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Use of native Mesenchymal Stromal Cells from the knee joint towards regenerating articular cartilage.

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Abstract

The existing technique for the use of stem cell based therapies for joint repair are based on time-consuming laboratory culture expansion protocols that are also expensive. The purpose of this thesis was to devise ways to exploit the discovery of synovial fluid mesenchymal stromal cells (MSC) towards the cost effective development of one stage cellular therapy development for knee osteoarthritis (OA). This work is based on the hypothesis that synovial fluid (SF) MSC that originate from the synovium, can be greatly increased in the joint cavity using a novel device. Secondly, it was hypothesised that knowledge of the joint *in vivo* environment following a microfracture procedure could be used to facilitate MSC adherence to, and migration towards a biological scaffold, similar to a microfracture, as a first step towards novel cartilage regeneration therapy development. First, a MSC releasing device was developed for effective cell release and minimal entrapment within the device. Secondly, using biological scaffolds, in an *in vitro* model, MSC were capable of adhering to, and migrating toward the scaffold, and that the scaffold composition could play an important function on migration. Finally it was shown that standard joint arthroscopy, washes resident joint fluid MSC away. It was then demonstrated in the laboratory that synovial brushing does indeed release cells that have the MSC immunophenotype (CD73, CD90, CD105) following culture expansion. In addition, released MSC demonstrated good chondrogenic, adipogenic and osteogenic potentials, further endorsing their use for articular cartilage repair. A prototype device was tested in humans and was shown to significantly increase fluid MSC *in vivo* in man. The thesis findings, demonstrate MSC augmentation, which provides the basis of testing novel regenerative strategies, from bench to operating theatre in orthopaedics, and supports the objective of a one stage cost effective procedure for joint repair.

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Chapter 1

1. Introduction

1.1 An introduction to the Knee Joint

The knee is a complex joint, comprising of bone, soft tissue and a neurovascular supply. It is the largest joint in the human body, consisting of articular surfaces between its three bones (femur, tibia and patella), two menisci and a number of large ligaments including the cruciates. The knee joint belongs to a family of joints known collectively as synovial joints, of which the knee is classed as a condyloid type (Moore *et al*, 2015). The hyaline cartilage aids in a smooth, painless joint movement. The knee joint synovium is involved in a variety of functions including lubrication and nutrient exchange (Moore *et al*, 2015) (see Figure 1).

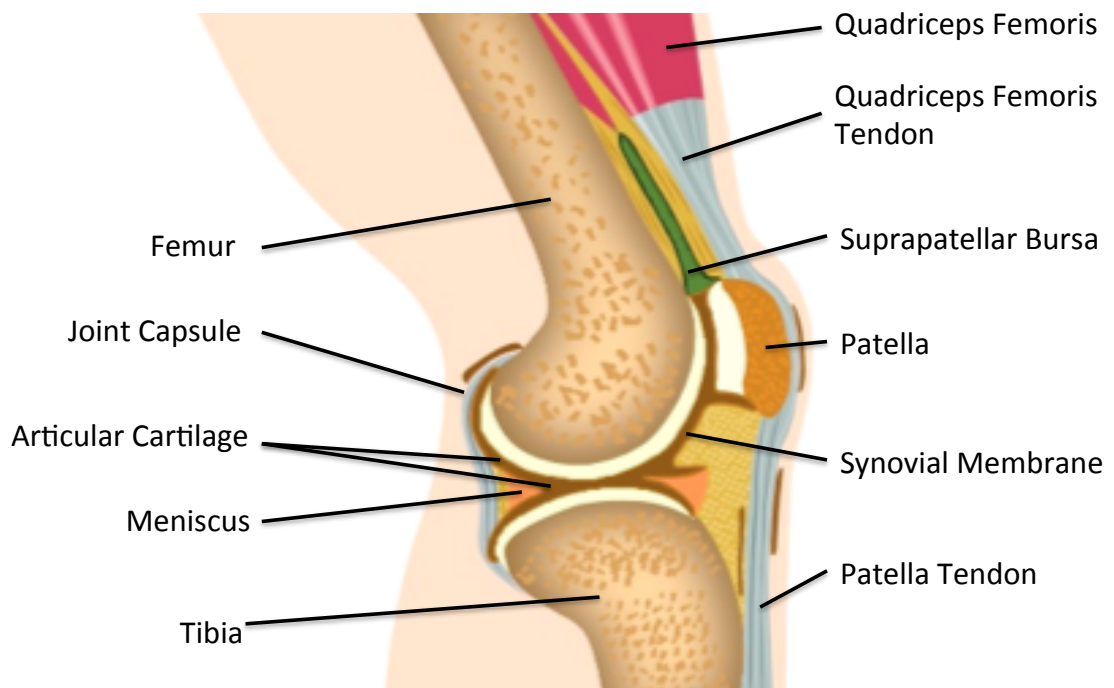


Figure 1: **The knee joint.** Diagram of the knee joint, including bone, and soft tissue components (original diagram adapted from: www.slideshare.net).

1.2 An introduction to the knee joint synovium

The synovium is a membranous connective tissue, lining the inner surface of the joint capsule. It comprises of lymphatics, nerve and blood vessels supply. The membrane is divided into two layers; the inner layer, known as the intima, and the outer layer know as the sub-intima (Stacey *et al*, 2007) (see Figure 2).

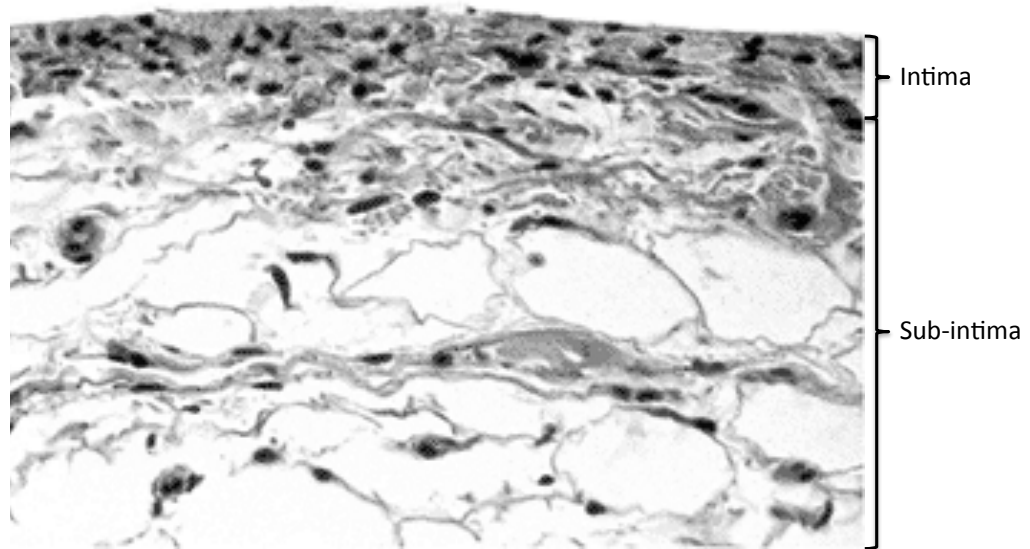


Figure 2. **Normal synovium.** Intima and sub-intimal layers, Image adapted from Stacy *et al*, 2007.

The sub-intimal region contains the blood and lymphatic supply of the membrane, which are involved in nutrition of joint structures such as the cartilage. Both layers of the synovial membrane act collectively to create a sealed system, this structure keeps the synovial fluid closed within the capsule, thus allowing for nutrient exchange. In addition, this system also allows for large compressive forces to be dissipated (Smith *et al*, 2011). Of particular relevance is that the synovium and the articular cartilages are embryologically derived from the same limb bud joint interzone region (Koyama *et al*, 2008). The knee joint synovium is particularly large due to the large suprapatellar pouch recess, which will be shown later in this thesis to be key to novel therapy development for stem cell based repair.

1.3 An introduction to synovial fluid (SF)

The ultrafiltration of blood plasma, coupled with hyaluronin, glycoproteins, lubricin, proteinases and collagenases contribute to creating SF. The unique composition of SF creates an environment where the SF viscosity contributes resistance against shear and compressive forces, in addition to providing vital lubrication of the joint lining, and nutrients and a supply of oxygen to important structures such as the cartilage (Blewis *et al*, 2007).

1.4 An introduction to cartilage

Cartilage is the connective tissue that plays a vital role within the body especially in stress resistance at the ends of bones: within a healthy knee the cartilage layer can range between 3-4mm in thickness. There are three types of cartilage, hyaline, fibro and elastic cartilage. Hyaline cartilage is the type that is found on the terminal ends of long bones within the joint cavity. Its structure allows it to provide resistance to compression and shear forces. Chondrocytes secrete the extracellular matrix, which consist of aggrecan or proteoglycan, which attract large volumes of water. The extracellular matrix also consists of collagen fibres that provide the tensile strength. As the chondrocyte produces the extracellular matrix, they themselves become engulfed within it. At this phase the fully matured chondrocytes develop and entrapped within a pit known as a lacunae (see Figure 3).

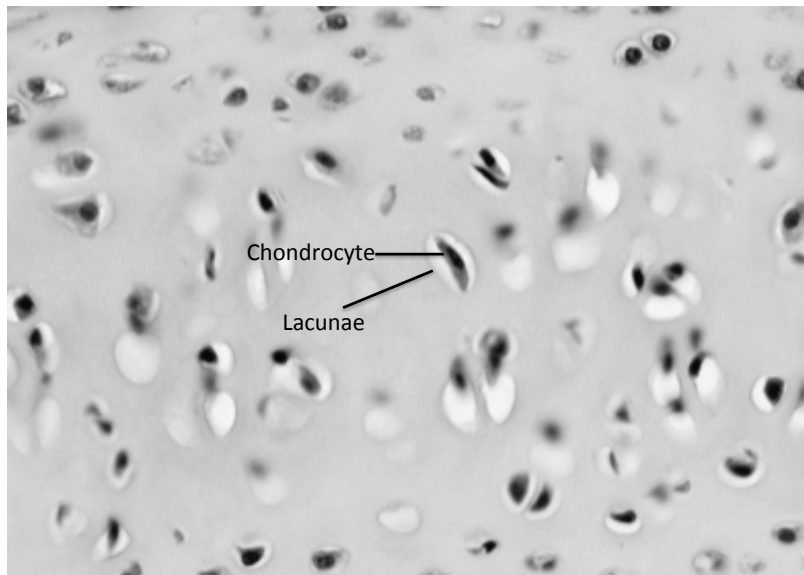


Figure 3: **Histology of a hyaline cartilage 1.** Section of hyaline cartilage, showing a chondrocytes, entrapped within a lacunae pit (original image adapted from www.pinterest.com)

Depending on the combination of key components such as, collagen, elastin fibres and proteoglycan, the type of cartilage present is determined. Within the knee, articular surfaces, hyaline cartilage is present; however, in the knee other types of cartilage are also resident, in structures such as the medial and lateral meniscus, where fibrocartilage predominates. The growth of hyaline cartilage is unique, as it is an avascular structure, and, thus, regeneration involves diffusional processes (Buckwalter *et al*, 2005). Indeed, growth of hyaline cartilage has been described in two differing ways: 1) interstitial growth in which chondrocytes divide and produce more extracellular matrix, and 2) appositional growth, where chondrocytes produce new layers of matrix from the top down (Hayes *et al*, 2001). This combination of growth provides hyaline cartilage with a layered structure, separated into zones. Articular hyaline cartilage comprises four zones; superficial, transitional, deep and the calcified zones, which are placed on top of the subchondral bone region (see Figure 4).

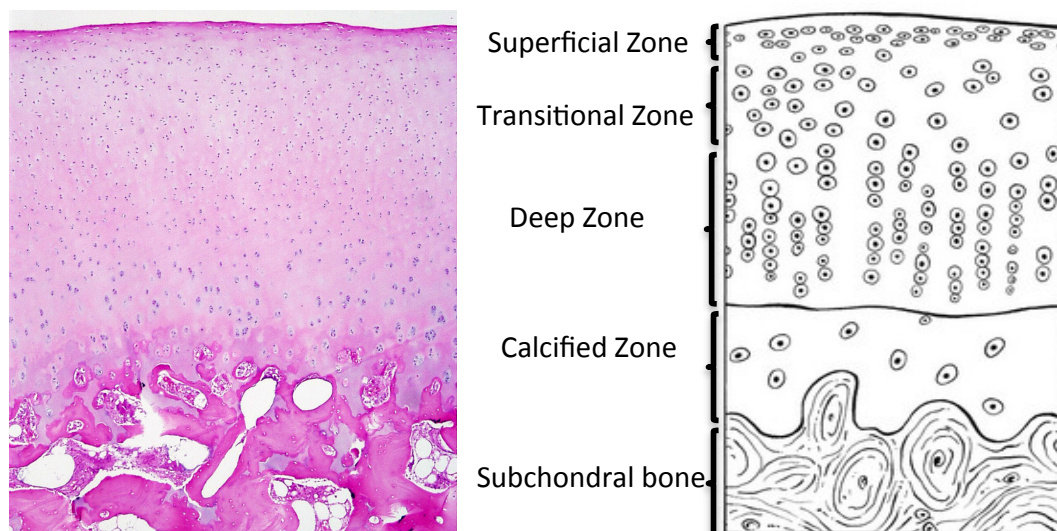


Figure 4: **Histology of a hyaline cartilage 2.** Four zones of hyaline articular cartilage; superficial, transitional, deep and the calcified zones, placed on top of the subchondral bone region. (original image adapted from clinialgate.com)

The superficial zone consists of two sub-layers, the upper layer has fibrillar sheet of collagen fibrils, and polysaccharide deposits, and the lower layer is the cellular layer, comprising of flattened chondrocyte cells. The superficial layer is the thinnest layer of the cartilage: however, due to its relative high concentration of collagen, it provides the ability to maintain smooth gliding of opposing terminal bone ends when a shear force is applied, this layer is vulnerable to changes with age (Gupta *et al*, 2006). The transitional layer contains mainly proteoglycans and some round chondrocytes. This

unique combination allows this layer to transition from shearing forces to compressive forces.

The largest layer of the articular cartilage is the deep layer, which is key in handling compressive forces, and this layer sits perpendicular to the subchondral plate, which contains the calcified cartilage layer. This region contains the tidemark layer, which is the actual boundary between cartilage and bone. The deep non-articular layer is the location where hyaline cartilage calcifies when it matures (Fox *et al*, 2009).

Due to the limitation of the knee joint blood supply, coupled with the avascular nature of cartilage, repair of injuries to the articular cartilage may be challenging, compared to other soft tissue structures. Injury or minor disruption to the homeostasis between the articular surface, synovium and synovial fluid, and other soft tissues (i.e. meniscus) may trigger a chain of events leading to the condition known as osteoarthritis (OA). Historically it was thought that articular cartilage was not capable of joint repair (Grande *et al*, 2013). Nevertheless, despite the apparent lack of vascularity there is increasing evidence from experimental and clinical settings that knee joint cartilage repair occurs (Koshino 2003; Grande 2013).

1.5 An introduction to osteoarthritis

Osteoarthritis (OA) from a pathophysiological perspective is a degenerative joint disease (Goldring *et al*, 2010). OA is a common condition in the UK, and up to 8.75 million people were treated for this condition in 2015, of which more than 50% are related to knee OA. OA is recognised to be a disease of the elderly population, mainly affecting people from 60+ years old. Currently 164,000 knee and hip replacements are undertaken in the UK per year, at the point when the disease is at its most destructive, costing the National Health Service (NHS) in the region of £1 billion (Arthritis Research UK). This disease is not limited to just the physical symptoms of pain for individuals, it also can play a role in the disruption of patients' psychosocial state, (Wise *et al*, 2010) which can increase the burden to the health service.

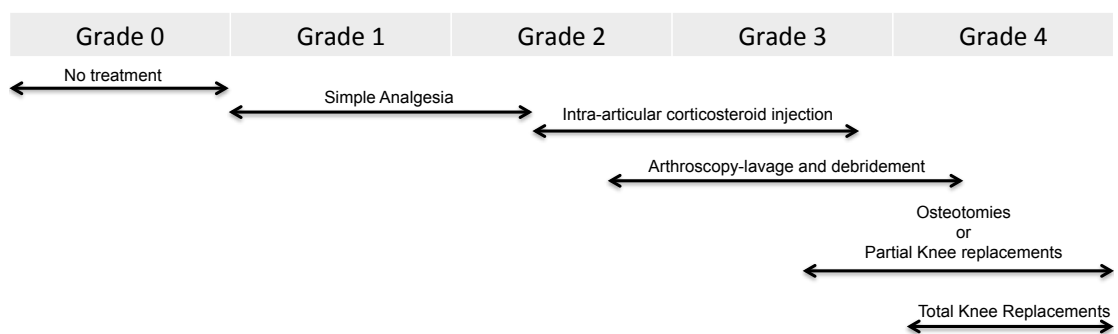
Early knee OA can be triggered from many causes; however, the main trigger is thought to be an injury causing an isolated condylar injury to one of the articular components of the knee, such as cartilage, meniscus, anterior or posterior cruciate ligaments or an intrarticular fracture (McKinley T, Giannoudis P, *et al*. 2010). These types of injury lead to a process of destruction and inflammation, which ultimately leads to end-stage OA. Aside from pain during a severe traumatic injury, initial symptoms of

knee OA, may present as stiffness, which gradually leads to pain. Pain intensity will gradually escalate, due to mechanical load imbalance, as the patient's gait is altered, known as an antalgic gait. As the disease progresses, subchondral inflammation occurs which leads to the development of subchondral cysts, common in advanced end-stage OA (Roemer *et al*, 2009). Indeed, end-stage OA is marked by abrasion of the cartilage. Joint effusion and subchondral bone bruising may arise, which lead to further pain and ongoing joint damage (van Dijk *et al*, 2010). Coupled with an increased life expectancy in Western populations, OA represents a growing healthcare burden (van Dijk *et al*, 2010; Adatia *et al*, 2012; Chen *et al*, 2012; Barry *et al*, 2013). Clinically, knee OA is classified using radiological visual grading, known as the Kellgren-Lawrence scale that was first proposed in 1957. There are 5 grades, from 0 = no radiographic features of OA, to 4 = extensive OA, comprising of osteophytes, marked joint space narrowing, and severe sclerosis (Emrani *et al*, 2008). Whilst joint replacement surgery is effective for established OA there is a lack of therapies available to combat early stages of the disease.

1.6 Current treatment options for early and advanced knee osteoarthritis

The table below outlines a generalized view of OA progression, using the Kellgren-Lawrence grading system combined with current treatments available, treatment option may vary according to local NHS protocols.

Table 1: An overview of current treatment options for OA



Initially patients are treated with oral analgesia, including paracetamol and codeine or non-steroidal anti-inflammatory drugs. Collectively, these strategies may alleviate pain but make no difference to the underlying disease process. Analgesia coupled with physiotherapy for quadriceps muscle strengthening and sensible weight loss, help

retain joint mobility, and may allow the patient to maintain good quality of life, this can lead to a delay in the progression of the disease. Intra-articular corticosteroid injections have also played a useful role, by controlling the initial inflammatory process, with patients showing transient improvement in function (Bellamy *et al*, 2005). Arthroscopic lavage and debridement is a minimally invasive surgical procedure to visualise the knee cavity to remove inflamed and damaged tissue; however, there is no long term benefit, as patients inevitably develop OA (Acebes *et al*, 2009; Rodriguez-Merchan *et al*, 2010; Thorlund *et al*, 2015).

1.7 Surgical techniques for joint regeneration

When patients present with isolated condylar injuries, a surgeon can use procedures such as microfracture, autologous chondrocyte implantation (ACI), osteochondral autograft transplantation/transfer system (OATS) and osteochondral allograft transplantation (OCA), in an attempt to restore and repair cartilage defects. Microfracture is a technique that was developed in the early 1990's, by Steadman (Steadman *et al*, 1999; Steadman *et al*, 2002; Mithoefer *et al*, 2009). It is an intermediate minimally invasive surgical technique for professional athletes with small (usually less than 2 cm) lesions within the femoral condyles. During knee arthroscopy, the surgeon identifies the lesion within the cartilage, and once the lesion has been debrided, the surgeon uses an awl to create small holes down to the subchondral bone and this is associated with subsequent repair tissue formation. It was considered for many years that this allowed blood and bone marrow mesenchymal stromal cells (BM MSC), that have cartilage repair potential, to migrate into the lesion (Neumann *et al*, 2008). The cartilage regeneration during microfracture is invariably fibrocartilage rather than articular cartilage. Patients that have undergone microfracture usually return within two years with pain recurrence. This is usually due to the fibrocartilage degeneration, in addition to delamination of the surrounding cartilage (Mithoefer *et al*, 2009) (see Figure 5).

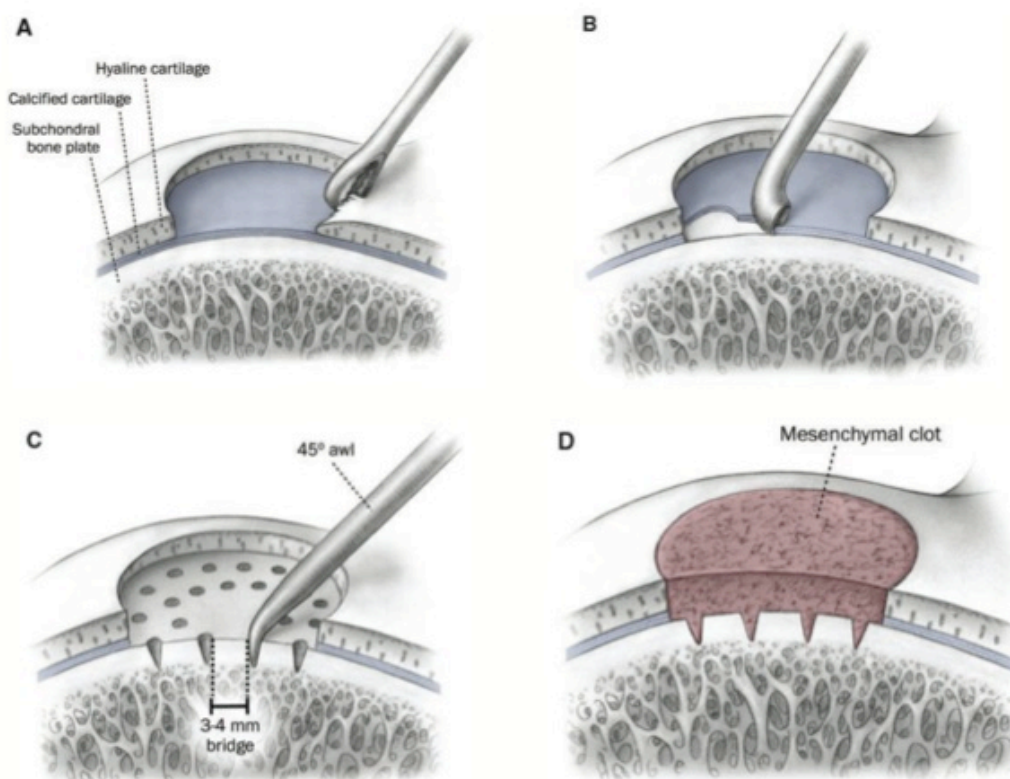


Figure 5: **Microfracture**. Steps in creating a microfracture. (A) Debridement of edges to the cavity. (B) Removal of unwanted cartilage layer. (C) Penetrating the subchondral bone, to create microfractures. (D) Filling of blood creating a clot (with BM MSC) which eventually become fibrocartilage (Mithoefer *et al*, 2009; Makris *et al*, 2014).

Brittberg and Peterson first introduced ACI in 1987 for treating high-level athletes (Viste *et al*, 2012), it provides an expensive alternative to microfracture, and is not currently available as a treatment option within the NHS. It involves a two-step surgical procedure where a sample of normal hyaline cartilage, approximately 200-300 mg in weight, is removed from a non-weight bearing region; chondrocytes are isolated and expanded over a period of up to six weeks within the laboratory, following which a second surgical procedure to re-implant the chondrocytes is performed. Studies comparing ACI and microfracture show that there is little benefit in undergoing ACI compared to microfracture (Knutsen *et al*, 2007).

Second generation ACI have been developed, where chondrocytes harvested from non-weight bearing regions of the knee (via arthroscopy) are loaded and cultured onto a three-dimensional biocompatible scaffold, commonly referred to as a matrix-induced autologous chondrocyte implantation (MACI) (Jacobi *et al*, 2011). These are used for larger technically difficult defects.

The OATS procedure replaces small defects that would not be suitable for microfracture, with healthy cartilage from a non-weight bearing surface region of the patients' knee (from the same knee). In larger cartilage defects, or when there are previous failed attempts at either microfracture, ACI or OATS, then OCA can be used successfully (Gracitelli *et al*, 2015). Donor articular surface from a cadaveric source is used. During the procedure the surgeon will use arthroscopy to visualise and remove damaged articular tissue, followed by insertion of a section of donor articular cartilage, including a section of the underlying bone, as this allows for anchorage of the "plug" replacement tissue to the recipients defect. This technique is useful to fill defects that are moderately sized, usually more than 2cm diameter, and has the benefit of no graft site morbidity; however, cadaveric sources grafts, in themselves, also have limitations. Complications can range from simple tissue mismatching to more serious implications, such as disease transmission, and tissue integration complications, thus leading to tissue rejection (Torrie *et al*, 2015). Most patients that undergo arthroscopy will, at some point, require further surgery in the form of knee osteotomy and eventually a total knee replacement (TKR).

1.8 Non cellular based treatments for OA

For advanced OA of the knee, osteotomy is a technique that aims to realign arthritic damage by changing the patient's axial load in the joint. In essence, it is a technique that involves removal of a "wedge" shaped section of bone from beneath the compartment of the knee that is not effected by cartilage damage, known frequently as a "high tibial osteotomy". In the majority of cases the osteotomy section is removed from the lateral side of the knee, in order to redistribute the axial load of the individual on to the medial compartment of the knee that usually has unhealthy cartilage. Survival rate for this procedure are 95% for 5 years and 79% for 10 years, demonstrating good patient satisfaction. The overall effect is to reduce pain within the knee of the individual thus delaying the time for the patient to undergo a TKR (Koshino *et al*, 2010; Hui *et al*, 2011), (see Figure 6).

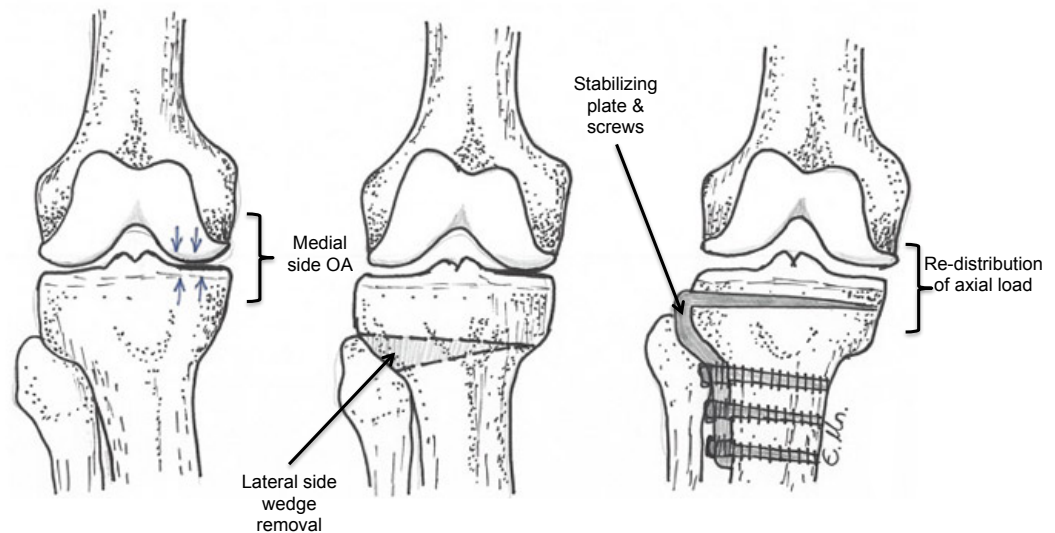


Figure 6: **Knee osteotomy.** Right knee medial compartment osteotomy. (Original image adapted from amirqureshi.co.uk/osteotomy)

Inevitably most patients progress to end-stage knee OA, where total knee replacement (TKR) is necessary. The numbers of TKR has surpassed that of total hip replacements, and is now the leading joint replacement surgery taking place in the UK (Birrell *et al*, 2011). It involves the surgical removal of the arthritic regions (commonly medial compartment in a partial knee replacement, and both medial and lateral compartments in a total knee replacement) and replaced with a metallic-on-plastic prosthesis. Total knee replacement implants have a 10 year survival of 93.8% and 20 year survival of 70.9% (Bae *et al*, 2012) Figure 7. Patients between 40-60 years old have poor results with treatments such as total knee replacement (section 1.6), as the prosthetic failure rate is high, meaning revision arthroplasty is required (Julin *et al*, 2010)

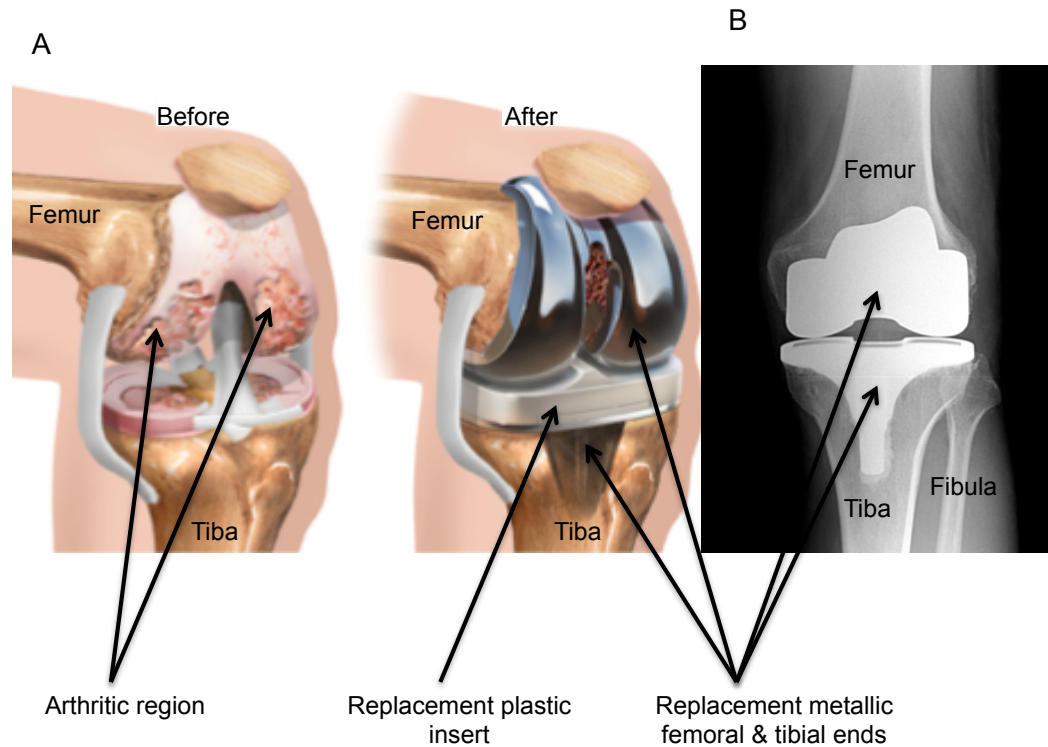


Figure 7: **Total knee replacement.** (A) Total knee replacement is a replaced with a metallic on plastic prosthesis. (image adapted from www.limbreconstructions.com) (B) Plain radiography of a left TKR (image adapted from www.hipandkneeAdvice.com).

1.9 Emerging treatment options for repair of cartilage defects

As seen above, early treatment options, such as microfracture, ACI, OATS, and OCA provide useful, though limited outcomes when attempting to repair isolated cartilage injuries. For example microfracture is a relatively convenient procedure, one that allows repair of the defect; however, the repair tissue consists of fibrocartilage (not the normal hyaline cartilage), coupled with multidirectional mechanical forces, this tissue usually wears away quickly, lasting as little as 2 years (Mithoefer *et al*, 2009). With low number of chondrocytes at the native cartilage site, it is not possible to obtain sufficient numbers of these cells to effectively fill the defect, thus ACI is used as a chondrocyte expansion procedure. However, it remains a costly two-step surgical process, and therefore is not used as a treatment option within the NHS. The challenge for researchers is to develop a procedure that amplifies the number of chondrogenic potent cells, within the knee, that are capable of repairing injured cartilage sites. Current approaches aim to increase number of cells that have the ability to promote chondrogenesis, such as MSC.

1.10 Mesenchymal Stromal Cells (MSC) and their potential role within an injured arthritic knee joint

Mesenchymal Stromal Cells (MSC) are capable of differentiating into chondrocytes, thus play a vital role in cartilage regeneration (Pountos *et al*, 2006; English *et al*, 2007; Koga *et al*, 2009; Nakamura *et al*, 2012; Anderson *et al*, 2014). Although originally thought to be bone marrow resident cells, MSC have now been documented to reside within multiple regions within the body, including the knee (Segawa *et al*, 2009; Via *et al*, 2012; Candela *et al*, 2014). These cells belong to the family of stem cells, which are defined by their ability to self-renew and differentiate into different cell types (Watt *et al*, 2000). MSC are somatic (or adult) stem cells, as opposed to embryonic stem cells that are totipotent (have the ability to differentiate into all cells types within an organism) whilst somatic MSC are multipotential (have a restrictive differentiated capabilities) (Lee *et al*, 2006).

The research history of MSC began with Friedenstein's (1976) (Friedenstein *et al*, 1976) original identification of bone marrow (BM) cells that formed fibroblastic colony forming units (CFU-Fs), they also displayed multipotency. Culture expanded daughter progeny of such cells have been termed mesenchymal stromal cells, mesenchymal stem cells, marrow stromal cells or multipotential stromal cells (MSC) have been studied in depth over the past few decades, and at each stage more of them have been discovered in multiple regions of body including, peripheral blood, umbilical cord cardiac tissue (Barry *et al*, 2004), adipose tissue (Zuk *et al*, 2002), trabecular bone (Tuli *et al*, 2003), synovium and periosteum (De Bari *et al*, 2001; De Bari *et al*, 2008), and synovial fluid (Jones *et al*, 2004). MSC have the ability to differentiate into mesodermal lineages such as osteoblasts, adipocytes, and importantly for cartilage regeneration, chondrocytes (Barry *et al*, 2013). Along with their differentiation properties, MSC also possess the ability to express cytokines such as Transforming Growth Factor beta (TGF β), Vascular Endothelial Growth Factor (VEGF), and Epidermal Growth Factor (EGF), that are involved in stimulating tissue repair, making MSC a useful tool in combating knee arthritic injuries. So how are MSC characterized and how are they sourced for use within such injuries?

