% of the molecular mass of the aggrecan molecule (Aspberg 2012). In articular cartilage, numerous aggrecan monomers non-covalently attach to hyaluronan in the presence of a link protein which stabilise this bond. Hyaluronan, also known as hyaluronic acid (HA) is a non-sulphated GAG and functions to interact with aggrecan and link protein to form macromolecular aggregates that are entrapped within the collagen networks (Hardingham 1981). HA also functions to link the ECM and chondrocytes through interaction with the HA cell surface receptor CD44, forming a direct link between the cells and their surrounding ECM. These aggregating proteoglycans reside in the interfibrillar space of the cartilage ECM (Heinegård 2009).

### ***Non-Collagenous matrix proteins***

Articular cartilage contains many non-collagenous proteins such as small leucine-rich proteoglycans (SLRPs), matrilins and cartilage oligomeric matrix protein (COMP), which are important for organizing and maintaining macromolecular structure of the ECM (Heinegård 2009). The SLRPs are an important set of molecules in the ECM and belong to the large family of leucine-rich proteins with multiple adjacent domains carrying a leucine-rich motif (Schaefer & Iozzo 2008). They can be classified into different sub-families based on their gene structure, the number of leucine-rich repeats and the type of GAG chain substituent. One class of SLRPs is represented by decorin and biglycan, both of which contain an 8 exon gene structure and dermatan sulphate chains. Another class of SLRPs represented by fibromodulin and lumican have a 3 exon gene structure and keratan sulphate side chains. The functions of SLRPs depend on their core protein and GAG chain (Roughley 2006). Their core protein allows them to interact with collagens during fibril formation and also help in cross-linking with other ECM molecules such as matrilins. Matrilins are multimeric proteins with three or four subunits, which have high affinity interactions with biglycan and decorin that in turn bind to collagen type VI beaded filament. Matrilin is interspersed between collagen type VI via SLRPs and collagen type II thereby interconnecting various entities of the ECM (Wagener *et al.*, 2005). Matrilin also binds to triple helical collagen domains of collagen type IX and another non-collagenous matrix protein called COMP. COMP is one of the five family members of the thrombospondin glycoproteins (Adams & Lawler 2004). COMP has five identical subunits that have the capacity to bind to five collagen molecules at the same time. This helps in bringing collagen molecules in close proximity to facilitate fibril formation. It also binds to collagen type IX and matrilin and in the adult cartilage primarily plays a role in stabilising the collagen network (Heinegård 2009).

### ***Chondrocytes***

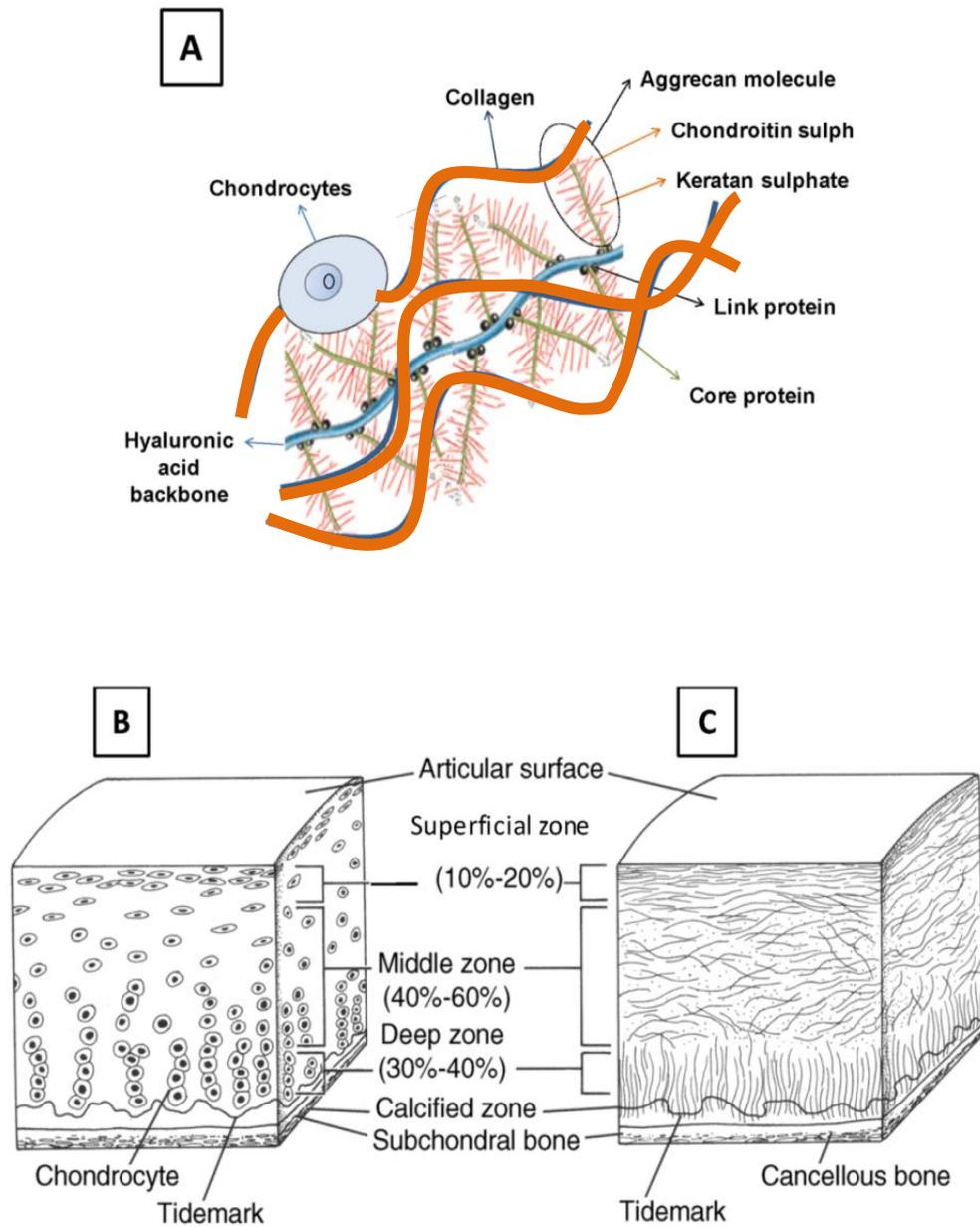
Chondrocytes are the resident cells of the articular cartilage and play a specialized role in the development and maintenance of the ECM. Their shape, number and size vary and depend on the anatomical regions of the articular cartilage. Chondrocyte physiology and its role in ECM turnover depend on a number of environmental factors such as matrix composition, growth factors and cytokines, and biophysical factors generated by the mechanical loading of the joint (reviewed by Freyria & Mallein-Gerin 2012). These specialised cells have limited potential for replication, a contributing factor for their limited intrinsic healing capacity and their survival depends on optimum chemical and mechanical environment (Wilusz *et al.*, 2014). Because cartilage is avascular, the immediate pericellular microenvironment of the chondrocytes plays an important role in regulating cell activity. Within the ECM, the chondrocytes are surrounded either as single cells or in groups by a narrow matrix called the pericellular matrix (PCM) that together with the enclosed cell is referred to as a chondron. A detailed understanding of the PCM function is currently unclear but it has been reported that PCM plays a role as a transducer of both biomechanical and biochemical signals for the chondrocytes (Guilak *et al.*, 2006). The chondrocyte primary cilium (single cytoplasmic organelle found in virtually all eukaryotic cells) extends into the PCM of the chondron and interacts with matrix molecules such as fibrillar collagen type II and non-fibrillar collagen type VI (McGlashan *et al.*, 2008). The PCM contains many of the same matrix molecular components as the ECM, however there is a distinct variation in the structure and composition of the PCM. A typical feature of the PCM is the lack of fibrillar collagens and the abundance of collagen type VI expression around the chondrocyte which co-localises with a large heparan sulphate proteoglycan called perlecan. Collagen type VI and perlecan have important functional roles in the maintenance of biochemical and biomechanical characteristics of the PCM. PCM is also rich other matrix components including fibronectin, aggrecan, HA, decorin, collagen type IX as well as cell membrane-associated molecule anchorin CII (Guilak *et al.*, 2006; Wilusz *et al.*, 2014). A normal matrix turnover is maintained through a balance between anabolic and catabolic factors secreted by chondrocytes. As chondrocytes are surrounded by PCM, all matrix

components secreted by chondrocytes must pass through the PCM. As growth factors and matrix protein pass through the PCM, they may be modified in their structure and action. It has been reported that matrilin-3 becomes pro-anabolic from anti-anabolic once it enters PCM (Vincourt *et al.*, 2012). In addition, changes in the PCM structure may have an effect on the response of enclosed chondrocytes to physical or chemical mediators. Therefore, PCM may have an important role in modulating the biochemical and biomechanical environment of chondrocytes (Guilak *et al.*, 2006). The matrix surrounding the PCM is the territorial matrix. Territorial region surrounds the PCM of single chondrocytes and in some parts the matrix of two or more chondrocytes. The fine collagen fibrils in this region form a basket-like network around the cells, which may have a protective role for the chondrocytes against mechanical stress during loading and deformation of the tissue. The interterritorial region makes up most of the volume of the articular cartilage ECM where the largest diameter of collagen fibrils and an abundant amount of proteoglycans is seen. This region consist of randomly oriented bundles of large collagen fibrils, arranged parallel to the superficial zone surface, obliquely angled in the middle zone and perpendicular to the joint surface in the deep zone (Buckwalter & Mankin 1997; Becerra *et al.*, 2010)

#### Zonal structure of articular cartilage

The ECM of articular cartilage consists of a dense stable network of collagen fibres embedded within a high concentration of a viscoelastic network of proteoglycans. The composition of the cartilage ECM varies with depth with regards to chondrocyte shape and size, differences in collagen fibre and proteoglycan network and also the water content (Figure 1.4). These differences are categorised into four layers or zones i.e. the superficial layer, the middle layer, the deep layer and the tide mark (Buckwalter *et al.*, 1988; Bhosale & Richardson 2008). Although the different zones have distinct morphological features, the borders in between the zones cannot be easily identified. However, studies have shown that the zonal organisation has functional importance (Becerra *et al.*, 2010).

The superficial zone is the thinnest zone (10% - 20% of total cartilage thickness), with the highest content of collagen and low levels of proteoglycans, although decorin and biglycan are found in higher concentrations (Fox *et al.*, 2009). This zone consists typically of two layers, a thin outer layer of fine fibrils with no chondrocytes and a layer of flattened ellipsoid shaped chondrocytes with little PCM that are oriented parallel to the joint surface. Chondrocytes here synthesize and secrete lubricin, an important joint lubricant responsible for providing a gliding surface to the articular cartilage (Flannery *et al.*, 2009). This zone is exposed to the highest tensile and compressive stresses. The parallel arrangement of collagen fibrils helps to resist shear forces generated during the joint movement. The zone next to superficial zone is the middle zone, which comprises 40% to 60% of total cartilage thickness. Collagen fibrils in this zone are less tightly packed with random orientation and have large diameters. Chondrocytes in this zone are spheroidal in shape and synthesize matrix that has high proteoglycan (aggrecan) content and low collagen and water content as compared to the superficial zone. In the deep zone (about 30% of the cartilage thickness), collagen fibres appear to be woven together into bundles and organise themselves perpendicular to the joint surface (Grogan *et al.* 2013). These bundles cross the tidemark (the border between calcified and non-calcified cartilage) to secure the attachment of cartilage onto the bone ends (Havelka *et al.* 1984). This zone contains spheroidal shaped chondrocytes with extensive PCM that align themselves in columns perpendicular to the joint surface and produce highest concentration of proteoglycans. The deepest zone is the calcified cartilage zone, which is the zone that separates the softer cartilage from the subchondral bone. The cells of this zone have a smaller volume compared to the other zones and are surrounded by calcified cartilage (Buckwalter *et al.* 1994; Fox *et al.* 2009).



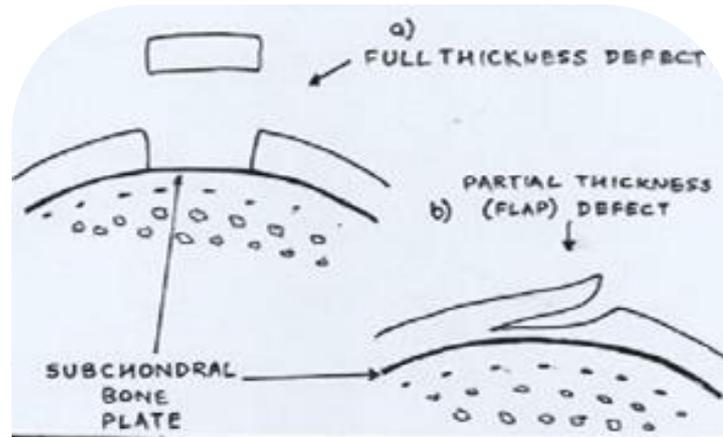
**Figure 1.4. Extracellular matrix molecules and zonal organisation of articular cartilage.** (A) Major constituents of the ECM of articular cartilage showing fibrils of collagen type II, aggrecan molecule, hyaluronic acid backbone and the chondrocytes; (B) zonal arrangement of chondrocytes; and (C) zonal arrangement of collagen fibers. Images adapted from Izadifar *et al.*, 2012 and Buckwalter *et al.*, 1994.

## **1.4 Articular cartilage defects**

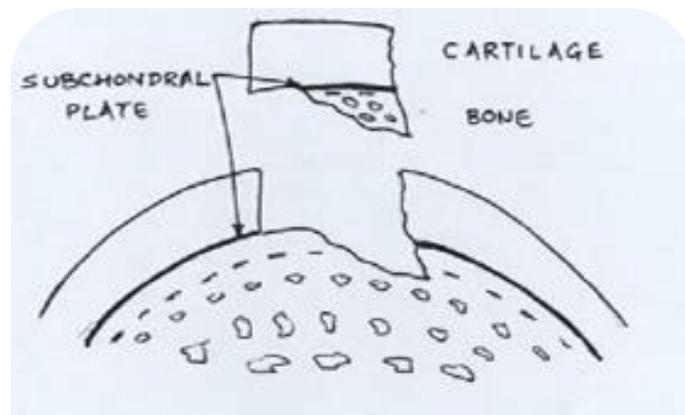
Degradation of articular cartilage can arise as a result of trauma, disease or continual mechanical loading. There are two broad categories of acute cartilage injuries: 1) direct mechanical trauma with a loss of matrix molecules without damage to the chondrocytes or the collagen meshwork; 2) blunt or penetrating trauma resulting in the mechanical destruction of chondrocytes and matrix (Bhosale & Richardson 2008; Memon & Quinlan 2012). However, these two categories can overlap as progressive loss of ECM macromolecules can cause mechanical disruption of the cartilage surface and that can further result in factors causing ECM degeneration. Acute injuries or trauma to the knee joint can cause focal damage to the cartilage, including fissures, chondral flaps or tears, and loss of a segment of articular cartilage. Because of the avascular nature of the tissue and the low number of cells present, the response to the injury is limited (Buckwalter 1998).

Depending on the depth of the defect, articular cartilage defects are classified as chondral or osteochondral. Chondral defects as the name suggest are the defects involving cartilage only, whereas osteochondral defects are the defects involving cartilage and the underlying subchondral bone. Chondral defects are further categorized as full-thickness where they extend all the way to the subchondral bone but do not penetrate it or partial thickness defects, which involves cartilage only (Redman *et al.*, 2005; Bhosale & Richardson 2008) (Figure 1.5). There are grading systems in place such as those devised by Outerbridge, ICRS and Bauer–Jackson to precisely define the defect and to approach them with suitable treatment options (Falah *et al.*, 2010). The grading system devised by Outerbridge is simple and clinically useful system to accurately describe the defects based on their location, size, shape, description of the wall and the depth of the defect (Outerbridge 1964).

(A) Chondral defects



(B) Osteochondral defects



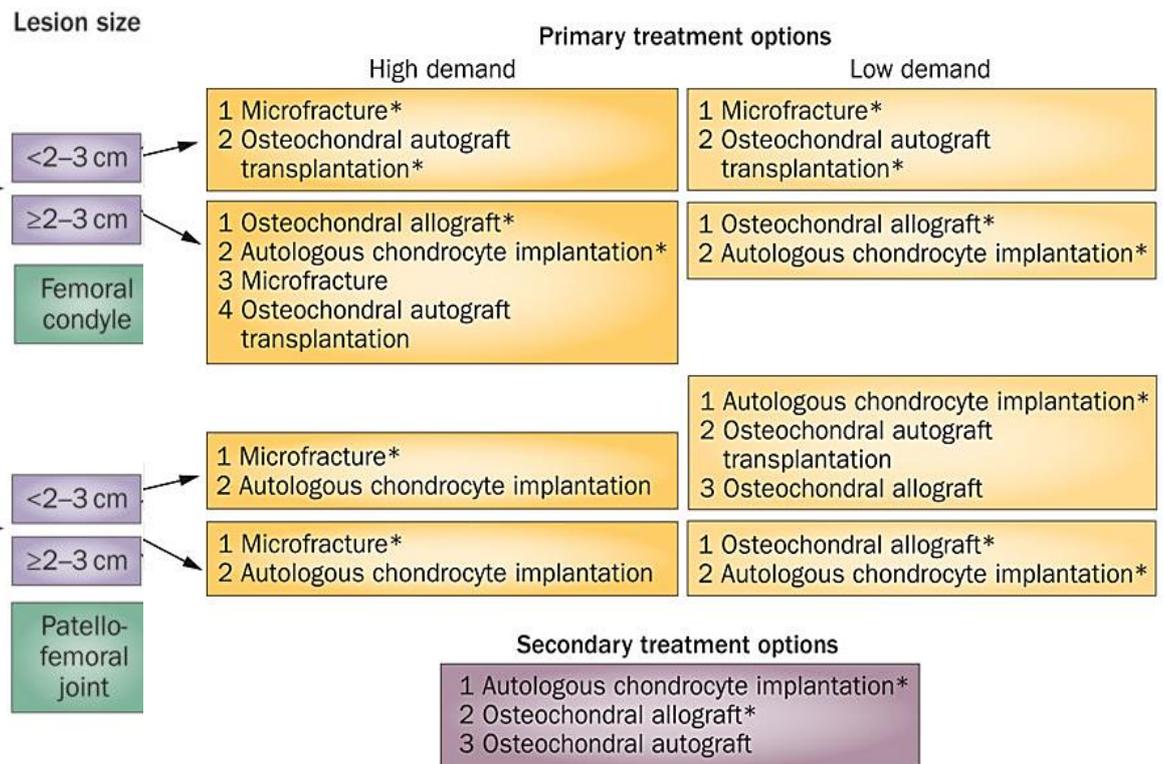
**Figure 1.5. A schematic representation of articular cartilage defects.** (A) Chondral defects can be classified as full-thickness where the defect extends down to the subchondral bone without penetrating it, or partial thickness where the damage is only reserved to cartilage; (B) Osteochondral defects damage the cartilage and penetrate the subchondral bone. Image adapted from Bhosale & Richardson, 2008.

The response to an injury of the articular cartilage depends upon numerous factors: the type of the injury, the severity of the injury, the condition of cartilage and joint as a whole at the time of the injury as well as the age of the patient (Buckwalter 1998). Immediately following a trauma, there is a structural damage to the joint tissue, which may lead to disruption in the collagen network and suppression of proteoglycan synthesis (DiMicco *et al.* 2004). Cartilage being a mechanosensitive tissue, senses the damage in the ECM by signalling the activation of chondrocyte surface receptors such as integrins, calcium ion channel and primary cilium (Vincent 2013). Articular chondrocytes and ECM work together to respond to an injury. The injury response of articular cartilage can be both catabolic and anabolic. The catabolic activity of chondrocytes includes the release of aggrecan-degrading enzyme disintegrin, metalloproteinase with thrombospondin motifs-5 (ADAMTS-5) and the collagenase matrix metalloproteinase-13 (MMP-13), which is specific to collagen type II (Burleigh *et al.* 2012). Various inflammatory mediators are also activated in injured cartilage such as interleukin (IL)-1 alpha ( $\alpha$ ), IL-6 and chemokine ligand 2 (CCL2). When the PCM is breached, various growth factors sequestered within the PCM are released for e.g. fibroblast growth factors (FGFs), connective tissue growth factor, insulin-like growth factor (IGF) and members of the TGF- $\beta$  superfamily (Vincent 2013). These growth factors participate in the catabolic and anabolic activities of chondrocytes. For e.g., FGF-2 through its interaction with fibroblast growth factor receptor (FGFR)-1, enhances ADAMTS-5 and MMP-13 activity (Ellman *et al.* 2013). This results in degradation of the damaged tissue and apoptosis mediated chondrocyte death (Kramer *et al.* 2011). In contrast, there is also evidence of an increase in the anabolic factors shortly after injury. These include chondroprotective genes such as tumor necrosis factor-inducible gene 6 protein (TSG-6), hyaluronan-binding anti-inflammatory molecule, tissue inhibitor of metalloproteinases-1 (TIMP-1) and activin A – a transforming growth factor beta (TGF- $\beta$ ) family member (Burleigh *et al.* 2012). Also, FGF-18 with FGFR-3 enhances chondrocyte viability and ECM formation (Ellman *et al.*, 2013). During the healing of articular cartilage, the growth factors, cytokines and chemokines released by chondrocytes

may cause a disruption in the balance between the catabolic and anabolic factors. Therefore, chondrocyte homeostasis is important for the quality of healing cartilage. (Kramer *et al.*, 2011)

### **1.5 Cartilage repair strategies**

Nearly three centuries ago, Hunter reported that “Ulcerated cartilage is a troublesome thing. . . once destroyed it is not repaired” (Hunter 1743). Efforts towards repairing damaged cartilage for the last few hundred years have been the major focus for scientists and clinicians in the field of cartilage regenerative medicine. Conventional treatment strategies include various medications, which give only temporary relief of symptoms rather than cure, and clinicians have sought various surgical procedures (Browne and Branch 2000). The repair techniques are mostly aimed at reducing pain in addition to restoring functionality to the tissue. The success of different treatment strategies depends on the long term performance of the knee joint along with the restoration of the damage with native-like cartilage tissue in terms of its composition and mechanical properties (Detterline *et al.*, 2005). To determine which repair approach to take, there are evidence based algorithms (Figure 1.6) depending on factors such as age, defect size and medical history (Cole *et al.*, 2009). These algorithms help to precisely define the defect and to approach them with suitable treatment options (Makris *et al.*, 2014). Current clinical treatments include arthroscopic repair procedures and cell and tissue transplantation procedures (reviewed by Matsiko *et al.*, 2013). These are described in the following sections of this thesis.



**Figure 1.6. Cartilage defect treatment algorithm.** Assessment is based on the size and the location of the lesion as well as the demand of the patient i.e. whether a more active (high demand) or sedentary (low demand) lifestyle is desired. \*First line treatment options.

Image adapted from Makris *et al.*, 2014.

### **1.5.1 Arthroscopic procedures**

Arthroscopy is a minimally invasive procedure in which an endoscope is inserted into the joint through a small incision for examination and treatment of cartilage injuries.

#### Debridement and lavage

Arthroscopic debridement and lavage is an approach whereby loose cartilage fragments that arise following an injury are removed from the joint surface (debridement) followed by an irrigation of the joint (lavage). It is mostly used for patients with less than 2 cm<sup>2</sup> defect sizes and is generally reserved for lower demand (patients with sedentary life style) older patients with limited symptoms (review by Detterline *et al.*, 2005). This procedure is believed to alleviate joint pain through an unknown mechanism, however it does not induce a repair response and it was reported that the reduction in the joint pain observed in the patients following this procedure could be no more than a placebo effect after surgery (Moseley *et al.*, 2003).

#### Microfracture

Microfracture is a reparative procedure for patients with small to moderate sized chondral lesions (1 to 5 cm<sup>2</sup>). The procedure is done arthroscopically and involves drilling 0.5-1 mm diameter holes in the subchondral bone to create bleeding within the defect, allowing cells from the bone marrow (BM) to enter the cartilage and promote healing of the defect (Steadman *et al.*, 1997; Gill and Macgillivray 2001; Steadman *et al.*, 2003). To achieve optimal results with this procedure continuous passive motion with limited weight bearing is required post-operatively (Detterline *et al.*, 2005). Clinical data at follow-ups has revealed that this procedure results in the formation of fibrocartilage that is biochemically and biomechanically inferior to hyaline articular cartilage (Mithoefer *et al.*, 2009; Saris *et al.*, 2009). In addition, due to the penetration of subchondral bone, intralesional osteophytes have been reported in 20-50% of the cases (Mithoefer *et al.*, 2009). Therefore, special indications

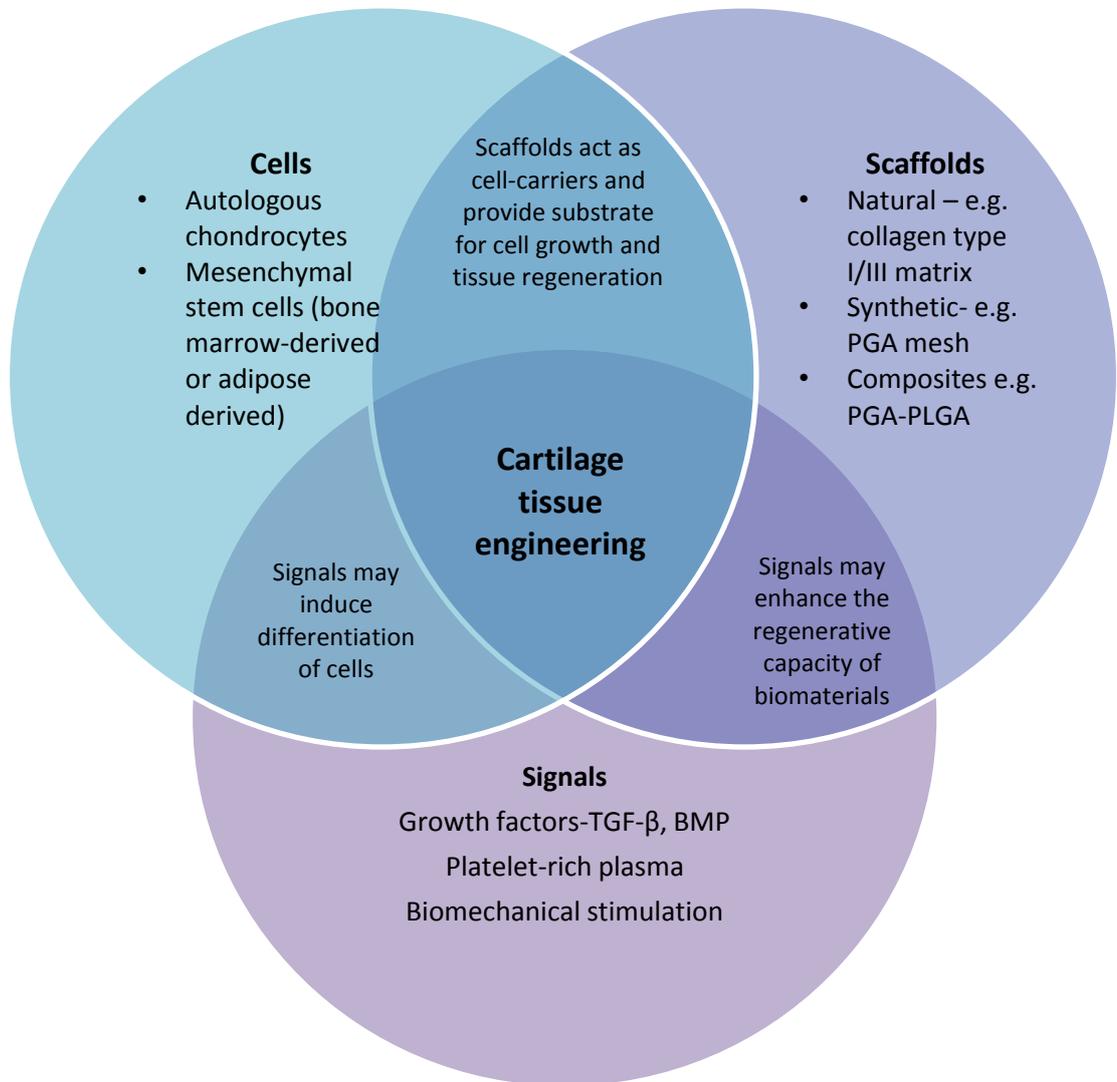
(shown in Figure 1.6) based on size, depth and location of the lesion, have been proposed for performing microfracture procedure (Makris *et al.*, 2014).

### Autografts

Treatment strategies including soft tissue grafts and osteochondral autograft transplantations have been employed clinically for intermediate to high demand patients (patients who require an active lifestyle) due to the failure of arthroscopic procedures. The soft tissue grafts can be employed in two techniques i.e., chondrocyte autograft transfer (OAT) or mosaicplasty (Memon & Quinlan 2012). In OAT, osteochondral autografts are harvested from the non-weight bearing area of the joint and placed in the prepared cylindrical defect. These autografts are round cylinders of full-thickness cartilage attached or plugged to its underlying bone. This procedure is used mainly for small to medium sized (0.5 to 3 cm<sup>2</sup>) defects as the amount of transplanting material is limited. This lead to the development of mosaicplasty in which multiple smaller sized osteochondral plugs are harvested for transplantation into the defect. Histological analysis of repair tissue following such grafting has revealed satisfactory clinical results with survival of transplanted hyaline cartilage as well as formation of fibrocartilage (Hangody *et al.*, 2008; Chow *et al.*, 2004). However there are some major limitations of this procedure including donor-site morbidity, the technical difficulty of donor-tissue grafting and the risk of joint incongruity in the case of multiple plugs (Huang *et al.*, 2004; Dettlerline *et al.*, 2005; Hangody *et al.*, 2008).

## **1.6 Tissue engineering based strategies for cartilage repair**

Currently, the standard surgical treatment for advance-stage degenerative joint pathology is total knee replacement. Early surgical interventions for cartilage defects as mentioned in the previous section of this thesis, whilst prevent further joint deterioration, they are not long-term clinical solutions (Makris *et al.*, 2014). This highlights a need for tissue engineering and regenerative medicine approaches to repair articular cartilage. Tissue engineering is an interdisciplinary field that adopts the principles of engineering and life sciences to biocompatible substitutes to restore and maintain tissue function (Langer & Vacanti 1993). Tissue engineering strategies are focused on three key aspects, which are the choice of cells, the cell carriers or scaffolds and the provision of the right signals (Figure 1.7). The cells are the key to the development and synthesis of native-like tissue. The cell-carriers or scaffolds provide a substrate for the cells to attach to and lay down matrix and potentially provide biological cues for encouraging cell differentiation. The signals help in directing differentiation of cells and assist in tissue synthesis (Matsiko *et al.*, 2013). These are described in more details in the following sections of the thesis.



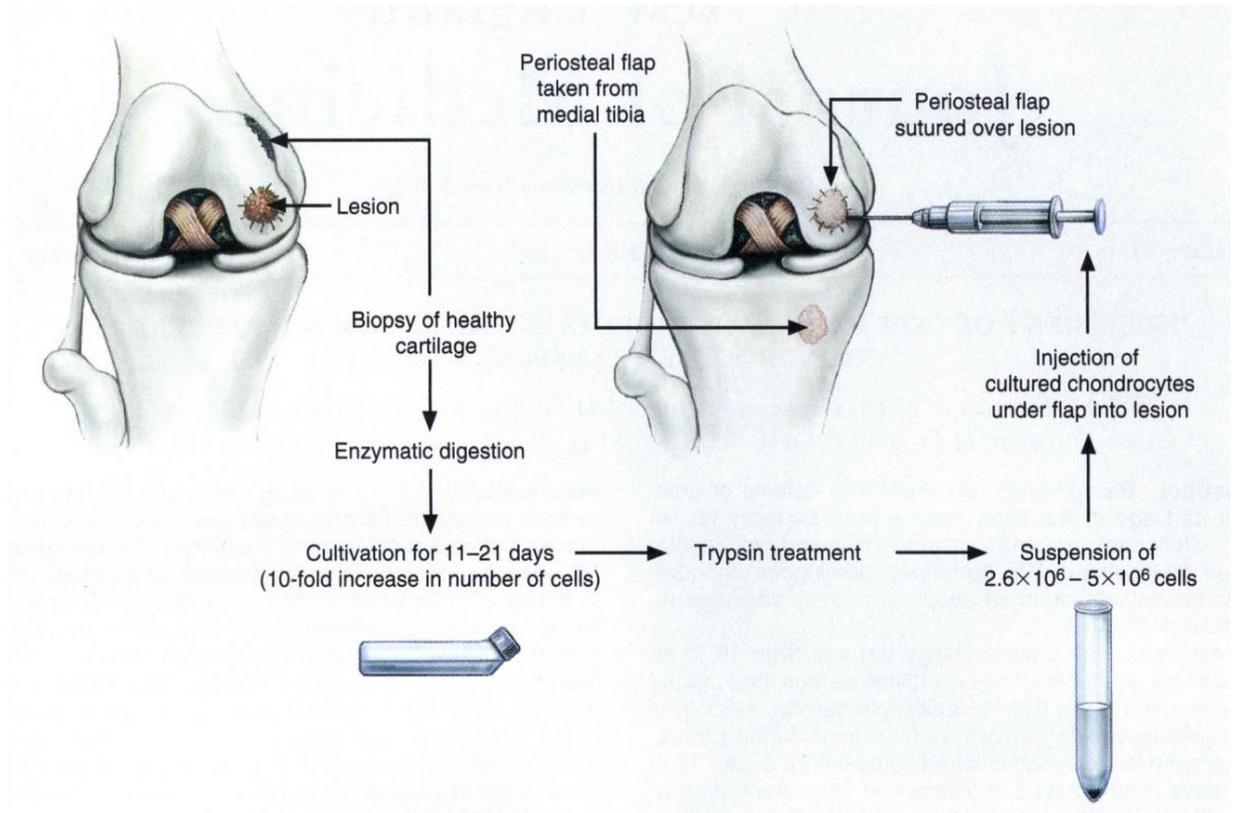
**Figure 1.7. The three key factors required for cartilage tissue engineering: cells, scaffolds and signals.** Cells, scaffolds and signals are the key elements for cartilage tissue engineering. For this purpose, many different combinations of cells, scaffolds and signals have been investigated. An example of each of the key elements is shown here. Abbreviations: TGF- $\beta$ , transforming growth factor- beta; BMP, bone morphogenetic protein; PGA, poly glycolic acid; PLGA, Poly-lactic-co-glycolic acid.

### 1.6.1 Cells for cartilage tissue engineering

Various different cell sources have been examined such as chondrocytes and adult stem cells, and in combination with scaffolds and growth factors for cartilage tissue engineering (Makris *et al.*, 2014), however a simplistic first approach for cartilage repair was to use just cell-transplants as described below.

#### Chondrocytes- Autologous chondrocyte implantation

Chondrocytes were used in a first-generation cell therapy procedure called autologous chondrocyte implantation (ACI). ACI is a two-step procedure (Figure 1.8) where during the first step a biopsy of cartilage is taken arthroscopically from the non-weight bearing area of the joint and enzymatically digested to isolate chondrocytes. The chondrocytes are then expanded in cell number by culturing the cells *in vitro* for two-three weeks to generate subsequent numbers for cell transplantation. The second step of the procedure involves an open arthrotomy to expose the defect, which is debrided to ensure the vertical circumferential walls of the defect have normal articular cartilage. A periosteal patch is harvested from the ipsilateral tibial shaft to act as a cover for the defect. The patch is sewn to the cartilage surface around the debrided defect and fixed with fibrin glue. The cultured chondrocytes are then injected under the sealed periosteal flap. This procedure was first used clinically in human patients by Brittberg and co-workers in 1987 (Brittberg *et al.*, 1994). They published the results of their first 23 patients with a mean follow-up of 39 months where good to excellent results were reported for 70% of the cases. Biopsies of the repair tissue were taken 12 months post-operation and 11 out of 15 had hyaline-like appearance. Since then a number of studies have reported that the resulting repair tissue from the ACI procedure is more durable and biomechanically similar to native tissue with better long-term outcomes than the other cartilage restoration procedures, like microfracture and mosaicplasty (Peterson *et al.*, 2010; Bentley *et al.*, 2012; Minas *et al.*, 2014). The ACI procedure is mainly reserved for intermediate to high demand patients (aged 20-50 years) who were previously unsuccessful with arthroscopic procedures. It is used for larger sized defects (2 to 10 cm<sup>2</sup>) involving both femoral condyles and the patella (Detterline *et al.*, 2005).



**Figure 1.8. Autologous chondrocyte implantation.** A biopsy of healthy cartilage from a non-weight bearing area of the knee joint is digested enzymatically to isolate chondrocytes which are then culture expanded and injected back into the defect with the help of a periosteal flap harvested from tibia. Image adapted from Brittberg *et al.*, 1994.

### ***Generations of autologous chondrocyte implantation procedures***

A range of complications are associated with the use of periosteal flaps in ACI procedures. Nearly 40% of the cases have reported symptomatic hypertrophy of the periosteal flap at one year follow-up (Gooding *et al.*, 2006). In addition, studies have reported the major complications of the procedure to be graft failure and delamination, tissue hypertrophy, chondromalacia, formation of a loose body and arthrofibrosis (Driesang and Hunziker 2000; Niemeyer *et al.*, 2008; Harris *et al.*, 2011). In the light of addressing these limitations, the traditional technique of ACI has undergone a series of major advances and developments including the use of alternative membranes to seal the cartilage defect. In 2004, Haddo and co-workers (Haddo *et al.*, 2004), reported on a small study of 31 patients treated with ACI using a porcine collagen type I/III membrane called Chondro-Gide® (Giestlich Biomaterials) instead of a periosteal flap. This study reported graft hypertrophy in only one case at 1 year follow up (Haddo *et al.*, 2004). In 2006, these results were reproduced on a larger scale by Gooding *et al.*, where they reported on 68 patients treated by ACI in which the defects were covered Chondro-Gide® (ACI-C) and compared it with defects covered with periosteal flaps (ACI-P). They reported that greater than 36.4% of the ACI-P cases showed signs of graft hypertrophy compared to none of the ACI-C cases and suggested the use of collagen membrane in future for the success of the technique (Gooding *et al.*, 2006). One year later, Steinwachs and Kreuz reported on 63 patients treated by ACI-C. Patients were evaluated at six, 18 and 36 months after surgery and showed significant improvement at all evaluation time-points. Not even a single case presented with symptomatic graft hypertrophy. Therefore, they also concluded that graft hypertrophy can be avoided by using a collagen membrane instead of periosteal flap (Steinwachs and Kreuz 2007). Despite the success of ACI-C, an open surgical procedure with sutures was still required. Furthermore, the traditional ACI-C or ACI-P techniques required the injection of chondrocytes underneath the periosteal or collagen membrane, which still risks an uneven distribution within the defect and leakage of chondrocytes (reviewed by Hunziker *et al.*, 2015). These factors lead to the development of a third generation approach called

matrix-induced autologous chondrocyte implantation (MACI), combining chondrocytes and a matrix/scaffold e.g., Chondro-Gide® (Brittberg 2010). The rationale for this approach was that the scaffold will provide a structure that would facilitate chondrocyte adhesion and expansion whilst maintaining their chondrocytic phenotype. In this procedure, the chondrocytes are seeded directly onto the porous side of Chondro-Gide® rather than being injected underneath the membrane like in the second generation ACI. Chondrocytes are pre-seeded onto Chondro-Gide® and following debridement and sizing of the defect, the cell-seeded membrane is then fixed onto the defect with the help of fibrin glue. Reported benefits of this technique are that there is no periosteal flap harvesting, no sutures are used and there is good stability of the implant. In a two-year clinical and histological follow-up study, it was reported that MACI was associated with pain relief, restoration of knee function and generated hyaline-like cartilage (D'Anchise *et al.*, 2005). Behrens *et al.*, in 2006 reported five-year follow up data after a MACI treatment and concluded that MACI was a suitable treatment for local cartilage defects of the knee joint (Behrens *et al.*, 2006). Zheng *et al.*, in 2007 also reported similar results of hyaline-like cartilage formation in 75% of biopsies from a group of 11 patients at 6 months follow-up following MACI (Zheng *et al.*, 2007). In a review by Brittberg on the MACI procedure, it was suggested that MACI is a safe and clinically effective procedure for the treatment of moderate to large symptomatic cartilage defects. The data reviewed is also suggestive of hyaline-like tissue repair with this procedure (Brittberg 2010).

In spite of the successful clinical outcomes of ACI, ACI-C and MACI, a major disadvantage of these techniques is that the surgical intervention requires a two-step procedure which requires long-term patient planning and costly cell-culture expansion. Not only this, the quantity of tissue that can be harvested as a biopsy to isolate chondrocytes is limited due to the risk of creating new cartilage defects (reviewed by Hunziker *et al.*, 2015). Although a small biopsy is taken, studies have reported that it could heighten the risk for osteoarthritis (OA) (Lee *et al.*, 2000; Hjelle *et al.*, 2002). Moreover, chondrocytes lose their chondrogenic phenotype during the *in vitro* culture expansion step in a process termed as

chondrogenic dedifferentiation. This causes several changes in the synthesis pattern implying a shift from production of type II collagen to type I collagen, a typical marker of fibroblast like cells such as tendon and skin (Benya *et al.*, 1978; Benya and Shaffer 1982). Consequently, the need for alternative cell sources for cartilage repair has been widely investigated and the following sections will explore the potential cell types for this application.

### Mesenchymal stem cells

As stated above, one of the main hurdles for successful ACI is obtaining vital and differentiated chondrocytes. Due to the pre-requisite expansion of chondrocytes in culture, they are forced to give up their round shape and attain a fibroblast-like morphology with a loss of their chondrogenic potential (von der Mark *et al.*, 1977; Benya & Shaffer 1982). Although some studies have shown that chondrocytes can re-differentiate *in vitro* with (Barbero *et al.*, 2003) and without (Anderer and Libera 2002) the addition of growth factors, the need for an alternative cell source with stable chondrogenic potential becomes necessary. An undifferentiated progenitor cell that possesses chondrogenic differentiation potential would be ideal for cartilage tissue engineering. Here, multipotent mesenchymal stem or stromal cells (MSCs) present themselves as a promising cell source (Caplan 1991; Caplan & Goldberg 1999).

MSCs are a heterogeneous subset of stromal cells that can be isolated from various tissues. The presence of regenerative cells in BM was first hypothesized by Cohnheim who believed that these regenerative cells were involved in wound healing throughout the body (Cohnheim 1867). In the 1960s and 1970s, Friedenstein *et al.*, pioneered the methods of isolation of BM-derived stromal cell by adherence to tissue culture plastic and reported their colony forming capacity, i.e. an ability to form colonies of fibroblasts or CFU-F (colony forming unit-fibroblast) after cell mitosis on the culture dishes, and their role in bone formation (Friedenstein *et al.*, 1966; Friedenstein *et al.*, 1970; Friedenstein *et al.*, 1976). Since then numerous groups have further investigated the potential of MSCs in regenerative medicine. Following their successful identification in the BM, human MSCs were isolated from various other tissues, including adipose tissue, umbilical cord and peripheral blood (reviewed by Hass *et al.*, 2011). *In vitro* and preclinical animal studies suggest that MSCs can provide an alternative to autologous chondrocytes for the regeneration of cartilage, as they possess chondrogenic differentiation potential, are obtainable from a number of tissue sources

and can be culture expanded *in vitro* to provide increased cell numbers for transplant therapies (Mitchell and Shepard 1976; Caplan and Goldberg 1999; Pittenger 1999).

A stem cell is an unspecialized progenitor cell that can self-renew or multiply whilst maintaining its potential to differentiate into other cells with specialised functions. These cells reside in tissue niches from where they can be recruited to replenish specific tissue cells that have died (Caplan and Dennis 2006). Stem cells are found in the embryo known as embryonic stem cells, as well as in adults known as adult stem cells. Embryonic stem cells are pluripotent in that they have the potential to form cells of all the three germ-layers, i.e. endoderm, ectoderm and mesoderm (Doss *et al.*, 2004). Adult stem cells, on the other hand, are tissue specific as they have limited differentiation potential and produce only a set of specialised cells of a particular tissue (Pittenger 1999).

The term MSCs was coined by Arnold Caplan in 1991 where he described how these MSCs divide and their progeny becomes committed to a particular, distinctive phenotypic pathway (Caplan 1991). In 1999, Pittenger and co-workers demonstrated their ability to differentiate down the three mesodermal lineages of osteoblasts, adipocytes and chondrocytes (Pittenger 1999). Since then, MSCs have been extensively studied as an alternative cell source for tissue engineering. Whilst Caplan popularised the term MSCs, some researchers refrained the reference to a stem cell identity when publishing pre-clinical or clinical results in the late 1990s (Horwitz & Keating 2000). In 2000, at the meeting of the International Society for Cellular Therapy (ISCT), many leading investigators concluded that convincing data to support the 'stemness' of the unfractionated plastic adherent cells was lacking. Thereafter, Horwitz *et al.*, published a position statement to address the inconsistency between nomenclature and the biologic properties of these cells (Horwitz *et al.*, 2005). They suggested that the multipotent plastic adherent cells derived from mesenchymal tissues should be termed mesenchymal stromal cells, regardless of the tissue origin. The term stem cell should be used to describe a subset of cells showing distinct characteristics, including (1) adherence to tissue culture plastic; (2) multipotent differentiation potential and (3) their ability to self-renew.

Nonetheless, the terminology has persisted and the acronym MSC is used to describe the mesenchymal stem or stromal cells that are isolated from mesenchymal tissues and have the multipotent differentiation capacity (Horwitz *et al.*, 2005).

MSCs are known to secrete an array of trophic factors such as cytokines and growth factors which influence cellular activity from cell survival, growth and differentiation to immunological response. They express mitogenic proteins such as transforming growth factor-alpha (TGF- $\alpha$ ), TGF- $\beta$ , hepatocyte growth factor (HGF) and FGF (Haynesworth *et al.*, 1996). It has been hypothesized that the expression of and reaction to these cytokines and growth factors is dependent on an individual's genotype giving credence to the philosophy of personalized medicine (Murphy *et al.*, 2013). Studies have also shown that MSCs can modulate immune responses *in vitro* by secreting factors that inhibit interleukin-1 (IL-1), IL-2, IL-12, tumor necrosis factor-alpha (TNF- $\alpha$ ) and interferon-gamma (IFN- $\gamma$ ) secretion (Beyth *et al.*, 2005; Iyer and Rojas 2008; Pittenger 2008). In an inflamed environment, MSCs are shown to have anti-inflammatory effects by restoring the T helper cells 1(T<sub>H</sub>1)/T<sub>H</sub>2 balance through inhibiting INF- $\gamma$  secretion and increasing IL-4 and IL-10 (Aggarwal and Pittenger 2005). These properties of MSCs offer a great advantage for their utilization in regenerative medicine and could have a considerable impact on the future of tissue engineering interventions (Shi *et al.*, 2010).

### ***Mesenchymal stem cells from bone marrow***

Amongst the various tissue sources, BM derived MSCs are the most extensively studied and investigated both *in vitro* and *in vivo*. Since the work of Johnstone *et al.*, Pittenger *et al.*, and Barry *et al.*, it is well-known that BM MSCs can undergo chondrogenesis *in vitro* through the induction of a cocktail of growth factors (Johnstone *et al.*, 1998; Pittenger 1999; Barry *et al.*, 2001). MSCs derived from the BM in humans are mainly isolated from the iliac crest. They are an attractive source for cartilage tissue engineering due to their ability to expand to high numbers *in vitro* as well as their potential towards chondrogenic differentiation (Boeuf & Richter 2010). However, the current term defining MSCs does not represent a homogeneous

population of cells. MSCs are a heterogeneous population of cells and their frequency of CFU-F is approximately 1 per 10,000- 100,000 mononuclear cells in BM (Friedenstein *et al.*, 1976). This number varies from donor to donor and declines with age. A newly born child has approximately 1 MSC per 10,000 cells, a teen approximately 1 in 100,000 and a 50 year old adult, 1 in 400,000 BM mononuclear cells (Caplan 2007). Due to the heterogeneity in mononuclear cell populations, the ISCT had listed three main criteria for cells to be regarded as MSCs, these are; their ability to adhere to plastic, their ability to differentiate into osteoblasts, adipocytes and chondrocytes and their expression of CD73, CD90 and CD105 (Dominici *et al.*, 2006).

Proof-of-concept for the use of BM MSCs for cartilage repair has been shown in various animal models of chondral and osteochondral defects and has showed improved repair of the defects when compared with the no MSC control groups (Wakitani *et al.*, 1994; Im *et al.*, 2001; Guo *et al.*, 2004; Koga *et al.*, 2008). Moreover, BM cells have already been employed in clinical trials where the cells have either been directly used as mononuclear cell concentrates (Wakitani *et al.*, 2002; Gigante *et al.*, 2011; Gobbi *et al.*, 2011; Skowroński *et al.*, 2013) or the MSCs have been culture expanded and pre-seeded on to cell-carriers or scaffolds to contain the cells with subsequent transplantation into the defects (Adachi *et al.*, 2005; Haleem *et al.*, 2010; Kasemkijwattana *et al.*, 2011). Most of these cases reported on clinical outcomes at follow-ups and where histological assessment was made, a mixture of hyaline-like and fibrocartilaginous repair tissue has been observed. In 2007, Kuroda *et al.*, treated a 31 year old judo player suffering from a full-thickness cartilage defect with autologous BM MSCs embedded within a collagen gel. At seven months follow-up, histology of the repair tissue revealed hyaline-like tissue and the patient reported significant improvement in clinical symptoms (Kuroda *et al.*, 2007). In a study of 72 patients, BM MSCs were compared to first generation ACI procedure and the results have been reported to show that BM MSCs are as effective as chondrocytes, although the use of BM MSCs is cost-effective with no damage to the normal articular cartilage (Nejadnik *et al.*, 2010). Despite the

success of clinical outcomes, BM MSCs are not fully-established for their use as candidate-cell types for cell-based cartilage repair treatments. The painful iliac crest biopsies accompanied with donor site morbidity as well as the limited number of MSCs in the BM are some of the hurdles in their successful therapeutic application (reviewed by Ahmed and Hincke 2014). In addition, it has also been shown that the chondrogenic differentiation potential of rat MSCs decline with age (Zheng *et al.*, 2007). This may have a major impact on the therapeutic capacity of autologous MSCs as a majority of orthopaedic pathologies present in older individuals in humans.

### ***Mesenchymal stem cells from adipose tissue***

Over the last decade, it has been recognised that fat, apart from being an energy reservoir is also a rich source of multipotent stem cells (Baer & Geiger 2012). Similar to BM, adipose tissue (AT) is also derived from embryonic mesenchyme and consists of a stroma that can be easily harvested (Ugarte *et al.*, 2003). The initial method to isolate adipose MSCs was first described by Rodbell and co-workers in the 1960s where they minced rat fat pads, digested the minced tissue with collagenase and centrifuged the digest to separate mature adipocytes from the pelleted stromal vascular fraction (SVF). The SVF is a heterogeneous population of cells including blood cells, fibroblasts, pericytes, endothelial cells as well as pre-adipocytes and adipocyte progenitors (Rodbell 1966). Subsequently, this method was modified for the isolation of human AT mesenchymal stem or stromal cells (AT MSCs) (reviewed by Bunnell *et al.*, 2008). Initially, the AT was minced manually, however with the advent of the liposuction technique, this procedure has been simplified. During this procedure, the AT is infused with a saline solution via a cannula and the tissue is harvested with the saline solution under suction (Illouz 1983). In 2001, Zuk and co-workers described a putative population of multipotent stem cells that they termed processed lipoaspirate cells or PLA cells due to their derivation from processed lipoaspirate tissue obtained through cosmetic surgery (Zuk *et al.*, 2001). These cells were shown to be capable of differentiating into adipocytes, osteoblasts and chondrocytes upon induction with specific growth factors (Zuk *et al.*, 2002). Nonetheless, a

multipotent adherent stromal cell population can be generated from liposuction tissue as well as from the SVF of manually minced AT (Schreml *et al.*, 2009).

There has been some confusion with regards to the nomenclature of the plastic adherent cell population from collagenase digested AT. Various terms have been used to describe the same population of cells including adipose-derived stem/stromal cells (ASCs), adipose derived adult stem cells (ADAS), adipose derived stromal cells (ADSC), adipose stromal cells (ASC), adipose mesenchymal stem cells (AdMSC) and processed lipoaspirate (PLA) cells (Bunnell *et al.*, 2008). Due to the variation in the terms used to describe the same population of cells, the International Fat Applied Technology Society (IFATs) reached a consensus to adopt the term “adipose-derived stem cells” (ASCs) to identify the plastic-adherent multipotent cell population (Gimble *et al.*, 2007). As per the ISCT, the term stem should be used for cells that meet a certain criteria including, adherence to plastic, ability to differentiate into different cell type and the ability to self-renew long term *in vivo*. Despite the absence of data confirming the last characteristic, the IFATs conference adopted the term ASC, or Adipose-derived Stem Cell, to describe plastic-adherent, cultured multipotent stromal cells isolated from the SVF (Bourin *et al.*, 2013). Throughout this thesis, the acronym AT MSCs will be used to describe mesenchymal stem or stromal cells isolated from AT. Furthermore, the ISCT recently proposed criteria to define AT MSCs similar to BM MSCs in that, they should be plastic adherent and should differentiate down the three mesodermal lineages as well as display CD profile negative for haematopoietic markers (CD11b and CD45) and positive for stromal cell markers such as CD13, CD73 and CD90 (Bourin *et al.*, 2013).

The application of AT MSCs for tissue engineering purposes has a number of advantages compared to BM MSCs in that AT contains a large number of multipotent cells, a necessary pre-requisite for cell-based therapies. It has been shown that the multipotent stromal cells in the SVF of AT constitutes up to 3% of the mononuclear cells, which is 2,500 fold higher than the frequency of MSCs in the BM (Fraser *et al.*, 2008). Other studies have also

reported on similar findings regarding the higher numbers of MSCs obtained from AT compared with BM. Approximately 6 million mononuclear cells are present per millilitre (ml) of BM aspirate, of which only 0.001- 0.01% are MSCs. In contrast, in a gram of AT, approximately 0.5-2 million mononuclear cells are present, of which 1 to 10 % are MSCs (Ugarte *et al.*, 2003; Aust *et al.*, 2004; Zhu *et al.*, 2008). The percentage varies from donor to donor, the age of the donors and on the tissue harvesting procedure (Oedayrajsingh-Varma *et al.*, 2006; Choudhery *et al.*, 2014). In addition, the adipose depots can be found ubiquitously in human body and are easily accessible with minimally invasive procedures. Liposuction is a safe and well-tolerated procedure yielding large quantities of tissue. It is also cheaper and less-invasive than BM aspiration for MSC isolation. In addition, AT harvested during cosmetic surgeries or other procedures is generally regarded as a medical waste product, hence representing a readily available source for stem cell isolation for tissue engineering purposes (Bunnell *et al.*, 2008).

Various animal studies have provided the proof-of-concept for the potential of AT MSCs for cartilage repair *in vivo*. Studies in rabbits of full-thickness osteochondral defects have shown promising results when transplanted with AT MSCs compared to the control groups (Nathan *et al.*, 2003; Masuoka *et al.*, 2006; Dragoo *et al.*, 2007). Some studies have reported the inferior chondrogenic potential of AT MSCs when compared with BM MSCs (Koga *et al.*, 2008; Im and Lee 2010; Li *et al.*, 2011). Nonetheless, according to the most recent systematic review performed by Perdisa *et al.*, all 28 preclinical animal studies investigating the potential of AT MSCs for cartilage repair have reported that AT MSCs have a regenerative effect on cartilage repair (Perdisa *et al.*, 2015). A few human case reports have also been published for the use of AT MSCs in degenerative joint pathologies including chondromalacia patellae and OA. Most of the human trial studies have used the intra-articular injection of freshly isolated SVF cells in combination with growth factors such as platelet rich plasma (PRP) and hyaluronic acid (HA). Follow ups from three to 30 months have reported significant improvement in functional outcomes as well as pain relief (Pak 2011; Koh and

Choi 2012; Pak *et al.*, 2013; Bui *et al.*, 2014; Koh *et al.*, 2014). The site of AT harvest for these studies has either been subcutaneous AT from the abdominal area or the buttocks or the infrapatellar fat pad in the knee joints. Infrapatellar fat pad is also a promising source of multipotent cells, particularly advantageous for orthopaedic applications (Khan *et al.*, 2008). These AT MSCs have shown potential for cartilage tissue engineering due to their chondrogenic differentiation potential, ease of harvest during knee surgeries and limited morbidity to patients (Dragoo *et al.*, 2003; Buckley *et al.*, 2010). Moreover, it has been shown that intra-articular injection of AT MSCs isolated from infrapatellar fat pad results in pain relief and an improvement in knee functions in patients suffering from knee OA (Koh and Choi 2012; Koh *et al.*, 2013). Furthermore, only a single study of 18 patients has been published to date, to test the safety and efficacy of culture expanded autologous AT MSCs for knee OA. Patients were injected with low-dose ( $1.0 \times 10^7$  cells), mid-dose ( $5.0 \times 10^7$ ), or high-dose ( $1.0 \times 10^8$ ) of AT MSCs. The results at 6 months follow-up showed improved function and pain of the knee joint with histology of the repair tissue revealing hyaline-like tissue in patients receiving high dose of AT MSCs (Jo *et al.*, 2014).

#### ***Other sources of mesenchymal stem cells***

With the increasing evidence of MSCs found in various tissues, the question arises as to which cell source is the most advantageous for cartilage repair? Whilst a single ideal cell source has not yet been defined, a few noteworthy studies point towards the use of BM MSCs compared to AT MSCs (Im *et al.*, 2005; Jakobsen *et al.*, 2010; Strioga *et al.*, 2012). In addition, MSCs from synovial tissue (ST MSCs) have also been reported to have multipotent progenitor cells that have considerable potential to synthesize cartilage matrix *in vitro* (De Bari *et al.*, 2001). They express MSC surface markers and have the ability to maintain linear growth over 30 population doublings (reviewed in Fan *et al.*, 2009). A study comparing the different cell sources *in vitro* including, synovium, BM, AT, periosteum and muscle tissue have demonstrated the superiority of ST MSCs regarding their proliferation and chondrogenic differentiation (Yoshimura *et al.*, 2007). Another study in rabbits reported that transplanted

autologous BM and ST MSCs gave rise to a significantly higher *in vivo* chondrogenic response than AT MSCs and muscle-derived MSCs (Koga *et al.*, 2008). The reason for enhanced chondrogenic potential of ST MSCs over other MSCs is not fully understood; however, it has been shown that synovium tissue may be a tissue-specific stem cell niche for cartilage repair since the healing response of the meniscus and articular cartilage tissue may be largely attributed to the synovium (Hunziker and Rosenberg 1996). There is also growing evidence that a population of progenitor cells exists within the articular cartilage. These progenitor cells play a role in supporting appositional cartilage growth from the surface. Although, the need to harvest large amounts of tissue i.e cartilage or synovium to obtain sufficient cell number is a major drawback of these cell sources (Dowthwaite *et al.*, 2004).

#### Freshly isolated versus culture expanded cells

Despite the successful clinical outcomes using chondrocytes or MSCs, these techniques are limited in their clinical use due to the need for two operations. In addition, the success of MSCs for cartilage repair relies not only on the intrinsic capability of the cells but also on the interaction between a cell and its surrounding microenvironment. It is also well established that MSCs secrete bioactive factors that are immunomodulatory and have regenerative capacity (Somoza *et al.*, 2014). As per this paradigm, it was proposed that MSC selection and culture expansion may not be necessary, allowing the development of a one-step procedure (Giannini *et al.*, 2009). Furthermore, the *in vitro* culture and expansion of MSCs could be associated with the risk of trans-differentiation or reprogramming of the cells as well as the possibility of infection, contamination and spontaneous transformation (Song & Tuan 2004; Herberts *et al.*, 2011; Neri *et al.*, 2013). These considerations prompted the use of autologous BM aspirate concentrate (BMAC) for the treatment of chondral defects. The rationale for this approach was that the concentrated BM would not only contain MSCs but also other precursor and accessory cells as a source of regenerative tissue (Cavallo *et al.*, 2013). In 2009, Giannini *et al* were the first to use such an approach for the treatment of talar osteochondral lesions (Giannini *et al.*, 2009). They concentrated the BM aspirate using a commercially available

system and combined it with collagen powder or hyaluronic acid membrane and platelet gel. No control group was used in this study. The results at a mean follow up of 29 months showed functional improvement with both scaffolds and the histology of the biopsy revealed hyaline-like cartilage. They concluded that although longer term follow-up is needed to ensure the validity of the repair over-time, the one-step arthroscopic technique is advantageous when compared with previous techniques (Giannini *et al.*, 2009). An animal study of full-thickness chondral defects in minipigs compared culture expanded BM MSCs with freshly isolated mono-nuclear cells (MNCs). The result at 8 weeks showed no significant difference between the repair tissue of the two cell-treated groups which led them to conclude that MNCs may be more economical and convenient for cartilage repair in clinical applications as they obviate the need for culture expansion (Zhang *et al.*, 2011). At the same time, Gobbi *et al.*, published clinical results of 15 patients suffering from chronic large full-thickness cartilage lesions (Gobbi *et al.*, 2011). In this study, the BM aspirate was concentrated and using batroxobin enzyme was transformed into a sticky clot. The clot was pasted into the defect and covered with collagen-based membrane and fixed in place with fibrin glue. At 6, 12 and 24 months follow-up, patients showed significant improvement in functional scores and histology revealed hyaline-like cartilage (Gobbi *et al.*, 2011). Although the technique of BMAC is utilized in these clinical studies, only a limited *in vitro* data on chondrogenesis is currently available to support its use in clinical practice (Kasten *et al.*, 2008; Cavallo *et al.*, 2013).

The use of freshly isolated cells from the SVF of AT (AT SVF) cells is more established than that of the MNCs from BM. Most of the clinical studies reported have tested the uncultured freshly isolated MSCs from the SVF in combination with PRP for knee OA and reported an improvement in clinical outcomes (reviewed by Perdisa *et al.*, 2015). The concept of using AT SVF cells was already tested *in vitro* and showed promising results confirming the ability of AT SVF cells to adhere to a poly (L-lactic-co-epsilon-caprolactone) scaffolds in a short time, and their capacity to undergo chondrogenic differentiation (Jurgens *et al.*, 2009; Jurgens *et al.*, 2011). The safety and feasibility of this technique was further tested for the

treatment of osteochondral defects *in vivo*. Here, the authors compared freshly isolated SVF cells with culture expanded AT MSCs seeded on collagen I/III scaffolds and showed that after 1 month and 4 months post-transplantation in goat models of osteochondral defects, similar regeneration was found between freshly isolated and culture expanded cells (Jurgens *et al.*, 2013).

#### *Pre-culture selective isolation of mesenchymal stem cells*

Human MSCs from different tissue sources constitute a heterogeneous population of cells comprising sub-populations each having its own particular phenotype and functional characteristics. These sub-populations presenting different cell surface markers may parallel their stage of differentiation, their location within the tissue niche and their ultimate functional fate (Somoza *et al.*, 2014). Therefore, current research in the field of cartilage tissue engineering is also focussing on identifying markers to select sub-populations from the pool of MSCs that may have higher chondrogenic potential compared to the uncultured heterogeneous population (Battula *et al.*, 2009; Arufe *et al.*, 2010). Pre-culture identification markers would ensure higher purity of chondrogenic MSCs than that obtained from plastic adherence and could potentially be used in a one-step procedure for cartilage repair. It has been shown that cells isolated using CD44, CD105 and CD29 as markers have greater chondrogenic potential over other MSC populations (Rada *et al.*, 2011). In addition, it was also shown that isolating MSCs that are positive for the CD271 marker express higher levels of collagen type II and aggrecan compared with CD73 and CD106 positive MSCs (Arufe *et al.*, 2010). A recent *in vivo* study confirmed these *in vitro* findings for the potential of CD271 positive cells to show therapeutic superiority over cells selected by plastic adherence alone (Mifune *et al.*, 2012). CD271 has been increasingly used as specific marker for the characterization and purification of human BM MSCs (Flores-Torales *et al.*, 2010). It is also known as the low-affinity nerve growth factor receptor (LNGFR), nerve growth factor receptor (NGFR), or neurotrophin receptor (p75 NTR). In 2002, Quirici and co-workers reported that anti-CD271 antibody is specific for a population of multipotent cells and suggested the use of this marker for selective

isolation of MSCs from BM (Quirici *et al.*, 2002). Work by Jones *et al* has since confirmed these findings and have demonstrated consistently that CD271 antigen (followed by CD146, CD106, D7-FIB, CD13 and CD166) is the most selective marker for the enrichment of multipotent progenitor cells from BM (Jones *et al.*, 2002; Jones *et al.*, 2006; Jones and McGonagle 2008). Numerous other groups have also published similar results supporting the idea of CD271 as a marker to selectively isolate MSCs from BM (Bühring *et al.*, 2007; Flores-Torales *et al.*, 2010; Kuçi *et al.*, 2010). Despite the availability of CD271 as a marker of MSCs from BM, which could potentially be used in a one-step treatment for orthopaedic pathologies, a major limitation for its use in autologous therapies is that both the number of MSCs and their differentiation potential decreases with age (Stolzing *et al.*, 2008). AT may be better suited for this purpose. AT MSCs were first isolated using P75NTR/ CD271 antibody in mice (Yamamoto *et al.*, 2007). Following this Quirici *et al*, isolated CD271 positive MSCs from AT in a similar way as had been done from BM. They reported that CD271 positive MSCs from AT showed higher clonogenic and differentiation potential compared to MSCs isolated with plastic adherence alone (Quirici *et al.*, 2010). Furthermore, researchers investigated the relationship between age and the CD271<sup>+</sup> cell yield and they found that the highest cell yield was seen in donors that were 30 to 40 years old; in addition, although the number of CD271<sup>+</sup> MSCs decline with age, the ability to obtain these cells was maintained through all age ranges with a yield higher than that reported from BM. Therefore, the authors proposed the use of CD271 positive MSCs as the primary choice for autologous cell-based therapies in older patients (Duran *et al.*, 2013). These findings prompted researchers to isolate CD271 positive MSCs from other sources including synovium, umbilical cord and placenta (reviewed by Álvarez-Viejo 2015). However to date, only one pre-clinical animal study has been published to show the potential of CD271 positive MSCs for cartilage repair (Mifune *et al.*, 2012). This highlights the need for further *in vivo* investigation into the use of CD271 as a marker for cell-based therapies.