1.11 MSC definition and characterization

MSC (Figure 8) *in vitro* are defined by three main characteristics: plastic adherence, *in vitro* differentiation into multi-lineages (osteoblasts, chondrocytes and adipocytes) and cell surface markers (CD73, CD90 and CD105) and the absence of other cell markers (CD11b or CD14, CD19 or CD79a, CD34, CD45 and HLA-DR) (Lv *et al*, 2014). Colony forming unit-fibroblasts (CFU-F) assays are used in the enumeration of MSC as a tool for quantifying the frequencies of MSC (Jones *et al*, 2002; Jones *et al*, 2004). The international Society of Cellular Therapy (ISCT) recommend that MSC express $\geq 95\%$ of CD73, CD90, CD105 markers, and $\leq 2\%$ of CD11b or CD14, CD19 or CD79a, CD34, CD45 and HLA-DR (Dominici *et al*, 2006). Mesenchymal stromal cells possess a great capacity for self-renewal, in terms of clonogenicity, and are capable of maintaining their multipotency. However, their ability to proliferate and differentiate decreases with age (Wang *et al*, 2014). CFU-Fs is a method used for quantifying MSC in cell preparations (Pochampally *et al*, 2008) in which MSC are plated on to culture plates, and adhere within 24 hours. Within a few days cells begin to multiply and form colonies, that latter can be used as a quantification tool.

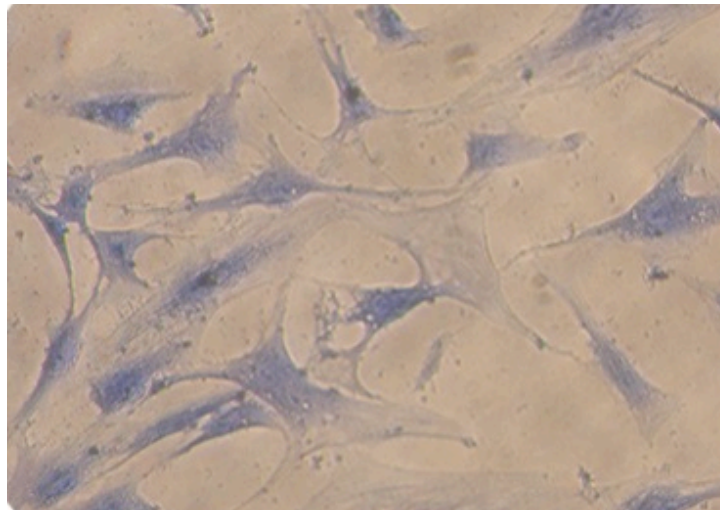


Figure 8: **Mesenchymal Stromal Cells.** Characteristic fibroblast-like morphology of synovial MSC (stained in methylene blue) at x20 magnification

1.12 Sources of MSC for repair of articular defects or OA

As previously mentioned, MSC have been identified in multiple regions of the body; however, a reliable source of MSC that can be used for regenerative purposes has been difficult to establish. Bone marrow MSC (BM MSC) have been extensively researched as a potential source of MSC to fill cartilage defects and aid in repair, and, indeed, as mentioned above, microfracture utilises the BM MSC that trickle through into the cartilage defect to repair into fibrocartilage. However, the number of BM MSC aspirates is low, and typical yields of BM MSC from a bone marrow aspirates are in the region of 0.001-0.002% (Peng *et al*, 2008). Higher numbers of BM MSC are potentially available; however, they require release by means of digestion by using enzymes such as collagenase (Jones *et al*, 2010). In such cases, BM MSC need to be expanded *in vitro* for regular use, by means of amplification, which usually can take up to 3 weeks. Currently this method of augmentation is useful in combating the issue of relatively low harvest of endogenous MSC; however, MSC do gradually lose progenitor properties (Banfi *et al*, 2000) resulting in impaired cell function as part of the senescence pathway (DiGirolamo *et al*, 1999). Another source of MSC, with a more impressive yield, is adipose-MSC (AD MSC), which boast harvests of up to 7% (Kern *et al*, 2006). The AD MSC appear to be an attractive source of MSC; however, the gain in encouraging cell numbers, may have associated unfavourable outcomes also, especially when needed to repair cartilage defects, as shown by Peng *et al*. (2008). They showed that chondrogenic abilities of both BM MSC and AD MSC are inferior to cartilage-MSC. Umbilical cord MSC (UC MSC) do offer an alternative source of cells, to be utilised for regenerative purposes, due to their rather attractive proliferation and differentiation capacities, (Baksh *et al*, 2007), which make them an appealing allogeneic cell type to use. However, coupled with ethical constraints, these cells lack incentives for local health care providers. Injecting MSC into defect sites is in its infancy, have only being investigated within the research setting thus far, and has no treatment option available within the NHS. Applications used for cartilage defects have been researched, using either BM MSC or peripheral blood MSC (PB MSC), both of which were supplemented with hyaluronic acid (a major constituent of synovial fluid found within the knee). In two separate studies, following microfracture, patients were injected with culture expanded MSC from either BM or PB (Lee *et al*, 2012; Saw *et al*, 2013); in a clinical setting, the 2 year follow-up results demonstrate positive outcomes with decreasing patient pain scores, and better repair visualised using MRI imaging. Injection of MSC in an OA knee is also being investigated, using either BM MSC or AD MSC. During a routine arthroscopic procedure in two studies, culture expanded BM MSC were injected into

the joint, and 0.5-1 year follow-ups indicated improvements in patient knee pain scores (Centeno *et al*, 2008; Orozco *et al*, 2013). Similar results, of improvement in pain scores, such as the Western Ontario and McMaster University Osteoarthritis Index (WOMAC) were achieved, when the authors used culture expanded AD MSC, supplemented with PRP that was injected into an OA knee following arthroscopic debridement (Koh *et al*, 2012; Koh *et al*, 2013; Filardo *et al*, 2015; Ornetti *et al*, 2016). Autologous MSC are an appealing source of MSC cells due to their differentiation capacities; however, the consistently low yields of cells, the necessity to culture expand cells, coupled with a surgical revisit to the site of injury, will ultimately present obstacles towards a successful low cost, one step repair of cartilage defects. This emphasises the need for a technique that provides good yields of autologous MSC that are cost effective, and uses only minimal surgical interventions, and are easily accessible by the surgeon. One potential region of the body where a source of autologous MSC can be located is the knee itself. As discussed previously, BM MSC percolate into a microfracture site and repair tissue as fibrocartilage. However, the numbers of cells are low, and, thus, other sources of MSC within the knee are required. Indeed, the knee exhibits multiple sites of endogenous MSC in the form of synovial fluid MSC (SF MSC), synovial MSC (SY MSC) and intrapatellar fat pad MSC (IFP MSC). SF MSC, phenotypically, are similar to BM MSC (Jones *et al*, 2008; Sekiya *et al*, 2012), and demonstrate better chondrogenic capabilities, compared to BM MSC. A further advantage of SF MSC is the greater number of cells present in arthritic knees, compared to non-arthritic knees (Jones *et al*, 2004). Another source of endogenous autologous MSC, are SY MSC (De Bari *et al*, 2001). Comparable to SF MSC (Baboolal *et al*, 2014), SY MSC demonstrate chondrogenic abilities, and express better chondrogenic potentials than BM MSC and IFP MSC (Sakaguchi *et al*, 2005; Fan *et al*, 2009), and would be easily accessible by the surgeon. Cell-surface markers provide a useful tool in recognizing similarities between MSC type within the knee joint (Barry *et al*, 2013) expanded MSC typically express CD73, CD90 and CD105 (Figure 9); however, cell surface markers may differ when comparing source tissue with expanded, for example down-regulation of surface marker CD271 does occur following culture expansion (Boxall S, *et al* 2012). SY MSC demonstrate promising results in animal models. Studies using a porcine model, involved harvesting synovium and digesting it in collagenase. Following culture expansion, the SY MSC were re-injected into the articular defect, to differentiate into chondrocytes and regenerate into cartilage compared to non-treated controls (Nakamura *et al*, 2012).

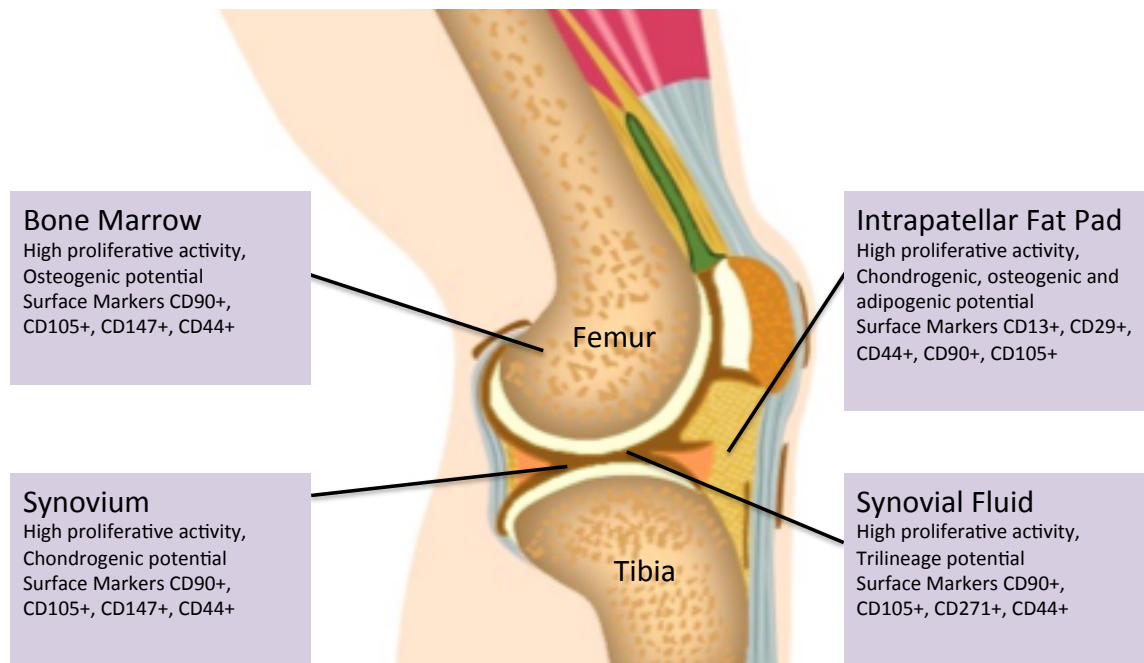


Figure 9: **Endogenous supply of MSC within the knee joint.** Characteristics, culture phenotypes and cell-surface markers of MSC located within the knee (Barry *et al*, 2013).

It is essential that future use of MSC within the injured knee meet the following conditions, to make it more available to patients within the NHS: 1) easy to obtain, ideally utilizing endogenously situated MSC; 2) minimize the need for expansion, thus maintaining their phenotypical identity; 3) require minimal surgical operating time and numbers of procedures, thus reducing mortality and morbidity rates for patients; 4) a process by which cells reach sites of injury for repairing; 5) cost effective process, thus allowing it to be available to all patients who will benefit from regenerative applications. In addition to augmenting cell numbers, there is a need for a process of loading MSC within injured sites. Growth factors (GF) are natural chemical stimulants that encourage articular cartilage production, and aid in cell homing.

1.13 Growth factor as stimulants for articular repair

Articular cartilage is regulated by an interaction between mechanical properties of a joint and the chemical stimulus in the form of Growth Factors (GFs). Growth factors such as Transforming Growth factor (TGF- β), Insulin-like Growth factors (IGFs) and Fibroblast Growth factor (FGF) encourage articular cartilage production. Each type of GF play a unique role: TGF- β : maintains chondrocytes phenotype, promotes cell differentiation. Whereas IGFs encourage chondrocyte proliferation (Chaipinyo *et al*,

2002), in addition, they also play a preventative role in cartilage degradation, by binding to proteoglycans, during periods of catabolism. To maintain the delicate homeostatic balance, FGF aid in cartilage suppression, by reducing synthesis of proteoglycan by up regulating matrix-degrading enzymes production (Li *et al*, 2012; Ellman MB, *et al*. 2013) and inhibiting articular chondrocytes (Kato *et al*, 1990), thus maintaining the homeostatic balance. Research has demonstrated improvements of articular cartilage damage with use of such products, as PRP intra-articular injection (Van Pham *et al*, 2013; Ornetti *et al*, 2016). In clinical practice, an effective method of delivering GF within a joint is by using PRP. Enriched with a host of GF, such as TGF- β , (Mollon *et al*, 2013) simple centrifugal processing steps of patients peripheral blood, enables clinicians to extract this chondrogenic-promoting constituent, and inject it into an injured knee. Augmentation of endogenous autologous MSC for use within the injured knee articular surface so there is an emerging scientific rationale for endogenous MSC manipulation towards OA therapy development. Previous animal studies have demonstrated cartilage regeneration of condylar defects even without the introduction of transplanted MSC (Lee CH, *et al* 2010). A key aspect of this thesis is to exploit knowledge of joint resident stem cells for OA therapy. First, the synovium and articular cartilage share the same embryonic origin and of course the synovial cavity exists because of these two structures. Secondly, the fact that a stem cell population resides in both the synovium and the synovial fluid, thus having ready access to superficial articular cartilage, leads to novel ways of thinking about joint repair. The superficial transitional area of cartilage has stem cells activity that has been shown in an animal model (Dowthwaite *et al*, 2004), in addition chondrocyte differentiation takes place from the “top down” or by so called appositional growth (Kozhemyakina, *et al*. 2015). All of these factors point to the notion of how SY MSC and SF MSC might have a role in cartilage regeneration from superficial layers.

MSC have shown to be a useful tool in the repair of cartilage, thus there is a clinical need for a technique that provides good yields of autologous MSC. This treatment would be desirable for isolated articular cartilage knee injuries, as well as for more advanced knee arthritic conditions such as joint osteoarthritis. As stated previously, one potential region of the body were a source of autologous MSC, can be located is the knee itself. Current clinical studies applying autologous MSC into cartilage defects focus on the use of BM MSC, AD MSC, with limited opportunities for use of SF or SY MSC as seen in a review by Vonk *et al* (2015). In addition Vonk states that umbilical cord derived MSC are the common source of allogeneic MSC used in clinical studies for cartilage repair. This leads to believe that there is a requirement for use of an

endogenous source of MSC, to fill this clinical vacuum. An endogenous supply of MSC can be in the form of SF MSC, and SY MSC, phenotypically are similar to BM MSC (Jones *et al*, 2008; Sekiya *et al*, 2012), and to each other (Baboolal *et al*, 2014), and demonstrating better chondrogenic capabilities compared to BM MSC. If a technique was developed in which SF MSC were supplemented with released SY MSC from the synovial lining to provide a good yield of autologous MSC, it would potentially lead to enhancing the quality of articular cartilage repair. This would have benefits for both the patients and the healthcare provider. This technique would also negate the need to use allogeneic MSC, thus helping to prevent infections.

1.14 Purpose made device to release SY MSC

The focus of this thesis was to develop a novel device design in an attempt to increase the total MSC within a joint cavity, thus potentially creating an environment that would favour high quality articular cartilage repair. As mention earlier, (De Bari *et al*, 2001) the synovium offers great potential in providing an abundant endogenous supply of SY MSC. Coupled with endogenous SF MSC, this will further aid in increasing the number of native MSC for repair of articular defects. Mechanically dislodging MSC from the synovial lining will potentially increase the number of SY MSC without the need of culture expansion. A purpose made device to release SY MSC, will be the ideal instrument to aid in the release of these cells. In addition, these released SY MSC coupled with resident SF MSC potentially will home towards and adhere to a arthroscopically created microfracture (biological scaffold), within the articular defects, using local chemo-attractants (such as growth factors, chemokines) released from the biological scaffolds (Vanden *et al*, 2014), thus leading to a more cost effective repair of an arthritic defect.

2. Hypotheses

This work tested the hypothesis that synovial fluid MSC were removed inadvertently by existing arthroscopic surgical techniques, but their numbers could be restored by release of MSC from the adjacent synovium using a purpose-built device. Secondly, such released cells would retain MSC functionality and may migrate into sites of injury as determined by an *in vitro* clot assay model.

3. Aims and Objectives

The overarching aim of this study is to use the philosophy that endogenous MSC can be used to regenerate articular cartilage within in the knee. The first aim was to augment synovial fluid with newly released MSC from the synovium. The second aim of this study was to explore biological scaffolds and their effect on MSC adherence and migration, thus providing a novel strategy for the future testing of cost effective therapy to treat early stages of joint degeneration in OA. These aims were addressed using the following objectives:

3.1 Obtaining MSC from the synovium *ex vivo*

- To use colony forming unit - fibroblast assay (CFU-Fs) to enumerate MSC released from the synovium.
- To develop a purpose made device to release SY MSC, and to demonstrate an increased MSC release compared to cells trapped in the brush with results confirmed by CFU-F assay and DNA analysis.
- To demonstrate trilineage differentiation of released MSC from synovial tissue following culture expansion.

3.2 Adhesion and migration of MSC with a biological scaffold

- To use clot based biological scaffolds to show that synovial derived MSC rapidly adhered to this structure.

3.3 Obtaining MSC from the synovium *in vivo*

- To show that synovial brushing generated functional colony forming unit – fibroblasts (CFU-Fs)
- To develop a purpose made device to release SY MSC and show that the released cell fraction was greater than trapped cells in brush bristles, confirmed by CFU-F and DNA analysis.
- To show that released MSC from synovial tissue following expansion demonstrated trilineage differentiation, and Immunophenotyping using Flow cytometry.

Chapter 2

This work initially used a Papanicolaou smear brush (PAP brush) to assess the feasibility of release MSC from the synovium. Excised synovium was used from human knee joints following elective total knee replacement procedures. In addition porcine synovium from freshly slaughtered pigs obtained from the local abattoir were used. Both sources of synovium were used in initial *ex vivo* experiments, to assess the feasibility of release MSC, and to determine an optimal device design, which allowed maximum release and minimal trapping of cells during synovial brushing. To further explore MSC release, human *in vivo* experiments were undertaken initially using the PAP brush to evaluate *in vivo* MSC release, during an elective arthroscopy procedure. The final experiments were focused on use of the purpose built MSC releasing device (conclusive design) within an *in vivo* setting; this was undertaken during an elective arthroscopy procedure. The numbers of cells released using the purpose built device were compared to cells released, during use of the PAP brush *in vivo*.

4. Materials and Methods

Please refer to appendix 1 for materials and media used.

4.1 Obtaining MSC from the synovium *ex vivo*

4.1.1 Device development

The design of the purpose made device to release SY MSC, was continuously improved, using quantification methods described below. The approach was focused on optimal cell release and minimal cell entrapping.

4.1.2 Collection of porcine synovium

Porcine hind legs freshly slaughtered were obtained from the local abattoir and transported within 4 hours to the laboratory, where processing took place within 2 hours. The synovium was dissected out of the knee, with the patella intact for ease of use when sectioning. Dissection was undertaken in sterile conditions using laminar airflow. The tissue was stored in PBS (Dulbecco's phosphatase buffered saline, Gibco®, Invitrogen) until further use (n= 11 porcine legs).

4.1.3 Collection of human synovium

Ethical approval was granted by the National Research Ethics Committee Yorkshire and the Humber – South Yorkshire. All patients provided informed consent (refer to appendix 2). Patients with a median age of 71.25 years old (50.5-89.5) undergoing elective total knee arthroplasty participated in this study (n= 22 donors). The synovium (placed into sterile 0.9% saline) was transported within an hour of extraction to the research laboratory for processing in sterile conditions.

4.1.4 Release of MSC from the human and porcine synovium

Under laminar flow the synovium was cut into sections approximately 1cm². Each section of human synovium was placed into 10mL of StemMACS MSC expansion media. Using forceps to anchor the synovium, 15 vertical strokes of a device were applied to release MSC from the synovial lining; this step was repeated for each device prototype using a different synovium section, after which CFU-F assays were set up (section 4.2). For each assay, 850 µL/well of the expansion media that contained released human MSC from each device, was placed into a 6 well plate in triplicates and was supplemented with 2 mL of expansion media. Cultures had twice weekly half media changes for up to 14 days. Porcine synovium MSC were released into 10mL of PBS, and the released cells were analyzed using a DNA quantification method as described in section 4.6 and 4.7.

4.2 Colony forming unit - fibroblast assay (CFU-F)

CFU-F assays were performed on released MSC from human synovium for quantification. Cells were cultured for 14 days at 37 °C, 5% CO₂ with twice weekly half media changes. Media was removed after 14 days and the plates were washed twice with PBS. The colonies were fixed with 3.7% formaldehyde (3 mL) at room temperature (RT) for 15 min. After removing the formaldehyde, 3 mL of 1% methylene blue in 10mM borate buffer (pH 8.8) was added for 10 min to stain the colonies. After washing with PBS, plates were left overnight to dry before counting the colonies. CFU-F numbers from triplicates were averaged, and plotted, comparing device prototypes.

4.3 Expansion for human SY MSC

Following CFU-F assay, the remaining cells released from each donor were pooled and incubated at 37°C, 5 % CO₂ in a 150 cm² flask in 20mL expansion media. Twice weekly half media changes took place until cells reached 90% confluence, at which point cells were passaged, until adequate cells for trilineage differentiation were

obtained. Passaging of MSC was performed by removing the expansion media and washing carefully with PBS to remove any non-adhered cells. After removing the PBS, 5 mL 0.05% trypsin/EDTA solution was placed into the flask for up to 5 min at 37°C to detach the MSC from the plastic. Trypsin containing MSC was supplemented with a further 5mL of DMEM (Dulbecco's modified eagle's medium) supplemented with 10% FCS (foetal calf serum) and standard antibiotic mixture (100 units/ml penicillin and 100 µg/ml streptomycin P/S) (refer to Appendix 1). Washing the flask with an equal volume of DMEM supplemented with 10% FCS and P/S, was used to collect remaining MSC in the flask. MSC were centrifuged for 5 min at 500 x g. The pellet was re-suspended in 1 mL of DMEM and cells counted (section 4.4). MSC were expanded to passage 3-5, at which point they were cryopreserved (section 4.5).

4.4 Cell counting

Cells were re-suspended in 1 mL DMEM then a 1:1 dilution with 0.4% trypan blue was made and placed onto a haemocytometer. Cells were counted in three different grids, and used to calculate the number of cells per ml of cell suspension.

$$[\text{Mean number of cells} \times \text{dilution factor}] \times 10,000 = \text{number of cells/ml}$$

4.5 Cryopreservation and Recovery of MSC

Freezing media consisting of FCS and 10% dimethyl sulfoxide (DMSO) was used for cryopreservation. Following trypsinisation MSC were pelleted by centrifugation at 500 x g for 5 min and re-suspended in 1 mL of the freezing media. Mean number of cells within a cryovial was approximately 1.0×10^6 . The cells were placed into a “Mr Frosty” (Nalgene container) containing isopropanol and placed in -80°C. Recovery of frozen cells was done by placing the cryovials into a water bath (37 °C), once thawed cells were placed into 10 mL of warm 90 %DMEM + 10% FCS (P/S), centrifuged and counted (section 4.4).

4.6 Cell lysis for DNA extraction

To obtain DNA for cell quantification, cells released from the relevant synovium were centrifuged at 1000 x g for 5 min in 10mL of PBS. The supernatant was discarded and cell pellet was re-suspended in 100µL lysis buffer, supplemented with 600µg/mL Proteinase K (refer to appendix 1). Cells that were trapped within the bristles of the device head were also submerged overnight in a 1000µL lysis buffer.

4.7 Phenol-cholorform DNA extraction

Following cell lysis (section 4.6) 500 μL of cells in lysis buffer was supplemented with 50 μL (2M) sodium acetate DEPC (diethylpyrocarbonate), 500 μL acid phenol (pH 5), and 100 μL red chloroform isoamylalcohol 24:1. Samples were vortexed and placed on ice for 10 minutes, this step was repeated. Samples were centrifuged at 13,000 x g for 20 minutes at 4⁰C the top aqueous layer was carefully removed (approximately 450 μL) to which an equal volume of chilled (-20⁰C) Propan-2-ol was added, and mixed thoroughly. All samples were placed in -20⁰C for 2 hours, to allow for DNA to precipitate. Samples were centrifuged at 13,000 x g for 30 minutes at 4⁰C, following which, the supernatant was discarded and 400 μL of chilled (-20⁰C), pellets were washed using 70 % ethanol. Pellets were allowed to air-dry at RT for up to 60 minutes. Once dry 50 μL of DEPC treated water was added to each sample. DNA quantification was undertaken using the NANO-drop reader. DNA extracted samples were incubated for 30 minutes in 55⁰C water bath and vortexed. Analysis of DNA quantification used 1 μL per sample, on a NanoDrop® ND-1000 Spectrophotometer and the samples analyzed using Thermo Scientific (NanoDrop 1000 3.8.1) software, which gave readings in ng/ μL . To adjust for difference in volume between those cells released and trapped on the brush head” Released cell sample were multiplied by a factor of 5, to account for dilution

4.8 MSC trilineage differentiation

MSC released from human synovium for each device prototypes were culture expanded separately for each donor, as described section 4.3. Trilineage differentiation into osteogenic, adipogenic and chondrogenic lineages was performed.

4.8.1 Osteogenic differentiation

Osteogenic differentiation required 3.0×10^4 MSC per well within a six well plate. Basic media was prepared (refer to appendix 1) and supplemented with dexamethasone (100nM) at the time of media induction and changes. Half media changes took place twice a week. Alkaline phosphate (an early osteoblast marker) staining was undertaken at 14 days for qualitative analysis. Media was removed, and cells in the wells were fixed using fixative (citrate working solution (2 mL citrate concentrate + 98 mL dH₂O) and acetone at 2:3 ratio), for 30 seconds and washed with deionised water. Fast blue salt solution (48mL of dh₂o + 2mL of 0.25% Naphthol + 1 FAST blue tablet) was added to each well and incubated at RT for 30 minutes in the dark. Wells were wash twice with dh₂o, then 2mL of Mayer’s haematoxylin was added for 10 minutes, and washed

with PBS. Wells were visualised using a light microscope and each well was digitized using a flatbed scanner. Alizarin Red (evaluates calcium-rich deposits produced by osteoblasts) staining was undertaken at 21 days for qualitative analysis. Media was removed and cells in the wells were fixed with 2mL ethanol (cooled at -20°C) and incubated for 60 minutes. Ethanol was removed and wells were carefully washed three times with distilled water. Alizarin red solution (684mg in 50mL of distilled water, pH adjusted to 4.1) was placed in each well and incubated for 10 minutes before being washed twice with distilled water. Wells were visualised using a light microscope and each well was scanned for data storage. "

The analysis of total calcium production for quantitative analysis of osteogenesis took place at 21 days. Media was removed from each well and washed twice with calcium-free PBS. To extract deposited calcium, 1 ml of 0.5M hydrochloric acid was added to each well and incubated for 5 minutes at room temperature (RT). Using a cell scraper, the well content was removed and transferred into a 1.5 ml Eppendorf tube. The sample was mixed at 4°C for four hours, using a circular rotating machine. Calcium assay was performed using a commercial available kit (refer to appendix 1) using calcium standards of 0, 1.5625, 3.125, 6.25, 12.5, 25, 50 and 100 µg/µL. Working dye solution was made with reagent 1 and reagent 2 (5:2 ratio) from the sentinel kit, from this 196 µL was placed into a 96 well plate, and to this 4 µL of a sample was added. This was repeated for all samples and standards, and was incubated for 10 minutes at RT in the dark. The samples with the 96 well plate had their optical density read at 570nm using the GENios microplate reader. Total calcium content was calculated from the standard curve generated using calcium standards. Each donor had assays undertaken in triplicate; the coefficient of variation between triplicates was 27.74 %, thus demonstrating satisfactory assay performance

4.8.2 Adipogenic differentiation

Adipogenic basic media was prepared (refer to appendix 1) and supplemented with hydrocortisone (500 µM) at time of induction and half media changes twice a week. For qualitative experiments, 5.0×10^4 MSC were seeded in a 24 well plate to undergo Oil Red-O staining. Oil Red-O staining is a method of assessing adipogenesis, at day 21 from induction. For this, adhered cells were first fixed with 3.7% formaldehyde. Filtered 0.5% Oil Red was next placed into each well for 10 minutes at RT, following a wash with PBS, filtered haematoxylin was added for 45 seconds, and then washed in cold tap water. Stained cell lipid vacuoles were visualised using the Olympus CKX41 light

microscope. Quantitative experiments were undertaken using 4.0×10^4 MSC seeded in triplicate in 48 well plates, on which a Nile red assay at 21 days was performed, using Nile red:DAPI ratio to assess lipid content per cell. Nile red dye is almost non-fluorescent in water and other polar solvents, but undergoes fluorescence enhancement and large absorption and emission (blue shift) in non-polar environments such as lipids (excitation 535). Using DAPI (4',6-diamidino-2-phenylindole) that binds to DNA. Large Nile red:DAPI ratios denote high lipid concentration per cell. Cells were fixed in 3.7% formaldehyde after being washed with 500 μ L of PBS, baseline readings were taken using the Berthold plate reader. After discarding each wells content, 200 μ L of 0.2% Saponin (in PBS) was placed into each well and supplemented with 2 μ L of DAPI and 2 μ L Nile red stock (working dilution of 1:2000 in PBS), and incubated for 15 minutes, in the dark. Contents were removed from each well, and washed three times with PBS. Finally, 200 μ L PBS was placed in each well and the plates were covered in foil, to incubate. Readings of DAPI and Nile red were taken in the Berthold plate reader. The background reading was subtracted from Nile red and DAPI readings from the same wells, giving a true reading in each well, Nile red: DAPI ratio were next calculated and plotted.

4.8.3 Chondrogenic differentiation

Chondrogenic basic media was used (refer to appendix 1) and supplemented with transforming growth factor beta 3 (TGF β 3) (2 μ g/mL), dexamethasone (100nM), and insulin transferrin sodium solution (ITS+) at the time of media induction and changes. Half media changes took place three times a week, for 21 days. To maintain gas exchange, lids on the Eppendorf used for chondrogenic cultures, were loosened. Cell pellets were created using 2.5×10^5 MSC in 1.5 mL microtubes by centrifuging for 5 min at 2000 x g. For qualitative analysis pellets were frozen in optimal control temperature reagent (OCT), sectioned and stained with toluidine blue, which stains cartilaginous extracellular matrix purple, and fibrous tissue blue. Glycosaminoglycan (GAG) assay was performed for quantitative analysis of produced GAGs. Their concentrations were measured against a standard curve, and interpolated to give a total GAG/pellet concentration. For GAG measurements, triplicate pellets of each donor were digested in 200 μ L of papain enzyme digestion solution (100mM Sodium buffer, 5mM EDTA, 10mM L-cysteine, 0.125 mg/ML papain) at 65°C overnight, then were centrifuged at 15,000 x g for 10 minutes and re-suspended in 100 μ L of deionized water. From each sample 50 μ L was analyzed for their GAG content using Blyscan™ Sulfated Glycosaminoglycan assay kit equipped with GAG standards of 1.0, 2.0, 3.0, 4.0, 5.0 μ g per 100 μ L of deionized water. Blyscan dye reagent (1mL) was added to

each tube and mixed by repetitive inversion for 30 minutes, at which point sulphated GAG-dye complex forms and precipitates out from the soluble unbound dye. The sample was next centrifuged at 12,000 x g for 10 minutes, and the supernatant removed, after which 500 µL of dissociation reagent was added to the remaining pellet, to dissolve the dye, and centrifuged again at 12,000 x g for 10 minutes. From each sample 200 µL was transferred into a 96 micro-well plate for analysis using the Berthold plate reader, measuring absorbance (656nm).

4.9 Adhesion and migration of MSC with relevant biological scaffolds

So far the study has focused on the ability to release MSC from the synovial lining. The following methods were next utilized to study how these released MSC were able to attach to and migrate through biological scaffolds, aiming to imitate an environment similar to one following a microfracture procedure (section 1.7).

4.10 Formation of platelet poor plasma (PPP) and platelet rich plasma (PRP) scaffold

Healthy volunteers with no hematological disorders were recruited and informed consent was obtained (refer to appendix 2) From each donor, 15 mL of venous blood was collected into 3.2% sodium citrate anticoagulation blood collection tube. Samples were centrifuged at 250 x g for 10 min. The supernatant was removed and centrifuged again at 1500 x g for 10 min (Araki *et al*, 2012). Following the second centrifugation, the supernatant was considered to be platelet poor plasma (PPP). PRP was created by centrifuging 4/5th of the collected PPP supernatant at 1500 x g for 10min and mixing the platelets formed at the bottom of the tube with a 1/5th of PPP total volume. The scaffold was prepared in the lid of an Eppendorf tube, with 200 µL PRP supplemented with 4 µL of 5M calcium chloride (CaCL) and 10 µL thrombin (1000U/mL), to allow for rapid scaffold formation.

4.11 Formation of whole blood (WB) scaffold

Each donor had 5 mL of venous blood collected into a 3.2% sodium citrate anticoagulation blood collection tube. The scaffold was prepared in the lid of an Eppendorf tube, with 200 µL WB supplemented with 4 µL of 5M CaCL and 10 µL thrombin (1000U/mL), to allow for rapid scaffold formation.

4.12 Formation of Fibrin glue (FG) scaffold

Fibrin glue scaffolds was prepared in the lid of an Eppendorf tube, with 23.7 µL/ml fibrinogen supplemented with expansion media, up to a volume of 200 µL, to which 4 µL of 5M CaCL and 10 µL thrombin (1000U/mL) was added. The common coagulation pathway describes that supplementation of fibrinogen with thrombin and calcium, produces a stable fibrin scaffold.

4.13 Formation of human platelet lysate (hPL) scaffold

PRP from three healthy individuals was used to prepare hPL. All three PRP samples were pooled and were frozen at -80°C for 24 hours and thawed at 4 °C for 24 hours, this cycle was repeated once, which allowed the platelets to lyse. Scaffolds of 200 µL hPL supplemented with 4 µL of 5M CaCL and 10 µL thrombin (1000U/mL), were next made.

4.14 Sectioning and staining of scaffolds

Scaffolds were placed in optimal cutting temperature solution (OCT) and frozen using liquid nitrogen and stored -80 °C until use for cells counting. Using a Cryostat, 10µm sections were made and placed onto glass slides and H&E staining was performed so that cell nuclei could be visualised and counted using Image-J 1.48V software.

4.15 Imaging and MSC cell quantification in scaffolds

Images were taken across the extent of the scaffold, to assess for its size, and shape at x4 and x10 magnification. The number of cells/mm² was calculated using Image-J 1.48V software. H&E stained cells were counted within scaffolds and on the edge of the scaffolds and compared for each scaffold type (number of cells/mm² was plotted against scaffold type).

4.16 Adhesion of MSC to relevant biological scaffolds

Following preparation of the three biological scaffolds, PRP (n=3 donors), WB (n = 3 donors) and FG (n = 1) (section 4.10, 4.11 and 4.12), 1mL of expansion media containing 10,000 MSC (culture expanded SY MSC from donor 4, see table 2) was placed into an Eppendorf tube, with the scaffolds formed in the Eppendorf lid. After closing the lid, the Eppendorf tube was inverted to allow cells to adhere to each scaffold type (see Figure 10). Non-adherent cells in the supernatant were removed and placed in culture to attach to tissue culture plastic overnight. Thereafter, cells were fixed in 3.7% formaldehyde (1 mL) at room temperature (RT) for 15 min. After removing the formaldehyde, 1 mL of 1% methylene blue in borate buffer pH 8.8 was added for 10 min to stain the cells allowing for it to be visualised and counted using Image-J 1.48V software, this was repeated in triplicates at each time point for each scaffold type. The percentage of adhered MSC was calculated, using the following formula:

$$\% \text{ Adhered cells} = (\text{initial MSC seeded for adherence} - \text{non-adhered MSC}) \times 100$$

MSC were counted for each biological scaffold, and subtracted from initial MSC number. The percentage of adherence was then plotted as a percentage to determine if adhesion was affected by MSC donor variation, the above assay was repeated using three MSC donors, with one FG scaffold type.

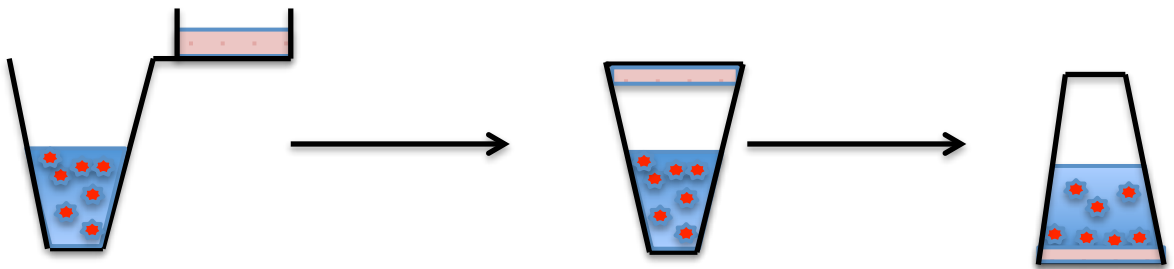


Figure 10. **Adhesion assay:** Eppendorf set up with MSC suspended in expansion media, inverted for a set period of time, and non-adhered cells placed in culture to attach to tissue culture plastic overnight, fixed in 3.7% formaldehyde, stained with 1% methylene blue and quantified using Image-J 1.48V software.

4.17 Migration of MSC towards relevant biological scaffolds

Migration abilities towards biological scaffolds such as PRP and hPL were investigated. Preparation of PRP (section 4.10), hPL (section 4.13) in liquid form (without supplementation of CaCl and thrombin), as well as negative control (DMEM + 0.5% foetal calf serum FCS) and positive control (DMEM + 10% FCS) media were used. Each chemokine, as well as the positive and negative controls (750 μ L) were placed into a separate well of 24 trans-well plates. The transwell insert with 8 μ m pores containing 10^5 MSC/insert, in serum starved media (DMEM + 0.5% FCS), from a single donor (p4) was inserted into each well. Migration was allowed to take place over 4 and 24 hours. This was repeated in duplicates with different MSC and PRP donors, to take in account of donor variations (see Figure 11). Following migration period the membrane of the trans-well, was fixed in 3.7% formaldehyde, stained in hematoxylin and eosin (H&E) solution, and quantified using Image-J 1.48V software.

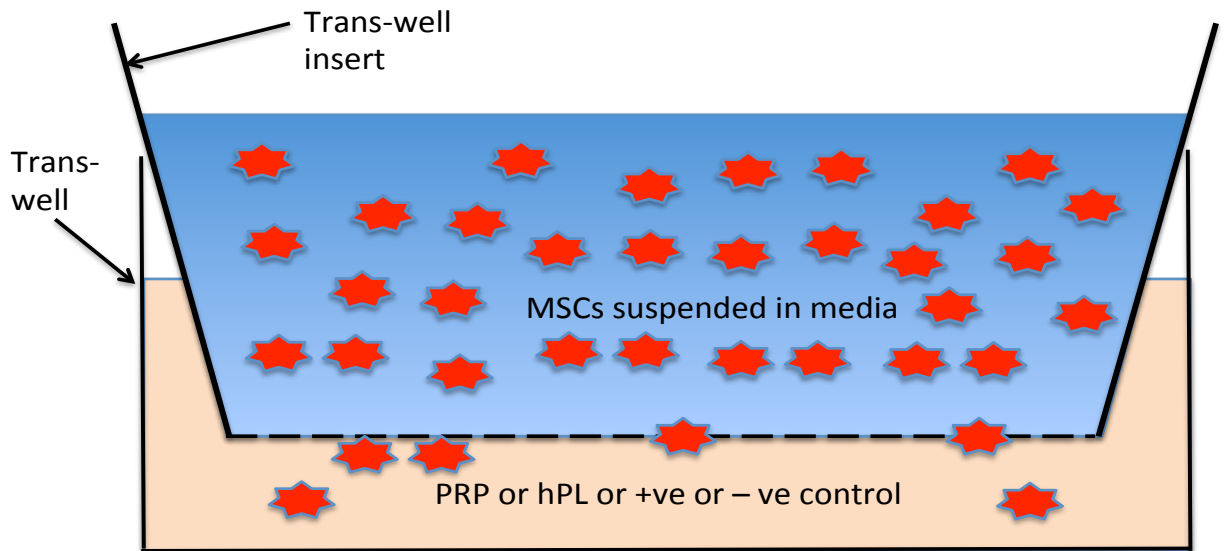


Figure 11: **Modeling MSC migration toward biological scaffold.** Trans-well migration assay within a well of 24 well plate and trans-well inserts. Wells were seeded with 10^5 MSC/insert allowing for MSC in serum-starved media to migrate toward liquid constituents of differing biological scaffolds (PRP or hPL or positive control or negative control). Following migration period of either 4 and 24 hours, the membrane of the trans-well, was fixed in 3.7% formaldehyde, stained in H&E solution and quantified using Image-J 1.48V software

4.18 Migration of MSC through relevant 3D biological scaffolds

The focus of this part of the study was to determine if released MSC could migrate into and through biological scaffolds. To allow cells to migrate from below the scaffold, sterile molds were created, using 1 mL cylinders (cut from a Pasteur pipette) placed on a paraffin wax wrap. Platelet poor plasma (PPP) and PRP scaffolds to the volume of 200 μ L were formed inside these molds (refer to 4.10), and placed on top of a previously prepared mono-layer of MSC (10^5 per well), in a six well plate containing expansion media (prepared 24 hours prior to scaffold placement, to allow for adhesion of MSC to the plastic). Twice weekly media changes took place for all formations for a total of 10 days at which point scaffolds were fixed (Mendelson *et al*, 2011). Scaffolds were fixed in 3 mL of 3.7% formaldehyde, sectioned and stained in H&E. Cell nuclei could be visualised and counted using Image-J 1.48V software; this was performed in triplicate (see Figure 12).

To allow cells to migrate from above the scaffold cells were seeded using the following method. Agarose gel wells were made, using 3 mL of 4% agarose in a 6 well plate. Once the gel set, 8 mm diameter wells were created using a borer; PRP and PPP scaffolds of 200 μ L were formed within this 4% agarose well and MSC were seeded at a density of 10^5 MSC/well in expansion media (Mendelson *et al*, 2011) (see Figure 13).

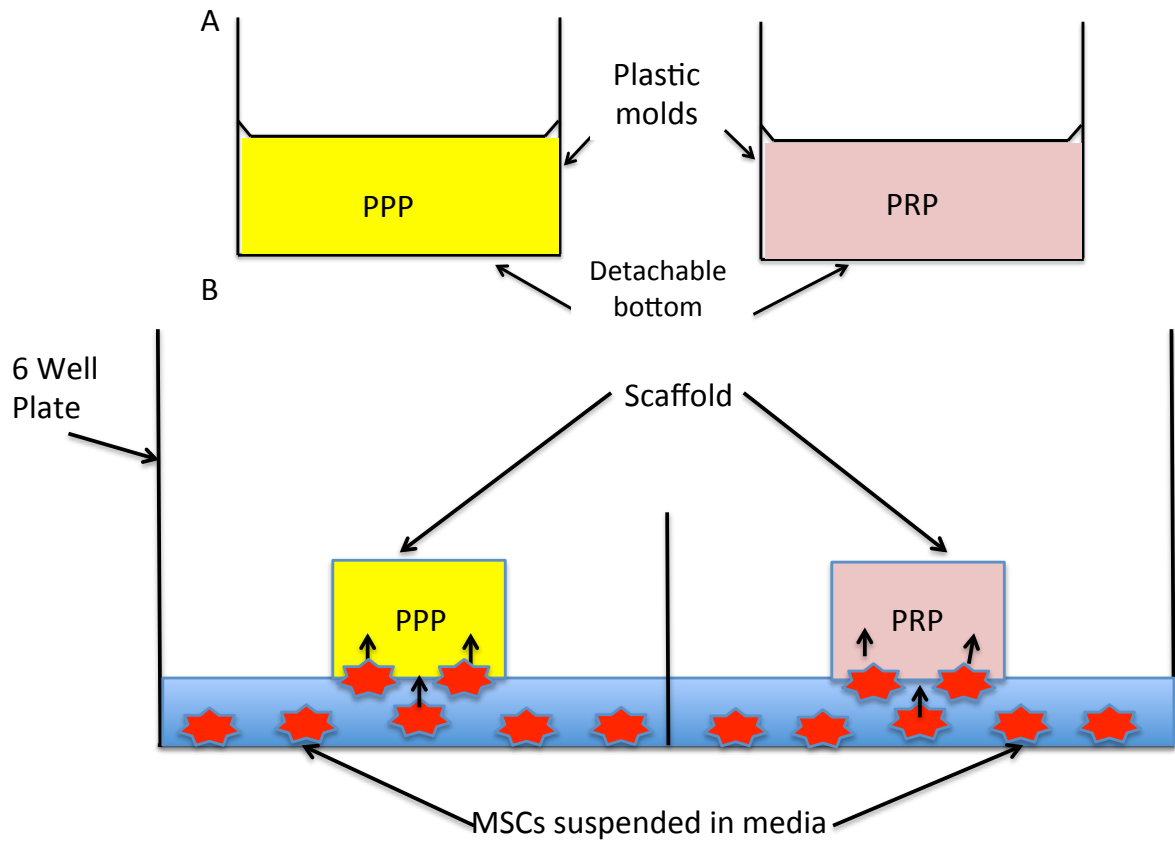


Figure 12: **Migration through biological scaffolds (from below)**. (A) PRP and PPP formed using a plastic molds. (B) PPP and PRP scaffolds placed on top of a monolayer of 10^5 MSC/well in expansion media. Following 10 days of migration, scaffolds were fixed in 3.7% formaldehyde, sectioned and stained in H&E solution. Number of cells was quantified using Image-J 1.48V software.

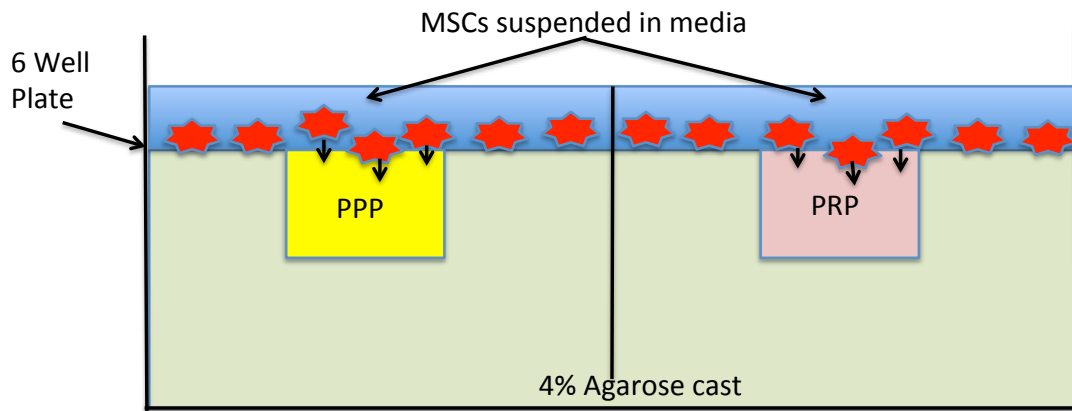


Figure 13: **Migration through biological scaffolds (from above)**. PPP and PRP scaffolds within constructed agarose cast constructed. MSC (10^5) seeded from above in expansion media. Following 10 days of migration, scaffolds were removed from the cast, fixed in 3.7% formaldehyde, sectioned and stained in H&E solution (Number of cells was quantified using Image-J 1.48V software).

4.19 Investigation of cell type, migrating through relevant 3D biological scaffolds

To investigate the type of cells migrating through a 3D biological scaffold, the following experiment was undertaken. MSC (10^5 MSC/well) were seeded in a 6 wells plate, to migrate through a PRP (n = 2 donors) and an hPL (n=1) scaffold for a time period of 2, 5 and 10 days. As a control, the PRP scaffolds were placed in wells containing no MSC. MSC from culture-expanded TKR synovium were used as the migrating cells. At 2, 5 days and 10 days scaffolds were taken out of the wells, fixed in 3 mL of 3.7% formaldehyde, sectioned and stained in H&E staining performed, so that cell nuclei could be visualised and counted using Image-J 1.48V software, this was repeated in triplicate at each time point (see Figure 14).

4.20 Dual-staining of SY MSC and leucocytes with surface marker specific primary antibodies

Using PRP, from one healthy individual, a 3D biological scaffold was created, and culture expanded SY MSC from one donor, were embedded into each scaffold. The scaffolds were fixed with 3.7% formaldehyde, placed in OCT and frozen using liquid nitrogen, until sectioning (section 4.14). Sectioned PRP scaffolds were stained with CD73 (for MSC) and CD45 (for leukocytes) primary antibodies using the EnVision™ G|2 Doublestain system, (see appendix 1 for full protocol). This specific dual staining experiment has not been undertaken previously. However, previous studies using primary antibodies staining method (Letouzey *et al*, 2015), were used as a guide, coupled with the instruction from the staining kit (refer to appendix 1). Stained cells were visualised using the Olympus CKX41 light microscope.

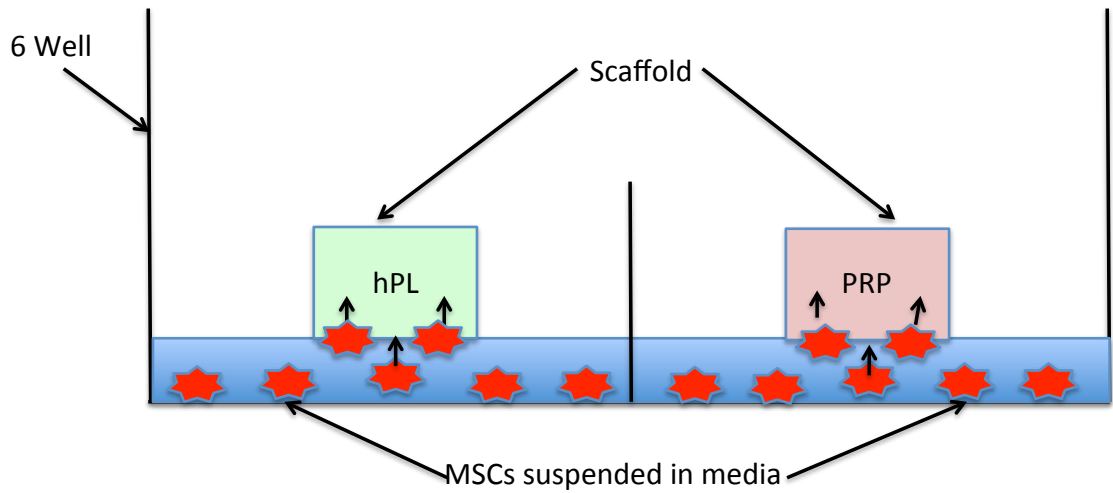


Figure 14: **Investigation of type of cells migrating.** Migration of MSC through a hPL and PRP scaffold. Wells were seeded with either 10^5 MSC/well or not seeded, as a control. Following 2, 5 and 10 days of migration period, scaffolds were removed from wells, fixed in 3.7% formaldehyde, sectioned and stained in H&E solution. Cell numbers was measured using Image-J 1.48V software.

4.21 MSC migration through 3D biological scaffolds, using dual-staining of MSC (CD73) and leucocytes (CD45) with surface marker specific primary antibodies

Biological scaffolds (PRP and PPP), were set up as described in section 4.10. Scaffolds were placed on a monolayer of MSC, as described in section 4.18, allowing for migration to occur. In addition, a PRP scaffold was placed in a well with no monolayer of MSC, as a negative control. After 10 days, all scaffolds were dual stained as described in section 4.20.

In vivo experiments were undertaken using two devices, during elective knee arthroscopies in different donors for each device (refer to table 2 and 3). The first set of *in vivo* experiments was undertaken using the Papanicolaou smear brush (PAP brush), to assess *in vivo* release of MSC from the synovium. The second set of *in vivo* experiments was undertaken using the purpose built MSC releasing device (refer to appendix 1 for images of both devices). Both experiments involved these devices being used in human knee joints

4.22 Collection of MSC from human synovium during arthroscopy using the PAP brush *in vivo*

Ethical approval was granted by the National Research Ethics Service Committee Yorkshire and the Humber – South Yorkshire. All patients provided informed consent (refer to appendix 2). Patients with a median age of 29.5 years old (18.1-52.0) undergoing elective knee diagnostic/therapeutic arthroscopies participated in this study (n=9). Three samples were collected from each donor, these consisted of 1) synovial fluid (SF), which in some cases was supplemented with saline, to aid in extraction. 2) Irrigation fluid, which was collected following the planned procedure (i.e. debridement) and 3) post brushing of the synovium with the device. SF was collected in a sterile container, with a mean volume of 37mL (5-50mL), the irrigation sample was collected, mean volume 39mL (25-50mL), and finally following brushing of the synovium (to release of MSC), the post brushing sample was collected, mean volume 53mL (50-70mL), all samples were transported within an hour of extraction to the research laboratory for processing in sterile conditions. Samples were centrifuged at 500xg for 5 minutes and re-suspended in 10mL of expansion media. Each sample, had 2 mL of cell suspension placed in a 10cm petri dish, which was supplemented with 8 ml of expansion media (1/5 dilution factor). This was done in duplicate, for each sample. CFU-F assay was used to quantify released numbers of MSC (section 4.2). The

remaining suspensions were pooled for each sample, and culture expanded as per standard protocol (section 4.3). Expansion of cells took place with twice weekly half media changes, until samples reached 90% cell confluence, at which point cells were passaged, as described in section 4.4, to a minimum of 1.0×10^6 per sample, and were cryopreserved for later use (section 4.5).

4.23 MSC trilineage differentiation of *in vivo* released synovial MSC

Osteogenic differentiation was conducted using standard method as described above (section 4.81). Adipogenic differentiation was conducted using standard method as describe above (section 4.82). Chondrogenic differentiation was conducted using standard method described above (section 4.83). However, for MSC released using *in vivo* PAP brush commercially supplied Chondrogenic media StemMACS ChondroDiff Media (Miltenyl Biotec, MACS[®]) was used.

4.24 Surface phenotype of cultured SF MSC and SY MSC by flow cytometry

Flow cytometry phenotyping of SF MSC (SF) and SY MSC (post-brushed) was performed using a LSRII four laser flow cytometer (BD Biosciences, Oxford, UK) with appropriate isotype controls. Synovial fluid MSC passage 5 and SY MSC passage 7 (n=1 donor) were trypinised and re-suspended at 1×10^6 cells/ml. To block non-specific antibody binding, cells (1×10^6) were incubated for 15 minutes with blocking buffer (10% mouse serum, 1% Human IgG in FACS buffer) supplemented with (FACS buffer PBS + 0.1%BSA) at RT. The following commercially available antibodies were used at manufactures recommended dilutions: anti-CD34- allophycocyanin-cyanine (APC), anti-CD19- phycoerythrin (PE), anti-CD45- phycoerythrin-cyanine (PE-Cy7), anti-CD14- fluorescein isothiocyanate (FITC), anti-CD73- phycoerythrin (PE), anti-CD90- phycoerythrin-cyanine (PECy7), anti-CD105- phycoerythrin (all from BD Biosciences). Cells were stained with 4',6- diamidino-2-phenylindole (DAPI) (Sigma-Aldrich) as a cell viability marker and in order to gate out dead cells. At least 10,000 live cell events were collected for each antibody combination (Baboolal *et al*, 2014).

4.25 Obtaining MSC from the SF and SY using the purpose made device to release SY MSC, *in vivo*

The following experiments were aimed to demonstrate *in vivo* release of MSC from human synovium using a purpose built synovial device. Ethical approval was granted by the National Research Ethics Service Committee Yorkshire and the Humber – West Yorkshire. All patients provided informed consent (refer to appendix 2). Patients with a

median age of 33.7 years old (22.0-53.7) undergoing elective knee diagnostic/therapeutic arthroscopy participated in this study (n=10). The SF was collected through the arthroscopy ports. In some cases SF was not easily expelled through the ports, in such cases a standard volume (20ml) of sterile 0.9% saline was introduced via the ports site, and the SF/saline mix was collected. During the patient's planned arthroscopy procedure, a sample of initial irrigated knee content was collected, which contained the SF MSC. After completing the planned procedure (for example microfracture) the surgeon would irrigate the joint, with saline, to wash away any debris created, following which, a sample would be collected. The surgeon then introduced the synovial device brush into the knee via the port site and brushing of the synovium took place, allowing for the release of the SY MSC. The knee was irrigated and a post-brushed sample was collected via the arthroscopic ports, this sample would contain SY MSC. The three samples were transported within an hour of extraction to the research laboratory for processing in sterile conditions.

4.26 Sample processing of SF and SY MSC from *in vivo* device brushing

All three samples: SF, irrigation and post-brush samples were centrifuged at 500 x g. Pellets were re-suspended in 1mL of DMEM media and cell number counted (section 4.4). All three samples were re-suspended in 9mL of StemMACS MSC expansion media (Miltenyi Biotec, UK). Each sample, had 2 mL of cell suspension placed in a 10cm petri dish, which was supplemented with 8 ml of expansion media (1/5 dilution factor). This was done in duplicate for each sample. CFU-F assay was used to quantify released numbers of MSC (section 4.2). The remaining suspension of cells in SF, irrigation and post-brushed were culture expanded until a minimum of 1×10^6 cells were attained, and then cryopreserved (section 4.5), for future use in differentiation assay.

5. Results

5.1 Obtaining MSC from the synovium *ex vivo*

The knee has an endogenous supply of MSC in the form of SF MSC. SF MSC, phenotypically are similar to BM MSC (Jones *et al*, 2008; Sekiya *et al*, 2012), and demonstrate better, more consistent chondrogenic capabilities. A further advantage of SF MSC is the greater number of cells present in arthritic knees, compared to non-arthritic knees (Jones *et al*, 2008). However, in a knee arthroscopy procedure SF MSC will be lost during irrigation. To replace these cells, another source of endogenous autologous MSC can be used, such as SY MSC (De Bari *et al*, 2001). Comparable to SF MSC, SY MSC demonstrate chondrogenic abilities, and express better chondrogenic potentials than BM MSC and IFP MSC (Sakaguchi *et al*, 2005; Fan *et al*, 2009). Previous studies undertaken in Leeds MSC group laboratory, showed effective *ex vivo* release of MSC from the synovial surface using a PAP brush. The focus of this study was to optimize the release and minimize the entrapment of cells within the bristles of a device, as well as design a new purpose built device to release SY MSC from the synovial surface. This purpose built device was designed for use in man, and its main function was aimed at the augmentation of the total number of endogenous MSC within the knee following an arthroscopy procedure.

Using the PAP brush as a control, a purpose made device to release SY MSC, was developed, using both porcine and human synovium as a source for SY MSC. Porcine synovium provides a consistent aged synovium, and was the synovium of choice for DNA analysis to minimize variability. Porcine synovium was used for quantification of cells; released and trapped within the device bristles. Human synovium was used to undertake CFU-Fs assays for quantification of released MSC. Due to the variation in section size of human synovium samples collected, not all devices could be used to brush the synovium during consecutive sample, please refer to table below (see Table 2).

Table 2: An overview of human synovial donors, used for the development of the purpose made device to release SY MSC. Highlighted rows (n=6 donors), are SY MSC donors used in trilineage differentiation experiments. (OA =osteoarthritis, TKR = total knee replacement).

Donor	Age	Gender	Disease Type	Surgery	Device Concept Type Used							
					PAP	C1	C2	C3	C4	C6	C7	C8
1	73.7	F	OA	TKR	✓		✓		✓	✓	✓	
2	71.4	F	OA	TKR	✓		✓		✓	✓	✓	
3	82.7	F	OA	TKR	✓		✓		✓	✓	✓	
4	50.5	F	OA	TKR			✓		✓	✓	✓	
5	70.8	M	OA	TKR			✓					
6	67.6	F	OA	TKR	✓		✓		✓			
7	72.8	M	OA	TKR	✓	✓	✓	✓	✓			
8	86.8	F	OA	TKR	✓	✓	✓	✓	✓			
9	58.1	F	OA	TKR	✓	✓	✓					
10	66.5	M	OA	TKR	✓	✓						
11	79.2	M	OA	TKR	✓					✓	✓	
12	77.9	M	OA	TKR	✓							✓
13	62.4	F	OA	TKR	✓							✓
14	64.2	F	OA	TKR	✓						✓	✓
15	57.6	F	OA	TKR	✓						✓	✓
16	85.3	F	OA	TKR		✓	✓	✓	✓			
17	79.1	M	OA	TKR		✓	✓	✓	✓			
18	64.2	F	OA	TKR		✓	✓	✓	✓			
19	89.5	F	OA	TKR		✓	✓	✓	✓			
20	64.8	M	OA	TKR		✓	✓	✓	✓			
21	89.1	F	OA	TKR	✓						✓	✓
22	71.1	F	OA	TKR	✓						✓	✓

5.2 Quantification released and trapped cells within the device bristle, using cells from porcine synovium

Freshly slaughtered porcine hind legs were obtained from the local abattoir. Under sterile conditions the synovium was dissected out of the knee with the patella intact for ease of use when sectioning. The number of cells that were released from the synovium and those trapped within the bristles of three prototypes (PAP, C2 and C4) (see Figure 15) were quantified by proportionally measuring the cellular content which is either released or trapped, in terms of DNA content. This gave an indication of cell numbers. The DNA was purified using phenol:chloroform extraction method, and was quantified using the NANO-Drop method described in sections 4.6 and 4.7), (n=11 donors).

Initially it was determined to what degree the prototype synovial brushing devices released or trapped synovial cells. Two devices used (PAP and C4) showed no statistical significance between DNA content of released cells from the synovium, and those trapped within the bristles of the device. However, device C2 showed statistical significance, where more cells were trapped within the bristles compared to release by this device (* = $p < 0.02$, paired t-test) (see Figure 16).

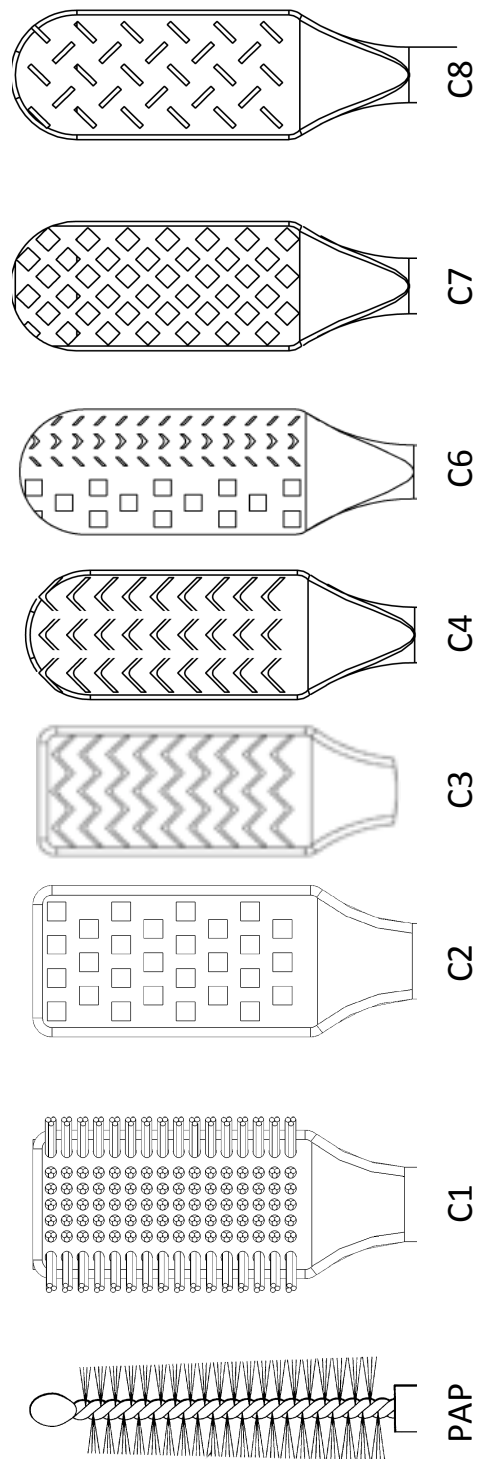


Figure 15: **SY MSC releasing device prototypes.** Variations of prototype brush ends. Prototypes C1-C4 are designs, used in initial SY MSC releasing experiments (phase 1). C6-C7 subsequent phase prototypes designed from initial experiment outcomes (phase 2). C8 is the final design used in *in vivo* experiments (final phase). PAP device used in all *ex vivo* and *in vivo* work.

5.3 Quantification of released MSC from human synovium using CFU-F assays

To determine the number of viable MSC released from excised human synovium, colony forming unit-fibroblast (CFU-F) assays, were used as a method of quantification. CFU-F assays were performed with each device by brushing the surface of the synovium section of each donor. MSC released from the synovium, formed colonies and were fixed and stained at 14 days, these were then counted (see Figure 17). Results revealed no statistical significant difference between the device prototypes used. This data (section 5.2 and 5.3) was used to further develop the prototype design.

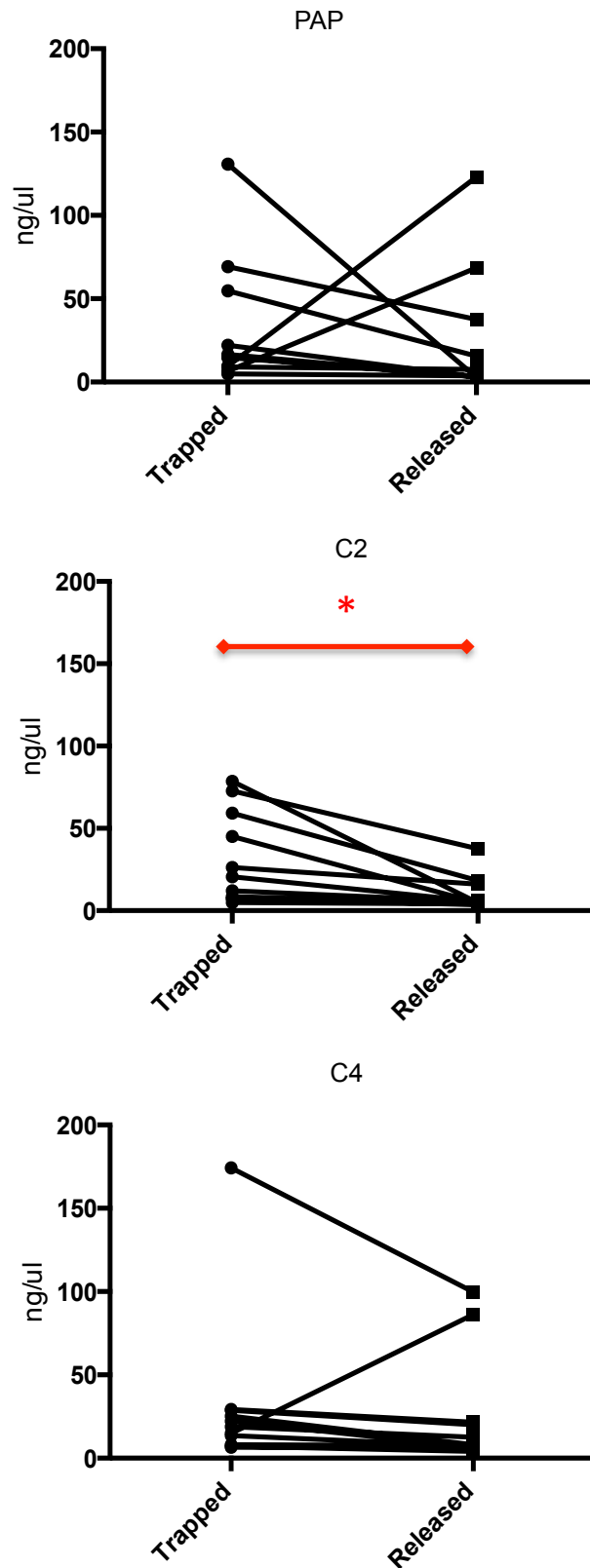


Figure 16: **Release of cells from porcine synovium, phase 1.** DNA content of cells (ng/ μ L) released from 1 cm² sections excised porcine synovium *ex vivo* experiment, and those trapped within the bristles of the device, using PAP and two brushing prototypes (C2 and C4). (n=11 donors). C2 (*=p<0.02, paired t-test).

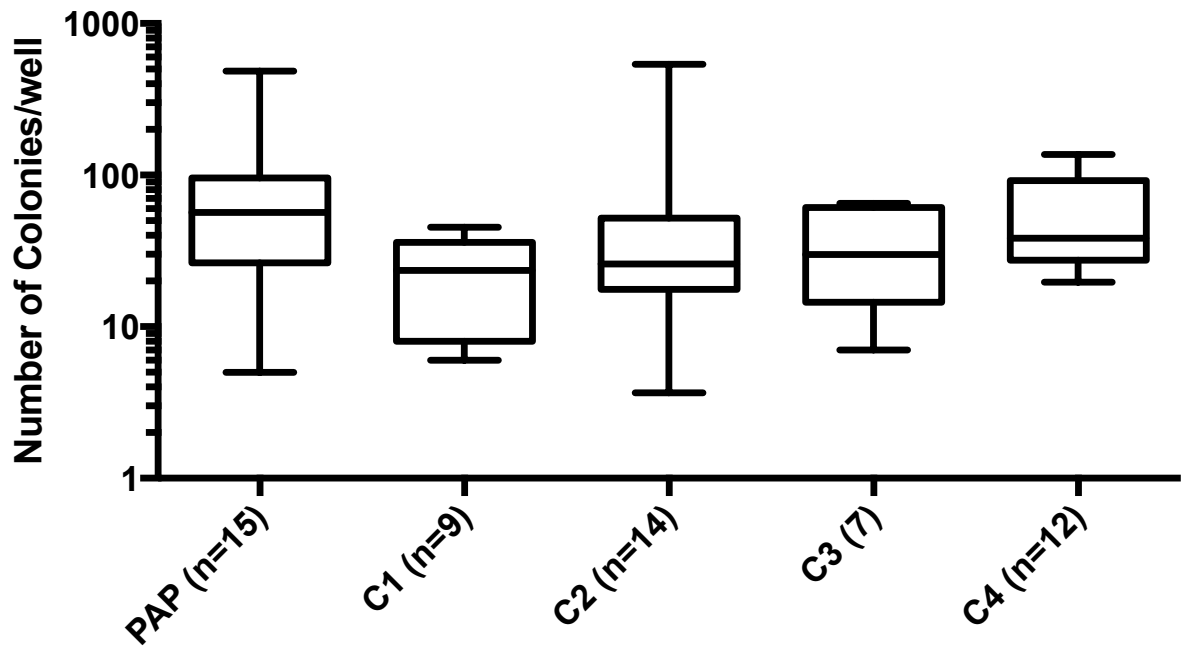


Figure 17: **Release of MSC from human synovium, phase 1.** CFU-F/well (6 well plate) data from excised 1 cm² sections human synovium *ex vivo* experiment indicating released MSC using PAP and the different prototypes (C1, C2, C3, and C4). Data represented by median value per device, with upper and lower quartiles and minimum and maximum values.