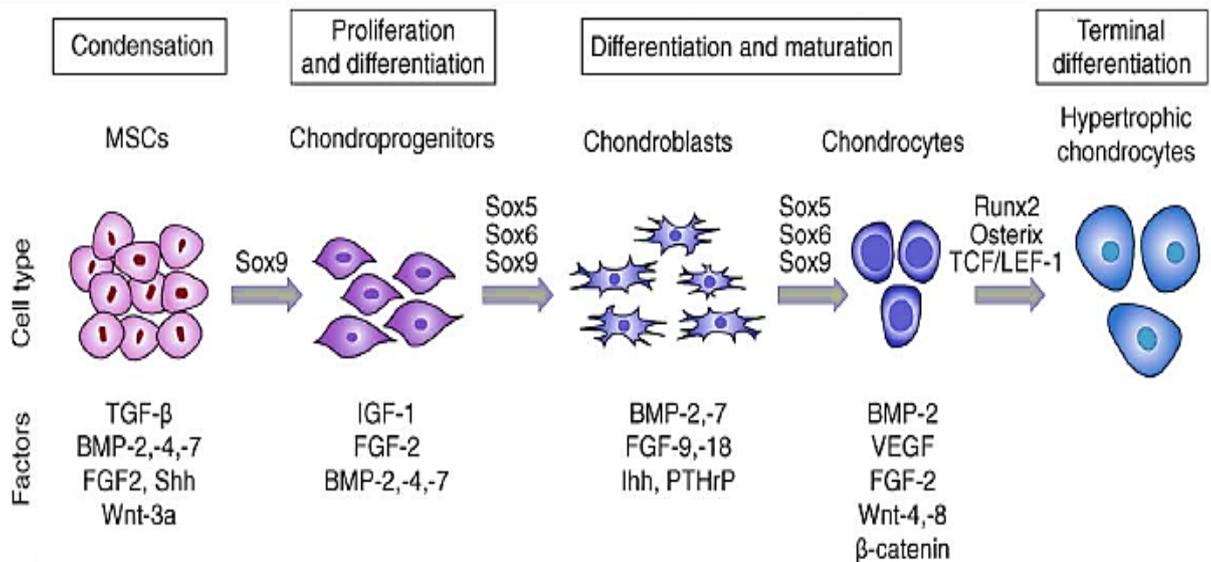
### 1.6.2 Signals for chondrogenesis

Present knowledge and success of *in vitro* differentiation of MSCs is based on the understanding of the mechanism of cartilage formation and its regulation *in vivo*. Chondrogenesis in the limb bud is initiated by sonic hedgehog signalling, which induces bone morphogenetic proteins (BMPs) to induce MSCs towards chondrogenesis (Lefebvre & Smits 2005). One of the earliest markers expressed in the mesenchymal condensations is sex determining region-Y-box 9 (Sox 9). Sox9 is one of the key transcription factors in chondrocyte differentiation (Lefebvre *et al.*, 1998). Two other Sox family members, L-Sox5 and Sox6 are co-expressed with Sox9 and their activity is required for Sox9-mediated differentiation of MSCs into chondroblasts. These Sox family members maintain the chondrocyte phenotype in the mature cartilage by controlling the expression of various cartilage-specific genes including collagens (*Col2a1*, *Col9a1* and *Col11a1*) and aggrecan (*Acan*) (Huang *et al.*, 2001). In addition, the ECM plays a crucial role in determining the fate of mesenchymal cells as well as the development of chondrocytes. The expression of cartilage specific genes is under strict control and ECM helps in the regulation of signalling pathways for co-ordination of cartilage formation (Bi *et al.*, 2005). The members of TGF- $\beta$  superfamily play a key role in directing MSCs towards chondrogenic pathway (Kingsley 1994). ECM integrates several signalling pathways including the fibroblast growth factor (FGF), BMP and the indian hedgehog (IHH) pathway (Bi *et al.*, 2005) (Figure 1.9).

It has been proposed that for *in vitro* chondrogenic differentiation, the following is required (1) MSCs should be grown in a three-dimensional (3D) culture format, (2) culture conditions should consist of serum-free nutrient medium, (3) induction with a member of TGF- $\beta$  superfamily (Johnstone *et al.*, 1998; Tanaka *et al.*, 2004). The 3D culture system for MSC chondrogenesis is typically a micromass pellet culture which was first described by Johnstone *et al* (Johnstone *et al.*, 1998). The micromass pellets cause cell condensation and allow cell-cell interactions thereby recapitulating pre-chondrogenic condensations during embryonic development (Barry *et al.*, 2001). In response to these specific conditions, the cells

lose their fibroblast morphology and undergo a sequence of cellular and molecular events resulting in a series of morphological alterations. At first, the spindle shaped MSCs aggregate and form junctional complexes. A week into the induction period, the cell pellets comprise three layers: the superficial layer of elongated, fibroblast-like cells, the middle zone with apoptotic cells and the deep zone consisting of chondrocyte-like cells. During the early stages of chondrogenesis, progressive alterations in the sulphation patterns of chondroitin-6-sulphate are also observed in that its ratio becomes four-fold higher than chondroitin-4-sulphate. Similar shifts in the ratio of chondroitin sulphates are also observed during maturation of human articular cartilage (Bayliss 1990). After two weeks, the middle zone is no longer present and after three weeks the elongated cells in the superficial layer also disappears and the chondrocyte-like cells in the deep zone are surrounded by ECM (Sekiya *et al.*, 2002). During the early stages of chondrogenesis, ECM molecules like cadherins, versican, fibromodulins and COMP are expressed, whereas the intermediate stage is defined by the synthesis of aggrecan, decorin and biglycan and subsequently type II collagen and chondroadherin (Barry *et al.*, 2001; Koga *et al.*, 2008). Members of TGF- $\beta$  superfamily especially TGF-  $\beta$ 1, TGF-  $\beta$ 2 and TGF-  $\beta$ 3 have the ability to induce this response. Numerous reports have described the role of Smads, in particular the Smad3-mediated TGF- $\beta$  signalling for chondrogenesis. Smad3 induces Sox9 transcriptional activity and stimulates TGF- $\beta$ 1 signalling and together with Smad2, Smad4 and Sox9 forms a transcriptional complex to activate the expression of *Col2a1* gene for the synthesis of collagen type II (Li *et al.*, 2006; Furumatsu *et al.*, 2009).

In addition to the response to biological signals, mechanical stimuli may also play an important role in directing MSC chondrogenesis since native articular cartilage tissue is subject to various mechanical stimuli, such as hydrostatic pressure, as well as compressive and shear strain. For this purpose, bioreactors have been designed in which mechanical stimuli and fluid flow can be applied in a controlled setting to assess chondrogenesis of cell-seeded scaffolds (Chung *et al.*, 2014).



**Figure 1.9. A schematic representation of the sequence of events during MSC chondrogenesis.** The main transcriptional and growth factors involved from the condensation step to the terminal differentiation step are shown. Abbreviations: TGF- $\beta$ , transforming growth factor-beta; BMP, bone morphogenetic protein; FGF, fibroblast-like growth factor; IGF, insulin-like growth factor, VEGF, vascular endothelial growth factor; Shh, sonic hedgehog; Ihh, indian hedgehog; PTHrP, parathyroid hormone-related protein; Wnt, wntless family; Runx2, Runt-related transcription factor 2; TCF- transcription factor; LEF- Lymphoid enhancer-binding factor. Image adapted from Vinatier *et al.*, 2009.

### 1.6.3 Scaffolds for cartilage repair

It is well known that *in vivo* cells reside, proliferate and differentiate within a 3D environment. Chondrocytes in the native tissue are embedded within an abundant ECM comprising of collagens and proteoglycans. As stated above, when cultured in a two-dimensional (2D) environment *in vitro*, these chondrocytes lose their phenotype and dedifferentiate (Benya and Shaffer 1982). However, this process is reversible as dedifferentiated chondrocytes can recover their differentiated phenotype when grown in 3D environments (Bonaventure *et al.*, 1994). These observations confirm that a 3D environment is a pre-requisite for the success of cartilage tissue engineering approaches. 3D environments have therefore been used in the form of scaffolds to support chondrocyte growth and stabilize their phenotype in culture. Micromass pellets also provide a 3D environment for MSCs to aggregate and mimic mesenchymal condensation observed during the developmental stages of chondrogenesis (Johnstone *et al.*, 1998; Barry *et al.*, 2001).

For functional tissue engineering, the scaffold used to provide a 3D environment should fulfil three main requirements: (1) it should be biocompatible and bioresorbable, with a degradation rate that ideally matches regeneration of new tissue; (2) it should have interconnected network to allow nutrient diffusion; (3) it should allow for cell attachment, proliferation and differentiation. In addition, the architectural structure of the scaffold can have an effect on the mechanical properties of the scaffold, cell-seeding and diffusion of nutrients. The biomaterial that the scaffold is made from is vital to the success of tissue engineering as it can either facilitate or impede cell attachment, proliferation and synthesis of the new tissue. These biomaterials can be classified as natural, synthetic and composites. Natural biomaterials are found naturally in living organisms and can be extracted and utilized as functional biomaterials. Synthetic biomaterials are created artificially using chemical processes and therefore have the advantage of customization of the required structural and mechanical properties. Composite scaffolds are a combination of two or more biomaterials that may have special characteristics intrinsic to each constitute material (Athanasίου *et al.*,

2010). The following sections of this introduction will describe natural and synthetic scaffolds in more details.

### Natural scaffolds

Natural biomaterials are relatively well established for their use in biological applications because they elicit little or no immune response and are believed to facilitate cell adhesion due to the presence of natural ligands that support cellular interactions. Various natural scaffolds used in cartilage tissue engineering are derived from components of the ECM. Their structure can vary from a hydrogel (i.e. colloidal gel-like form containing water as the dispersion media) to fibrous and porous constructs. Collagens and hyaluronic acid-based materials are some of the most widely used natural biomaterials in addition to alginate, agarose, fibrin glue and chitosan. ECM polypeptides and polysaccharides are most widely used as they are biodegradable and produce non-toxic agents upon degradation (Athanasίου *et al.*, 2010).

### *Collagens*

Collagen type I is the traditionally used collagen-based scaffold in tissue engineering and studies have shown that this scaffold can facilitate cartilaginous tissue formations under the influence of mechanical compression (Hunter *et al.*, 2002) or when cross-linked with GAGs (Van Susante *et al.*, 2001; Lee *et al.*, 2004). However, collagen type II is the predominant collagen found in the ECM of articular cartilage and some studies have proven its superiority in making cartilaginous tissue compared to collagen type I (Mueller *et al.*, 1999; Bosnakovski *et al.*, 2006). For clinical application, collagen type I/type III membranes have been used for MACI including MACI® (Verigen, Leverkusen, Germany), Maix® (Matricel, Hezzenrath, Germany) and Chondro-Gide® (Geistlich Biomaterials, Wolhusen, Switzerland)(Cherubino *et al.*, 2003). A collagen type I gel called Atelocollagen® (Koken Co. Ltd, Tokyo, Japan) has been utilized for culture and *in vivo* implantation of human autologous chondrocytes (Ochi *et al.*, 2002) and of BM MSCs (Kuroda *et al.*, 2007). The utilization of these collagen-based gels and matrices has shown promising results clinically.

### *Hyaluronic acid (HA)*

HA or hyaluronan is a non-sulphated GAG found in the ECM of articular cartilage. This polysaccharide has been used to create biocompatible scaffolds for cartilage tissue engineering. It can be cross-linked to form a porous matrix or it could be used as an injectable gel. It has been commercially fabricated into a matrix called HYAFF II® (Fidia Advanced Biopolymers, Italy) which has been used mainly for osteochondral application (Solchaga *et al.*, 1999). This matrix is a derivative of HA formed by esterification of carboxyl groups of glucuronic acid with benzyl alcohol and the scaffold has been shown to support cellular growth, proliferation and chondrogenic differentiation *in vitro* (Campoccia *et al.*, 1998). For *in vivo* application, HYAFF II® was seeded with autologous chondrocytes and this tissue-engineered graft for the treatment of full-thickness cartilage defects was referred to as Hyalograft C® (Grigolo *et al.*, 2001; Marcacci *et al.*, 2005). In a recent systematic review of clinical trials with Hyalograft C®, it was reported that the quality of the repair tissue was predominantly of a hyaline-like in nature (Wylie *et al.*, 2015). Despite the success of this scaffold for the treatment of chondral and osteochondral defects, the European Medical Association (EMA) expressed concerns regarding its manufacturing and the company withdrew their EU application for further approval (McGowan and Stiegman 2012).

### *Synthetic scaffolds*

Synthetic scaffolds have been produced commercially and can be customized according to their physical and chemical properties. One of the major advantages of these scaffolds are that they are pathogen-free and can therefore have low potential for immunological rejection. They can be tightly controlled for their mechanical strength and degradation profile as per the demand of the tissue they are designed to repair or regenerate. Poly-glycolides, poly-lactides, and their co-polymers are extensively used for designing synthetic scaffolds. They can be conformed into porous matrices, non-woven meshes, or felts which allow flexibility to shape and architecture. The most commonly used synthetic materials in cartilage tissue engineering include polycaprolactone (PCL), poly L-lactic acid (PLLA), poly(DL-lactic-co-glycolic acid)

(PLGA) and polyglycolic acid (PGA) (reviewed by Matsiko *et al.*, 2013). PCL scaffolds have been used to investigate the *in vitro* chondrogenic potential of MSCs and the results indicated chondrogenic gene expression and matrix deposition (Kim *et al.*, 2010). PGA based porous felt has been evaluated *in vitro* and *in vivo* for repair of cartilage defects (Endres *et al.*, 2012). It has shown successful results when transplanted with autologous chondrocytes in a porcine model of chondral defects (Liu *et al.*, 2002). ECM production has been consistently observed using PGA scaffolds, which, along with predictable degradation rates, makes PGA attractive for cartilage tissue engineering purposes (Freed *et al.*, 1994; Freed *et al.*, 1993; Kreuz *et al.*, 2013). PLA is another polymer used extensively in the medical field and along with PGA, has been approved by the FDA for application in humans. PLA generally degrades slower than PGA with a total degradation time ranging from twelve months to over two years (Middleton and Tipton 2000). PLGA is a copolymer composed of PGA and PLA monomers. It has been used to assess chondrogenesis of MSCs *in vitro* (Zheng *et al.*, 2011) and *in vivo* (Han *et al.*, 2008) and has shown successful cartilage regeneration. The controllable degradation rate of these polymeric scaffolds is advantageous for cartilage tissue engineering purposes, however a major drawback of the synthetic materials is that their degradation products are highly concentrated acids that can lead to localised inflammation and apoptosis *in vivo* (reviewed by Hutmacher 2001). A synthetic bilayered product licensed as TRUFIT CB® is composed of poly-(DL-lactide-co-glycolide or PDLG) and calcium sulphate and is commercially available for chondral and osteochondral tissue repair applications (Carmont *et al.*, 2009)

The microarchitecture of these biomaterials is a key factor in determining the adhesion and proliferation of cells as well as the retention of newly synthesized matrix. Porosity and pore size can have a major impact on cell infiltration and matrix production. The pores need to be large enough to permit cell migration, but also small enough to provide sufficient surface area for cell adhesion (Zeltinger *et al.*, 2001). The mechanical properties of the scaffold play a significant role in the regeneration of neo-cartilage. These biomaterials must be able to support cell growth whilst possessing adequate durability to remain uncompromised by normal joint

functioning until sufficient new tissue regenerates. To truly mimic articular cartilage, scaffolds must be able to withstand high compressive loads (Matsiko *et al.*, 2013). It has been previously shown that the mechanical properties of a biomaterial can have an effect on the differentiation of MSCs where scaffolds with stiff compressive modulus encouraged osteogenic differentiation and softer more compliant scaffolds supported chondrogenic differentiation (Murphy *et al.*, 2012). Furthermore, for the success of tissue engineered constructs to regenerate cartilage, they should be retained at the implantation site. Not only this, constructs should allow for the integration of the regenerated tissue with the native tissue (Risbud and Sittinger 2002).

Despite the various different natural and synthetic scaffolds developed for the restoration of damaged cartilage, their clinical use is limited due to either a lack of preclinical animal studies or their risk of disease transmission and immune reaction. As a result, collagen- and HA-based matrices are the most popular natural biomaterials in clinical use nowadays, as they offer a substrate naturally found in native cartilage tissue (Iwasa *et al.*, 2009; Wylie *et al.*, 2015).

#### *Clinically available scaffolds used for cartilage repair*

Several different biomaterials have been utilized clinically for restoration of chondral defects including NeoCart (Histiogen- ics Corporation, Waltham, MA), CaReS-1S (ArthroKinetics, Esslingen, Germany), Hyalograft C (Fidia Farmaceutia, Italy), Cartipatch (TBF Tissue Engineering, Bron, France), BST-CarGel (Bio- syntech, Quebec, Canada), Bioseed-C (BioTissue Technologies, Freiburg, Germany), Chondro-Gide® (Geistlich Biomaterials, Wolhusen, Switzerland), MACI® (Genzyme, Cambridge MA), and Novocart (TETEC AG, Reutlingen, Germany) (Getgood *et al.*, 2009; Brittberg 2010). Table 1.1 represents some of the current scaffolds used for clinical trials for cartilage repair. In this study, three clinical grade scaffolds have been investigated for their potential towards chondrogenesis, namely; Chondro-Gide®, Alpha Chondro Shield® and Hyalofast™.

### *Chondro-Gide®*

Chondro-Gide® is a bilayered structured scaffold consisting of collagen type I and collagen type III. The external layer is a compact surface with good mechanical strength and the internal layer is a porous surface that supports cell attachment and matrix synthesis. The compact surface acts as a barrier and prevents cell leakage. *In vitro* and *in vivo* studies have demonstrated the excellent capacity of this scaffold for cell attachment, maintenance of chondrocyte phenotype and encapsulation of the cells within the defect (Fuss *et al.*, 2000; Gigante *et al.*, 2007). This scaffold has also been clinically applied in MACI procedure for over a decade (reviewed by Iwasa *et al.*, 2009). As per a recent systematic review on the effects of matrices on cartilage repair, it was shown that Chondro-Gide® was one of the two most common membranes used with the ACI procedure. Clinical outcomes at follow-ups have shown significant improvement in functional scores as well as regeneration of a hyaline-like repair tissue or hyaline-like and fibrocartilage repair tissue (Wylie *et al.*, 2015).

### *Alpha Chondro Shield®*

Alpha Chondro Shield® is a synthetic non-woven mesh of PGA. It is intended for use in microfracture procedures as an easy-to-use, off-the-shelf and cost-effective implant. So far, only one pre-clinical animal study has been performed using this scaffold in combination with HA in an ovine chondral defect model with promising outcomes revealing cartilaginous repair tissue 3 months after transplantation (Ergelet *et al.*, 2007).

### *Hyalofast™*

Hyalofast™ (Fidia Advanced Bio- polymers, Abano Terme, Italy) is a non-woven HA-based mesh intended for use as a cell-free implant for the *in situ* entrapment of MSCs for the treatment of chondral and osteochondral lesions. So far only one clinical study has been published where this scaffold was filled with BM concentrate and covered with a layer of platelet-rich-fibrin in a one-step procedure. The authors reported overall good results in terms of the functional improvement, graft integration and defect fill at a 2 year follow-up (Buda *et al.*, 2010).

Product name	Biomaterial & cell type	Trials
NeoCart (Histiogenics Corporation, Waltham, MA)	<ul style="list-style-type: none"> <li>Bovine-type I collagen</li> <li>Chondrocytes</li> </ul>	<ul style="list-style-type: none"> <li>Phase II studies complete</li> <li>Ongoing phase III study</li> </ul>
CaReS-1S (ArthroKinetics, Esslingen, Germany)	<ul style="list-style-type: none"> <li>Rat-tail type I collagen</li> <li>Cell free</li> </ul>	<ul style="list-style-type: none"> <li>Animal trials</li> <li>Case study of 15 humans</li> <li>No comparative human trials to date</li> </ul>
Hyalograft C (Fidia Advanced Biopolymers, Abano Terme, Italy)	<ul style="list-style-type: none"> <li>Hyaluronic acid (HA)</li> <li>Chondrocytes</li> </ul>	<ul style="list-style-type: none"> <li>Multiple comparative studies with microfracture showing improved results at 5 years</li> </ul>
Cartipatch (Tissue Bank of France)	<ul style="list-style-type: none"> <li>Agarose-alginate</li> <li>Chondrocytes</li> </ul>	<ul style="list-style-type: none"> <li>Phase II multicentre study showed significant improvements</li> <li>Ongoing phase III trials comparing mosaicplasty and microfracture</li> </ul>
BST- CarGel (Biosyntech, Quebec, Canada)	<ul style="list-style-type: none"> <li>Chitosan-glycerol phosphate buffer</li> <li>Cell free</li> </ul>	<ul style="list-style-type: none"> <li>Phase II clinical trials show improved scores for pain, stiffness and function</li> </ul>
Bioseed C (BioTissue Technologies GmbH)	<ul style="list-style-type: none"> <li>Fibrin glue – co-polymer</li> <li>Chondrocytes</li> </ul>	<ul style="list-style-type: none"> <li>Phase III trials comparing with ACI showed improved outcome scores</li> <li>Caution in that ACI-only group had larger defect and longer follow-up</li> </ul>
BioCart II (ProChon BioTech Ltd., Ness Ziona, Israel)	<ul style="list-style-type: none"> <li>fibrinogen and HA</li> <li>Chondrocytes</li> </ul>	<ul style="list-style-type: none"> <li>Phase II clinical trial versus microfracture on-going</li> </ul>
Chondro-Gide® (Geistlich Biomaterials, Wolhusen, Switzerland)	<ul style="list-style-type: none"> <li>Collagen type I/III</li> <li>Chondrocytes (MACI) and cell-free (AMIC)</li> </ul>	<ul style="list-style-type: none"> <li>Positive clinical improvement and MRI improvement when used as adjunct to microfracture.</li> </ul>

**Table 1.1. Scaffolds used for clinical applications.** A variety of scaffolds have been developed for the restoration of cartilage defects. This table provides a description of the commercially available scaffolds, their composition, cell type they are implied to use with and the on-going or completed clinical trials. Table adapted from Ryan and Flanigan, 2013.

## **1.7 Current perspective**

Articular cartilage is an avascular, load bearing connective tissue, which has limited intrinsic healing capacity. Chondral or osteochondral defects and OA represents major clinical challenges. As per the ICRS, there are numerous emerging therapies for restoring damaged cartilage and some of these have become available in the last five years. The microfracture procedure, ACI and osteochondral autografting procedures have the capacity to stimulate cartilage repair and restoration, but the results are highly variable. In addition, clinical outcomes may be greatly influenced by patient-specific and defect-specific factors (Harris *et al.*, 2010). Tissue engineering approaches with chondrocytes and MSCs are now considered to be promising for the repair of articular cartilage lesions. There is a significant need for novel methods and procedures that can provide sufficient cell numbers, either using chondrocyte or MSCs, which are capable of effective cartilage repair. The wide availability of MSCs from different sources makes their use for cartilage repair an attractive option (reviewed by Mobasher *et al.*, 2014). Although tissue engineering approaches for cartilage repair using MSCs appear promising, the question of which cell source is the most suitable source still remains. Future efforts in cartilage tissue engineering should focus on addressing the question of which cell type and which scaffold is most suited for cartilage repair.

## **1.8 Aims and objectives**

The overriding aim of this thesis was to investigate cell sources for cartilage repair using commercially available and clinically applicable cell scaffolds. In particular, the thesis examined the chondrogenic differentiation potential of primary human MSC populations from BM and AT in three clinical scaffolds, namely Chondro-Gide®, Alpha Chondro Shield® and Hyalofast™. It has also examined the chondrogenic and cartilage repair potential of plastic adherent versus CD271 selected MSCs.

In order to address the overriding aim, the following objectives were determined:

- To examine *in vitro* the incorporation, growth and chondrogenic differentiation potential of culture expanded MSCs versus freshly isolated cells from BM and AT. This study has been presented in Chapter 3.
- To further examine *in vitro* the chondrogenic differentiation potential of AT MSCs selected using CD271 as a marker compared to MSCs selected on the basis of plastic adherence alone. This study has been presented in Chapter 4.
- To test *in vivo* the potential of CD271 positive AT MSCs versus AT MSCs selected on the basis of plastic adherence for cartilage repair. This study has presented in Chapter 5.

The findings of these studies have been discussed in Chapter 6.

## **Chapter 2: Materials and methods**

## **2.1 Routine cell culture**

### 2.1.1 Isolation and culture of primary bone marrow-derived mesenchymal stem cells (BM MSCs)

Bone marrow aspirates were kindly provided by Mr. Martyn Snow at the Royal Orthopaedic hospital, Birmingham. Following local research ethical committee (LREC) approval (LREC number 12/EE/0136 and 06/Q2601/9) and informed consent, BM was harvested from the posterior superior iliac crest of patients having a knee-reparative surgery. BM-MSCs were isolated from BM aspirates by density gradient centrifugation (Ficoll-Paque™ Plus). Briefly, 2-5 ml of aspirate was mixed with 5ml phosphate buffered saline (PBS) (PAA, Yeovil, Somerset, UK), and was gently layered onto 5 ml of Ficoll-Paque™ Plus (GE Healthcare, Buckinghamshire, UK) in 15 ml tubes (SLS, East Riding of Yorkshire, UK) and centrifuged at 900g for 20 minutes. After this time a “buffy coat” containing mononucleated cells would appear, which was harvested with sterile plastic pasteur pipettes and washed in standard culture media, i.e., Dulbecco’s Modified Eagle’s Medium (DMEM)/F-12, supplemented with 10% (v/v) fetal calf serum, 1% (v/v) penicillin (50U/ml) and 1% (v/v) streptomycin (50µg/ml) (All from PAA, Yeovil, Somerset, UK), followed by centrifuging at 750g for 10 minutes. The resulting pellet was re-suspended in standard culture media when a viable cell count was performed by trypan blue exclusion [See section 2.1.3(b)]. These freshly isolated cells were referred to as BM mononucleated cells (BM MNCs). BM MNCs were either used directly for experiments (see section 2.3.1) or were plated out at a density of  $2 \times 10^7$  cells per  $75\text{cm}^2$  flask in 20 ml of standard media and incubated at 37 °C in humidified atmosphere containing 5% CO<sub>2</sub>. After 24 hours the non-adherent cells were washed off gently with PBS and the adherent cells referred to as BM MSCs were subsequently culture expanded until they reached ~70% confluence. At 70% confluency the cells were passaged and sub-cultured as described below in section 2.1.3 (a).

### 2.1.2 Isolation and culture of primary adipose-tissue derived mesenchymal stem cells (AT MSCs)

Following ethical approval and informed consent (LREC number 12/EE/0136), AT MSCs were harvested from the excised infrapatellar fat pad of the knee joints of patients having a knee replacement surgery. The stromal cells were isolated from the AT as previously described (Bunnell *et al.*, 2008). Briefly, the tissue sample was minced and treated with 0.075% collagenase type I (Sigma, Dorset, UK) for 30 minutes to one hour at 37 °C and 5% CO<sub>2</sub>. After collagenase digestion, DMEM with 20% (v/v) FCS and 1% (v/v) penicillin (50 U/ml) and 1% (v/v) streptomycin (50 µg/ml) was added to neutralise collagenase activity and the digest was centrifuged at 1200 rpm for 10 minutes. Next, the supernatant was discarded and the pellet was washed in DMEM containing 20% (v/v) FCS and 1% (v/v) penicillin (50 U/ml) and 1% (v/v) streptomycin (50 µg/ml) followed by centrifugation at 1200 rpm for 10 minutes. The re-suspended cell pellet was filtered through 70µm cell strainers. These cells were referred to as freshly isolated AT stromal vascular fraction (AT SVF) cells. The AT SVF cells were either used directly for experiments (see section 2.3.1) or were cultured in standard culture media at 37 °C and 5% CO<sub>2</sub>. After 24-48 hours the non-adherent cells were washed off and the adherent cells referred to as AT MSCs were subsequently culture expanded until they reached ~70% confluence. At 70% confluency the cells were passaged and sub-cultured as described below in section 2.1.3 (a).

### 2.1.3 Expansion and storage of cells

#### **2.1.3 (a) Passaging monolayer of cells**

Monolayer of cells in tissue culture flasks were washed with PBS and incubated with 0.25% (w/v) trypsin-ethylendiamine tetra-acetic acid (EDTA) (PAA) for 5-10 minutes at 37°C. After cell detachment, trypsin activity was neutralised by adding an equal volume of standard culture media. This cell suspension was centrifuged for 10 minutes at 1000 rpm to form a cell pellet. The supernatant was discarded and the cell pellet was re-suspended in standard culture media. Following this a viable cell count was performed using trypan blue exclusion [see section 2.1.3(b)]. The cells were then seeded into fresh tissue culture flasks at  $5 \times 10^3$  cells/cm<sup>2</sup> or used at the desired seeding density for different experimental procedures.

#### **2.1.3 (b) Viable cell counting by trypan blue exclusion**

Trypan blue selectively stains dead cells or membrane damaged cells due to its unique property of not passing through intact cell membranes of live cells (Altman *et al.*, 1993). A 20 µl sample of cell suspension was mixed with 20 µl of trypan blue dye in an eppendorf tube. After this, 20 µl of the cell sample and dye mix was loaded onto a standard improved Neubauer Haemocytometer (Fisher Scientific, Loughborough, Leicester, UK). Using an X10 objective, trypan blue negative cells were counted as viable cells and trypan blue positive cells were counted as non-viable cells. The numbers of both viable and non-viable cells within an area corresponding to a volume of one square of the haemocytometer i.e.  $1 \times 10^4$  cells/ml were counted to give a ratio of viable to non-viable cells. The concentration of viable cells per ml in the original cell suspension was calculated by multiplying the viable cell count by 2.

#### **2.1.3 (c) Cryopreservation of cells**

Cells were harvested by trypsinisation and the cell pellet after centrifugation was re-suspended in cold 10% (v/v) dimethyl sulphoxide (DMSO- Sigma) in FCS. Cryovials containing 1-2 ml per vial were then placed in a designated cryofreezing container called Mr Frosty (Genta Medical, York, UK) that contains isopropyl alcohol (IPA) which is stored at -80°C. This

container allows slow freezing at the rate of  $-1^{\circ}\text{C}/\text{minute}$ , the optimal rate for cell preservation. The cryovials were then stored in  $-80^{\circ}\text{C}$  freezers or liquid nitrogen for long-term storage.

### ***2.1.3 (d) Thawing of cells***

Cells were recovered from  $-80^{\circ}\text{C}$  storage by rapidly thawing the cryovials under a running hot tap water. 1 ml of cold standard culture media was then added to the thawed cell suspension drop wise over a minute and the resultant diluted cell suspension was transferred to a 15 ml centrifuge tube. After this, 5 ml of cold standard culture media was added drop wise over a period of 5 minutes. The cell suspension was left to stand for 5 minutes after which, a further 4 ml of cold standard culture media was added slowly over a period of one minute. The cells were subsequently pelleted by centrifugation for 10 minutes at 1,000 rpm. The pellets were re-suspended in warm standard culture media, counted and then cultured at a seeding density of  $5 \times 10^3$  viable cells/cm<sup>2</sup> in  $37^{\circ}\text{C}$  at 5% CO<sub>2</sub>.

#### 2.1.4 Mycoplasma testing of primary cells

Mycoplasma are a large group of intracellular micro-organisms that can contaminate cell cultures, and affect experimental data. They lack a cell wall and therefore are unaffected by  $\beta$ -lactam antibiotics such as penicillin used in cell culture media, as these antibiotics target bacterial cell wall synthesis. These bacteria have the ability to induce remarkable changes in cell behaviour with regards to their growth and metabolism and can also cause chromosomal aberrations and deprivation of cell culture growth (Chernov *et al.*, 2014). In response to this potential problem, cell cultures were screened for contamination with mycoplasma whenever a primary cell sample was acquired and then routinely during subsequent culture expansion of primary cells every 3-6 months. The EZ-PCR mycoplasma test kit protocol (Geneflow, Lichfield, Staffordshire, UK) was used as per manufacturer's instructions to screen cell cultures. For each culture tested, 0.5-1.0 ml of cell culture supernatant was transferred into a 2ml micro-centrifuge tube and cellular debris was pelleted by centrifugation at 250 g for 1 minute. The supernatant was transferred into a fresh micro-centrifuge tube and centrifuged at 15,000 g for 10 minutes to sediment any mycoplasma. The supernatant was carefully decanted and the pellet was re-suspended with 50  $\mu$ l of the Buffer Solution and mixed thoroughly with a micro-pipet and heated to 95°C for 3 minutes. The reaction mix was prepared by combining 35  $\mu$ l of H<sub>2</sub>O, 10  $\mu$ l of reaction mix and 5  $\mu$ l of test sample in sterile PCR tubes. A positive template control was also prepared by adding 1  $\mu$ l of positive template in place of the 5  $\mu$ l sample. All tubes were placed into a DNA thermal cycler and the following parameters were set to amplify the DNA: 30 seconds at 94°C, then 35 cycles of; 30 seconds at 94°C then 120 seconds at 60°C then 60 seconds at 72°C, followed by 30 seconds at 94°C, 120 seconds at 60°C and finally 5 minutes at 72°C. The amplified products were analysed using gel electrophoresis by loading 15  $\mu$ l of the amplified PCR product and running the samples in a 2% (w/v) agarose gel at 100 volts for 1 hour, alongside a known DNA ladder of 100-1013 base pairs (HyperLadder™ 100bp, Bioline, London, UK). The gel was then visualised under UV light and digitised images were taken using Syngene imaging software (Syngene,

Cambridge, UK). The positive control for mycoplasma infection gave a visible band at 270bp. Mycoplasma positive cultures were omitted from further experimentations and have not been included in any analysis presented in this thesis.

## **2.2 Phenotypic characterisation of culture expanded mesenchymal stem cells**

### 2.2.1 Immunophenotyping by flow cytometry

The isolated stromal cells from BM and AT were characterised as per the guidelines proposed by the ISCT (Dominici *et al.*, 2006). The stromal cells at passage II-III were harvested by trypsinisation and suspended in PBS containing 2% bovine serum albumin (BSA) (Sigma-Aldrich). The cells were then initially blocked with 10% normal human immunoglobulin (Ig) (Grifols, Cambridge, UK) prior to incubation at 4 °C with one of the following phycoerythrin (PE) conjugated mouse monoclonal anti-human antibodies: CD34, CD45, CD105 (Immunotools, Friesoythe, Germany), CD73 or CD90 (BD Biosciences, UK). Non-specific fluorescence was determined by incubating cells with isotype-matched control phycoerythrin-conjugated antibodies IgG2a and IgG1 (Immunotools). Immunoreactivity for each CD marker was assessed by flow cytometry using a Beckman Coulter FC500 flow cytometer and data were analysed using Kaluza® Flow Analysis Software (Beckman Coulter, High Wycombe, UK)

### 2.2.2 Tri-lineage differentiation of primary stromal cells

#### **2.2.2 (a) Osteogenic differentiation**

Passage II-IV MSCs were cultured in a 24-well plate and allowed to reach 70-80% confluency. The cells were then maintained under osteogenic conditions, consisting of standard culture medium supplemented with 10 nM dexamethasone (DEX), 10 mM  $\beta$ -glycerophosphate and 50  $\mu$ M ascorbic 2-phosphate (all from Sigma), as previously described (Bajada *et al.*, 2009) for 28 days at 37°C and 5% CO<sub>2</sub>. Control cultures were maintained in standard culture media with carriers alone which were methanol as a control for DEX, sterile cell culture water for  $\beta$ -glycerophosphate and ascorbic 2-phosphate. The media was completely replaced every 2–3 days in either osteogenic or control conditions. Differentiation along the osteogenic lineage was evaluated by increased amount of alkaline phosphatase activity in differentiated cells compared to undifferentiated cells [See section 2.2.3(a)]

### **2.2.2 (b) Adipogenic differentiation**

Passage II-IV MSCs were cultured in a 24-well plate and allowed to reach 70-80% confluence. The cells were then maintained under adipogenic conditions, consisting of standard culture medium supplemented with; 1  $\mu$ M DEX, 0.5 mM 3-isobutyl-methylxanthine (Sigma-Aldrich), 1% insulin, transferrin and selenium (ITS-X 100 pre-mix; PAA) and 100  $\mu$ M indomethacin (Sigma-Aldrich), for 28 days at 37°C and 5% CO<sub>2</sub> as previously described (Bajada *et al.*, 2009). Control cells were maintained in complete media with carriers alone which were methanol for DEX and IBMX, and DMSO for indomethacin. Both adipogenic and control medium were completely replaced every 2–3 days. Differentiation along the adipogenic lineage was evaluated by cellular accumulation of neutral lipid vacuoles by staining with Oil Red O [see section 2.2.3(b)]

### **2.2.2 (c) Chondrogenic differentiation**

A cell suspension of  $25 \times 10^4$  cells was prepared per 1ml of standard culture medium. 1ml of the cell suspension was transferred into a 1.5 ml eppendorf and pelleted by centrifugation at 500g for 5 minutes (Johnstone *et al.*, 1998). The standard culture medium was then replaced with chondrogenic induction medium consisting of DMEM/High glucose supplemented with 100nM DEX, 37.5 $\mu$ g/ml ascorbate-2-phosphate, ITS-X and 10 ng/ml transforming growth factor- $\beta$ 1 (TGF- $\beta$ 1) (PeproTech Ltd., London, UK), and antibiotics. The control cultures were treated with DMEM/High glucose media with carriers alone which were methanol for DEX, sterile water for ascorbate-2-phosphate and 0.1% bovine serum albumin (BSA) solution for TGF- $\beta$ 1. Pellets were maintained at 37°C and 5% CO<sub>2</sub> for 28 days. Both chondrogenic and control medium was replaced every 2-3 days, after which the differentiation status of the pellets was examined histologically by fixing the pellets, embedding and staining sections with toluidine blue histological stain [see section 2.2.3 (c)].

### 2.2.3 Assessment of MSC differentiation potential

#### **2.2.3 (a) Alkaline phosphatase activity for osteoblastic differentiation**

Alkaline phosphatase is an extracellular enzyme which is extensively used as a marker for osteoblastic differentiation. It was detected by alkaline phosphatase mediated hydrolysis of substituted naphthol ester substrate (Naphthol AS-MX phosphate- Sigma), which liberates a naphthol derivative that in turn reacts with a diazonium salt (Fast red TR) to produce an insoluble azo dye at the site of presence of the active enzyme (Jaiswal *et al.*, 1997). A staining solution was prepared immediately prior to staining as follows; 10 mg of Fast red TR was added to 8.4 mls of 0.1M Tris HCl buffer, then 1.6 mls of naphthol was added and filtered (Whatman No.1 filter paper, Maidstone, UK) just before use. Differentiated and control cultures were fixed with 4% formaldehyde for 1 minute and then washed with Tris-Buffered Saline and Tween 20 (TBS-T) twice for 1 minute at each wash. To each well of a 24 well plate, 500 µl of staining solution was added and then the plates were incubated at room temperature for 3-5 minutes. Alkaline phosphatase activity was indicated by the formation of dark pink cellular precipitates.

#### **2.2.3 (b) Oil Red O staining of lipid vacuoles for adipogenic differentiation**

Oil Red O is a dye that binds to lipids such as triglycerides and was used as a marker to assess adipogenic differentiation as described previously (Ramírez-Zacarías *et al.*, 1992). The Oil Red O staining solution was prepared by dissolving 60mg of Oil Red O powder (Sigma) to 20 mls of isopropanol and then mixing 6 mls of this stock solution with 4mls of distilled water. This solution was then filtered using Whatman filter paper No.1. Differentiated and control cultures were washed and fixed with 4% formaldehyde for 30 minutes at room temperature. To each well of a 24-well plate was added 500 µl of staining solution and the plates were incubated for 1 hour at room temperature. After this time, any unbound dye was washed off and lipid accumulation was indicated by red staining of the intracellular lipid vacuoles.

### ***2.2.3 (c) Toluidine blue staining of extra cellular matrix for chondrogenic differentiation***

Toluidine blue is an acidophilic metachromatic dye that selectively stains negatively charged tissue components i.e, sulphates, carboxylates, and phosphate radicals. In cartilage, this cationic dye binds to negatively charged sulphates attached to glycosaminoglycan chains in the extracellular matrix and gives a purple colour (Sridharan and Shankar 2012). Pellets were harvested at week 4, fixed with 10% neutral buffered formalin for 24 hours and then processed in graded strengths of alcohol and paraffin embedded and sectioned at 5 micron thickness. The sections were dewaxed three times in xylene for 4 minutes each and then rehydrated in graded strengths of ethanol i.e, 100%, 96% to 70% for 2 minutes in each and then rinsed in tap water for 1 minute. Then 0.4% staining solution of toluidine blue (Sigma) in 0.2 M sodium acetate buffer (pH 3.75 to 4.25) was added to the sections for 10 minutes. After 10 minutes, the slides were rinsed in tap water for 1 minute and then briefly (10 dips) rehydrated through a graded alcohol series followed by clearing in two changes of xylene for 5 minutes in each and then mounted in Pertex mounting media (Sigma).

## **2.3 *In vitro* experimental procedures**

### 2.3.1 Cell seeding into 3-D scaffolds

In initial experiments to assess the incorporation and growth of different cell sources in different scaffolds, the freshly isolated cells from BM (BM MNCs) and AT (AT SVF) were used directly for cell seeding following isolation and without culture expansion. The culture expanded cells from BM (BM MSCs) and AT (AT MSCs) were used after II-IV passages in culture following trypsinisation as previously described [section 2.1.3 (a)]. All cell types were seeded at a density of  $5 \times 10^4$  cells per  $3 \times 3 \text{ mm}^2$  piece of Chondro-Gide®, Alpha Chondro Shield® or Hyalofast™ in a volume of 30  $\mu\text{l}$ . The cell-seeded scaffolds were left to incubate at 37°C and 5% CO<sub>2</sub> to allow cell attachment and then 1ml of standard culture media was added to each well. This was done in non-tissue culture coated plates to avoid cell adhesion to the well surface. All cell-seeded scaffolds were fed with standard culture media, three times a week for a period of four weeks, unless they were used for chondrogenic assessment where they were treated with chondrogenic versus control conditions.

### 2.3.2. Cell viability assay

At day 1, day 7, day 14 and day 28, the cell-seeded scaffolds were assessed for cell incorporation and viability using the live/dead cell double staining kit (Sigma-aldrich). The staining solution was prepared by mixing 10  $\mu\text{l}$  of calcein AM and 5  $\mu\text{l}$  of propidium iodide (PI) in 5 mls of PBS. Calcein-AM, is highly lipophilic and cell membrane permeable. The intracellular esterase activity of viable cells hydrolysis Calcein AM that generates calcein which emits a strong green fluorescence ( $\lambda_{\text{ex}}$  490 nm,  $\lambda_{\text{em}}$  515 nm). Alternatively, the nuclei staining dye PI cannot pass through a viable cell membrane and only intercalates with nucleic acid in the nucleus. When the cell membrane is damaged, it emits red fluorescence ( $\lambda_{\text{ex}}$  535 nm,  $\lambda_{\text{em}}$  617 nm). At each of the time points mentioned, the media was replaced with 1ml of the live/dead staining solution and incubated in the dark for 30 minutes at 37°C in 5% CO<sub>2</sub>. After incubation, the staining solution was removed and the cell seeded scaffolds were washed

in PBS. The live and dead cells were visualised by fluorescence imaging and confocal microscopy (see section 2.3.4).

### 2.3.3 Cell viability scoring

At each time point, the number of live and dead cells was counted by randomly selecting two different regions within each cell-seeded scaffold. Cells were counted throughout the depth of the scaffold taking in four fields of view from when the first cell was seen to when the last cell was seen. The count of number of cells within the four fields of view was performed for each of the two regions. The cell viability data is plotted as number of cells/mm<sup>2</sup>. This was calculated by measuring the area of the field of view using x10 lens and then dividing the number of cells in that field of view by the area of that field of view to calculate the number of cells/mm<sup>2</sup>. If x number of cells were counted in a single field of view, the number of cells/mm<sup>2</sup> were calculated as shown below:

Area of field of view =  $\pi r^2$ , where r is the radius of the field of view calculated using a 10x lens.

$$\text{Cells/mm}^2 = \frac{x}{\pi r^2}$$

### 2.3.4 Confocal microscopy

Confocal microscopy was performed with the Leica SP5TCSII microscope (Leica, Wetzlar, Germany). All images were acquired using the 10X dipping objective. The Z-stacks were acquired at a resolution of 1024x1024 pixels firing at 200Hz with a 2  $\mu\text{m}$  to 3.21  $\mu\text{m}$  distance between each z-stack slice depending on the scaffold. Following acquisition of the z-stack, images were compressed into a maximum projection using the Leica imaging software (Leica Microsystems DM6000B – SP5TCS).

### 2.3.5 Chondrogenesis of MSC-seeded scaffolds

To induce chondrogenesis of MSCs seeded within Chondro-Gide®, Alpha Chondro Shield® and Hyalofast™, the cell-seeded scaffolds were treated with inducers of chondrogenic differentiation, consisting of DMEM-High glucose supplemented with 100 nM DEX, 1µM ascorbic acid (Sigma), 1% ITS-X, 10 ng/ml TGF-β1 (PeproTech). The control cultures were maintained in DMEM-High glucose with carriers alone. These were cultured for a period of 28 days at 37°C and 5% CO<sub>2</sub>. Pellet cultures were used as a control for chondrogenesis. In all cases, media was replaced 2-3 times per week.

### 2.3.6 Histological processing, paraffin embedding and sectioning of cell-seeded scaffolds

Cell-seeded scaffolds were washed twice in PBS and then fixed in 10% neutral buffered formalin (Sigma) for 24 hours. After 24 hours, formalin was discarded and replaced with PBS. The samples were then processed by the staff at Department of Musculoskeletal Pathology, Birmingham, UK. Following tissue processing, the scaffolds were embedded in paraffin in an automated tissue embedder. Briefly, the processed cell-seeded scaffold were placed in molten wax in a metal container and then the wax was allowed to set at 4°C and a paraffin wax block was generated. The blocks were then inserted into the block holder of a standard rotary microtome and 5 micron thin sections were taken and were left to air dry for 15 minutes before incubation of the slides at 65°C for 60 minutes. The sections on glass slides were then stained using different histological stains.

### 2.3.7 Haematoxylin and eosin staining of paraffin embedded sections of cell-seeded scaffolds

Haematoxylin and Eosin (H&E) staining of paraffin embedded sections was performed according to the standard operating protocol from the Department of Musculoskeletal Pathology, Birmingham, UK. Briefly, sample sections were dewaxed with xylene and rehydrated in serial dilutions (100%-70%) ethanol and then washed in tap water. Sections were then stained with Ehrlich's haematoxylin, washed in tap water and differentiated in 0.3% acid alcohol (0.3% hydrochloric acid in 70% ethanol). Following this, sections were washed in running tap water and counterstained with 1% eosin, washed again with running tap water and finally dehydrated. They were then "cleared" and a coverslip was mounted onto the sections with Pertex mounting media. The H&E stain indicates nuclei with dark blue colour and cytoplasm with varying shades of pink.

### 2.3.8 Toluidine blue staining of paraffin embedded sections of cell-seeded scaffolds

For staining of GAGs in the cartilage ECM, 5 µm thick sections were cut and deparaffinized, rehydrated through a series of graded alcohols, and stained with 0.4% toluidine blue stain as previously described [see section 2.2.3 (c)]. Toluidine blue stains cartilage GAGs purple and cytoplasm and other connective tissue varying degrees of blue.

### 2.3.9 Collagen type II immunohistochemistry

Collagen type II immunolocalisation was used to identify articular cartilage extracellular matrix. Antigen retrieval was performed by incubating sections in 0.1% hyaluronidase and 0.2% trypsin in hyaluronidase buffer (comprising of 0.02 M phosphate buffer at pH 7, with 77 mM sodium chloride and 0.01% BSA: all from Sigma) for 60 minutes at 37°C in a humidified chamber. After antigen retrieval, the sections were washed three times in PBS and then incubated with a solution of 6.5 µg/ml of collagen type II antibody (Clone CIIC1; Developmental Studies Hybridoma Bank, Iowa City, IA) for 60 minutes at room temperature. The primary antibody was rinsed off and washed three times in PBS. Visualisation of positive collagen type II immunostaining was performed using a commercial kit (Vecta Stain Elite ABC kit, Vector labs, Peterborough, UK). Briefly, sections were incubated with a biotinylated secondary anti-mouse IgG for 30 minutes at room temperature. The sections were then washed three times in PBS and endogenous peroxidase activity was blocked using 0.3% hydrogen peroxide in methanol for 30 minutes at room temperature. During this incubation step, the Vecta ABC reagent (Vector labs) was prepared and was allowed to stand for 30 minutes before use as per the manufacturer's instructions. After blocking the endogenous peroxidase activity, sections were washed three times in PBS and incubated with ABC reagent for 30 minutes at room temperature. Following this, DAB chromogen (Vector labs) was added for 6-8 minutes depending on the intensity of colour desired. Sections were then washed in PBS and dehydrated through a series of ethanol (70-100%), cleared with xylene and mounted in Pertex mounting media.

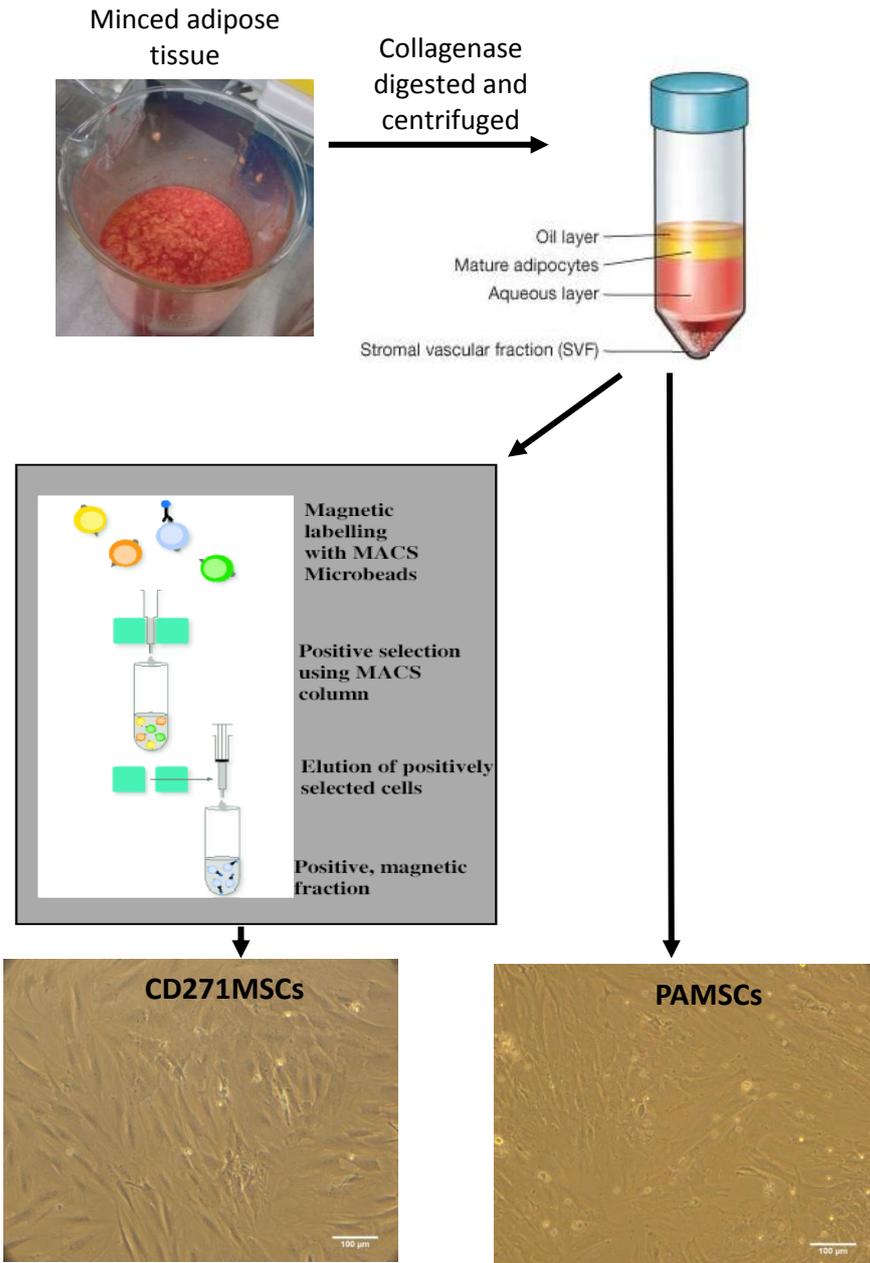
#### 2.3.10 Dimethylmethyline blue (DMMB) assay for proteoglycans

The DMMB assay protocol was adapted from the method of Farndale *et al.*, 1986 as follows: (i) the DMMB dye solution was prepared by adding 3.04g of glycine, 2.37g of NaCl and 16 mg of 1,9 DMMB to 1 litre of deionized water; (ii) the pH was adjusted to 3.0 with hydrochloric acid and the dye solution was stored in a brown bottle; (iii) 50µl aliquots of culture medium harvested from the cell-seeded scaffolds at day 28 were added in triplicate to a 96 well plate; (iv) 200µl of the DMMB dye solution was added to the culture medium and the absorbance was assessed at 540 nm immediately. Chondroitin sulphate (CS) from shark cartilage (Sigma) was used to provide a standard curve of absorbance (0-40 µg/ml, CS) from which the GAG content in the samples of culture medium was calculated. The levels of absorbance for GAG content in the samples of culture medium were normalized to account for the background absorbance resultant from the presence of phenol red within the medium by dissolving the standards in DMEM medium.

#### 2.3.11. Selective isolation of MSCs using CD271 as a marker

Following ethical approval and informed consent (LREC number 12/EE/0136), a total of 61.15 grams of subcutaneous AT and 300 ml of lipoaspirate was used from waste products of an abdominoplasty procedure. The AT was minced into small pieces and treated with 0.3 U/ml of collagenase (Sigma) for two hours at 37°C. The lipoaspirate sample was digested with 0.3 U/ml of collagenase for 30 minutes on a pre-warmed orbital shaker at a rotation of 250 rpm 37 °C. After collagenase digestion, the enzyme was neutralised by adding an equal volume of DMEM/20% (v/v) FCS and 1% (v/v) penicillin and streptomycin. The digested preparation was transferred to fresh sterile conical bottom tubes and spun at 600g for 10 minutes. The resulting cell pellet (i.e, the SVF) was re-suspended in 10 ml of standard culture media and was passed through a 100µm cell strainer (BD Biosciences) to remove undigested connective tissue. The filtrate was collected in a fresh sterile 15 ml conical bottom tube and was centrifuged again at 600g for 10 minutes. The resulting cell pellet was re-suspended in 5ml standard culture media and passed through a 40µm cell strainer and a cell count was

performed using trypan blue exclusion technique. If red blood cells were observed then the cell suspension was treated with one times dilution of erythrocyte lysis buffer (pre-made-Miltenyi) constituting 1.55M  $\text{NH}_4\text{Cl}$ , 100mM  $\text{KHCO}_3$  and 1mM EDTA at pH7.3, for 10 minutes at room temperature. The single cell suspension was then used for magnetic associated cell sorting (MACS) to isolate  $\text{CD271}^+\text{MSCs}$ . Half of the cell suspension was culture expanded as plastic adherent MSCs (PA MSCs) at a seeding density of  $5 \times 10^3$  cells/cm<sup>2</sup> in tissue culture flasks and the rest was used for  $\text{CD271}^+\text{MSCs}$  isolation using Miltenyi Biotec's CD271 MicroBead kit with the magnetic separators (Miltenyi, Surrey, UK), as per manufacturer's instructions. Briefly, a cell suspension of up to  $10^7$  cells was transferred to fresh sterile 15 ml conical bottom tube and centrifuged at 300 g for 10 minutes. The cells were then re-suspended in 60  $\mu\text{l}$  of PBS containing 0.5% BSA and 2mM EDTA at pH 7.2 (MACS buffer). Then 20  $\mu\text{l}$  of FcR blocking reagent and 20  $\mu\text{l}$  of CD271 MicroBeads were added to the suspension and incubated for 15 minutes in the refrigerator (2-8°C). The unbound MicroBeads were then washed off by adding 1-2 ml of MACs buffer and centrifuged at 300 g for 10 minutes. The supernatant was aspirated completely following centrifugation and the cells were re-suspended in 500  $\mu\text{l}$  of MACs buffer. Then a MACS column was prepared by placing the column in the magnetic field of a MACS separator and rinsing with 3 ml of MACS buffer. The cell suspension of 500  $\mu\text{l}$  was applied to the column and the flow-through was collected as the negative fraction. A further 3 ml of MACS buffer was passed through the column three times to collect unlabelled cells. Then, using a plunger, 5 ml of MACS buffer was flushed through the column and the flow-through was collected as the positive fraction referred to as  $\text{CD271}^+\text{MSCs}$ . The recovery of  $\text{CD271}^+\text{MSCs}$  (n=2) was  $0.3\% \pm 0.14\%$ .



**Figure 2.1.** A schematic representation of the isolation of CD271<sup>+</sup>MSCs and PAMSCs. Adipose tissue was minced and collagenase digested, after subsequent centrifugation and washing steps, the SVF cell pellet was used for either magnetic labelling with CD271 microbeads (to generate CD271 MSCs) or directly added to tissue culture flasks to select MSCs based on their ability to adhere to plastic (PAMSCs).

### 2.3.12 Scanning electron microscopy

SEM was performed in collaboration with the University of Fukui, Japan and the College of Medical and Dental Sciences, Birmingham, UK using their standard operating procedures. Briefly, the cell-seeded scaffolds were washed in PBS and then fixed in 2% glutaraldehyde in 0.1M phosphate buffer (pH 7.4) for 2 hours. Following this, the samples were washed in 0.1M phosphate buffer and then treated with 1% osmium tetroxide for 1 hour. The samples were then dehydrated through a graded series of ethanol solution from 50% to 75% to 90% to 95% and finally 100% for 10 minutes in each. This was followed by treating the samples with transition solvent, t-butyl alcohol for 30 minutes in order to allow freeze-drying in the same solvent or they were dried overnight in hexamethyldisilazane (HMDS). After the samples were freeze-dried, they were coated with gold palladium and then imaged using a JSM-6390 (JEOL, Tokyo, Japan) scanning electron microscope or a Zeiss EVO10 scanning electron microscope (Carl Zeiss, Cambridge, UK).

## **2.4 *In vivo* experimental procedures**

The *in vivo* study was conducted as a collaborative project with Dr Kenzo Uchida and Prof. Hisatoshi Baba along with other members of the surgical team at the Department of Orthopaedic and Rehabilitation Medicine, University of Fukui, Fukui, Japan.

### 2.4.1 Animal study design

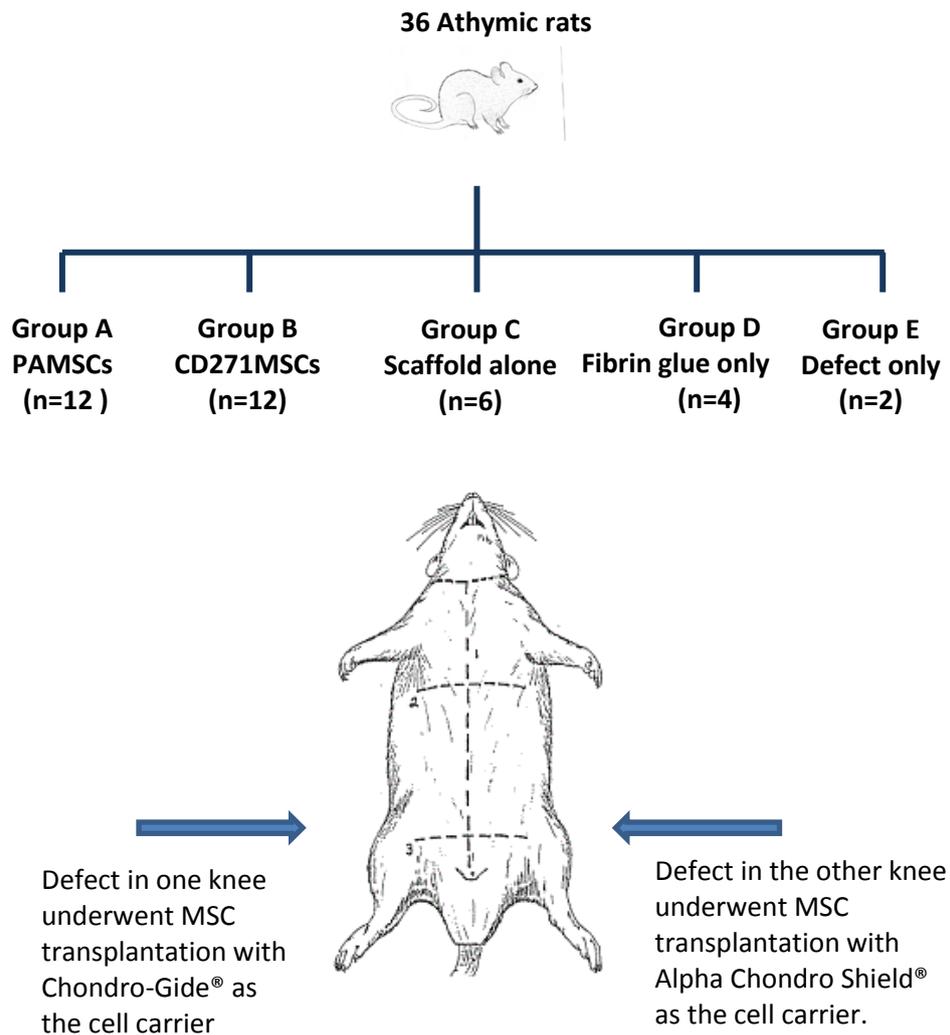
A total of 36 female athymic nude rats (F344/N Jcl rnu/rnu, CLEA Japan, Inc. Tokyo, Japan) age 6 to 10 weeks and weighing 150 to 170 grams were divided into five groups as; group A with PA MSCs (n=12), group B with CD271 MSCs (n=12), group C with scaffold alone control group (n=6), group D with fibrin glue alone control group (n=4), group E with no intervention (n=2). Bilateral osteochondral defects were created in the patellar groove of each animal and either Chondro-Gide® or Alpha Chondro Shield® was used as a cell carrier or scaffold. At 3 weeks and 6 weeks post-transplantation, animals were sacrificed to examine the extent of wound repair macroscopically (section 2.4.6) and histologically (section 2.4.8). An overall representation of the animal study design is shown in Figure 2.3.

The rats were fed a standard maintenance diet and provided water freely. The Institutional Animal Care and Use Committees of Fukui University, Department of Orthopaedics and Rehabilitation Medicine approved all animal procedures (Ethical Approval Number 25-053).

### 2.4.2 Femoral osteochondral defect model

The rats were anaesthetized by exposure to 3% isoflurane in O<sub>2</sub> gas in an anaesthetic chamber and during surgery were maintained at 1.5% isoflurane in O<sub>2</sub> gas delivered through the inhalation mask. After sterilising the knees using 70% ethanol, a medial parapatellar skin incision was made followed by dissecting through the muscle and then exposing the knee joint by lateral dislocation of the patella. A reproducible model of osteochondral articular cartilage defect of 2 mm diameter and 1 mm depth was created in the patellar groove of each femur using a 2mm diameter surgical drill. Although the plan was to create full-thickness chondral defects, on later histological inspection it was clear that the injury penetrated beyond the

subchondral bone and into the trabecular spaces on several occasions. Therefore, the study has been designated as osteochondral defect to reflect this extent of injury.



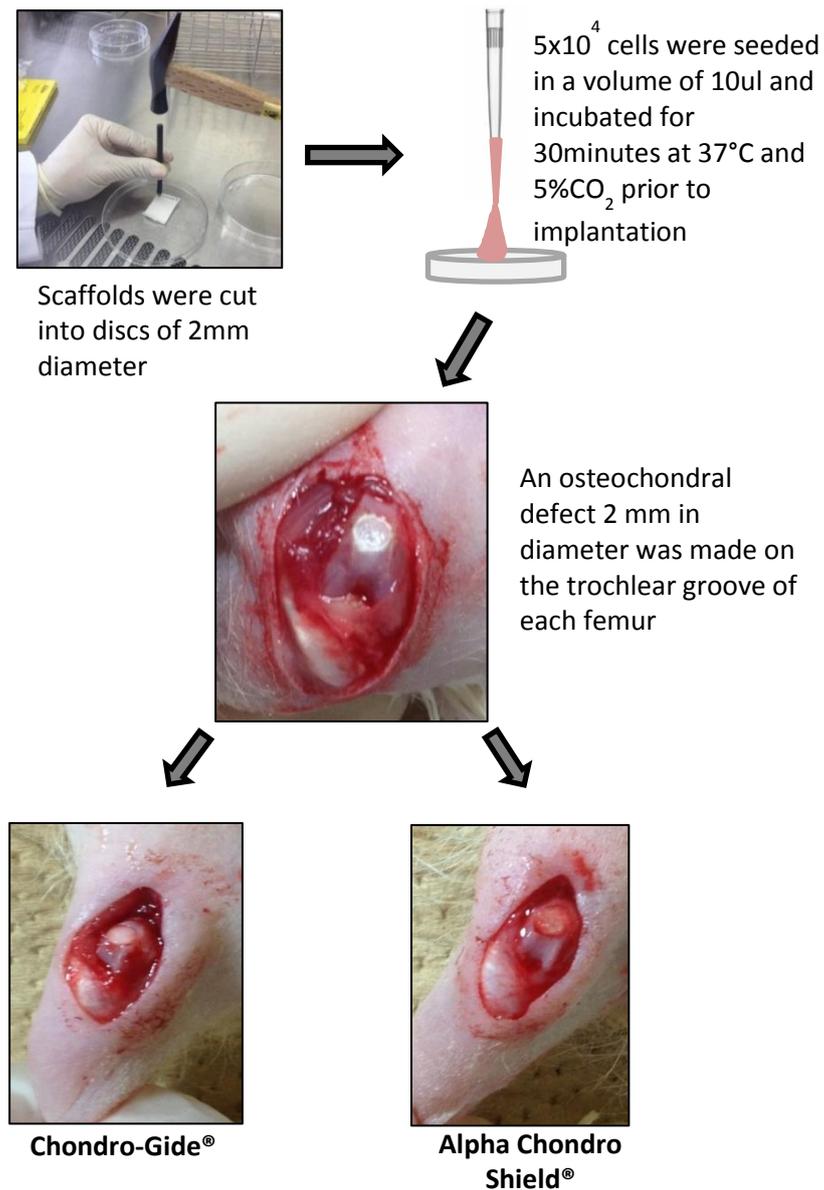
**Figure 2.2. A schematic representation of the *in vivo* study design.** A total of 36 athymic female rats were divided into five groups. Bilateral osteochondral defects were created in the patellar groove of each animal. For animals in groups A and B, one knee received MSCs seeded on Chondro-Gide® as the cell-carrier and the other knee received MSCs seeded on Alpha Chondro Shield® as the cell-carrier. For animals in group C, both knees received scaffolds alone. For animals in groups D and E, both knees received fibrin glue only or had no intervention, respectively.