5.4 Quantification of cells released using modified device prototypes (phase 2)

Phase 2 of *ex vivo* experiments using DNA analysis on excised porcine synovium. Each synovium was sectioned into multiple 1 cm² sections, and brushed with phase 2 modified devices termed C6 and C7 (see Figure 15). Device C6 was developed using a combination design of two previous prototypes C2 and C4 and their respective results (see Figure 15 and 17). Device C7 was a new design that coupled the bristles design feature of both C2 and C4, in a diamond orientation. Results demonstrated no statistical significant differences between the two types of devices. (see Figure 18). Excised human synovium obtained from patients undergoing elective total knee replacement (table 2) was used. Each human synovium section was brushed with either C6 or C7 on a donor-matched 1cm² section of synovium (see Figure 19).

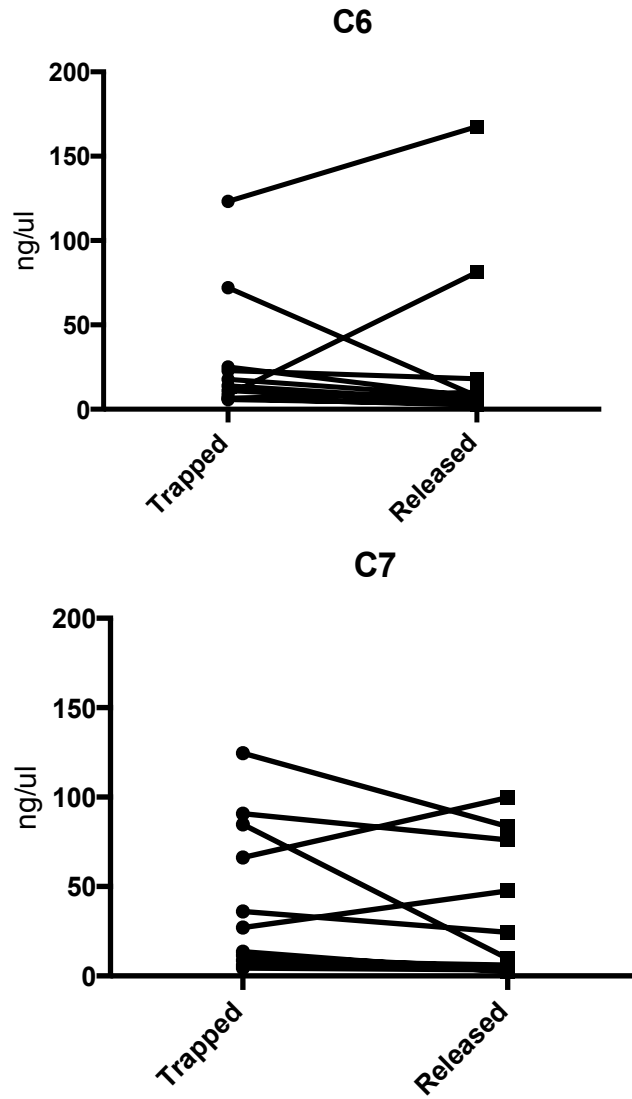


Figure 18: **Release of cells from porcine synovium, phase 2.** DNA content (ng/ μ L) of cells from excised 1 cm² porcine synovium *ex vivo* experiment, that were released, and trapped within device bristles C6 and 7 (n=11 donors).

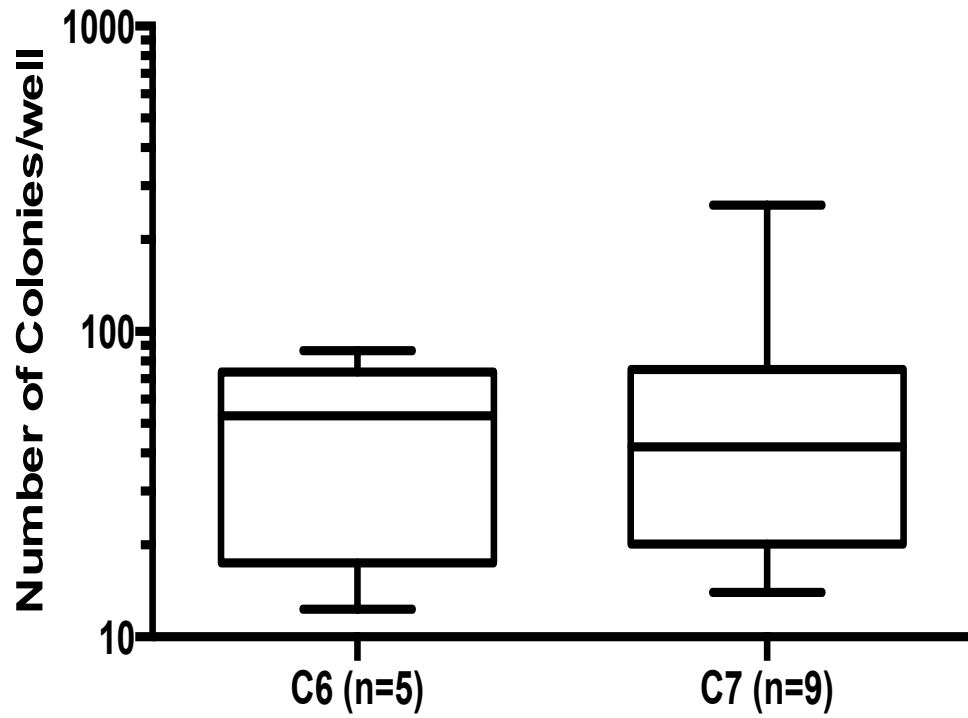


Figure 19: **Release of MSC from human synovium, phase 2.** Quantification of released MSC from excised human synovium, using CFU-F assays. CFU-F/well (6 well plate), data showing released MSC using the device prototypes C6 (n=5 donors) and C7 (n=9 donors). Data represented by median value per device, with upper and lower quartiles and minimum and maximum values.

Results from both human and porcine synovium in phase 2 of device design, demonstrated no statistical significant difference between the two types of devices. This data was used to further develop the prototype bristle design, in the final phase of the study.

5.5 Quantification of cells released using modified device prototypes (final phase)

Using the data from phase 2 of device concept design, a final device design C8, was developed, and compared to the PAP brush as a control. C8 (see Figure 15) was design based on the diamond shaped configuration as C7, allowing for cell release to occur when multiple brushing directions are used, rather the only a front to back motion, thus allowing a greater cell release. In addition, spacing between bristles were increased, as data from phase 2, demonstrated that similar numbers of cells were being released as well as trapped. So it was proposed that larger gaps between bristles would reduce cells being entrapped (see Figure 20).

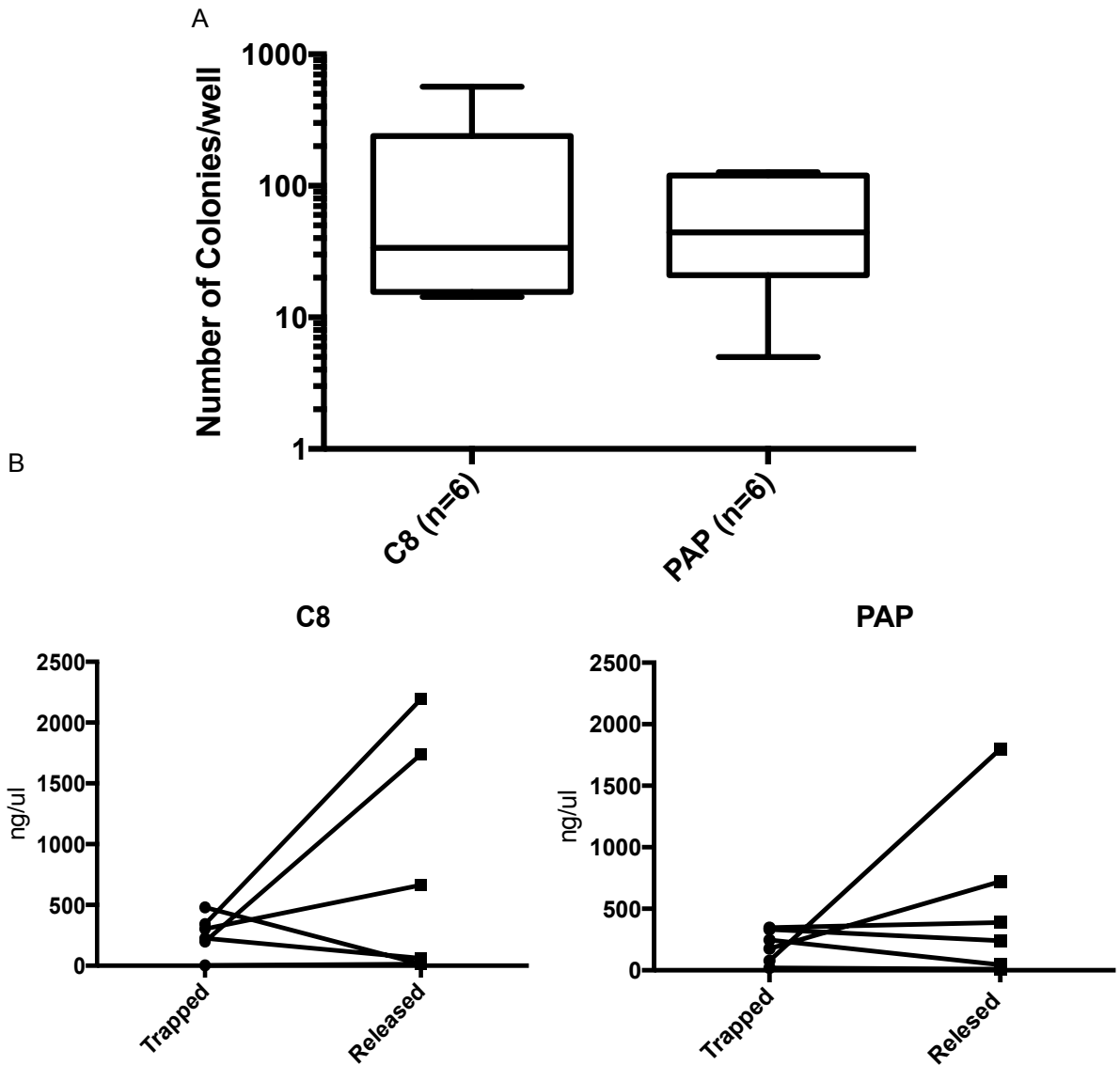


Figure 20: **Release of MSC and cells from human synovium, final phase** (A) Quantification of released MSC using CFU-F assays. CFU-F/well (6 well plate), data showing released MSC using the device prototypes C8 (n=6 donors) and PAP (n=6 donors). Data represented by median value per device, with upper and lower quartiles and minimum and maximum values. (B) DNA content (ng/ μ L) of cells that were released from excised human synovium, and trapped within device bristles (n=5 donors).

The above results demonstrated no statistical significance when comparing MSC and cells release from human synovium, during brushing with device C8 and the PAP brush. Although improvements have been made in the device cell releasing capabilities when compared to trapping, there seems to be no significant advantage for the use of C8 prototype over PAP brush within an *ex vivo* setting. However, it is sensible to acknowledge that this device demonstrates an effective cell release, which might be further enhanced when it is developed into a purpose build device tailored to arthroscopy. Dr. Baboolal made all design specifications for each concept with the aid of the above results that were obtained by my work. The final purpose built device was designed with feedback in consultation with Mr Wall and Mr Calder our orthopaedic surgeons.

5.6 Trilineage differentiation of cultures expanded, SY MSC released from human synovium *ex vivo*

Cells released from each human synovium were pooled separately for each donor and culture expanded. Following expansion these pooled cells were used to demonstrate trilineage differentiation into osteogenic, adipogenic and chondrogenic lineages, performed on passage 3-5 cells. Osteogenesis was evaluated both qualitatively and quantitatively for the SY MSC in triplicate. Alkaline phosphatase staining at day 14 and alizarin red at day 21 were performed. Total amount of calcium produced was used to quantify osteogenesis at day 21 (section 4.8.1)(see Figure 21).

All three donors demonstrated ALP at 14 days. At 21 days calcium deposits were also identified in all three donors by Alizarin Red staining. Total calcium production over 21 days was quantified colorimetrically using a standard calcium assay kit, and showed calcium production values consistent with SY MSC (Jones *et al*, 2010).

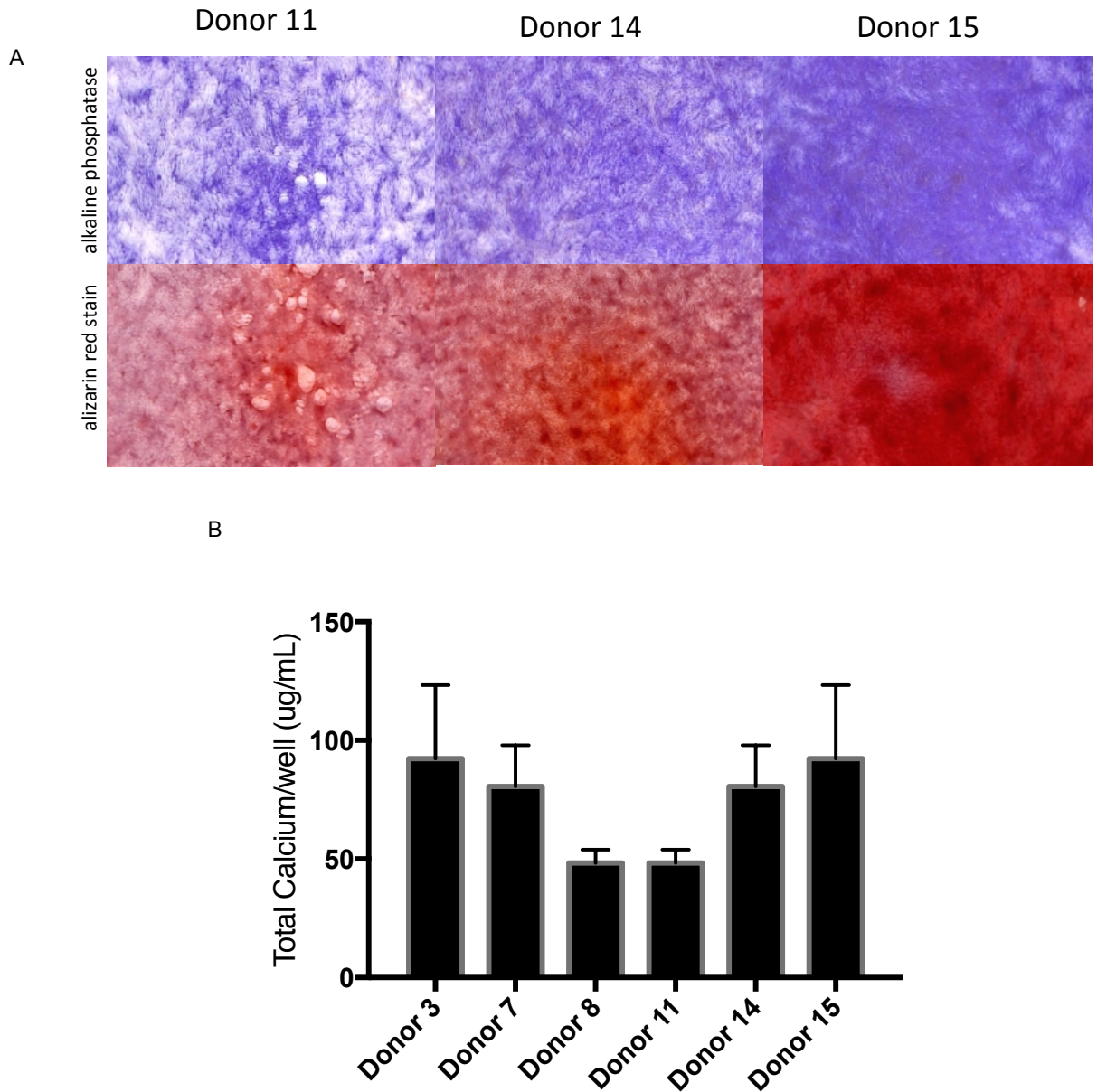


Figure 21: **Osteogenesis of ex vivo released SY MSC** (A) Examples images of alkaline phosphatase and alizarin red staining (n=3 donors). (B) Total calcium/well ($\mu\text{g/mL}$) production (n=6 donors). Each bar represents readings calculated from triplicates per donor as mean and standard deviation. These findings demonstrate acceptable osteogenic capabilities.

Adipogenesis was evaluated both qualitatively and quantitatively, for the released passage 3-5 cell cultures in triplicate. At day 21, oil red-O staining was performed to visualise lipid-containing vesicles produced by the MSC. Nile red-DAPI ratio was used to quantify adipogenesis, where large Nile red:DAPI ratios denote high lipid concentration per cell (see Figure 22).

Lipid filled vesicles were observed using oil-red-O staining, and ratios of Nile red against DAPI, displayed good yields of adipogenesis (Aldridge *et al*, 2013), thus adipogenic potential was demonstrated in all three donors.

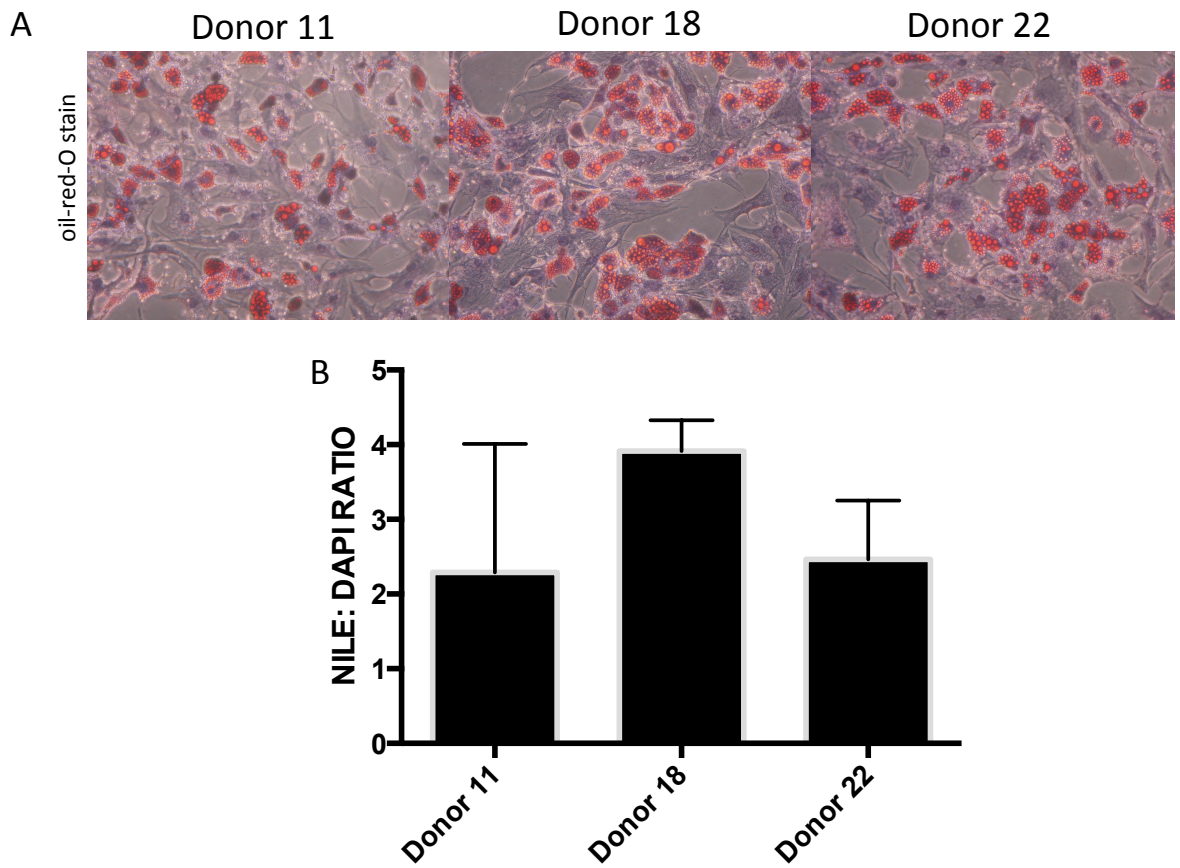


Figure 22: **Adipogenesis of ex vivo released SY MSC** (A) Oil-red-O staining at x20 magnification for all three donors shows lipid filled vesicles. (B) Quantification of adipogenesis assays, using Nile:DAPI ratio, mean value of triplicates, including SD (n=3 donors). These findings demonstrate acceptable adipogenic capabilities.

Chondrogenesis was evaluated both qualitatively and quantitatively. Pellets were cultured for 21 days from passage 3-5 cultures of five donors in triplicates. For qualitative analysis chondrogenic pellets were sectioned and stained with toluidine blue. This stain highlights cartilaginous extracellular matrix purple, and fibrous tissue blue. To quantify chondrogenesis, the remaining pellets were digested in papain. Sulfated-GAG (s-GAG) precipitate was quantified using the Blyscan™ s-GAG assay (see Figure 23).

Chondrogenic potential was demonstrated using GAG quantitative measurements. Donor-to-donor variation was observed within the chondrogenic assay; however, overall results demonstrate chondrogenesis consistent with SY MSC (Jones *et al*, 2010). In summary, overall results for trilineage differentiation of *ex vivo* culture expanded MSC released from human synovium, do indeed show their trilineage differentiation capabilities.

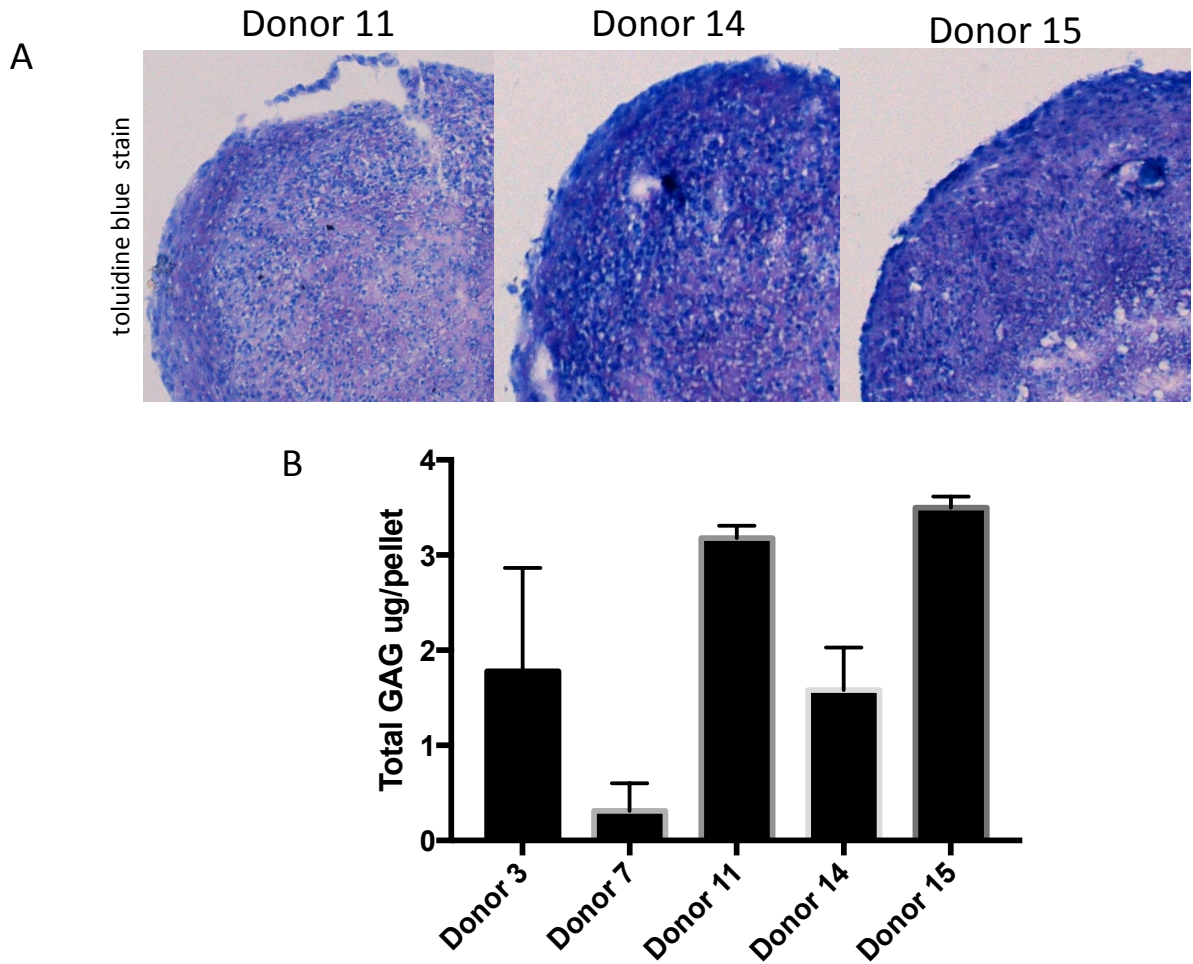


Figure 23: **Chondrogenesis of ex vivo released SY MSC.** (A) Chondrogenic pellets stained with toluidine blue showed GAG stained in purple, and fibrous tissue stained blue for three donors. (B) Total GAG content ($\mu\text{g}/\text{pellet}$) for each sample following papain digestion, mean value of triplicates, including SD ($n=5$ donors). These findings demonstrate acceptable chondrogenic capabilities.

5.7 Adhesion and migration of MSC within relevant biological scaffolds

The study so far has demonstrated the ability to release MSC from the synovium during an *ex vivo* experiment. These MSC proliferated *ex vivo* and had trilineage differentiation potential. Previous research has identified these cells as a source of highly chondrogenic MSC (Sakaguchi *et al*, 2005). Current models for using these types of cells require a two stage surgical procedure, and cell culture expansion. So far this study has shown that these cells can be accessed with a single surgical process, and thus become an attractive option in the repair of articular cartilage injuries.

The focus of this section of the thesis was to evaluate the interaction of SY MSC when they are allowed to attach and migrate through biological scaffolds. Biological scaffolds such as whole blood scaffold (WB) is created by the orthopaedic surgeon during an arthroscopy procedure (known clinically as a microfracture) (section 1.6), it allows for repair of isolated articular injuries; Fibrin glue (FG), is an effective scaffolds for focused MSC delivery within articular cartilage defects (Kim *et al*, 2015). Platelet rich plasma (PRP), known for their delivery of growth factors (GF), that aid in tissue repair (Van Pham *et al*, 2013; Ornetti *et al*, 2016) and Human platelet lysates (hPL), a more immediate source of GF that is released due to lysis of platelets. This can be a quicker source of GF compared to PRP which releases its GF content in a slower manner (Jonsdottir-Buch *et al*, 2013; Radtke *et al*, 2014; Sellberg *et al*, 2016).

5.8 Adhesion of MSC to relevant biological scaffolds

To quantify MSC adhesion onto biological scaffolds, culture expanded SY MSC from donor 4 (table 2) were used. Following the method describes in section 4.16, non-adhered cells were stained with methylene blue, after being plated overnight to allow for plastic adherence. Cells were counted using Image-J 1.48V software. The percentage of adhered MSC was plotted using the calculation described in methods (section 4.16) (see Figure 24).

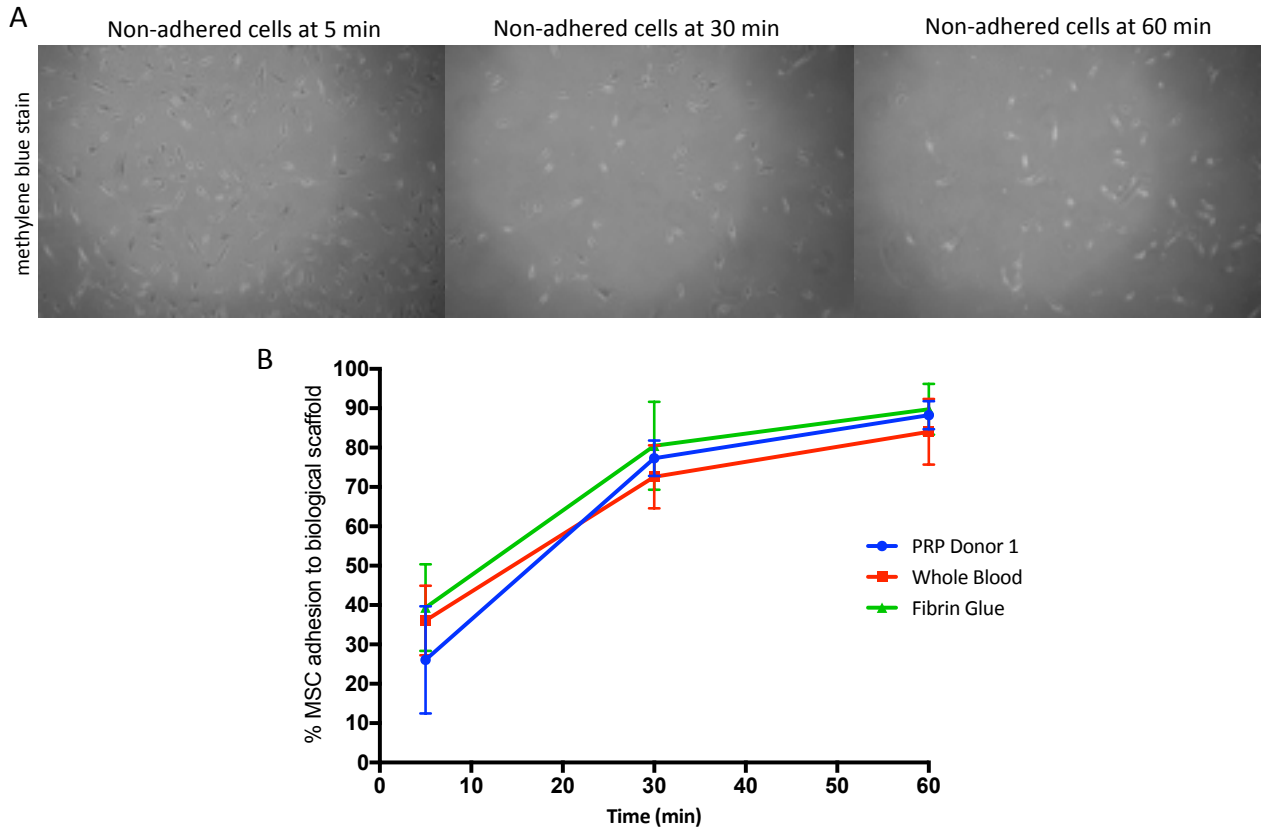


Figure 24: **Adhesion of MSC to biological scaffolds.** (A) After adhesion to a biological scaffold, non-adhered MSC from 1 donor were plated overnight, then subsequently fixed and stained with methylene blue. Images taken at x10 magnification. (B) The percentage of adhered MSC to all three biological scaffolds was plotted against 5, 30 and 60 minutes. These findings show a rapid adhesion of the MSC to all biological scaffolds.

It was demonstrated that 25-35% of MSC adhered within 5 minutes, 65-75% of adherence took place within 30 minutes, and >80% adhered after 60 minutes, with no variation between PRP (n=3 blood donors), WB (n=3 blood donors) and FG scaffold.

To account for potential variation in SY MSC between different donors in their adhesion abilities to a biological scaffold, this experiment was repeated with use of a FG scaffolds (section 4.12), and three different culture expanded MSC donors (donor 5, 6 and 7, see table 2). Following the method describes in section 4.16, the percentage of adhered MSC was plotted against 5, 30 and 60 minutes (see Figure 25).

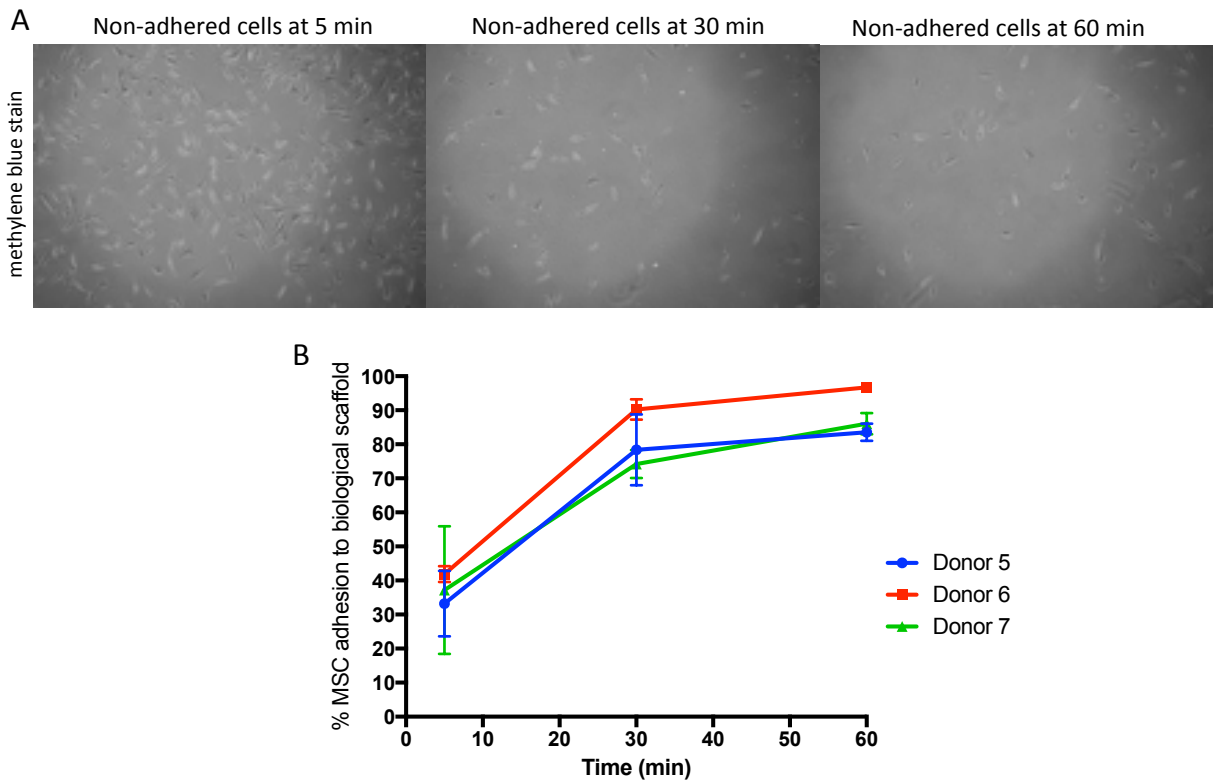


Figure 25: **Adhesion of MSC to FG scaffold** (A) Non-adhered MSC were plated overnight, then subsequently fixed and stained with methylene blue (representative donor). Images taken at x10 magnification. (B) The percentage of adhered MSC to FG scaffold was plotted against 5, 30 and 60 minutes. These findings show a rapid adhesion of the MSC to FG scaffolds.

It was demonstrated that 30-40% of MSC adhered within 5 minutes, 75-90% of adherence took place within 30 minutes, and no marked change of adherence levels up to 60 minutes (75-90%), this trend was similar for donor 5, donor 6 and donor 7 SY MSC adherence to FG scaffold. These rapid kinetic interactions are compatible with an almost immediate potential for MSC interactions *in vivo* following MSC release.

Results demonstrate that SY MSC adhesion to biological scaffolds is rapid, with more than 65% occurring within 30minutes. In addition, it was shown that no significant SY MSC donor variation occurred on adhesion times, when three different donor SY MSC were allowed to adhere to a FG scaffold.

5.9 Migration of MSC towards relevant biological scaffolds

The focus of this study now will be to investigate migration of SY MSC toward biological scaffolds, in the form of PRP and human platelet lysates (hPL). As stated in the introduction (section 1.13), PRP is currently used as a therapeutic treatment within an arthritic joint, due to its release of growth factors (GF), which aid in regenerative articular repair (Mollon *et al*, 2013). Within a clinical setting, PRP can easily be made using the patient own blood. To quantify migration of SY MSC towards biological scaffolds with high GF content, PRP and hPL were used. FG was not used for migration studies, as one of the aims of this study was to use the patients' own supply of biological scaffold, thus making this procedure cost effective in the clinical setting. In addition, WB was not selected for migration studies due to previous studies demonstrating an increase level of GF in PRP compared to WB (Lee *et al*, 2013). As hPL is known to have a more immediate supply of GF (Jonsdottir-Buch *et al*, 2013; Radtke *et al*, 2014; Sellberg *et al*, 2016), it was an ideal scaffold to be used to compare migration with PRP. Using a transwell plate, cultured SY MSC from donor 8 (n=1 donor, see table 2) were allowed to migrate through 8 µm pores towards liquid form of either 10% PRP, 10% hPL scaffold (pooled from 3 donors) or a positive control (10% FCS + DMEM) or negative control (0.5 % FCS + DMEM) media, (see Figure 26).

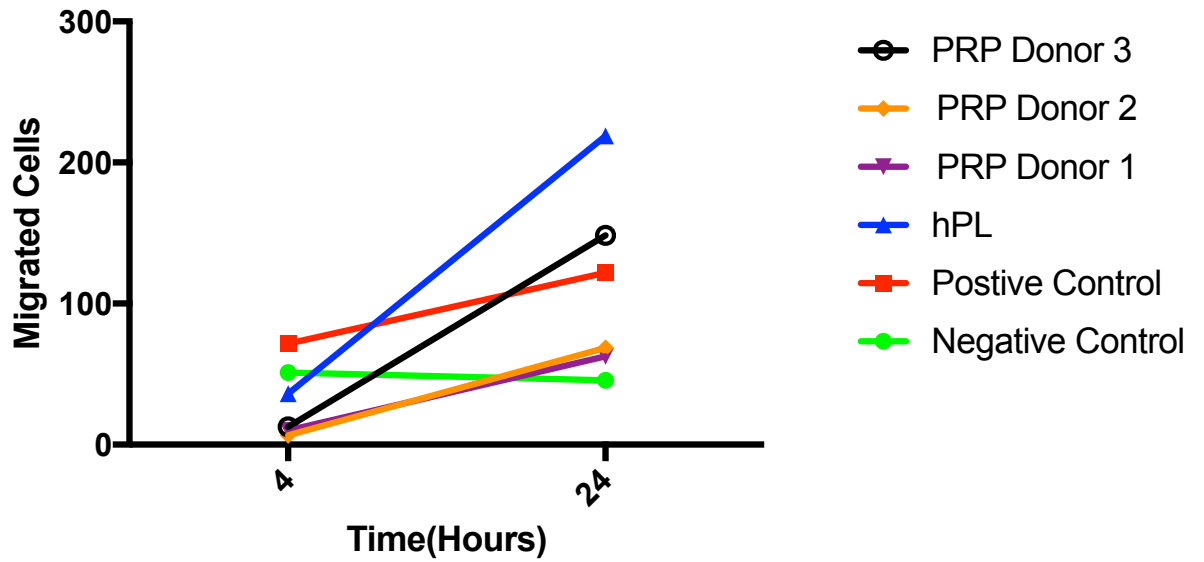


Figure 26: **Migration of cultured SY MSC towards PRP and hPL.** Migration of cultured SY MSC (from donor 8, table 3) with 10% pooled hPL compared to 10% PRP (three hematological healthy donors), and positive (10% FCS) and negative (0.5% FCS) controls, at both 4 and 24 hours. Data represented by mean value of triplicate wells of migrated cells. These findings demonstrate favorable migration towards PRP and hPL at 24 hours.

The results demonstrate SY MSC migration towards both PRP and hPL that occurs better at 24 hours compared to 4 hours. In addition, both PRP and hPL demonstrated better migration of SY MSC compared to negative control. This study will now investigate migration of SY MSC through 3D biological scaffolds.

5.10 Migration of MSC through relevant 3D biological scaffolds

Next the migration of SY MSC through 3D biological scaffolds was investigated utilizing PRP and PPP. As described earlier, PRP has a supply of GF, and it is expected that the lack of platelets within PPP, potentially showed less MSC migration compared to PRP, due to the lack of GF in PPP. Migration was allowed to take place over a ten days period, following which the scaffolds were sectioned and stained with H&E, allowing for migrated cells to be counted (section 4.18), (see Figure 27).

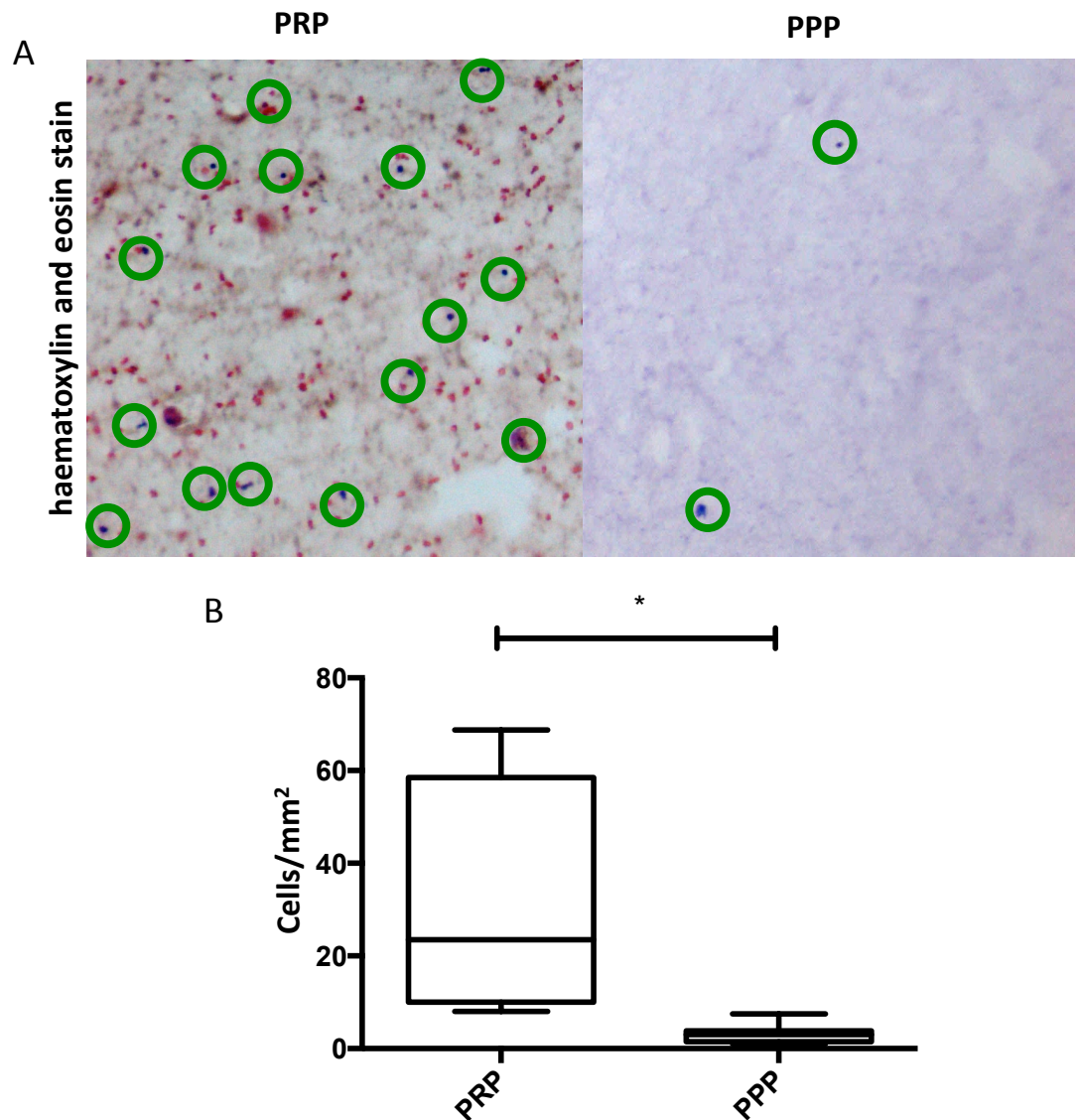


Figure 27: Migration of cultured SY MSC through relevant biological scaffolds (3D model). (A) Image of putatively migrated SY MSC through PRP and PPP scaffolds following 10 days assay (x10 magnification). Green circles highlight some putatively migrated cells. (B) This demonstrated higher numbers of cells in PRP scaffolds compared to PPP scaffolds (*= $p < 0.02$, Wilcoxon test). Data represented by median value of triplicate sections, with upper and lower quartiles and minimum and maximum values ($n = 7$ blood donors).

These results demonstrated a statistical increase in cells present in PRP biological scaffolds compared to PPP biological scaffold, at 10 days of migration. However, it was observed that some cells were present in PPP biological scaffolds. As there is some platelets remaining in PPP following processing, these platelets could potentially be contributing to some GF release, thus could be a cause for a small number of cells to migrate through a PPP biological scaffold. Furthermore, it would be important to consider that cells that are being counted may not be MSC. Instead these cells could be constituents of PRP, for example leukocytes which remained in PRP following its preparation (Amable *et al*, 2013; Lee *et al*, 2013). To test this, SY MSC in scaffolds were investigated further.

5.11 Investigation of cell type, migrating through relevant biological scaffolds (3D model)

As stated above, higher levels of cell counts in a PRP scaffold compared to a PPP scaffold were evident. To verify these cells were MSC, a migration assay was performed, to compare PRP scaffolds seeded with MSC and PRP scaffolds not seeded with MSC. As a control, hPL was used due to its high GF content, but lack of leukocytes. Migration took place over a ten-day period. Two hematological healthy PRP donors were used and each donor had one PRP scaffold seeded with cultured SY MSC, and another PRP scaffold not seeded with MSC. After the migration period, scaffolds were sectioned and stained with H&E, and cells visualised and counted. (section 4.19), (see Figure 28).

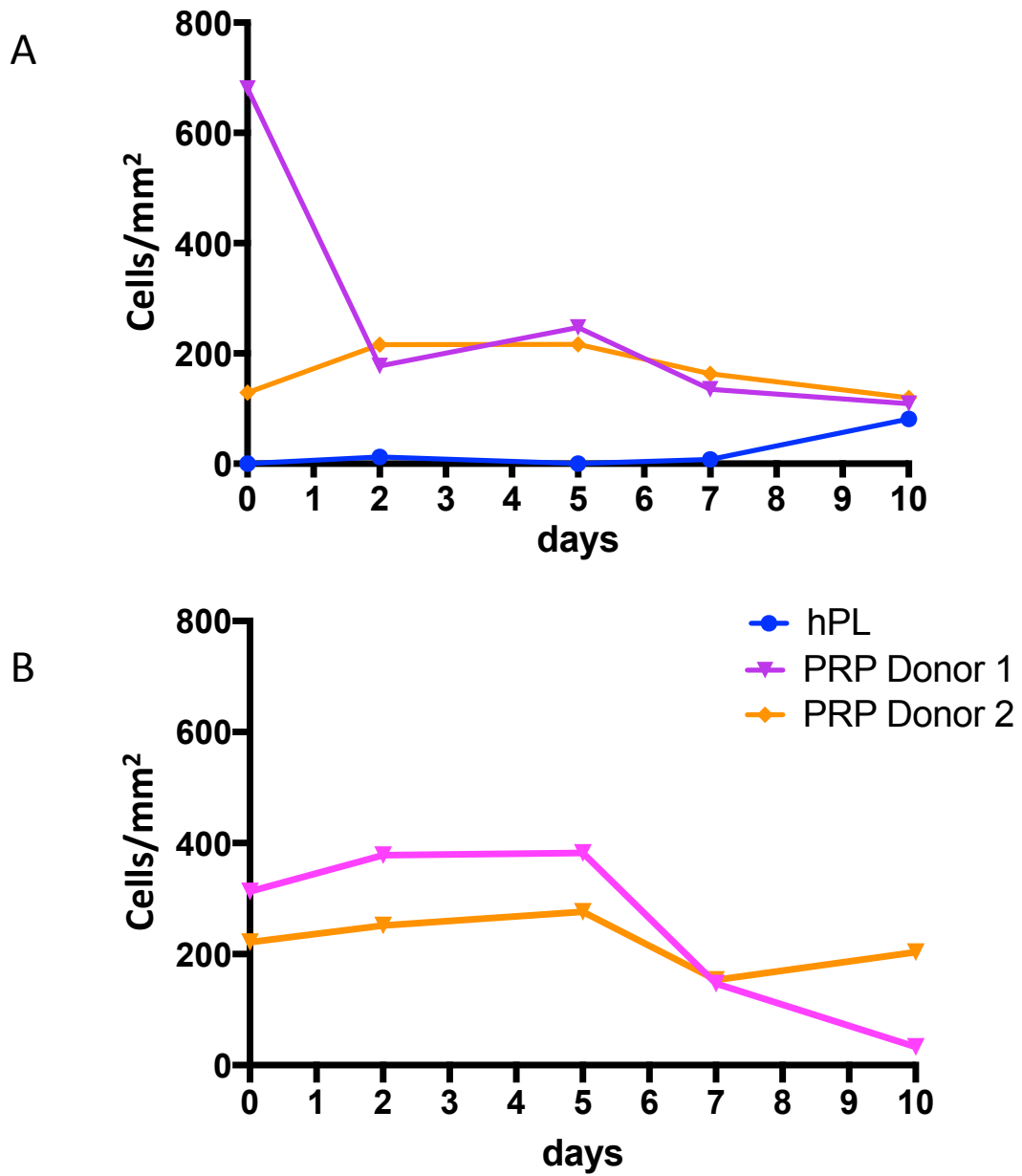


Figure 28: **Cells counted within relevant 3D biological scaffolds.** (A) PRP donor 1 and 2, seeded with SY MSC, demonstrate a gradual decline in cell numbers over a 10 days period. No cells are counted in hPL until day 7. (B) PRP donor 1, not seeded with SY MSC, demonstrates a gradual decline in cell numbers over a 10 days period; however, PRP donor 2, not seeded with SY MSC, demonstrates a gradual decline in counted cell numbers over a 7 days period followed by then, an increase of cell numbers.

Results demonstrated a gradual decline in the number of cells over a 10 day period, in both PRP donors, seeded with SY MSC, in addition a similar gradual decline in cell numbers was observed within PRP donor 1 scaffold, not seeded with SY MSC, and up to 7 days in PRP donor 2 scaffold, after which a small increase is observed. These results indicate that the cells being counted could indeed be another cell type, such as leukocytes, which can exist in PRP fractions (Amable *et al*, 2013) and not migrated SY MSC. This was further supported by the lack of cells counted initially, in the hPL scaffold, which was expected, followed by an increase in cells numbers after 7 days. Altogether, these data indicated that SY MSC migration through biological scaffolds are likely to be a slow process (after 7 days).

To confirm this, a dual staining assay was set up, to label surface markers typically seen on MSC (CD73) as opposed to leukocytes (CD45), using this method of staining, accurate cell identification was achieved (section 5.12).

5.12 Dual staining of SY MSC and leucocytes with specific surface marker primary antibodies

Using PRP from one healthy individual, a 3D biological scaffold was created, and culture expanded SY MSC from one donor, were embedded into each scaffold, to ensure cells would be visualised once stained (section 3.13). The primary antibodies, namely anti-CD73 for MSC and anti-CD45 for leucocytes were used to stain the sectioned biological scaffolds. Due to the novelty of this type of dual stain, previous studies using a similar method were used as a guide (Letouzey *et al*, 2015), (see Figure 29).

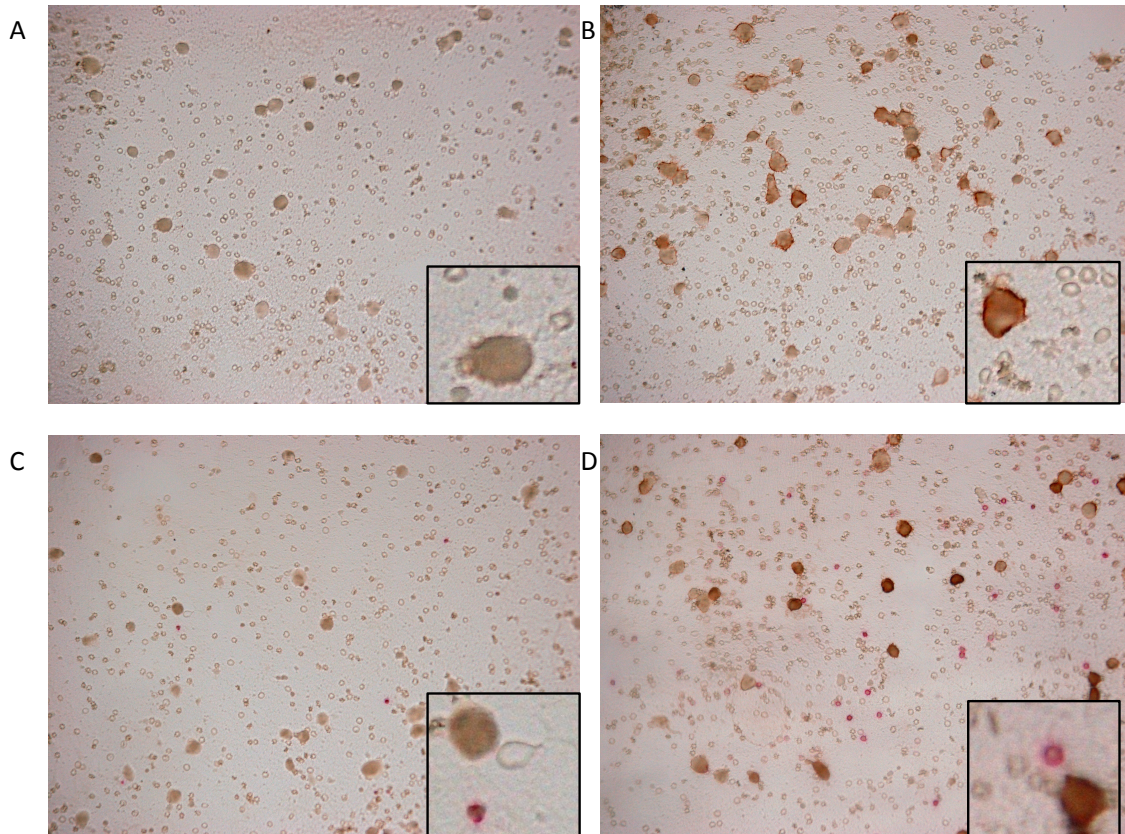


Figure 29: **Dual staining of PRP scaffold embedded with cultured SY MSC.** (A) Negative isotype control stained PRP scaffold (B) PRP scaffold stained with CD73 primary antibody; embedded MSC appearing as large deep brown stained cells, highlighted in the inserted image. (C) PRP scaffold stained with CD45 primary antibody; leukocytes present in PRP appearing as small red stained cells, highlighted in the inserted image. (D) PRP scaffold dual stained with CD45 and CD73 primary antibodies, leukocytes and SY MSC appearing as small red and large deep brown stained cells, respectively highlighted in the inserted image. Original magnification x 10 (x 20 for inserts).

The above images provided a reference for how SY MSC within a biological scaffold look morphologically and phenotypically (large, CD73 positive cells). The investigation progressed to identify these cells by dual staining method following migration (section 4.21), and to decide if indeed the cells being counted in sections 5.10 and 5.11 were migrated MSC, and not leukocytes present in PRP. Using PRP and PPP from one healthy individual, biological scaffolds were created. Culture expanded SY MSC from one donor, were seeded in a monolayer, and migration was allowed to take place over a 10 day period. Similar to previous migration study (section 4.14 and 4.20), scaffolds were sectioned and dual stained, and were visualised using the Olympus CKX41 light microscope. A control, PPP scaffold was created, which had no MSC seeded for migration, over a 10 day period, (see Figure 30).

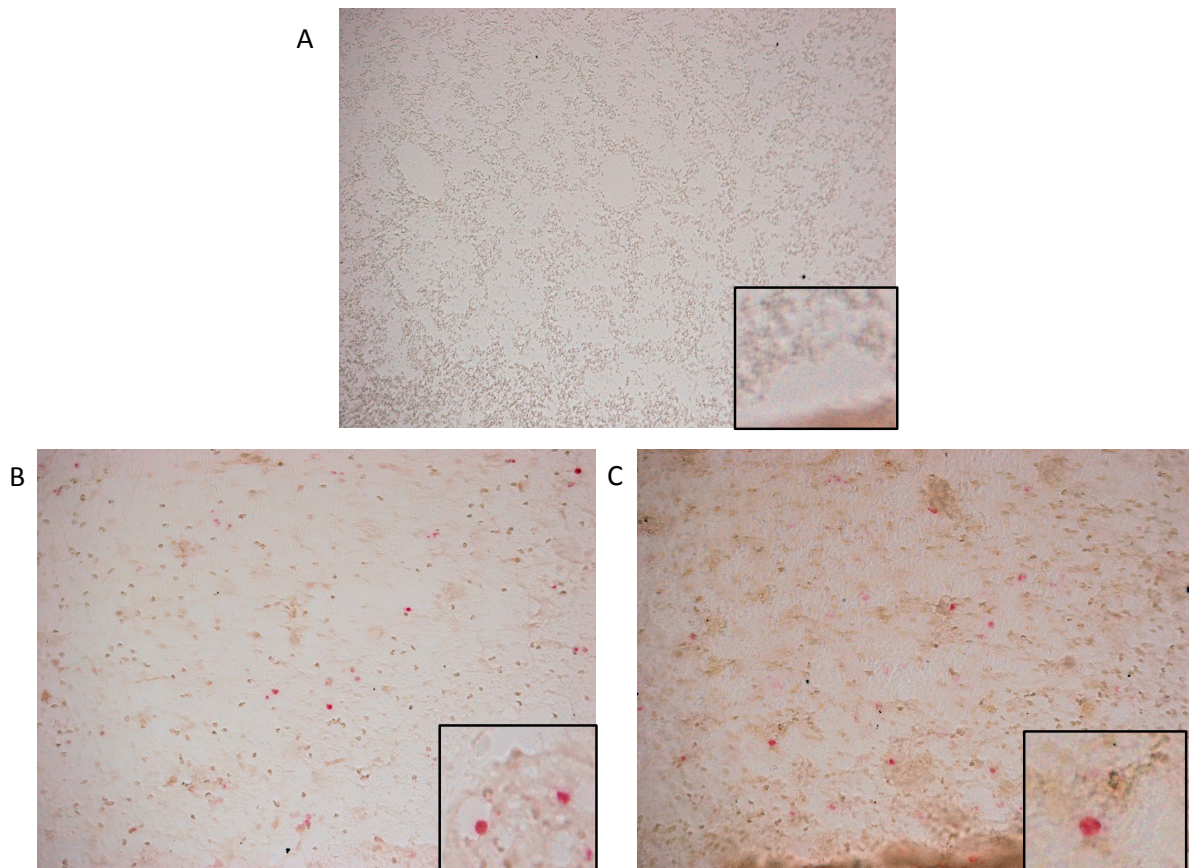


Figure 30: **Dual staining of PRP and PPP scaffold following 10 days of migration with cultured SY MSC.** (A) Negative control, PPP scaffold with cultured SY MSC. PPP scaffold dual stained with CD45 and CD73 primary antibodies, no CD45 stained or CD73 stained cells were identified, highlighted in the inserted image. (B) PRP scaffold with cultured SY MSC. PRP scaffold dual stained with CD45 and CD73 primary antibodies, small CD45 stained cells identified, with no CD73 stained cells observed, highlighted in the inserted image. (C) PRP scaffold with no cultured SY MSC. PRP scaffold dual stained with CD45 and CD73 primary antibodies, CD45 stained cells identified, with no CD73 stained cells observed, highlighted in the inserted image. Original magnification x 10 (x 20 for inserts)

The above images suggest that the cells counted in previous section of this study, investigating SY MSC migration through a relevant biological scaffold, were not SY MSC. The cells counted were likely leucocytes as they have been identified in similar quantities through scaffolds from both MSC seeded and non-seeded migrational studies, and been similarly stained with CD45 primary antibody. It is highly likely that previous section of this study (section 5.10 and 5.11) where cells being counted as migrated cells, were in fact leukocytes, and the MSC did not migrate through these biological scaffolds during chosen experimental timeframes (up to 10 days).

In summary of SY MSC adherence and migration investigations, the results supported the hypothesis that SY MSC adhered and migrated towards biological scaffold; however, SY MSC migration through a biological scaffold did not occur in given experimental conditions. This suggests that SY MSC can be used for regeneration of the damaged articular cartilage; however, they may need to be placed in position of the articular defect, rather than being released and allowed to self-migrate towards and through a biological scaffold.

5.13 Obtaining MSC from the synovium using the PAP brush *in vivo*

The following experiments aimed to demonstrate *in vivo* release of MSC from human synovium using the PAP brush. The focus of this part of the study was to use the knowledge gained from previous *ex vivo* experiments within an *in vivo* setting. MSC released from the synovium and those resident in SF were investigated using CFU-F assays. Flow cytometry was performed on cultured MSC (n=1 donor) as described in section 4.24, to demonstrate MSC phenotype. Trilineage potential of released and SF MSC was investigated using differentiation assays. Table 3 shows a patient overview.

Table 3: An overview of patients participating in the *in vivo* PAP brushing study. Highlighted rows (n=5 donors) identify patients where culture expanded MSC were used for trilineage differentiation.

Donor	Age (yrs)	Gender	Injury Type	Surgery
23	25.4	F	Left Knee Lateral Meniscal Tear	Arthroscopic Left Knee Lateral Meniscal Tear Repair
24	39.0	M	Left Knee Anterior Cruciate Rupture	Arthroscopic Left Knee Anterior Cruciate Rupture Repair
25	40.4	M	Right Knee Medial Meniscal Tear	Arthroscopic Right Knee Medial Meniscal Tear Repair
26	18.1	M	Right Knee Lateral Meniscal Tear + Anterior Cruciate Rupture	Arthroscopic Right Knee Lateral Meniscal Tear + Anterior Cruciate Rupture Repair
27	19.3	M	Left Knee Lateral Meniscal Tear	Arthroscopic Left Knee Lateral Meniscal Tear Repair
28	19.9	F	Left Knee Lateral Partial Meniscal Tear	Arthroscopic Left Knee Lateral Partial Meniscal Tear Repair
29	52.0	M	Left Knee Medial Partial Meniscal Tear	Arthroscopic Left Knee Medial Partial Meniscal Tear Repair
30	36.0	M	Right Knee Anterior Cruciate Rupture	Arthroscopic Right Knee Anterior Cruciate Rupture Repair
31	29.5	M	Right Knee Anterior Cruciate Rupture	Arthroscopic Right Knee Anterior Cruciate Rupture Repair

5.14 Obtaining MSC from the synovial fluid and synovium using the PAP brush *in vivo*

Patients with a median age of 29.5 years old (18.1-52.0) undergoing elective knee diagnostic or therapeutic arthroscopies participated in this study (n= 9 donors, Table 3). Three samples were collected from each donor, these consisted of 1) synovial fluid (SF), which in some cases was supplemented with saline, to aid in extraction; 2) Irrigation fluid, which was collected following the planned procedure (i.e. debridement) and 3) fluid collection after brushing of the synovium with the PAP brush (referred as post-brushing). All samples were transported within an hour of extraction to the research laboratory for processing in sterile conditions. SF with a mean volume of 37.2mL (5-50mL), irrigation with a mean volume of 39.1mL (25-50mL), and post brushing with a mean volume of 53.1mL (50-70mL) were all collected in separate sterile container. Although all samples had differences in their volumes when received from the operating theatre, total number cells for each sample were normalized. This was achieved by centrifuging each cells sample into a pellet, and then re-suspending the pellet of cells in the same volume (10mL) of expansion media for each sample. CFU-F assays were performed on matched samples and data presented as colonies per sample, (see Figure 31)

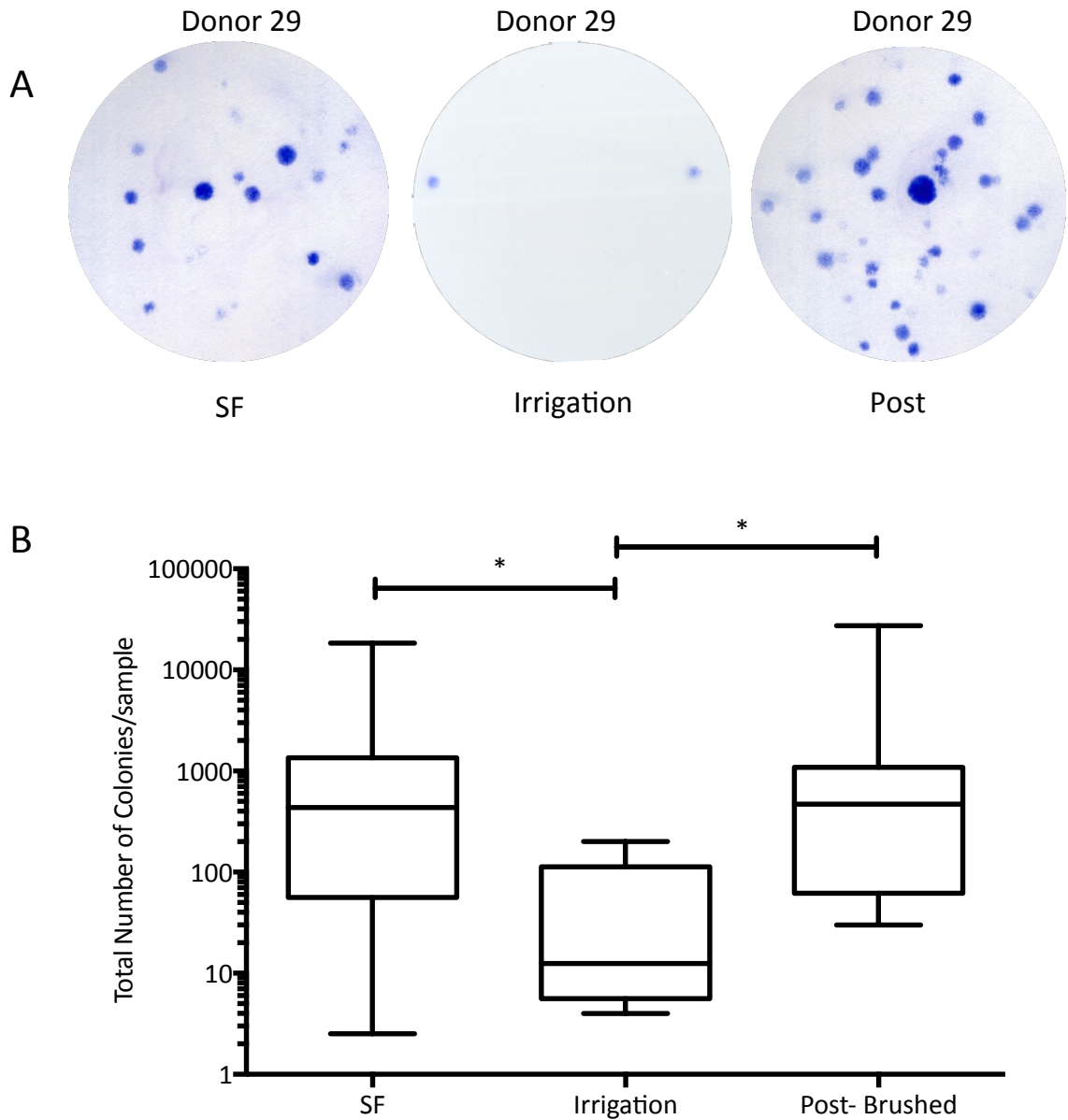


Figure 31: **Release of MSC from human synovium using *in vivo*, using PAP brush.** (A) Examples of CFU-F assays for SF, irrigation and post brushing samples (n=1 donor) stained with methylene blue. (B) CFU-F quantification data for the three arthroscopy samples: SF, irrigation and post-brushing. Data represented by median; (SF =432.5, irrigation = 12.5, post brushing = 470 colonies/sample) with upper and lower quartiles and minimum and maximum values (* = p<0.02, paired Wilcoxon test) (n=9 donors).

These CFU-F assays quantify MSC numbers from SF, irrigation and post synovial brushing, following an arthroscopy procedure and clearly demonstrated replacement of SF MSC, lost during irrigation in a standard arthroscopic procedure, with MSC from the post brushing phase, demonstrating no change (+0.09 fold increase) in colonies/sample. The PAP brush is not a device that is built to be used for such as procedure, thus one would expect an increase in released cell numbers when using a purpose made device to release SY MSC. Of particular note, the operating surgeon found this device unwieldy and difficult to manoeuvre in the suprapatellar pouch during brushing.

5.15 Cell surface phenotype of culture expanded SF and post brushed cells

Flow cytometry was performed on culture expanded SF cells (n=3 donor) and post brushing released MSC (n=3 donor), passage 5-7, as described in section 4.24. Percentage of positive population for both phenotypically positive markers for MSC (CD73, CD90, CD105) and negative markers for MSC (CD14, CD19, CD34, CD45) consistent for expanded MSC (Jones *et al*, 2010; Harvanová *et al*, 2011; Baboolal *et al*, 2014) were plotted (see Figure 32).