#### 2.4.3 Preparation of scaffolds for cell seeding

2mm diameter disks of sterile Chondro-Gide® and Alpha Chondro Shield® were cut out using a sterile 2mm diameter punch and a hammer. After the scaffolds pieces were cut, they were UV treated on both sides for 15 minutes each to further sterilise the pieces.

#### 2.4.4 Cell seeding and transplantation

AT derived, PA MSCs and CD271<sup>+</sup> MSCs were trypsinised at confluency as previously described in section 2.1.3(a). A viable cell count was performed using the trypan blue exclusion technique and  $5 \times 10^4$  viable cells were seeded in a volume of 10  $\mu$ l of standard culture medium onto the 2 mm diameter disks of each of Chondro-Gide® and Alpha Chondro Shield® and were left to promote cell adherence and incorporation at 37°C and 5% CO<sub>2</sub> for 30 minutes before transplantation. Scaffolds were imaged using SEM to examine the extent of cell attachment at this time using separate samples. The cell-seeded scaffolds were transplanted into the osteochondral defects created in the patellar groove of each rat knee joint and then fixed in place with fibrin glue, which was allowed to set for about 10-20 seconds (Figure 2.4). The patella was then relocated and the connective tissue was sutured followed by the suturing of the skin with nylon sutures. The inhalation mask was then removed and rats were transferred back to their cage and were allowed to move freely following recovery. The rats were inspected for any signs of infection or inflammation every day.



**Figure 2.3. A schematic representation of MSCs transplantation for full-thickness osteochondral defects.** Both scaffolds were cut into 2mm diameter sized disks and were sterilised using UV prior to cell-seeding. Culture expanded MSCs were trypsinised and seeded onto the disk shaped scaffolds using a simple pipetting technique in a volume of 10µl. Subsequently the cell-seeded scaffolds were transplanted into the defects and fixed in place using fibrin glue.

#### 2.4.5 Animal sacrifice and perfusion

At 3 weeks and 6 weeks post-transplantation, 18 animals (comprising of n=6 animals in PA MSCs; n=6 animals in CD271<sup>+</sup>MSCs; n=3 animals in scaffold alone control group; n=2 animals i.e, n=4 knees in fibrin glue alone control group and n=1 animal i.e., n=2 knees for no- intervention control group) were sacrificed by overdoes of 3% isoflurane. Once the animals had reached the surgical plane of anaesthesia, they were placed on the surgical tray and a toe pinch-response test was conducted to determine depth of anaesthesia and to ensure that the animals were unresponsive. A 5-6 cm medial incision was made in the middle of the rib cage and the skin was separated using the blunt scissors. Then a small lateral incision was made through the integument and abdominal wall just beneath the rib cage and the liver was carefully separated from the diaphragm. The diaphragm was carefully excised using blunt scissors along the entire length of the rib cage to expose the pleural cavity. The sternum was then lifted away, carefully trimming any tissue connecting to the heart and the major vessels of the heart were clearly viewed. A small incision was then made to the posterior end of the left ventricles using sharp scissors. A 15 gauge blunt needle was passed through the cut ventricle into the ascending aorta. A haemostat was used to clamp the heart and an incision was made in the right atrium to create an outlet of flow without damaging the descending aorta. At this point, the animal was ready to be perfused. This was done by attaching the outlet port of the tube containing 4% paraformaldehyde to the needle and opening the port to allow perfusion of paraformaldehyde through the animal's heart until the outlet flow from the atrium runs clear. Once the animal was fixed, the knees were surgically opened in a similar way as at the time of surgery.

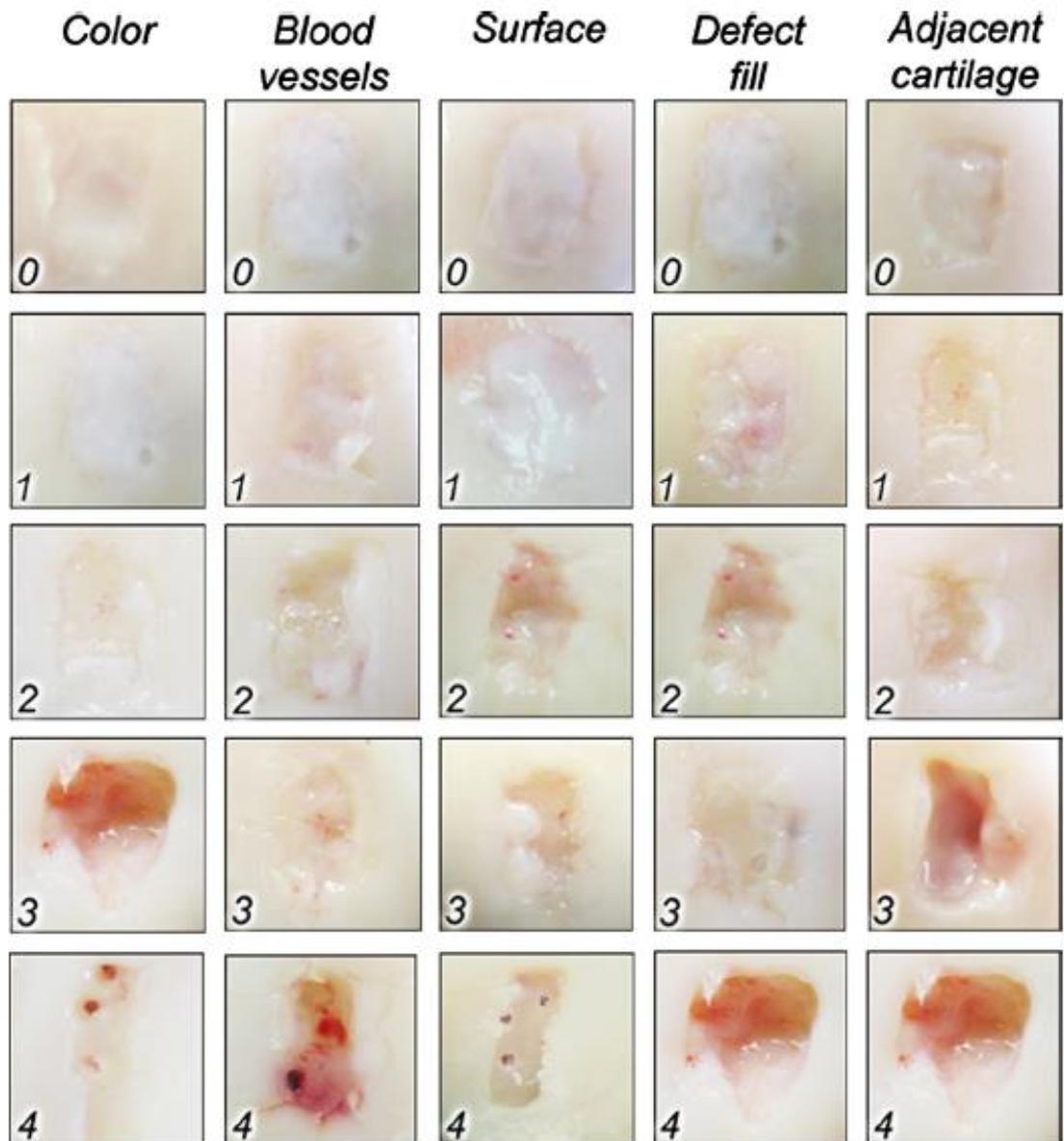
#### 2.4.6 Macroscopic scoring

All n=18 animals at each time point were assessed macroscopically as follows. After exposing the knee joint, the defect was observed and scored macroscopically using an established macroscopic scoring system for smaller animal models (Goebel *et al.*, 2012). Table 2.1 shows the parameters used for macroscopically scoring the extent of wound repair. The degree of

repair was scored from 0 points (for the best outcome) to 4 points (for the worst outcome) for each of the parameters examined. For total scores, the points were added and the degree of best repair was represented with the lowest score from a total score of 20 (Table 2.1). Figure 2.4 shows the representative example images for each of the parameters.

Newly developed macroscopic scoring system		
Parameter	Description	Points
Colour of the repair tissue	Hyaline or white	0
	Predominantly white (>50%)	1
	Predominantly translucent (>50%)	2
	Translucent	3
	No repair tissue	4
Presence of blood vessels in the repair tissue	No	0
	Less than 25% of the repair tissue	1
	25-50% of the repair tissue	2
	50-75% of the repair tissue	3
	More than 75% of the repair tissue	4
Surface of the repair tissue	Smooth, homogeneous	0
	Smooth, heterogeneous	1
	Fibrillated	2
	Incomplete new repair tissue	3
	No repair tissue	4
Filling of the defect	In level with adjacent cartilage	0
	>50% repair of defect depth or hypertrophy	1
	<50% repair of defect depth	2
	0% repair of defect depth	3
	Subchondral bone damage	4
Degeneration of adjacent articular cartilage	Normal	0
	Cracks and/or fibrillations in integration zone	1
	Diffuse osteoarthritic changes	2
	Extension of the defect into the adjacent cartilage	3
	Subchondral bone damage	4
<b>Total points</b>		<b>20</b>

**Table 2.1. Parameters utilized for macroscopic scoring of the repair tissue at 3 weeks and 6 weeks post-transplantation.** The degree of repair tissue was scored 0 points for the best outcome and 4 points for the worst outcome for each of the parameters assessed. On total the worst repair would get a score of 20 points and the best repair would get a score of 0 points. Parameters adapted from Goebel *et al.*, 2012.



**Figure 2.4. Representative examples for macroscopic scoring of the defects.**

The images display representative examples for the scores (bottom left) for each of the parameters assessed. Image adapted from Goebel *et al.*, 2012.

#### 2.4.7 Histology

Out of the 18 animals sacrificed at each time point, 9 animals and one knee from no intervention control group were used for histological assessment. The study was designed to use the rest 9 animals and one knee for RNA isolation, which was unsuccessful. Therefore, the extent of defect repair was only assessed qualitatively using histological assessments. The number of n's presented in Chapter 5 of this thesis for histological scoring reflects the number of knees. For histological assessment at 3 weeks and 6 weeks, the n's were, n=3 in PA MSCs group for each of the scaffolds, n=3 in CD271<sup>+</sup>MSC group for each of the scaffolds, n=2 for each of the scaffolds in scaffold alone control group, n=2 for fibrin glue only, n=1 knee for no intervention control group.

The animal knees were fixed in 10% neutral buffered formalin (Sigma) for 48 hours and then fixed in K-CX decalcifying solution (FALMA, Tokyo, Japan) for 24-48 hours at 4°C. K-CX is a rapid decalcification solution constituting mainly hydrochloric acid along with other chelating agents. Following this, the rat knees were washed overnight in running tap water and then processed and embedded in paraffin wax blocks ready for sectioning. Tissue sections were cut at 5 micron thickness in a standard rotary microtome and were then stained with H&E stain and toluidine blue histological stain and collagen type II immunolocalisation as described in sections 2.3.7, 2.3.8 and 2.3.9, respectively. The tissue sections were scored for the extent of cartilage repair using a modified Wakitani scoring system as described in the section below.

#### 2.4.8 Histological scoring

A modified Wakitani scoring system was used to assess the extent and quality of cartilage repair (Wakitani *et al.*, 1994). The original scoring system was modified to add two more parameters, to examine the presence of blood vessels and foreign body giant cells (FBGCs). Hence, the scoring system comprised of seven different parameters to examine the histological quality of the repair tissue. For each of these parameters (presented in Table 2.2), the lowest

score represented the 'best' repair and a highest score represented the 'worst' repair .The overall scores were added and then compared between groups.

Modified Wakitani Scoring system		
Parameter	Description	Points
<b>Cell morphology</b>	Hyaline cartilage	0
	Mostly hyaline cartilage	1
	Mostly fibrocartilage	2
	Mostly non-cartilage	3
	Non-cartilage only	4
<b>Matrix staining (metachromasia)</b>		
	Normal (compared with host adjacent cartilage)	0
	Slightly reduced	1
	Markedly reduced	2
	No metachromatic stain	3
<b>Surface regularity (total smooth area compared with entire area of cartilage defect)</b>	Smooth ( $> 3/4$ )	0
	Moderate ( $> 1/2-3/4$ )	1
	Irregular ( $1/4-1/2$ )	2
	Severely irregular ( $< 1/4$ )	3
<b>Thickness of cartilage (compared with that of surrounding cartilage)</b>	$> 2/3$	0
	$1/3-2/3$	1
	$< 1/3$	2
<b>Integration of donor with host adjacent cartilage</b>	Both edges integrated	0
	One edge integrated	1
	Neither edge integrated	2
<b>Blood vessels</b>	0-5	0
	5-10	1
	10-20	2
	20-30	3
	$>30$	4
<b>Foreign body giant cells</b>		
	0	0
	0-5	1
	5-10	2
	$>10$	3
<b>Total points</b>		<b>21</b>

**Table 2.2. Modified Wakitani scoring system for histological assessment of repair tissue.**

The best repair tissue was scored 0 points and the worst repair was scored either 2,3 or 4 depending on the parameter assessed (Wakitani *et al.*, 1994).

#### 2.4.9 Immunohistochemistry for human mitochondrial antigen staining

The sections were stained with anti-human mitochondrial antigen (HMA) antibody (Clone 113-1: Abcam, Cambridge, UK) to assess the presence of human MSCs. The sections were first immersed in citric acid-based antigen retrieval solution (Vector Labs Ltd ) at pH 6 and incubated at 60°C in a waterbath overnight (minimum 12 hours). Following this antigen retrieval step, the slides were washed in three changes of PBS and incubated for 20 minutes with 2.5% horse serum (Vector Labs Ltd) at room temperature to prevent non-specific binding. The sections were then incubated with the mouse anti-HMA antibody (1:400 dilution in PBS) for 1 hour at room temperature in a humidified chamber. Following this, any unbound antibody was washed off in three changes of PBS gently and the sections were incubated with biotinylated anti-mouse IgG for 30 minutes at room temperature. The sections were washed three times in PBS and endogenous peroxidase activity was blocked using 0.3% hydrogen peroxide in methanol for 30 minutes at room temperature. During this incubation step, the Vecta ABC reagent (Vector Labs Ltd) was prepared and was allowed to stand for 30 minutes before use as per the manufacturer's instructions. After blocking the endogenous peroxidase activity, sections were washed in three times PBS and incubated with the ABC reagent for 30 minutes at room temperature. Following this, a DAB chromogen (Vector labs) was added for 6-8 minutes depending on the intensity of colour desired. Sections were then washed and dehydrated through series of ethanol (70-100%), cleared with xylene and mounted in Pertex. Chondrogenic pellet of human MSCs was used as a positive control. Negative control included chondrogenic pellet where the primary antibody was omitted.

## 2.5 Statistical analysis

For results presented in Chapter 3, data was generated from a minimum of  $n=2$  independent donors for each BM and AT. For  $n=2$  donors, e.g., live/dead scoring for BM and AT cells in scaffolds, data was pooled from internal replicates and has been presented as means  $\pm$  standard deviation of the mean (SD). For  $n=3$  donors, e.g., CD profiling of BM and AT MSCs, data has been presented as means  $\pm$  SEM.

For results presented in Chapter 4, with the exception of SEM, data was generated from  $n=2$  MSC donors. This was pooled from internal replicates of GAGs analysis and has been presented as means  $\pm$  SDs.

For results presented in Chapter 5, data was generated from a single MSC donor, but from multiple animals that were included in different experimental groups. For macroscopic scoring for e.g., this varied from  $n=6$  for PA MSC group to  $n=3$  for scaffold alone control groups. Here, data has been presented as means  $\pm$  SDs for all groups except for the no intervention control group where only 1 animal was assessed.

Statistical analysis was performed using GraphPad Prism6 software (GraphPad Software, Inc. CA, USA) for data presented within Chapter 5 only for groups that had a minimum of  $n=3$  animals. Data presented in Chapters 3 and 4 were excluded from statistical analysis due to small sample size. For macroscopic scoring of repair tissue in different animal groups, a non-parametric one-way ANOVA in conjunction with Dunn's multiple comparison test was performed. For histological scoring of cell-treated groups A and B, a non-parametric Mann Whitney U test was performed. *P* values of  $<0.05$  were considered significant.

**Chapter 3: An *in vitro* investigation of the incorporation, growth and chondrogenesis of freshly isolated versus culture expanded cells from human bone marrow and adipose tissue in clinically used scaffolds**

### 3.1 Background and aims

ACI is the most extensively studied and practised cell-based therapy for chondral defects. This procedure requires the expansion of autologous chondrocytes in the laboratory followed by subsequent cell transplantation in a second surgery (Brittberg *et al.*, 1994). The long term (3-9 years) clinical outcomes of ACI has shown variable results with some studies indicating a production of durable cartilage-like repair tissue (Bartlett *et al.*, 2005; Peterson *et al.*, 2010) and others reporting the production of fibrous cartilage (Horas *et al.*, 2003; Roberts *et al.*, 2003). Despite the success of a few clinical studies for the use of autologous chondrocytes, one of the main obstacles to successful cartilage repair is the de-differentiation of chondrocytes to a fibroblast-like state during cell culture expansion (Benya *et al.*, 1978). This phenotypic shift is accompanied by alterations in collagen expression patterns with a loss of type II collagen, the major extracellular matrix collagen found in hyaline cartilage (Schnabel *et al.*, 2002), and a decreased synthesis of proteoglycan. Although, chondrocyte re-differentiation in appropriate 3D culture systems has been shown *in vitro* (Benya and Shaffer 1982), there is less clear evidence of this occurring after transplantation (Roberts *et al.*, 2003; Bartlett *et al.*, 2005). In addition, the chondrocytes that are used for ACI are derived from biopsies of tissue obtained from the articular joint, which may be associated with some donor site morbidity (Matricali *et al.*, 2010). Therefore, a current focus in regenerative medicine for the loss of cartilage is to identify alternative potential therapeutic cell sources that form cartilage repair tissue with the characteristics and functionality of native hyaline articular cartilage.

Several preclinical *in vitro* and animal studies have suggested that MSCs can potentially provide an alternative to autologous chondrocytes for the regeneration of cartilage as they have the ability to differentiate towards chondrogenic lineage (Mitchell and Shepard 1976; reviewed by Caplan and Goldberg 1999; Pittenger 2008). BM is the most extensively studied source of MSCs, however, AT also appears to be a good source of MSCs (Zuk *et al.*, 2001). Moreover, MSCs from AT may have certain advantages over those derived from BM,

for example, AT can be obtained by minimally invasive procedures compared to highly invasive iliac crest biopsy for BM aspirate. Also, the yield of MSCs from AT is markedly greater than other sources (Kern *et al.*, 2006; Helder *et al.*, 2007).

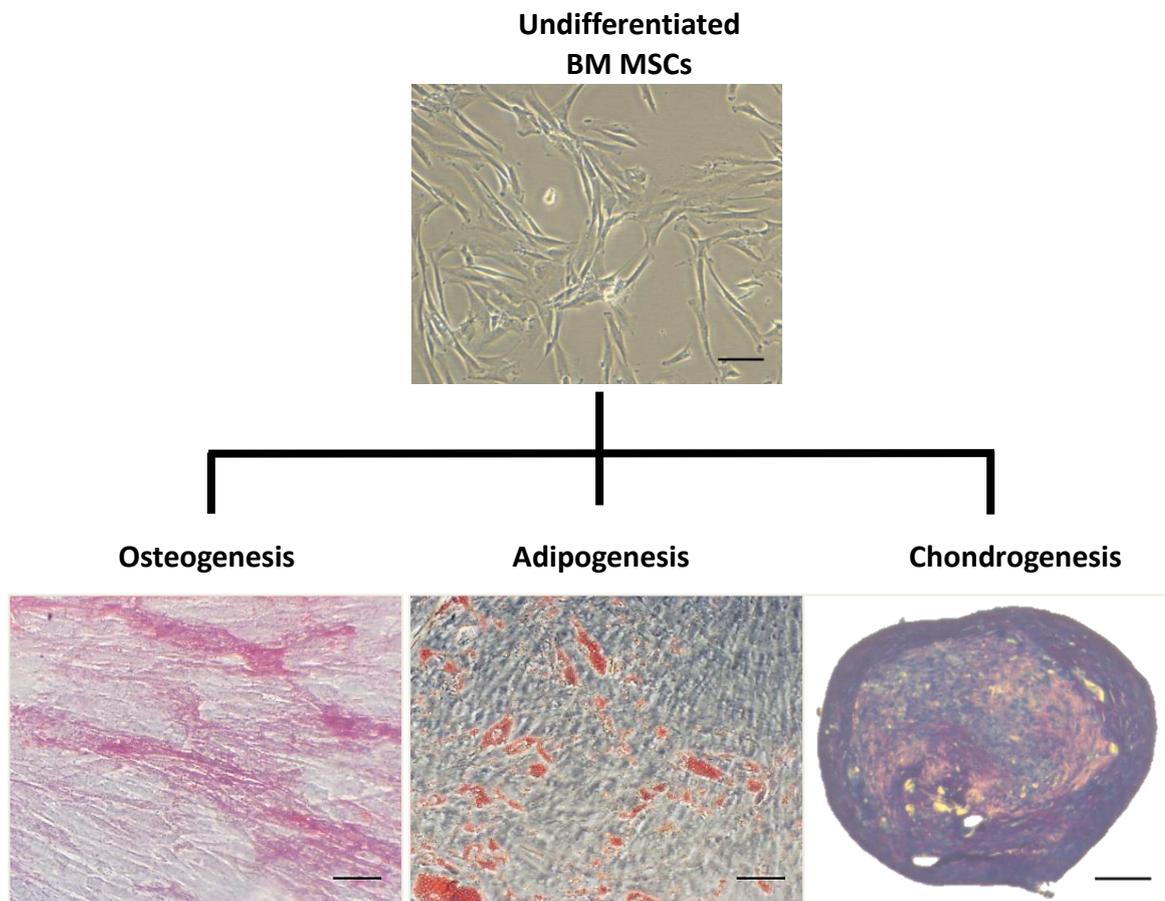
Whether or not AT MSCs are equivalent to BM MSCs in terms of their chondrogenic differential potential is still a matter of debate, where some studies suggested that AT MSCs may have inferior potential for chondrogenesis (Im *et al.*, 2005; Lee *et al.*, 2012) whilst others reported on the successful multilineage differentiation potential of AT MSCs that included chondrogenesis (Danišovič *et al.*, 2007; reviewed by Strem *et al.*, 2005). Therefore, it is important to consider both cell sources when examining and identifying potential therapeutic targets for cartilage repair.

In addition, the uptake of cell therapies for cartilage repair is limited, due, in part, to the perceived need for a costly phase of cell culture expansion, whether using chondrocytes or MSCs, in order to generate enough cells for transplantation. An alternative approach has recently gained some attention, where freshly isolated cells have been used in transplantation studies, as BM concentrate, without a period of culture (Giannini *et al.*, 2009; Buda *et al.*, 2010; Gobbi *et al.*, 2011). Using freshly isolated cells obviates the need for culture expansion, thereby reducing the costs of cell therapies for cartilage repair, as cells can be obtained and administered in a one-step procedure, so long as there is a suitable cell scaffold/delivery system.

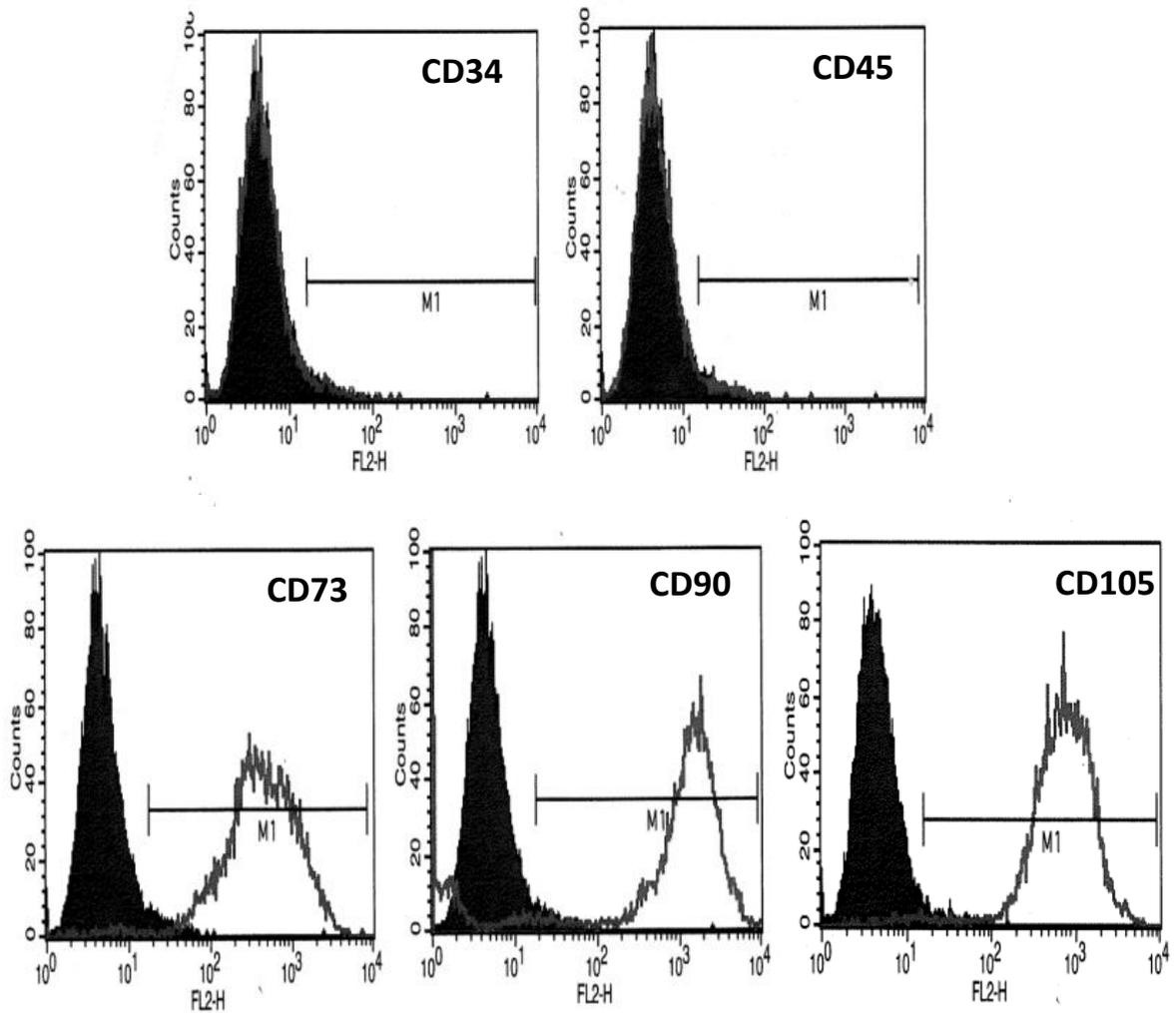
This chapter has focussed on looking at a variety of potential therapeutic human cell sources in combination with three clinically useable cell carriers or scaffolds; Chondro-Gide®, Alpha Chondro Shield® and Hyalofast®. The work described was performed to assess the incorporation and viability of each of the cell types examined, and to establish the potential of the cell-seeded scaffolds for chondrogenesis. The different cell sources were cell culture expanded MSCs from BM (BM MSCs) and from AT (AT MSCs), compared with freshly isolated mononucleated cells from BM (BM MNCs) and from AT, the AT SVF.

### **3.2. Phenotypic characterisation of BM MSCs**

Mononucleated cells isolated from BM were selected according to their ability to adhere to tissue-culture plastic and culture expanded to passage II-III. These culture expanded cells demonstrated a phenotype consistent with the three main criteria proposed by the ISCT. As show in Figure 3.1, the adherent culture expanded cells of stromal appearance differentiated down the three mesodermal lineages as indicated by positive alkaline phosphatase staining for osteogenesis, positive Oil Red O staining of lipid vacuoles for adipogenesis and metachromatic toluidine blue staining of paraffin-sections of cell pellets for chondrogenesis. The BM MSCs also showed immunopositivity for MSC specific cell-surface antigens, which were CD73, CD90 and CD105 and immunonegativity for haematopoietic markers, which were CD34 and CD45 (Figure 3.3).  $2.2\% \pm 0.3\%$  cells were CD34 positive,  $2.7\% \pm 0.7\%$  cells were CD45 positive,  $90.4\% \pm 5.1\%$  cells were CD73 positive,  $84.8\% \pm 4\%$  cells were CD90 positive, and  $97.8\% \pm 0.6\%$  cells were CD105 positive, respectively. Table 3.1 shows the percentage expression of positive and negative markers in BM MSCs (n=3).



**Figure 3.1. Tri-lineage differentiation potential of BM MSCs.** BM MSCs differentiated into osteoblasts, adipocytes and chondrocytes as indicated by the presence of alkaline phosphatase positive pink osteoblasts (osteogenesis), Oil Red O positive red lipid vacuoles (adipogenesis) and purple metachromatic staining of cartilage ECM (chondrogenesis), respectively. BM MSCs, n=3. Original magnification x10. Scale bar = 100  $\mu$ m.



**Figure 3.2. Flow cytometry for CD-cell surface antigen characterisation of BM MSCs.** The grey lined histogram shows immunopositivity for each indicated marker whereas the black histogram indicates immunolabelling with an isotype-matched control antibody. The x- axis represents the fluorescence intensity and the y-axis represents the number of cells counted. The marker M1 indicates positive events. BM MSCs, n=3.

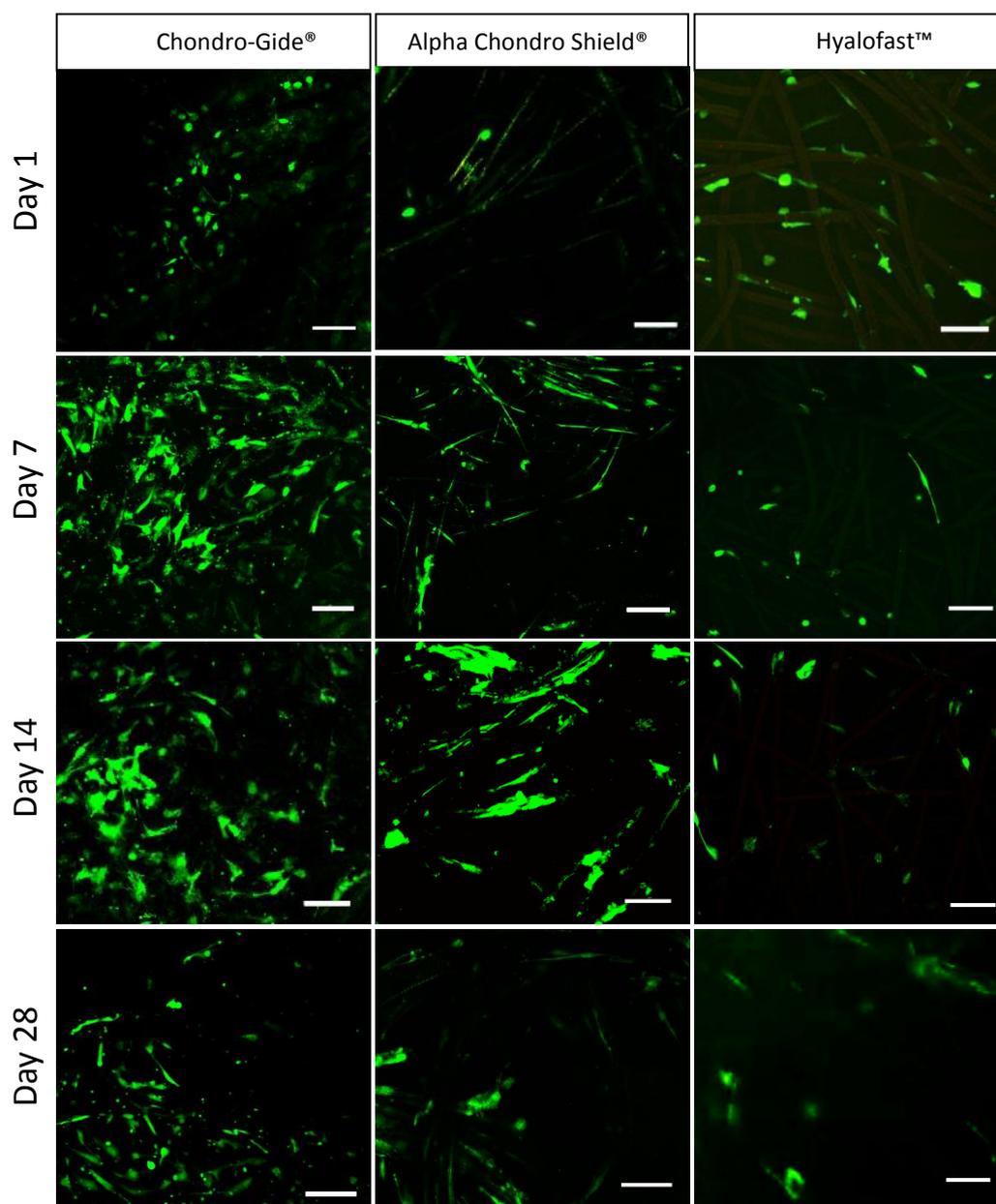
		Percentage expression of CD markers				
Cell type	Patient ID	CD34	CD45	CD73	CD90	CD105
BM MSCs	12-067	2.46	2.74	99.19	84.24	99.07
	BMS 050	1.72	3.84	90.67	92.23	97.37
	BMS 101	2.69	1.54	81.48	78.16	97.15

**Table 3.1. Immunoreactivity of BM MSCs for each CD marker.** The percentage of cells that were immunopositive for CD34, CD45, CD73, CD90 and CD105 are shown. 2.2%  $\pm$  0.3% cells were CD34 positive, 2.7%  $\pm$  0.7% cells were CD45 positive, 90.4%  $\pm$  5.1% cells were CD73 positive, 84.8%  $\pm$  4% cells were CD90 positive, and 97.8%  $\pm$  0.6% cells were CD105 positive. Data has been presented as means  $\pm$  SEM of n=3 patient donors.

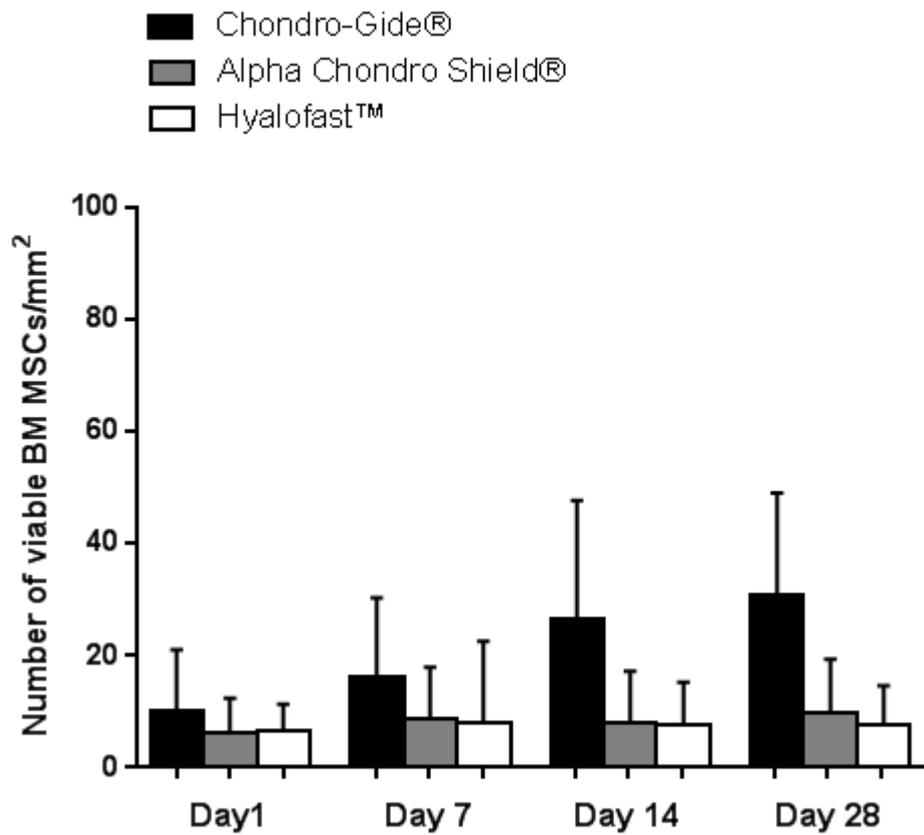
### **3.3 The incorporation and viability of culture expanded BM MSCs versus freshly isolated BM MNCs in the three clinically relevant scaffolds**

The BM MSCs (n=2) adhered and incorporated in all three scaffolds with a greater initial incorporation of cells in Chondro-Gide® compared to Alpha Chondro Shield® and Hyalofast™. Many cells were lost during cell-seeding and only  $0.8\% \pm 0.1\%$  of BM MSCs retained in Chondro-Gide® compared with  $0.5\% \pm 0.4\%$  of cells retained in Alpha Chondro Shield® and  $0.5 \pm 0.3\%$  of cells in Hyalofast™ at day 1. With time in culture after initial cell seeding, the prevalence of BM MSCs became markedly greater in Chondro-Gide® than the other two. The mean number of BM MSCs per mm<sup>2</sup> in Alpha Chondro Shield® and Hyalofast™ did not appear to increase with time in culture. The morphology of BM MSCs in Chondro-Gide® and Alpha Chondro Shield® appeared to be fibroblast-like whereas the cells in Hyalofast™ showed a mixed morphology of elongated fibroblast-like cells and round cells. Cell viability remained greater than 95% at all-time points and in all the scaffolds. Data has been presented as means  $\pm$  SDs of 16 internal replicates pooled from two patient donors (Figure 3.3 and Figure 3.4).

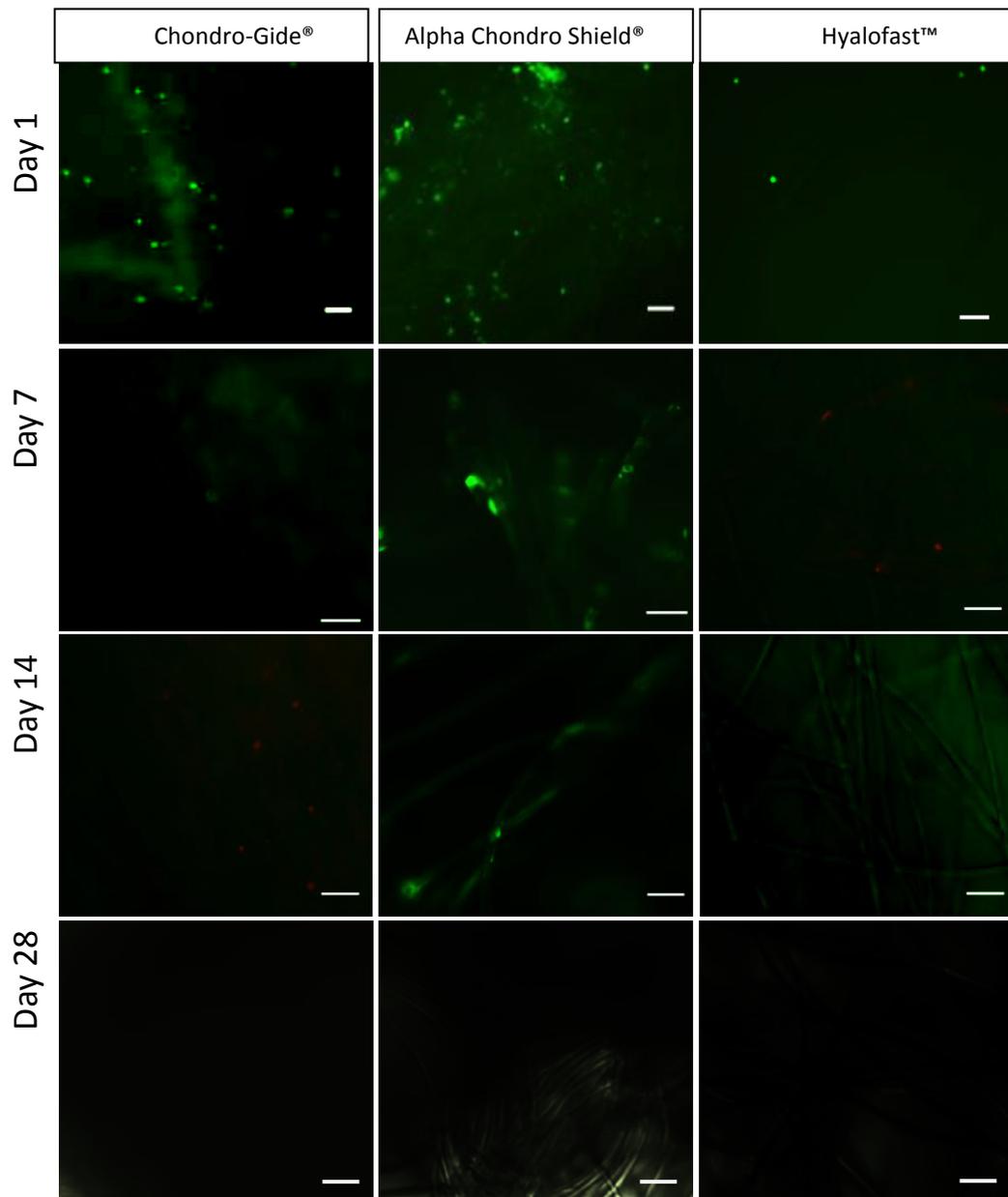
Freshly isolated BM MNCs (n=2) showed poor incorporation and adhesion in all three scaffolds. The number of cells initially incorporated were lower compared to the culture expanded MSCs. With time in culture, no viable BM MNCs were seen at day 7 in Chondro-Gide® and Hyalofast™ and only a few BM MNCs were seen in Alpha Chondro Shield®. Morphologically, these cells appeared round and did not show a fibroblast-like appearance. By 28 days post-seeding, no viable BM MNCs were seen in any of three scaffolds (Figure 3.5 and Figure 3.6).



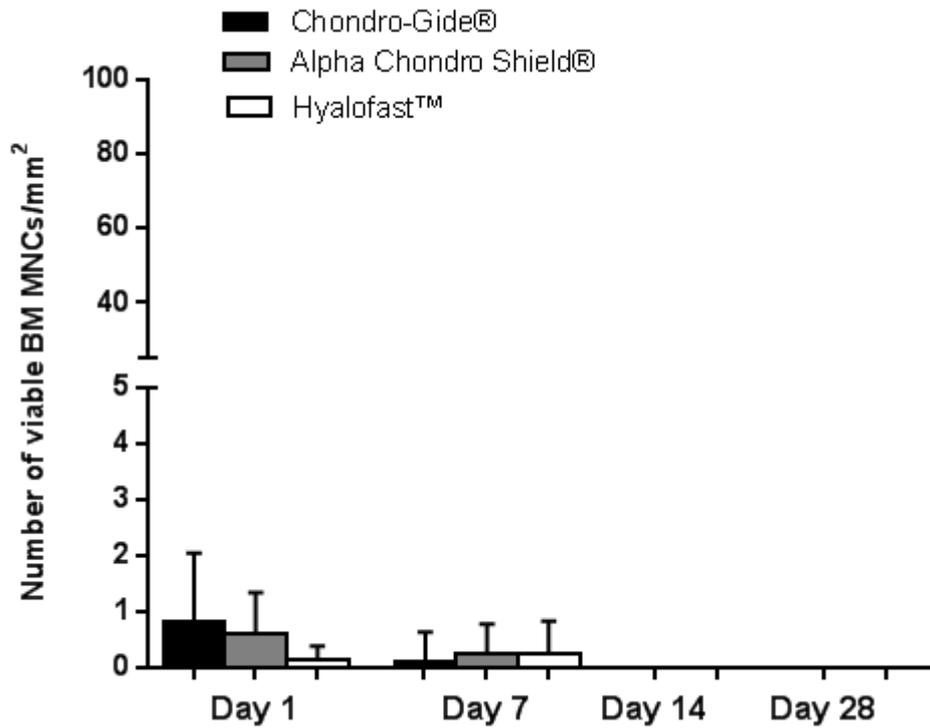
**Figure 3.3. Growth and viability of BM MSCs in Chondro-Gide®, Alpha Chondro Shield® and Hyalofast™ over 28 days in culture.** Representative confocal z-stacks are shown of BM MSCs following live/dead staining. There appeared to be a greater prevalence of viable BM MSCs in Chondro-Gide® compared to Alpha Chondro Shield® and Hyalofast™ over a 28 day period. Overall BM MSCs were over 95% viable in all three scaffolds during these long term cultures. Viable cells = green fluorescence, non-viable cells = red fluorescence. BM MSCs, n=2. Scale bar = 100  $\mu$ m.



**Figure 3.4. The number of viable BM MSCs in Chondro-Gide®, Alpha Chondro Shield® and Hyalofast™ over 28 days in culture.** The prevalence of BM MSCs was greatest in Chondro-Gide® compared to Alpha Chondro Shield® and Hyalofast™ at all time points studied. An increase in the number of cells was seen in Chondro-Gide® only with time in culture. All data have been presented as means  $\pm$  SD of 16 internal replicates pooled from n=2 patient donors.



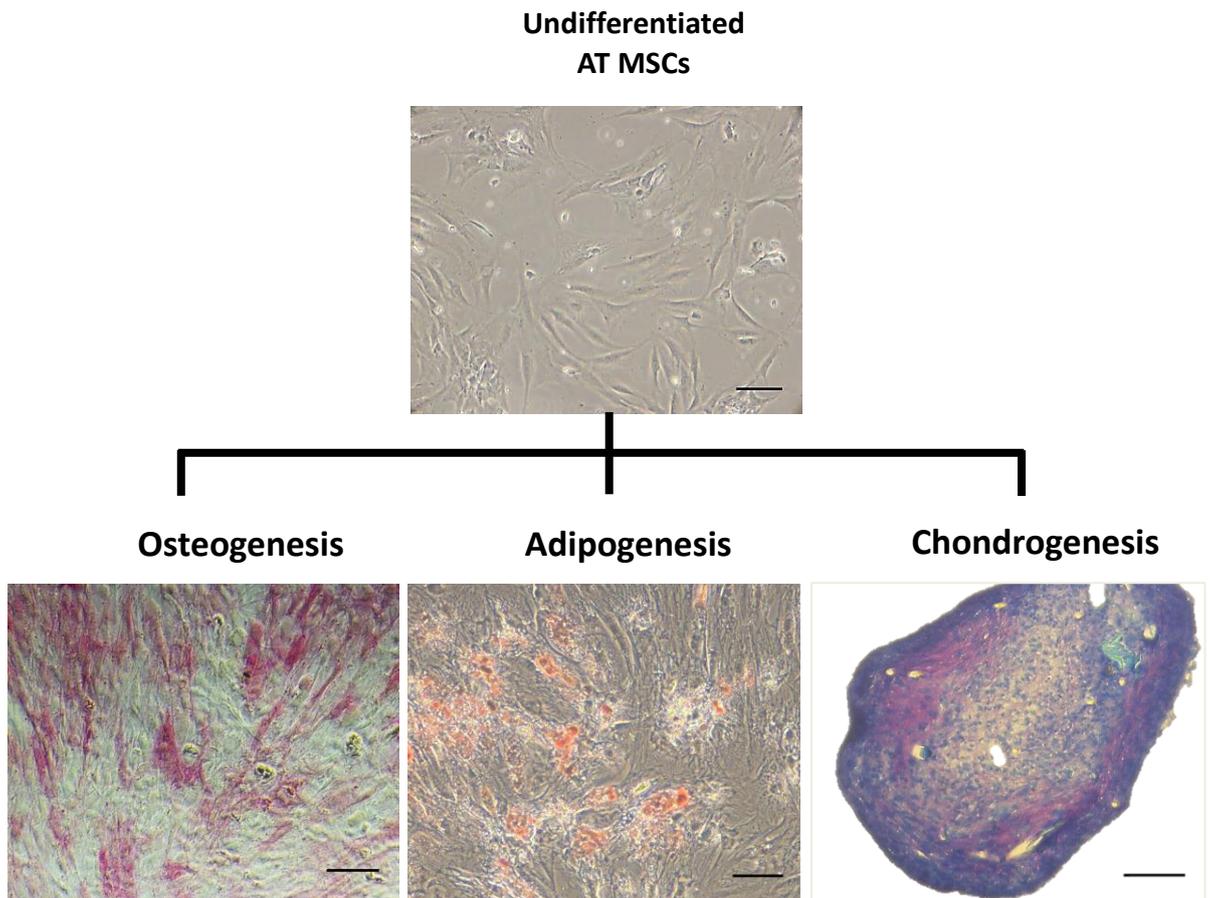
**Figure 3.5. Growth and viability of BM MNCs in Chondro-Gide®, Alpha Chondro Shield® and Hyalofast™ over 28 days in culture.** Representative confocal z-stacks are shown of BM MNCs following live/dead staining. The prevalence of BM MNCs was poor in all three scaffolds. At day 1, a few cells were seen in Chondro-Gide® and Alpha Chondro Shield®, however no cells were seen in any of the three scaffolds during long term cultures. Viable cells = green fluorescence, non-viable cells = red fluorescence. BM MNCs, n=2. Scale bar = 100  $\mu$ m.



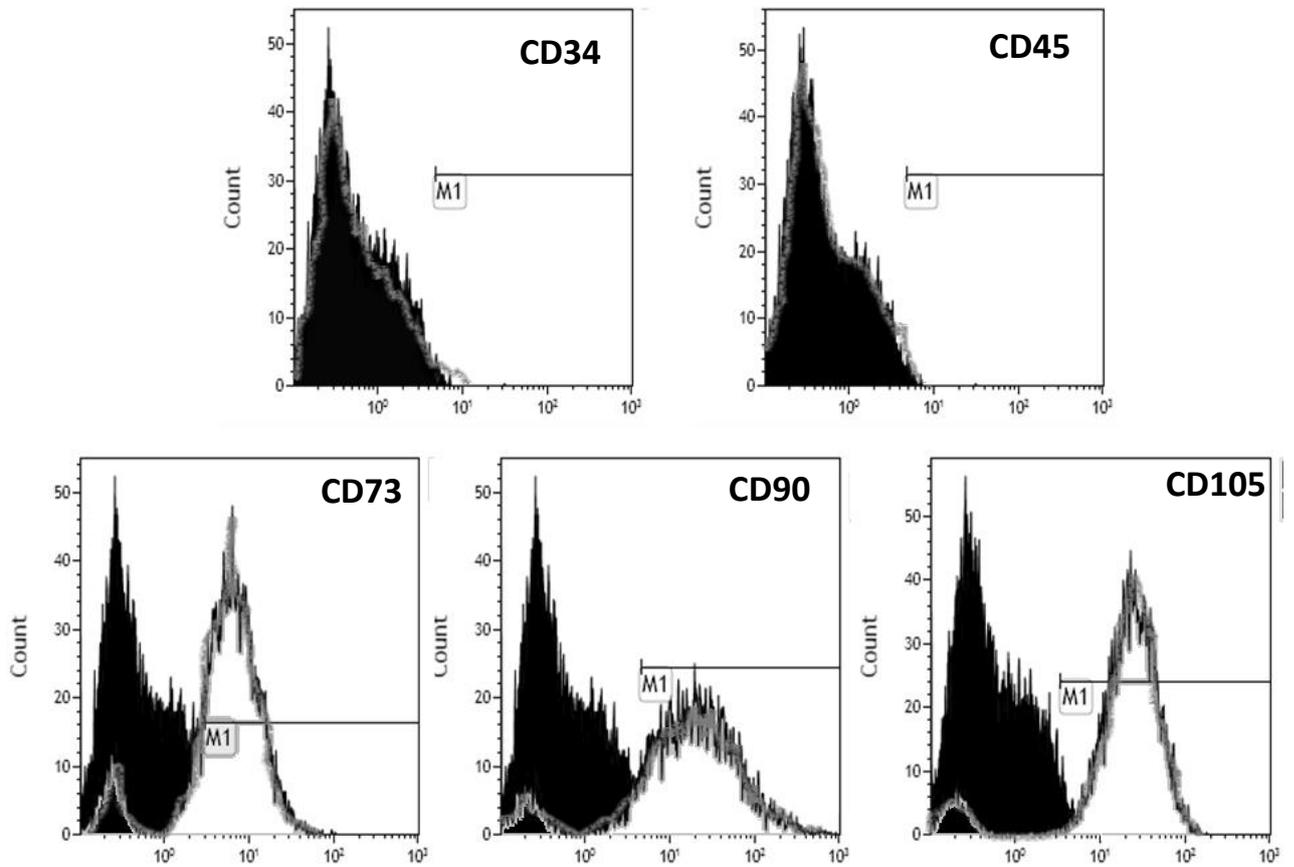
**Figure 3.6. The number of viable BM MNCs in Chondro-Gide®, Alpha Chondro Shield® and Hyalofast™ over 28 days in culture.** The prevalence of BM MNCs was greater in Chondro-Gide® followed by Alpha Chondro Shield®, however during long term cultures (over 7 days), no cells were seen in any of the three scaffolds. Data has been presented as means  $\pm$  SD of 16 internal replicates pooled from n=2 patient donors.

### **3.4 Phenotypic characterisation of AT MSCs**

Mononucleated cells isolated from the SVF of AT were selected according to their ability to adhere to tissue-culture plastic and culture expanded to passage II-III. These culture expanded cells demonstrated a phenotype consistent with the three main criteria proposed by the ISCT. As show in Figure 3.7, the adherent culture expanded cells of stromal appearance differentiated down the three mesodermal lineages indicated by the positive alkaline phosphatase staining for osteogenesis, positive Oil Red O staining of lipid vacuoles for adipogenesis and metachromatic toluidine blue staining of paraffin-sections of cell pellets for chondrogenesis. The AT MSCs also showed immunopositivity for MSC specific cell-surface antigens, which were CD73, CD90 and CD105 and immunonegativity for haematopoietic markers, which were CD34 and CD45 (Figure 3.8).  $3\% \pm 1.1\%$  cells were CD34 positive,  $3\% \pm 1.2\%$  cells were CD45 positive,  $92.4\% \pm 4\%$  were CD73 positive,  $90.4\% \pm 3\%$  cells were CD90 positive and  $93.8\% \pm 2.8\%$  cells were CD105 positive, respectively. Table 3.2 shows the percentage expression of positive and negative markers in AT MSCs (n=3).



**Figure 3.7. Tri-lineage differentiation potential of AT MSCs.** AT MSCs differentiated into osteoblasts, adipocytes and chondrocytes as indicated by the presence of alkaline phosphatase positive pink osteoblasts (osteogenesis), Oil Red O positive red lipid vacuoles (adipogenesis) and purple metachromatic staining of cartilage GAGs (chondrogenesis), respectively. AT MSCs, n=3. Original magnification x10. Scale bar = 100 $\mu$ m.



**Figure 3.8. Flow cytometry for CD-cell surface antigen characterisation of AT MSCs.**

The grey lined histogram shows immunopositivity for each indicated marker whereas the black histogram indicates immunolabelling with an isotype-matched control antibody. The x-axis represents the fluorescence intensity and the y-axis represents the number of cells counted. The marker M1 indicates positive events. AT MSCs, n=3.

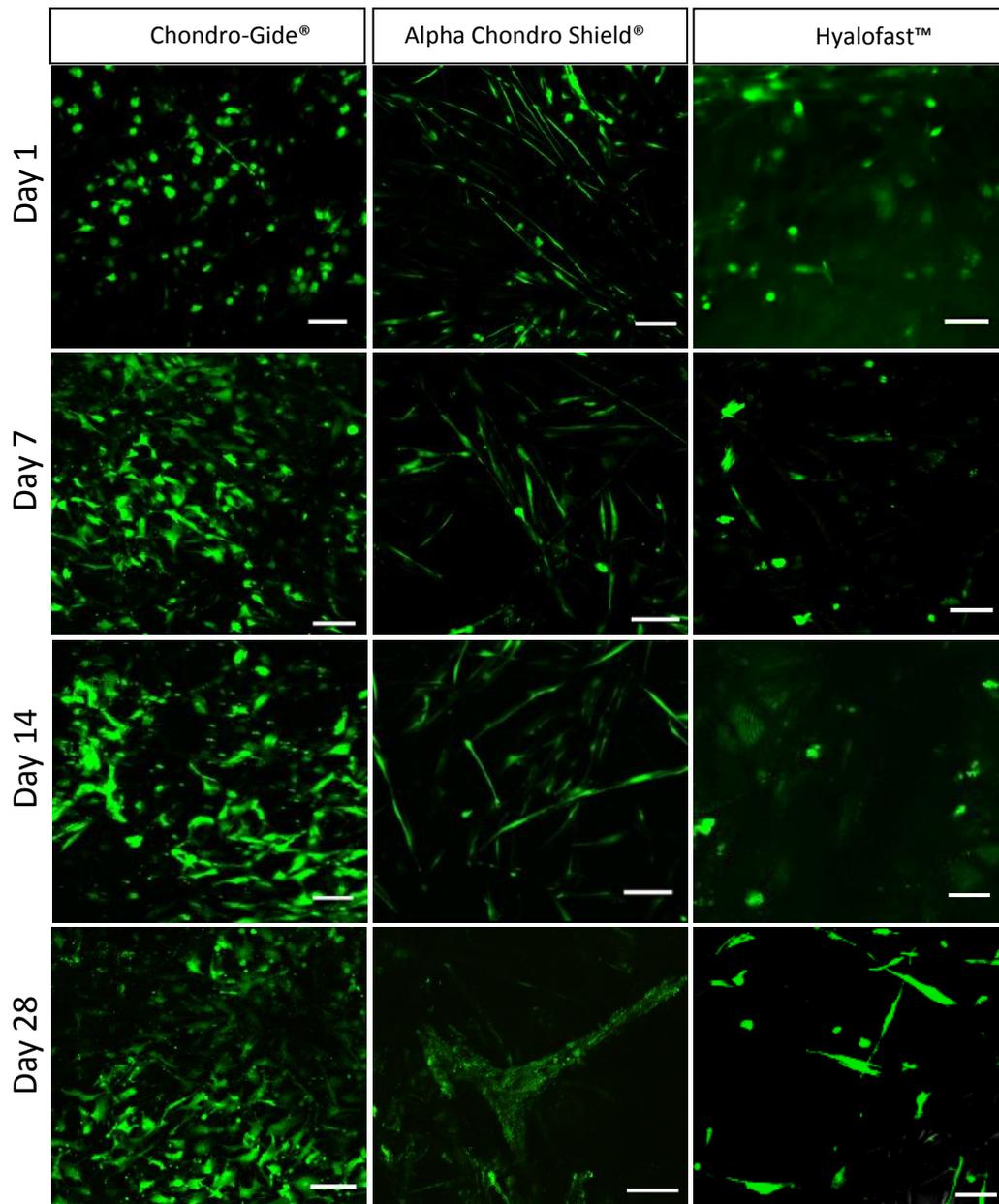
		Percentage expression of CD markers				
Cell type	Patient ID	CD34	CD45	CD73	CD90	CD105
AT MSCs	BMS 003	1.30	1.26	84.77	92.07	88.30
	BMS 006	2.27	1.63	97.92	84.54	98.09
	BMS 010	5.11	5.32	94.78	94.67	95.07

**Table 3.2. Immunoreactivity of AT MSCs for each CD marker.** The percentage of cells that were immunopositive for CD34, CD45, CD73, CD90 and CD105 are shown. 3% ± 1.1% cells were CD34 positive, 3% ± 1.2% cells were CD45 positive, 92.4% ± 4% were CD73 positive, 90.4% ± 3% cells were CD90 positive, and 93.8% ± 2.8% cells were CD105 positive. Data has been presented as means ± SEM of n=3 patient donors.

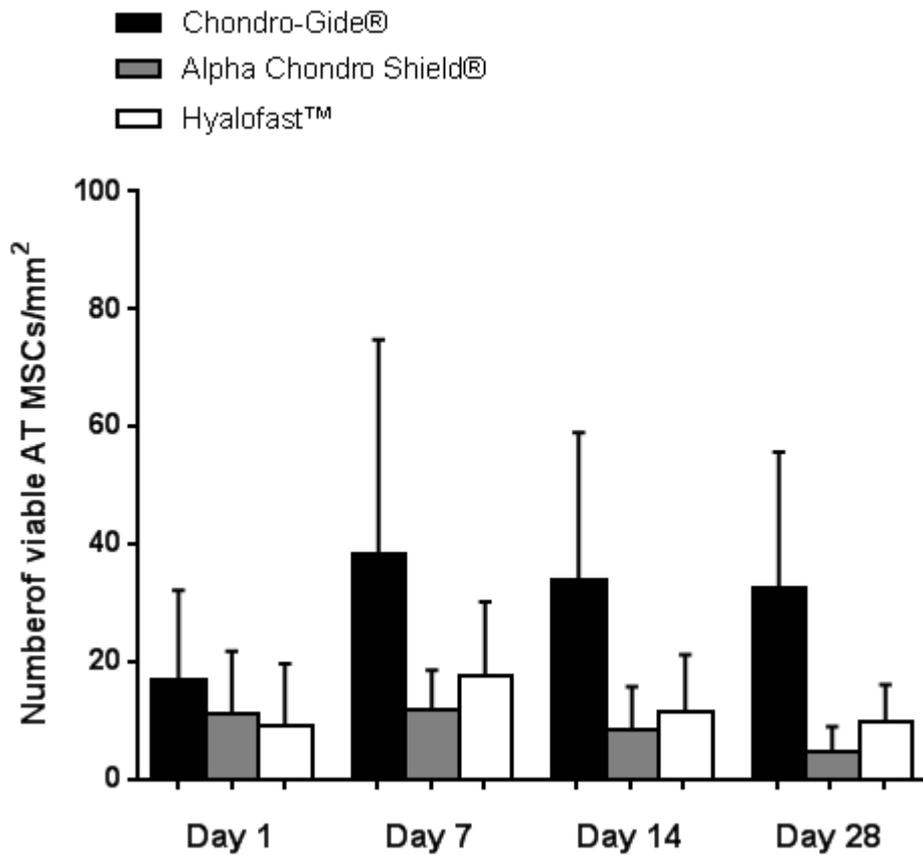
### **3.5 A comparison of the incorporation and viability of culture expanded AT MSCs versus freshly isolated AT SVF cells in the three clinically relevant scaffolds**

Similar to BM MSCs, AT MSCs (n=2) also incorporated and adhered to all three scaffolds and there was a greater initial incorporation of AT MSCs in Chondro-Gide® compared to Alpha Chondro Shield® and Hyalofast™, and this difference in the prevalence of cells became increased considerably by day 7 in culture. Many cells were lost during cell-seeding and only  $1.2\% \pm 0.1\%$  of AT MSCs retained in Chondro-Gide® compared with  $0.7\% \pm 0.4\%$  of cells retained in Alpha Chondro Shield® and  $0.6 \pm 0.4\%$  of cells in Hyalofast™ at day 1. AT MSCs in Alpha Chondro Shield® and Hyalofast™ remained 100% viable during short and long term cultures; however, the number of cells incorporated in the two scaffolds did not show an increase with time in culture. Morphologically, AT MSCs were similar in appearance to BM MSCs, which was fibroblast-like in Chondro-Gide® and Alpha Chondro Shield® where no round cells were seen and a mixed morphology of round and elongated cells in Hyalofast™ alone. Data has been presented as means  $\pm$  SDs of 16 internal replicates pooled from two patient donors (Figure 3.9 and Figure 3.10).

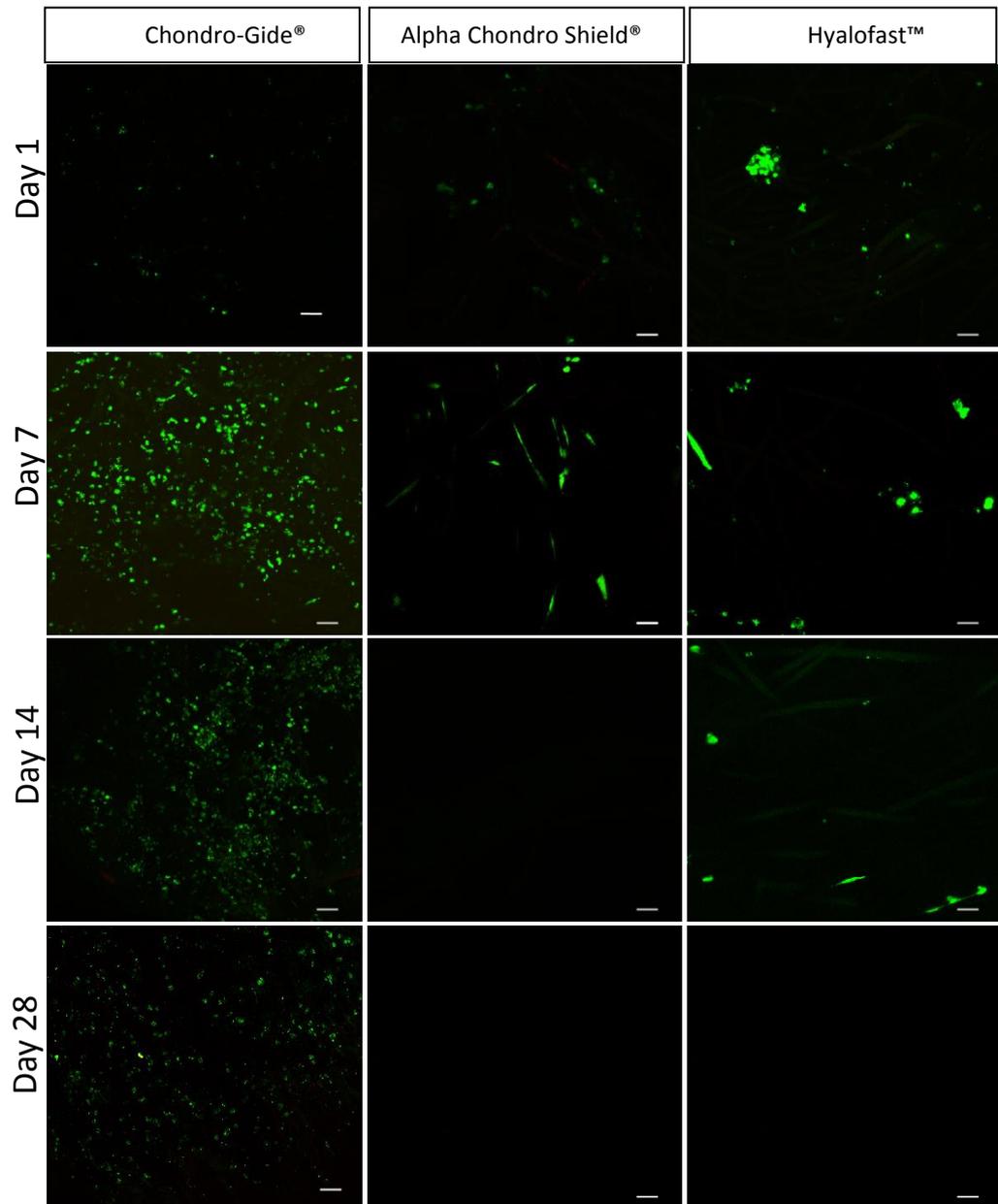
Freshly isolated AT SVF cells (n=2) showed better incorporation into the cell scaffolds than the BM MNCs. However, only Chondro-Gide® showed cellular incorporation during long term cultures. No AT SVF cells were seen in Alpha Chondro Shield® past day 7 and in Hyalofast™ past day 14. AT SVF cells appeared mostly round with very few fibroblast-like cells where cell incorporation was seen. No cell death was observed in any of three scaffolds (Figure 3.11 and Figure 3.12).



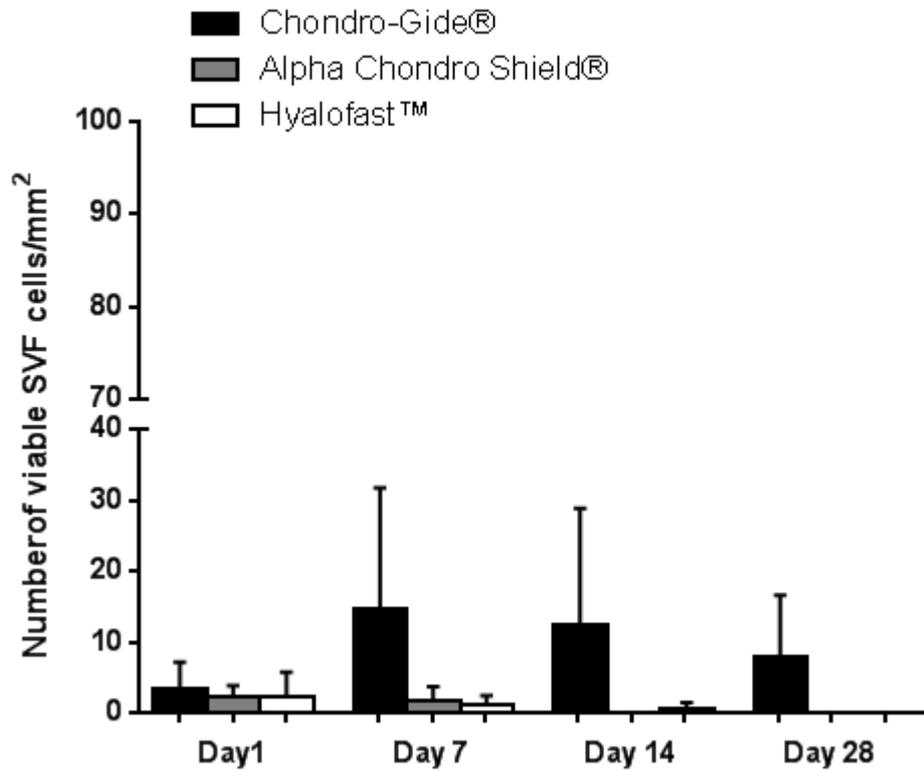
**Figure 3.9. The viability of AT MSCs in Chondro-Gide®, Alpha Chondro Shield® and Hyalofast™ over 28 days in culture.** Representative confocal z-stacks are shown of AT MSCs following live/dead staining. There appeared to be a greater incorporation of viable AT MSCs in Chondro-Gide® compared to Alpha Chondro Shield® and Hyalofast™ over a 28 day period. Overall AT MSCs were over 95% viable in all three scaffolds during these long term cultures. Viable cells = green fluorescence, non-viable cells = red fluorescence. AT MSCs, n=2. Scale bar = 100µm.



**Figure 3.10. The number of viable AT MSCs in Chondro-Gide®, Alpha Chondro Shield® and Hyalofast™ over 28 days in culture.** The prevalence of AT MSCs was greatest in Chondro-Gide® compared to Alpha Chondro Shield® and Hyalofast™ at all time points studied. An increase in the number of AT MSCs was seen Chondro-Gide® and Hyalofast™ up to day 7 whereas AT MSCs in Alpha Chondro Shield® did not increase with time in culture. During long term cultures of day 14 and day 28, AT MSCs within all three scaffolds decreased in numbers. All data are presented as means  $\pm$  SD of 16 internal replicates pooled from n=2 patient donors.



**Figure 3.11. The viability of AT SVF cells in Chondro-Gide®, Alpha Chondro Shield® and Hyalofast™ over 28 days in culture.** Representative confocal z-stacks are shown of AT SVF cells following live/dead staining. There was a greater prevalence of AT SVF cells in Chondro-Gide® compared to Alpha Chondro Shield® and Hyalofast™. Overall AT SVF cells were over 95% viable where cell incorporation was seen and appeared mostly round. Viable cells = green fluorescence, non-viable cells = red fluorescence. AT MSCs, n=2. Scale bar = 100µm.



**Figure 3.12. The number of viable AT SVF cells in Chondro-Gide®, Alpha Chondro Shield® and Hyalofast™ over 28 days in culture.** The prevalence of AT SVF cells was greatest in Chondro-Gide® from early time points (day 1 and day 7) through to long term cultures of day 14 and day 28. The incorporation of cells diminished in Alpha Chondro Shield® and Hyalofast™ after day 7 and no cells were seen in these two scaffolds during long term cultures. All data are presented as means  $\pm$  SD of 16 internal replicates pooled from n=2 patient donors.

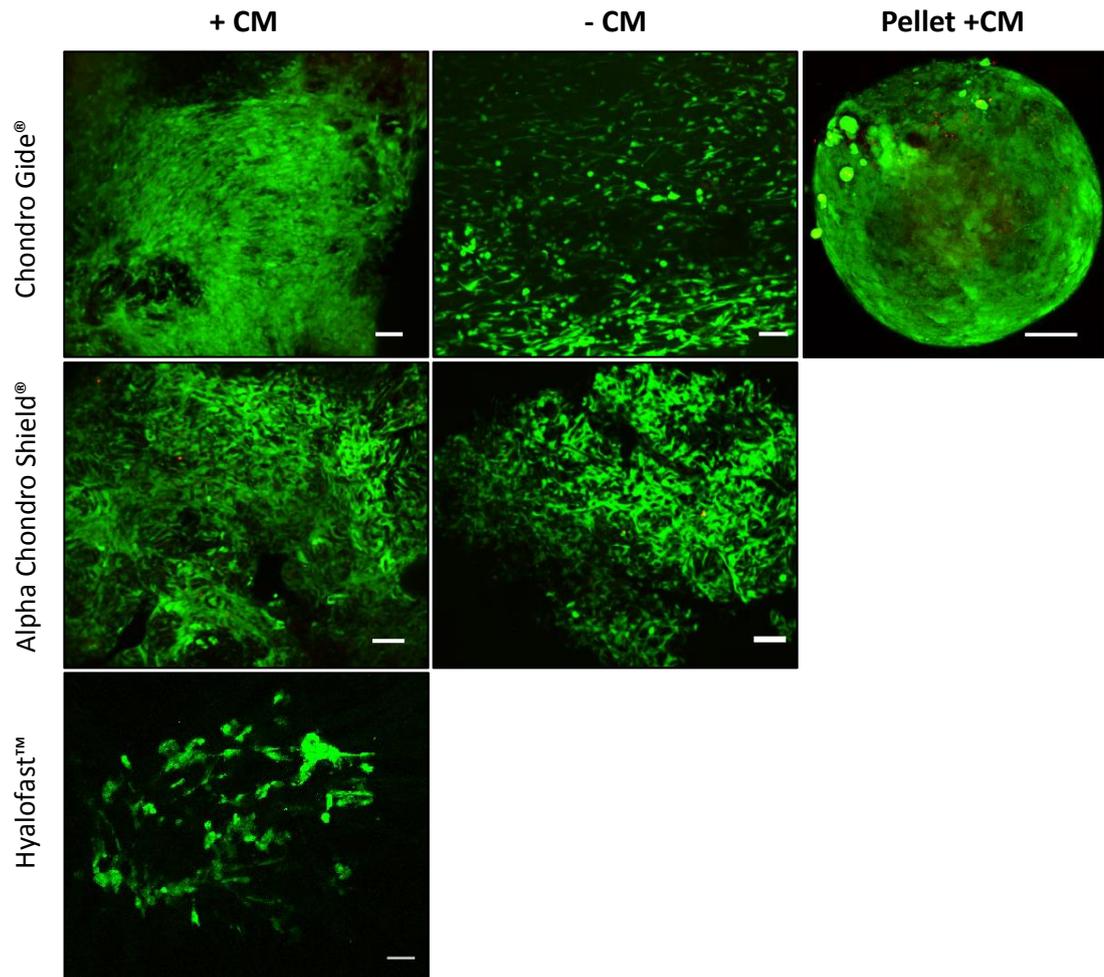
### **3.6 Chondrogenesis of culture expanded MSCs in the three clinically relevant scaffolds**

The initial studies on cell incorporation and growth of culture expanded versus freshly isolated cells from BM and AT demonstrated that for both cell sources, the culture expanded cells appeared to incorporate and grow better than the freshly isolated cells. Therefore, further experiments were performed to test whether these culture expanded cells (MSCs) could be induced towards chondrogenesis within the same scaffolds.

#### 3.6.1 BM MSCs

##### *Viability*

BM MSCs (n=2) incorporated, adhered and remained viable over a 28 day culture in Chondro-Gide® and Alpha Chondro Shield® under both chondrogenic (+CM) and non-chondrogenic (-CM) conditions and only under +CM conditions in Hyalofast™. Hyalofast™ had disintegrated under -CM conditions. Under +CM conditions, in Chondro-Gide® and Alpha Chondro Shield®, BM MSCs were mostly round with a very few elongated or fibroblast-like cells, and there was the appearance of dense networks or aggregates of cells throughout the depth of the scaffolds. In control -CM conditions, BM MSCs in Chondro-Gide® were mostly individually dispersed and grew sparsely throughout the scaffold with an appearance mostly of fibroblast-like cells and a few round cells that did not aggregate together. In Alpha Chondro Shield® under -CM conditions, BM MSCs grew in a less dense network with an appearance of mainly elongated and fibroblast-like cells. The Hyalofast™ scaffold showed clusters of cells which were mainly round in morphology with few elongated cells that grew along the length of the fibres under +CM conditions (Figure 3.13).



**Figure 3.13. The viability of BM MSCs in Chondro-Gide®, Alpha Chondro Shield® and Hyalofast™ under chondrogenic (+CM) and non-chondrogenic (-CM) conditions.** Representative confocal z-stacks are shown of BM MSCs seeded scaffolds following live/dead staining after 28 days in culture. BM MSCs remained over 95% viable under both +CM and –CM conditions in Chondro-Gide® and Alpha Chondro Shield®. Hyalofast™ degraded during these long term cultures under –CM conditions and viable cells were seen only under +CM conditions. BM MSCs pellet showed mostly viable cells with a few dead cells seen towards the centre of the cell pellet. BM MSCs, n=2. Scale bar = 100  $\mu$ m.

### ***H&E staining***

The haematoxylin and eosin (H&E) staining of paraffin embedded sections of Chondro-Gide® and Alpha Chondro Shield® revealed cell penetration throughout the scaffolds (Figure 3.14). Under +CM conditions, cells within the Chondro-Gide® scaffold showed characteristic rounded shape of mature chondrocytes embedded within ECM on the porous side of the scaffold, whereas cells within Alpha Chondro Shield® showed a more elongated morphology through the scaffold with little ECM deposition. BM MSCs showed a more elongated fibroblast-like morphology with no matrix deposition under –CM conditions in both Chondro-Gide® and Alpha Chondro Shield®. The Hyalofast™ scaffold did not survive histological processing and this precluded further histological examination of chondrogenesis with this scaffold.

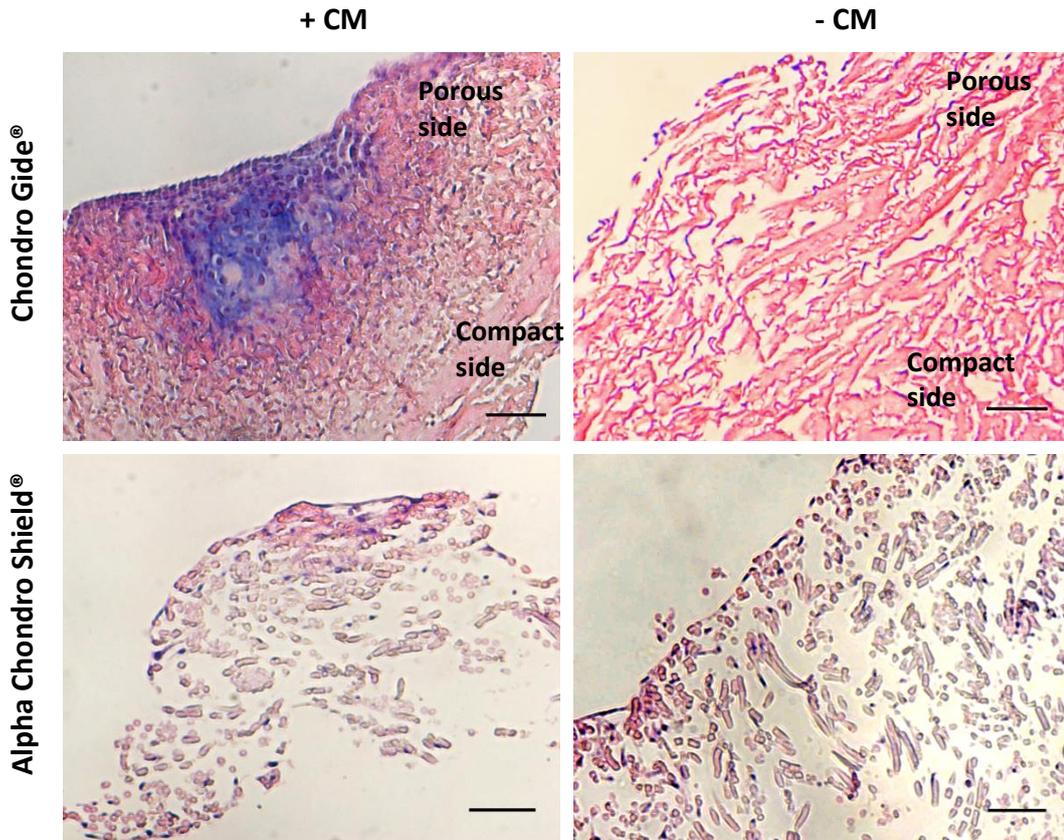
### ***Toluidine blue staining***

In +CM conditions, toluidine blue staining of Chondro-Gide® and Alpha Chondro Shield® scaffold showed varying degrees of ECM deposition with the greatest evidence of purple metachromatic staining seen in Chondro-Gide® consistently with the cells of mature chondrocytic (round or oval) morphologies. Although there was ECM deposition observed in Alpha Chondro Shield® seeded with BM MSCs, there were no clearly chondrocytic round-shaped cells present. BM MSCs seeded within both scaffolds under –CM conditions showed no ECM deposition (Figure 3.15).

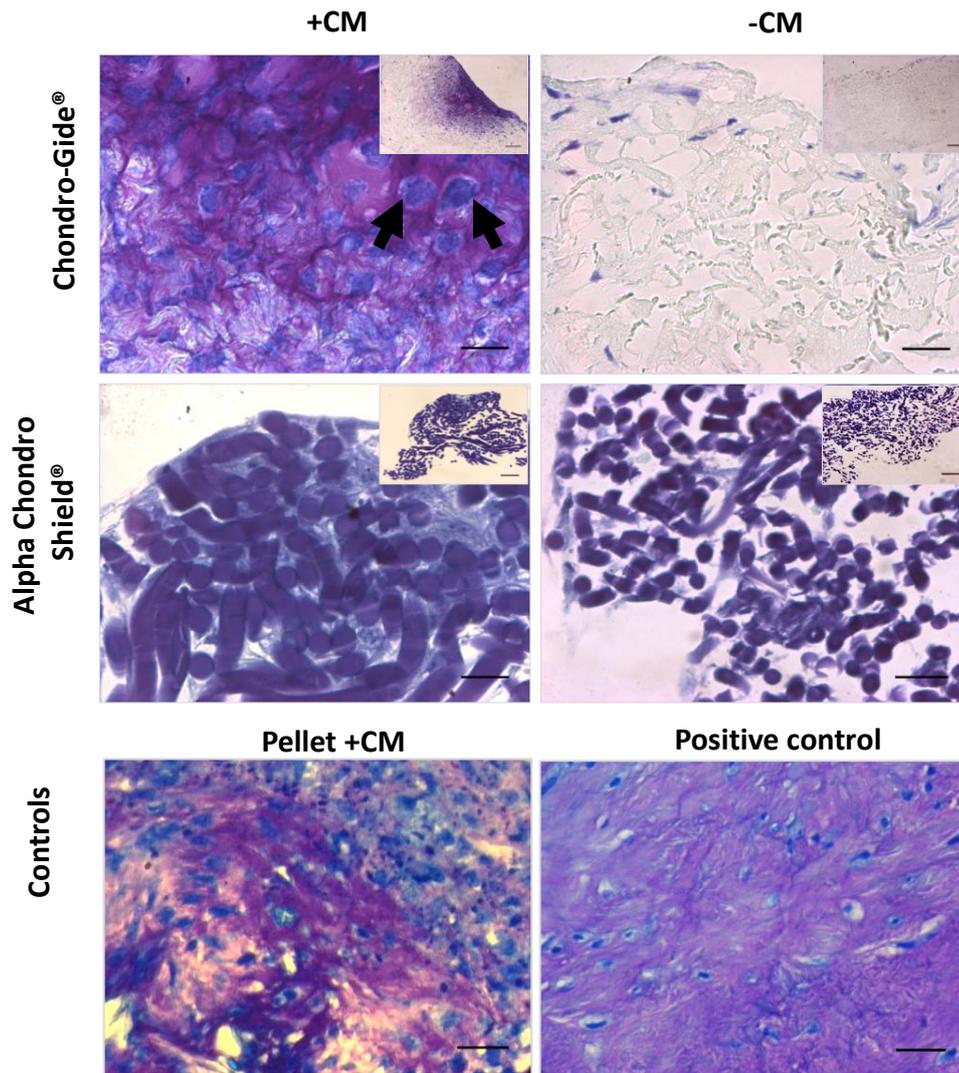
### ***Soluble GAG analysis***

BM MSCs in Chondro-Gide® secreted an average of 13 µg/ml ± 7 µg/ml of GAGs in +CM conditions compared to 4 µg/ml ± 2 µg/ml under –CM conditions. In Alpha Chondro Shield®, BM MSCs secreted an average of 7 µg/ml ± 2 µg/ml of GAGs under +CM and 5 µg/ml ± 3 µg/ml under –CM conditions. In Hyalofast™, BM MSCs secreted an average of 14 µg/ml ± 2 µg/ml of GAGs under +CM conditions compared to 7 µg/ml ± 4µg/ml of GAGs under –CM

conditions. BM MSCs when cultured as pellets secreted lower levels of GAGs ( $3\mu\text{g/ml} \pm 2.5\mu\text{g/ml}$ ) than BM MSCs seeded within the three scaffolds under +CM conditions (Figure 3.16).

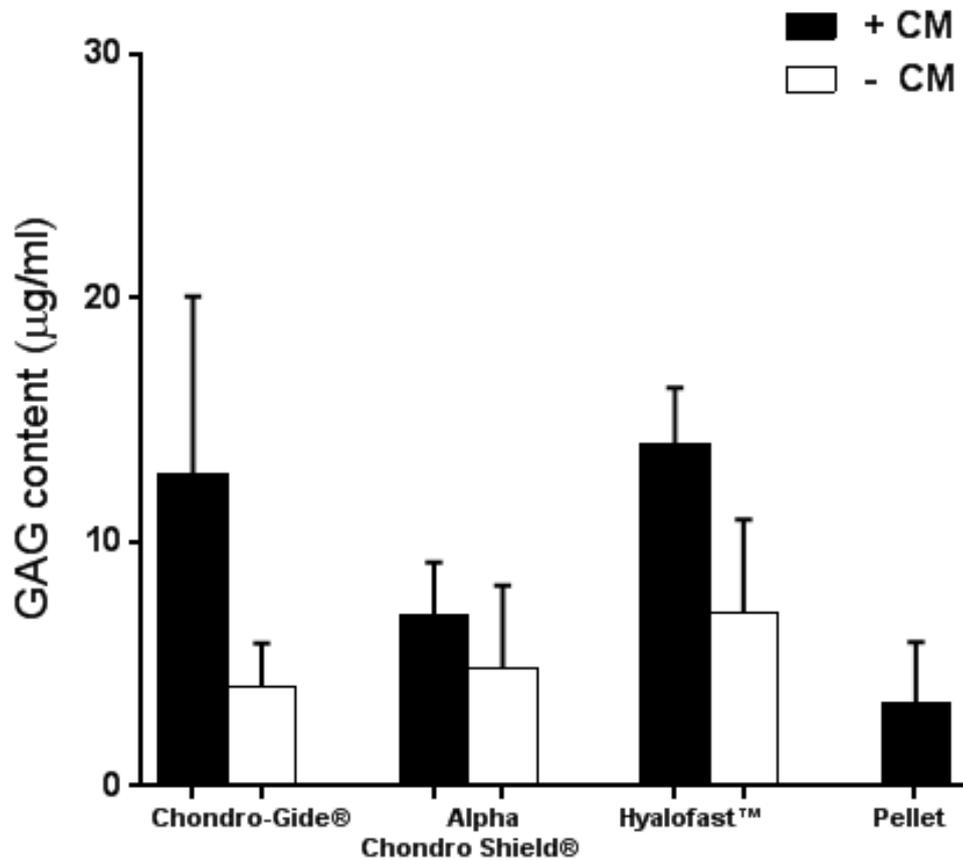


**Figure 3.14. The incorporation and distribution of BM MSCs in Chondro-Gide® and Alpha Chondro Shield® under chondrogenic (+CM) and non-chondrogenic conditions (-CM).** Representative haematoxylin and eosin (H&E) staining of BM MSCs seeded scaffolds is shown. Purple haematoxylin staining of cell nuclei was observed in both the scaffolds under both +CM and –CM conditions. In Chondro-Gide®, cells showed a characteristic rounded morphology, indicative of chondrocytes, under +CM conditions. In Alpha Chondro Shield®, cells were seen along the length of the fibres with little evidence of ECM deposition under +CM conditions. In both scaffolds under –CM conditions, cells showed dispersed penetration through the scaffolds. BM MSCs, n=2. Original magnification x10. Scale bar =100 µm.



**Figure 3.15. GAG deposition by BM MSCs in Chondro-Gide® and Alpha Chondro Shield® under chondrogenic (+CM) and non-chondrogenic (-CM) conditions.**

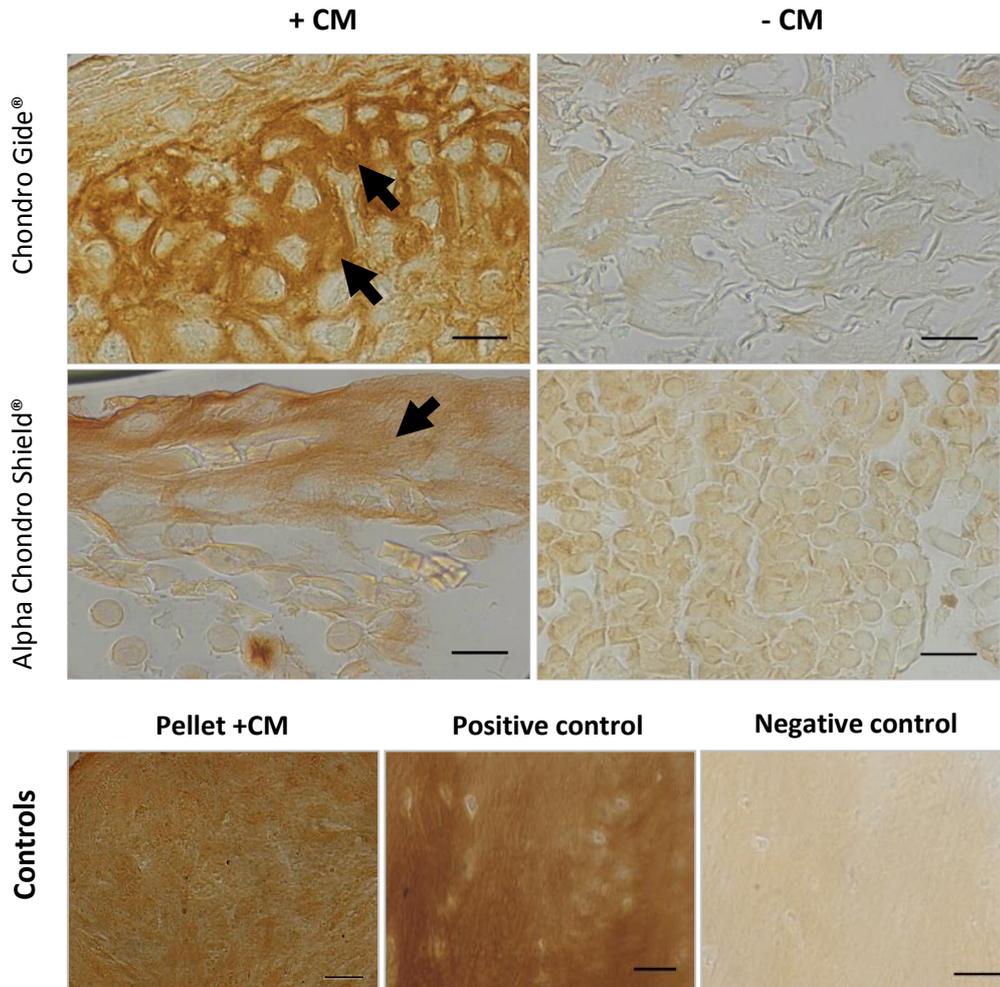
Representative images are shown of toluidine blue stained BM MSCs seeded scaffolds after 28 days in culture. BM MSCs in Chondro-Gide® showed greater GAG deposition than BM MSCs in Alpha Chondro Shield® under +CM conditions. BM MSCs in Chondro-Gide® showed a differentiated morphology of distinct round cells (arrowed) embedded within the metachromatic purple ECM under +CM conditions only. BM MSCs in Alpha Chondro Shield® did not show distinct round shaped cells and the purple metachromatic staining was less marked. BM MSC pellet and a section of human knee cartilage were used as controls. BM MSCs, n=2. Insets represents x10 image of the same sample; scale bar = 100  $\mu$ m. Original magnification x40; scale bar = 25  $\mu$ m.



**Figure 3.16. GAG secretion by BM MSCs within Chondro-Gide®, Alpha Chondro Shield® and Hyalofast™ under chondrogenic (+CM) and (-CM) conditions.** BM MSCs seeded within all three scaffolds secreted greater levels of GAGs in +CM conditions compared to -CM conditions. BM MSCs seeded within these scaffolds also secreted greater levels of GAGs than the cell pellet alone. BM MSCs seeded within Alpha Chondro Shield® secreted lower levels of GAGs compared to BM MSCs seeded within Chondro-Gide® and Hyalofast™. Data shown are means  $\pm$  SD of 6 internal replicates pooled from n=2 patient donors.

### ***Collagen type II immunostaining***

As shown in Figure 3.17, BM MSCs under +CM conditions deposited an ECM that was immunopositive for collagen type II. This immunopositive staining for collagen type II was absent under –CM conditions. There was marked and more widespread immunopositivity for collagen type II within the porous layers of Chondro-Gide® than in Alpha Chondro Shield®. Some positive staining for collagen type II was seen in the pellets of BM MSCs; however, it was not as marked as that seen in Chondro-Gide®. The strongest collagen type II staining was seen in sections of human articular cartilage used as controls.



**Figure 3.17. Collagen type II immunolocalisation in BM MSCs seeded Chondro-Gide® and Alpha Chondro Shield® under chondrogenic (+CM) and non-chondrogenic conditions (-CM).** BM MSCs within Chondro-Gide® scaffold under +CM conditions showed collagen type II immunostaining staining of ECM (black arrowed). BM MSCs within Alpha Chondro Shield® showed no obvious mature chondrocytes, however localised darker brown staining was seen around the scaffold fibres (black arrowed). BM MSCs pellets showed some positivity for collagen type II. The positive control was a tissue section of human knee articular cartilage and the negative control was the same section with the omission of primary antibody. BM MSCs, n=2. Original magnification x40. Scale bar = 25  $\mu$ m.

### 3.6.2 AT MSCs

#### ***Viability***

AT MSCs (n=2) remained viable in all three scaffolds under +CM conditions and –CM conditions, with the exception of Hyalofast™ that has degraded under –CM conditions. Similar to BM MSCs, AT MSCs appeared condensed and formed confluent layers of cells in Chondro-Gide® and Alpha Chondro Shield® only under +CM conditions. AT MSCs appeared to be individually dispersed under –CM conditions. Morphologically, cells appeared round within Chondro-Gide® and Alpha Chondro Shield® with a few fibroblast-like cells seen under +CM conditions compared to a greater number of elongated and fibroblast-like cells seen under –CM conditions. AT MSCs in Hyalofast™ condensed to form clusters of cells in round shaped nodules which were evenly distributed throughout the scaffold under +CM conditions only (Figure.3.18).

#### ***H&E staining***

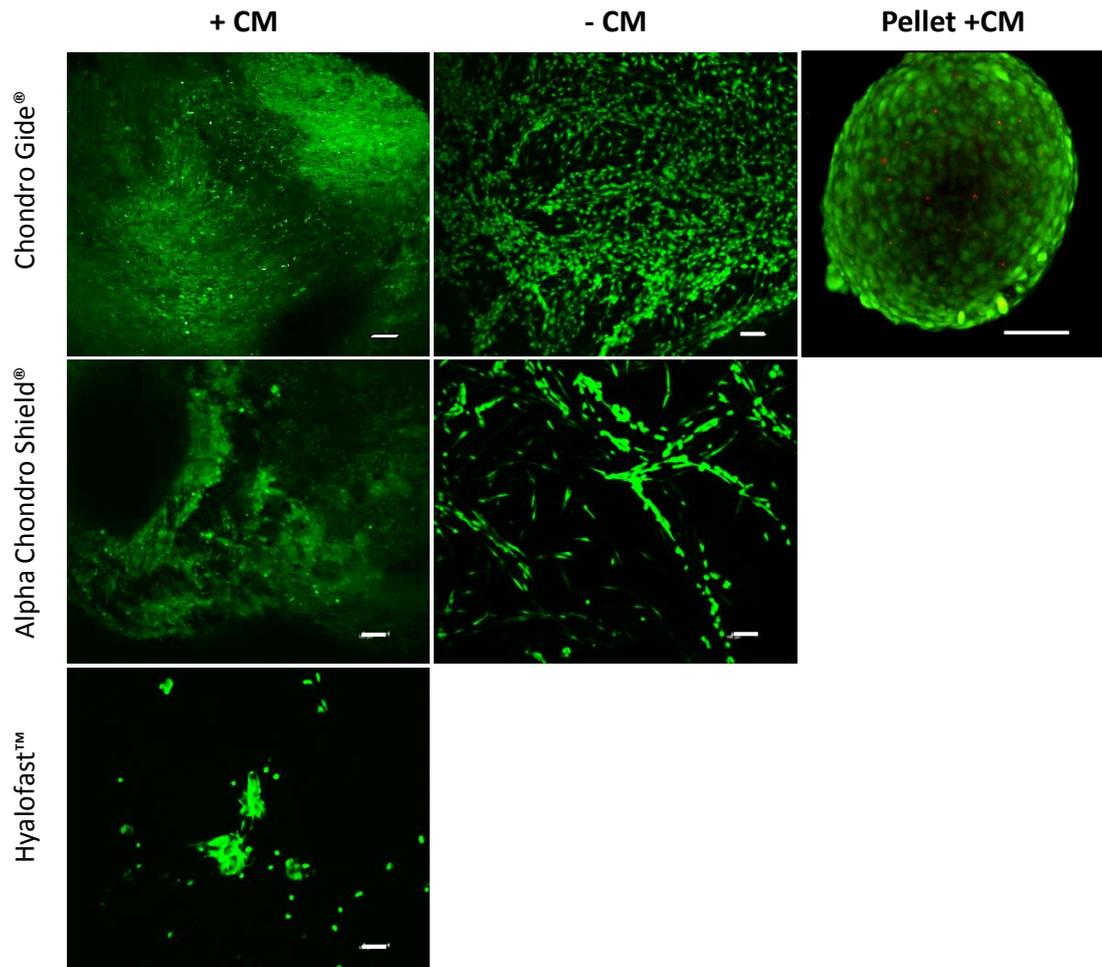
H&E staining of sections of the cell-seeded scaffolds revealed cell penetration throughout the scaffold in Chondro-Gide® in both +CM and –CM conditions. In Alpha Chondro Shield®, AT MSCs were seen in clusters along the length of the scaffolds mostly under +CM conditions (Figure 3.19). Similar to the studies of BM MSCs, the Hyalofast™ scaffold did not survive the histological processing and therefore no histological analyses were possible for this scaffold.

#### ***Toluidine blue staining***

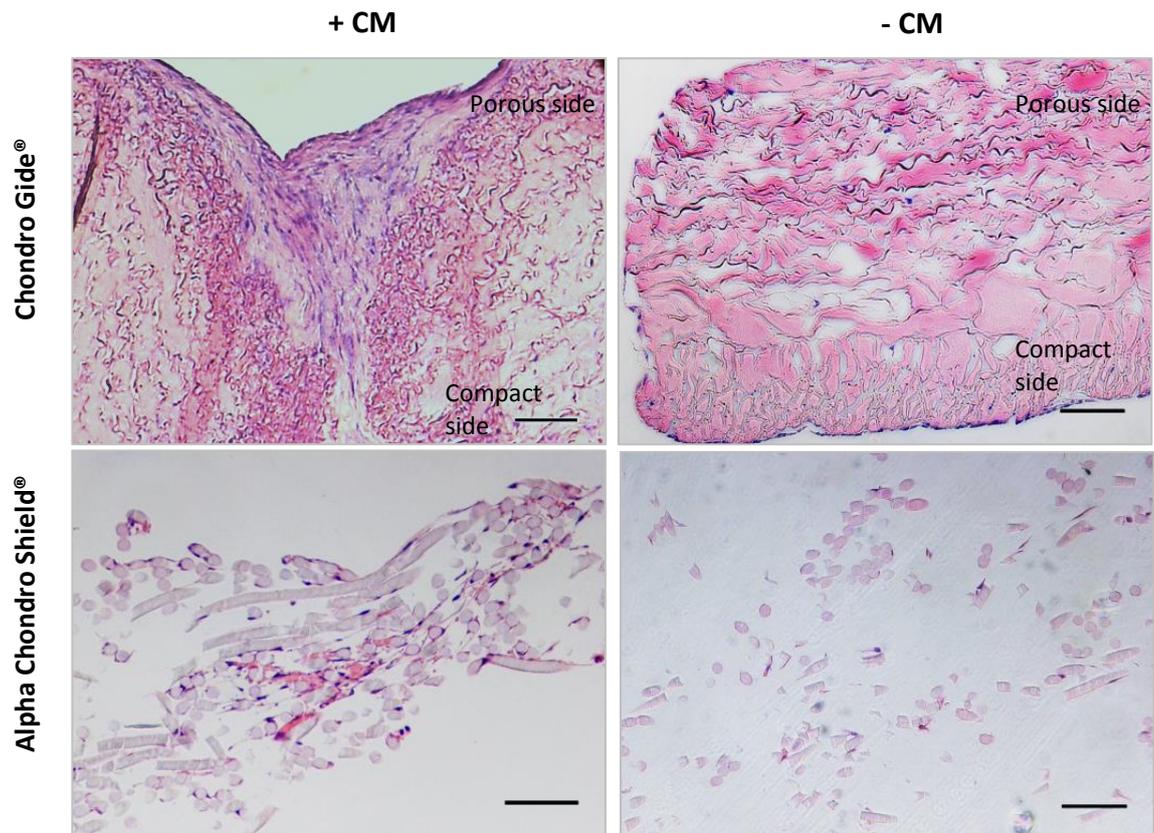
ECM deposition was observed to varying degrees under +CM conditions with the greatest amount of deposition observed in Chondro-Gide®, followed by Alpha Chondro Shield®. AT MSCs in Chondro-Gide® showed localised areas of purple metachromatic toluidine blue staining only under +CM conditions. Under –CM conditions no ECM deposition was seen in both the scaffolds (Figure 3.20).

### ***Soluble GAG analysis***

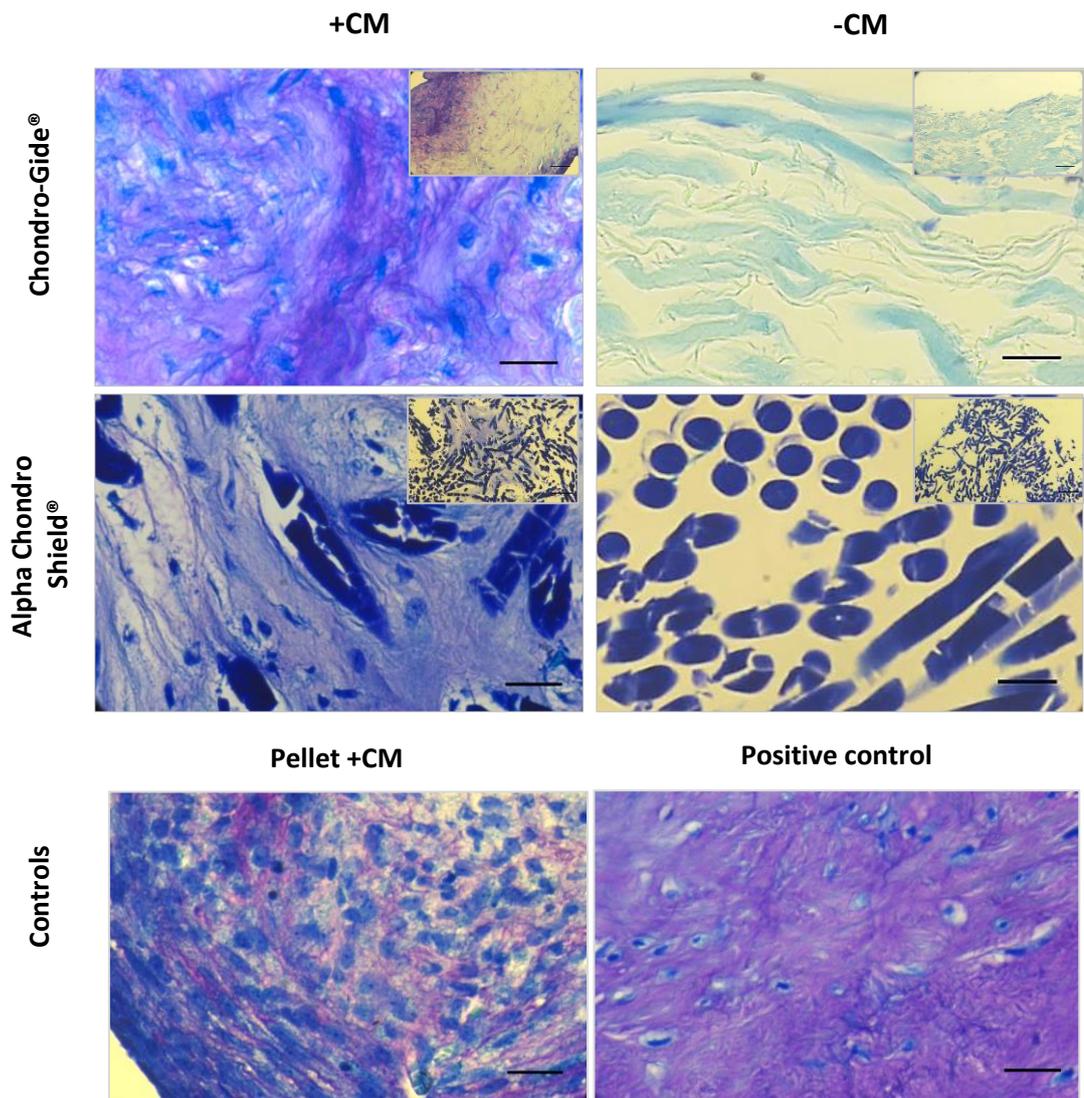
AT MSCs in Chondro-Gide® secreted an average of  $18 \mu\text{g/ml} \pm 10 \mu\text{g/ml}$  of GAGs in +CM conditions compared to  $4 \mu\text{g/ml} \pm 3 \mu\text{g/ml}$  under –CM conditions. In Alpha Chondro Shield®, AT MSCs secreted an average of  $12 \mu\text{g/ml} \pm 6 \mu\text{g/ml}$  of GAGs under +CM and  $4 \mu\text{g/ml} \pm 4 \mu\text{g/ml}$  under –CM conditions. In Hyalofast™, AT MSCs secreted an average of  $14 \mu\text{g/ml} \pm 7 \mu\text{g/ml}$  of GAGs under +CM conditions compared to  $6.5 \mu\text{g/ml} \pm 1 \mu\text{g/ml}$  of GAGs under –CM conditions. Similar to the GAG studied on BM MSCs, AT MSCs when cultured as pellets secreted lower levels of GAGs ( $1 \mu\text{g/ml} \pm 0.8 \mu\text{g/ml}$ ) than AT MSCs seeded within the three scaffolds under +CM conditions (Figure 3.21).



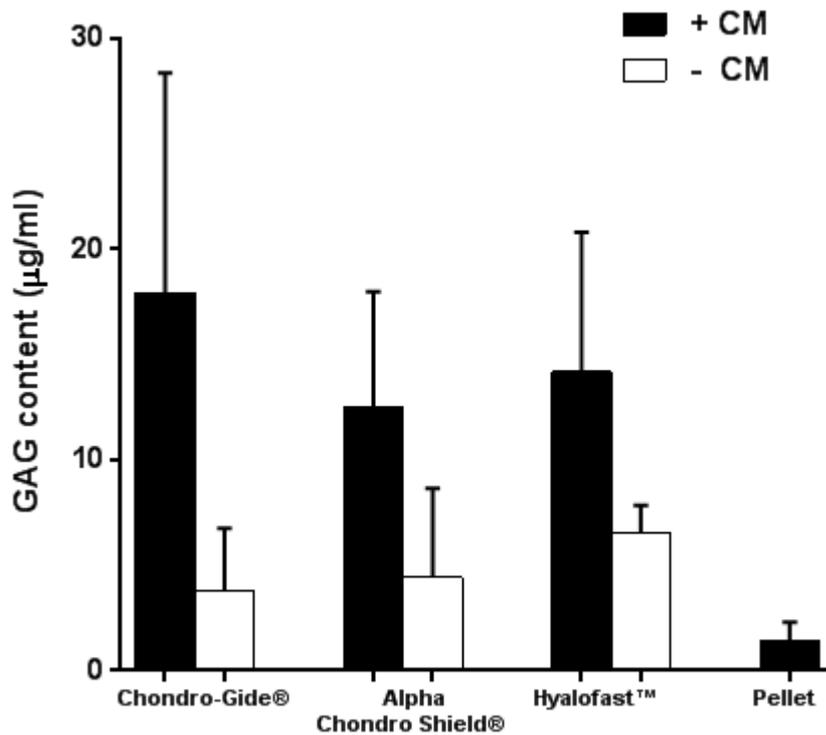
**Figure 3.18. The viability of AT MSCs in Chondro-Gide®, Alpha Chondro Shield® and Hyalofast™ under chondrogenic (+CM) and non-chondrogenic -CM) conditions.** Representative confocal z-stacks are shown of AT MSCs seeded scaffolds following live/dead staining after 28 days in culture. AT MSCs remained over 95% viable under +CM and -CM conditions in Chondro-Gide® and Alpha Chondro Shield®. Hyalofast™ degraded during these long term cultures under -CM conditions and viable cells were seen only under +CM conditions. AT MSCs pellet showed mostly viable cells with a few dead cells seen towards the centre of the cell pellet. AT MSCs, n=2. Scale bar = 100  $\mu$ m.



**Figure 3.19. The incorporation and distribution of AT MSCs in Chondro-Gide®, and Alpha Chondro Shield® under chondrogenic (+CM) and non-chondrogenic conditions (-CM).** Representative images are shown of H&E stained AT MSCs seeded scaffolds. Purple haematoxylin staining of cell nuclei was observed in all the scaffolds under both +CM and -CM conditions. In Chondro-Gide®, AT MSCs distributed evenly through the porous side of the scaffold. In Alpha Chondro Shield® cells were seen along the length of the fibres with evidence of ECM deposition in +CM conditions only. AT MSCs, n=2. Original magnification x10. Scale bar =100µm.



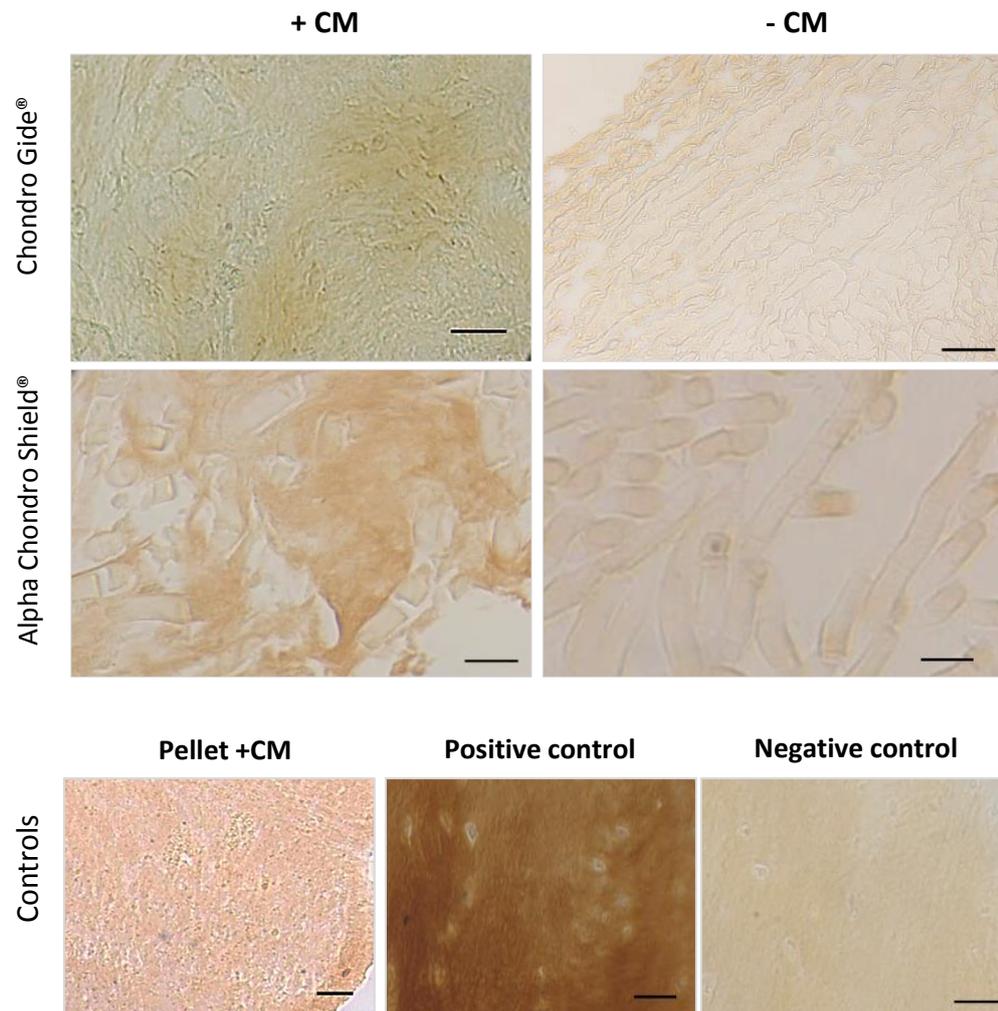
**Figure 3.20. GAG deposition by AT MSC in Chondro-Gide® and Alpha Chondro Shield® under chondrogenic (+CM) and non-chondrogenic (-CM) conditions.** Representative images are shown of toluidine blue stained AT MSCs seeded scaffolds after 28 days in culture. AT MSCs in Chondro-Gide® showed greater GAG deposition as indicated by more marked purple metachromatic staining in Chondro-Gide® than in Alpha Chondro Shield® under +CM conditions only. AT MSC pellet and a section of human knee cartilage were used as controls. AT MSCs, n=2. Insets represents x10 image of the same sample; scale bar = 100  $\mu$ m. Original magnification of each image is x40; scale bar = 25  $\mu$ m.



**Figure 3.21. GAG secretion by AT MSCs seeded within Chondro-Gide®, Alpha Chondro Shield® and Hyalofast™ under chondrogenic (+CM) and (-CM) conditions.** AT MSCs seeded within all three scaffolds showed greater levels of GAGs under +CM conditions compared with -CM conditions. AT MSCs seeded within the three scaffolds also secreted increased levels of GAGs than the cell pellet alone. The highest amounts of GAGs were secreted by AT MSCs seeded within Chondro-Gide®. Data shown are means  $\pm$  SD of 6 internal replicates pooled from n=2 patient donors.

### ***Collagen type II immunostaining***

In contrast to the results seen with BM MSCs, there was little immunopositivity for collagen type II observed in Chondro-Gide® seeded with AT MSCs under +CM or –CM conditions. however, some collagen type II immunopositivity was seen around the fibres of the Alpha Chondro Shield® scaffolds seeded with AT MSCs, but only under +CM conditions. The cell pellet of AT MSCs under +CM conditions stained weakly for collagen type II. The strongest staining was observed in the sections of human articular cartilage used as controls (Figure 3.22).



**Figure 3.22. Collagen type II immunolocalisation in AT MSCs seeded in Chondro-Gide®, and Alpha Chondro Shield® under chondrogenic (+CM) and non-chondrogenic (-CM) conditions.** AT MSCs seeded within Chondro-Gide® and Alpha Chondro Shield® showed localised collagen type II under +CM conditions only. AT MSCs cell pellet did not stain strongly positive for collagen type II. The positive control was a section of human knee articular cartilage and negative control is the same section with the omission of primary antibody. AT MSCs, n=2. Original magnification of each image is x40. Scale bar = 25 µm.

### 3.7 Discussion

MSCs have been studied as a potential alternative cell source to chondrocytes for repairing damaged cartilage for over a decade now (reviewed by Csaki *et al.*, 2008). Preclinical animal studies suggest the potential of various different cell types or sources to address this need (reviewed by Leijten *et al.*, 2013). This study was therefore designed to examine the *in vitro* incorporation, viability and chondrogenesis of several different cell sources in three clinical and commercially available scaffolds or cell carriers used for cartilage repair.

In this set of experiments, it was demonstrated that culture expanded BM MSCs and AT MSCs incorporate, adhere and proliferate more readily in clinical scaffolds tested than freshly isolated cells from both cell sources. Both BM and AT MSCs showed the best incorporation within Chondro-Gide® scaffolds where the cells attained a fibroblast-like morphology and showed penetration throughout the scaffolding network. This bilayer structured scaffold has a porous side for cell attachment and a compact side to prevent cell leakage. It has been the scaffold of choice for MACI since 2004 (Haddo *et al.*, 2004). One reason for the greater incorporation of cells within Chondro-Gide®, which is composed of type I and type III porcine collagen, could be the ability of MSCs to bind to the scaffold fibres through the integrin based family of receptors, specifically the  $\alpha 2\beta 1$  integrins, which is the major receptor for type I collagen and other fibril-forming collagens (Jokinen *et al.*, 2004). The  $\alpha 2\beta 1$  integrin has also been shown to increase ECM synthesis and turn over which is fundamental to chondrogenesis (Riikonen *et al.*, 1995). Alpha Chondro Shield® on the other hand is a synthetic scaffold made of pure PGA. The scaffold is an absorbable non-woven fleece that lacks specific cellular adhesion sites and therefore, the adhesion of cells to the scaffold is aided by extracellular molecules adsorbed on the polymer surface from the serum in culture medium (Lamba *et al.*, 1998). Previous studies have utilized integrin binding peptide domains like Arg-Gly-Asp (RGD) coatings to facilitate further adhesion of cells to the polymer based scaffolds (Kim and Park 2006; Huang *et al.*, 2010). However, in this study Alpha Chondro Shield® scaffold was used uncoated primarily because it is commercially

available as an easy-to-use, off-the-shelf product for cartilage repair therapies. Hyalofast™ is made of fibres of HA. HA is the major component of the ECM of articular cartilage and therefore, may serve as a successful scaffold for articular cartilage regeneration (Solchaga *et al.*, 1999). MSCs are known to express CD44 which is a membrane bound receptor with HA (Peach *et al.*, 1993; Goodison *et al.*, 1999; Cristino *et al.*, 2005). Migration of cells across a HA substrate via CD44 cell-mediated movement has previously been reported (Thomas *et al.*, 1992). It has also been shown that interaction of CD44 with HA can cause cells to aggregate or round up (Green *et al.*, 1988), which is consistent with the findings in this chapter, however MSCs showed a mixed morphology of round and elongated cells when grown in the Hyalofast™ scaffold.

MSCs in Alpha Chondro Shield® and Hyalofast™ remained 100% viable during short and long term cultures but within Chondro-Gide®, some cell death was observed after 14 days in culture. This could be due to excessive proliferation of MSCs and subsequent cell death due to nutrients and oxygen deprivation.

AT MSCs showed a markedly greater incorporation in all of the scaffolds compared to BM MSCs. If AT MSCs are consistently shown to incorporate into cell scaffolds more readily than BM MSCs (with further donor analysis), there may be reasons for such differential incorporation. Strioga *et al* suggested that AT MSCs have a higher proliferative capacity and express greater levels of CD49d (integrin  $\alpha 4\beta 1$ ) and CD54 (intercellular adhesion molecule-1, ICAM-1) compared to BM MSCs (Strioga *et al.*, 2012). The  $\alpha 4\beta 1$  integrins have long been known to play a role in cell-cell and cell-matrix interactions (Takada *et al.*, 1989). One of the ligands for  $\alpha 4\beta 1$  is fibronectin (Wayner *et al.*, 1989), which is present in media containing serum (Swisher and Rannels 1997). The increased retention of AT MSCs compared to BM MSCs within Chondro-Gide® may be due, in part, to the adsorption of serum proteins especially to the collagen fibers (Mosher *et al.*, 1991); hence this may enhance the adhesion of AT MSCs via  $\alpha 4\beta 1$  integrins. On synthetic polyglycolic acid scaffold, the cells adhere by non-specifically adsorbing proteins from their microenvironment (Lamba *et al.*, 1998). Fibronectin

being one of the key ligands for  $\alpha 4\beta 1$  integrins may play a key role in facilitating increased AT MSCs adhesion to the PGA fibres. For the HA based, Hyalofast™ scaffold, the increased adhesion of AT MSCs may likely be due to ability of  $\alpha 4\beta 1$  integrins to act as a co-receptor with CD44 (Verfaillie and Benis, 1994; Acharya *et al.*, 2008)

Structural properties of the scaffold like porosity, pore size, fibre thickness, topography as well as scaffold stiffness can directly influence cell behaviour and colonization (reviewed by Lawrence and Madihally 2008). It has been previously shown by Nuernberger *et al.*, that collagen based fleeces supports a polygonal morphology of chondrocytes (Nuernberger *et al.*, 2011) whereas the HA based scaffold supports both elongated and round morphology of chondrocytes. They showed that in scaffolds with widely spaced fibres and thickness less than the cell diameter, chondrocytes adopt a spherical morphology whereas, if the scaffold fibres are tightly packed and have thickness more than the cell diameter then the cells adopt a polygonal or elongated morphologies (Nuernberger *et al.*, 2011). The findings of this chapter are consistent with the study by Nuernberger and co-workers in that the fibrous Alpha Chondro Shield® and Hyalofast™, the cells showed a mixed morphology of both round and elongated cells depending on whether the surface the cells adhere to diameter larger the cell type or not. These findings have also been observed by Schlegel and co-workers in 2008 using chondrocytes. The chondrocytes developed a fibroblast-like morphology when seeded onto a type I and type III collagen scaffolds whereas cells on a HA scaffold showed a mix of round and elongated morphologies (Schlegel *et al.*, 2008). The molecular mechanism behind this difference in morphologies of the same cell type grown on different scaffolds remains unknown.

The differences in MSC proliferation that were observed between the scaffolds may be attributed to their differing degradation rates. BM MSCs proliferated in Chondro-Gide® throughout the time course of the experiments. Conversely, there was no increase in the BM MSC numbers in Alpha Chondro Shield® or Hyalofast™ with time in culture. The collagens in Chondro-Gide® are slow to degrade compared to PGA fibres in Alpha Chondro Shield® ,

which begins to lose mechanical integrity over a 12 day period (Vunjak-Novakovic *et al.*, 1998) and degrades to about 50% of its initial mass by 28 days (Freed *et al.*, 1994). In the absence of matrix production or a blood-clot (generated *in vivo*), it is likely that the fast degrading Alpha Chondro Shield® does not provide a suitable environment for cells to grow and proliferate and, therefore, this results in cell loss. In addition, whilst PGA based scaffolds provide a good substrate for chondrocyte adhesion, cell proliferation during long term cultures may be significantly affected by acidic products during scaffold degradation (Rampichová *et al.*, 2010). The Hyalofast™ scaffold disintegrated during long term cultures and this loss of scaffold integrity may have contributed to the lack of growth of cells within the scaffold.

Although MSCs generally adhered and incorporated in all three scaffolds, overall the cell retention in all three scaffolds was poor, with only ~ 0.1-1.5% of the MSCs that were seeded attached to the scaffolds following a 2 hour incubation period. The effectiveness of the cell-seeding process is a crucial step, which could have a significant effect on the number of cells delivered to a cartilage lesion and thus the clinical outcome of any cell therapy. For MACI procedures, chondrocytes pre-seeded onto Chondro-Gide® have been grown for four weeks prior to implantation (Behrens *et al.*, 2006), whereas ACI procedures have been adapted to pre-seed Chondro-Gide® with chondrocytes for a recommended time of only 10-15 minutes prior to transplant (Steinwachs *et al.*, 2012). Studies have previously examined the use of spinner flasks to encourage more efficient cell seeding in porous scaffolds (Vunjak-Novakovic *et al.*, 1998) or of using polymerizing gels as a delivery vehicle for rapid cell seeding within collagen sponges (Radisic *et al.*, 2003). However, in this study, a simple cell-seeding strategy was utilized to replicate the clinical setting, with the results probably representing the best case scenario given an incubation period that is in excess (2 hours) of what would be clinically acceptable for a one-step procedure. The small size of the scaffolds used in this study may have contributed to the low incorporated cell numbers as they could have been of insufficient size to initially retain the total volume of medium used for cell-seeding. Hence, cells may have

initially leaked out of the scaffolds into the wells. Although a potential weakness of the study, this scenario may commonly reflect the clinical situation.

The clinical use of MSCs is limited due to the need for culture expansion to generate enough cells for transplantation. Therefore, current research is focusing on developing one step procedures for the use of freshly isolated population of MSCs as a potential for cartilage repair. In this *in vitro* study, freshly isolated and minimally manipulated populations of cells from the BM or the SVF cells of AT showed poor incorporation and growth within all three scaffolds compared to the culture expanded MSCs. The BM mononucleated cell prep is a heterogeneous population containing differentially matured haematopoietic cells, endothelial cells and MSCs. Literature suggests that only 0.01-0.00001% of the BM mononucleated cell population are MSCs (Caplan 1994). This could explain the poor incorporation and growth of the adherent cell population of MSCs from BM MNCs as the cell count used for cell-seeding experiments constituted all the mononucleated cells and not just the MSCs. Although these findings suggest that BM MNCs may be unsuitable as a cell source for one-step cell-based therapies for cartilage repair, a study conducted on human patients by Buda *et al.*, using Hyalofast™ and BM concentrate fixed into the defect with platelet-rich fibrin, reported clinical improvement at 2 years follow up with 80% graft integration and 70% defect fill (Buda *et al.*, 2010). Similarly, Gobbi *et al.* used Chondro-Gide® with clotted BM concentrate to fill chondral defects that were subsequently covered with fibrin glue, and also reported promising results at 2 years follow-up, with MRI showing good defect fill and histology of biopsy samples showing hyaline like tissue (Gobbi *et al.*, 2011). These clinical studies demonstrate that *in vitro* experiments are not very effective in mimicking the situation *in vivo* and therefore, preclinical animal studies maybe required to better examine the potential of different cell sources for the treatment of cartilage defects.

The AT SVF cells incorporated in all three scaffolds during short-term cultures, but only proliferated in Chondro-Gide®. Morphologically, these cells appeared mostly round with very few fibroblast-like cells in Chondro-Gide®. Alpha Chondro Shield® showed very few

elongated cells that did not survive through the long-term cultures. This may have been due to disintegration of scaffold structure, which was initially observed at day 14. It has been shown previously that cell-free PGA scaffolds after 28 days of incubation in PBS lose their integrity (Li and Mak 2005). AT SVF cells in Hyalofast™ were mostly of a round morphology with very few elongated cells present and the scaffold was observed also to have disintegrated during longer-term cultures. The problem of scaffold disintegration was one of the limitations of the experiments conducted in this study. The scaffolds used are all commercially available and are stored in air tight packs ready to use clinically. In this study, the scaffolds were exposed to atmospheric gases and studies have shown that this can alter the chemical and mechanical properties of the PGA based scaffolds particularly (Ma and Langer 1995) This was only observed for Alpha Chondro Shield® and Hyalofast™ scaffold and not for Chondro-Gide® scaffold. Nonetheless, AT SVF cells showed better incorporation than BM MNCs over all. This may be because there is a greater number of MSCs present within the SVF of adipose tissue. Literature suggests that the MSC population in the uncultured SVF usually amounts to up to 3% of the cells present, and this is 2,500-fold more than the frequency of stem cells in BM (Fraser *et al.*, 2008).

Therefore, the first part of this chapter suggests that the use of culture expanded MSCs from both BM and AT is favoured over that of freshly isolated cells with Chondro-Gide® proving an effective cell delivery system.

The next part of this chapter focussed on looking at chondrogenic differentiation potential of BM and AT MSCs in Chondro-Gide® and Alpha Chondro Shield® only. As stated earlier, the Hyalofast™ scaffold disintegrated during long term culture and therefore, no chondrogenic assessment was possible for this scaffold. BM MSCs seeded on Chondro-Gide® showed distinct rounded shape of mature chondrocytes that deposited matrix rich in type II collagen which was not seen in Alpha Chondro Shield® under +CM conditions. AT MSCs on the other hand showed round shaped cells with matrix deposition in Chondro-Gide® and Alpha Chondro Shield® and localised positivity of type II collagen was observed within these

two scaffolds under +CM conditions. BM MSCs showed an increased amount of metachromatic staining with toluidine blue under +CM conditions in both Chondro-Gide® and Alpha Chondro Shield® compared to -CM conditions. This result was consistent with the soluble GAG biochemical analysis. The soluble proteoglycans secreted into the media were analysed as an indirect measure of ECM synthesis because it has been shown that the removal of proteoglycans from the ECM in cartilage explant cultures is in balance with the deposition of newly synthesised GAGs (Ilic *et al.*, 1995). In addition, another study has shown that the kinetics of GAG release into culture media by chondrocytes seeded within agarose, collagen and PGA constructs were comparable to the GAGs found in the cell-seeded constructs (Mouw *et al.*, 2005). The reason why elevated soluble GAGs were seen in Hyalofast™ scaffold may be due to disintegration of fibres of HA and loss of potential ECM and their subsequent binding to DMMB dye. Overall, AT MSCs produced similar amounts of GAGs in the three scaffolds and secreted comparatively more GAGs in response to chondrogenic inducers as compared to controls when seeded within Chondro-Gide® and Alpha Chondro Shield®. With time in culture the PGA based scaffold Alpha Chondro Shield® and HA based scaffold Hyalofast™ will degrade faster than the collagen based scaffold Chondro-Gide® resulting in a potential of loss of deposited matrix with continuous media changes in culture (Ma and Langer 1995, Lee and Lee 2006). Therefore, it was expected that cells within Alpha Chondro Shield® and Hyalofast™ may show decreased levels of soluble GAGs at day 28 compared to Chondro-Gide®. Chondro-Gide® proved to be a superior scaffold for BM MSC differentiation as determined by the increased expression of type II collagen under +CM conditions. In Alpha Chondro Shield® there was a weak and nonhomogeneous ECM staining which is likely due to the relatively short culture period of 28 days. PGA based scaffolds have previously been successful for *in vitro* chondrogenesis of chondrocytes after 40 days in culture (Freed *et al.*, 1993).

In summary, the major findings of this chapter suggest that culture expansion of MSCs and the use of the Chondro-Gide® scaffold is favoured as an effective cell-delivery

system. Furthermore, BM MSCs successfully underwent chondrogenesis when seeded within Chondro-Gide® scaffold and AT MSCs when seeded within Alpha Chondro Shield® scaffold. Therefore, further studies in this thesis have focused on Chondro-Gide® and Alpha Chondro Shield® scaffolds and MSCs rather than freshly isolated cells.

Furthermore, the work presented in this chapter indicates the need for culture expansion of MSCs for chondrogenesis; however, further work is also needed to optimise the incorporation and growth of mononucleated cells, possibly by selectively purifying the MSC progenitors from heterogeneous cell populations present.

**Chapter 4: An *in vitro* investigation of the chondrogenic differentiation potential of adipose tissue derived CD271 selected MSCs versus standard plastic adherent MSCs in clinically used scaffolds**

#### 4.1 Background and aims

BM has been shown to be a promising source of MSCs for cartilage repair with evidence of hyaline like cartilage formation both *in vitro* (Johnstone *et al.*, 1998; Jakobsen *et al.*, 2010) and *in vivo* (Im *et al.*, 2001; Zhang *et al.*, 2011). In comparison only a few studies support the use of MSCs derived from AT for their use in cartilage repair. From the previous chapter, even with the limited donors investigated, the MSCs from BM were a better source for *in vitro* chondrogenesis as indicated by the deposition of ECM that was positive for collagen type II. However, there are advantages to using AT as a source of MSCs as it has been shown that up to 3% of the SVF population are MSCs which is 2500 fold more than the frequency of MSCs in the BM (Fraser *et al.*, 2008). In addition, the method for AT harvest is simple and less invasive than that for BM, and in most orthopaedic surgical procedures often regarded as medical waste.