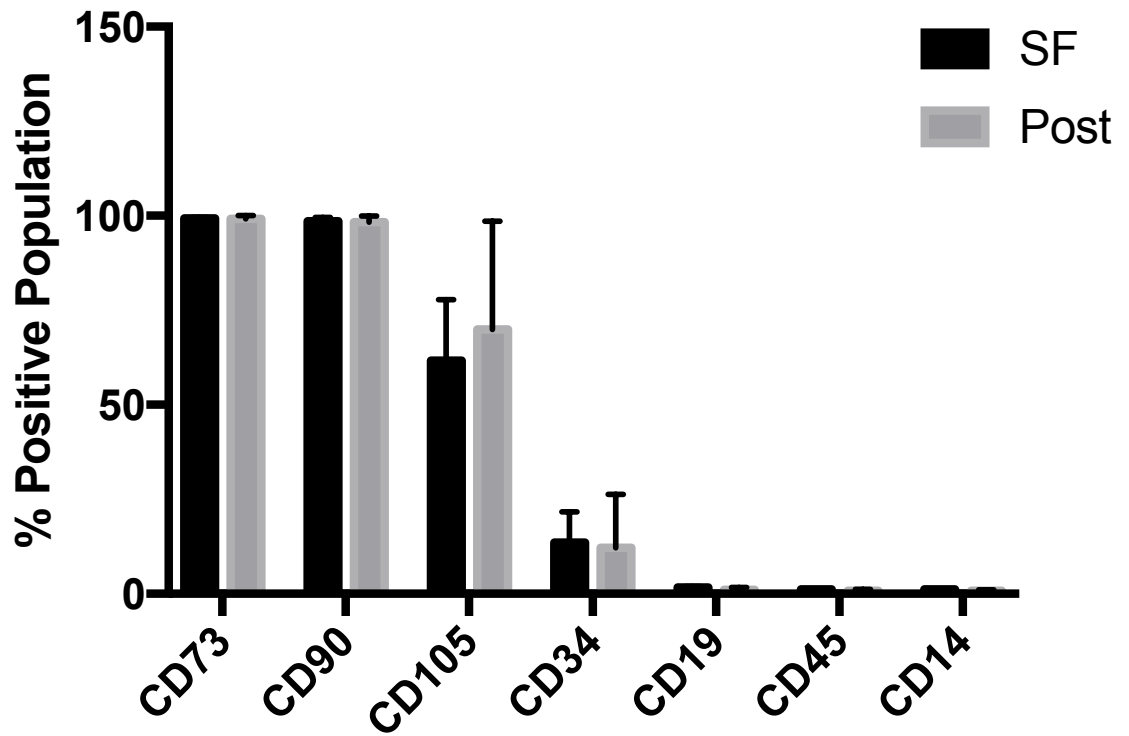


Figure 32: **Surface phenotype of culture expanded SF and Post brushed cells.** Percentage positive population for known positive and negative MSC surface markers for SF cells (n=3 donor) and post brushed cells (n=3 donor). Error bars represent standard deviation.

The results demonstrated that both SF cells and those released from post synovial brushing, were phenotypically are MSC.

5.16 Trilineage differentiation of cultured MSC obtained from human synovium brushing *in vivo* study

Investigation of SF MSC and SY MSC was undertaken using culture expanded cells passage 4-7, for trilineage differentiation. Osteogenesis was evaluated both qualitatively and quantitatively for five donors, undertaken in triplicate, as described in section 4.8.1. Alkaline phosphatase (an early osteoblast marker) staining at day 14 and alizarin red (demonstrating calcium deposits) at day 21 were performed. Total amount of calcium produced was quantified colorimetrically using a standard calcium assay kit for osteogenesis at day 21, (see Figure 33).

Total calcium values were comparable to previous studies (Jones *et al*, 2010). No statistical significance between total calcium production from SF MSC and SY MSC was observed, suggesting that both cells have similar osteogenic properties.

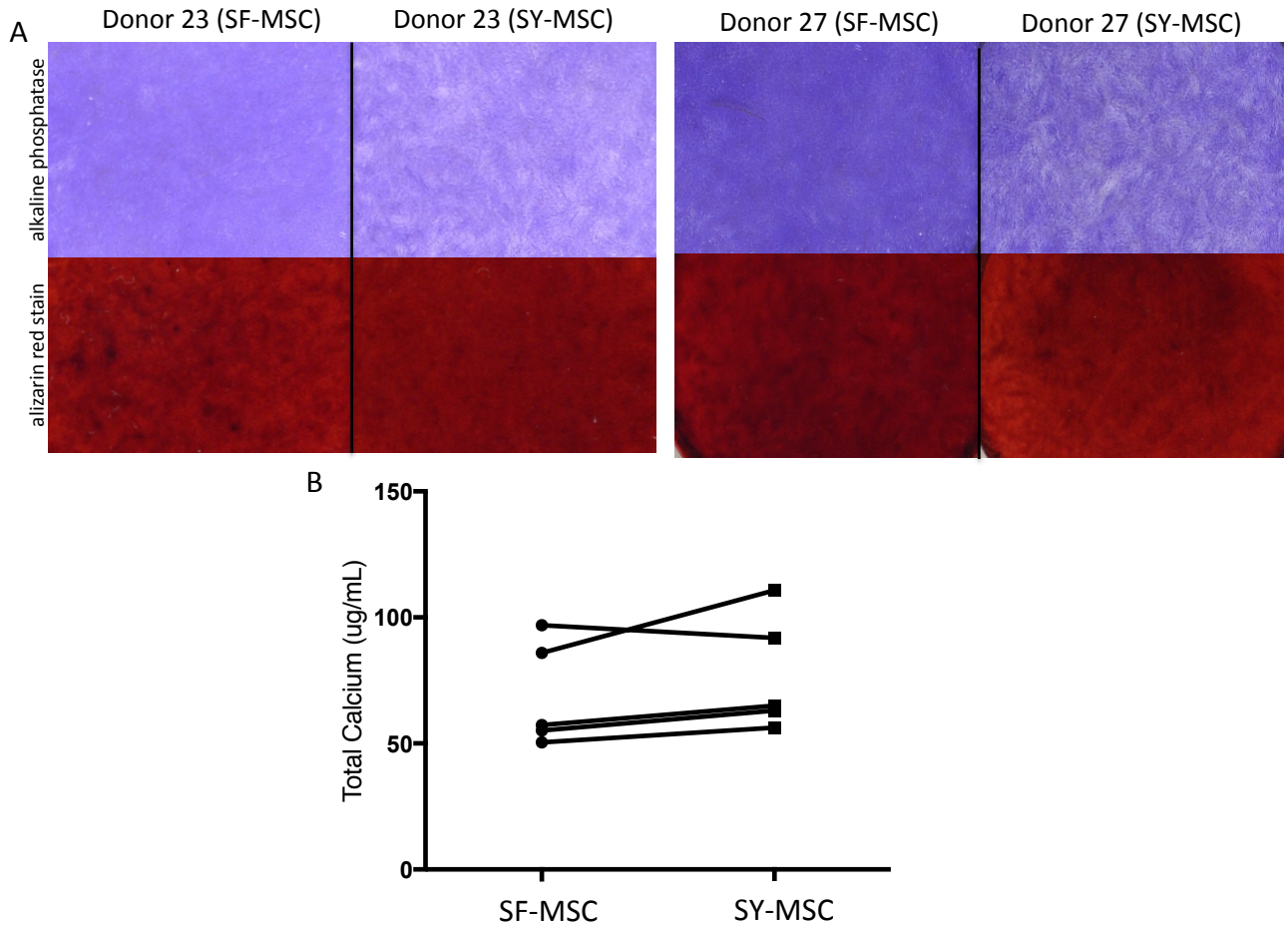


Figure 33: **Osteogenesis of *in vivo* released and cultured, donor matched SF MSC and SY MSC.** (A) Examples of alkaline phosphatase and alizarin red staining, for SF MSC and SY MSC (n=2 donors) (scanned images). (B) Total calcium/well ($\mu\text{g/mL}$) production for SF MSC and SY MSC (n=5 donors). These findings demonstrate acceptable osteogenic capabilities.

Adipogenesis was evaluated both qualitatively and quantitatively, using cultured SF MSC and SY MSC for five donors, as described in section 4.8.2. At day 21, Oil red-O staining was performed; showing lipid-containing vesicles produced by the MSC. Nile red-DAPI ratio was used to quantify adipogenesis. Large Nile red:DAPI ratios denote high lipid concentration per cell, (see Figure 34).

Adipogenic potential was demonstrated from SF MSC and SY MSC. These results were comparable to those observed previously in this study and other studies (Aldridge *et al*, 2013). Lipid filled vesicles was observed using oil-red-O staining, and ratios of Nile red against DAPI, displayed good yields of adipogenesis. No statistical significance was observed for total lipid production between SF MSC and SY MSC, implying that both cells have similar adipogenic properties.

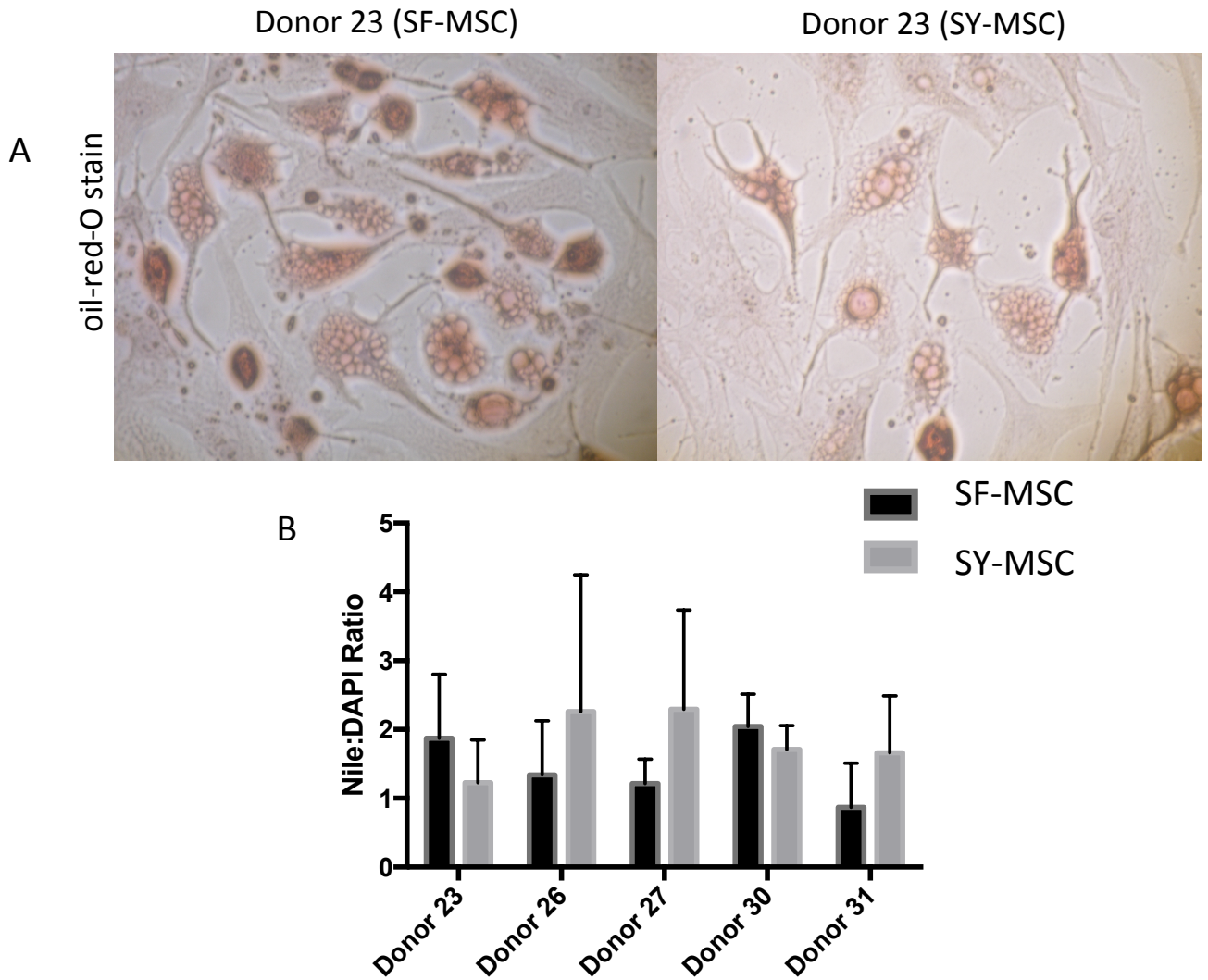


Figure 34: **Adipogenesis of *in vivo* released and cultured, donor matched SF MSC and SY MSC.** (A) Oil red-O stain at x20 magnification of SF MSC and SY MSC (n=1 donor). (B) Quantification of adipogenesis using Nile:DAPI ratio, mean values of triplicates. Error bars represent standard deviation (n=5 donors). These findings demonstrate acceptable adipogenic capabilities.

Chondrogenesis was evaluated both qualitatively and quantitatively as described in section 4.8.3. Chondrogenic pellets were cultured for 21 days, for both SF MSC and SY MSC. Quantitative analysis was performed using the Blyscan™ s-GAG assay kit for five donors (in triplicate for each donor), at day 21, and three donor cultures were used for qualitative assay (see Figure 35).

Chondrogenic potential was demonstrated from the SF MSC and SY MSC. Both quantification and qualification was comparable to those observed in previous studies (English *et al*, 2007; Jones *et al*, 2010). No statistical significance between total GAG production from SF MSC and SY MSC was observed, implying that both cells types have similar chondrogenic properties.

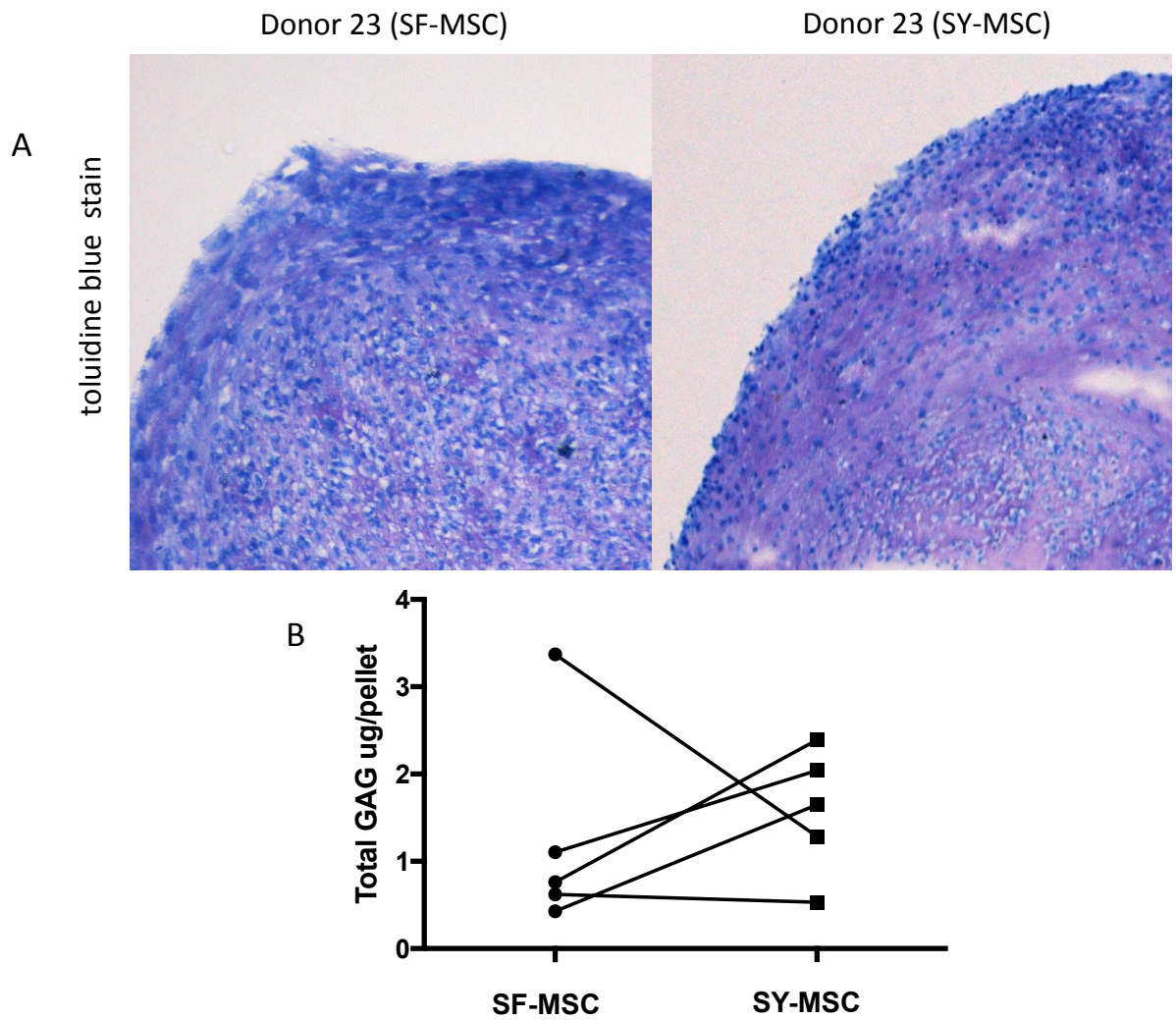


Figure 35: **Chondrogenesis of *in vivo* released and cultured, donor matched, SF MSC and SY MSC.** (A) Chondrogenic pellets stained with toluidine blue, showed GAG stained in purple, and fibrous tissue stained blue (n=1 donor). (B) Total GAG content in µg/pellet for each sample following papain digestion (n=5 donors). Data represented by mean value of the triplicate assays (total GAG content) per samples, for each donor. These findings demonstrate acceptable chondrogenic capabilities.

The overall results for trilineage differentiation of *in vivo* released (using PAP brush) SF MSC and SY MSC demonstrated that resulting cultures do indeed show trilineage differentiation capabilities, both qualitatively and quantitatively. In three out of five donors, SY MSC demonstrated overall higher levels of GAG and lipid production compared to resident SF MSC. Calcium production was similar between both MSC sources. In summary, experiments demonstrated encouraging results in terms of *in vivo* released SY MSC and their use for cartilage defect repair.

As previously mentioned, the design of the PAP brush is not built for the purpose of arthroscopy, hence the consideration of the purpose made device that would further increase and augment SY MSC numbers. For this reason, a tailored synovial MSC releasing device was developed based on prototype C8 (see Figure 15), with design opinions from expert orthopaedic surgeons who would use this device during an arthroscopic procedure, thus providing in depth knowledge, of device design requirements. The following set of experiments substituted the PAP brush with the purpose built device; however, maintained the same experimental protocols, allowing both devices to be relatively compared.

5.17 Obtaining MSC from the synovium using a purpose made device to release SY MSC *in vivo*

The following experiments aimed to demonstrate *in vivo* release of MSC from human synovium using a purpose made device (based on prototype C8, see Figure 15). Quantification of released cells and those originating within the synovial fluid, were compared, to consolidate the hypothesis that MSC lost during the irrigation phase of an arthroscopy procedure can be replaced using a purpose made device that releases SY MSC. Patients with a median age of 33.7 years old (22.0-53.7) undergoing elective knee diagnostic or therapeutic arthroscopy participated in this study (n= 10) Table 4. Similar arthroscopy procedure to that described in the previous section 4.22 was used, with the PAP brush being substituted with a purpose made device. Three samples were collected from each donor, these consisted of 1) SF, 2) Irrigation fluid, and 3) post brushing of the synovium samples. SF was collected, with a mean volume of 38.7mL (5-50mL), the irrigation sample was collected, with a mean volume of 40.5mL (25-50mL), and the post brushing sample was collected, with a mean volume of 53.4mL (50-70mL), all samples were collected in a sterile container and transported within an hour of extraction to the research laboratory for processing in sterile conditions. Similar to the previous experiment using the PAP brush, all samples in this experiment had different initial volumes, total number cells within each sample were quantified by centrifuging each sample and re-suspended in 10mL of expansion media. CFU-F assays were performed on matched samples and data presented as colonies per sample (section 4.22 and 4.25), (see Figure 36).

Table 4: An overview of patients participated in the *in vivo* purpose built *synovial* device brushing study.

Donor	Age (yrs)	Gender	Injury Type	Surgery
32	41.3	M	Right Knee Medial Osteochondral Fragment + Medial Meniscal Tear	Arthroscopic Right Knee Medial Osteochondral Fragment + Medial Meniscal Tear Repair
33	26.2	M	Right Knee Medial Meniscal Tear + Anterior Cruciate Rupture	Arthroscopic Right Knee Medial Meniscal Tear + Anterior Cruciate Rupture Repair
34	45.3	F	Left Knee Medial Condylar Lesion	Arthroscopic Left Knee Medial Chondroplasty
35	27.1	M	Left Knee Medial Condylar Lesion	Arthroscopic Left Knee Medial Chondroplasty
36	22.0	M	Left Knee Partial Lateral Meniscal Tear	Arthroscopic Left Knee Partial Lateral Meniscal Tear Repair
37	25.2	M	Unknown Right Knee Injury	Diagnostic Arthroscopic of Right Knee
38	53.7	M	Right Knee Medial Condylar Lesion + Medial Meniscal Tear	Arthroscopic Right Knee Medial Chondroplasty + Medial Meniscal Tear Repair
39	25.4	M	Left Knee Anterior Cruciate Rupture + Medial Meniscal Tear	Arthroscopic Left Knee Anterior Cruciate Rupture + Medial Meniscal Tear Repair
40	40.3	M	Left Knee Lateral Collateral Ligament Rupture	Arthroscopic Left Knee Lateral Collateral Ligament Rupture Repair
41	42.1	M	Unknown Right Knee Injury	Diagnostic Arthroscopic of Right Knee

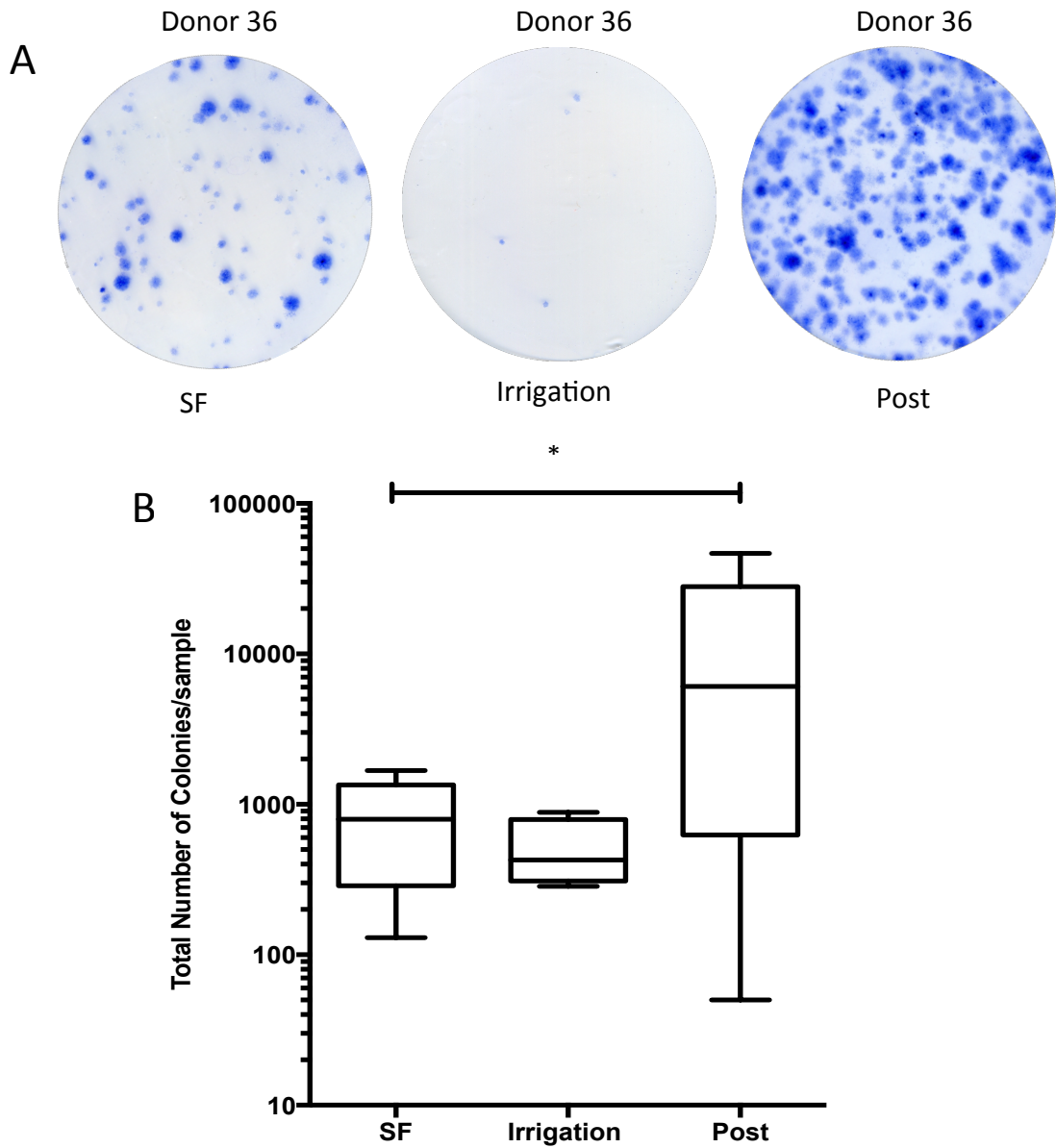


Figure 36: **Release of MSC from human synovium using *in vivo*, a purpose built device.** (A) Representative examples of CFU-F assays for SF, irrigation and post brushing samples (n=1 donor). (B) This demonstrated a reduction of MSC during irrigation, and a replacement of MSC following synovial brushing. Data represented by median; (SF =797.5, irrigation = 426, post brushing = 6075 colonies/sample) with upper and lower quartiles and minimum and maximum values. Statistical significant increase of MSC numbers between SF and post-brushed groups observed with a 6.6 fold increase (* = $p < 0.05$, paired Wilcoxon test). (n= 10 donors)

The results from section 5.13 demonstrated that MSC were released from the synovium during an arthroscopy procedure in which a PAP brush was used. The results from section 5.13 also demonstrated a statistically significant loss in MSC numbers from SF to irrigation. There was also a statistical significant increase in MSC number when comparing irrigation with post-brushing. Comparing MSC numbers between SF and post brushing cell numbers, we observed no change (+0.09 fold increase). Trilineage differentiation assays (section 5.16) demonstrated that MSC found within the SF and those released during synovial brushing were similar in respect to osteogenic, adipogenic and chondrogenic capabilities.

It must be noteworthy that the PAP brush is not a device built to be used in an arthroscopy procedure, thus a larger increase would be expect in released cell numbers when using a purpose made device to release SY MSC. Indeed, this was observed in section 5.17, were the purpose made device was used *in vivo*, a statistically significant increase in number of MSC from SF to post brushing, demonstrating a 6.6 fold increase in colonies compared to SF *see Figure 36). Indeed when comparing MSC released from both PAP and the purpose made device, we observed a 12-fold increase in MSC numbers. Although the results were from two separate sets of data, and have been implicitly compared as both *in vivo* MSC brushing protocol was followed for both. The only significant difference between the two *in vivo* experiments was that of the device used intra-operatively. (see Figure 37).

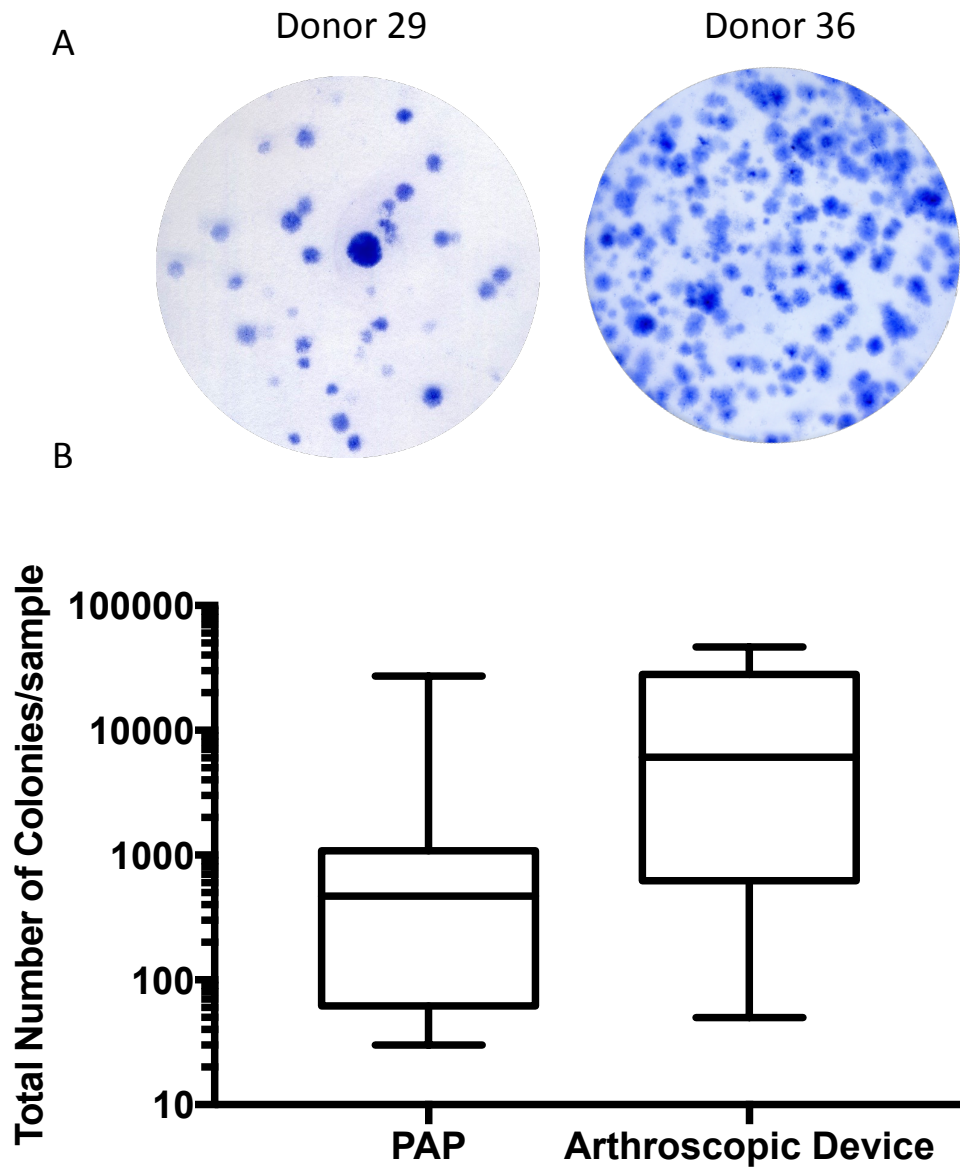


Figure 37: Comparison of released MSC *in vivo*, using a PAP and purpose built device. (A) Representative examples of CFU-F assays for SF, irrigation and post brushing samples (n=1 donor). (B) Demonstrates a 12 fold increase of MSC using the purpose built arthroscopy device compared to PAP brush. Data represented by median; PAP = 470 colonies/sample (n=9 donors) and purpose built arthroscopy device = 6075 colonies/sample (n=10 donors) with upper and lower quartiles and minimum and maximum values.

Cartilage destruction is a key feature of OA (section 1.4 and 1.5) with chondrogenesis playing a key role in providing a supply of GAG that is needed for cartilage homeostasis and repair. The findings in the present studies endorse, the use of SY MSC as a potential source of cells for cartilage repair, and as observed above, SY MSC can be released *in vivo* in high numbers. In summary, this study supports the hypothesis that, a purpose made device to release SY MSC, would not only replace lost MSC, but would rather augment their number. These cells, in the correct mechanical and correct chondrogenic environment could contribute to cartilage regeneration in injured arthritic knee joints.

Chapter 3

6. Discussion

6.1 Review of aims and hypothesis

The overarching aim of this study was to use the philosophy that endogenous MSC could be used to regenerate knee joint articular cartilage. The strategy of using a novel purpose built device that is able to release large numbers of endogenous MSC, has been developed within this study. These outcomes provide a new cost effective concept of MSC obtainability towards new proof of concept studies in OA.

The first part of this study was focused on developing a brushing device that released MSC rather than trap them within its bristles. Differing design prototypes were used to release MSC from the synovium under *ex vivo* porcine and human tissue conditions. MSC were expected to develop from released cells of the synovium tissue (De Bari *et al*, 2001), and a standard CFU-F assay was used in quantification. This assay is commonly used to measure the MSC (Mochizuki *et al*, 2006) in the synovium and other tissues, such as synovial fluid and bone marrow MSC (Jones *et al*, 2004) and cardiac MSC (Chong *et al*, 2011). Using DNA analysis, released cells from the synovium and those trapped within the device bristles were quantified, which aided in the development of a purpose made device to release SY MSC. While DNA analysis was used as an indicator of cellular material released from the synovium and also trapped within the prototypes bristles, CFU-F assay was used to measure MSC released from the synovium. Finally, released MSC from synovial tissue were expanded in culture and expected to demonstrate typical trilineage differentiation abilities (English *et al*, 2007; Baksh *et al*, 2007; Solchaga *et al*, 2011; Aldridge *et al*, 2013)

The second part of this study investigated natural biological scaffolds, and their effects on the adherence and migration of SY MSC. Once MSC are released intra-articular, the role of endogenous factors that might bring released MSC to the regions of cartilage damage is desirable. Posited methods to achieve this include either MSC loading onto a biological scaffold and then placed into defect or by adherence and migration to naturally formed scaffolds such as a microfracture.

The types of scaffolds tested were based on a fibrin clot, commonly used in cartilage tissue engineering studies (Stafford *et al*, 2011; Kim *et al*, 2015), without or with the addition of platelet-based. The latter were added based on the evidence that hPL or its predecessor PRP contain a cocktail of growth factors (GF) and inflammatory mediators, potentially effective in cartilage repair (Xie *et al*, 2014). It was hypothesized that first, SY MSC would adhere to biological scaffolds and second, that scaffolds containing PRP or hPL would demonstrate favorable MSC migration properties. This was based on previous literature showing the high content of GF in hPL (Radtke *et al*, 2014) and PRP (Mollon *et al*, 2013; Van Pham *et al*, 2013; Ornetti *et al*, 2016) that aid in articular repair.

The third part of this study was to use the knowledge gained from the *ex vivo* study, including both PAP brush and the chosen best performing purpose-made brushing device (C8), to be used within an *in vivo* surgical setting. The aim of which was to release enough SY MSC that would replace (or augment numbers) of SF MSC, lost during the irrigation phase of an arthroscopy procedure. This would potentially create an environment to aid in the repair of an arthritic knee. CFU-Fs were expected to develop from released cells of the synovium tissue. In this work, the released cells had a SY MSC nature and proliferated and demonstrated typical trilineage differentiation capabilities. Furthermore, cultures from released synovial were confirmed phenotypically as MSC, using flow cytometry based on ISCT phenotype used in previous studies (Jones *et al*, 2010; Harvanová *et al*, 2011; Baboolal *et al*, 2014). It was hypothesized that a purpose made device to release SY MSC, would release more MSC than the PAP brush, and would therefore better replace lost MSC following irrigation phase of an arthroscopy.

6.2 Obtaining MSC from the synovium *ex vivo*

Previous studies (De Bari *et al*, 2001; Sakaguchi *et al*, 2005; Fan *et al*, 2009; Harvanová *et al*, 2011; Ogata *et al*, 2015) have shown that the synovium holds great potential in providing an abundant endogenous supply of SY MSC. It was expected that mechanically dislodging MSC from the synovial lining, potentially increased the number of SY MSC within the knee cavity, without the need of culture expansion.