AT is a suitable reservoir of potentially regenerative cells as this tissue is abundant and easy to harvest in human adults. The cells present within the SVF of AT can be easily isolated by tissue digestion and subsequent washings and centrifugation steps, followed by the outgrowth of a plastic adherent fraction referred to as AT MSCs (Zuk *et al.*, 2001). The SVF is a highly heterogeneous population consisting of AT MSCs along with many other cell types such as preadipocytes, adipocytes, endothelial cells, fibroblasts, monocytes, macrophages and lymphocytes (Baer & Geiger 2012). The plastic adherent population of MSCs has been extensively characterised for their expansion and differentiation potential; however, the reproducibility of the methods used is questionable due to the heterogeneity of the starting population and the variability in the stem cell recovery among different donors and different tissue sources (Van Harmelen *et al.*, 2004; Schipper *et al.*, 2008). Therefore, there is a need to investigate and identify pre-culture markers that would ensure higher purity than that obtained by selection based on adherence to plastic.

A focus of current research in regenerative medicine has been examining the potential of defined subpopulations of MSCs from the heterogeneous pool of cells from different tissue

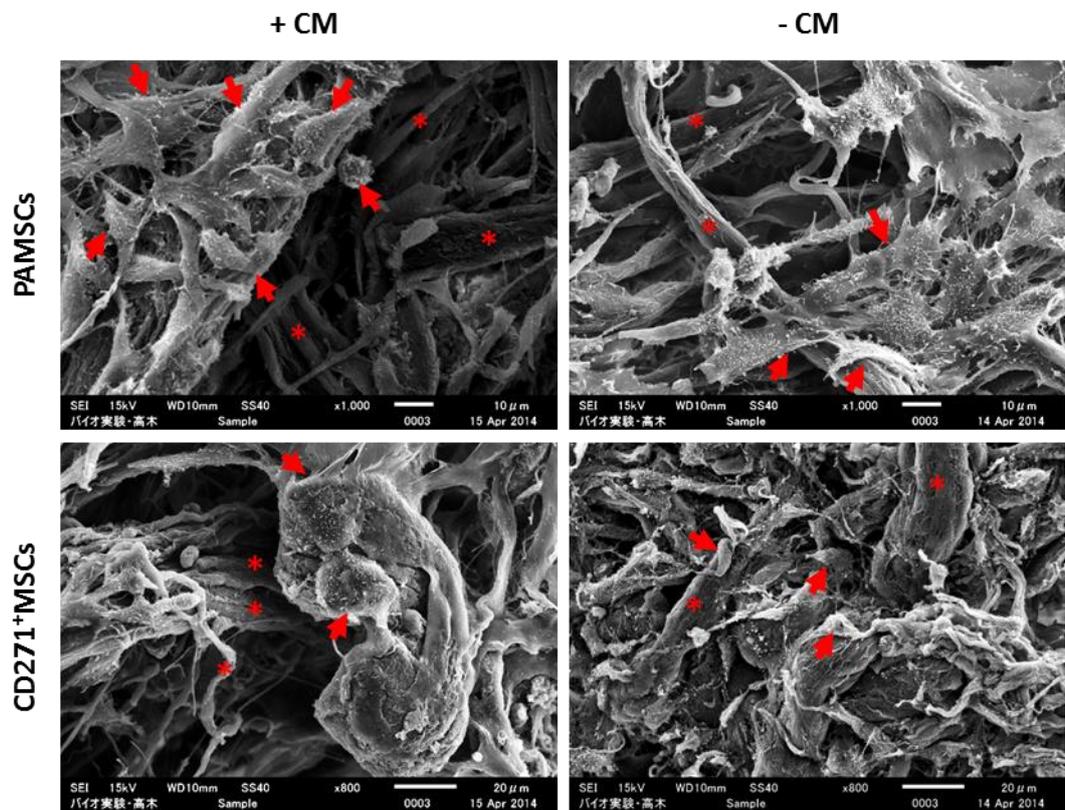
sources towards a defined lineage (Jones *et al.*, 2002; Psaltis *et al.*, 2010; Rada *et al.*, 2011; Churchman *et al.*, 2012). CD73 and CD105 are shown to have better chondrogenic differentiation potential compared to the unsorted population of MSCs (Arufe *et al.*, 2009; Rada *et al.*, 2011). In this regard, CD271 has been described as a specific markers for the purification of BM MSCs (Álvarez-Viejo 2015). CD271 is the low affinity nerve growth factor receptor (LNGFR), also referred to as P75 neurotrophin receptor (P75NTR) and belongs to the tumor necrosis factor superfamily (Thomson *et al.*, 1988). CD271 has been extensively studied as a suitable marker for selectively isolating MSCs from BM (Quirici *et al.*, 2002; Jones *et al.*, 2002; Jones *et al.*, 2006; Jones and McGonagle 2008). However, only a single study has so far reported on the enhanced capacity of CD271<sup>+</sup> MSCs from AT towards chondrogenesis. In a study by Quirici and co-workers, CD271<sup>+</sup>MSCs were isolated from AT using magnetic microbeads and were compared for their clonogenicity and differentiation potential with PA MSCs (Quirici *et al.*, 2010). They showed that CD271<sup>+</sup> MSCs had a greater differential potential towards osteogenesis, adipogenesis and chondrogenesis compared to PA MSCs (Quirici *et al.*, 2010). The results reported in the previous chapter of this thesis are indicative of inferior chondrogenic potential of AT MSCs when compared to BM MSCs. The heterogeneity in the SVF of AT may contribute to this effect.

Therefore, the rationale of this chapter was based on the hypothesis that CD271<sup>+</sup>MSCs have an increased chondrogenic differentiation potential compared to the MSCs isolated by plastic adherence. The aim of this chapter was to investigate the chondrogenic differentiation potential of MSCs selected on the basis of plastic adherence (PA MSCs) versus CD271 selected MSCs (CD271<sup>+</sup>MSCs) in Chondro-Gide® and Alpha Chondro Shield® *in vitro*. These two scaffolds were selected because Hyalofast™ had not been suitable for longer term (28 days) *in vitro* analysis.

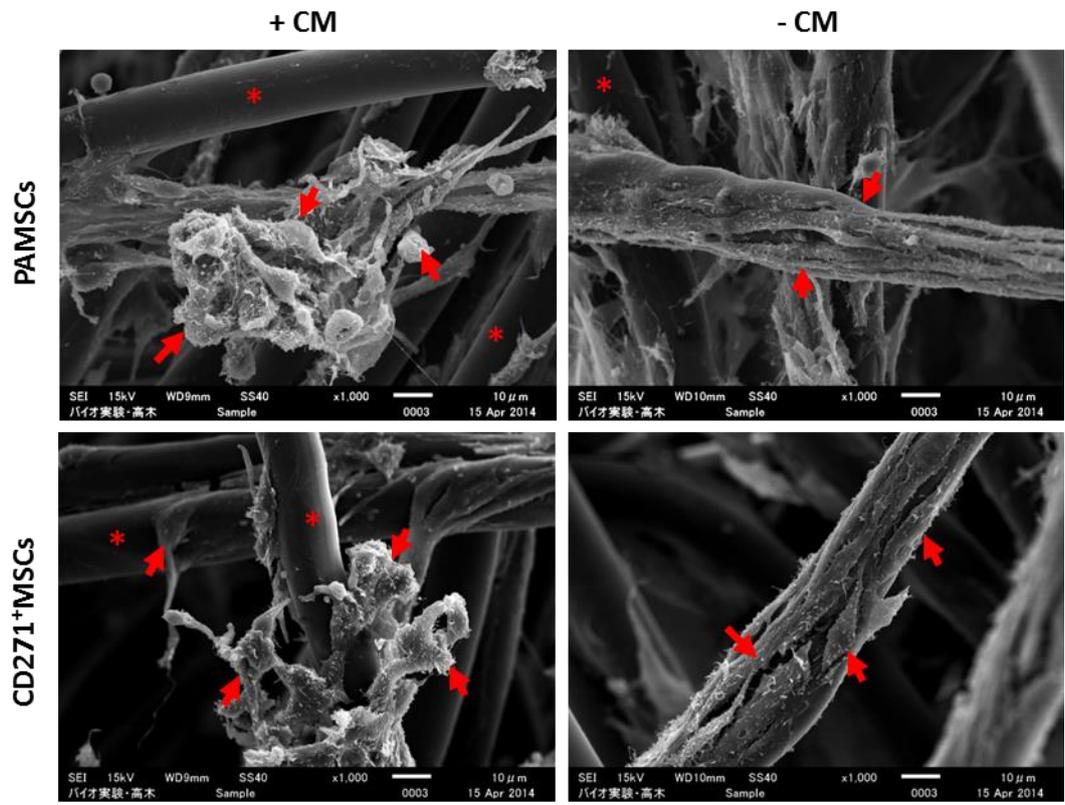
#### **4.2 The morphology and growth of PA MSCs and CD271<sup>+</sup>MSCs under chondrogenic (+CM) and non-chondrogenic (-CM) conditions**

No major differences were observed between PA MSCs and CD271<sup>+</sup>MSCs with regards to their morphology. PA MSCs and CD271<sup>+</sup>MSCs grew in dense networks in Chondro-Gide® compared to MSCs in Alpha Chondro Shield® where they grew along the length of the fibres and appeared to be less dense. Under +CM conditions only, at day 7, cell condensation and aggregation was seen to some extent in Chondro-Gide® (Figure 4.1) whereas, such condensations and aggregations were seen to a greater extent in Alpha Chondro Shield® (Figure 4.2) compared to the flattened morphology of cells observed in both the scaffolds under -CM conditions.

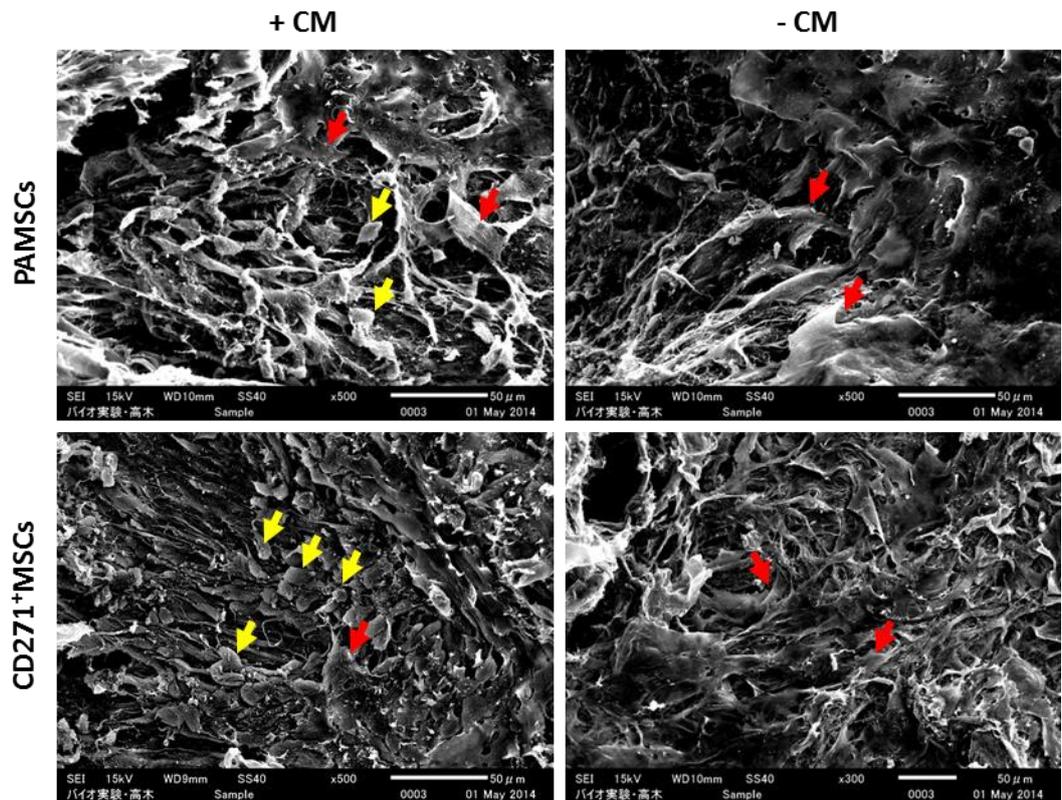
Also from SEM, it appeared that MSCs in Chondro-Gide® (Figure 4.3) and Alpha Chondro Shield® (Figure 4.4) proliferated under both +CM and -CM conditions. At day 28, MSCs appeared denser and covered the entire surface of the scaffold. Therefore, it was difficult to assess any morphological differences between PA and CD271<sup>+</sup>MSCs especially in Alpha Chondro Shield® where the scaffold was covered entirely by a dense layer of cells and matrix. In Chondro-Gide®, a few round-shaped cells were distinguishable in both PA and CD271<sup>+</sup>MSCs seeded scaffolds under +CM conditions only.



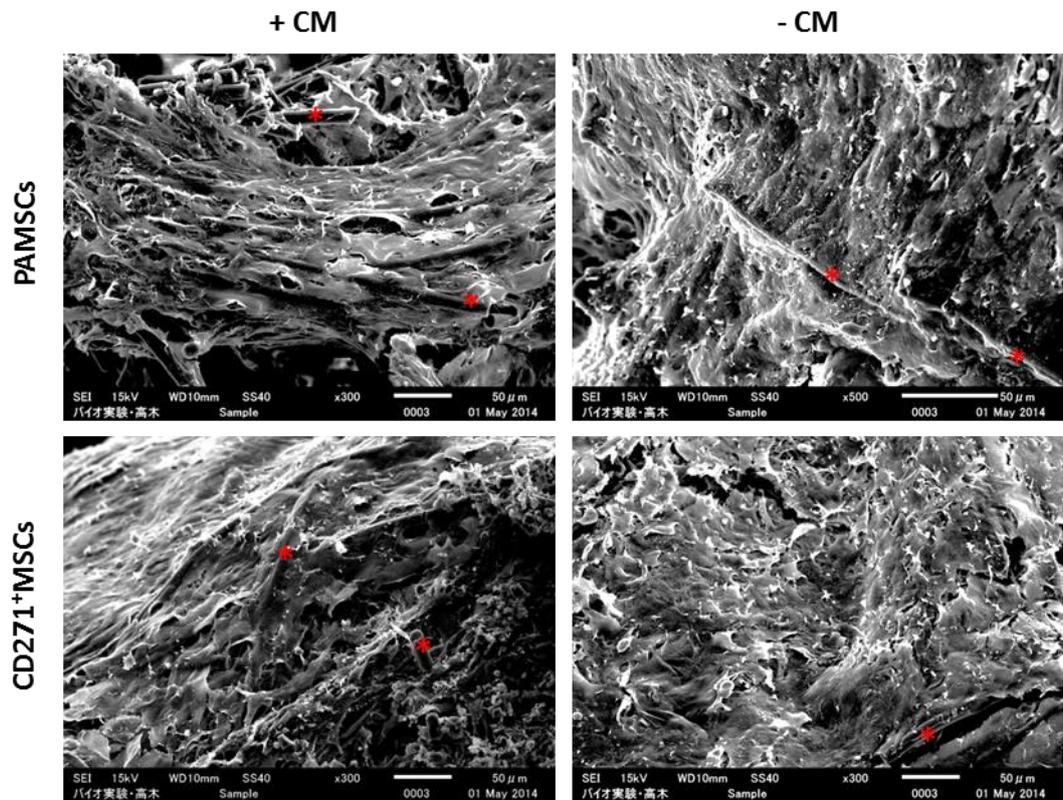
**Figure 4.1.** The morphology of PA MSCs and CD271<sup>+</sup> MSCs in Chondro-Gide® under chondrogenic (+CM) and non-chondrogenic (-CM) conditions. Representative SEM images are shown of PAMSCs and CD271<sup>+</sup> MSCs in Chondro-Gide® 7 days post-seeding. Both PA MSCs and CD271<sup>+</sup> MSCs were seen as flattened cells and rounded cells under +CM and -CM conditions, however cell aggregation into clumps was most prominently seen under +CM conditions. Arrows represent cells and asterisks represent scaffold fibres. PA MSCs and CD271<sup>+</sup> MSCs, n=1 matched donor.



**Figure 4.2.** The morphology of PA MSCs and CD271<sup>+</sup> MSCs in Alpha Chondro Shield® under chondrogenic (+CM) and non-chondrogenic (-CM) conditions. Representative SEM images are shown of PAMSCs and CD271<sup>+</sup> MSCs in Alpha Chondro Shield® 7 days post-seeding. PA MSCs and CD271<sup>+</sup> MSCs were mostly condensed and aggregated around the fibres of PGA under +CM conditions and appeared to be flat and elongated along the length of the fibres under -CM conditions. Arrows represent cells and asterisks represent scaffold fibres. PA MSCs and CD271<sup>+</sup> MSCs, n=1 matched donor.



**Figure 4.3.** The morphology of PAMSCs and CD271<sup>+</sup>MSCs in Chondro-Gide® under chondrogenic (+CM) and non-chondrogenic (-CM) conditions. Representative SEM images are shown PAMSCs and CD271<sup>+</sup>MSCs in Chondro-Gide® 28 days post-seeding. PA MSCs and CD271<sup>+</sup>MSCs showed dense growth patterns under +CM and -CM conditions. Under +CM conditions a mixed morphology of flat and round cells was seen with both PA MSCs and CD271<sup>+</sup>MSC seeded Chondro-Gide®, whereas under -CM conditions the cells appeared mostly flat and spread. Red arrows represent flat cells and yellow arrows represent round cells. PA MSCs and CD271<sup>+</sup>MSCs, n=1 matched donor.

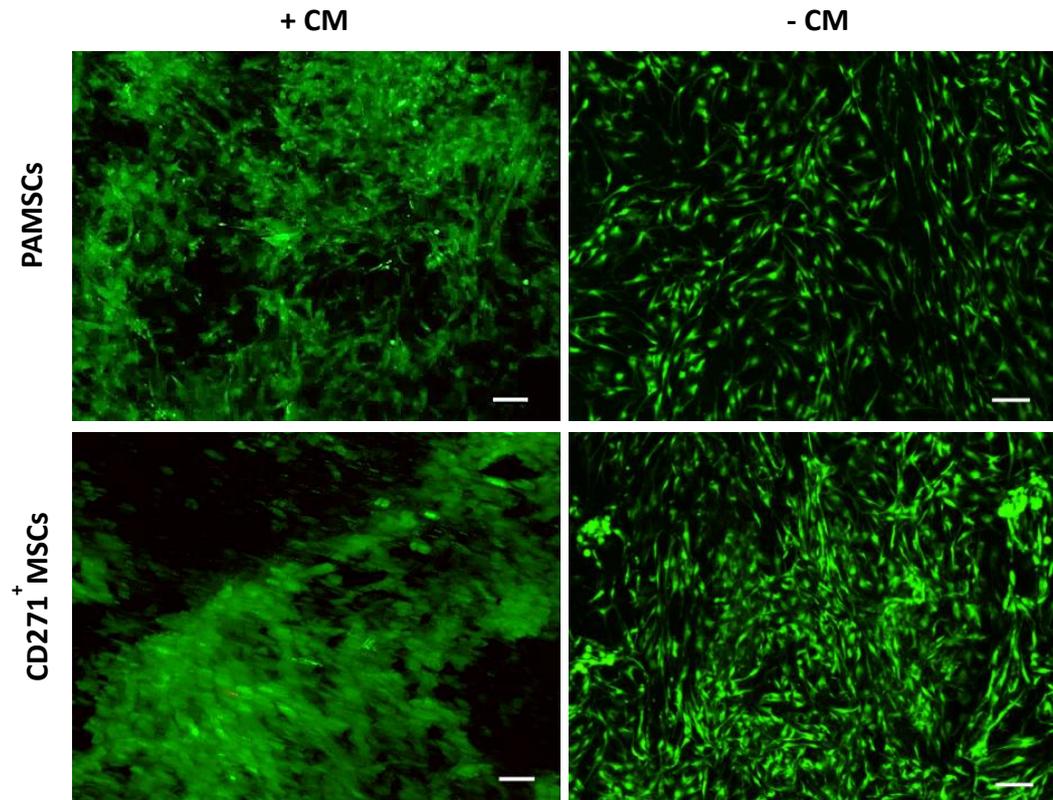


**Figure 4.4. The morphology of PAMSCs and CD271<sup>+</sup>MSCs in Alpha Chondro Shield® under chondrogenic (+CM) and non-chondrogenic (-CM) conditions.** Representative SEM images are shown PAMSCs and CD271<sup>+</sup>MSCs in Alpha Chondro Shield® 28 days post-seeding. PA MSCs and CD271<sup>+</sup>MSCs showed dense growth patterns under +CM and -CM conditions. The entire scaffold surface was covered with a dense growth of cells. Due to the extent of the growth of cells, any morphological differences were difficult to assess. Asterisks represent fibres of the scaffold. PA MSCs and CD271<sup>+</sup>MSCs, n=1 matched donor.

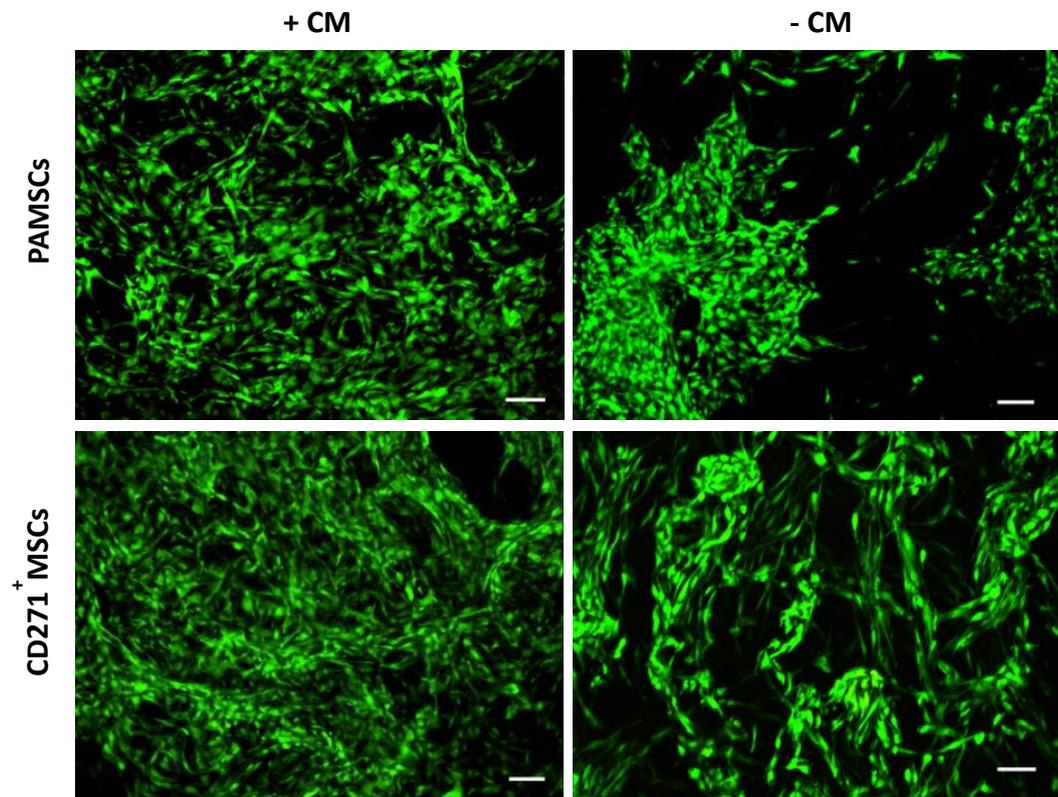
### **4.3 The viability of PA MSCs and CD271<sup>+</sup>MSCs in Chondro-Gide® and Alpha Chondro Shield® under +CM and –CM conditions**

No cell death was observed in either Chondro-Gide® or Alpha Chondro Shield® scaffolds under any condition. A homogenous distribution of PA and CD271<sup>+</sup>MSCs was seen throughout the depth of the scaffolds after live/dead staining and 3D confocal microscopy. Similar to the SEM results, cell aggregations were observed in Chondro-Gide® under +CM conditions. Under –CM conditions, MSCs appeared to be individually dispersed and showed a fibroblast-like appearance. No cell aggregation was observed under –CM conditions in Chondro-Gide® (Figure 4.5).

In Alpha Chondro Shield®, a dense growth of MSCs was observed under +CM conditions whereas under –CM conditions the cells grew sparsely along the length of the fibres and appeared elongated along the length of the fibres. No cell aggregation was observed under –CM condition (Figure 4.6).



**Figure 4.5. The viability of PAMSCs and CD271<sup>+</sup> MSCs in Chondro-Gide® under chondrogenic (+CM) and non-chondrogenic (-CM) conditions.** Representative confocal z-stacks are shown of PA MSCs and CD271<sup>+</sup> MSCs following live/dead staining after 28 days in culture. PA MSCs and CD271<sup>+</sup> MSCs remained viable at day 28 in both scaffolds and under +CM and –CM conditions. MSCs under +CM conditions appeared aggregated and grew in dense layers compared to –CM conditions where they appeared to be individually dispersed throughout the depth of the scaffold. PA MSCs and CD271<sup>+</sup> MSCs, n=2 matched donor. Scale bars = 100  $\mu$ m.



**Figure 4.6. The viability of PAMSCs and CD271<sup>+</sup> MSCs in Alpha Chondro Shield® under chondrogenic (+CM) and non-chondrogenic (-CM) conditions.** Representative confocal z-stacks are shown of PA MSCs and CD271<sup>+</sup> MSCs following live/dead staining after 28 days in culture. PA MSCs and CD271<sup>+</sup> MSCs remained viable at day 28 in both scaffolds and under +CM and -CM conditions. MSCs under +CM conditions appeared more dense and aggregated along the length of the fibres compared to -CM conditions, where they grew in less dense layers and appeared elongated along the length of the fibres of Alpha Chondro Shield®. PA MSCs and CD271<sup>+</sup> MSCs, n=2 matched donors. Scale bars = 100  $\mu$ m.

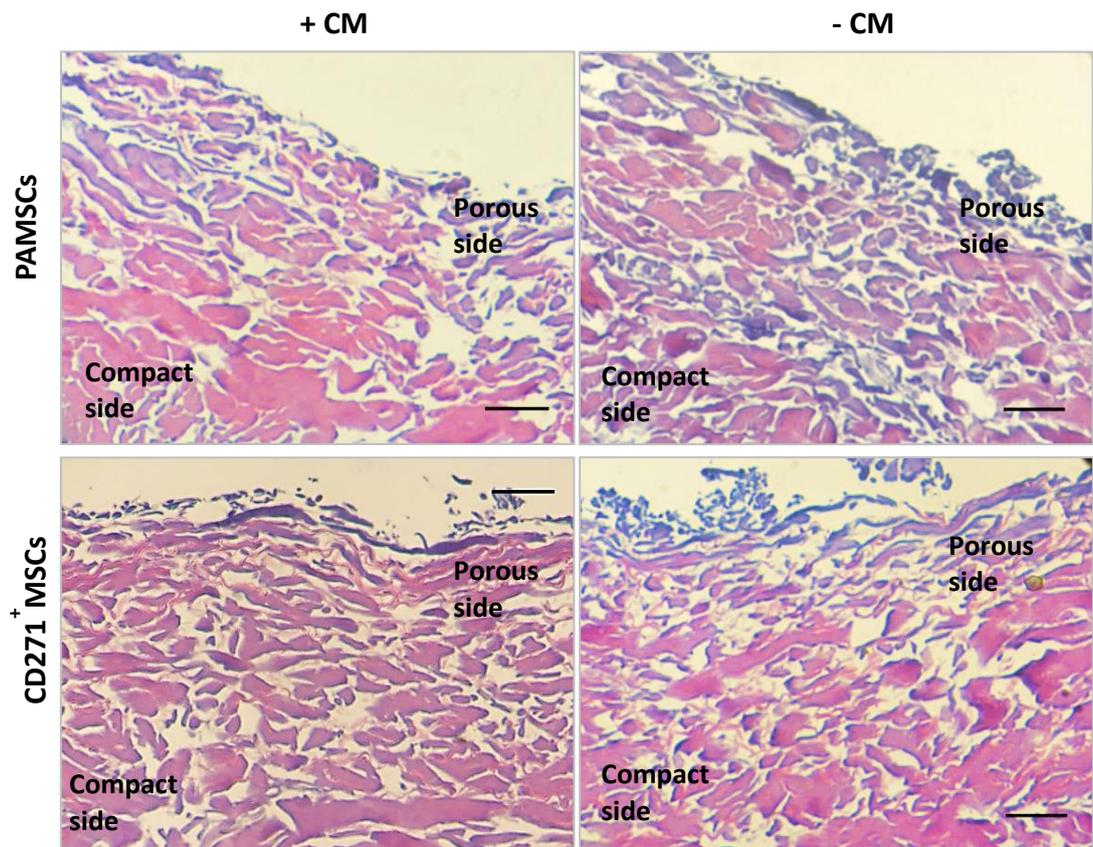
#### **4.4 Histology of PA MSCs and CD271<sup>+</sup>MSCs in Chondro-Gide® and Alpha Chondro Shield® under +CM and –CM conditions**

There were no differences observed with regards to the distribution and growth of PA and CD271<sup>+</sup> MSCs in both the scaffolds. H&E staining of sections of Chondro-Gide® demonstrated that PA and CD271<sup>+</sup>MSCs were evenly distributed throughout the porous side of Chondro-Gide® scaffold under both +CM and –CM conditions; however, it was difficult to distinguish between matrix derived from MSCs and the collagenous fibres of the scaffold (Figure 4.7).

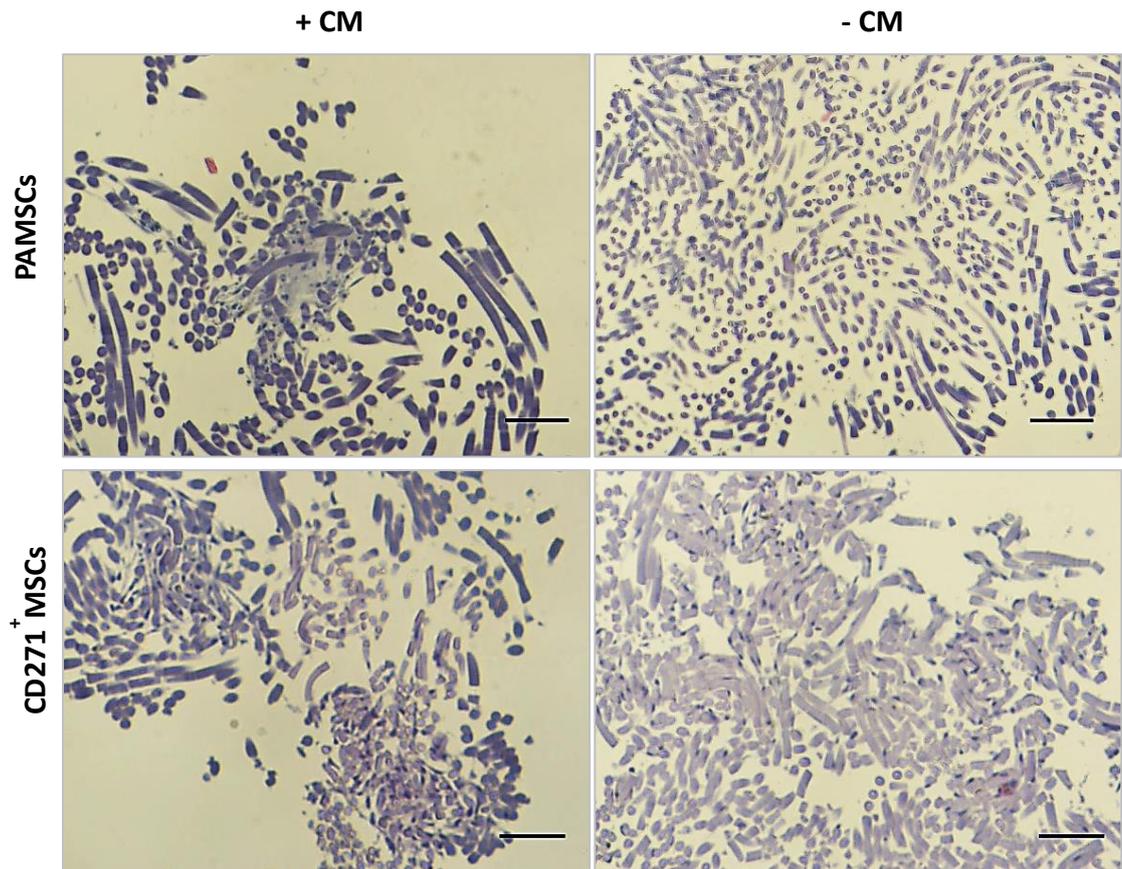
In Alpha Chondro Shield®, H&E staining revealed the distribution of cells to be homogenous under both +CM and –CM conditions; however, ECM deposition was only seen under +CM conditions and cells were individually dispersed under –CM conditions (Figure 4.8).

Toluidine blue staining of proteoglycans revealed a greater amount of ECM staining in Chondro-Gide® seeded with CD271<sup>+</sup>MSCs only compared to no ECM deposition seen in Chondro-Gide® seeded with PA MSCs or with both cell types under –CM conditions (Figure 4.9). Similarly, in Alpha Chondro Shield® stained with toluidine blue, ECM deposition was only seen with CD271<sup>+</sup>MSC seeded scaffolds compared to PA MSCs seeded scaffolds. No ECM deposition was seen in MSC-seeded Alpha Chondro Shield® under –CM conditions (Figure 4.10).

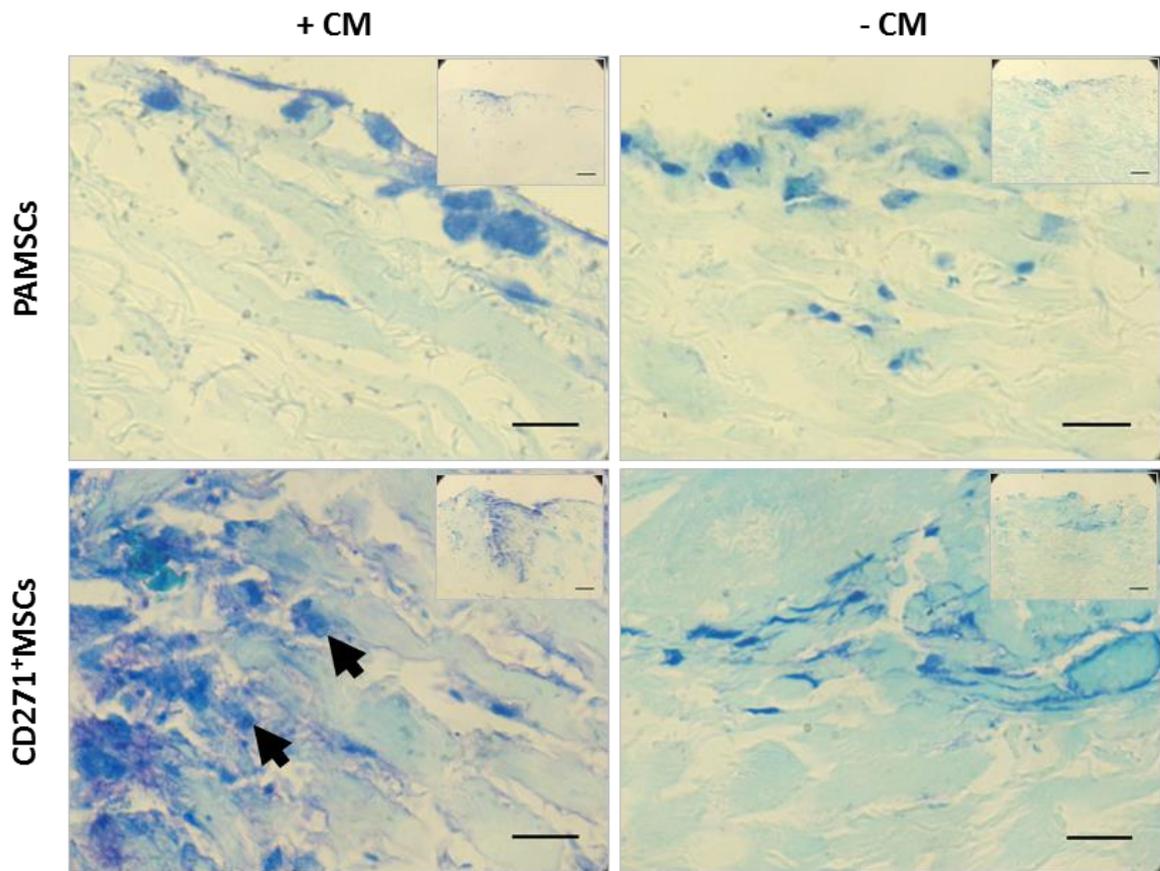
Pellets of PA MSCs and CD271<sup>+</sup>MSCs both showed purple metachromatic staining with toluidine blue. However, an ECM positive for collagen type II was only seen in the pellets of CD271<sup>+</sup>MSCs (Figure 4.11).



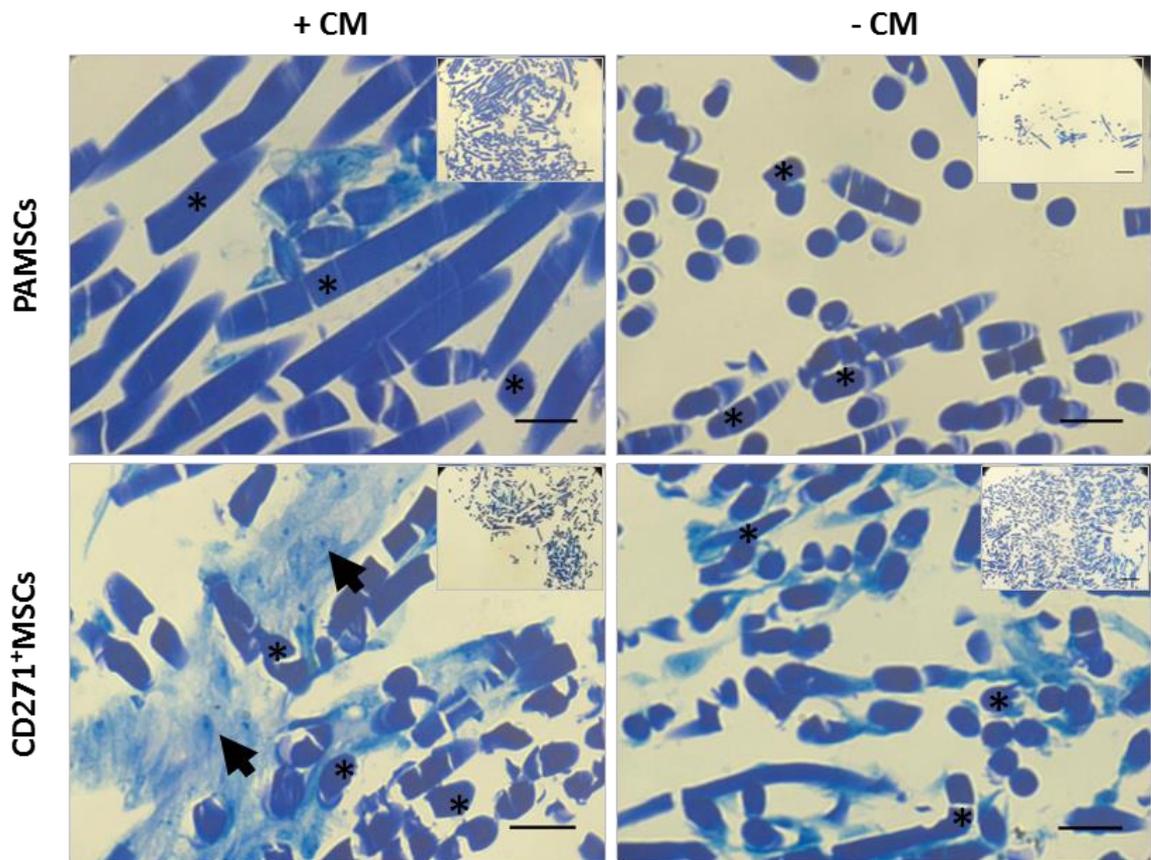
**Figure 4.7. The incorporation and distribution of PA MSCs and CD271<sup>+</sup> MSCs in Chondro-Gide® under chondrogenic (+CM) and non-chondrogenic (-CM) conditions.** Representative images are shown of H&E stained PA MSCs and CD271<sup>+</sup> MSCs seeded Chondro-Gide® after 28 days in culture. PA MSCs and CD271<sup>+</sup> MSCs were evenly distributed along the porous side of the scaffold as indicated by the purple haematoxylin staining of the cell nuclei. The collagen fibres of the scaffold stained pink with eosin. PA MSCs and CD271<sup>+</sup> MSCs, n=2 matched donors. Original magnification x10. Scale bar =100  $\mu$ m.



**Figure 4.8. The incorporation and distribution of PA MSCs and CD271<sup>+</sup> MSCs in Alpha Chondro Shield® under chondrogenic (+CM) and non-chondrogenic (-CM) conditions.** Representative images are shown of H&E stained PA MSCs and CD271<sup>+</sup> MSCs seeded Alpha Chondro Shield® after 28 days in culture. Dark purple haematoxylin staining of cell nuclei was observed under both +CM and -CM conditions. Under +CM conditions, little ECM deposition was observed with either PA MSCs or CD271<sup>+</sup> MSC seeded scaffolds. Under -CM conditions, cells appeared dispersed homogenously along the length of the scaffold fibres. PA MSCs and CD271<sup>+</sup> MSCs, n=2 matched donors. Original magnification x10. Scale bar = 100 µm.

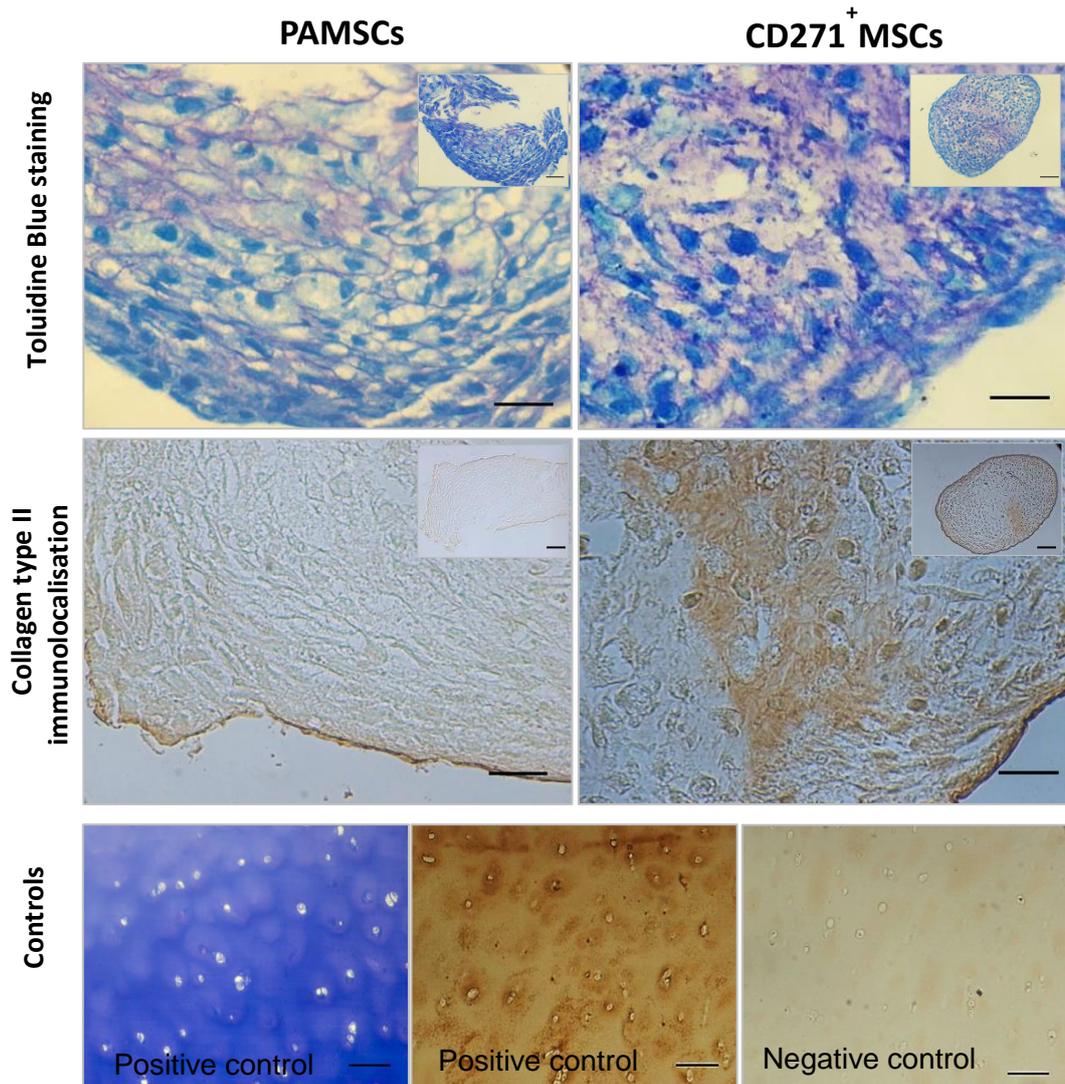


**Figure 4.9. GAG deposition by PA MSCs and CD271<sup>+</sup>MSCs in Chondro-Gide® under chondrogenic (+CM) and non-chondrogenic (-CM) conditions.** Representative images are shown of toluidine blue stained PA MSCs and CD271<sup>+</sup>MSCs in Chondro-Gide® after 28 days in culture. GAG deposition was indicated by toluidine blue metachromasia only in CD271<sup>+</sup>MSC seeded scaffolds under +CM conditions. A round morphology of cells (black arrowed) was also observed under +CM condition within the CD271<sup>+</sup>MSC seeded scaffolds. No GAG deposition was seen with PA MSCs seeded scaffold under +CM conditions or in PA MSC or CD271 MSC seeded scaffolds under – CM conditions. PA MSCs and CD271<sup>+</sup>MSCs, n=2 matched donors. Insets represent images at x10 magnification, scale bar = 100 µm. Original magnification x40, scale bar = 25µm.



**Figure 4.10. GAG deposition by PA MSCs and CD271<sup>+</sup> MSCs in Alpha Chondro Shield® under chondrogenic (+CM) and non-chondrogenic (-CM) conditions.**

Representative images are shown of toluidine blue PA MSCs and CD271<sup>+</sup> MSCs in Alpha Chondro Shield® after 28 days in culture. GAG deposition was observed only in CD271<sup>+</sup> MSC seeded scaffolds with a few round shaped cells (black arrowed) seen only under +CM conditions. No matrix deposition was seen with PA MSCs seeded scaffold under +CM conditions or in PA MSC or CD271<sup>+</sup> MSC seeded scaffolds under -CM condition. Black asterisks represent the fibres of the scaffold. PA MSCs and CD271<sup>+</sup> MSCs, n=2 matched donors. Insets represent images at x10 magnification, scale bar = 100  $\mu$ m. Original magnification x40, scale bar = 25  $\mu$ m.



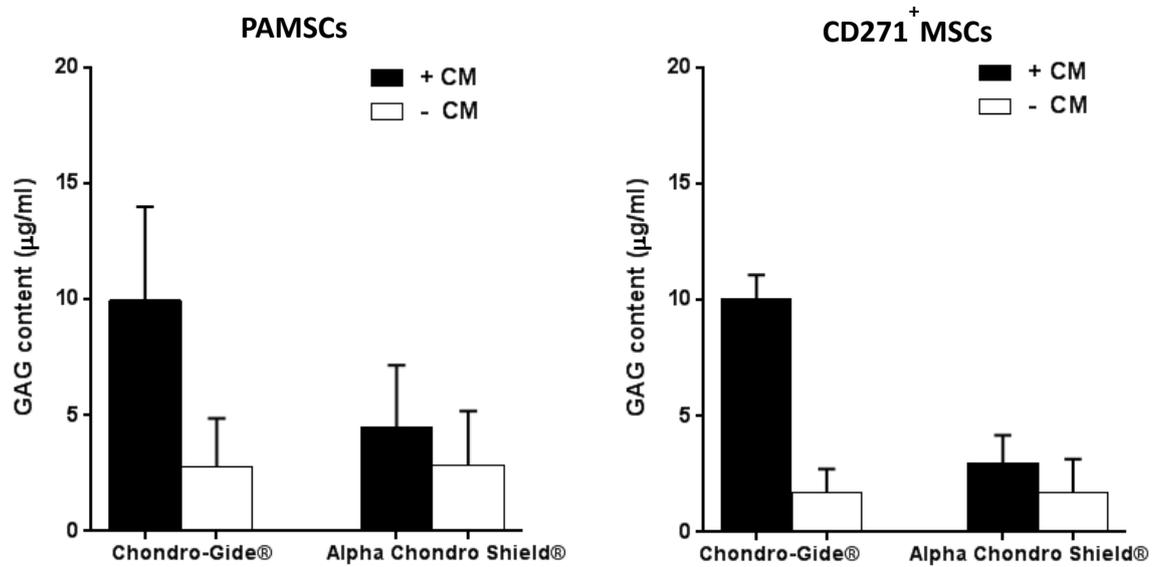
**Figure 4.11. GAG deposition and collagen type II immunolocalisation in PA MSCs and CD271<sup>+</sup> MSCs pellets under chondrogenic (+CM) conditions.** GAG deposition was seen in PA MSCs and CD271<sup>+</sup> MSCs pellets as indicated by the metachromatic staining of these pellets after toluidine blue staining (top panels). Collagen type II immunolocalisation was observed in the ECM deposited by CD271<sup>+</sup> cell pellet only (bottom panels). The positive control was a section of human knee articular cartilage and negative control is the same section with the omission of primary antibody. PA MSCs and CD271<sup>+</sup> MSCs, n=2 matched donors. Insets represent images at x10 magnification, scale bar = 100  $\mu$ m. Original magnification x40, scale bar = 25  $\mu$ m.

#### **4.5 Glycosaminoglycan (GAG) production by PA MSCs and CD271<sup>+</sup>MSCs seeded within Chondro-Gide® and Alpha Chondro Shield® under +CM and –CM conditions**

There was greater GAG production by PA and CD271<sup>+</sup>MSCs in both of the scaffolds tested under +CM conditions compared with –CM conditions (Figure 4.12). PA MSCs secreted  $9.9 \pm 4.0$  µg/ml of GAGs within Chondro-Gide® and  $4.5 \pm 2.6$  µg/ml of GAGs within Alpha Chondro Shield® under +CM conditions, compared to  $2.8 \pm 2.1$  µg/ml of GAGs within Chondro-Gide® and  $2.8 \pm 2.3$  µg/ml of GAGs within Alpha Chondro Shield® under –CM conditions.

CD271<sup>+</sup>MSCs secreted  $10 \pm 1.0$  µg/ml of GAGs within Chondro-Gide® and  $3.0 \pm 1.2$  µg/ml of GAGs within Alpha Chondro Shield® under +CM conditions, compared to  $1.7 \pm 1.0$  µg/ml of GAGs within Chondro-Gide® and  $1.7 \pm 1.4$  µg/ml of GAGs within Alpha Chondro Shield® under –CM conditions.

There was no marked difference in levels of GAG production between the two cell types in either of the scaffolds or conditions.



**Figure 4.12. GAG secretion by PA MSCs and CD271<sup>+</sup> MSCs seeded within Chondro-Gide® and Alpha Chondro Shield® under chondrogenic (+CM) and non-chondrogenic (-CM) conditions.** Greater levels of GAGs were secreted by both cell types and in both scaffolds under +CM conditions compared to -CM conditions. The GAG levels secreted by PA MSCs and CD271 MSCs were similar and no major differences were seen in between the two cell types with either scaffold. Data has been presented as means  $\pm$  SD of 6 internal replicate values pooled from n=2 matched patient donors.

## 4.6 Discussion

There were no differences seen between PA and CD271<sup>+</sup> MSCs from AT with regards to the cell morphology and growth. However, cell condensation and aggregation was observed under +CM conditions as early as day 7 in all cell-seeded scaffolds. Cell-rounding and aggregation may be indicative of chondrogenesis, which involves condensation of the MSCs followed by their differentiation into chondrocytes and subsequently chondrocyte maturation and ECM production during long-term cultures (Goldring *et al.*, 2006). CD271<sup>+</sup> MSCs showed greater chondrogenesis in both scaffolds compared to PA MSCs as indicated by greater metachromatic staining by toluidine blue. In addition, collagen type II immunostaining was more intense in CD271<sup>+</sup> MSC pellet compared to the pellets of PA MSCs. These findings are consistent with a recent report in which synovium derived CD271<sup>+</sup>MSCs showed significantly more intense staining of collagen type II at day 28 of chondrogenesis compared to cells selected with CD73 or CD106 (Arufe *et al.*, 2010). Arufe and co-workers suggested that the enhanced chondrogenic potential of CD271<sup>+</sup>MSCs could be attributed to the higher co-expression of CD105 marker in the CD271<sup>+</sup> cells compared to CD73<sup>+</sup> cells (Arufe *et al.*, 2010). CD105, also known as endoglin, is a TGF- $\beta$ 1 and TGF- $\beta$ 3 co-receptor and has been shown to modulate TGF- $\beta$  signalling in endothelial cells (Dijke *et al.*, 2008) and chondrocytes (Parker *et al.*, 2003). Since TGF- $\beta$  is one of the most important factors for the induction of chondrogenesis in MSCs, CD105 co-expression linked to TGF- $\beta$  signalling may induce greater chondrogenesis in CD271<sup>+</sup>MSCs (Jiang *et al.*, 2010).

With regards to GAG secretion, greater levels of GAGs were secreted by both PA and CD271<sup>+</sup>MSC seeded scaffolds under +CM conditions only. In this set of experiments an increase in GAG synthesis of PA MSCs was observed without much evidence of ECM deposition within the scaffold. The higher levels of GAGs released into the media allied to the toluidine blue staining of the ECM may be indicative of the inferior chondrogenesis of PA MSCs compared to CD271<sup>+</sup> MSCs. This may be associated with the low levels of collagen type II deposition by the PA MSCs, as proteoglycans interact with the fibrillar collagens (as

reviewed by Roughley 2006). In the absence of collagen type II in the ECM deposited by PA MSCs, greater levels of GAGs may be released into the medium. In contrast, CD271<sup>+</sup>MSCs deposit ECM that contained more collagen type II. Therefore, in addition to retaining the GAGs within the ECM, GAG secretion is also observed in the media.

Overall, limited or poor chondrogenesis was observed in these cell-seeded scaffolds as indicated by a sparse and irregular deposition of ECM. The limited chondrogenesis observed in these set of experiments maybe influenced by the age of the donors. The samples used were isolated from patients in the age range of 53 to 69 years old. BM MSCs harvested from patients with advance stage OA with an age range of 59-82 years have a reduced chondrogenic activity (Murphy *et al.*, 2002). A recent study also investigated the age-related effect of AT MSCs on their expansion and differentiation and reported that MSCs from older patients (over 46 years of age) had significantly reduced expressions of aggrecan and collagen type II compared to young donors of under 30 years of age (Choudhery *et al.*, 2014). It was also reported that the frequency of CD271<sup>+</sup> cells in AT decreases with age, however the osteogenic and adipogenic differentiation potential of these cells was not affected by age (Duran *et al.*, 2013).

The results reported in this chapter indicate that CD271<sup>+</sup>MSCs may have a greater chondrogenic potential than that of PA MSCs. Therefore, selectively isolating CD271<sup>+</sup> cells from the heterogeneous SVF into a purified population may increase their capacity towards chondrogenic differentiation. In the case of PA MSCs, which are a more heterogeneous population that includes those cells expressing CD271 during early cultures, the chondrogenic capacity of these CD271<sup>+</sup> cells within the PA MSC population may be limited due to the presence of other subpopulations of cells and because the expression of CD271 is lost during culture expansion (Jones *et al.*, 2002). The results presented within this chapter are limited due to *in vitro* analysis of chondrogenesis of cell-seeded scaffolds. Further work would require testing these cell-scaffolds combinations *in vivo* to adequately examine the potential of selectively isolated cells for cartilage repair.

**Chapter 5: An *in vivo* investigation of the chondrogenic differentiation potential of adipose tissue derived CD271 selected MSCs versus standard plastic adherent MSCs in clinically used scaffolds**

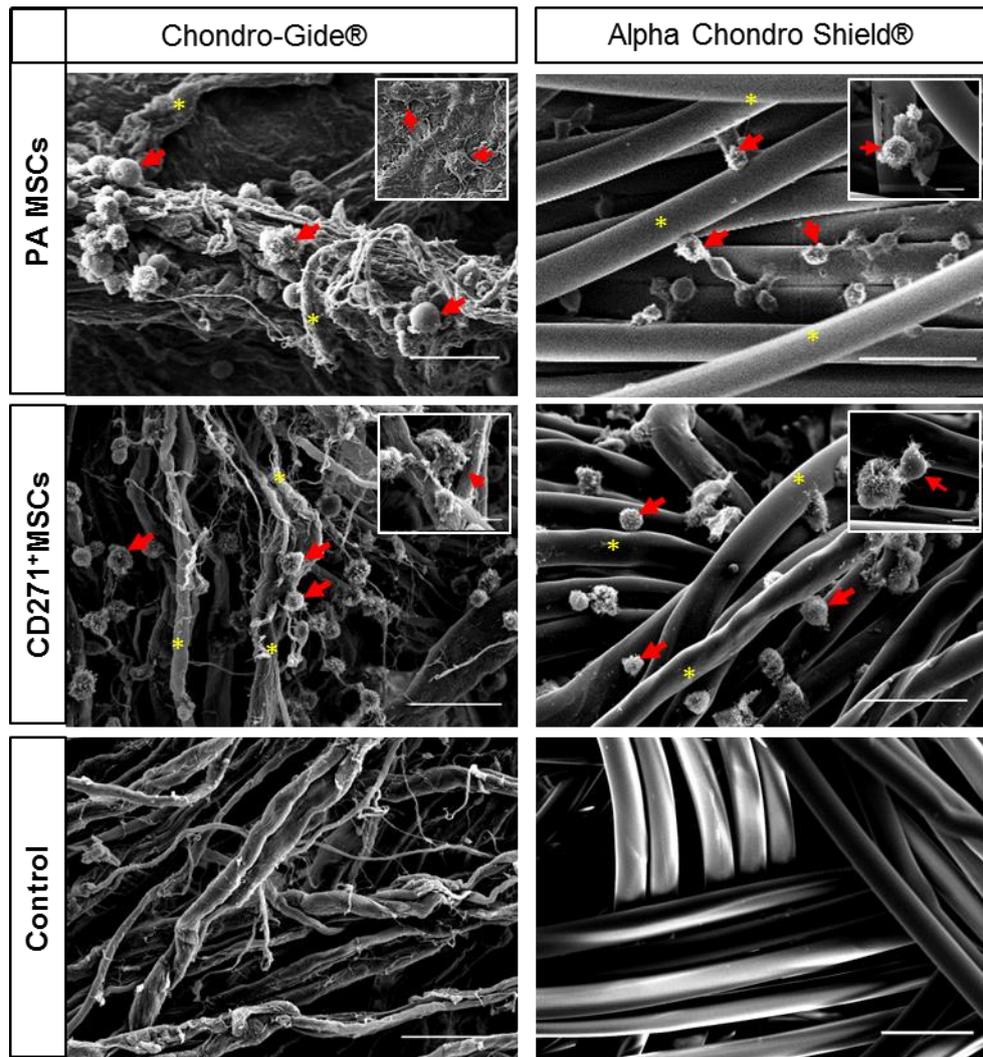
## 5.1 Background and aims

Studies have shown that selectively isolating MSCs from the heterogeneous population of cells from BM, AT and synovium can enrich for cells with improved differentiation potential towards mesenchymal lineages compared to the unsorted population (Arufe *et al.*, 2010; Kuçi *et al.*, 2010; Rada *et al.*, 2011; Álvarez-Viejo 2015; Cuthbert *et al.*, 2015). In the previous chapter, it was shown that selectively isolated CD271<sup>+</sup>MSCs from the SVF of AT deposited an ECM that was positive for collagen type II compared to PA MSCs in pellet cultures.

Similar findings of CD271<sup>+</sup> MSCs having a higher chondrogenic potential than PA MSCs have been reported *in vitro* (Quirici *et al.*, 2010; Arufe *et al.*, 2010); however, only one study has reported that CD271 MSCs have a better chondrogenic potential *in vivo* (Mifune *et al.*, 2012). Mifune *et al.*, reported that CD271 selected MSCs from BM are a superior cell source for cartilage repair compared to PAMSCs in a rat model of a full-thickness chondral defect (Mifune *et al.*,2012). No studies have been published to date, regarding the chondrogenic potential of adipose derived CD271<sup>+</sup> MSCs versus PA MSCs *in vivo*. Therefore, and on the basis of the results shown in Chapter 4, the rationale for the experiments reported in this chapter was to test whether CD271<sup>+</sup> MSCs from AT may also have a greater potential for repairing cartilage compared to PA MSCs from AT *in vivo*. This involved creating osteochondral defects in athymic female rats and transplanting the defects with human AT derived CD271<sup>+</sup>MSCs or PA MSCs in Chondro-Gide® and Alpha Chondro Shield®. Athymic nude rats are a common model for xenograft research as they lack functionally mature T cells and therefore are deficient in initiating T cell-mediated immune responses (Rolstad 2001). Transplantations of the cell scaffolds alone were also performed to assess tissue repair and scaffold biocompatibility in the absence of MSCs.

## **5.2 Incorporation of PA MSCs and CD271<sup>+</sup>MSCs within Chondro-Gide® and Alpha Chondro Shield® prior to transplantation**

SEM demonstrated that PA MSCs and CD271<sup>+</sup>MSCs had incorporated within the two scaffolds within 30 minutes of seeding. This time point was used to ensure cell adhesion prior to implantation of the cell-seeded scaffolds into the defects created simultaneously in the athymic rats. There was no difference between the prevalence of PA MSCs and CD271 MSCs within the two scaffolds. Both cell types showed firm attachment to the fibres of collagen in Chondro-Gide® and the fibres of polyglycolic acid in Alpha Chondro Shield® 30 minutes after incubation. There was evidence of penetration of cells into the inner parts of both the scaffolds. Morphologically, the cells still appeared round with more cells showing flat adherent morphology in Chondro-Gide® than in Alpha Chondro Shield®. Scaffolds without cells were used as controls (Figure 5.1).



**Figure 5.1. The morphology of PA MSCs and CD271<sup>+</sup> MSCs in Chondro-Gide® and Alpha Chondro Shield® after 30 minutes of incubation prior to transplantation.**

Representative SEM images are shown of PA MSCs and CD271<sup>+</sup> MSCs seeded scaffolds. PA MSCs and CD271<sup>+</sup> MSCs (red arrowed) were seen to be attached to the fibres of collagen (Chondro-Gide®) and PGA (Alpha Chondro Shield®). Asterisks display the fibres of the scaffolds. Insets show high magnification images of flattened cell morphology in Chondro-Gide® and round cell morphology in Alpha Chondro Shield®, scale bar = 10 µm. The scaffold alone controls are also shown. PA MSCs and CD271<sup>+</sup> MSCs, n=1 matched donor. Scale bar = 50 µm.

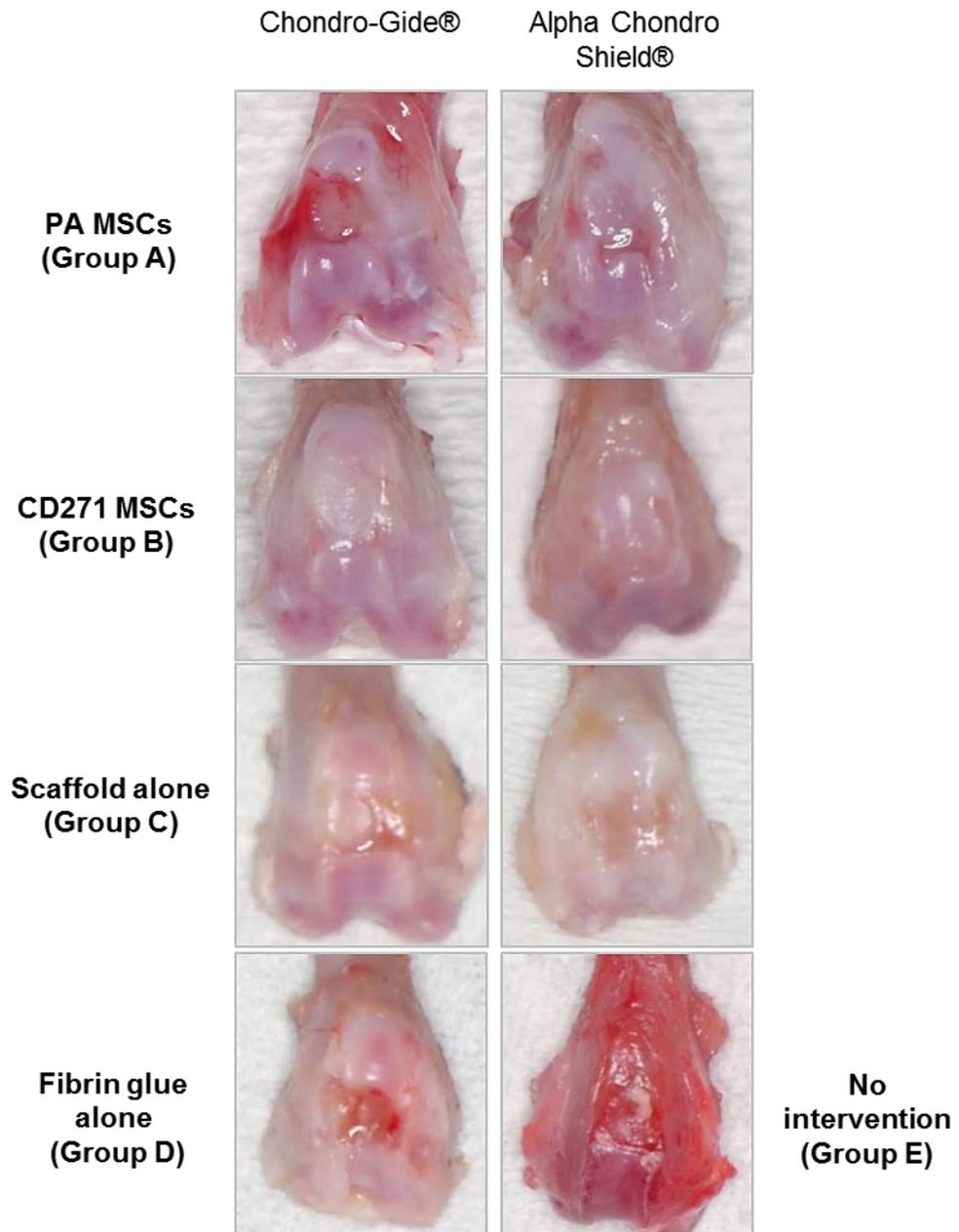
### **5.3 The effects of PA MSCs and CD271<sup>+</sup>MSCs seeded scaffolds on cartilage repair: gross morphology at 3 weeks post-transplantation**

Gross examination of the defects at 3 weeks post-transplantation revealed a well-integrated glossy white repair tissue in the defects transplanted with CD271<sup>+</sup>MSCs (group B) seeded within both Chondro-Gide® and Alpha Chondro Shield®. In the defects transplanted with PA MSCs (group A) with either Chondro-Gide® or Alpha Chondro Shield®, a depressed repair tissue with distinct defect edges was observed. Moreover, the border area of the defect was clearly distinguishable and depressions were obvious in the defects of the control groups of scaffolds alone (group C), fibrin glue alone (group D) and the no intervention control (group E) (Figure 5.2).

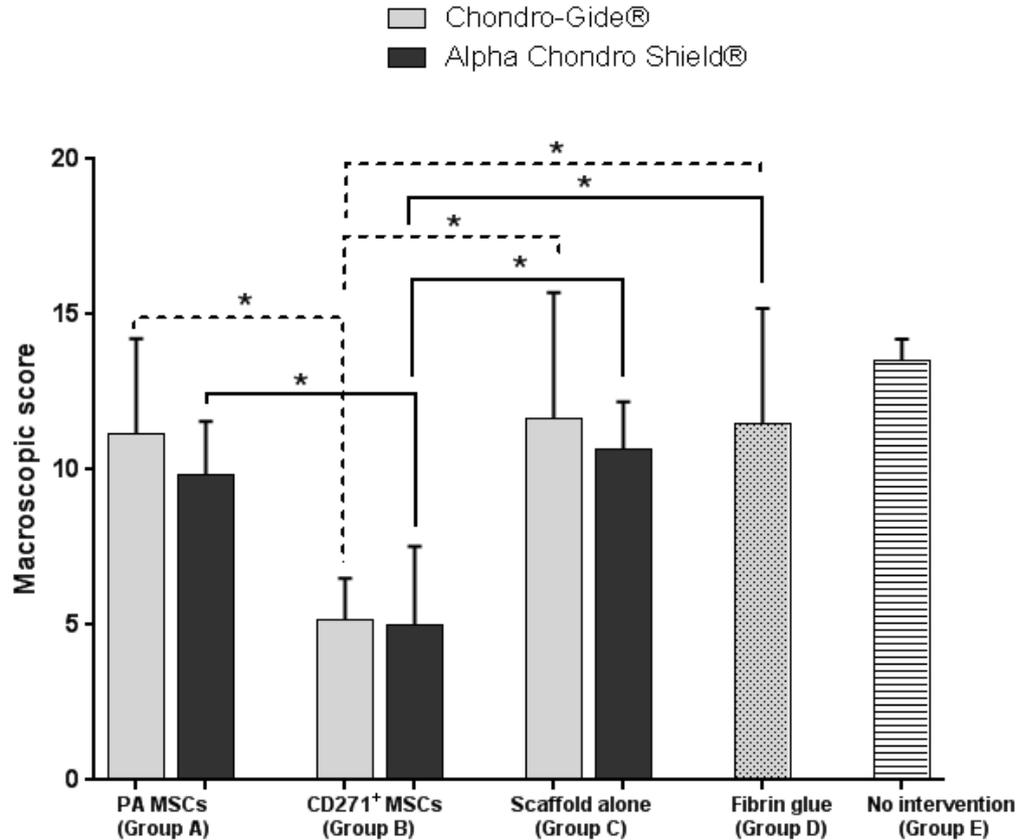
The scoring system used to macroscopically assess cartilage repair considers five main parameters; (i) the colour of the repair tissue; (ii) the presence of blood vessels in the repair tissue; (iii) the surface of the repair tissue; (iv) filling of the defect; (v) the degeneration of adjacent host cartilage. An overall score of 0 represents the best outcome indicative of complete repair and an overall score of 20 represents the worst outcome indicative of incomplete repair. As per this macroscopic scoring system, defects transplanted with CD271<sup>+</sup>MSCs (group B) had a significantly better repair compared to the defects in group A, C and D (p value < 0.05; Dunn's multiple comparison test). Defects in group B were scored at  $5.2 \pm 1.3$  with Chondro-Gide® and  $5.0 \pm 2.5$  with Alpha Chondro Shield®, whereas, defects transplanted with PA MSCs (group A) were scored at  $11.2 \pm 3.1$  with Chondro-Gide® and  $9.8 \pm 1.7$  with Alpha Chondro Shield®. Defects transplanted with scaffolds alone (group C) were scored at  $11.6 \pm 4.0$  with Chondro-Gide® and  $10.6 \pm 1.5$  with Alpha Chondro Shield® alone. The fibrin glue (group D) and the no intervention (group E) control groups were scored at  $11.5 \pm 3.7$  and  $13.5 \pm 0.7$ , respectively. All data has been presented as means  $\pm$  SDs of n=6 for defects transplanted with PA MSCs (group A) and CD271<sup>+</sup>MSCs (group B), n=3 for defects transplanted with scaffold alone (group C), n=4 for defects that were treated with fibrin glue

only (group D) and n=2 for defects that had no intervention (group E). Group E was excluded from the statistical testing due to a small sample size (see also Figure. 5.3).

For each of the parameters examined for the macroscopic assessment of the defects, an individual score of 4 represents the worst repair and an individual score of 0 would represent the best repair (Table 5.1). The repair tissue in the CD271<sup>+</sup>MSC transplanted animals (group B) was predominantly white coloured, whereas the repair tissue present in the defects in the other groups were predominantly translucent coloured. Less than 25% of the repair tissue in group B showed the presence of blood vessels, whereas 25 -75% of the repair tissue in all of the other groups contained blood vessels. A smooth surface of the repair tissue was only seen in group B and with either Chondro-Gide® or Alpha Chondro Shield®. The defects transplanted with PA MSCs (group A) or scaffold alone (group C) had fibrillated surface of repair tissue, whereas those transplanted with fibrin glue only (group D) or had no intervention (group E) showed an incomplete new repair tissue that had not fully resurfaced the defect. The defect fill was in line with the adjacent cartilage in the defects transplanted with CD271<sup>+</sup>MSCs (group B) with either of the scaffolds. In Alpha Chondro Shield® alone (group C) over 50% of the defect depth was filled. In the defects transplanted with PA MSCs with either scaffolds (group A), or Chondro-Gide® alone (group C) or the fibrin glue alone control group (group D) and the no intervention control (group E), less than 50% of the defect was filled with a repair tissue. Few fibrillations or cracks were seen at the integration zone of defects with the host tissue for group B with either scaffold, whereas the defects of group A, C and D showed degenerative changes at these integration sites with the extension of the defect into the adjacent host cartilage.



**Figure 5.2. Gross morphology of the defects at 3 weeks post-transplantation.** Representative images are shown of the gross morphology of the defects in groups A, B, C, D and E. Gross examination of defects at 3 weeks revealed glossy white and well-integrated repair tissue in the animals that received CD271<sup>+</sup> MSCs (Group B) and not in the animals that received PA MSCs (Group A), or scaffold alone (Group C), or fibrin glue alone (group D), or had no intervention (Group E).



**Figure 5.3. Macroscopic scores for cartilage repair at 3 weeks post-transplantation.**

The macroscopic mean scores of the defects transplanted with CD271<sup>+</sup> MSCs (group B) were significantly better than the defects transplanted with PA MSCs (group A) with Chondro-Gide® ( $p$  value = 0.015), and Alpha Chondro Shield® ( $p$  value = 0.043) as well as the defects that received scaffold alone (group C) ( $p$  value = 0.040 for Chondro-Gide® and  $p$  value = 0.039 for Alpha Chondro Shield®). The defects transplanted with CD271<sup>+</sup> MSCs (group B) also showed significantly lower scores than defect treated with fibrin glue alone (group D) ( $p$  value = 0.022 for Chondro-Gide® and  $p$  value = 0.032 for Alpha Chondro Shield®). Data are presented as means  $\pm$  SD of  $n=6$  for group A and B,  $n=3$  for group C,  $n=4$  for group D and  $n=2$  for group E. Group E was excluded from statistical analysis due to a small sample size.  $p$  value  $<0.05$ ; Dunn's multiple comparison test. Dotted lines indicate significance for Chondro-Gide® and intact lines indicate significance for Alpha Chondro Shield®.

	PA MSCs (Group A)		CD271 <sup>+</sup> MSCs (Group B)		Scaffold alone (Group C)		(Group D)	(Group E)
	Chondro- Gide®	Alpha Chondro Shield®	Chondro- Gide®	Alpha Chondro Shield®	Chondro- Gide®	Alpha Chondro Shield®	Fibrin Glue alone	No intervention
<b>Colour of the repair tissue</b>	<b>2.8 ± 0.4</b>	2.3 ± 0.5	<b>1.3 ± 0.5**</b>	1.3 ± 0.8	2.7 ± 0.6	2.3 ± 0.6	2.3 ± 0.5	3.0 ± 0.0
<b>Blood vessels in the repair tissue</b>	2.2 ± 1.2	1.7 ± 0.8	1.2 ± 0.8	<b>0.8 ± 0.8*</b>	2.7 ± 1.2	2.7 ± 0.6	<b>2.8 ± 1.0</b>	2.5 ± 0.7
<b>Surface of the repair tissue</b>	2.3 ± 1.0	<b>2.2 ± 0.4</b>	1.0 ± 0.0	<b>1.2 ± 0.4*</b>	2.3 ± 1.2	2.0 ± 0.0	<b>2.5 ± 0.6</b>	3.0 ± 0.0
<b>Defect fill</b>	1.5 ± 0.9	1.7 ± 0.5	<b>0.5 ± 0.5*</b>	0.5 ± 0.5	<b>2.3 ± 0.6</b>	1.3 ± 0.6	2.0 ± 1.2	2.0 ± 0.0
<b>Degeneration of adjacent cartilage</b>	2.3 ± 0.8	2.0 ± 0.6	1.2 ± 0.4	1.3 ± 0.5	1.7 ± 1.2	2.3 ± 0.6	2.0 ± 0.8	3.0 ± 0.0

**Table 5.1. Macroscopic scores for the individual parameters of cartilage repair at 3 weeks post-transplantation.** The colour of the repair tissue in the defects transplanted with CD271<sup>+</sup> MSCs (group B) had a significantly lower score than that of the defects transplanted with PA MSCs (group A) using Chondro-Gide® (*p* value = 0.006). Blood vessel scores were significantly lower in the macroscopic repair tissue of the defects of group B with Alpha Chondro Shield® compared to the defects treated with fibrin glue alone (group D) (*p* value = 0.041). The surface of the repair tissue was also significantly better in these defects of group B as compared to the defects of group A with Alpha Chondro Shield® only (*p* value = 0.039) and defects of group E (*p* value = 0.009). Data are presented as means ± SD (n=6 for group A and B, n=3 for group C, n=4 for group D and n=2 for group E). Group E was excluded from statistical analysis due to a small sample size. *p* value <0.05; Dunn's multiple comparison test. Bold text indicates statistical significance with asterisks displayed on the lower scores.

## **5.4 The effects of PA MSCs and CD271<sup>+</sup>MSCs seeded scaffolds on cartilage repair: overall histological outcomes at 3 weeks post-transplantation**

### 5.4.1 H&E staining

A greater extent of defect fill was observed in the defects transplanted with CD271 MSCs (group B) with Chondro-Gide® or Alpha Chondro Shield® compared to defects transplanted with PA MSCs (group A) (Figure 5.4.1). A firm attachment of the scaffold to the underlying trabecular bone was seen in all cases when transplanted using Alpha Chondro Shield®. However, Chondro-Gide® was present in two out of the three animals that received CD271<sup>+</sup>MSCs (group B) and one out of the three animals that received PA MSCs (group A). For gross morphology scoring, Chondro-Gide® was seen in all the animals; however there was no Chondro-Gide® present in several animals following processing of the rat knees for histology. Therefore, histological scoring of defect fill included any repair tissue present within the defect. Defects transplanted with scaffold alone (group C) were covered with the scaffolds and were infiltrated with a cell-rich fibrous tissue in the case of Alpha Chondro Shield® and a development of a fibrous layer of tissue in between the scaffold-bone interphase in the case of Chondro-Gide®. The development of a cell-rich fibrous layer overlying the trabecular bone was seen in the defects treated with fibrin glue only (group D) and the defects that had no intervention (group E) (Figure 5.4).

### 5.4.2 Toluidine blue staining

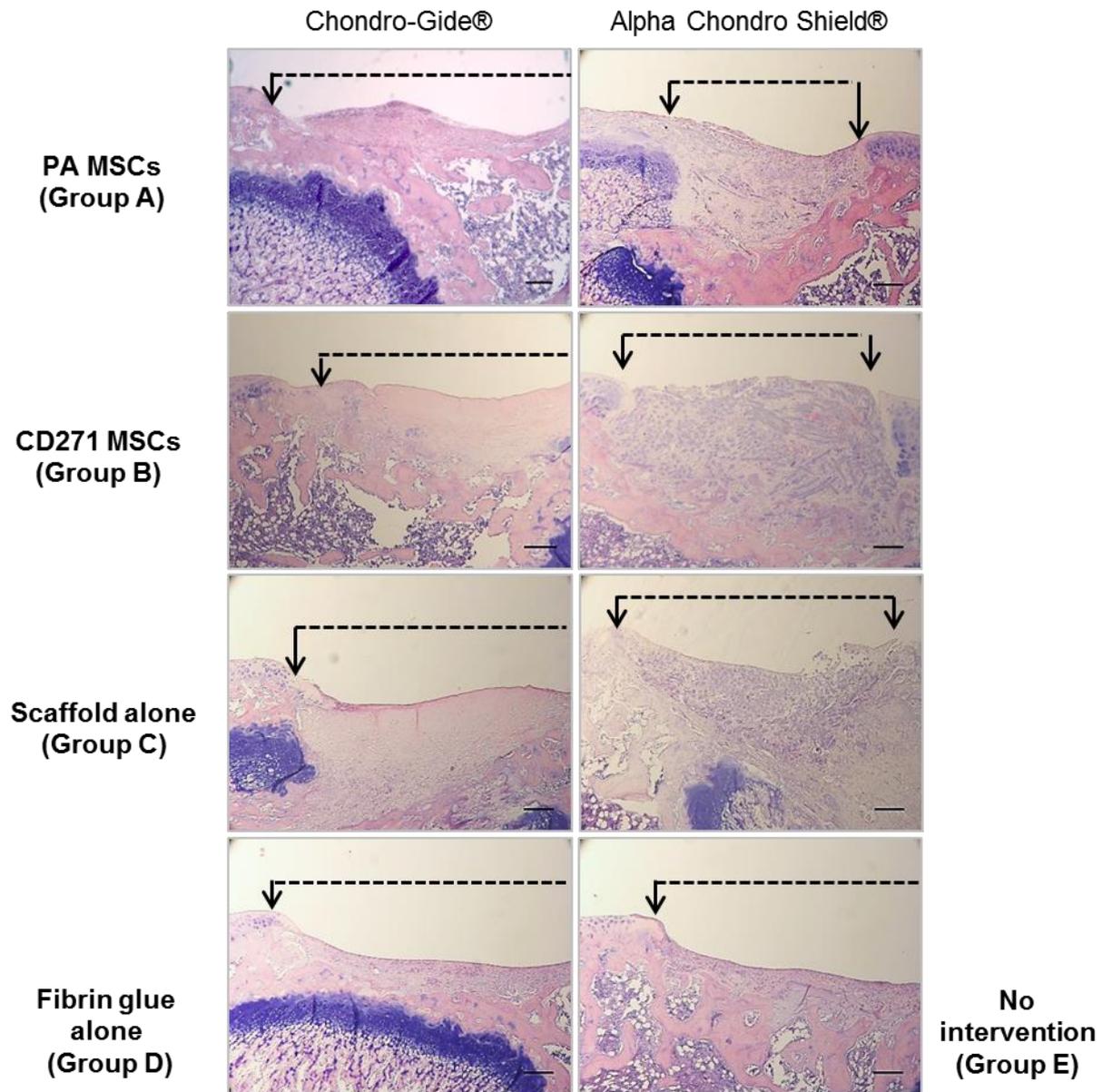
The repair tissue within the defects that received PA MSCs (group A) and CD271<sup>+</sup>MSCs (group B) when transplanted with Alpha Chondro Shield® and the repair tissue at the scaffold-bone interphase in the case of Chondro-Gide® showed localised toluidine blue metachromasia. The cell-rich fibrous tissue in the defects transplanted with scaffolds alone (group C) or fibrin glue only (group D) or the no intervention control (group E) also showed no metachromasia with toluidine blue dye (Figure 5.5).

#### 5.4.3 Collagen type II immunostaining

Localised collagen type II immunostaining was seen at the scaffold-bone interphase in the CD271<sup>+</sup> MSC group (group B) transplanted with Chondro-Gide® and within the repair tissue of defects transplanted with CD271<sup>+</sup>MSCs and Alpha Chondro Shield®. No collagen type II production was seen in the repair tissue of the defects of all the other groups (Figure 5.4.3).

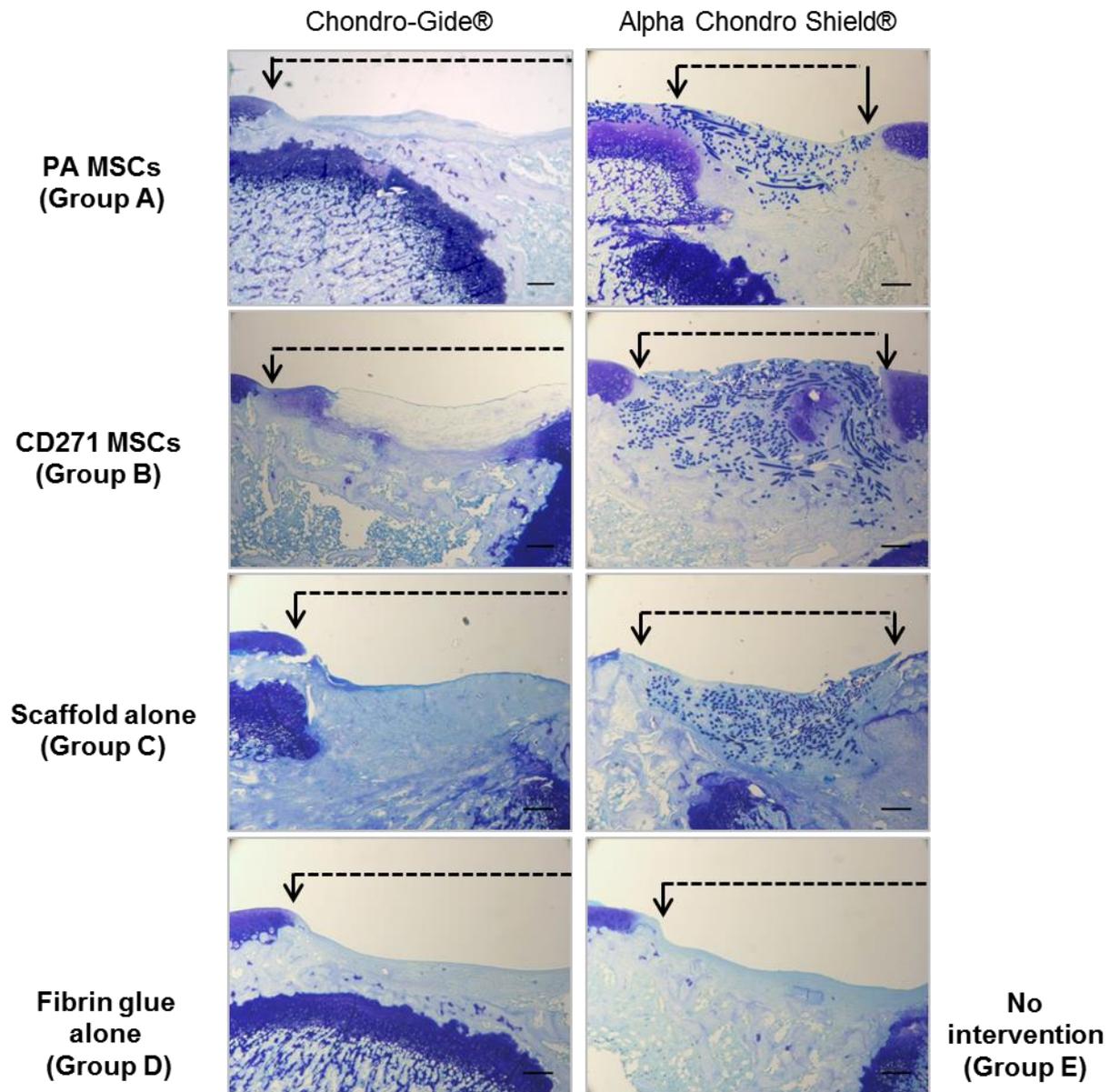
#### 5.4.4 Overall histological scores

The extent of repair was evaluated in each group using a modified Wakitani's score (Wakitani *et al.*, 1994). As described in the methods section of this thesis, the scoring system is composed of eight parameters with a score range from 0 to 20 points, where a lower score represented a superior repair tissue and a higher score represented a poor repair tissue. The mean score of the repair tissues for CD271<sup>+</sup>MSC transplants in Chondro-Gide® or Alpha Chondro Shield® were lower than the scores for PA MSCs transplants in the respective scaffolds (CD271<sup>+</sup>MSCs: Chondro-Gide® =  $9.3 \pm 1.5$ , Alpha Chondro Shield® =  $9.2 \pm 1.0$ ; PA MSCs: Chondro-Gide® =  $12.3 \pm 1.6$ , Alpha Chondro Shield® =  $12.3 \pm 0.8$ ). Defects treated with scaffolds alone (group C) were scored at  $12.5 \pm 0.7$  with Chondro-Gide® and  $15.5 \pm 0.7$  with Alpha Chondro Shield®. Defects in the control groups treated with either fibrin glue alone (group D) or the control groups that had no intervention (group E) were scored at  $14.5 \pm 0.7$  and  $16.5$ , respectively. The histological scores were consistent with the macroscopic scores where defects transplanted with CD271<sup>+</sup>MSCs in combination with either cell-carrier obtained the lowest overall scores. All data has been presented as means  $\pm$  SDs of n=3 for PA MSCs group (group A) and CD271<sup>+</sup>MSCs group (group B) and n=2 for each of the control groups i.e. scaffold alone group (group C) and fibrin glue only group (group D). Data for no intervention control group (group E) has been presented as the overall score of n=1 animal. There were no statistical differences between any of the groups (See also Figure 5.7).



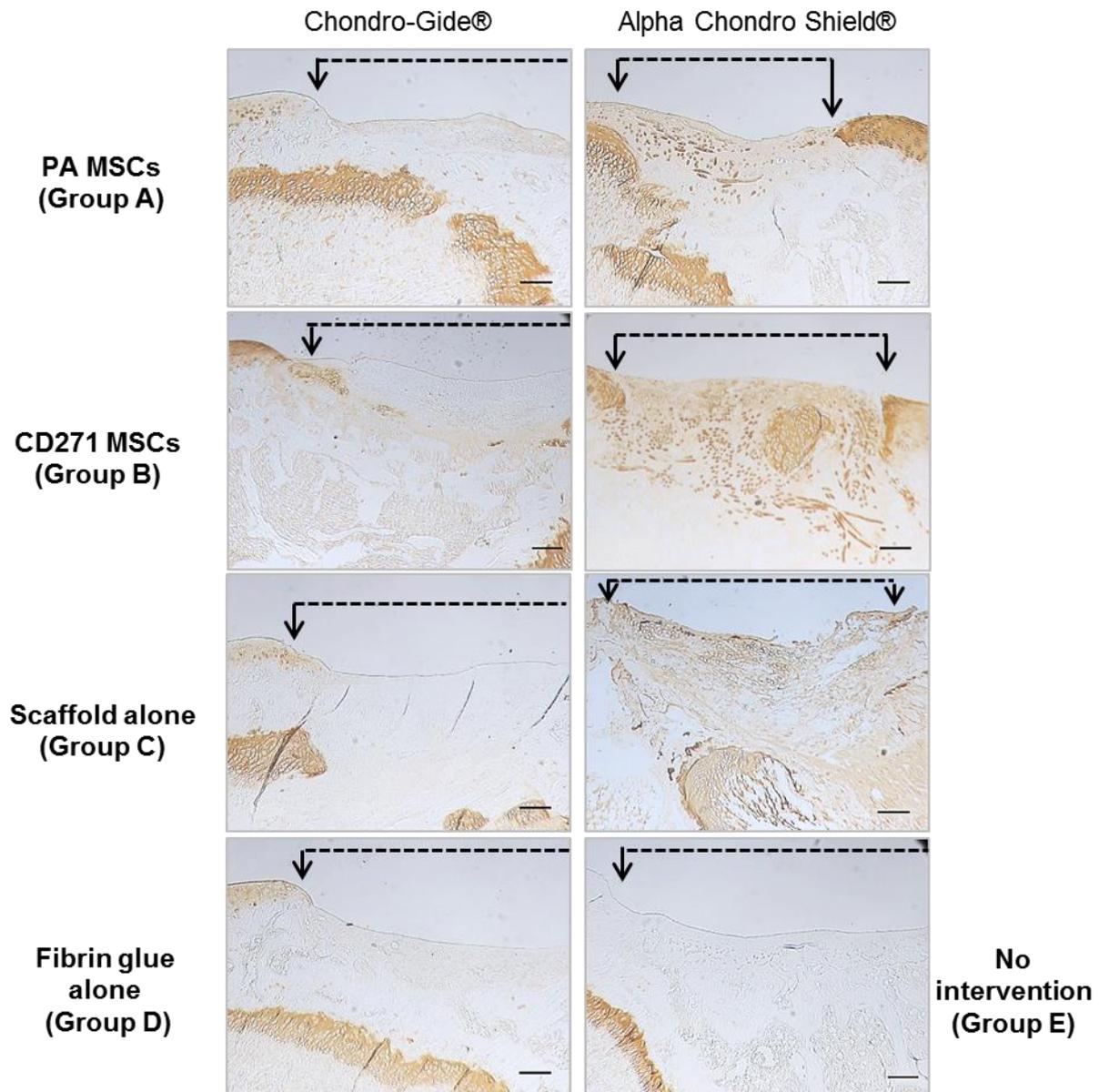
**Figure 5.4. Histology of rat knee tissue sections at 3 weeks post-transplantation.**

Representative images are shown of the H&E stained tissue section of the osteochondral defects. A greater extent of defect fill was observed in defects transplanted with CD271<sup>+</sup> MSCs (group B) with both scaffolds as compared to the defects in group A, C, D or E. Original magnification x4. All scale bars = 200 μm. Dotted line with arrows indicates the margins of the defect.

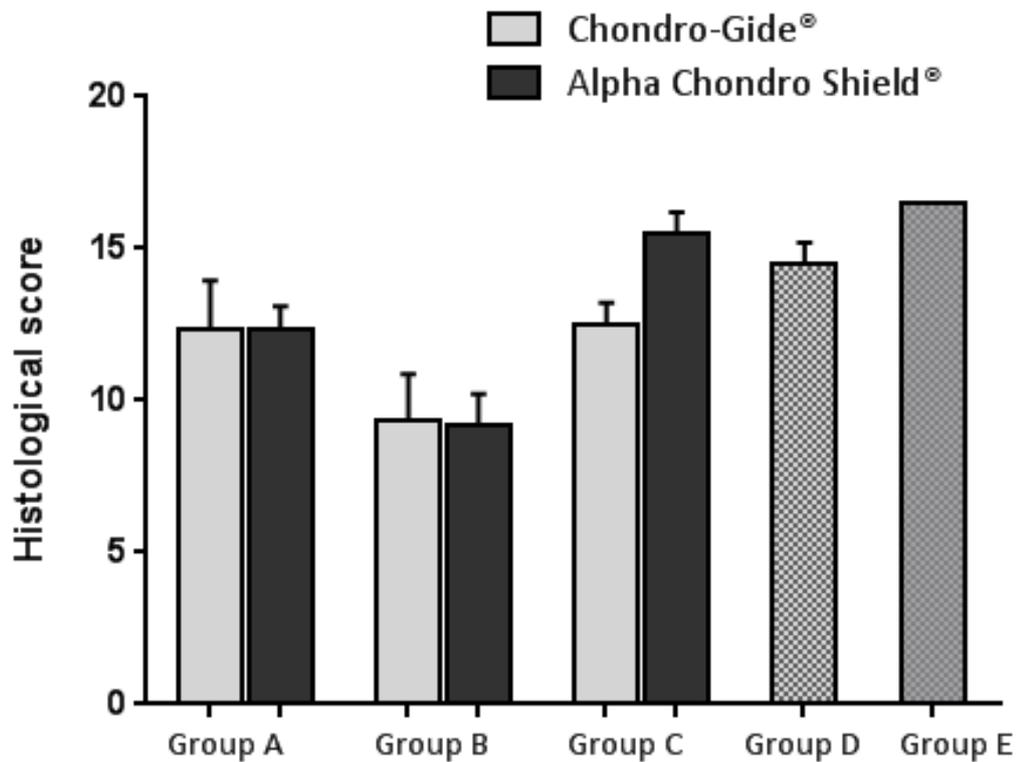


**Figure 5.5. GAG deposition in the defects at 3 weeks post transplantation.**

Representative images are shown of toluidine blue stained tissue sections of the osteochondral defects. GAG deposition was seen in PA MSCs (group A) and CD271<sup>+</sup> MSCs (group B) and no obvious metachromatic staining indicative of GAG content was seen in the three control groups (group C, D and E). Original magnification x4. All scale bars = 200  $\mu$ m. Dotted line with arrows indicates the margins of the defect.



**Figure 5.6. Collagen type II immunolocalisation in the defects at 3 weeks post-transplantation.** Localised collagen type II immunoreactivity was seen in the defects treated with CD271<sup>+</sup> MSCs (group B) in the fibrous layer between Chondro-Gide® and the underlying bone, and in the repair tissue around the fibres in Alpha Chondro Shield®. No type II collagen was seen within the repair tissue of the defects of groups A,C,D and E. Original magnification x4. All scale bars = 200 µm. Dotted line with arrows indicates the margins of the defect.



**Figure 5.7. Histological scoring of the repair tissue at 3 week post-transplantation.**

The defect transplanted with CD271<sup>+</sup>MSCs (group B) using Alpha Chondro Shield® as the cell-carrier achieved a lower overall histological score; however, no significant differences were observed in the histological scores between the cell-treated groups. Data are presented as means  $\pm$  SD n=3 for group A and B, n=2 for group C and D, n=1 for group E). The Mann-Whitney U test was used to compare histological scores of group A versus group B for each cell-carrier. Group C, D and E were excluded from statistical analysis due to the small sample size.

## **5.5 The effects of PA MSCs and CD271<sup>+</sup>MSCs seeded scaffolds on cartilage repair: detailed histological outcomes at 3 weeks post-transplantation**

Differences observed in the various histological parameters that were assessed have been described below and are summarised in Table 5.2:

### 5.5.1 Cell morphology

Cell morphology was graded from 0 for round or oval shaped cells (i.e. chondrocytic in shape) to 4 points when no round or oval shaped cells were present within the repair tissue. The repair tissue of the defects transplanted with PA MSCs (group A) and CD271<sup>+</sup>MSCs (group B) with Alpha Chondro Shield® only showed the presence of both round-shaped cells and elongated fibroblast-like cells in the repair tissue indicative of fibro-cartilaginous repair tissue; whereas those with Chondro-Gide® showed elongated spindle shaped cells indicative of mostly non-cartilaginous repair tissue. Cell infiltration within Chondro-Gide® was limited, whereas Alpha Chondro Shield® allowed greater cell infiltration. Cell morphology in the repair tissue of the defects transplanted with scaffolds alone (group C) with both Chondro-Gide® and Alpha Chondro Shield® and of the defects in fibrin glue only group (group D) and the no intervention control group (group E) were also indicative of mostly non-cartilaginous repair tissue (Figure 5.8).

### 5.5.2 Matrix staining

The extent of metachromatic staining by toluidine blue in the repair tissue was compared with the adjacent normal cartilage. Toluidine blue metachromasia was reduced compared to the adjacent cartilage in the defects of animals transplanted with PA MSCs (group A) and CD271<sup>+</sup>MSCs (group B) and with both scaffolds, but it was more markedly reduced in the defects of the control groups, scaffold alone (group C), fibrin glue alone (group D) and no intervention control (group E). None of these groups showed metachromatic staining equivalent to the surrounding host cartilage.

### 5.5.3 Surface regularity

Surface regularity referred to the defect surface that appeared smooth compared to the whole defect surface. Defects transplanted with either PA MSCs (group A) or CD271<sup>+</sup>MSCs (group B) using Alpha Chondro Shield® showed a moderately smooth surface of the repair tissue compared to an irregular surface of the repair tissue seen in the control groups of scaffold alone (group C) and no interventional group (group E). Defects in PA MSC transplanted animals (group A) and CD271<sup>+</sup>MSC transplanted animals (group B) with Chondro-Gide® also showed an irregular surface of the repair tissue. In contrast, defects in the fibrin glue control group (group D) showed a smooth surface of repair tissue.

### 5.5.4 Thickness

Cartilage thickness was scored from 0 (over two-third of the defect filled with a repair tissue) to 2 (less than 1/3 of the defect was filled with a repair tissue). Defect fill was greater in the defects transplanted with either PA MSCs (group A) or CD271<sup>+</sup>MSCs (group B) with Alpha Chondro Shield®, which showed over 2/3 repair of the defect depth compared with all the other groups. In the scaffold alone control group (group C), between 1/3-2/3 of the defects was filled with a repair tissue with either Chondro-Gide® or Alpha Chondro Shield®. Defects in the fibrin only group (group D) and the no intervention control group (group E) showed less than 1/3 of the defect was filled with a repair tissue. In defects where Chondro-Gide® was absent, the repair tissue within the defect was scored for thickness (Figure 5.9).

### 5.5.5 Integration of the repair tissue with the host adjacent cartilage

Integration of repair tissue with adjacent host tissue was scored from 0 (no gap between the repair tissue and host cartilage) to 2 (incomplete integration of the edges of the repair tissue). Both the PA (group A) and the CD271<sup>+</sup>MSCs (group B) treated groups and the scaffold alone control group (group C) showed a good integration of the scaffold with the surrounding host cartilage when transplanted using Alpha Chondro Shield®. Since Chondro-Gide® was either absent or delaminated within the defects, the score for the defects transplanted using Chondro-

Gide® was higher than those transplanted using Alpha Chondro Shield®. In defects where Chondro-Gide® was absent, the repair tissue within the defect was scored for thickness. Due to a poor defect fill, the defects treated with fibrin glue only (group D) or had no intervention (group E) scored the worst as the surface of the repair tissue was not seen to be integrating with the adjacent host cartilage.

#### 5.5.6 Vascularisation within the defect

The presence of blood vessels was graded from 0 to indicate the absence of blood vessels to 4 to indicate the presence of 30+ blood vessels within the repair tissue. Defects transplanted with either PA MSCs (group A) showed greater vascularisation within the defect compared to the defects transplanted with CD271<sup>+</sup>MSCs (group B) with either of the scaffolds. Defects treated with Alpha Chondro Shield® alone (group C), or with fibrin glue only (group D) or those that had no intervention (groups E) showed enhanced vascularisation within the repair tissue compared to the cell treated groups. The repair tissue of the defects transplanted with PA MSCs (group A) using Chondro-Gide® had less vascularisation compared those that were transplanted using Alpha Chondro Shield®. The blood vessels were observed in the fibrous tissue layer in between Chondro-Gide® and the underlying trabecular bone and no blood vessels were seen within Chondro-Gide® (Figure 5.10).

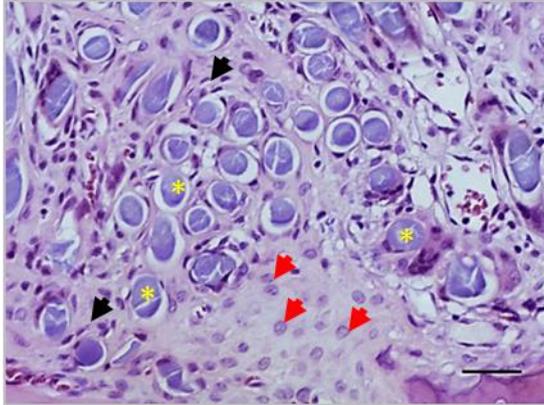
#### 5.5.7 Foreign body giant cells

Only the repair tissue of the defects transplanted with Alpha Chondro Shield® showed an inflammatory response where FBGCs were seen to be engulfing the fibres of the scaffold. In comparison, defects transplanted using Chondro-Gide® showed no FBGC reaction. Also, no FBGC reaction was observed in the repair tissue of the defects treated with fibrin glue alone (group D) or no intervention control (group E) (Figure 5.11).

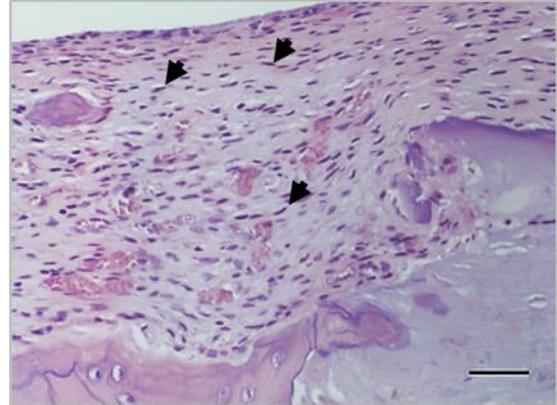
	PA MSCs (Group A)		CD271 <sup>+</sup> MSCs (Group B)		Scaffold alone (Group C)		(Group D)	(Group E)
	Chondro- Gide®	Alpha Chondro Shield®	Chondro- Gide®	Alpha Chondro Shield®	Chondro- Gide®	Alpha Chondro Shield®	Fibrin Glue alone	No intervention
<b>Cell morphology</b>	2.5 ± 0.0	2.0 ± 0.0	2.7 ± 0.3	2.0 ± 0.0	3.0 ± 0.0	3.0 ± 0.0	3.5 ± 0.7	4.0
<b>Matrix staining</b>	1.8 ± 0.3	1.5 ± 0.0	2.0 ± 0.0	1.7 ± 0.3	2.5 ± 0.5	2.0 ± 0.0	2.0 ± 0.0	2.5
<b>Surface regularity</b>	1.7 ± 0.8	1.3 ± 0.3	1.7 ± 0.8	1.5 ± 0.5	2.0 ± 0.0	2.0 ± 0.0	1.5 ± 0.7	2.5
<b>Thickness</b>	1.5 ± 0.5	1.0 ± 0.5	0.5 ± 0.9	0.0 ± 0.0	1.0 ± 0.0	1.0 ± 0.0	2.0 ± 0.0	2.0
<b>Integration</b>	1.8 ± 0.3	0.7 ± 0.3	1.5 ± 0.5	0.3 ± 0.3	0.5 ± 0.7	0.5 ± 0.7	2.0 ± 0.0	2.0
<b>Blood vessels</b>	3.0 ± 1.0	3.7 ± 0.6	1.0 ± 1.0	1.0 ± 1.0	2.0 ± 0.0	4.0 ± 0.0	4.0 ± 0.0	4.0
<b>Foreign body giant cells</b>	0.0 ± 0.0	2.2 ± 0.6	0.0 ± 0.0	2.3 ± 0.8	0.0 ± 0.0	2.5 ± 0.7	0.0 ± 0.0	0.0

**Table 5.2. Histological scores of the individual parameters at 3 weeks post-transplantation.** Defects of group B showed better overall defect fill as indicated by low scores of thickness and little overall vascularisation as indicated by a lower score for blood vessels. Data are presented as means ± SD (n=3 for group A and B, n=2 for group C and D and n=1 for group E). No significant differences were observed in the individual histological scores of the cell-treated groups. Groups C, D and E were excluded from statistical analysis due to a small sample size. The Mann Whitney U test was used to test for statistical significance.

**Group A**  
**(PAMSCs with Alpha Chondro Shield®)**  
**Mean score = 2.0 ± 0.0**



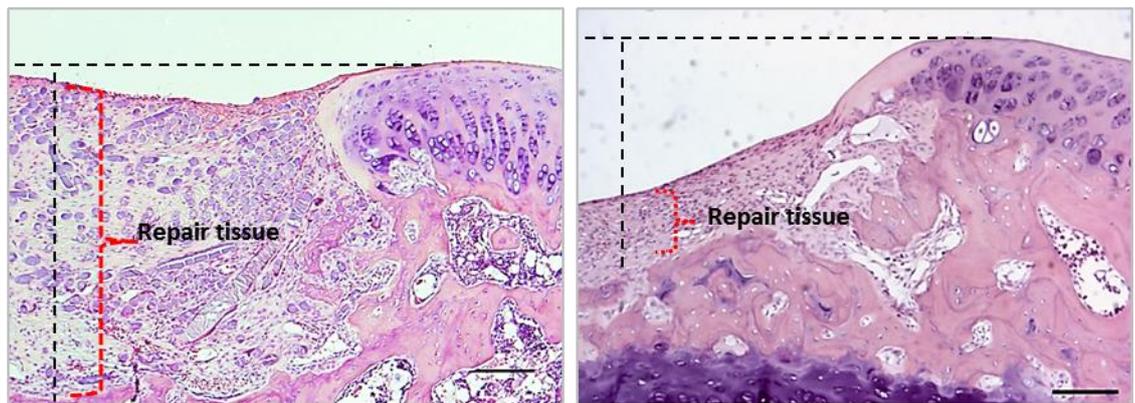
**Group E**  
**(Defect alone control)**  
**Overall score = 4.0**



**Figure 5.8. Representative H&E stained images are shown to demonstrate the difference in cell morphology of the repair tissue.** The image on the left demonstrates the cell morphology occasionally observed in defects treated with PA MSCs (group A) in combination with Alpha Chondro Shield®, i.e. showing mostly round or oval shaped cells in the repair tissue (red arrowed) with a few elongated cells (black arrowed); whereas defect of the no intervention control group (group E) showed mostly flat elongated cells in the repair tissue. Yellow asterisks represent the fibres of Alpha Chondro Shield®. Scores have been presented as means  $\pm$  SD for n=3 in group A and overall score of n=1 for group E. Original magnification x40. Scale bar = 25 $\mu$ m.

**Group B**  
**(CD271<sup>+</sup>MSCs with Alpha Chondro Shield®)**  
**Mean score = 0.0 ± 0.0**

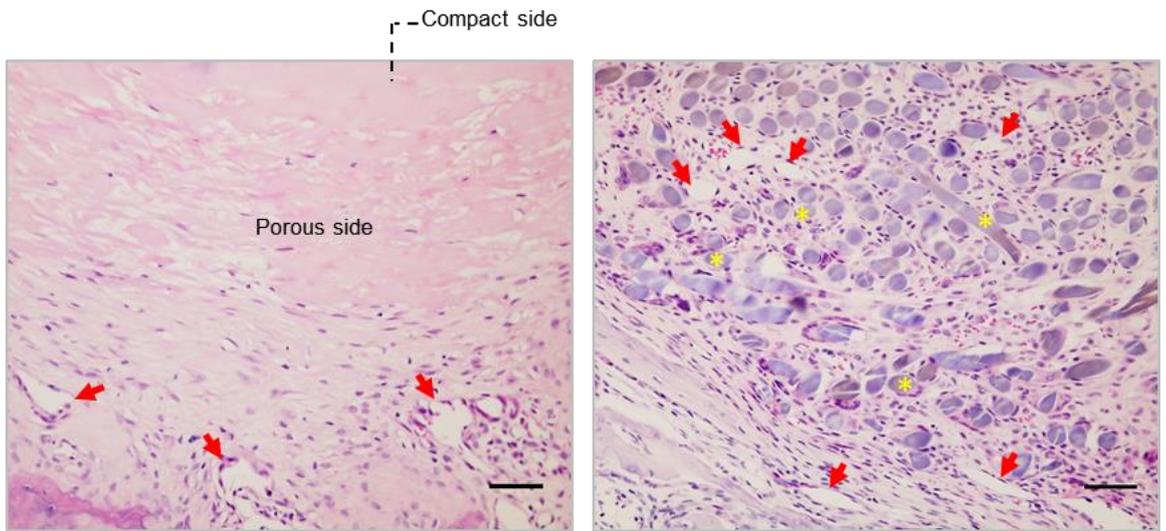
**Group D**  
**(Fibrin glue alone control)**  
**Mean score = 2.0 ± 0.0**



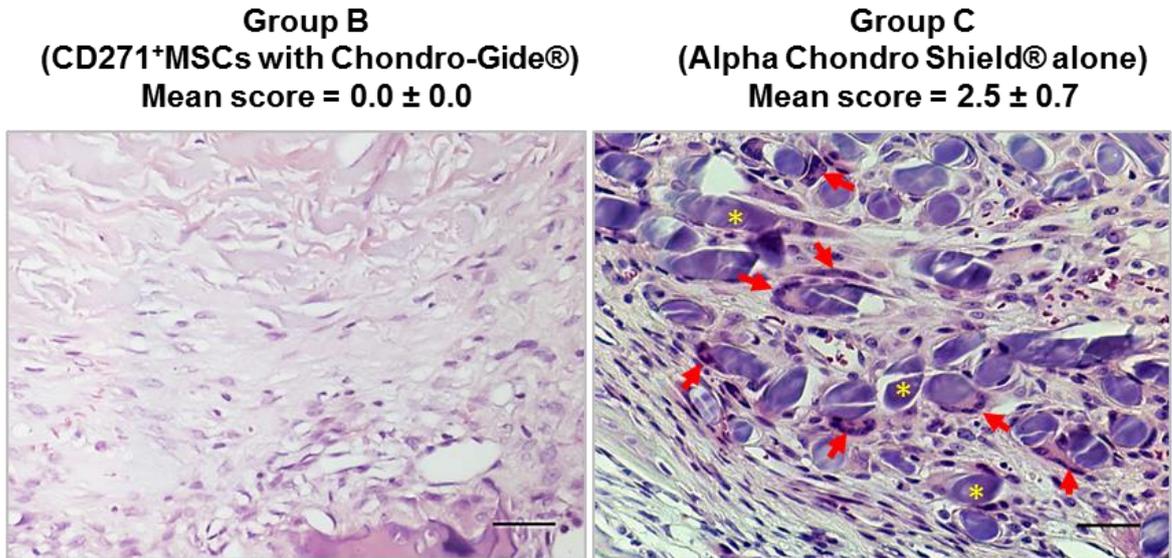
**Figure 5.9. Representative H&E stained images are shown to demonstrate the difference in the defect fill or thickness of the repair tissue.** The image on the left demonstrates a near-complete defect fill where over 2/3 of the defect depth was filled with a repair tissue in the case of CD271<sup>+</sup> MSCs with Alpha Chondro Shield® (group B). In contrast, the image on the right demonstrates an incomplete defect fill with less than 1/3 of the defect depth filled with a repair tissue in the case of the fibrin glue alone control group (group D). Black dotted lines indicates the depth of the defect and red dotted single brackets indicates the depth of the repair tissue. Scores have been presented as means ± SD of n=3 in group B and n=2 in group D. Original magnification x10. Scale bar = 100 µm.

**Group B**  
**(CD271<sup>+</sup>MSCs with Chondro-Gide®)**  
**Mean score = 1.0 ± 1.0**

**Group C**  
**(Alpha Chondro Shield® alone)**  
**Mean score = 4.0 ± 0.0**



**Figure 5.10. Representative H&E stained images are shown to demonstrate the differences in the extent of vascularisation of the repair tissue.** The image on the left demonstrates a low number of blood vessels (red arrowed) in the repair tissue of the defects transplanted with Chondro-Gide® (group B) compared to the number of blood vessels (red arrowed) in the defects treated with Alpha Chondro Shield® alone (group C) (right image). Blood vessels were observed in the fibrous tissue between the scaffold-bone interphase in the case of Chondro-Gide®, whereas blood vessels were present throughout the repair tissue in the case of Alpha Chondro Shield®. Yellow asterisks represent the fibres of Alpha Chondro Shield®. Scores have been presented as means ± SD of n=3 in group B and group C. Original magnification x20. Scale bar = 50 µm.

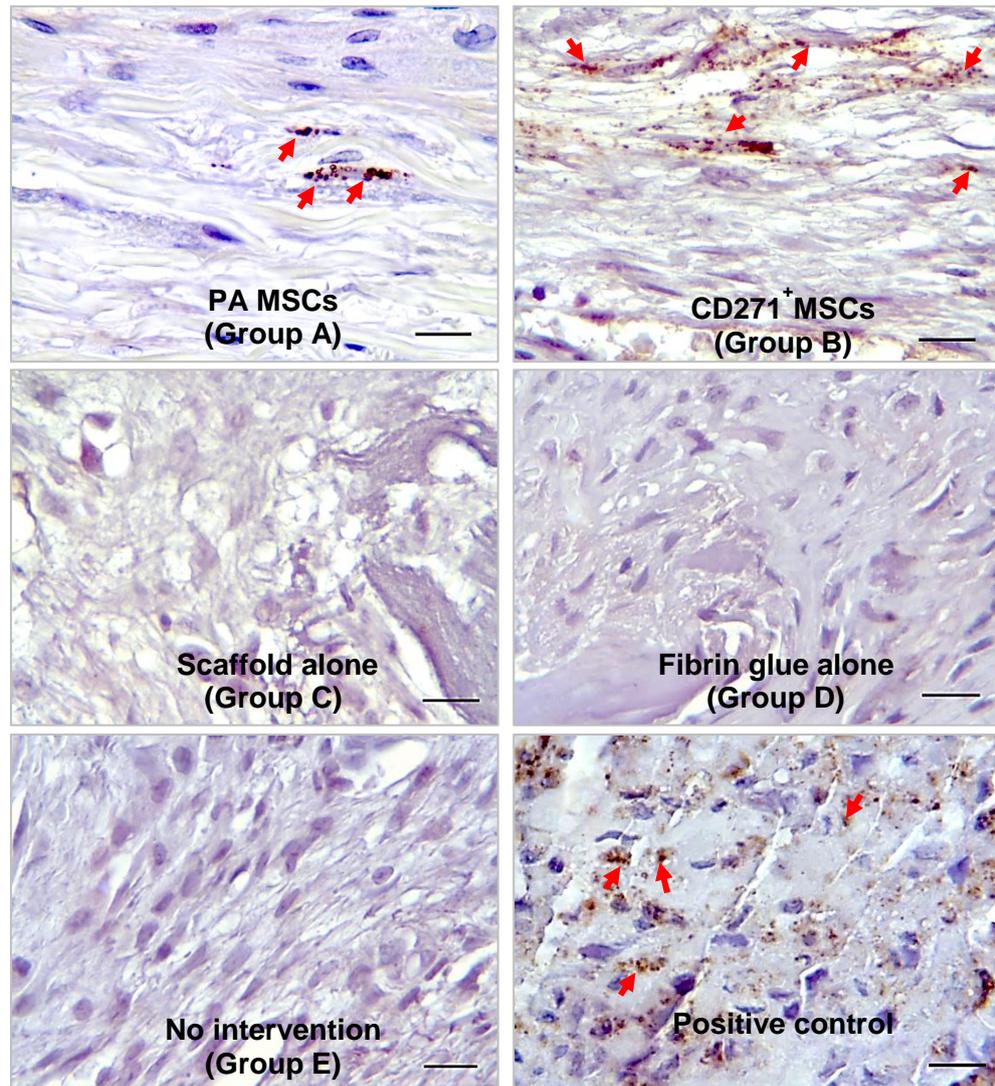


**Figure 5.11. Representative H&E stained images are shown to demonstrate the differences in FBGC reaction in the repair tissue.** FBGC reaction was only seen in the defects that were transplanted with Alpha Chondro Shield® (right image) compared to those that were transplanted using Chondro-Gide® (left image). FBGCs (red arrowed) were seen to be engulfing the fibres of Alpha Chondro Shield®. Yellow asterisks represent the fibres of Alpha Chondro Shield®. Scores have been presented as means ± SD of n=3. Original magnification x40. Scale bar = 25 µm.

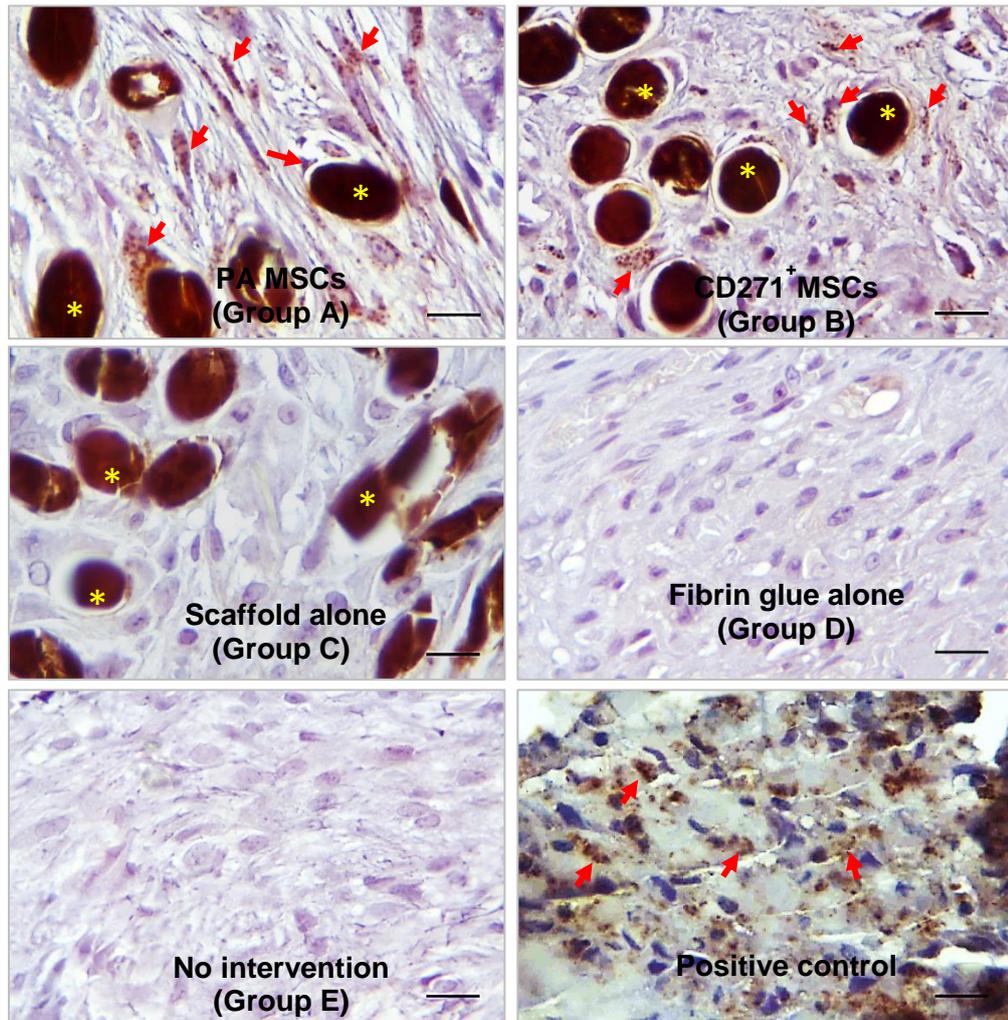
## **5.6 Human mitochondrial staining at 3 weeks post-transplantation**

Immunolocalisation for human mitochondrial antigen (HMA) was observed in the repair tissues of both PA MSCs (group A) and CD271<sup>+</sup>MSC groups (group B) with both Chondro-Gide® and Alpha Chondro Shield®. HMA expression was more marked in the defects transplanted using Alpha Chondro Shield® than Chondro-Gide® with both PA and CD271<sup>+</sup> MSCs. In animals where Chondro-Gide® was present in the defects, the HMA expression of PA or CD271<sup>+</sup>MSCs was mostly detected in the fibrous tissue layer in the junction between the scaffold and the underlying trabecular bone and was minimally detected within the scaffold also. In animals where Chondro-Gide® was absent, little HMA expression was detected in the fibrous repair tissue in the defects. Between PA and CD271<sup>+</sup>MSCs treated animals using Chondro-Gide®, HMA expression was more marked in the CD271<sup>+</sup>MSCs transplanted group.

In animals treated with PA or CD271<sup>+</sup>MSCs with Alpha Chondro Shield®, HMA expression was detected in the ECM surrounding the scaffold fibres. Similar extent of HMA expression was detected in both PA and CD271<sup>+</sup>MSC group. Due to the nature of mitochondrial staining, the exact number of cells was difficult to quantify. There was no evidence of HMA immunolocalisation in any of the controls groups that were not transplanted with human MSCs, i.e groups C, D and E. Greatest HMA expression was detected in the pellet cultures of human MSCs used as controls (Figures 5.12 and 5.13).



**Figure 5.12. Immunolocalisation of human mitochondrial antigen (HMA) in the repair tissue of the defects at 3 week post-transplantation with Chondro-Gide®.** HMA immunoreactivity, indicated by brown punctate staining (red arrowed), was observed in the repair tissue of the defects treated with PAMSCs (group A) and CD271<sup>+</sup> MSCs (group B). The positive expression was seen at the bottom of the defect in the fibrous tissue layer formed between the scaffold and the trabecular bone. No HMA expression was detected in the defects of group C,D and E. Haematoxylin was used as a counterstain. Human MSC pellets were used as positive controls. Original magnification x100; scale bar = 10  $\mu$ m.



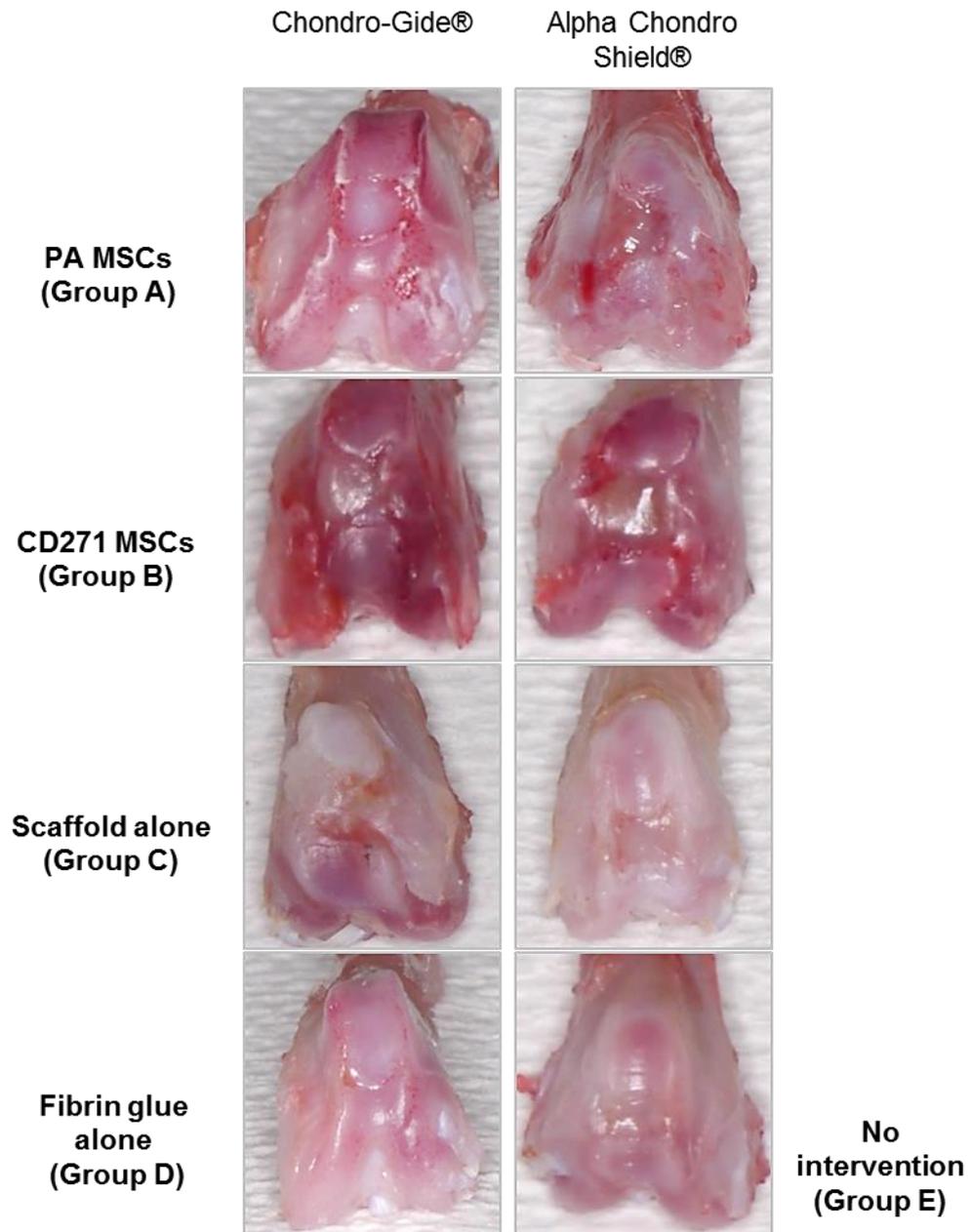
**Figure 5.13. Immunolocalisation of human mitochondrial antigen (HMA) in the repair tissue of the defects at 3 week post-transplantation with Alpha Chondro Shield®.** HMA immunoreactivity indicated by brown punctate staining (red arrowed), was observed in the repair tissue of the defects treated with PAMSCs (group A) and CD271<sup>+</sup> MSCs (group B). The positivity was localised either around the fibres of the scaffolds or within the repair tissue itself. No HMA expression was detected in the defects of group C, D and E but there was dark brown artifactual staining (yellow asterisks) of the fibres of Alpha Chondro Shield®. Haematoxylin was used as a counterstain. Human MSC pellets were used as positive controls. Original magnification x100; scale bar = 10 µm.

### **5.7 The effects of PA MSCs and CD271<sup>+</sup>MSCs seeded scaffolds on cartilage repair: gross morphology at 6 weeks post-transplantation**

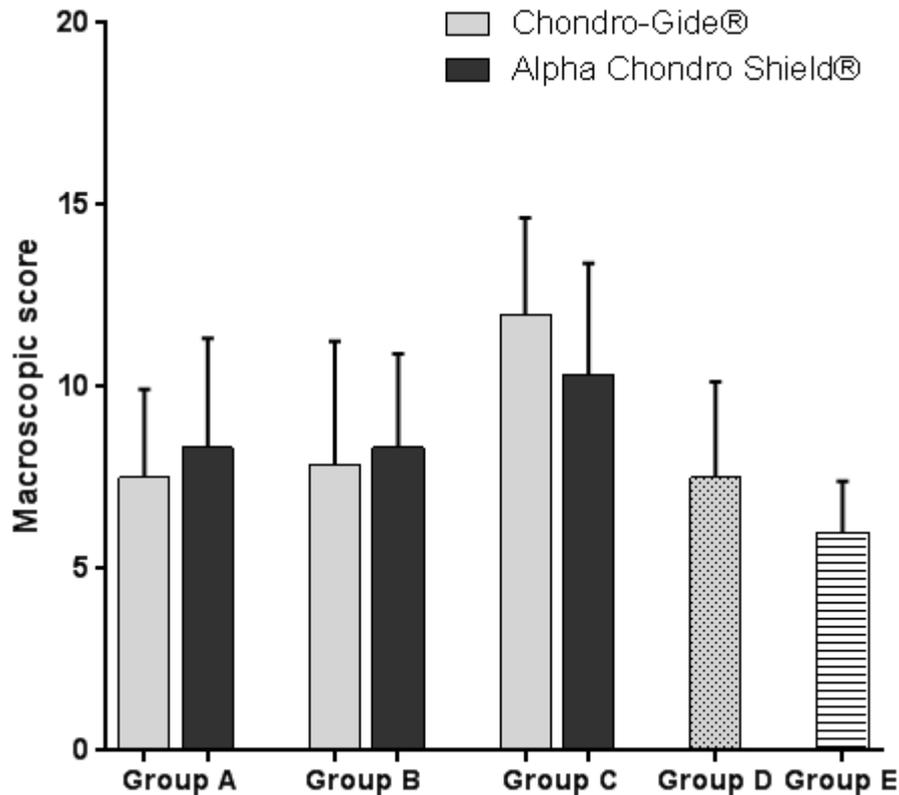
Gross examination of the defects at 6 weeks post-transplantation revealed no differences in the quality of the repair tissue of the defect when transplanted with either PA MSCs (group A) or CD271<sup>+</sup>MSCs (group B). Both group A and group B showed a poorly integrated repair tissue with the edges of the defects clearly visible. The scaffold alone control group (group C), showed no repair tissue and had severe degeneration of the adjacent cartilage in the case of Chondro-Gide®. In contrast, the control groups with fibrin only (group D) or no intervention (group E) appeared to have a well-integrated repair tissue, especially in the no intervention control group (group E) where a glossy pale-white repair tissue was seen and the edges of the defects were difficult to distinguish from the surrounding cartilage (Figure 5.14).

As per the macroscopic scoring system, defects transplanted with PA MSCs (group A) or CD271<sup>+</sup>MSCs (group B) had comparatively lower scores with either Chondro-Gide® or Alpha Chondro Shield® than defects that were treated with scaffolds alone (group C). Defects in group A were scored at  $7.5 \pm 2.4$  with Chondro-Gide® and  $8.3 \pm 3.0$  with Alpha Chondro Shield® and defects in group B were scored at  $7.8 \pm 3.4$  with Chondro-Gide® and  $8.3 \pm 2.6$  with Alpha Chondro Shield®. In contrast, defects in group C were scored at  $12 \pm 2.6$  for Chondro-Gide® and  $10.3 \pm 3.1$  with Alpha Chondro Shield®. The defects treated with fibrin glue only (group D) had similar scores ( $7.5 \pm 2.6$ ) as the two cell-treated groups, group A and B. The no intervention control group (group E), was scored at  $6 \pm 1.6$ , which was the lowest compared to all the other groups. However, no statistical significant differences were seen in the overall histological scores between any of the groups. All data has presented as means  $\pm$  SDs of n=6 for defects transplanted with PA MSCs (group A) and CD271<sup>+</sup>MSCs (group B), n=3 for defects transplanted with scaffold alone (group C), n=4 for defects that were treated with fibrin glue only (group D) and n=2 for defects that had no intervention (group E) (see also Figure. 5.3).