In these experiments CFU-F assay was used as a tool to measure MSC numbers, (Jones *et al*, 2010). Supplementary quantification methods were used to provide quicker results for the total released versus trapped cellular content, such as phenol-cholorform DNA extraction. This method removes DNA from the nuclei of cells. The

DNA content is measured using a NANO-drop spectrophotometer. However, this method provided quantification of all cellular material released or trapped, and was not specific to MSC, therefore was used as an indication for prototype development. A series of device prototypes, in which bristles were positioned in a series of configurations, aimed at maximal cell release and minimal cell trapping was tested. For example C1 consisted of thin multiple soft bristles, whereas C2 was designed with thicker more ridged bristles (see Figure 15). The focus was to optimize the released number of cells from the synovium, whilst attempting to reduce the number of cells trapped within the bristles, these were compared to the PAP brush (control).

During the initial phase of device development, using human synovium, with CFU-F quantification, MSC release using a purpose build device was achieved. However, in the devices used, no statistical significance was observed, between numbers of MSC released. Although C2 and C4 demonstrated a marginally increased released numbers of MSC, in terms of CFU-F assays, compared to other device designs (see Figure 17). In addition, comparing number of trapped cells against released cells, two devices C2 and C4 were tested. Device C2 did show statistically significant number of cells were trapped more than released in *ex vivo* excised porcine synovium experiments (see Figure 16). This information was used to further develop devices C6 and C7.

Device C6 was developed using combination of design features from C2 and C4, both of which releasing marginally higher MSC compared to other device prototypes. C7 was a new design prototype that coupled the bristles shaped design feature of C2 with the diamond shaped orientation of C4. The results revealed no statistical significant difference between these two device prototypes during *ex vivo* excised porcine and human synovium experiments (see Figure 18 and Figure 19).

The final phase of device development was to use the diamond shaped configuration as seen in C7, with the rationale that cell release will be improved if multiple brushing directions were used. In addition, spacing between bristles was increased, in an attempt to allow for a reduction in trapped cells. When C8 was compared to the PAP brush, in regards to MSC release, no statistically significant difference in released MSC numbers observed in *ex vivo*, 1 cm² excised human synovium was observed. (see Figure 20a). However, improvements had been observed with these new designs, which were demonstrated by a reduction of cell trapped, compared to released, when used to brush *ex vivo* 1 cm² excised porcine synovium (see Figure 20b).

Although results from both porcine and human synovium *ex vivo* experiments, demonstrated overall no statistical significance, it felt sensible to recognize, that the device used during an arthroscopy procedure should be a purpose built device, suited for this type of surgery.

Trilineage differentiation potential of *ex vivo* cultured SY MSC, was satisfactory demonstrated. Osteogenesis was assessed using recognised analysis techniques (Pittenger *et al*, 1999; Jones *et al*, 2002; English *et al*, 2007). Total calcium production over 21 days of osteogenic induction was quantified colorimetrically using a standard calcium assay kit (English *et al*, 2007). Alkaline phosphatase (ALP) activity, an early marker of osteogenic progression (Tsai *et al*, 2009), which is peaked at the initial 14 days of differentiation, was confirmed by staining with Fast RR blue salt. Finally, after 21 days calcium deposits were stained using alizarin red dye which evaluates calcium-rich deposits produced by osteoblasts (Pittenger *et al*, 1999). Qualitative and quantitative analysis demonstrated tripotential MSC released from all six donor synovium samples (see Table 2); however, total calcium values (see Figure 21) were less than those described in other studies using SF MSC from arthritic joints (Jones *et al*, 2004). A possible explaining for this could be due to the synovium used during *ex vivo* experiments was from donors of an average age of 71.25 years. Age has been shown to a down-regulate MSC osteogenesis (De Girolamo *et al* 2009; Beane *et al*, 2014).

Adipogenesis was assessed using recognised analysis techniques in which fat cells were stained with oil red-O (Sekiya *et al*, 2012; Aldridge *et al*, 2013). Adipogenic potential was demonstrated for all cultures in the *ex vivo* experiments (see Figure 22). Both staining and fat quantification was comparable to those observed in previous studies (Aldridge *et al*, 2013), which state Nile red/DAPI ratios of 2-12, compared to 2-4 found in this study. These ratios of Nile red against DAPI, displayed good levels of adipogenesis and lipid filled vesicles were present using oil-red-O staining.

Chondrogenesis was assessed using a recognised pellet culture model, of *ex vivo* MSC released from the synovium (Jones *et al*, 2002; English *et al*, 2007). Chondrogenic potential was demonstrated using GAG measurements as well as sectioning of pellets followed by staining with toluidine blue which stains cartilaginous extracellular matrix purple, and fibrous tissue blue (see Figure 23). Although using a different GAG quantification kit from that in previous studies, comparable results of GAG content were demonstrated (English *et al*, 2007) which state values of 2.5-

10µg/pellet, compared to 1-9 µg/pellet found in this study. Pellet staining also demonstrated equivalent results (Jones et al, 2010; Segawa *et al*, 2009; Ogata *et al*, 2015).

Donor-to-donor variation was observed in the chondrogenic assay. A possible explanation for this difference could be due to variation in levels of synovial inflammation observed between donors, which is known to effect chondrogenesis (Jones et al, 2010). However, overall chondrogenic results in this study were satisfactory in demonstrating chondrogenesis.

The overall results for trilineage differentiation of *ex vivo* released MSC demonstrated that resulting cultures did indeed show trilineage differentiation capabilities, both qualitatively and quantitatively. These findings occurred despite the robust mechanical release of MSC.

6.3 Adhesion and migration of MSC with relevant biological scaffolds

The interaction of culture expanded SY MSC when allowed to attach, migrate towards and through biological scaffolds was tested. One of the scaffolds tested was made from whole blood, and was supposed to mimic natural scaffold formed as a result of microfracture. Currently microfracture (section 1.7) is used as a treatment option for isolated articular injuries. It is believed that following a microfracture BM MSC and the constituents of the blood, namely platelets, from the sub-chondral bone migrate into a defect, and over time differentiate and mature into fibrocartilage (Steadman *et al*, 2002; Neumann *et al*, 2008; Chen *et al*, 2011; McIlwraith *et al*, 2011; Haugom *et al*, 2014) If released SY MSC could attach to such a naturally formed scaffold, this would point towards a possibility of using synovial brushing arthroscopy in conjunction with microfracture. The rationale for testing the effects of PRP was based on available clinical treatments showing PRP intra-articular injection to be beneficial compared to placebo (Patel *et al*, 2013), this could be possibly due to the chemo-attractive and chondro-inductive substances present in PRP (Mollon *et al*, 2013;.Zhu *et al*, 2013). Should stronger SY MSC migration toward PRP scaffold be seen, this would point towards a directed delivery of PRP scaffold in a defect area, in the same procedure as synovial brushing. Overall, this part of the study was to observe the interactions between SY MSC and these biological scaffolds that are potential useful adjunct to arthroscopic SY MSC therapy. All biological scaffolds were based on clots that were additionally supplemented with: a) whole blood scaffold (resembling a microfracture, section 1.7), FG (described as an effective scaffold for focused MSC delivery) (Kim *et*

al, 2015), b) Platelet rich plasma scaffold (known for their delivery of chondro-inductive growth factors) (Van Pham *et al*, 2013; Mollon *et al*, 2013; Ornetti *et al*, 2016) and c) human platelet lysate scaffold (rich source of growth factors from thermally burst platelets) (Radtke *et al*, 2014).

These results demonstrated that SY MSC adhesion to the aforementioned three biological scaffolds was rapid, with more than 65% attachment occurring within 30 minutes. In addition, it was shown that no significant variation occurred on adhesion times, when SY MSC cultures from three different donors were used to adhere to a FG scaffold, suggesting that adhesion of MSC is non-specific to currently available scaffold types. Experiments investigating MSC migration towards biological scaffolds, demonstrated better migration at 24 hours compared to 4 hours when using hPL, which is consistent with a previous study (Marrazzo *et al*, 2016) that showed optimal migration period to be 24 hours when using hPL. In addition, both PRP and hPL scaffolds induced better migration of SY MSC compared to negative controls, suggesting that human platelets do indeed release constituents that improve migration (Leotot *et al*, 2013; Zhu *et al*, 2013). Within a clinical setting, platelet based biological scaffolds could be used a method of delivering MSC directly to the defect.

Given SY MSC are able to migrate towards and attach to a biological scaffold, it would be advantageous if they could evenly migrate through the scaffold and populate to whole area with chondrogenic precursor cells. In an experimental conditions used in this study, SY MSC migration through a 3D biological scaffold was not observed, this was demonstrated by using dual stain technique with primary antibodies specific to MSC (CD73) and leucocytes (CD45). Previous studies have shown SY MSC migration through a synthetic collagen-based 3D scaffold laced with growth factors transforming growth factor β 3 (TGF- β 3) or human stromal derived factor 1 β (SDF-1 β) (Mendelson *et al*, 2011). It is likely that scaffold pore size between this and Mendelson *et al* study were different and that they may have a strong effect on MSC migration through a 3D scaffold (Murphy M, *et al*. 2010). The biological scaffolds used in this study potentially could have differing structural properties amongst each other, for example the matrix structure possibly could be too tight in a PRP scaffold, therefore creating small pore sizes that are too small to allow for MSC to migrate through, and could be a reasonable explanation for lack of migration. This experiment would require further work for example, by testing biological scaffolds that are supplemented with constituents that could alter its matrix layout, for example, a polymer creating a layered bed of fibres, thus modifying MSC migration through the scaffold, or another option may be to use a

collagen or GAG scaffold embedded with MSC released from brushing, in an attempted to keep the materials homogenous (Lawrence B, *et al.* 2008; Meng X, *et al.* 2014).

In summary these results strengthened the hypothesis, that released SY MSC could adhere to and migrate towards platelet based biological scaffolds, which can be used for regeneration of the articular cartilage damaged, potentially removing the need to create a microfracture.

6.4 Obtaining MSC from the synovial fluid and synovium using the PAP brush *in vivo*

The PAP brush is a medical device that is used in human as a method of harvesting cells from superficial, deep serosal or mucosal surfaces (Al-Abbadi *et al.*, 2011). Its main use is within a gynecological setting, where it is used to gather cells for cytopathology. In this study we used the concept of the PAP brush, and implemented it towards designing a brush that would release SY MSC *in vivo*. The CFU-F assays were used to quantify cells from SF, irrigation and post synovial brushing (Jones *et al.*, 2010). These results demonstrated a statistically significant replacement of SF MSC, lost during irrigation with SY MSC released during post synovial brushing, in a standard arthroscopic procedure. Comparing MSC numbers between SF and post-brushing, comparable results were observed; however, it must be noted that the PAP brush is not designed for this type of procedure, thus an increase in released synovial cell numbers when using a purpose made device was expected. In addition, MSC from the SF, irrigation and post synovial brushing fraction, did adhere to plastic and formed colonies, which is consistent with MSC; however, to further verify these cells as being MSC, flow cytometry was used on selected cultures.

Flow cytometry was performed on culture expanded SF cells (n=3 donors) and culture expanded post synovial brushing released cells (n=3 donors). As stated previously (Dominici M, *et al.* 2006), flow cytometry is an established method of confirming MSC identify of plastic adherent fibroblastic cultures. Previous studies that have characterized SF-MSC and SY-MSC using markers for both phenotypically positive (CD73, CD90, CD105) and negative (CD34, CD19, CD45, CD14) for expanded MSC (Jones *et al.*, 2010; Harvanová *et al.*, 2011; Baboolal *et al.*, 2014). Surface markers CD73 and CD90, are well-recognised MSC markers, and were uniformly expressed in all cultures. Expression of CD105 in both SF and post brushing cells, is significant due to it being suggestive of the cells responses to chondrogenic induction (English *et al.*, 2007). As previously mentioned, SF MSC and SY MSC share similar phenotypes, and

this was found to be the case in this study, thus further suggesting that these cells may be from a similar source. However, cell surface markers expression is not stable, and conditions such as inflammation MSC can potentially alter their phenotype (Lv et al, 2014), or culture expansion can indeed make MSC phenotype similar. For this reason, further investigation of SY MSC and SF MSC from donor matched, non-cultured samples, would aid in the characterization of these cells.

Trilineage differentiation of *in vivo* cultured SY MSC, was satisfactory demonstrated. Osteogenesis in all five donors showed acceptable levels of ALP, alizarin red and calcium deposits Adipogenic potential was also demonstrated for cultures from the cells released, and were comparable to those observed in previous studies (Sekiya et al, 2012; Aldridge et al, 2013). Chondrogenic potential were comparable to results observed in previous studies (Jones et al, 2002; English et al, 2007; Segawa et al, 2009; Ogata et al, 2015). In all three domains (CFU-F assays, phenotyping and trilineage differentiation) this study demonstrates these cells to have MSC capabilities.

In summary, these experiments demonstrated encouraging results in terms of *in vivo* released SY MSC. In addition both SF MSC and SY MSC have similar osteogenic, adipogenic and chondrogenic capabilities, thus further supporting the hypothesis for SY MSC use as a potential source of MSC for cartilage repair.

The next part of the thesis brought together the knowledge gained from results of MSC release, using device prototypes and PAP brush, and was coupled with design opinions from orthopaedic surgeons. This resulted in a tailored synovial cell-releasing device being developed. The design feature encompassed a robust design that is personalized to this type of procedure. These features of the device included a long robust arm, which can enter easily through an arthroscopic port for robust brushing of the suprapatellar pouch. The material used was an inert poly-ethylene, in addition the brush head was angulated at 30 degrees on an extended arm allowing for control when brushing the synovium.

6.5 Obtaining MSC from the synovium using a purpose made device to release SY MSC, *in vivo*

A similar process was used to previous *in vivo* experiments described in section 4.22 to release MSC during an arthroscopy procedure; however, in this part of the study the PAP brush was replaced with a purpose made device to release SY MSC (based on prototype C8, see Figure 15). CFU-F assays were used to quantify cells from SF,

irrigation and post synovial brushing and demonstrated a statistical increase in number of cells from SF to post-brushing. In addition we observe a 12 fold increase of MSC when using the purpose built device compared to the PAP brush (see Figure 37). This further supports the hypothesis that SY MSC can be successfully released from the lining of the synovium to augment MSC numbers within the joint cavity, during an arthroscopy. With an increase number of MSC released post brushing compared to SF cells, this would potentially set an environment where an amplified number of endogenous MSC would be available to regenerate cartilage defects.

6.6 Study Limitations

This study uses a novel approach to release joint resident MSC, providing a new approach for healthcare providers toward the assessment of endogenous MSC towards articular cartilage repair. Due to the studies' originality the difficulties faced were unique. During device prototype development, there were some delays in phase 2 prototypes production. This had an effect on the number of samples used for quantification, as some patients that could be included in the study, were unfortunately not recruited, three donors were lost due to this delay.

Using human synovial tissue from patients with advanced OA resulted in large variations in consistency of the synovium. The synovial sizes that were harvested were varied and depended on the individual's anatomy. It was therefore difficult to section the synovial sample so that all the device prototypes could be tested. This resulted in differing donor numbers for CFU-F assays.

The study on adherence and migration using biological scaffolds was limited, as conditions did not truly replicate typical *in vivo* environments. It would have been ideal if experiments could have recreated the dynamic movements of MSC within the joint, alongside the mechanical forces. In addition SF effects, due to its viscosity (Baboolal et al, 2014) were not replicated, and could have implication on adherence and migration.

Biological scaffolds made from whole blood (WB) were used in the study, due to its resemblance to clots formed during a microfracture. However, unlike microfracture, WB does not have bone marrow MSC (BM MSC) present. In order to create an *ex vivo* microfracture model, it would have been useful to have BM MSC embedded within a WB scaffold, to assess potential variability. While the presence of embedded BM-MSC would not likely affect SY-MSC attachment, it might improve on the quality of cartilage repair.

The biological scaffolds were used to assess MSC migration through 3D structures did not demonstrate migration of SY-MSC through these scaffolds. Constructing the various biological scaffolds was difficult, due to the inconsistency in the scaffold formation. This could have been due to many factors, for example variations in the PRP preparation methods, which regularly provide differing platelet and leukocyte quantities, and is quite a common outcome in other studies (Amable *et al*, 2013; Araki *et al*, 2012; Lee *et al*, 2013; Van Pham *et al*, 2013).

Due to the fragility of scaffold constructs, it led to difficulties in the sectioning of the scaffold. Once stained, counting the cells within the scaffold was also difficult, despite counting several sections for the better statistical averages.

Further work is needed to understand migration of MSC within a scaffold; for example, by labelling MSC with specific fluorescent tracker dyes (Baboolal T *et al*, 2016; Zou L, *et al*, 2013) that would clearly identify MSC. In addition, the difficulty with biological scaffold sectioning could also be addressed by use of such fluorescent imaging techniques using a confocal microscope. Changing scaffolds' consistency to observe potential effects of pore sizes on migration, for example by using differing concentration of fibrinogen, maybe useful in determining a consistent scaffold for MSC migration.

These results can be used as an indication to adopt an approach of cartilage regeneration using native MSC, such as releasing SY-MSC and then loading them into a scaffold to be directly placed into the defect; this could negate the need to rely upon natural MSC migration and homing into defects. This can be achieved by capturing released SY-MSC using irrigation of the cells into a reservoir, then a in theatre centrifugation system to pellet the SY MSC. SY MSC can be then loading in to the defect with a PRP scaffold using a dual syringe system (Haleem M, *et al*. 2016). For more advanced OA, there is evidence that without scaffolds, MSC can adhere to sites of injured cartilage, so the augmentation of MSC in that setting might naturally lead to good joint repair by mechanisms not dependent on scaffolds (Baboolal T, *et al* 2016).

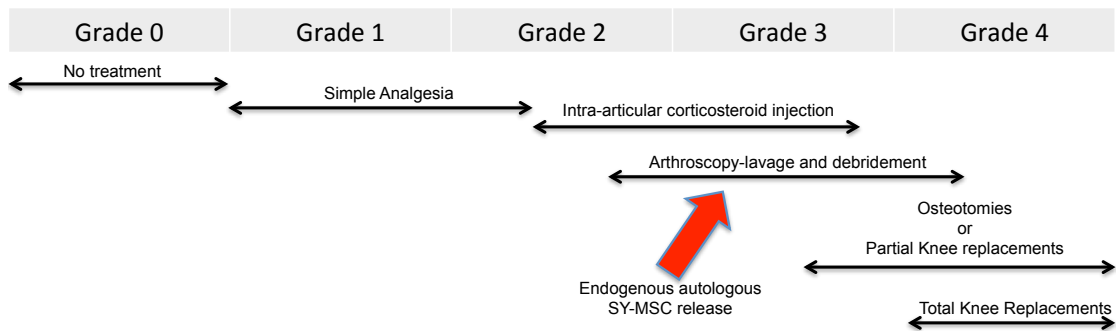
6.7 Clinical relevance and future clinical directions

Future proof of concept studies that increased numbers of MSC within the knee joint cavity may aid in repair, and reduce pain that is indicated by better patient pain outcomes are due to start in 2017. These will employ clinical outcomes including the visual analog score (VAS), or the Western Ontario and McMaster University Osteoarthritis Index (WOMAC) in clinical practice (Lamo-Espinosa JM, *et al.* 2016). Autologous MSC are an appealing source of MSC cells due to their lack of tissue rejection or cross infection; however, the consistent low yields of cells, and the necessity to culture expand these cells, coupled with a two stage surgical procedure, will ultimately not be cost effective. This emphasises the need for a technique that provides good yields of autologous MSC that are obtained cost effectively, and uses only minimal number of surgical interventions.

This study demonstrates significant increase of SY MSC released from the synovium using a purpose made device. There was a 12 fold increase in the number of SY-MSC released, when the surgeon uses the purpose made device compared to PAP brush. In summary, this study demonstrates a novel device that can be used cost effectively by healthcare providers to augment the number of autologous joint resident MSC within the knee, without the need to culture expand MSC. In addition this process can be undertaken within the time limits of current arthroscopy procedures, which negates the requirement for extra anesthetic time. Knowing that cells can adhere rapidly and migrate well towards a scaffold, the augmentation of SY MSC can be used in conjunction with, but not limited to, procedures such as microfracture. Post-surgical rehabilitation is also not affected, as patients usually are non-weight bearing following many arthroscopy procedures for times that are beyond what is taken for released cells to adhere and migrate.

The release of MSC from the synovium, using the purpose made device, can be used in combination with existing therapeutic procedures. The current treatment of articular disease such as OA (shown below and in section 1.6) could be amended to include treatment with endogenous autologous SY MSC (Table 5).

Table 5: An overview of current treatment options for OA, with the addition endogenous autologous SY MSC release treatment.



For isolated articular injuries, microfracture (section 1.7) is a therapeutic procedure used, that relies on BM MSC to fill the defect, repairing the cartilage with fibrocartilage. As BM MSC are relatively low in numbers (Peng *et al*, 2008), these could be augmented with the released SY MSC allowing more cells to be available for repair. A further enhancement of an arthroscopic microfracture can be developed, using biological scaffolds, such as PRP sourced from the patients' own blood. During arthroscopy, where an isolated defect is identified and debrided, and the synovium brushed with the purpose built device to release SY MSC, the region can have a biological scaffold applied to it with the aid of the dual-syringe system loaded with released SY MSC. This would potentially have three benefits; 1) it will use endogenous autologous MSC; 2) it will withhold the use of drilling holes within the already damaged articular region, allowing for less site morbidity and 3) it will focus chondro-inductive agents (i.e. autologous PRP) directly at the site of injury, this potentially would create better quality cartilage. In addition to improving the outcome for the patients, this new procedure would also be considered advantageous for the healthcare provider, as it would be cost effective, as this new technique requires no externally sourced MSC. However, there may be drawbacks with this method, for example, usual arthroscopy operating times may be extend due to biological scaffold preparation times. Coupled with the process of SY MSC embedding into the scaffold this could drastically increase operation times, which would inevitably increase costs. To overcome this, a more simple protocol could be developed where a biological scaffold such as PRP, would be prepared, prior to surgery, using the patient own blood source. Following debridement of the defect, the scaffold can be applied to the defect, and then the synovium brushed, so that SY MSC can be augmented within the cavity. Although this would not focus the SY MSC to the sight of injury, this technique would be a quicker one-stage surgical procedure, thus, potentially cost saving.

As shown in this thesis, natural migration of released SY MSC through biological scaffold may be difficult (section 5.10-5.12). Based on these findings one can propose one further advance of the above procedure, i.e. to load the scaffold with the newly released SY MSC, with a simple in theatre centrifugation procedure; however, this may increase anaesthetic times, and potentially costs.

For advanced osteoarthritis (OA-grades 3-4), this purpose built device can be used in procedures that are currently being researched, as an alternative to total knee replacement (TKR). Knee joint distraction (KJD) is currently available within the Netherlands and USA (see Figure 38). It involves a technique whereby the joint is separated using an external fixation method. This altered mechanics results in unloading the joint for a period of time (six weeks) (Wiegant *et al*, 2013), whilst still permitting the patient to fully weight bear through the joint. Separation and reduction of joint forces, allows the joint articular surface to regenerate, possibly by allowing resident SF MSC to attach to injured sites. The mechanism by which the cartilage regenerates has hitherto not been defined. In its infancy of being a viable treatment option for knee OA, long-term outcomes are not comprehensively assessed; however, in a stimulated study comparing KJD and TKR, cost effectiveness ranks highly with KJD over TKR (Van der Woude *et al*, 2016). If SY MSC were released and augmented with other resident MSC (i.e. SF MSC,) during the distraction period, potentially more MSC would be able to attach to defected regions, thus accelerating regeneration of the articular cartilage (Van Roemund PM, et al. 1999; Intema F, et al 2011; Baboolal T, et al. 2014, Baboolal T, et al. 2016).

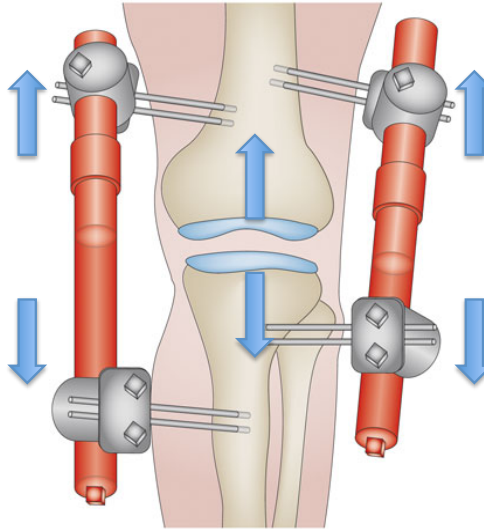


Figure 38: **Knee joint distraction.** The knee is distracted; demonstrate by the arrows direction, resulting in joint surfaces unloading (original images adapted from Mastbergen S, et al. 2013).

Although the purpose built device has shown encouraging results with regards to MSC augmentation, and chondrogenesis, being a newly developed concept, there could be potential apprehensions for its use. For example using the device to assertively release MSC from the synovium may in fact damage the synovium itself. However, it has been documented for some time that the synovium has the capabilities to regenerate in both animal models (Bentley *et al*, 1975; Theoret *et al*, 1996) and in humans, following an arthroscopic synovectomy (Ostergaard *et al*, 2001). Another suggestion could be that in established OA there are already increased numbers of SF MSC (Jones *et al*, 2008), therefore is there any advantage of releasing more cells? This would indicate that indeed established OA, which involves damage to many structures (ligaments, meniscus, fat pad, synovium etc), would require further interventions, rather than just increasing MSC, such as a change in the joint loading mechanics as demonstrated by joint distraction (Mastbergen *et al*, 2013; Van der Woude *et al*, 2016; Wiegant *et al*, 2013) would alter the mechanical environment of the joint, thus allowing for all released MSC to adhere and repair sites of damage.

6.8 Conclusion

Existing orthopaedic procedures naturally wash away endogenous knee joint MSC. Endogenous MSC can be significantly increased in numbers within the knee joint, using a purpose built device to release MSC from the synovium, during an arthroscopy procedure. Released MSC demonstrate phenotypic characteristics as described in previous studies, verifying their identification as MSC. In addition these released cells display good potentials into osteogenic, adipogenic and chondrogenic lineages. Synovial MSC adhere to relevant biological scaffolds rapidly, and demonstrate migration towards them.

The limitations related to this study have been noted; however, further work into these areas will determine whether this novel procedure is useful within the clinical setting. These findings provide the basis for the testing of novel regenerative strategies for orthopaedics, in which low numbers of joint resident MSC can be augmented, to aid in the regeneration of damaged articular cartilage and other joint tissues. This thesis work represents a bench to operating theatre strategy, for knee joint repair in a one stage cost effective procedure, based on knowledge of *in vivo* MSC.

7. References

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8. List of abbreviations

- (3D)** Three dimensional
- (ACI)** Autologous Chondrocyte implantation
- (AD MSC)** Adipose mesenchymal stromal cells
- (ALP)** Alkaline phosphatase
- (ALZ)** Alizarin red
- (BM)** Bone marrow
- (BM MSC)** Bone marrow mesenchymal stromal cells
- (CaCl)** Calcium chloride
- (CFU-F)** Colony forming unit – fibroblast
- (C1)** Concept device design 1
- (C2)** Concept device design 2
- (C3)** Concept device design 3
- (C4)** Concept device design 4
- (C6)** Concept device design 6
- (C7)** Concept device design 7
- (C8)** Concept device design 8
- (DAPI)** 4',6-diamidino-2-phenylindole
- (DEPC)** Diethylpyrocarbonate
- (DMEM)** Dulbecco's modified eagle's medium
- (DMSO)** Dimethyl sulfoxide
- (dsDNA)** Double stranded DNA
- (DNA)** Deoxyribonucleic acid
- (EGF)** Epidermal Growth Factor
- (FCS)** Fetal calf bovine serum
- (FG)** Fibrin glue
- (GAG)** Glycosaminoglycan

- (GF)** Growth factors
- (H&E)** Hematoxylin and eosin stain
- (hPL)** Human platelet lysate
- (IFP MSC)** Intrapatellar fat pad mesenchymal stromal cells
- (IgG)** Immunoglobulin G
- (ISCT)** International Society for Cellular Therapy
- (KJD)** Knee joint distraction
- (MACI)** Matrix-induced autologous chondrocyte implantation
- (MF)** Microfracture
- (MSC)** Mesenchymal stromal cells
- (n)** Number of sample/donors
- (NHS)** National Health Service UK
- (OA)** Osteoarthritis
- (OATS)** Osteochondral autograft transplantation/transfer system
- (OCA)** Osteochondral allograft transplantation
- (OCT)** Optimal control temperature
- (PAP)** Papanicolaou smear brush
- (PB MSC)** peripheral blood mesenchymal stromal cells
- (PBS)** Phosphate buffered solution
- (PRP)** Platelet Rich Plasma
- (PPP)** Platelet Poor Plasma
- (RT)** Room temperature
- (SF MSC)** Synovial fluid mesenchymal stromal cell
- (SDF-1 β)** Human stromal derived factor 1 β
- (SY MSC)** Synovial mesenchymal stromal cells
- (TKR)** Total knee replacement
- (TGF- β 3)** Transforming growth factor β 3
- (TGF β)** Transforming Growth Factor beta

(UC MSC) Umbilical cord mesenchymal stromal cells

(VAS) Visual analog score

(VEGF) Vascular Endothelial Growth Factor

(WB) Whole blood

(WOMAC) Western Ontario and McMaster University Osteoarthritis Index

(Yrs) Years

Appendix 1

Media, buffers & materials

Media or buffer	Constituents
Lysis buffer 1	20mM Tris-HCL, pH 8, 20mM NaCL, 20mM EDTA, 1% SDS
Lysis Buffer 2	150mM Nacl, 10mM EDTA, add Proteinase K (600 µg/mL) (supplemented with Proteinase K)
Agrose	4%
Fibrinogen	23.7 mg/mL
Thrombin	1000 units/mL
Calcium chloride (CaCl) (H&E)	5M Hematoxylin and eosin staining
Adipogenic basic media	DMEM, 10% FCS, 0.5mM Isobutylmethyxantine, 60 µM Indomethacine.
Chondrogenic basic media	DMEM (high Glucose), Ascorbic-2-Phosphate (As2P) 200mM, Sodium Pyruvate, Proline, BSA, P/S.
Osteogenic basic media	DMEM, Foetal Calf Serum (FCS) 10%, As2P 100 µM, β-Glycerophosphate 10mM

Media & Reagents (manufactured)

Media / Reagents	Product code	Company
Dulbecco's modified eagle's medium DMEM(1X) + GlutaMAX™-I	61965-026	Gibco®, Invitrogen
Stromal macs MSC expansion media	130-091-680	Miltenyi Biotec
Dulbecco's phosphatase buffered saline DPBS(1X)	14190-094	Gibco®, Invitrogen
Dimethyl sulfoxide (DMSO)	M81802	Sigma
Trypsin/EDTA solution	15400-054	Invitrogen
Penicillin/Streptomycin solution	14140	Gibco®, Invitrogen
Boric acid	B0252	Sigma
Trypan blue	T8154	Sigma
Methylene blue stain	66720	Fluka
Fetal calf serum (FCS)	A3059-500g	Sigma
Formaldehyde	BP531-500	Fischer Scientific
Quanti-iT™ dsDNA Assay Kit	1458609	Invitrogen
Sulfated glycosaminoglycan assay kit	B1000	Blyscan™
Calcium assay kit	17667	Senital Diagnostics
G 2 Doublestain system Rabbit/Mouse	K5361	EnVision™

Equipment

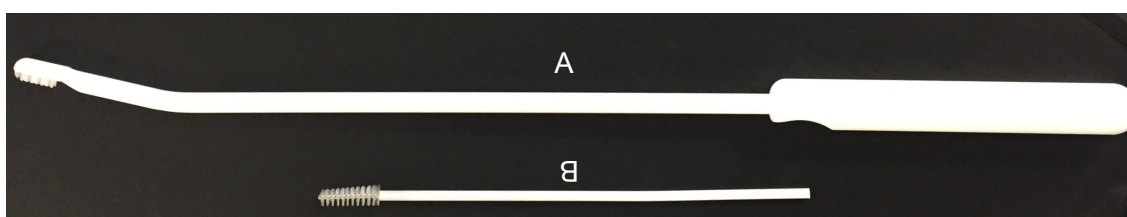
Equipment	Product code	Company
Inverted microscope	DMIL090-135.001	Leica MicrosyStromals
Inverted microscope	CKX41	Olympus
Digital camera	C-7070	Olympus

Mechanical freezer -80°C	ULTF 80	Dairel
Liquid nitrogen	NA	NA
Centrifuge	PK 130 R	ACL
Centrifuge	5810 R	Eppendorf
Centrifuge	Z 206 A	Hermle
Sterile surgical dissection kit	NA	NA
PAP brush	NA	CERVIBRUSH™ + CE
Devices C1, C2, C3, C4, C6, C7	NA	NA
Incubated at 37 °C, 5% CO ₂	50100783	Sanyo
Haemocytometer	BS.748	Hawksley
8 mm Diameter Core Extruder	NA	Arthrex OATS™
3.2% sodium Citrate anticoagulation blood collection tube	A1403IES	Vacurette®
37 °C water bath	NA	Grant

Plastic devises

Plastic-ware	Product code	Company
Pasteur pipettes sterile 1mL	1170915	Gilson
Pipette tips (1000 µL)	F167104	Gilson
Pipette tips (200 µL)	F167103	Gilson
Pipette tips (10 µL)	F167101	Gilson
Stripette (25 mL)	4251	Corning
Stripette (10 mL)	4101	Corning
Stripette (5 mL)	4051	Corning
96 well plate (round bottom)	3799	Corning
6 well plates	3516	Corning
48 well plate	3548	Corning
15 mL Falcon tubes	430790	Corning
50 mL Falcon tubes	430828	Corning
Flask (25 cm ²)	430639	Corning
Flask (75 cm ²)	430641	Corning
Flask (150cm ²)	430825	Corning
Petri-dish (10cm diameter)	430167	Corning
Cryovials (2 mL)	72.380	Sarstedt
Cryovials (1.2 mL)	72.377.005	Sarstedt
Cryo boxes	93.872.625 and 93.876.281	Sarstedt
Syringe (10 mL)	302188	BD
Centrifuge tubes (15 mL)	430790	Corning
Centrifuge tubes (50 mL)	430828	Corning

brush verses purpose built device.