For individual parameters examined, the defects in groups D and E showed a predominantly (>50%) white repair tissue, whereas the defects transplanted with PA MSCs (group A) and CD271<sup>+</sup>MSCs (group B) and cell-free groups (group C) revealed a predominantly translucent repair tissue. Macroscopically, the presence of blood vessels was reduced in the cell-treated groups (group A and B) with both the scaffolds as well as in the defects that received fibrin glue only (group D) or had no intervention (group E). Between 25-50% of the repair tissue in all the groups contained blood vessels except for the scaffold alone control group (group C), which showed up to 75% of blood vessels in the repair tissues examined. A smooth heterogeneous surface of the repair tissue was seen only in group D and E. In PA MSCs transplanted animals (group A) with both scaffolds, there was a heterogeneously smooth to a somewhat fibrillated surface. The defects in the CD271<sup>+</sup>MSC transplanted animals (group B) with both scaffolds and the scaffold alone control group (group C) showed mostly fibrillated or an incomplete surface of the repair tissue. Over 50% of the defect depth was filled in the defects that received PA MSCs (group A) and CD271<sup>+</sup>MSCs (group B) with both the cell-carriers and in the defects treated with fibrin glue only (groups D) or had no intervention (group E). The defects treated with scaffolds alone (group C) with both the cell-carriers showed less than 50% of the defect fill. Cracks or fibrillations were observed at the integration zone of the repair tissue in the defects transplanted with PA MSCs (group A) with Chondro-Gide® and in the defects treated with fibrin glue only (group D) or had no intervention (group E). In contrast, the defects in group A with Alpha Chondro Shield® as the cell carrier and CD271<sup>+</sup>MSCs group (group B) and scaffold alone control group (group C) showed diffuse degenerative changes in the adjacent articular cartilage of the host (Table 5.3).



**Figure 5.14. Gross morphology of the defects at 6 weeks post transplantation.** Representative images are shown of the gross morphology of the defects in groups A, B, C, D and E. Gross examination of defects at 6 weeks revealed a translucent coloured and poorly integrated repair tissue in both the cell treated groups (group A and B) and in the animals that received scaffold alone (group C). The animals that received fibrin glue alone (group D) or had no intervention (group E) showed a predominantly white and well-integrated repair tissue.



**Figure 5.15. Macroscopic scores for cartilage repair at 6 weeks post-transplantation.** Mean macroscopic scores for animals transplanted with PA MSCs (group A) or CD271<sup>+</sup> MSCs (group B) were lower than the scaffold alone control group (group C) and were similar to the fibrin glue alone control (group D) but the no intervention control group (group E) showed the lowest scores compared to all the other groups. However, no significant differences were observed in the macroscopic mean scores of group A, B, C and D. Group E was excluded from statistical analysis due to small sample size. Data are presented as means  $\pm$  SD (n=6 for group A and B, n=3 for group C, n=4 for group D and n=2 for group E).

	PA MSCs (Group A)		CD271+MSCs (Group B)		Scaffold alone (Group C)		(Group D)	(Group E)
	Chondro- Gide®	Alpha Chondro Shield®	Chondro- Gide®	Alpha Chondro Shield®	Chondro- Gide®	Alpha Chondro Shield®	Fibrin Glue alone	No intervention
<b>Colour of the repair tissue</b>	2.2 ± 0.8	2.2 ± 0.8	2.3 ± 0.5	2.5 ± 0.5	<b>3.3 ±</b> <b>0.6</b>	2.7 ± 0.6	1.5 ± <b>0.6*</b>	1.5 ± 0.7
<b>Blood vessels in the repair tissue</b>	1.8 ± 0.8	1.8 ± 1.0	1.7 ± 0.8	1.7 ± 0.8	2.3 ± 0.6	2.3 ± 0.6	1.8 ± 0.5	1.5 ± 0.7
<b>Surface of the repair tissue</b>	1.5 ± 0.8	1.5 ± 0.5	2.3 ± 0.5	1.7 ± 0.8	2.3 ± 0.6	2.0 ± 1.0	1.5 ± 0.6	1.0 ± 0.0
<b>Defect fill</b>	1.0 ± 0.0	0.8 ± 0.4	1.3 ± 0.5	1.0 ± 0.6	1.7 ± 1.2	1.7 ± 0.6	1.0 ± 0.8	1.0 ± 0.0
<b>Degeneration of adjacent cartilage</b>	1.5 ± 0.5	1.8 ± 0.8	2.5 ± 0.5	2.3 ± 0.5	2.7 ± 0.6	2.0 ± 1.0	1.0 ± <b>0.0*</b>	1.0 ± 0.0

**Table 5.3. Macroscopic scores for the individual parameters of cartilage repair at 6 weeks post-transplantation.** The colour of the repair tissue in the defects treated with fibrin glue only (group D) was significantly better than that of the defects that were transplanted with Chondro-Gide® alone (group C,  $p$  value = 0.02). There was also a significant difference in the scores for degeneration of adjacent cartilage in defects transplanted with CD271+MSCs (group B) with both the scaffolds ( $p$  value = 0.030, Chondro-Gide® and Alpha Chondro Shield®) and when transplanted with Chondro-Gide® alone (group C) compared to fibrin glue alone group (group D,  $p$  value = 0.024). Data are presented as means  $\pm$  SD (n=6 for group A and B, n=3 for group C, n=4 for group D and n=2 for group E). Group E was excluded from statistical analysis due to a small sample size. ( $p$  value <0.05; Dunn's multiple comparison test). Bold text indicates statistical significance with asterisks displayed on the lower scores.

## **5.8 The effects of PA MSCs and CD271<sup>+</sup>MSCs seeded scaffolds on cartilage repair: overall histological outcomes at 6 weeks post-transplantation**

### 5.8.1 H&E staining

A poor degree of defect fill was observed in the defects of the MSC transplanted (group A and B) and scaffold alone transplanted animals (group C) regardless of the type of scaffold. Whilst the animals transplanted with Alpha Chondro Shield® showed good integration with the host adjacent cartilage and a firm attachment to the underlying trabecular bone, animals that were transplanted with Chondro-Gide® either contained delaminated scaffold or the scaffold detached during histological processing. In PA MSCs transplanted animals (group A), Chondro-Gide® was delaminated in one out of the three animals and was not present in the other two animals; in addition, in CD271<sup>+</sup>MSCs transplanted animals (group B), Chondro-Gide® delaminated in all three animals. Defects that were treated with fibrin glue alone (group D) showed a fibrous repair tissue with a partial defect fill; whereas the defects that had no intervention (group E) showed the greatest degree of defect fill compared to the defects of other groups. Bone matrix deposition was seen at the bottom of the defects in cell-treated groups (group A and B) or the scaffold alone control group (group C) with Alpha Chondro Shield® scaffold only. No bone was seen in the defects that were transplanted using Chondro-Gide® (Figure 5.16).

### 5.8.2 Toluidine blue staining

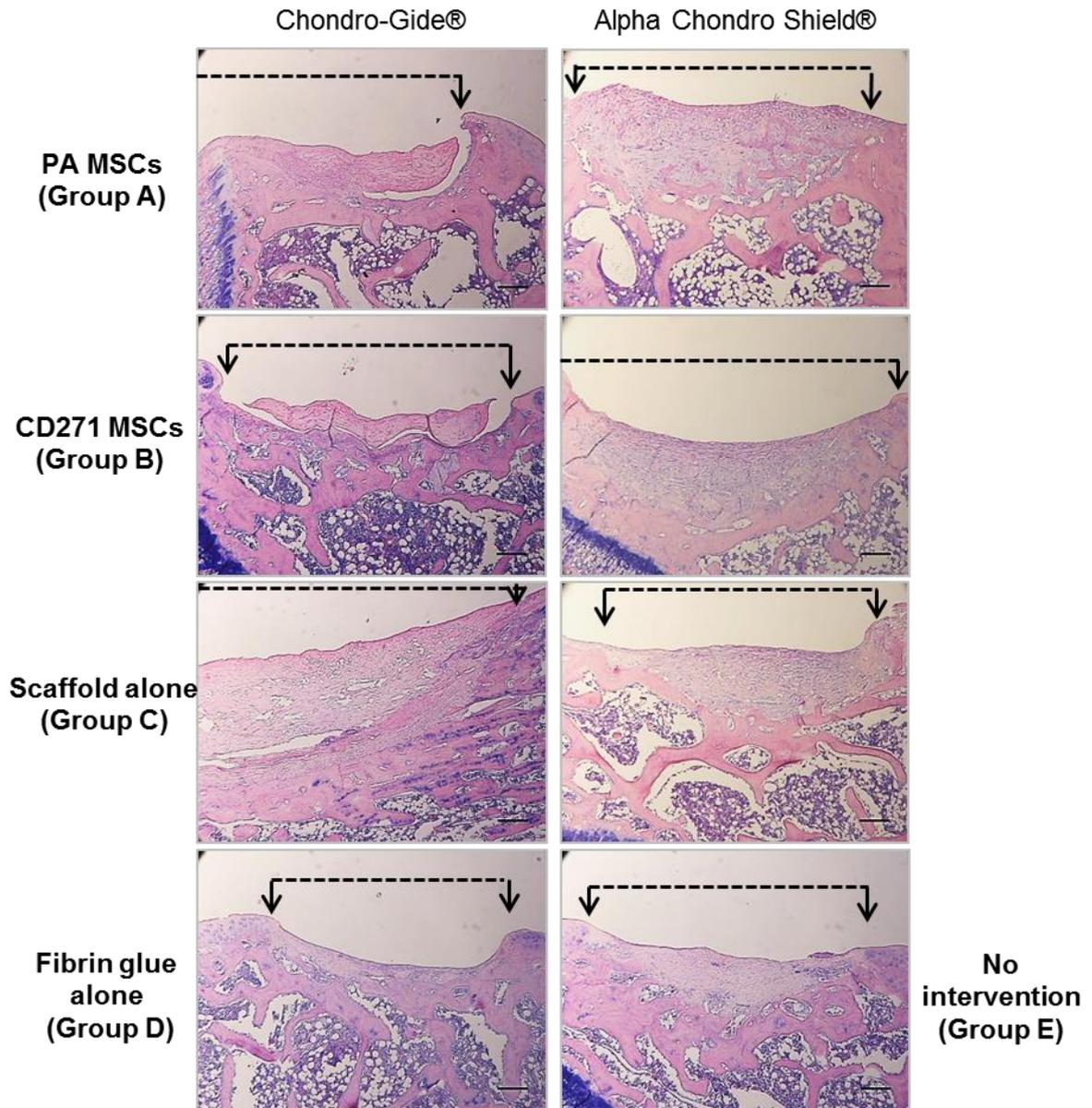
The repair tissue within the defects of PA MSC (group A) and CD271<sup>+</sup>MSC (group B) transplanted animals with Alpha Chondro Shield® only and the repair tissue of the no intervention control group (group E) showed localised toluidine blue metachromasia. In contrast, defects of any group transplanted with Chondro-Gide® showed no metachromasia. The fibrous tissue in the defects of the fibrin glue only control group (group D) also showed no metachromasia with toluidine blue dye (Figure 5.17).

### 5.8.3 Collagen type II immunostaining

No collagen type II production was observed in the repair tissues of the defects of cell-treated groups (group A and B) or the scaffold alone and fibrin glue only control groups (group C and D). However, collagen type II was only seen in the defects of the no intervention control group (group E) (Figure 5.4.3).

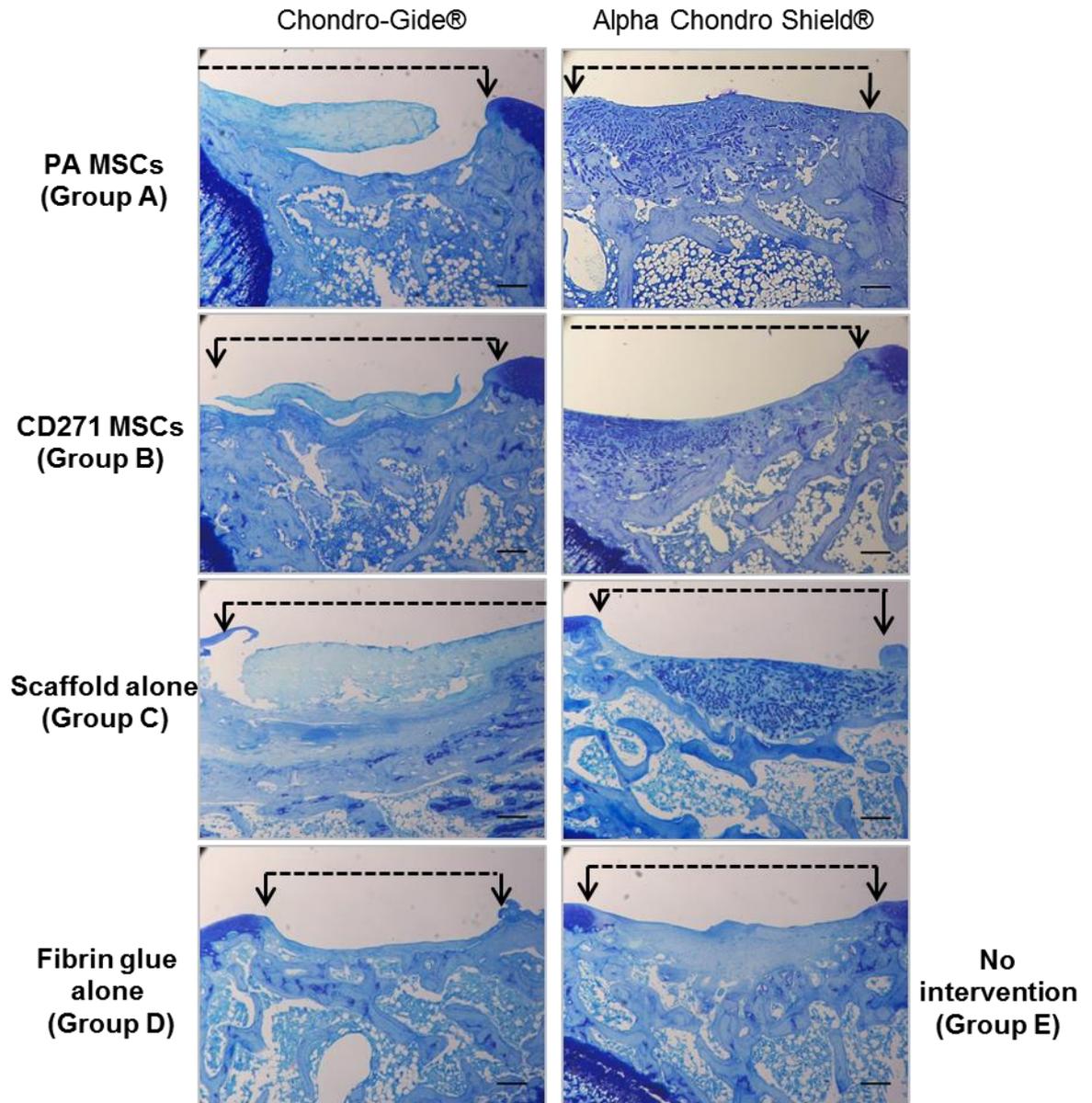
### 5.8.4 Histological scoring of the repair tissue

The histological scores for the PA MSC (group A) and CD271<sup>+</sup>MSC (group B) transplanted groups were similar, irrespective of the cell scaffold used. When Chondro-Gide® was used, the repair tissue was scored at  $12.5 \pm 2.3$  for PA MSC transplanted animals (group A) and  $11.5 \pm 3.0$  for CD271<sup>+</sup>MSC transplanted animals (group B). When Alpha Chondro Shield® was used, the repair tissue was scored at  $11.2 \pm 0.7$  for PA MSCs group and  $11.5 \pm 0.5$  for CD271<sup>+</sup>MSCs group. Defects treated with scaffolds alone (group C) were scored at  $15.0 \pm 1.4$  with Chondro-Gide® and  $15.5 \pm 0.7$  with Alpha Chondro Shield®. The defects treated with fibrin glue only (group D) were scored at  $15.5 \pm 0.7$ . The no intervention control group (group E) was scored at 9.5. No significant differences were observed in the histological outcomes of the cell-treated groups. All data has been presented as means  $\pm$  SDs of n=3 PA MSCs (group A) and CD271<sup>+</sup>MSCs (group B), n=2 for defects transplanted with scaffold alone (group C) and fibrin glue only (group D). Data for no intervention control group (group E) has been presented as the overall score of n=1 animal. Group C, D and E were excluded from stats analysis due to a small samples size (Figure 5.19).



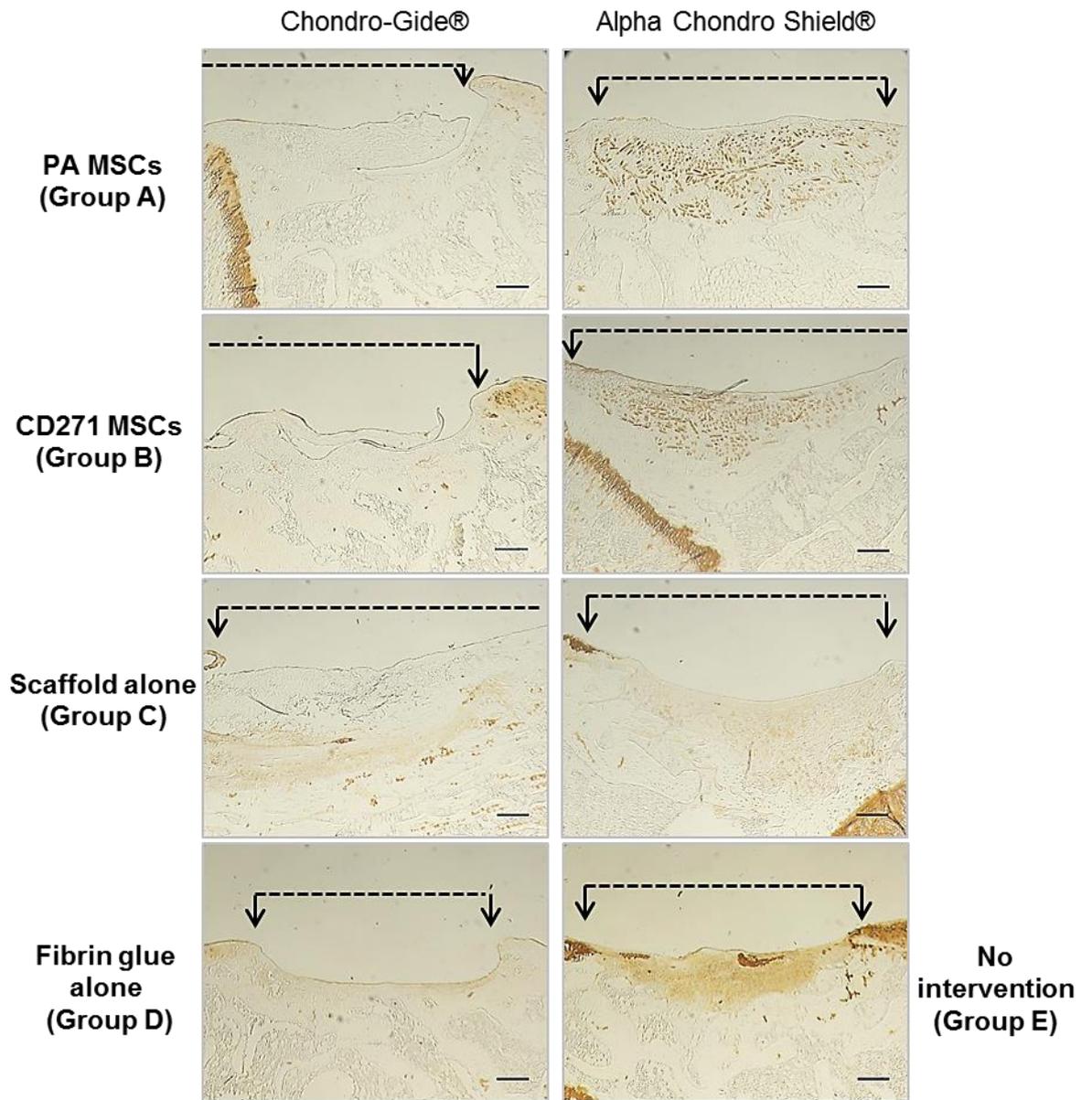
**Figure 5.16. Histology of rat knee tissue sections at 6 weeks post transplantation.**

Representative images are shown of the H&E stained tissue sections of the osteochondral defects. A poor degree of defect fill was observed in the animals of all groups except for the no intervention control group (group E) where the defect was spontaneously resurfaced with fibrous tissue. Original magnification x4. All scale bars = 200µm. Dotted line with arrows indicates the margins of the defect.

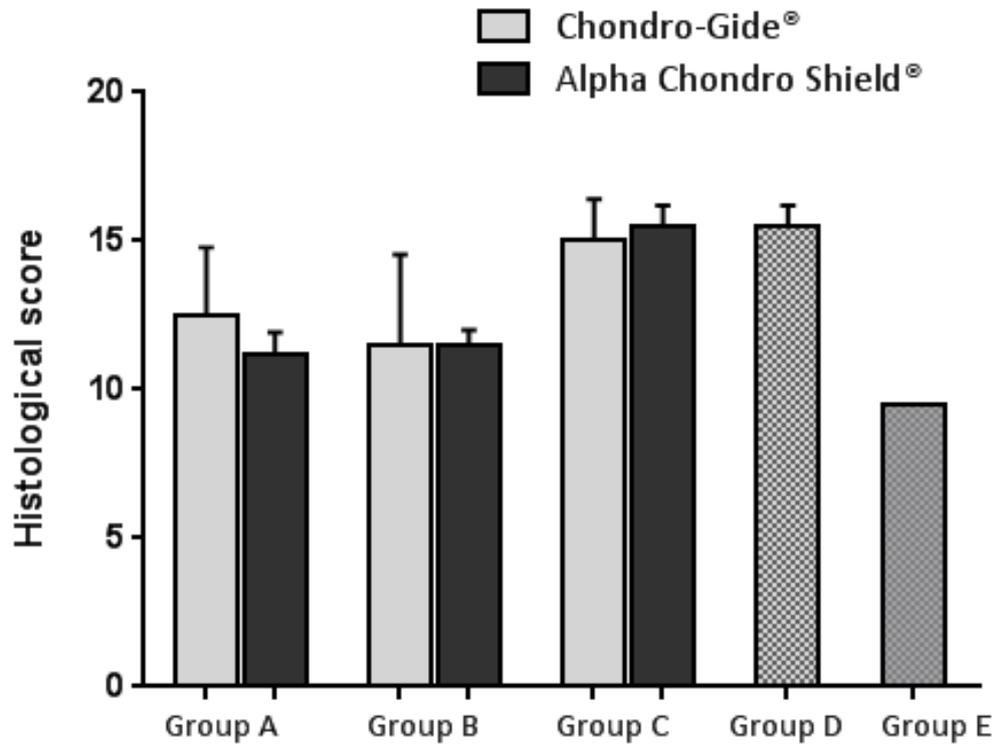


**Figure 5.17. GAG deposition in the defects at 6 weeks post-transplantation.**

Representative images are shown of toluidine blue stained tissue sections of the osteochondral defects. GAG deposition was seen in the repair tissue of the defects treated with PA MSCs (group A) or CD271<sup>+</sup> MSCs (group B) with Alpha Chondro Shield® only and in the no intervention control group (group E). Defects transplanted with either cell type using Chondro-Gide®, or the control groups C and D showed no metachromatic staining. Original magnification x4. All scale bars = 200µm. Dotted line with arrows indicates the margins of the defect.



**Figure 5.18. Collagen type II immunolocalisation in the defects at 6 weeks post-transplantation.** No collagen type II was seen within the repair tissue of the defects of either cell treated groups (groups A and B) or of no cell group (group C). Collagen type II immunopositivity was only seen in the repair tissue of the defects that had no intervention (group E). Original magnification x4. All scale bars = 200µm. Dotted line with arrows indicates the margins of the defect.



**Figure 5.19. Histological scoring of the repair tissue at 6 week post-transplantation.**

The cell-treated groups (group A and B) achieved lower histological scores compared to scaffold alone control group (group C) and fibrin blue alone control (group D). The defect in group E achieved a lower overall score compared to the other groups. There were no significant differences observed in the overall histological scores of the defects in group A and B. Data are presented as means  $\pm$  SD (n=3 for group A and B, n=2 for group C and D, n=1 for group E). The Mann-Whitney U test was used to compare histological scores of group A versus group B for each cell-carrier. Group C, D and E were excluded from statistical analysis due to the small sample size.

## **5.9 The effects of PA MSCs and CD271<sup>+</sup>MSCs seeded scaffolds on cartilage repair: detailed histological outcomes at 6 weeks post-transplantation**

Differences observed in the various histological parameters that were assessed have been described below and are summarised in Table 5.4:

### 5.9.1 Cell morphology

The repair tissue of the defects transplanted with PA MSCs (group A) and CD271<sup>+</sup>MSCs (group B) irrespective of the scaffold showed the presence of mostly elongated fibroblast-like cells with a few round-shaped cells indicative of mostly fibro-cartilaginous repair tissue. Bone matrix deposition was seen only in the repair tissue of the defects transplanted with PA and CD271 MSCs with Alpha Chondro Shield® alone (Figure 5.20). The defects in the controls groups of scaffold alone (group C) and fibrin glue alone (group D) showed mostly elongated spindle shaped cells indicative of non-cartilaginous repair tissue. The repair tissue of the defects that had no intervention (group E) showed a mix of round shaped and elongated cells.

### 5.9.2 Matrix staining

Metachromatic toluidine blue staining was markedly reduced compared with the host adjacent cartilage in the defects transplanted with PA MSCs (group A) or CD271<sup>+</sup>MSCs (group B) with either of the cell scaffolds. The defects of the scaffold alone group (group C) and the fibrin glue control group (group D) showed no metachromatic staining in the repair tissue whereas, the no intervention control group (group E) showed metachromatic toluidine blue staining that was reduced compared to the adjacent cartilage.

### 5.9.3 Surface regularity

A severely irregular surface of the repair tissue was seen in the defects of PA MSC and CD271<sup>+</sup>MSC transplanted animals (group A and B, respectively) with both scaffolds, and the scaffold alone control group (group C) as well as the fibrin glue only control group (group D). In contrast, the defect alone control group (group E) showed a comparatively less irregular

surface of the repair tissue. In defects where Chondro-Gide® was absent, the surface of the repair tissue constituted the fibrous repair tissue within the defects.

#### 5.9.4 Thickness

Defect fill was greater in the defects transplanted with either PA MSCs (group A) or CD271<sup>+</sup>MSCs (group B) with Alpha Chondro Shield® only and in the no intervention control group (group E) that showed between 1/3 to 2/3 of the defects were filled with a repair tissue. In the scaffold alone control group (group C) and fibrin glue only control group (group D), less than 1/3 of the defects was filled with a repair tissue with either Chondro-Gide® or Alpha Chondro Shield®. In defects where Chondro-Gide® was absent, the repair tissue within the defect was scored for thickness.

#### 5.9.5 Integration of the repair tissue with the host adjacent cartilage

Integration of the repair tissue within the defects transplanted with either PA MSCs (group A) or CD271<sup>+</sup>MSCs (group B) in combination with Chondro-Gide® was poor as either only one edge or neither of the edges were seen to be integrated with the host cartilage. Similarly, poor integration was observed in the scaffold alone control group (group C) with Chondro-Gide and in the defects treated with fibrin glue only (group D). Defects in which PA or CD271<sup>+</sup>MSCs were transplanted with Alpha Chondro Shield® showed good integration with both edges of the repair tissue integrated with the surrounding cartilage. This was also the case with defects that had no intervention (group E). In defects where Chondro-Gide® was absent, the repair tissue within the defect was scored for integration.

#### 5.9.6 Vascularisation within the defect

An increased number of blood vessels were seen in the defects transplanted with PA MSCs (group A) or CD271<sup>+</sup>MSCs (group B) using Alpha Chondro Shield® compared to those transplanted using Chondro-Gide®. The blood vessels penetrated the repair tissue of the defects transplanted with MSCs using Alpha Chondro Shield®, whereas they were only present in the fibrous tissue layer between Chondro-Gide® and the underlying trabecular

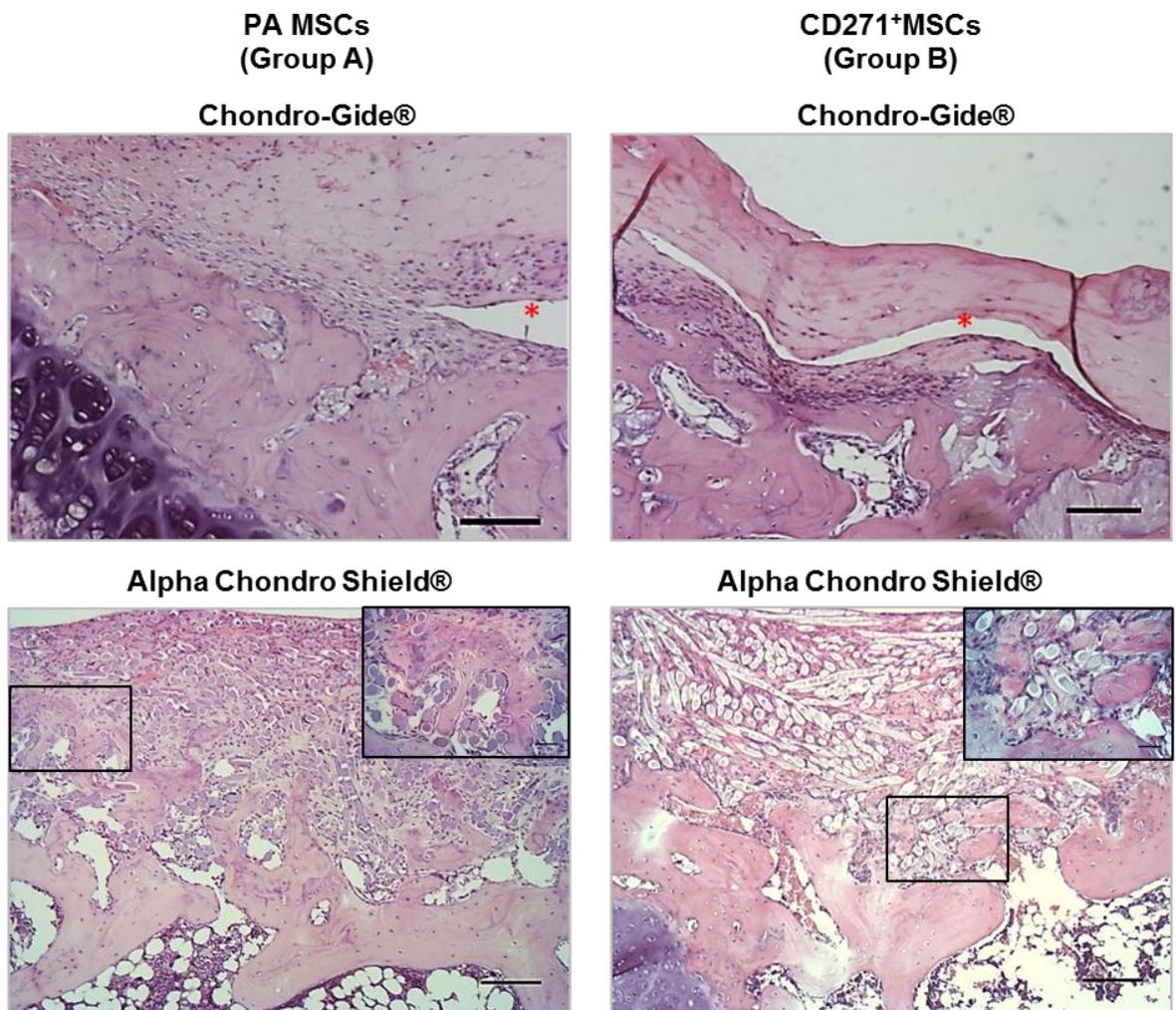
bone. The blood vessels were less frequently seen in the PA or CD271<sup>+</sup>MSC transplanted animals and the animals that were treated with fibrin glue alone (group D) than the animals that were treated with scaffolds alone (group C) or had no intervention (group E).

#### 5.9.7 Foreign body giant cell reaction

Similar to the 3 week results, FBGC reaction was seen only in the defects that were transplanted with Alpha Chondro Shield® with or without PA MSCs (group A) or CD271<sup>+</sup>MSCs (group B). FBGCs were observed engulfing the fibres of Alpha Chondro Shield®. Where Chondro-Gide® was used as the scaffold no FBGCs were present in the repair tissue. This was also true for control groups that were treated with fibrin glue only (groups D) or those that had no intervention (group E).

	PA MSCs (Group A)		CD271 <sup>+</sup> MSCs (Group B)		Scaffold alone (Group C)		(Group D)	(Group E)
	Chondro- Gide®	Alpha Chondro Shield®	Chondro- Gide®	Alpha Chondro Shield®	Chondro- Gide®	Alpha Chondro Shield®	Fibrin Glue alone	No intervention
<b>Cell morphology</b>	2.3 ± 0.6	2.0 ± 0.0	2.3 ± 1.0	2.2 ± 0.3	2.5 ± 0.7	2.5 ± 0.7	3.0 ± 0.0	2.0
<b>Matrix staining</b>	2.0 ± 0.5	1.6 ± 0.6	2.0 ± 0.5	1.8 ± 0.3	2.5 ± 0.7	2.5 ± 0.7	3.0 ± 0.0	1.5
<b>Surface regularity</b>	2.5 ± 0.5	2.3 ± 0.3	2.8 ± 0.8	2.5 ± 0.0	3.0 ± 0.0	3.0 ± 0.0	3.0 ± 0.0	2.0
<b>Thickness</b>	1.5 ± 0.5	0.3 ± 0.6	1.5 ± 0.5	1.0 ± 0.5	2.5 ± 0.7	1.5 ± 0.7	2.0 ± 0.0	0.5
<b>Integration</b>	1.3 ± 0.6	0.0 ± 0.0	1.2 ± 0.8	0.0 ± 0.0	1.5 ± 0.7	0.0 ± 0.0	2.0 ± 0.0	0.0
<b>Blood vessels</b>	2.0 ± 1.7	2.7 ± 1.2	1.7 ± 0.6	2.0 ± 0.0	3.5 ± 0.7	4.0 ± 0.0	2.5 ± 0.7	3.0
<b>Foreign body giant cells</b>	0.0 ± 0.0	2.2 ± 0.3	0.0 ± 0.0	2.0 ± 0.0	0.0 ± 0.0	2.0 ± 1.4	0.0 ± 0.0	0.0

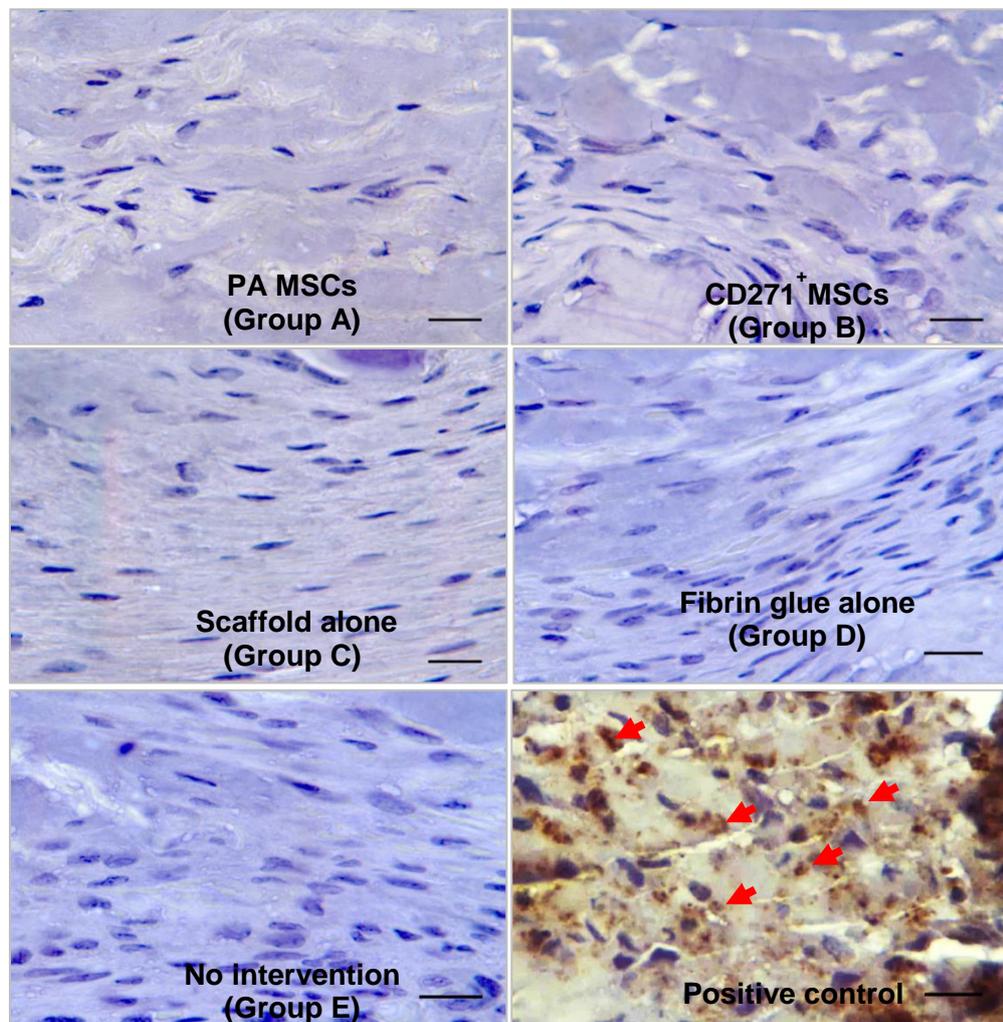
**Table 5.4. Histological scores of the individual parameters at 6 weeks post-transplantation.** The no intervention control group (group E) achieved lower scores for most of the parameters assessed excluding blood vessels. Data are presented as means ± SD (n=3 for group A and B and n=2 for group C and D, n=1 for group E). There was no statistical difference between the repair tissue of the defects of group A and B. Group C, D and E were excluded from statistical analysis due to the small sample size. The Mann-Whitney U test was used to test statistical significance.



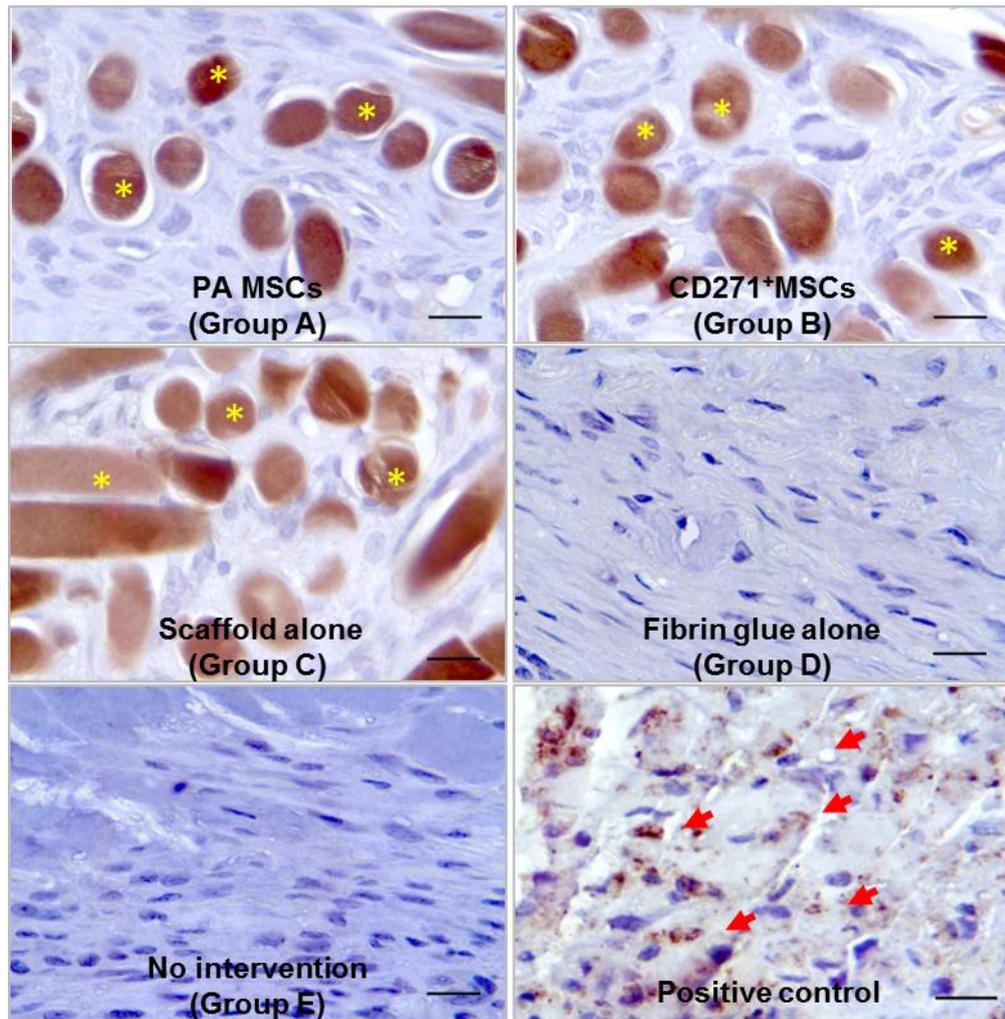
**Figure 5.20. Representative images to bone matrix within the repair tissues of the defects at 6 week post transplantation.** Bone matrix deposition was not observed in the repair tissue of the defects that received Chondro-Gide®; the space between the scaffold and the underlying bone is shown by red asterisks. In contrast, bone matrix was observed in the repair tissue of the defects treated with either cell-type using Alpha Chondro Shield® as the cell-carrier. Black boxes indicate areas where ECM in the repair tissue has been replaced with bone matrix. Original magnification x10; scale bars = 100µm. Insets show x40 magnification of areas shown in black boxes; scale bar 25 µm.

### **5.10 Human mitochondrial staining**

Only one out of the three animals transplanted with PA MSCs using Alpha Chondro Shield® showed HMA expression. There was no HMA immunolocalisation in the repair tissues in the CD271<sup>+</sup>MSC (group B) transplanted animals or any of the control groups (group C, D and E) at 6 weeks post-transplantation. HMA expression was only detected in the human MSC pellets that were used as controls (Figure 5.21 and 5.22)



**Figure 5.21. Immunolocalisation of human mitochondrial antigen (HMA) in the repair tissue of the defects at 6 week post-transplantation with Chondro-Gide®.** HMA immunoreactivity was absent in the repair tissue of the defects treated with PAMSCs (group A) and CD271<sup>+</sup> MSCs (group B). HMA expression was also absent in the control groups (group C,D and E). Positive expression as indicated by brown punctate staining (red arrowed) was only seen in the human MSC pellets used as positive controls. Haematoxylin was used as a counterstain. Original magnification x100. Scale bar = 10µm.



**Figure 5.22. Immunolocalisation of human mitochondrial antigen (HMA) in the repair tissue of the defects at 6 week post-transplantation with Alpha Chondro Shield®.** HMA immunopositivity was absent in the repair tissue of the defects treated with PAMSCs (group A) and CD271<sup>+</sup>MSCs (group B). Positive expression as indicated by brown punctate staining (red arrowed) was only seen in the human MSC pellets used as a positive control. There was dark brown artifactual staining (yellow asterisks) of the fibres of Alpha Chondro Shield®. Haematoxylin was used as a counterstain. Original magnification x100; scale bar = 10  $\mu$ m.

## 5.11 Discussion

CD271 selected MSCs from various sources including BM (Cuthbert *et al.*, 2015), AT (Quirici *et al.*, 2010; Duran *et al.*, 2013), and synovium (Arufe *et al.*, 2010) have shown an immense potential for their use in regenerative medicine. These *in vitro* studies suggest that isolating cells immune-positive for CD271 results in a homogenous population that also co-expresses CD73, CD90 and CD105 (Arufe *et al.*, 2010; Cuthbert *et al.*, 2015; Jones and McGonagle 2008). CD271<sup>+</sup>MSCs from BM have previously been shown to give rise to superior cartilage formation *in vivo* compared to PA MSCs (Mifune *et al.*, 2012). In this study, the defects transplanted with AT derived CD271<sup>+</sup>MSCs (group B) at 3 weeks post-transplantation showed macroscopically superior repair tissue compared to the defects transplanted with PA MSCs (group A) and all the other control groups (groups C, D and E). Histologically, all defects showed fibrous cartilaginous repair tissue, but localised metachromatic toluidine blue staining was seen in the repair tissue of the defects treated with CD271<sup>+</sup>MSCs (group B). For complete healing of the full-thickness defects, it was essential that the trabecular and subchondral bones were fully restored followed by resurfacing of the defect with articular cartilage. Therefore, the candidate cell type must have the ability to differentiate into both bone and cartilage. There was indication of bone matrix deposition at the proximal end of the defects transplanted with PA MSCs (group A) and CD271<sup>+</sup>MSCs (group B) using Alpha Chondro Shield® only at 6 weeks post-transplantation. Wakitani *et al.* have previously listed the sequence of events that are likely to take place following transplantation of cell-scaffolds into a full thickness defect (Wakitani *et al.*, 1994). They hypothesized that vascular infiltration into the defect causes host-derived MSCs to undergo osteogenesis and restore the osseous tissue. This happens only up to the natural junction between the host cartilage and subchondral bone, as the distal part of the defect is under the influence of the factors present in the synovial cavity where the osteochondral progenitor cells give rise to the cartilage (Wakitani *et al.*, 1994). In this study, vascular infiltration was seen at 3 weeks followed by bone matrix deposition at 6 weeks in

both PA and CD271<sup>+</sup>MSC group. Human cells were seen only in one animal of the PA MSC group at 6 weeks and in this animal no evidence of bone matrix was seen.

Repair tissue in the defects at 3 week or 6 weeks and in any of the MSC transplanted groups (group A and B) did not show a hyaline-like repair tissue at the surface of the defect and this may be due to the use of AT as cell source. Previous reports suggest that AT is not a suitable source of MSCs for cartilage regeneration (Huang *et al.*, 2005; Im *et al.*, 2005; Lee, *et al.*, 2012). Studies have also shown that the chondrogenic differentiation potential of AT MSCs maybe dependent on the site of harvest of AT (Mochizuki *et al.*, 2006; Lopa *et al.*, 2014). Mochizuki and co-workers reported that subcutaneous AT is not a suitable source of MSCs for cartilage repair as the subcutaneous fat MSCs have lower chondrogenic potential compared to synovium-derived MSCs (Mochizuki *et al.*, 2006). In addition, in a donor-matched comparison, infrapatellar fat pad MSCs were reported to have a higher chondrogenic potential than subcutaneous AT MSC (Lopa *et al.*, 2014). Furthermore, AT MSCs have previously been shown to secrete angiogenic factors that are inhibitory to cartilage-like ECM deposition (Lee *et al.*, 2012). The repair tissue of both cell-treated groups (groups A and B) with Alpha Chondro Shield® showed vascular invasion and that's may be why hyaline like ECM deposition was not seen. However, selective isolation of CD271<sup>+</sup>MSCs may have partially improved the outcome as a comparatively lower number of blood vessels were seen in the repair tissue of the defects transplanted with CD271<sup>+</sup>MSCs (group B) compared to PA MSCs (group A). In addition, localised metachromasia after staining with toluidine blue dye was more markedly seen in the repair tissue of the defects transplanted with CD271<sup>+</sup>MSCs. Studies have previously shown that articular cartilage GAGs inhibit endothelial cell adhesion (Fenwick *et al.*, 1999; Johnson *et al.*, 2005; Bara *et al.*, 2012). It may be that CD271<sup>+</sup>MSCs induced a cartilage-like repair response comprising of GAGs that inhibit blood vessel formation, whereas PA MSCs did not induce the deposition of GAGs. Further work is needed to explore the anti-angiogenic capacity of the CD271<sup>+</sup>MSCs.

Repair tissue of the defects treated with Alpha Chondro Shield® alone control (group C) showed an extensive cell infiltration and little ECM deposition compared to the cell-treated groups, where less cells and more ECM deposition was observed. This may suggest that the human MSCs were essential for tissue repair. Furthermore, HMA was detected in the cell-treated defects at 3 weeks post-transplantation indicative of human cell survival in the defects that started to lay down ECM. Human cell survival was observed in the repair tissues of the cell-treated groups; however, that did not reduce the FBGC in the defects that received MSCs with Alpha Chondro Shield. HMA immunolocalisation at 3 weeks was seen around the fibres of PGA in Alpha Chondro Shield, which is also where FBGCs were observed. This co-localisation of MSCs with FBGCs may suggest that implant rejection continued without evidence of an anti-inflammatory activity of the MSCs. In an inflamed environment, MSCs have been shown to have anti-inflammatory effects by restoring the  $T_H1/T_H2$  balance through inhibiting  $IFN-\gamma$  secretion and increasing IL-4 and IL-10 (Aggarwal and Pittenger 2005). However, athymic rats do not elicit a T-cell mediated immune response and therefore, MSCs may have been unable to induce a T-cell mediated immunomodulatory response. Moreover, only one animal in the PA MSCs treated group showed persistence of human cells at 6 weeks post-transplantation, all the other animals in the PA or CD271<sup>+</sup>MSC treated groups showed no evidence of human cell survival at 6 weeks post-transplantation as depicted by the absence of HMA immunopositivity. This may indicate that the inflammatory environment accompanying the FBGC reaction to Alpha Chondro Shield® may have contributed to the loss of human cells. However, there was also no HMA immunopositivity in the animals transplanted with MSCs and Chondro-Gide® at 6 weeks, in which no FBGC reaction was noted. This suggests that the human cells failed to persist within the defects irrespective of the FBGC reaction. In a recent study of xenogeneic transplantation of human articular chondrocytes into full-thickness cartilage defects in minipigs, it was reported that human cells failed to persist in the defects at 4 weeks following transplantation (Niemietz *et al.*, 2014). The study also reported an extensive host cell invasion followed by a decline in human cells to less than 5% of initial cell numbers as early as 2 weeks. Co-localisation of macrophages to the implanted cells was

suggested to cause this effect. On the other hand, Mifune *et al.*, have shown human cell survival within a rat model of full-thickness chondral defects at 4 and 8 weeks post transplantation (Mifune *et al.*, 2012). Sato *et al.*, have also shown human MSC survival in guinea pigs knee joints 5 weeks after transplantation (Sato *et al.*, 2012). Clearly, further research is needed to understand whether and how transplanted MSC survival is determined, particularly if the presence of the MSCs is associated with experimental (or clinical) outcome.

Alpha Chondro Shield® showed better integration and tissue ingrowth compared to Chondro-Gide® at both 3 weeks and 6 weeks. Alpha Chondro Shield® is a porous scaffold with an open pore structure that allows cellular access from all sides whereas Chondro-Gide® consist of a porous side for cell-attachment and a compact side to avoid cell-leakage. Cell-seeded Chondro-Gide® was fixed with its porous side facing down on to the defect and with its compact side lying adjacent to the host articular cartilage. The fixation of MSC-seeded Chondro-Gide® was technically challenging in the rat knee joints, which may have resulted in the delamination of Chondro-Gide® in some cases. Where Chondro-Gide® was seen in the defect, cellular infiltration was observed at the bottom of the defects with the development of a fibrous tissue layer in between the scaffold and the underlying trabecular bone. Alpha Chondro Shield® on the other hand showed cellular ingrowth from the sides as well as the bottom of the defects. At 6 weeks post transplantation, Chondro-Gide® showed poor integration and was delaminated within the defect whereas Alpha Chondro Shield® remained firmly attached to the donor tissue. Although Chondro-Gide® showed poor integration with the host tissue, it evoked no FBGC response and was not supportive of vascular invasion, whereas Alpha Chondro Shield® showed a greater degree of FBGC reaction and vascular invasion. Host reaction following implantation of a biomaterial can elicit a sequence of events including acute and chronic inflammation ultimately resulting in FBGC reaction and fibrous capsule formation (Anderson 2001). Depending on the composition of biomaterial, various different inflammatory mediators and signalling molecules such as cytokines, chemokines and growth factors evoke monocyte/macrophage migration towards the implantation site. During

chronic inflammation lymphocytes are recruited to the implantation site. Lymphocytes, especially T lymphocytes induce macrophage adhesion, fusion and cytokine secretion depending on the material composition of the biomaterial/scaffold. Interleukin-4 (IL-4) and IL-13 are cytokines that induce macrophages to gain an alternatively activated phenotype and fuse together to form FBGCs. It is believed that these cytokines are produced *in vivo* by T cells (Anderson *et al.*, 2008). In this study, female athymic nude rats were used to create full-thickness chondral defects; therefore, a T-cell mediated immune response is absent. The presence of FBGCs in the repair tissues of the defects transplanted using Alpha Chondro Shield® i.e. a synthetic scaffold, could potentially be due to other sources of IL-4 and IL-13 production as it has been previously shown that NK cells, mast cells, eosinophils and basophils are also capable of producing these cytokines (Al-Saffar *et al.*, 1998; Gessner *et al.*, 2005). Furthermore, it has also been shown that T cells are not necessary for eliciting a FBGC reaction to synthetic materials in nude mice. The authors concluded that T cells are a redundant source of IL-4 and/or IL-13 *in vivo* (Rodriguez *et al.*, 2009). Nevertheless, the consequence of FBGC reaction can cause the release of mediators of degradation such as reactive oxygen intermediates (ROIs), enzymes and acid. The biomaterials are then exposed to these degradative agents in the immediate microenvironment and the chemistry of the biomaterial greatly influences its susceptibility to degradation (Anderson *et al.*, 2008). The repair tissue of the defects that received Alpha Chondro Shield at 3 weeks and 6 weeks still showed fibres of PGA suggesting that the degradation rate of PGA based scaffold is longer than 6 weeks *in vivo*. Freed *et al.*, have previously shown when chondrocytes-seeded PGA are implanted within a rabbit model of full thickness chondral defect, the remnants of the scaffold are still visible at 6 months post-implantation (Freed *et al.*, 1993). Chondro-Gide® is a natural scaffold, which did not evoke a FBGC reaction. Natural biomaterials are usually thought to be biocompatible and suitable for biomedical applications. Studies have previously shown that coating materials with collagen type I reduces macrophage responses to the material and fibrosis (Ksander & Gray 1985; Cho *et al.*, 2005). Results presented within this chapter

support the previous studies with regards to the absence of FBGC reaction in the case of Chondro-Gide®.

Rats were chosen as the animal models of osteochondral defects in this study as they are cost effective in providing proof-of-concept data to build a bridge between *in vitro* experiments and expensive large animal studies. However, these smaller animals have thin cartilage which could be a limiting factor for investigating repair responses as it may heal faster than human cartilage and makes it difficult to draw robust conclusions. In addition, the small sized joints and thin cartilage also makes it practically difficult to investigate the suitability of surgical implants in this model (Constance *et al.*, 2010). In this study, it was technically very challenging to fix MSC-seeded Chondro-Gide® and Alpha Chondro Shield® into the defects, especially as the cell-seeded scaffolds tend to swell in size. The poor quality of repair in rat models of full-thickness cartilage defects has previously been shown by Anraku *et al.*, who reported that spontaneous cartilaginous repair responses fail to occur in larger sized defects (width 1.5mm) compared to hyaline-like cartilage regeneration observed in smaller sized defects (width 0.7mm) at 4 weeks after the creation of defects. The larger sized defects were covered only with fibrous scar tissue and showed no signs of subchondral bone restoration (Anraku *et al.*, 2009). In this study, although the defects were 2 x 2 x 1 mm in size, smaller sized defects would spontaneously heal and therefore, to investigate the repair responses of MSC transplanted animals, bigger sized defects were created.

In summary, the results presented in this chapter are suggestive of fibrocartilaginous repair tissue in the defects transplanted with either PA MSCs or CD271<sup>+</sup>MSCs derived from AT. There are some indications of improved repair using CD271<sup>+</sup>MSCs with regards to ECM deposition and a lower degree of neovascularisation compared to PA MSCs. Furthermore, the presence of cells with chondrocytic appearance observed in the repair tissue at 3 weeks in the CD271 group (group A) and the presence of bony matrix in the defects at 6 weeks also support the observation that CD271<sup>+</sup>MSCs may have beneficial healing potential. May be with longer time-points, the repair tissue would

restructure the sub chondral bone and subsequently re-surface with hyaline-like articular cartilage, as seen in previous studies in rats that analysed the repair responses at 8 weeks (Mifune *et al.*, 2012) or 16 weeks (Chung *et al.*, 2014) post transplantation.

In terms of the cell-carriers or scaffolds, it is difficult to draw conclusions with regards to Chondro-Gide® as it had either delaminated or was lost during histological processing from the defects of the knees of these smaller animal models; although when Chondro-Gide® was present, it showed no gross FBGC reaction. In contrast, Alpha Chondro Shield® showed good integration within the defects; however, it evoked a FBGC reaction. Nonetheless, Chondro-Gide® has been used in the clinics as the scaffold of choice for AMIC or MACI procedures and has shown promising clinical outcomes (Wylie *et al.*, 2015). To further assess the suitability of Alpha Chondro Shield®, bigger animal models are necessary.

## **Chapter 6: Discussion**

Damage to the knee articular cartilage is troublesome in that it lacks the capacity to repair itself and mostly presents symptomatically when the damage has extended to the subchondral bone. This progressive loss of knee articular cartilage is one of the major causes of degenerative joint pathologies such as OA (Buckwalter 1998). Efforts to treat symptomatic cartilage defects such as those described in the first chapter of this thesis only provide temporary pain relief. Therefore, various surgical procedures have sought to prevent the progression of cartilage damage (Falah *et al.*, 2010; Memon & Quinlan 2012). ACI is considered the gold standard for the treatment of cartilage defects, however, it has shown mixed results with the quality of the repair tissue as fibrocartilage more often than hyaline-like cartilage in clinical studies (reviewed by Harris *et al.*, 2010). In addition, there are some major drawbacks of ACI including the need for a two-step operative treatment procedure and the loss of a chondrocyte phenotype that is seen in monolayered cell culture (Makris *et al.*, 2014). Therefore, there is a need to investigate alternative cell sources for cell-based therapies that can be easily harvested and can potentially be used in a one-step procedure. As discussed in the first chapter, BM and AT MSCs have been extensively investigated both *in vitro* and *in vivo* for their potential to regenerate damaged articular cartilage (Boeuf & Richter 2010; Somoza *et al.*, 2014). Both freshly isolated and culture expanded cells from these two cell sources have been used in human patients; however, there is no consensus as to which cell type or cell source, in combination with which scaffolds or cell delivery systems, is the best for repairing damaged cartilage. With regards to scaffolds or the cell delivery systems, only limited *in vitro* and pre-clinical data is available on scaffolds that are currently in clinical use. In addition, culture expanded MSCs are heterogeneous, which is also a potential problem as not all isolated or culture expanded MSCs will have the same differentiation or reparative capacity (Phinney 2012). This becomes a key issue with regards to the use of MSCs for cell-based therapies as the isolated population may not be functionally homogenous and uniformly chondrogenic. The data presented within this thesis has addressed some of the key features of cell-based therapies for cartilage repair, which are:

1. To expand or not to expand autologous cell sources for cartilage repair?
2. To use BM or AT as a cell source?
3. To use Chondro-Gide® versus Alpha Chondro Shield® versus Hyalofast™ as a scaffold for chondrogenesis? – from an in vitro perspective
4. To optimise AT as a cell source by selectively isolating a prospective more homogeneous cell population?
5. To use Chondro-Gide® versus Alpha Chondro Shield® as a scaffold for cartilage repair? – from an in vivo perspective

### **6.1 To expand or not to expand autologous cell sources for cartilage repair?**

The results presented within Chapter 3 of this thesis sought to answer the first question by investigating the incorporation and growth of culture expanded MSCs versus freshly isolated cells from BM and AT in three clinical scaffolds, followed by analysis of chondrogenesis of the persistent cell type in the scaffolds examined. The findings of this Chapter suggest that culture expanded MSCs from both cell sources adhere, incorporate and grow in Chondro-Gide®, Alpha Chondro Shield® and Hyalofast™. In contrast, freshly isolated BM MNCs or AT SVF cells do not grow as well as culture expanded cells in any of the three scaffolds during long-term cultures. Unfortunately, that suggests that the proposition of one-step cell-based therapies for cartilage repair may be less favourable than using culture expanded MSCs in a two-step procedure. However, there may be ways to enhance and optimise the freshly isolated cell population by enriching for prospective MSCs. One of the ways to do this is described in Chapter 4. Interestingly, freshly isolated BM MNCs have already been applied in the treatment of full-thickness chondral lesions of the knee in human patients and have shown promising results, however the histological assessment was based on small number of biopsies (2-3) and the overall studies lacked control groups (Buda *et al.*, 2010; A. Gobbi *et al.*, 2011). Similarly, AT SVF cells have been applied clinically in the treatment of knee OA, and have shown successful clinical outcomes, although no histological results were reported (Pak *et al.*, 2013; Bui *et al.*, 2014). Also, a majority of the clinical trials with AT SVF cells have been

case series that lack control groups. Therefore, the success of these clinical studies, in terms of an improvement in function or reduction in pain, could be attributed to the placebo effect. Whilst freshly isolated cells have shown considerable promise in human patients, the results presented within Chapter 3 of this thesis do not show good *in vitro* incorporation and growth of freshly isolated cells from BM or AT in the three clinical scaffolds examined. This could be attributed to the *in vitro* cell seeding procedure and culture conditions compared to the clinical settings. For *in vitro* analysis, BM MNCs and AT SVF cells were seeded directly onto the scaffold and grown in culture dishes whereas during *in vivo* studies and clinical treatments, a number of different techniques have been utilized for cell seeding, including the mixing of concentrated BM MNCs with collagen powders or using collagen and HA membranes as cell carriers prior to their fixation into defects with platelet rich fibrin gel (Giannini *et al.*, 2009; Gobbi *et al.*, 2011). AT SVF cells have also been used as intra-articular injections in combination with platelet rich plasma (PRP) (Koh *et al.*, 2013; Bui *et al.*, 2014). During the *in vitro* analysis shown in this thesis, cells may have escaped the scaffolds after cell-seeding due to a lack of an enclosed environment, e.g. fibrin gel encapsulation. Future work should optimise cell seeding and culture conditions to better mimic the surgical procedure including encapsulation in fibrin gels. Therefore, the answer to question to culture expand or not to culture expand autologous cells is to expand cells, as per the findings of this thesis, but with further study warranted.

## **6.2 To use BM or AT as a cell source?**

Culture expanded MSCs from BM and AT were further examined for their chondrogenic differentiation potential. BM MSCs showed better potential for chondrogenesis than AT MSCs as indicated by increased collagen type II and GAG deposition in BM MSC seeded scaffolds. This indicates that BM may be a better source of MSCs for cartilage repair than AT. There is evidence in the literature that supports these findings (Im *et al.*, 2005b; Danišovič *et al.*, 2007; Boeuf & Richter 2010). However, a potential weakness of the studies presented within this thesis was that the BM MSCs and AT MSCs were obtained from different patient

donors, therefore, direct comparisons on which cell source is better for cartilage repair is limited due to the possibility of donor-to-donor variation. Studies have previously highlighted the variability in gene expression between different donors of AT, in addition to differences observed due to harvest site and isolation and culture conditions (Baer *et al.*, 2012; Lopa *et al.*, 2014). Ideally, BM and AT obtained from the same donor should have been compared to better establish the potential of these cells for chondrogenesis. In addition, TGF- $\beta$ 1 was used as an inducer for chondrogenic differentiation of MSCs from both cell sources, which may not be ideal for inducing chondrogenic differentiation in AT MSCs. For example, some studies have previously reported that TGF-  $\beta$ 1 is not as efficient at inducing chondrogenic differentiation of AT MSCs as BMP 6 (Estes *et al.*, 2006; Hennig *et al.*, 2007).

### **6.3 To use Chondro-Gide® versus Alpha Chondro Shield® versus Hyalofast™ as a scaffold for chondrogenesis? – from an *in vitro* perspective**

MSCs from both cell sources showed the best growth in Chondro-Gide®. Of the three scaffolds examined, Hyalofast™ disintegrated and was not suitable for long-term chondrogenic experiments whereas Chondro-Gide® and Alpha Chondro Shield® persisted. The chondrogenic differentiation of MSCs was evident in both Chondro-Gide® and Alpha Chondro Shield® but was most marked in Chondro-Gide® cultured with BM MSCs. Furthermore, there was increased collagen type II and GAG deposition in the ECM of BM MSC-seeded Chondro-Gide® cultures compared to AT MSC-seeded Chondro-Gide® cultures. Therefore, the findings of the chapter 3 favour the use of Chondro-Gide® for cell-based therapies for cartilage repair. Since Chondro-Gide® is an ECM based scaffolds, it holds a considerable promise for engineering cartilage-like tissue *in vitro*. If cartilage tissue can be engineered *in vitro* using the right cell-scaffold and growth factor combination, it would highly benefit the patients and clinicians in that it would obviate the need for highly invasive surgeries and prevent donor-site morbidity. However, articular cartilage is a structurally complex 3D environment consisting of different types of matrix molecules in which multiple bioactive growth factors are incorporated and even with the advances in the development of

appropriate scaffolds it is very difficult to reach this complexity (Benders *et al.*, 2013). Currently in the field of cartilage tissue engineering, efforts are being made to regenerate cartilage which would be structurally and functionally similar to the native tissue. However, what the current literature reports as hyaline cartilage seems to be far from the true native tissue in that, the proportions of the ECM molecules of the tissue engineered cartilage are likely to be far from hyaline cartilage, which will have negative implications with regards to its load bearing capacity. Additionally, the zonal arrangement and spatial organisation of the native cartilage is often not seen in tissue-engineered constructs, which contributes to the poor mechanical strength of the construct. ECM based scaffolds such as those used in this thesis i.e., Chondro-Gide® and Hyalofast™, may be more suited for this purpose since they already mimic a natural 3D environment for cells and may help in retaining growth factors in the ECM. The findings presented in this thesis are consistent with the current literature on the use of Chondro-Gide® as it has been the scaffold of choice for the MACI procedure for over a decade now (Haddo *et al.*, 2004; Wylie *et al.*, 2015). However, to date, no clinical data is available on the use of culture expanded MSCs with Chondro-Gide® in human patients, possibly due to a lack of robust *in vitro* and pre-clinical *in vivo* data demonstrating their chondrogenic differentiation potential. Furthermore, studies have also previously shown that synthetic scaffolds such as PGA based scaffolds can be optimised for cartilage tissue engineering by combining them with fibrin gels (Endres *et al.*, 2012), collagen gels or alginate gels (Hannouche *et al.*, 2007). Since PGA has been approved by the FDA for human clinical use, it should be further explored for its potential for tissue engineering cartilage constructs.

Attempts have been made through the isolation of chondrocytes from different layers of articular cartilage and encapsulation of cells in a multi-layered hydrogel system to recreate the stratification of native cartilage (Kim *et al.*, 2003). Only one recently published study has so far shown that by recapitulating the developmental events, MSCs can be induced to form mesenchymal cell bodies, which when seeded onto decellularised bone matrix give rise to functional cartilage tissue *in vitro* (Bhumiratana *et al.*, 2014). Overall, whilst it may be

possible to optimize culture conditions to recreate the composition of the native cartilage, the dynamic complexity required for the recreation of developmental events including an understanding of the role of the micro-environmental cues, such as soluble factors, ECM molecules, cell-cell and cell-matrix interactions in controlling lineage progression and stem cell fate still pose a significant challenge (reviewed by Somoza *et al.*, 2014).

#### **6.4 To optimise AT as a cell source by selectively isolating a prospective more homogeneous cell population?**

On the basis of cell growth and ECM deposition, the use of BM MSCs with Chondro-Gide® is favoured. However, due to the easy availability of MSCs from AT and a less-invasive tissue harvesting procedure, in addition to the lack of robust preclinical data available on the use of AT MSCs for chondrogenesis in clinical scaffolds, this thesis further examined the suitability of AT as a cell source for cartilage repair. Chapters 4 and 5 of this thesis focussed on investigating the potential of AT MSCs for their chondrogenic differentiation and cartilage repair, examining cells that were selected on the basis of plastic adherence (PA MSCs) compared to the cells selected on the basis of their expression of CD271. The rationale for this study was based on the hypothesis that selective isolation of MSCs from AT SVF using CD271 as a marker results in a more homogenous cell population that may have a superior potential for chondrogenic differentiation and cartilage repair compared to a heterogeneous population of MSCs selected on the basis of plastic adherence. This hypothesis was based on the evidence in the current literature regarding CD271 as a selective marker of MSCs from BM and their superiority for chondrogenesis *in vitro* and *in vivo* (Mifune *et al.*, 2012; Álvarez-Viejo 2015). The data presented in Chapter 4 suggested that CD271<sup>+</sup>MSCs upon induction with chondrogenic inducers deposited better and more ECM in the scaffolds compared to PA MSCs, which did not show as much ECM deposition. Also, the pellet cultures of CD271<sup>+</sup>MSCs showed a deposition of ECM that was richer in collagen type II compared to pellets of PA MSCs. This data is novel and supportive of the hypothesis that CD271 selection from AT is advantageous for the use of MSCs in cartilage repair.

With these promising *in vitro* results, the potential of CD271<sup>+</sup>MSCs was compared with PA MSCs in the healing of osteochondral defects in a rat model. The data presented in Chapter 5 showed superior macroscopic scores for cartilage formation in CD271<sup>+</sup>MSC transplanted animals compared to PA MSC transplanted animals and control groups at 3 weeks post-transplantation. However, histologically, a fibrous repair tissue was seen in all cases. The only difference observed between CD271<sup>+</sup>MSC and PA MSC treated groups was in the extent of vascularisation of the repair tissue, where CD271<sup>+</sup>MSCs showed comparatively fewer blood vessels compared to PA MSC treated groups. The lower number of blood vessels observed in the repair tissue of the CD271<sup>+</sup>MSCs group indicate that these cells may have anti-angiogenic activity that may be beneficial for the regeneration of cartilage. For bone regeneration, MSCs with angiogenic activity may be more favourable as angiogenesis supports bone formation during fracture healing (Dimitriou *et al.*, 2011). A recent study has shown that CD271<sup>+</sup>MSCs from BM have elevated gene expression for bone formation and vascularisation (Cuthbert *et al.*, 2015). Arufe *et al.*, have also shown increased expression of RUNX2, a transcription factor involved in osteochondral differentiation by CD271<sup>+</sup>MSCs and deposition of collagen type II and aggrecan in the pellets of a CD271 selected cell population from synovial tissue (Arufe *et al.*, 2010). Mifune *et al.*, also reported on the superior chondrogenic potential of BM derived CD271<sup>+</sup>MSCs *in vitro* and *in vivo* compared to unselected MSCs (Mifune *et al.*, 2012). All these findings indicate that MSCs from different tissue sources display intra-population heterogeneity with different subpopulations comprising different phenotypic and functional characteristics (Phinney 2012). The evidence regarding the functional heterogeneity of MSCs obtained from clonal studies have shown that clones from immortalized human MSCs contain tri-potent progenitor that yield more restricted bi-potent progenitors such as adipo-chondrogenic progenitors, adipo-osteogenic progenitors or osteochondrogenic progenitors (Okamoto *et al.*, 2002). Likewise, Muraglia *et al.*, showed that only one-third of the MSC clones derived from BM were able to display tri-lineage differentiation potential (Muraglia *et al.*, 2000). In addition, even with the selective isolation of cells using a single marker, not all isolated MSCs may be uniformly tri-potent. In a recent clonal analysis

on CD271<sup>+</sup>MSCs it was reported that 13.3% of clones differentiated into osteoblasts, adipocytes and chondrocytes (tri-potent), 20% differentiated into chondrocytes and osteoblasts (bi-potent) and 7% differentiated into adipocytes and osteoblasts (Kuçi *et al.*, 2013). So, in addition to the selective isolation using a single marker, primary MSCs cultures should be established at the clonal level. However, still only a fraction of these clonal MSCs will display chondrogenic differentiation. Unfortunately, no markers are currently available to distinguish multipotent MSCs from bi-potent or uni-potent MSCs in humans (Bianco *et al.*, 2008). In a recent study conducted on mice, a mouse skeletal stem cell population, which is multipotent and has the ability to self-renew, was identified. Through lineage tracing experiments, it was shown that this skeletal stem cell population can differentiate into more lineage-restricted progenitor cells, which further produce uni-potent progenitors such as pro-chondrogenic progenitors or pro-osteogenic progenitors. In this study, the immunophenotype of each of these progenitors was identified (Chan *et al.*, 2015). If similar studies can be conducted in humans, it would represent a paradigm shift in the therapeutic regeneration of skeletal tissues where specific uni-potent sub-populations of progenitor cells can be isolated for differentiation towards a particular tissue type. CD271<sup>+</sup>MSCs show considerable promise for enrichment of MSCs with osteogenic and chondrogenic capacities, however, future work should further examine AT derived CD271<sup>+</sup>MSCs to investigate their potential for cartilage and bone repair, e.g. by examination at the transcriptional level.

## **6.5 To use Chondro-Gide® versus Alpha Chondro Shield® as a scaffold for cartilage repair? – from an *in vivo* perspective**

Where Chondro-Gide® was used as the scaffold for MSC delivery *in vivo* there was a difference in the results observed compared to the *in vitro* study. The scaffold was either delaminated or absent in the defect at both time points. This poor outcome may well be related to the disadvantages associated with using smaller animal models. For example, in the rat model tested there was cartilage thickness of less than 0.5 mm, used to test the suitability of clinical scaffolds that are intended to be used on human knee cartilage with a thickness between 2.2-2.5 mm (Frisbie *et al.*, 2006; Cook *et al.*, 2014). Due to this likely technical issue, it was particularly difficult to make firm conclusions on the suitability of Chondro-Gide® for repairing damaged cartilage. Nonetheless, Chondro-Gide® has been extensively used in ACI procedures and has shown successful results (Haddo *et al.*, 2004). Previously, in small animal models of osteochondral defects, different combinations of hydrogels have been used as cell-delivery systems, including collagen-based hydrogels (Zhang *et al.*, 2011), HA and alginate-based hydrogels (Chung *et al.*, 2014) or collagen type I sponge (Mifune *et al.*, 2012). Although PGA-based porous scaffolds have been studied in animal models before (Erggelet *et al.*, 2007), Chondro-Gide® and Alpha Chondro Shield® have not previously been tested in combination with MSCs for their potential in healing osteochondral defects. One conclusion that can be drawn from this *in vivo* study is that whilst Chondro-Gide® generally showed poor integration, no FBGC reaction was observed where it was seen to be integrated. That may favour the use of Chondro-Gide® as the scaffold for autologous cell-therapies compared to Alpha Chondro Shield® as an FBGC reaction can ultimately result in graft failure (Anderson *et al.*, 2008). The FBGC reaction observed in the defects that were transplanted using Alpha Chondro Shield® could also negatively affect articular cartilage repair due to the release of inflammatory cytokines and ROS, and stimulate hypertrophic differentiation in chondrocytes (Morita *et al.*, 2007). A pre-requisite for the success of cell transplantation therapies for articular cartilage repair using scaffolds is that the scaffold must maintain its properties and functions and regenerate tissue even when exposed to a compromised environment, e.g. as in

the case of FBGC reaction (Iwasa *et al.*, 2009). In the osteochondral defects of the rat knee joints, vascularisation was needed to reform bone, followed by re-surfacing with articular cartilage. Whilst, some indications of trabecular bone restoration were observed in the repair tissue of cell-treated groups at 3 weeks and 6 weeks, no hyaline articular cartilage formation was seen at the surface of these defects. With longer time point analysis, maybe Alpha Chondro Shield® would have degraded with subsequent subchondral bone restoration and resurfacing with articular cartilage. It has also been shown in human patients that repair tissue following ACI procedure showed a fibrocartilage morphology at 12 months follow up whereas the repair tissue showed hyaline-like morphology at 19.8 month follow up (Roberts, I. McCall, *et al.*, 2003). It was suspected that the fibrous repair tissue may remodel with time to form hyaline-like cartilage. Future work should examine the potential for cartilage repair of CD271 selected MSCs from AT in larger animal models and with longer time point analysis post-transplantation.

## 6.7 Limitations of the study

There are various limitations to this study. One of the limitations is the small number of patient donors assessed. Experimental data on the incorporation and chondrogenesis that was presented within Chapters 3 and 4 of this thesis has been derived from two patient donors for each data set. Limited donor availability is a drawback faced by researchers in the field of tissue engineering using primary human cells. Further research is required to examine more MSC donors from both tissue sources to ensure the reproducibility of the results presented within this thesis and to examine mechanisms of increasing the efficiency of cell seeding into these scaffolds and their optimal application in cell transplantations for cartilage repair.

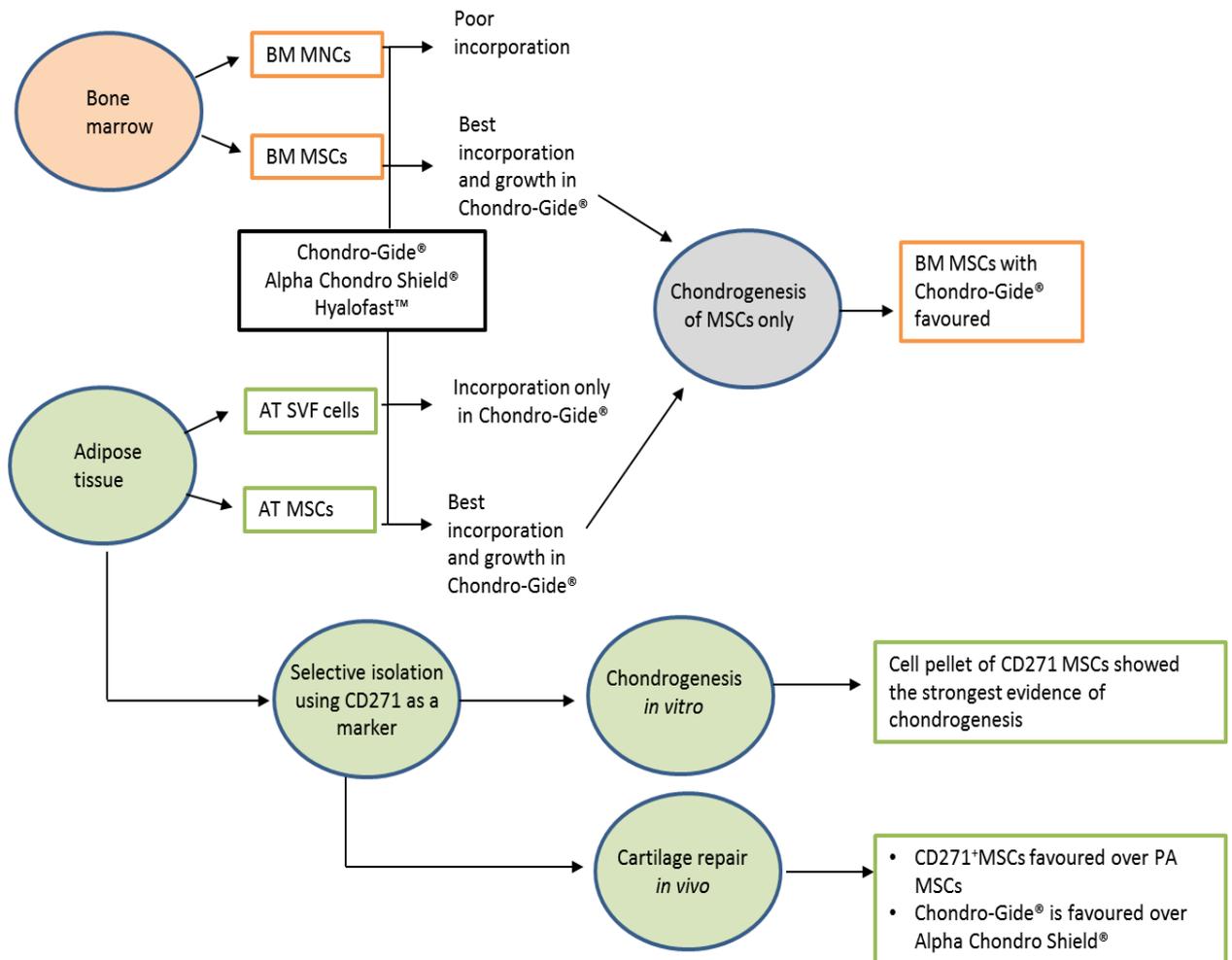
In addition, the source of AT used for the experiments in Chapter 3 and 4 (both infrapatellar fat pad) was different to the AT used in the experiments in Chapter 5 (subcutaneous AT). As stated earlier in this Chapter, variability in gene expression of AT MSCs from donor-to-donor as well as in between different harvest sites has previously been reported (Baer and Geiger 2012). Another study stated that AT MSCs from infrapatellar fat pad showed enhanced levels of cartilage specific genes compared to MSCs from subcutaneous fat around the knee (Lopa *et al.*, 2014). Future studies should compare BM and AT from the same patient. Furthermore, even with the same site of harvest of AT MSCs, discrepancies were observed between MSC samples used in Chapter 3 versus Chapter 4. That also indicates that MSCs display donor -to-donor variation. Indeed, the outcome of any autologous MSC-based therapies may be ultimately depend on the health, life style and age of the donor (Siddappa *et al.*, 2007; Choudhery *et al.*, 2014; Phinney 2012). In a recent review by Somoza and co-workers, it was emphasized that MSCs from different anatomical locations, or even subpopulations within the same tissue, are most likely to have different proliferation and differentiation potentials. They will also have varying requirements for, and responses to inducers of differentiation. Therefore, it may well be important to adopt standardised approaches for the isolation of MSCs from different sources in future (Somoza *et al.*, 2014).

Another limitation of this work is that the results of the *in vitro* studies reported herein are not directly comparable to the *in vivo* situation in a human knee joint due to differences in the environment. For example, the physiological oxygen concentration of cartilage in the human knee joint is considered to be 2%, which becomes elevated somewhat in the case of joint pathologies such as OA and rheumatoid arthritis (RA) (Henrotin *et al.*, 2005). During the *in vitro* culture conditions in this thesis, the cells were grown in 21% O<sub>2</sub> and it has been previously shown that MSCs cultured under hypoxic conditions (1% O<sub>2</sub>) showed enhanced expression of *SOX9*, *Col2a1* and *Acan* genes compared to atmospheric (21% O<sub>2</sub>) conditions (Lee *et al.*, 2013). It has also been shown that chondrocytes grown in hypoxic conditions show enhanced chondrogenic differentiation potential compared to hyperoxic or atmospheric conditions (Kay *et al.*, 2011). However, many different culture conditions are used for differentiating MSCs towards cartilage and certainly researchers and clinicians have not yet adopted a hypoxic approach as one of the standard conditions for growing MSCs for chondrogenesis (reviewed by Freyria and Mallein-Gerin 2012).

The data presented herein on chondrogenesis is based largely on qualitative histology results. Obtaining quantitative data from histological sections is challenging and subjective, but nonetheless the results presented in Chapter 5 would have benefited from gene expression analysis of cartilage specific genes to more accurately define differences in the quality of the repair tissue seen in different localities between the different groups examined. In addition, data on the biomechanical functions of the knee joint following treatments would also have helped to determine the success of the cell-therapies tested.

## 6.8 Summary

A summary of the major findings of this thesis is presented in Figure 6.1. Overall, the results presented suggest that the culture expansion of MSCs is better in cell-based cartilage repair than the use of freshly isolated cells. On the basis of cell growth and collagen type II production, the *in vitro* studies suggest the use of BM MSCs with Chondro-Gide®. Where selective isolation is concerned, this thesis has also shown that better differentiation of CD271 selected MSCs than PA MSCs towards chondrogenesis was observed *in vitro*; however, the outcomes of the *in vivo* studies were limited, with poor evidence of chondrogenesis by either cell type. The potential for BM derived CD271 selected MSCs for *in vivo* cartilage repair has only been shown in one study by Mifune and co-workers (Mifune *et al.*, 2012). Further work is required to examine AT derived CD271<sup>+</sup>MSCs and to investigate their secretome for the effects of trophic factors during cartilage repair. In addition, further work should examine cell-scaffold transplantation in bigger animal models of reproducible chondral defects.



**Figure 6.1 A Summary of the major findings.** The major findings of Chapter 3-5 are presented here in a summary to show that the results of chapter 3 suggest that MSCs are better for their incorporation and growth than freshly isolated cells from both BM and AT with Chondro-Gide® proving to be the most suitable scaffold for MSCs incorporation and growth. In terms of chondrogenesis, BM MSCs with Chondro-Gide® is favoured over other cell-scaffold combinations studied. The findings of Chapter 4 and 5 suggest that CD271 selected MSCs are superior for chondrogenesis compared to PA MSCs and that CD271 MSCs may be better suited for cartilage repair as compared to PA MSCs. With regards to the scaffolds, Chondro-Gide® is favoured over Alpha Chondro Shield®.

## 6.9 Concluding remarks

Cartilage tissue engineering is an emerging field that holds considerable promise towards the success of a cell-based therapy for repairing chondral and osteochondral defects. As researchers and clinicians gradually incorporate new data in this rapidly expanding field, the tissue engineering of this complex tissue can be perfected. This thesis contributes to the current knowledge in the field of cartilage tissue engineering and repair by helping to address two main questions, whether to culture expand or not culture expand autologous cells for articular cartilage repair and whether to or not to selectively isolate CD271<sup>+</sup> cells from AT for cartilage repair. From the findings presented, culture expansion of cells is favoured with BM being a more potent source of MSCs for chondrogenesis than AT. However, isolating CD271<sup>+</sup>MSCs from AT may have improved the potential of chondrogenesis of AT MSCs. Since the clinical grade anti-CD271 microbeads are already available and licensed for human use, CD271 can be easily translated for clinical use. Going forward, on the basis of the results presented within this thesis, a one-step procedure using CD271<sup>+</sup>MSCs from AT SVF maybe proposed for the treatment of chondral defects. This would require a minimally invasive liposuction procedure, followed by isolation of CD271<sup>+</sup>MSCs using clinical grade microbeads, which takes about 1 hour, with subsequent transplantation of CD271 immunopositive cells in combination with Chondro-Gide® as the scaffold, all in the same operating room. This would obviate the need for costly cell culture expansion and would greatly benefit the patients by reducing the risk of donor-site morbidity, as well as benefit clinicians who would not need to perform two highly invasive surgeries, which currently is standard practice.

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## **Appendix I**

### **List of publications**

Kohli, N., Wright, K.T., Sammons, R.L., Jeys, L., Snow, M. & Johnson, W.E.B., 2015. An In Vitro Comparison of the Incorporation, Growth, and Chondrogenic Potential of Human Bone Marrow versus Adipose Tissue Mesenchymal Stem Cells in Clinically Relevant Cell Scaffolds Used for Cartilage Repair. *Cartilage*.

# An *In Vitro* Comparison of the Incorporation, Growth, and Chondrogenic Potential of Human Bone Marrow versus Adipose Tissue Mesenchymal Stem Cells in Clinically Relevant Cell Scaffolds Used for Cartilage Repair

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## Abstract

**Aim.** To compare the incorporation, growth, and chondrogenic potential of bone marrow (BM) and adipose tissue (AT) mesenchymal stem cells (MSCs) in scaffolds used for cartilage repair. **Methods.** Human BM and AT MSCs were isolated, culture expanded, and characterised using standard protocols, then seeded into 2 different scaffolds, Chondro-Gide or Alpha Chondro Shield. Cell adhesion, incorporation, and viable cell growth were assessed microscopically and following calcein AM/ethidium homodimer (Live/Dead) staining. Cell-seeded scaffolds were treated with chondrogenic inducers for 28 days. Extracellular matrix deposition and soluble glycosaminoglycan (GAG) release into the culture medium was measured at day 28 by histology/immunohistochemistry and dimethylmethylene blue assay, respectively. **Results.** A greater number of viable MSCs from either source adhered and incorporated into Chondro-Gide than into Alpha Chondro Shield. In both cell scaffolds, this incorporation represented less than 2% of the cells that were seeded. There was a marked proliferation of BM MSCs, but not AT MSCs, in Chondro-Gide. MSCs from both sources underwent chondrogenic differentiation following induction. However, cartilaginous extracellular matrix deposition was most marked in Chondro-Gide seeded with BM MSCs. Soluble GAG secretion increased in chondrogenic versus control conditions. There was no marked difference in GAG secretion by MSCs from either cell source. **Conclusion.** Chondro-Gide and Alpha Chondro Shield were permissive to the incorporation and chondrogenic differentiation of human BM and AT MSCs. Chondro-Gide seeded with BM MSCs demonstrated the greatest increase in MSC number and deposition of a cartilaginous tissue.

## Keywords

bone marrow, adipose tissue, mesenchymal stem cells (MSCs), cell scaffolds, chondrogenesis

## Introduction

The regeneration of hyaline cartilage still poses a significant clinical challenge, with current available treatments resulting in a reparative tissue with inferior mechanical properties.<sup>1</sup> Cell therapy using autologous chondrocyte implantation (ACI) has been used to treat cartilage defects since 1987,<sup>2</sup> but has some disadvantages, such as the production of fibrous cartilage and donor site morbidity.<sup>3</sup> *In vitro* and preclinical animal studies suggest that multipotent mesenchymal stem or stromal cells (MSCs) can provide an alternative to autologous chondrocytes for the regeneration of cartilage, as they possess chondrogenic differentiation potential, are obtainable from a number of tissue sources

and can be culture expanded *in vitro* to provide increased cell numbers for transplant therapies.<sup>4,5</sup> Bone marrow (BM) is currently the most extensively studied source of MSCs. However, harvesting an adequate number of MSCs from

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BM is problematic because of the finite volume available at any one site. Hence, adipose tissue (AT) has recently been shown as an attractive alternative,<sup>6,7</sup> wherein 200 mL of lipoaspirate can readily be removed from patients, yielding  $4 \times 10^8$  nucleated cells of which more than 2% constitutes the MSC population.<sup>8,9</sup> The ready availability of AT MSCs is advantageous in autologous cell therapies as the time needed for costly culture expansion to generate a sufficient cell number for transplantation is considerably reduced when compared with BM. Moreover, harvesting AT through lipoaspiration makes AT MSCs an attractive cell source compared to more invasive and potentially painful iliac crest biopsies.

Whether or not AT MSCs are equivalent to BM MSCs in terms of their chondrogenic differential potential is a matter of considerable debate. Some studies have suggested that AT MSCs have inferior potential for chondrogenesis and hence use in cell therapies for cartilage repair,<sup>10,11</sup> while others have reported on successful multilineage differentiation of AT MSCs, including toward chondrogenesis.<sup>12,13</sup>

The aim of this *in vitro* study was to compare the incorporation, growth, and chondrogenic potential of BM versus AT MSCs in 2 commercially available cell scaffolds currently used for cartilage repair in humans, namely Chondro-Gide and Alpha Chondro Shield. *In vitro* studies have tested these scaffolds with BM MSCs and chondrocytes, but very little data are available on their use with AT MSCs in comparison.<sup>14,15</sup> Chondro-Gide (Geistlich Pharma AG, Wolhusen, Switzerland) is a bilayered scaffold, composed of type I and type III collagen, with one porous side for cell attachment and a compact side to prevent cell leakage, which has been extensively used in the clinic for autologous matrix induced chondrogenesis (AMIC) procedures and ACL.<sup>16,17</sup> Alpha Chondro Shield (Swiss Biomed Orthopaedics AG, Zurich, Switzerland) is intended to be used mainly as a cell-free cartilage implant to aid the migration and differentiation of mesenchymal progenitor cells from subchondral bone after a microfracture procedure. Alpha Chondro Shield is composed of fibers of polyglycolic acid (PGA) arranged in a homogeneous non-woven pattern; currently there is no clinical data available on its use with chondrocytes or MSCs, whether from BM or AT.

## Methods

Before commencement of the study ethical approval was obtained from the national review body (12/EE/0136 and 06/Q2601/9) and the study was conducted with the principles of the Declaration of Helsinki (World Medical Association).

## Isolation, Expansion, and Characterisation of MSCs

In total, MSCs were cultured from 3 BM donors (age range 19-80 years) and 4 AT donors (age range 27-75 years). BM was aspirated from the posterior superior iliac spine or harvested from excised femoral head during total hip replacement surgery. AT was harvested from the infrapatellar fat pad of the patients undergoing knee-reparative surgery. Mononuclear cells were isolated from BM aspirates by density gradient centrifugation using Ficoll-Paque Plus (GE Healthcare Life Sciences, Buckinghamshire, UK). AT samples were minced and treated with 0.1% collagenase type IA (Sigma; Poole, Dorset, UK) for up to 2 hours at 37°C and 5% CO<sub>2</sub>. After this enzymatic digestion, Dulbecco's modified Eagle medium (DMEM) supplemented with 20% (v/v) fetal calf serum (FCS) (PAA, Yeovil, Somerset, UK) was added to neutralize collagenase activity and the digest was centrifuged into a cell pellet, which was then subsequently washed in DMEM/F-12, supplemented with 10% (v/v) FCS, 1% (v/v) penicillin (50 U/mL), and streptomycin (50 µg/mL) (standard medium; all from PAA), and filtered through 70-µm cell strainers to remove undigested tissue. The BM mononuclear cells and adipose stromal vascular fraction (SVF) cells<sup>18</sup> were then plated out at a density of  $2 \times 10^7$  cells per 75 cm<sup>2</sup> flask in 20 mL of standard medium and incubated at 37°C in humidified atmosphere containing 5% CO<sub>2</sub>. After 24 to 48 hours, the nonadherent cells were washed off gently with phosphate-buffered saline (PBS; PAA) and the adherent cells were subsequently cultured until they reached approximately 70% confluence. Cells were routinely passaged at 70% confluence using 0.25% trypsin-EDTA (Life Technologies Ltd, Paisley, UK) and reseeded at  $10^4$  cells/cm<sup>2</sup> into fresh 75-cm<sup>2</sup> flasks to culture expand the adherent cell population. At passage II-III, culture expanded cells were characterised by their adherence to tissue culture plastic, by immunoprofiling for CD markers and by examining their differentiation potential to form osteoblasts, adipocytes, and chondrocytes. These criteria meet the MSC phenotype defined by the International Society for Cellular Therapy.<sup>19</sup>

## Cell Seeding Into Scaffolds and Chondrogenesis

Bone marrow MSCs and AT MSCs were seeded at a density of  $5 \times 10^4$  cells in 50 µL of standard culture medium per 9mm<sup>2</sup> piece of Chondro-Gide or Alpha Chondro Shield ( $n = 4$  scaffolds per MSC donor) in non-tissue culture coated plates. After 2 hours incubation at 37°C to permit cell adhesion to the cell scaffolds, an additional 1 mL of standard culture medium was added to each well. For initial assay of cell incorporation and growth, the cell-seeded scaffolds

were maintained in standard culture media for a period of 28 days. A further analysis to examine the comparative adhesion of MSCs to Chondro-Gide and Alpha Chondro Shield was performed using scanning electron microscopy, as follows: (1) the scaffolds were fixed in 2% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) for 2 hours; (2) the scaffolds were then dehydrated through a series of alcohols (20% to 100%) for 10 minutes in each solution; and (3) the scaffolds were dried overnight in hexamethyldisilazane (HMDS) and then gold sputtered and imaged using a Zeiss EVO10 scanning electron microscope (Carl Zeiss, Cambridge, UK).

In separate assays of chondrogenesis, the cell-seeded scaffolds were maintained in induction medium, consisting of DMEM (high glucose) supplemented with 2% FCS (Life Technologies Ltd), 100 nM dexamethasone (Sigma), 37.5 µg/mL ascorbate 2-phosphate (Sigma), insulin, transferrin and selenium (1% ITS-X; Sigma) and 10 ng/mL transforming growth factor-β1 (PeproTech Ltd., London, UK) (duplicate scaffolds for each MSC donor), or with control medium that contained carriers alone (duplicate scaffolds for each MSC donor) for the same period. Culture medium was replaced 2 to 3 times per week. The incorporation and viability of cells following these chondrogenesis experiments was assessed by DAPI counterstaining of sections of induced cultures harvested at day 28 and by Live/Dead staining, respectively. MSCs from 2 separate donors from both BM and AT were analyzed for the initial incorporation of cells into the cell scaffolds and MSCs from a further 2 separate donors of BM and AT were analyzed for the inductions of chondrogenesis.

### *Live/Dead Staining and Confocal Microscopy for Cell Viability/Growth*

Cell-seeded scaffolds were assessed for cell incorporation, viability and growth using Live/Dead cell staining according to the manufacturer's guidelines (Sigma), wherein live cells fluoresce green and dead cells fluoresce red. The staining procedure was performed in the dark for 30 minutes at 37°C and 5% CO<sub>2</sub>. Live and dead cells were visualized and scored by fluorescence imaging and confocal microscopy (Leica Microsystems DM6000B-SP57CS). This was performed by scoring the number of viable (green) and the number of dead (red) cells present in each of 4 fields of view taken through the depth of the scaffold over 2 separate regions for each MSC donor and each scaffold at each time point.

### *Dimethylmethylene Blue Assay for Glycosaminoglycans*

The dimethylmethylene blue (DMMB) assay protocol was adapted from previously published methods<sup>20</sup> as follows: (1) the DMMB dye solution was prepared by adding 3.04 g of glycine, 2.37 g of NaCl, and 16 mg of 1,9 dimethylmethylene

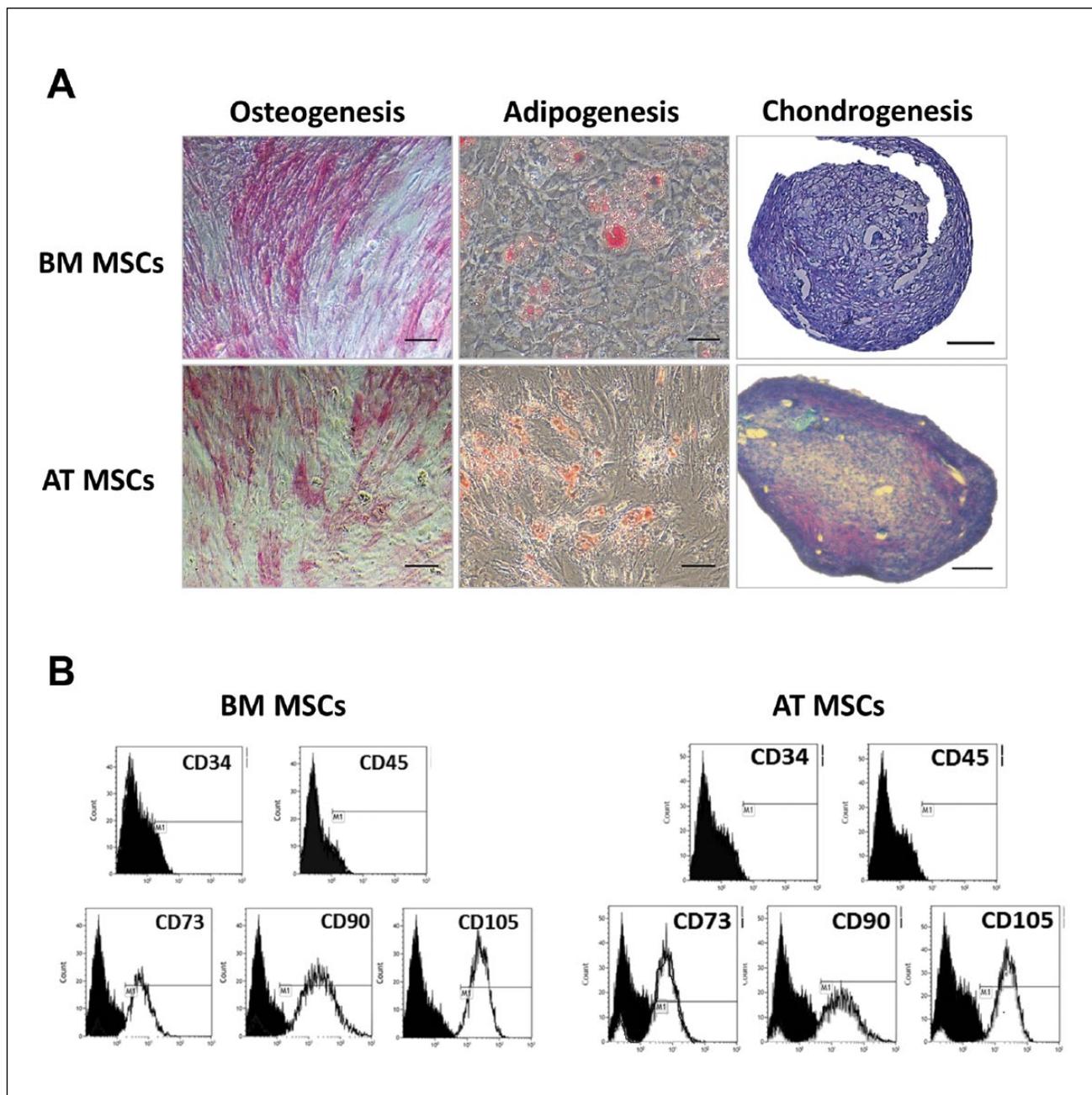
blue to 1 L of deionized water; (2) the pH was adjusted to 3.0 with hydrochloric acid and the reagent was stored in a brown bottle; (3) 50-µL aliquots of culture medium harvested from the cell-seeded scaffolds at day 28 were added in triplicate to a 96-well plate; (4) 200 µL of the DMMB dye was added and absorbance was assessed at 540 nm immediately. Chondroitin sulfate from shark cartilage (Sigma) was used to provide a standard curve (0-40 µg/mL) from which the glycosaminoglycan (GAG) content in the samples of medium was calculated. The levels of absorbance for GAG content in the samples of medium were normalized to account for the background absorbance resultant from the presence of phenol red within the medium. Replicate values from 2 independent donors of BM MSC versus 2 independent donors of AT MSCs for each experimental condition were pooled and GAG content has been presented as means ± standard deviations from these pooled data.

### *Histology and Immunohistochemistry*

Cell-seeded scaffolds were harvested at day 28 by fixation in 10% neutral buffered formalin for 24 hours, then processed and paraffin embedded. Toluidine blue staining was performed to reveal GAG content, whilst the presence of collagen type II was detected by immunolocalization as follows: (1) Antigen retrieval was performed by incubating sections in 0.1% hyaluronidase; (2) slides were incubated in a solution containing antibodies for collagen type II (6.5 µg/mL; CIIC1: Developmental Studies Hybridoma Bank, Iowa City, IA) for 60 minutes at room temperature (RT); (3) the omission of a primary antibody was used for control purposes; (4) slides were then incubated with a biotinylated secondary antibody for 30 minutes at RT; and (5) immunopositivity was revealed with a streptavidin-based tertiary step and 3,3'-diaminobenzidine (DAB) chromogen using a commercial labelling kit (Vector ABC Elite, Vector Laboratories Ltd, Peterborough, UK). Some sections of the harvested cultures were mounted in Vectamount containing DAPI (Vector Laboratories Ltd) to counterstain for cell nuclei.

### *Statistical Analysis*

Quantitative data for CD profiling have been presented as means ± standard error of the mean from 3 independent donors for BM MSCs and 3 independent donors for AT MSCs. Quantitative data for MSC incorporation, cell viability, and GAG content have been presented as means ± standard deviations, which derived from replicate values pooled from 2 independent donors for each MSC type, that is,  $n = 2$  BM MSC versus  $n = 2$  AT MSC donors for each experimental condition and time point.



**Figure 1.** Characterisation of bone marrow (BM) mesenchymal stem cells (MSCs) and adipose tissue (AT) MSCs. **(A).** Culture expanded and plastic adherent cells from BM and AT differentiated along mesenchymal lineages, as indicated by the presence of alkaline phosphatase positive osteoblasts or Oil Red O positive adipocytes in monolayer cultures and metachromatic staining for glycosaminoglycans (GAGs) in pellet cultures (scale bars represent 100  $\mu$ m). **(B).** Representative histograms are shown for positivity for CD markers in BM MSCs (left panels) and AT MSCs (right panels). The white histogram shows immunopositivity for each indicated marker, which is only clearly apparent when the extent of immunofluorescence is greater than that detected following immunolabeling with an isotype-matched control antibody, indicated by the black histogram.

## Results

### *Mesenchymal Stem Cell Characterisation*

Plastic-adherent stromal cells isolated and culture expanded from BM and AT differentiated down the three mesodermal lineages, as indicated by alkaline phosphatase staining for osteogenesis, Oil Red O staining of lipid vacuoles for adipogenesis and toluidine blue metachromatic staining of paraffin sections of cell pellets for chondrogenesis (**Fig. 1A**). Using flow cytometry, these cells were immunoreactive for MSC-specific cell surface antigens, that is, CD73, CD90, and CD105 and were not immunoreactive for non-MSC markers, that is, CD34 and CD45 (**Fig. 1B**). For BM MSCs,  $2.2\% \pm 0.3\%$  cells were CD34 positive,  $2.7\% \pm 0.7\%$  cells were CD45 positive,  $90.4\% \pm 5.1\%$  cells were CD73 positive,  $84.8\% \pm 4\%$  cells were CD90 positive, and  $97.8\% \pm 0.6\%$  cells were CD105 positive. For AT MSCs,  $3\% \pm 1.1\%$  cells were CD34 positive,  $3\% \pm 1.2\%$  cells were CD45 positive,  $92.4\% \pm 4\%$  were CD73 positive,  $90.4\% \pm 3\%$  cells were CD90 positive, and  $93.8\% \pm 2.8\%$  cells were CD105 positive.

### *Bone Marrow MSCs and AT MSCs Incorporated and Remained Viable in Chondro-Gide and Alpha Chondro Shield during Long-Term Cultures*

A greater number of MSCs from both tissue sources incorporated into Chondro-Gide than into Alpha Chondro Shield. Scanning electron microscopy at 30 minutes postseeding demonstrated that the cells more readily attached to the surface of the Chondro-Gide than to the surface of Alpha Chondro Shield (**Fig. 2A**). Many cells were lost during the cell seeding process. For the AT MSCs, only  $1.2\% \pm 0.1\%$  of cells were retained in Chondro-Gide compared with  $0.7\% \pm 0.4\%$  of cells retained in Alpha Chondro Shield at day 1. In comparison, for the BM MSCs only  $0.8\% \pm 0.4\%$  of cells were retained in Chondro-Gide and  $0.5\% \pm 0.4\%$  retained in Alpha Chondro Shield at day 1. Greater numbers of AT MSCs than BM MSCs appeared to incorporate in both of the scaffolds at day 1.

In longer term cultures, that is, from 7 days postseeding onward, both BM and AT MSCs appeared to become fibroblast-like in Chondro-Gide, whereas they also showed an elongated morphology and attached and spread along the length of the fibers in Alpha Chondro Shield (**Fig. 2B**). The number of viable MSCs from both cell sources increased from day 1 with further time in culture in Chondro-Gide. In contrast, the number of viable MSCs from either BM or AT did not increase in Alpha Chondro Shield (**Fig. 2C**). There were increased numbers of viable cells in Chondro-Gide compared with Alpha Chondro Shield from day 14 to day 28 for BM MSCs and from day 7 to day 28 for AT MSCs.

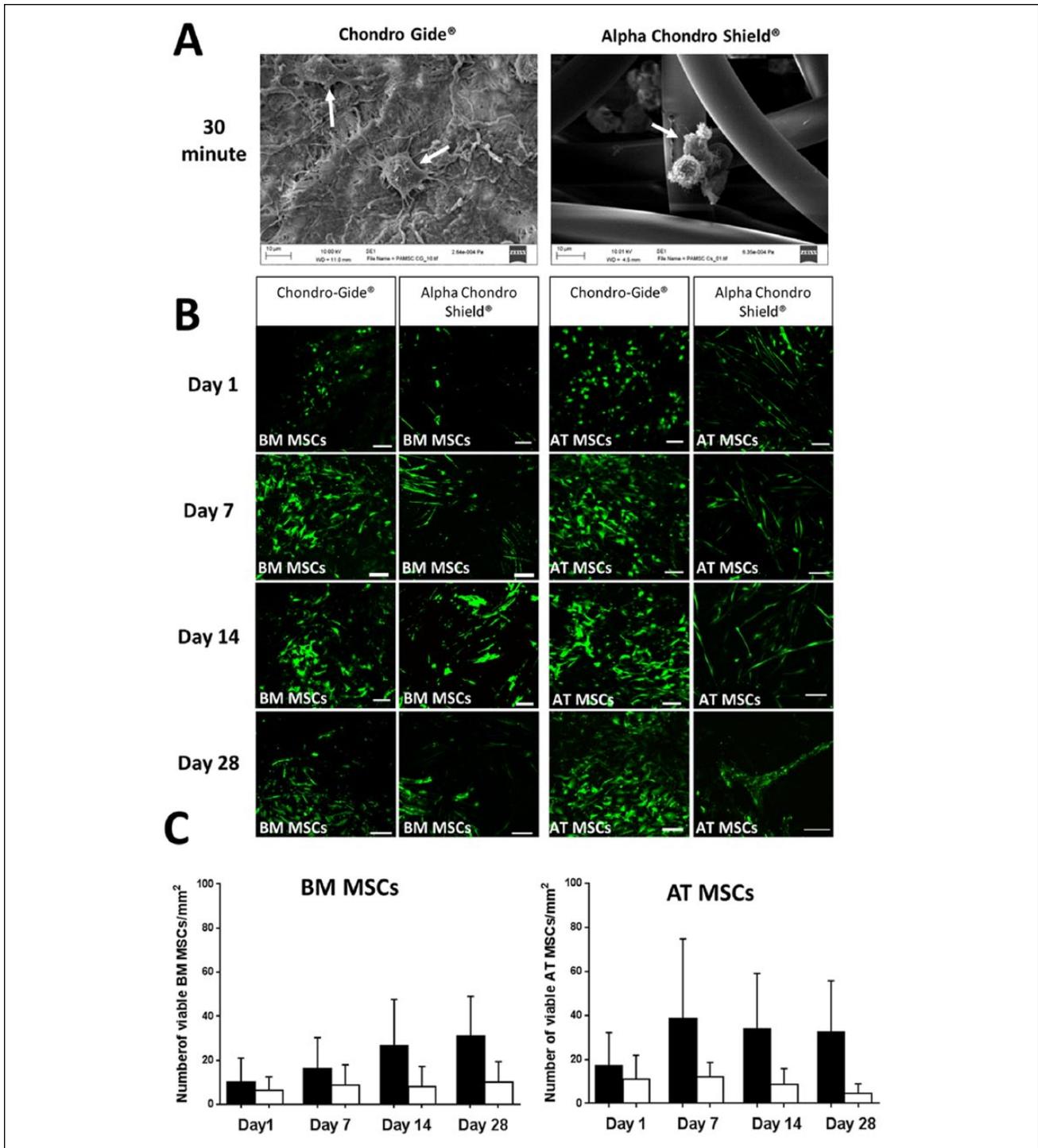
Fewer than 5% of cells were scored as nonviable (red) in both scaffolds and at all time points.

### *Mesenchymal Stem Cell Condensation under Chondrogenic Conditions in Chondro-Gide and Alpha Chondro Shield*

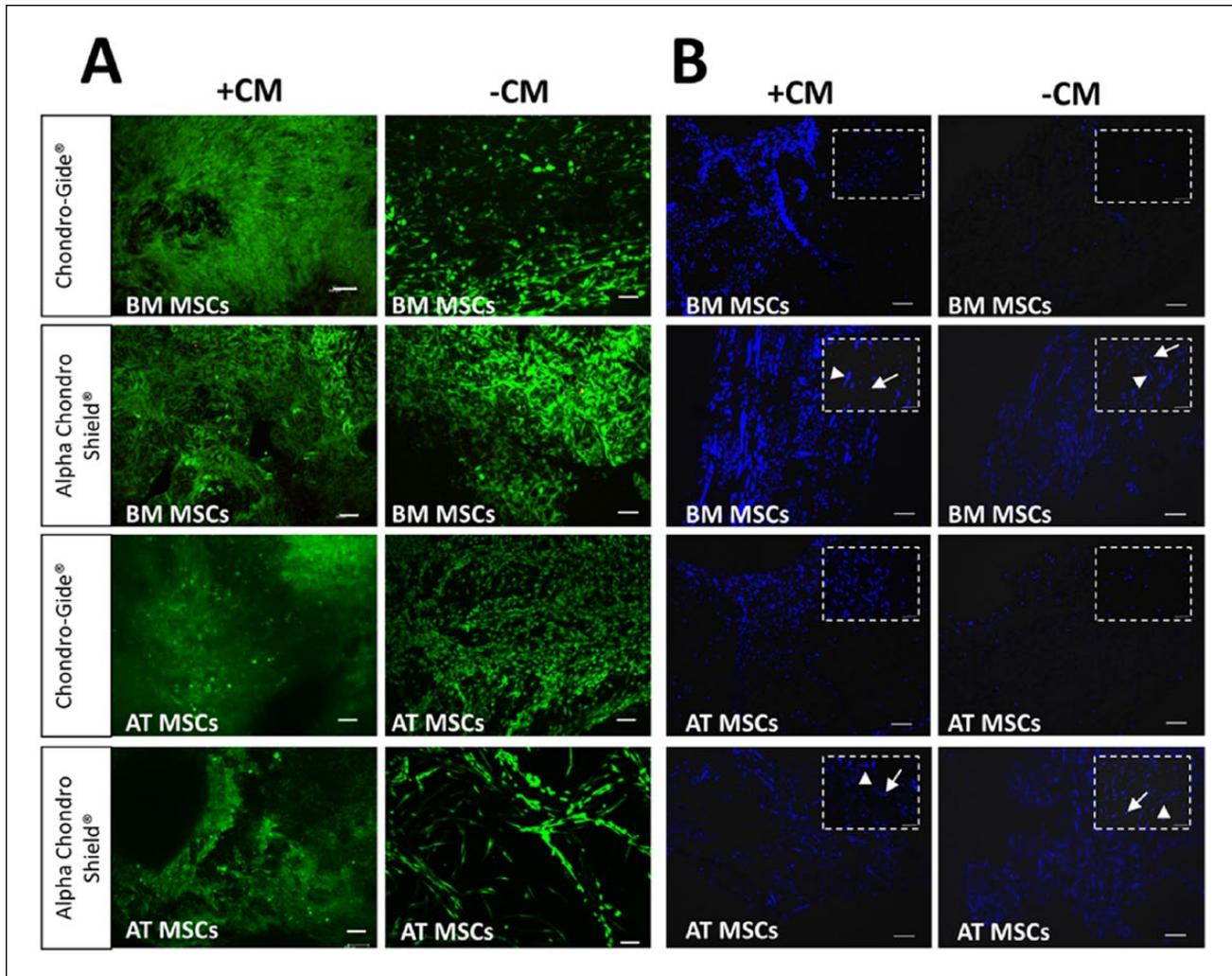
Under chondrogenic (+CM) and nonchondrogenic (-CM; control) conditions BM and AT MSCs remained greater than 95% viable over a 28-day culture in Chondro-Gide and Alpha Chondro Shield. Under chondrogenic conditions, there appeared to be a greater increase in the number of viable BM and AT MSCs in Chondro-Gide, where the cells appeared condensed and confluent compared with the less dense network of cells seen under control conditions. Because of this growth of cells, exact cell counts were not possible. In Alpha Chondro Shield, there also appeared to be an increase in the number of viable BM and AT MSCs under chondrogenic versus control conditions, with increased cell condensations apparent (**Fig. 3A**). The distribution of cells in these experiments was examined using DAPI staining of tissue sections from harvested cultures at day 28. This demonstrated that the MSCs were evenly distributed through the porous side of Chondro-Gide only, without any cells present within the nonporous side. In addition, there was an even distribution of MSCs within Alpha Chondro Shield (**Fig. 3B**).

### *Cartilage-Specific Extracellular Matrix Deposition in BM and AT MSCs Seeded Scaffolds Was Seen in Long-Term Cultures Following Chondrogenic Induction*

Histology and immunohistochemistry were performed to examine the deposition of cartilage-specific extracellular matrix (ECM) within MSC-seeded scaffolds. Metachromatic toluidine blue staining was seen in chondrogenic (+CM) treated scaffolds, which is indicative of the accumulation of GAGs. A greater amount of metachromatic staining in the ECM was seen in both BM and AT MSCs within Chondro-Gide compared with Alpha Chondro Shield. However, rounded cell morphologies surrounded by ECM, which is indicative of a mature chondrocytic phenotype was seen only in cultures of Chondro-Gide seeded with BM MSCs under chondrogenic conditions. None of the MSC-seeded scaffolds under non-chondrogenic conditions showed any ECM deposition. Collagen type II deposition was most markedly seen in Chondro-Gide cultures seeded with BM MSCs under chondrogenic conditions, with some collagen type II seen to a lesser extent in BM and AT MSCs in Alpha Chondro Shield, only under chondrogenic conditions (**Fig. 4A** and **4B**). Staining of human articular cartilage was



**Figure 2.** The incorporation and growth of bone marrow (BM) mesenchymal stem cells (MSCs) and adipose tissue (AT) MSCs in Chondro-Gide and Alpha Chondro Shield. **(A).** Representative images are shown of the appearance of AT MSCs following 30 minutes of incubation with Chondro-Gide and Alpha Chondro Shield. As shown, MSCs had already become firmly attached and spread out (arrowed) on Chondro-Gide, whereas they appeared mostly spherical in morphology and only projected 1 or 2 cell processes to attach to Alpha Chondro Shield. **(B).** Representative images are shown of BM MSCs (left panels) and AT MSCs (right panels) after Live/Dead staining. Scale bars represent 100  $\mu$ m. **(C).** The number of viable BM and AT MSCs in each cell scaffold over time. BM MSCs proliferated in Chondro-Gide over time. There were more BM MSCs in Chondro-Gide than Alpha Chondro Shield at 14 and 28 days in culture, whereas for AT MSCs this difference was noticeable after 7 days in culture. Data are presented as means  $\pm$  standard deviations. Black bars = Chondro-Gide, white bars = Alpha Chondro Shield.



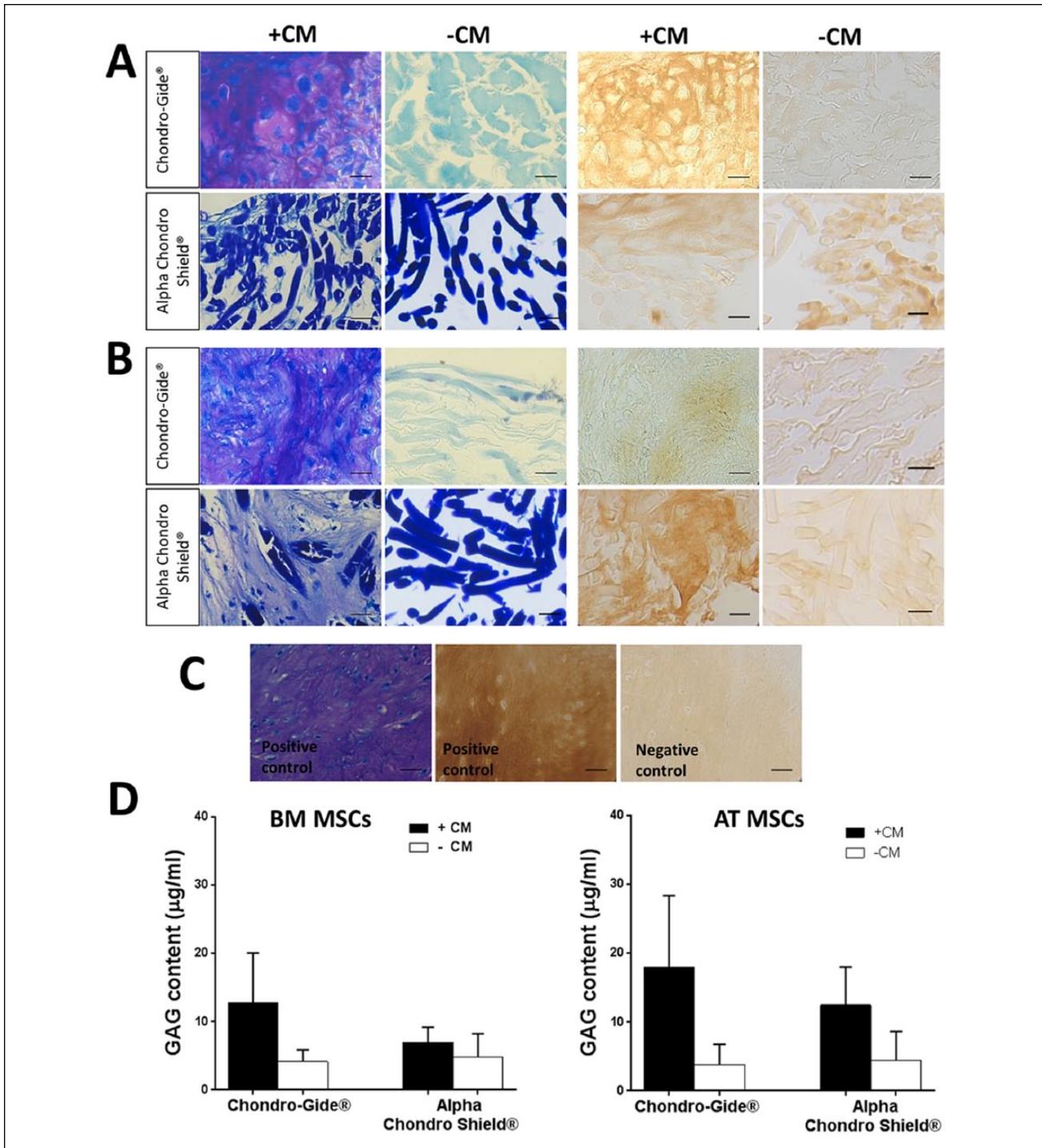
**Figure 3.** The viability of bone marrow (BM) mesenchymal stem cells (MSCs) and adipose tissue (AT) MSCs in Chondro-Gide and Alpha Chondro Shield under chondrogenic and nonchondrogenic conditions. **(A).** Representative images are shown of BM and AT MSCs at day 28 of culture in the presence (+CM) or absence (–CM, control) of chondrogenic inducers. More than 95% of MSCs were viable in Chondro-Gide and Alpha Chondro Shield under both conditions. Scale bar represents 100  $\mu$ m, digitized images of projected z stacks following confocal microscopy. **(B).** Representative images showing the distribution of BM MSCs and AT MSCs in Chondro-Gide and Alpha Chondro Shield after 28 days in culture under chondrogenic (+CM) versus control (–CM) conditions. Scale bars = 100  $\mu$ m for the main images and 50  $\mu$ m for the inset images. There was an even distribution of cells throughout the porous elements of both scaffolds. MSCs have been arrowed and the autofluorescence of the scaffold fibers in the Alpha Chondro Shield indicated by arrowheads.

used to demonstrate the specificity of the histological and immunohistochemical procedures (Fig. 4C).

#### *Higher Levels of Soluble GAGs Were Detected in Culture Medium under Chondrogenic versus Nonchondrogenic Conditions*

The presence of GAGs was analyzed in cell culture supernatants harvested at day 28, that is, from medium that was harvested from the last feed only (a period of 3

days). This biochemical analysis of GAG content showed that both BM and AT MSCs secreted markedly more GAGs in Chondro-Gide than in Alpha Chondro Shield. Under chondrogenic conditions, a greater amount of GAGs was released into the medium by BM and AT MSCs in Chondro-Gide and by AT MSCs in Alpha Chondro Shield compared with control conditions. For BM MSCs in Alpha Chondro Shield, an increase in soluble GAGs was also detected under chondrogenic conditions (Fig. 4D).



**Figure 4.** Histology and immunohistochemistry of mesenchymal stem cell (MSC)-seeded Chondro-Gide and Alpha Chondro Shield. (**A** and **B**). There was greater matrix deposition in Chondro-Gide cultures with bone marrow (BM) MSCs (**A**) and adipose tissue (AT) MSCs (**B**) than Alpha Chondro Shield, as shown by increased toluidine blue staining and collagen type II immunolocalization under chondrogenic conditions (+CM) compared with control conditions (-CM). Alpha Chondro Shield seeded with BM and AT MSCs only showed some localized positivity of collagen type II, which was not seen in control conditions. (**C**) A section of human knee cartilage was used as a control for both toluidine blue and collagen type II immunostaining. Scale bars represent 25 µm. (**D**) A greater level of soluble glycosaminoglycan (GAG) was detected in the culture supernatants of Chondro-Gide cultures seeded with BM MSCs and AT MSCs at day 28 under chondrogenic conditions (+CM) compared with control conditions (-CM). Data are presented as means  $\pm$  standard deviations.

## Discussion

To date, there are limited clinical data available for the use of MSCs in cell-based cartilage repair therapies. One potential reason is the lack of robust *in vitro* data demonstrating their chondrogenic differentiation potential in clinically available scaffolds. This study has suggested that Chondro-Gide provides a more suitable environment than Alpha Chondro Shield for the culture and chondrogenesis of MSCs and the formation of a cartilaginous tissue. MSCs isolated and culture expanded from BM and AT underwent chondrogenic differentiation in response to chondrogenic inducers. This was evident in both scaffolds but was most marked in Chondro-Gide cultured with BM MSCs.

Bone marrow MSCs and AT MSCs were initially more readily incorporated into Chondro-Gide than Alpha Chondro Shield. One reason for this greater incorporation of cells within Chondro-Gide, which is composed of natural type I and type III porcine collagen, could be the ability of MSCs to bind to the scaffold through integrin receptors, specifically  $\alpha 2\beta 1$  integrins, which is the major receptor for type I collagen and other fibril-forming collagens.<sup>21</sup> In contrast, Alpha Chondro Shield is a synthetic scaffold of pure PGA that lacks specific cellular adhesion sites, which may explain how the adhesion of cells to the scaffold was minimal. The morphologies of MSCs attached to Chondro-Gide and Alpha Chondro Shield revealed by scanning electron microscopy at an early time point postseeding would support such an interpretation. The synthetic nature of Alpha Chondro Shield may facilitate its adaptation to increase MSC incorporation, for example, other researchers have used the integrin-binding peptide Arg-Gly-Asp (RGD) into polymer-based scaffolds to facilitate cell adhesion.<sup>22,23</sup> Within Chondro-Gide, the increased retention and growth of cells is also possibly because of the decreased porosity of the scaffold caused by the presence of a compact surface that functions to prevent cell leakage. In contrast, Alpha Chondro Shield consists of large interconnected pores that aim to encourage cell growth and attachment *in vivo*, where blood clot formation from associated microfractures likely helps retain cells. Previous studies have shown that with time such porous scaffolds in fill with cartilaginous ECM deposition in synchrony with the degradation rate of the scaffold.<sup>24,25</sup> However, in the current *in vitro* study the increased porosity of Alpha Chondro Shield may have allowed cells to have escaped the scaffold.

The differences in MSC proliferation that were observed between the 2 scaffolds may be attributed to their differing degradation rates. BM MSCs proliferated in Chondro-Gide throughout the time course. Conversely, there was no increase in BM MSC numbers in Alpha Chondro Shield with time in culture. The collagens in Chondro-Gide are slow to degrade compared with PGA fibers in Alpha Chondro Shield, which begins to lose mechanical integrity

over a 12-day period<sup>26</sup> and degrades to about 50% of its initial mass by 28 days.<sup>25</sup> In the absence of matrix production or a blood clot (generated *in vivo*), it is likely that the fast degrading Alpha Chondro Shield does not provide a suitable environment for cells to grow and proliferate and, therefore, this results in cell loss. In addition, while PGA-based scaffolds provide a good substrate for chondrocyte adhesion, cell proliferation during long-term cultures may be significantly affected by acidic products during scaffold degradation.<sup>27</sup>

Overall, cell retention in both scaffolds was poor, with only approximately 0.1% to 1.5% of the MSCs attaching to the scaffolds following a 2-hour incubation period. The effectiveness of the cell-seeding process is a crucial step, which could have a significant effect on the number of cells delivered to a cartilage lesion and thus the clinical outcome of any cell therapy. For MACI procedures, chondrocytes preseeded onto Chondro-Gide have been grown for 3 days prior to implantation,<sup>28</sup> whereas ACI procedures have been adapted to preseed Chondro-Gide with chondrocytes for a recommended time of only 10 to 15 minutes prior to transplant.<sup>29</sup> Studies have previously examined the use of spinner flasks to encourage more efficient cell seeding in porous scaffolds<sup>26</sup> or of using polymerizing gels as a delivery vehicle for rapid cell seeding within collagen sponges.<sup>30</sup> However, in this study, a simple cell-seeding strategy was used to replicate the clinical setting, with the results probably representing the best case scenario given an incubation period that is in excess (2 hours) of what would be clinically acceptable. The small size of the scaffolds used in this study may have contributed to low incorporated cell numbers as they could have been of insufficient size to initially retain the total volume of medium used for cell seeding. Hence, cells may have initially leaked out of the scaffolds into the wells. Although a potential weakness of the study, this scenario commonly reflects the clinical situation. Of the two cell sources, there was better incorporation of AT MSCs into both scaffolds compared with BM MSCs. If AT MSCs are shown to incorporate into cell scaffolds more readily than BM MSCs, following analysis of increased numbers of MSC donors, there may be reasons for such differential incorporation. A recent review suggested that AT MSCs express greater levels of integrin  $\alpha 4\beta 1$  (CD49d) compared with BM MSCs.<sup>31</sup> The  $\alpha 4\beta 1$  integrins have long been known to play a role in cell-cell and cell-matrix interactions<sup>32</sup> and one of the ligands for CD49d is fibronectin,<sup>33</sup> which is present in serum-containing medium.<sup>34</sup> Hence, increased retention of AT MSCs compared with BM MSCs within Chondro-Gide may be due, in part, to the adsorption of serum proteins especially fibronectin<sup>35</sup> to the scaffolds via interaction with  $\alpha 4\beta 1$  integrins. Further research is required to examine more MSCs donors from both tissue sources to ensure the reproducibility of our observations and to examine mechanisms of increasing the efficiency of

cell seeding into these scaffolds and their optimal application in cell transplantations for cartilage repair.

It is known from previous studies that scaffold structure and properties like porosity, pore size, fiber thickness, topography, and scaffold stiffness directly influence cell behavior and colonization (reviewed by Lawrence and Madhally<sup>36</sup>). The cells in the Chondro-Gide scaffold attained a fibroblast-like morphology and showed progressive penetration throughout the collagen network. This has also been shown by Nuernberger *et al.*<sup>37</sup>, where the dense network of collagen fibers supported a flattened cell shape of chondrocytes. In the more fibrous Alpha Chondro Shield, the cells showed a mixed morphology of both round and elongated cells. These differences in cell morphology in similar types of cell scaffolds have been observed by Schlegel *et al.*<sup>38</sup> in 2008 using chondrocytes. The chondrocytes developed a fibroblast like morphology when seeded onto a type I/type III collagen scaffold, whereas the chondrocytes showed a mix of round and elongated morphologies on a scaffold composed of hyaluronic acid.<sup>38</sup> Adoption of a spherical morphology by MSCs and chondrocytes in 3-dimensional culture can influence their synthesis of cartilage-specific ECM components,<sup>39-41</sup> although it is unclear if cell shape is a critical factor in influencing chondrocyte differentiation.<sup>42</sup> In this study, the presence of round-shaped MSCs in Alpha Chondro Shield was not associated with increased chondrogenic differentiation compared with less rounded cell morphologies seen in Chondro-Gide, which proved to be a superior scaffold for chondrogenic differentiation.

We observed increased collagen type II and GAG deposition in the ECM of BM MSC-seeded Chondro-Gide cultures compared with AT MSC-seeded Chondro-Gide cultures. This indicates that BM may be a better source of MSCs for cartilage repair than AT. However, a potential weakness of this study was that the AT MSCs were derived from infrapatellar fat pad, which, although of use as an intraoperative cell source in the treatment of cartilage defects may be an inferior donor tissue, because cells from the damaged or diseased articular environment can possess pro-inflammatory characteristics.<sup>43</sup> Ideally, AT MSCs from peripheral fat sources and BM obtained from the same donor should have been compared to better establish the potential of these cells for chondrogenesis in autologous cell therapy for cartilage repair. In addition, TGF- $\beta$ 1 was used as an inducer for chondrogenic differentiation of MSCs from both cell sources, which may not be ideal for inducing chondrogenic differentiation in AT MSCs. For example, some studies have previously reported that TGF- $\beta$ 1 is not as efficient at inducing chondrogenic differentiation of AT MSCs as bone morphogenetic protein 6.<sup>44,45</sup> Moreover, the faster degradation rate of Alpha Chondro Shield makes it difficult to undertake long-term cultures in order to compare chondrogenic differentiation of BM MSCs

and AT MSCs. Future *in vitro* studies to examine chondrogenesis with this scaffold should focus on improving culture conditions for longer term analysis, as well as using advanced and effective cell-seeding procedures.

In conclusion, this study has demonstrated that BM MSCs and AT MSCs undergo chondrogenic differentiation *in vitro* in cell scaffolds that have been used clinically for cartilage repair. On the basis of cell growth and ECM deposition, the use of BM MSCs with Chondro-Gide is favored. However, further study is required to test the potential of these different cell types and scaffolds for cartilage repair *in vivo*.

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### Authors' Note

All the work presented was conducted at the School of Life and Health Sciences at Aston University and the Department of Musculoskeletal Pathology, Royal Orthopaedic Hospital, Birmingham, UK.

### Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

### Ethical Approval

Ethical approval was obtained from the national review body (12/EE/0136 and 06/Q2601/9).

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