(A) Calder Brush (purpose built device) (B) PAP Brush

Chondrogenic differentiation assay SOP

Equipment needed

Laminar flow cabinet
CO₂ incubator at 37°C
Waterbath
Small Eppendorfs with screw caps
Pastettes
Sterile pipettes
Gloves
Syringe and needle

Reagents required

Chondro medium:-

DMEM (Gibco/Life Tech 61965-059) 500mL
As2P (Sigma A8960, 5g) 200mM stock 0.5mL
Sodium Pyruvate (Sigma S8636) 100x concentrated stock 5mL
Proline (Sigma P5607) 20mg
BSA (Sigma a-7906) 500mg
Penicillin/Streptomycin ((Gibco/Life Tech 15140-122) 5mL
PBS (Gibco LifeTech 14190-169)
Papain (Sigma P4762)

Supplements added to Media

Dexamethasone (Sigma D1756, 25mg) 1µL
TGFβ3 (R&D System, 243-B3-002, 2µg) 50µg
ITS+ (Sigma 12521) 1:100 50µL

PBS (GIBCO LifeTech 14190-169)

Papain

Na₂ HPO₄
NaH₂PO₄
Acetyl cysteine
EDTA

sGAG assay eg Wieslab AB GAG 201

Method

Trypsinise cells, resuspend in 0.5 mL DMEM/2% FCS and count.
Each chondrogenic assay requires 0.25x10⁶ cells. Try to replicate at least twice (total require 0.5x10⁶ cells) .
Place cells in Eppendorf tubes and make up the volume to 0.5mL with DMEM/2 % FCS and spin at 2000rpm for 5 minutes.
Avoiding the pellet, gently remove the medium with a tip of a pipette or pastette, break the pellet by tapping and re-suspend in 0.5mL complete chondrogenic media.
Centrifuged for 2000rpm for 5minutes, observe the pellet, loosen the tops and incubate in CO₂ incubator at 37°C.

Half media changes three times per week (Mon, Weds and Fri) by removing 0.25mL of medium and replacing with 0.25mL fresh medium.

After 3 weeks, stop the assay by carefully removing the medium with a syringe and needle.

Wash twice with PBS.

Add 100µl papain, replace lid tightly and incubate at 65°C overnight in the waterbath. Mix contents well, spin at 5000rpm for 5 minutes and freeze at -20°C (do not remove supernatant, freeze whole tube).

Perform GAG assay according to manufacturers instructions.

PREPARATION OF STOCK SOLUTIONS

BASIC CHONDRO MEDIUM

DMEM (Gibco/Life Tech 61965-059)	500mL
As2P 200mM stock	0.5mL
Sodium pyruvate 100x stock	5mL
Proline	20mg
BSA	500mg
P/S	5mL

Weigh out BSA and proline, dissolve in a minimum of DMEM, filter into a sterile bottle. Aseptically add other reagents, mix well and aliquot in 5mL volumes, store at -20°C.

ASCORBIC-2-PHOSPHATE As2P (Sigma A8960, 5g)

Make 200mM stock by dissolving 2.895g in 50mL water. Aliquot at 60µL (for Osteo assay) and 0.5mL and freeze at -20°C. Add 0.5mL to 500mL DMEM.

SODIUM PYRUVATE (SIGMA S8636)

100Mm ie 100x concentrated. Stored at 4°C, use at 5mL per 500mL DMEM.

PROLINE (Sigma P5607)

Stored at room temperature.

BSA (SIGMA A-7906)

Stored at 4°C.

Penicillin/Streptomycin (Gibco/Life Tech 15140-122)

Aliquoted in 5mL vols and stored at -20°C.

Dexamethasone (Sigma D1756, 25mg)

As per Osteo assay:- Make 50mM stock solution by adding 1280µL 100% ethanol to 25mg. Aliquot in 10µL volumes, freeze at -20°C. Prior to use 10µL is added to 1mL medium to give 500µM stock and refrozen in 10µL aliquots. Then make a 1:5000 dilution to give a final concentration of 10nM.

TGFβ3 (R & D Systems, 243-B3-002 2µg)

a) Make a diluent of 4mM HCl containing 1mg/mL BSA :-

1M HCl – add 86.2% HCl to 1L water or 2.15mL to 25mL water.

Dilute by 1:250 to give a 4mM concentration ie 100µL of 1m in 25mL water.

b) Add 25mg BSA to 25mL 4mM HCl to produce desired diluent.

c) Dissolve 2 μ g TGF β 3 in 2mL of diluent to give 1 μ g /mL TGF β 3 stock solution. Aliquot in 20 μ L volumes and store at -20°C.

Supplements to be added immediately before use to produce complete Chondro medium

	Vol to add to 5mL base medium
ITS+ (Sigma 12521) 1:100	50 μ L
TGF β 3 1:100	50 μ g
Dexamethasone 500 μ M	1 μ L

PAPAIN (SIGMA P4762)

Prepare buffer:-

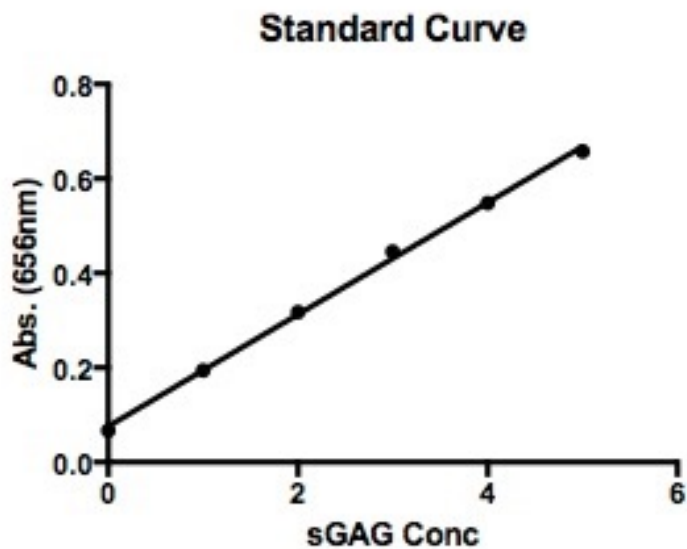
50mM sodium phosphate pH 6.5 :-mix 3mL of 1M Na₂HPO₄ (1.42g/10 mL) with 7mL of NaH₂PO₄ (1.56g/10mL) and top up to 200mL with water.

2mM Acetyl cysteine (Sigma A7250) :- prepare 100mM solution (1.63g/100mL water) and dilute 50 fold (use 4mL in 200mL of phosphate buffer).

2mM EDTA

Mix all reagents together, check pH = 6.5.

Dissolve 100mg papain in 100mL of the above buffer, filter through a 0.2 μ filter, aliquot in 1mL volumes and freeze at -20°C.



General Protocol

Detection Limit:
Time Required:

0.25µg
1 hour

Set Up Assay

Label a set of 1.5 ml microcentrifuge tubes. All samples, standards and blanks should be run in duplicate.

Prepare;

Reagent blanks - 100 µl of deionised water or the test sample buffer.

Glycosaminoglycan standards - use aliquots containing 1.0, 2.0, 3.0, 4.0 and 5.0 µg of the reference standard. Make each standard up to 100µl using the same solvent as the Reagent blanks. The standards and the reagent blank (0µg) are used to produce a calibration curve.

Test samples - use volumes between 10 and 100 µl.

Adjust the contents of all tubes to 100 µl with deionised water or appropriate buffer. Where there is no previous knowledge of the glycosaminoglycan (GAG) content 50µl of the test material is suggested for a trial run. Samples should not contain excessive salt that could interfere with the GAG-dye interaction; and GAG content must not exceed 5µg or dye-GAG saturation will not occur.

Commence Assay

To each tube add Blyscan dye reagent (1.0ml).

Cap tubes; mix by inverting contents and place tubes in a gentle mechanical shaker for 30 minutes, (or manually mix at 5 minute intervals).

During this time period a sulphated glycosaminoglycan-dye complex will form and precipitate out from the soluble unbound dye (Fig. 1a, see outside back cover).

Centrifuge

Transfer the tubes to a microcentrifuge and spin at 12,000r.p.m. for 10 minutes (Fig. 1b).

ASSAY PROTOCOL CONTINUED ON INSIDE BACK COVER

Origin of Blyscan Assay Name

The name for the assay was found using a computer based dictionary. Looking for three or more letters in sequence from 'glycan' we found 'blyscan'. Blyscan is an Old English word meaning 'to shine' and from which the word 'blush', (blushing), may have been derived. This was an appropriate choice as the Blyscan Assay contains a blue dye which turns bright pink when it binds to sulphated glycosaminoglycans.

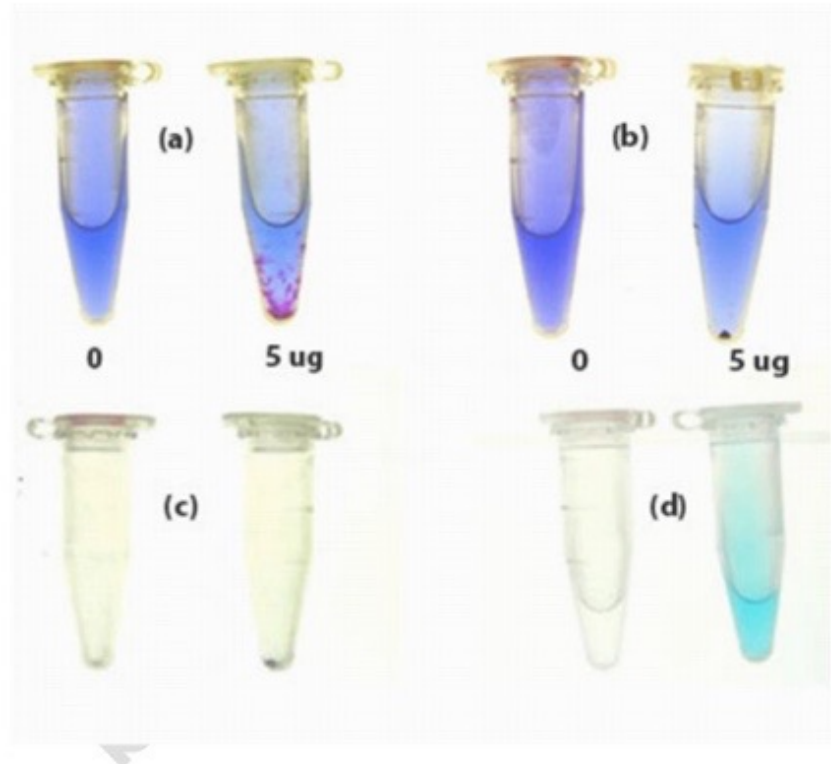


Fig. 1 Blyscan Assay; step-by-step

- (a) 0 and 5 μ g of sGAG and Blyscan Dye, (after 15 minutes mixing).
- (b) 30 min mixing and then centrifuged, (note the sGAG-Dye pellet).
- (c) The non-sGAG Dye was drained from tubes with pellet retained.
- (d) Dye released from sGAG using the Dye Dissociation Reagent.

PROTOCOL CONTINUED FROM INSIDE FRONT COVER

Draining

Important: firmly packing the insoluble sGAG-dye complex at the bottom of the tubes is required to avoid any pellet loss during draining of unbound dye. Carefully invert and drain tubes (Fig. 1c).

Any remaining droplets can be removed from the tubes by *gently* tapping the inverted tube on a paper tissue.

Do not attempt to physically remove any fluid that is in close contact to the deposit.

Release and Recovery of s-Glycosaminoglycan Bound Dye

Add dissociation reagent (0.5ml) to tubes (Fig. 1d).

Re-cap the tubes and release the bound dye into solution. A vortex mixer is suitable.

When all of the bound dye has been dissolved, (usually within 10 minutes), centrifuge at 12000 rpm for 5 mins to remove foam.

Keep the tubes capped until ready to measure absorbance.

Measurement

Transfer 200 μ l of each sample to individual wells of a 96 micro well plate. Avoid rapid pipetting as foaming can cause abnormal absorbance readings. Keep a record map of the contents of each well; A1 to H12.

Set the microplate reader to 656nm or the closest matching red filter.

Measure absorbance against water for the reagent blanks, standards and test samples.

Obtain s-glycosaminoglycan concentrations from the Standard Curve. Duplicates should be close to $\pm 5\%$ of their mean value.

If the sample absorbance value is at the top end of the standard curve the test sample should be diluted five or ten-fold and the assay repeated. The dye concentration available will not be sufficient to fully label more than 5 μ g of the GAG molecules present.

SEE MANUAL FOR SAMPLE EXTRACTION AND PREPARATION DETAILS

Osteogenic differentiation assay SOP

Equipment Needed

Laminar flow cabinet
CO₂ incubator
Haemocytometer
Pastettes
Sterile pipettes
Gloves
Sterile 12 or 6 well plates
1 mL syringe and 19/23G needle
1.5mL Eppendorf
Cell scrapers

Reagents needed

Osteogenic medium DMEM (GIBCO/Life Tech 14190-169)
Foetal calf serum (FCS) 10%
Ascorbic-2-phosphate (As2P) 100 μ M
 β glycerophosphate 10mM
Dexamethasone 100nM

DMEM / 2%FCS

Trypsin
0.5N HCl
PBS (NB must be Calcium-free)
Commercial calcium assay kit eg Sigma 587-A

PREPARATION OF STOCK SOLUTIONS

DEXAMETHASONE (SIGMA D1756 25mg)

(1M = 392.5mg/mL or 25mg/0.064mL)

25mg in 640 μ L = 100mM, or 25mg in 1280 μ L = 50mM

Dissolve 25mg in 1280 μ L of 100% ethanol to give 50mM stock solution. Aliquot this solution in 10 μ L volumes in sterile vials and store at -20° C.

10 μ L of 50mM is added to 1mL of medium to give a 500 μ M solution, which can be refrozen in 10 μ L aliquots. This is then added to medium just before use at a 1:5000 dilution ie 20 μ L to 100mL of medium.

ASCORBIC-2-PHOSPHATE (SIGMA A8960, 5g)

(1M = 257 mg/mL or 2.57g/10mL)

2.57g/100mL = 100mM.

Dissolve 2.57g in 50mL of water to give 200mM stock solution. This can be aliquoted in 1.0mL and 60 μ L volumes (and 0.5mL for chondro assay) and stored at -20° C. Use at a 1:2000 dilution ie 50 μ L of stock to 100mL of medium.

BETA-2-GLYCEROPHOSPHATE (SIGMA G6251, 10g)

(1M 216g/L or 10g/46mL)

Dissolve 10g in 23mL of water to give 2M stock. This can be frozen at -20° C in sterile vials. Use at a 1:200 dilution to give a 10mM dilution eg 0.5mL in 100mL of media.

Medium containing both glycerophosphate and ascorbic-2-phosphate can be prepared and frozen.

TRYPsin (GIBCO/LIFE TECH 34500-027)

Aliquoted in 2mL universals and stored at -20°C . Add 18mL PBS just before use.

FCS (GIBCO 10106-169)

Aliquoted in 10mL universals and stored at -20°C .

0.5N HCl

26.5mL 37% HCl + 473.5mL dd water.

Method

Steps 1 – 5 must be carried out aseptically in the laminar flow cabinet, wearing gloves.

Trypsinise cells, make up to 0.5mL in DMEM / 2% FCS, keep on ice while counting.

Calculate the volumes required.

6 well plate needs 0.03 million cells/well

12 well plate needs 0.01 million cells/well

3 replicates are essential

Add required medium to the wells

6 well plate requires 4 mL/well

12 well plate requires 2mL/well

Add the cells to the medium, swirl gently and incubate at 37°C in the CO_2 incubator.

The cells need feeding twice per week eg Monday and Friday. Remove half the volume of medium and replace with fresh medium.

The assay lasts for 3 weeks, for Calcium and Alizarin, and 2 weeks for Alkaline Phosphate assay.

Calcium Assay

To stop the assay, remove medium and wash twice with PBS.

Add 1mL for 6 wells, 0.3mL for 12 wells of 0.5N HCl, leave for 5 minutes at room temperature.

Using a cell scraper (large wells) or a pastette (small wells) gently scrape the cells off the surface

Aspirate with a 1mL syringe and 19G/23G needle

Transfer to 1.5mL Eppendorf (conical), ensure lids are tight.

Mix for 4hours at 4°C using rotator.

Transfer to -20°C freezer.

When ready to perform calcium assay:-

Thaw cells at room temperature

Centrifuge at 8000g for 5 minutes (8000rpm in small centrifuge)

Transfer to fresh Eppendorf

Perform Ca assay according to the manufacturers instructions Please see (To Measure Ca^{++})

Suggested lay out.

(Colour is stable for up to 1 hour therefore no absolute need to spread the standards across the plate).

	1	2	3	4	5	6	7	8	9	10	11	12
A	100 mg/mL			Sample 1								
B	50 mg/mL			Sample 2								
C	25 mg/mL			Sample 3								
D	12.5 mg/mL			etc								
E	6.25 mg/mL											
F	3.125 mg/mL											
G	1.5625 mg/mL											
H	BLANK											

Centrifuge thawed samples for 5 min at 8000rpm. Use the supernatant, by either plating 4µL directly into clear flat bottomed plate (not TC plate), in triplicate, or transfer to round bottomed plate to enable transfer by multichannel pipette.

Prepare standard curve from 1000mg/L stock. A1 – 3 is neat. Prepare the remainder by serial dilution, ensuring excess is removed from G. Dilutions can be made either in calcium free water, or in 0.5N HCl. Again, transfer 4 µL to each of the relevant wells.

Sentinel diagnostic (ref: 17667, Italy),

NOTE: Standard curve MUST be done on EVERY PLATE.

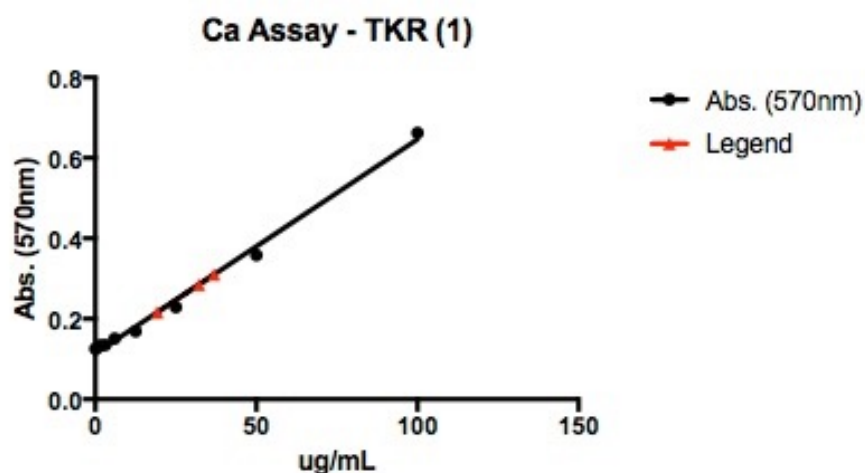
Prepare Calcium Liquid according to ratio below, enough for 200 µl per well. (Do not use weigh boats, they appear to contain calcium).

Reagent 1 : Reagent 2
5mL : 2mL

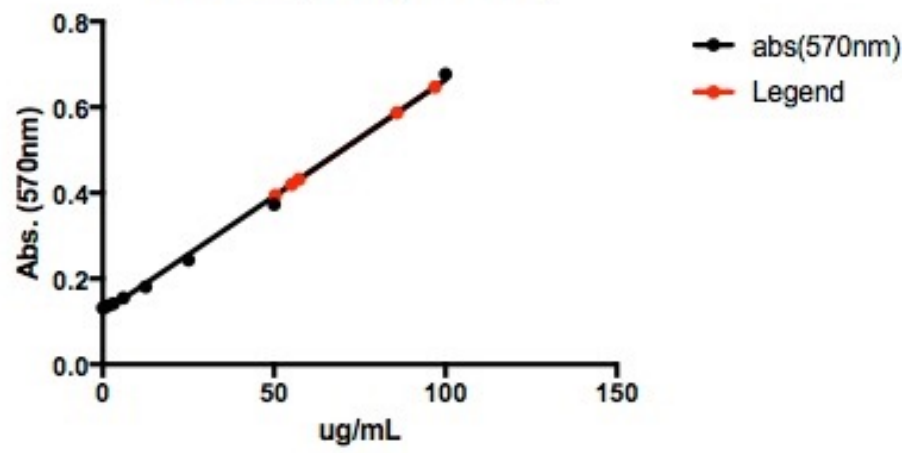
Pipette Calcium reagent using a multichannel pipette.

The colour change is immediate, measure the absorbance at 570 nm, ideally within 30 minutes.

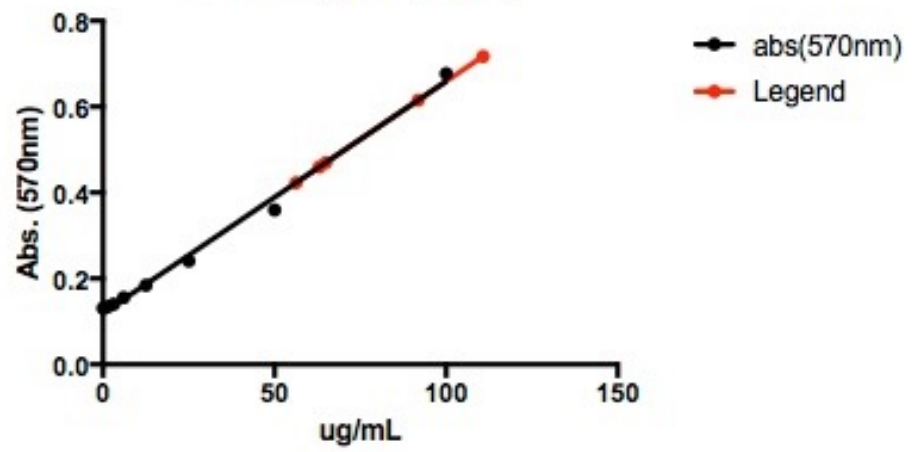
NOTE: If reading is outside that of the standard curve, the sample should be diluted (using the same diluent as above) and tested again on a fresh plate, including a standard curve.



Ca Assay -KB (WASH/SF)



Ca Assay-KB (POST)



Alkaline Phosphatase SOP

Fast blue RR Salt Sigma FBS-25 -20°
Naphthol AS-MX phosphate alkaline solution Sigma 85-5 +4°
Mayers Haematoxylin Sigma MHS-1
Citrate concentrated solution Sigma 85-4C
Ca⁺⁺ free PBS

Adapted for 3cm dishes.

Prepare citrate working solution – 2mL citrate concentrate to 98mL deionised water (scale down as necessary).

Prepare fixative, 2 volumes citrate working solution to 3 volumes acetone. Discard after use

Prepare Fast Blue Solution - Measure 48mL distilled water, bring to RT, dissolve 1 capsule Fast blue RR salt into the distilled water, wrap in foil to protect from light. (may need stirrer or warm in 37 degree waterbath). Once dissolved add 2mL Naphthol AS-MX phosphate alkaline solution to Fast Blue solution.

GENTLY aspirate media from dishes; GENTLY wash with PBS (x2).

Remove PBS and then add 2mL fixative for 30 seconds, wash in distilled water x2 - do not allow to dry.

Add Fast Blue dye mixture and incubate at RT for 30 minutes in the dark

Wash twice with deionised water

Add 2mL Mayer's haematoxylin for 10 minutes

Wash in deionised water (x2 or x3)

Adipogenic differentiation assay SOP

SOP Number: Alam

Equipment needed

Laminar flow cabinet
CO₂ incubator at 37°C
Hemocytometer
Inverted microscope
12 or 24 well plates
Fine tweezers
Glass coverslips, 16mm for 12 well plates, 13mm for 24 well plates
Leica microscope
Microscope slides
Sterile pipettes
Pastettes
Gloves
0.8µ pore filter
Reagents needed

Adipo medium :-

DMEM

10% foetal calf serum (FCS) (**GIBCO 10106-169**)

10% horse serum (**STEM CELL TECHNOLOGIES 06750**)

0.5 mM isobutylmethylxantine (**SIGMA I5879**)

60 µM indomethacin (**ICN**)

0.5 mM hydrocortisone (**SIGMA H2270**)

DMEM / 2% FCS

Trypsin

70% ethanol

Oil red

PBS (GIBCO/Life Tech 14190-169)

10% formalin

Harris's Haematoxylin, freshly filtered

Aquamountant (BDH 362262H)

Method

Steps 1 – 7 should be carried out aseptically in the laminar flow cabinet wearing gloves.

1. Trypsinise cells, re-suspend in 0.5mL DMEM / 2% FCS, leave on ice while counting.
2. Calculate the volume of cells required per well.
For a 12 well plate 0.1 million cells per well are needed.
For a 24 well plate 0.05 million cells per well are needed.
Ideally 3 replicates are required, but 1 is adequate if necessary.
3. Sterilise the coverslips in ethanol using the tweezers and place in the wells.
4. Wash the coverslips twice in PBS to remove the ethanol.
5. Add the medium to the wells.
1mL / well for 24 well plate, 2mL / well for 12 well plate.
6. Add cells to wells, swirl gently and incubate in CO₂ incubator at 37°C.
7. Feed cells twice a week by aseptically removing half the medium and replacing it with fresh medium on eg Monday and Friday. Continue for 3 weeks.
8. To stop the assay, remove the medium using a pastette and rinse twice with

PBS.

9. Fix with 10% formalin for 10 minutes.

10. Place Oil Red at 37°C for minimum of 30 minutes. Dilute the stock Oil Red in distilled water, 3 parts Oil Red to 2 parts water, then filter through a 0.8 μ pore filter followed by 0.2 μ filter to remove particles.

11. Remove formalin, wash twice with PBS, add filtered Oil Red 1mL / well for 10 minutes.

12. Remove Oil Red, wash twice with PBS.

13. Add filtered haematoxylin for 45 seconds then rinse in cold tap water.

14. Place 1 drop of mountant on a slide.

15. Remove coverslip with tweezers and place cell side down on the mountant.

16. Examine under the microscope.

PREPARATION OF STOCK SOLUTIONS

ISOBUTYLMETHYLXANTINE (SIGMA I5879)

(For a 1M solution use 222mg / mL)

Weigh out 200mg and dissolve in 1.8mL DMSO to make a 0.5M stock solution. Filter through a 0.2 μ pore size filter. This stock solution can be aliquoted in 100 μ L volumes and frozen at -20°C. 100 μ L is added to 100mL of medium (ie 1:1000) to give a final concentration of 0.5mM .

INDOMETHACINE (ICN)

(For 1M solution use 358 mg/mL or 100mg in 280 μ L)

Make up a 0.6M stock solution by dissolving 100 μ g in 467 μ L of DMSO. Filter sterilise and aliquot in 10 μ L volumes and freeze at -20°C. 10 μ L is added to 100mL of medium (ie 1:1000) for use.

HYDROCORTISONE (SIGMA H2270)

Make up 10⁻⁴M solution eg 4.845mg in 100mL of PBS. Filter sterilise and freeze at -20°C in 100 μ L volumes. Add to basic adipo medium immediately before use. 50 μ L per 10mL

HORSE SERUM (STEM CELL TECHNOLOGIES 06750)

Stored at -20°C in 10mL aliquots.

5. FOETAL CALF SERUM (GIBCO 10106-169)

Stored at -20°C in 10mL aliquots.

OIL RED (SIGMA O0625)

0.5% in isopropanol ie 50mg in 10mL

TRYPsin (GIBCO BRL 34500-027)

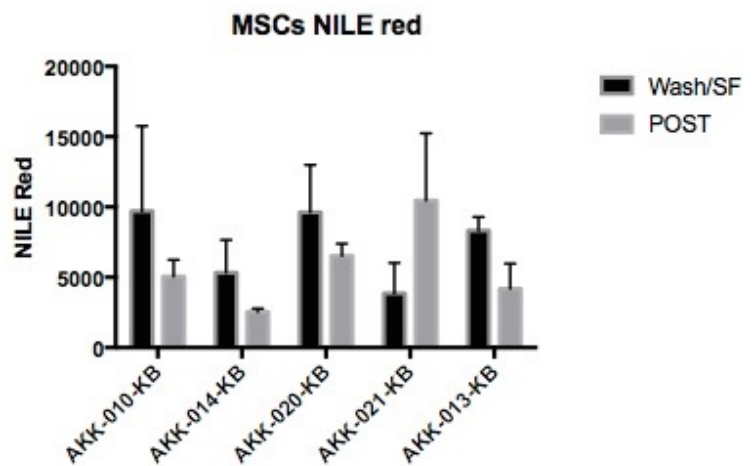
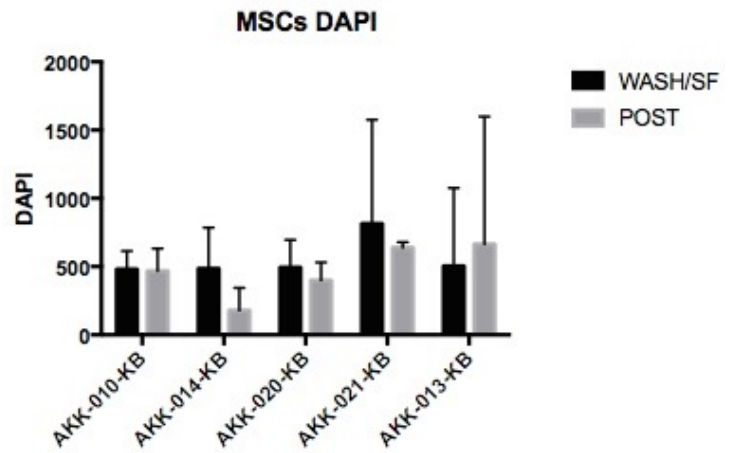
Aliquoted in 2mL volumes and stored at -20°C. For use add 18mL PBS. Use approximately 5mL per small flask.

Measurement Of Adipogenrc Differentiation by N110 Red on the plate reader

- 1) Design 48 well late to required layout below, do not use the outer rows/coloums of wells. In triplicate wells seed 40,000 MSC per test sample for both adipo and NH media.
2. Feed as per standard NH and adipo induction for 21 days. .
3. Make up Nile red stock solution of 10mg/mL in methanol aliquot and freeze at -20°C.
4. Remove media from wells.
5. Wash wells twice carefully in PBS.
6. Add 200ul 4% formalin in PBS and leave for 30min.
- 7 Make up 0.2% saponin in PBS (measure out in hood, and only remove from hood if covered, saponin powder is toxic). This needs to be fresh each time. ..
8. To Saponin solution add nile red and DAPI to a working concentration of (100uL of DAPI, and 100uL of NILE red stock), 1ug/ml, protect from light.
9. Remove formalin from wells.
10. Wash twice with PBS.
11. Add 200ul PBS to each well, be precise.
12. Read plate on Berthold plate reader (level 9) using DAPI and Nile red-FITC. The points should be set to 5 and area 2.38 for each reading. Check the plate layout before reading. This will give a background reading
13. Remove PBS from wells
14. Add 200ul 0.2% saponin/lug/ml nile red/lug/ml DAPI solution to each well.
15. Incubate for 15min in foil at room temperature.
16. Remove liquid from wells, and wash 3 times with PBS
17. Add 200ul PBS (careful precise pipetting is required), wrap plates in foil.
18. Read plate on Berthold plate reader (level 9) using DAPI and Nile red-FITC The points should be set to 5 and area 2.38 for each reading. Check the plate layout before reading. The filter slide is A (DAPI 355/460 and Nile Red 485/535)

19. Keep in fridge if fluorescent pictures are required. –

20. For analysis subtract the background reading and then do a ratio of Nile Red to DAPI



EnVision™ G|2 Doublestain System, Rabbit/Mouse (DAB+/Permanent Red) Code K5361

Protocol:

A. Reagent preparation

A.1 DAB+ Working Solution DAB+ Working Solution is prepared by thoroughly mixing 1 mL of DAB+ Substrate Buffer (Vial 3) with 1 drop (25-30 µL) of DAB+ Chromogen (Vial 4). Each 1 mL aliquot is sufficient for 10 tissue sections. Prepare only the volume required for the number of slides that shall be stained. Prepared DAB+ Working Solution is stable for approximately 5 days when stored at 2-8 °C. This solution should be mixed thoroughly prior to use. Any precipitate developing within 5 days in the solution does not affect staining quality. DAB+ Working Solution should be prepared in an Autostainer Reagent Vial when used on the Dako Autostainer instrument.

A.2 Permanent Red Working Solution Permanent Red Working Solution is prepared by thoroughly mixing 100 parts of Permanent Red Substrate Buffer (Vial 8) with 1 part of Permanent Red Chromogen (Vial 9), e.g. mix 1 mL Permanent Red Substrate Buffer (Vial 8) with 10 µL Permanent Red Chromogen (Vial 9). Prepare only the volume required for the number of slides that shall be stained. Use the solution within 30 minutes. Permanent Red Working Solution should be prepared in an Autostainer Reagent Vial when used on the Dako Autostainer instrument.

A.3 Guideline for optimal dilution of primary antibodies EnVision™ G|2 Doublestain System is compatible with suitably diluted Dako concentrated primary antibodies. Optimal dilution should be determined by the user.

B. Staining procedure for manual use

B.1 Procedural notes The following pre-treatment steps should be carried out before using EnVision™ G|2 Doublestain System on formalin-fixed, paraffin-embedded tissue sections. The specimen should be deparaffinized and rehydrated. Some specimens should be subjected to epitope demasking by heat-induced epitope retrieval or enzyme digestion. Please refer to the package insert of the individual primary antibody for specific information on specimen preparation. Following demasking, the specimen should be rinsed gently with wash buffer solution from a wash bottle (do not focus directly on tissue) and placed in a fresh wash buffer bath for 5 minutes.

All reagents should be equilibrated to room temperature (20-25 °C) prior to immunostaining. Likewise, all incubations should be performed at room temperature.

Do not allow specimens to dry out during the staining procedure. Dried specimens may display increased non-specific staining.

B.2 Staining protocol

Step 1: Vial 1, Dual Endogenous Enzyme Block Tap off excess wash buffer and carefully wipe around the specimen to remove any remaining liquid and to keep the reagent within the prescribed area. Apply 200 µL Dual Endogenous Enzyme Block to cover specimen. Incubate for 5 (± 1) minutes. solution from a wash bottle (do not focus directly on tissue) and place in a fresh wash buffer bath for 5 minutes.

Step 2: Primary antibody No. 1 or negative control reagent No. 1 Tap off excess wash buffer and wipe slides as before. Apply 200 µL mouse or rabbit primary antibody or

negative control reagent (see section A.3) to cover specimen. Incubate for 10 (\pm 1) minutes. Rinse specimen as described in Step 1.

Step 3: Vial 2, Polymer/HRP Tap off excess wash buffer and wipe slides as before. Apply 200 μ L Polymer/HRP to cover specimen. Incubate for 10 (\pm 1) minutes. Rinse specimen as described in Step 1, twice.

Step 4: DAB+ Working Solution Prepare DAB+ Working Solution as described in section A.1. Tap off excess wash buffer and wipe slides as before. Apply 200 μ L DAB+ Working Solution to cover specimen. Incubate for 5-15 minutes. Optimal incubation time may vary and should be determined by each individual laboratory. Rinse specimen gently with distilled or deionised water from a wash bottle (do not focus directly on tissue). Collect DAB+ Working Solution waste in a hazardous materials container for proper disposal. If the staining procedure must be interrupted, slides may be kept in a wash buffer bath following incubation of DAB+ Working Solution for up to one hour at room temperature (20-25 °C) without affecting staining result.

Step 5: Vial 5, Doublestain Block Tap off excess wash buffer and wipe slides as before. Apply 200 μ L Doublestain Block to cover specimen. Incubate for 3 (\pm 1) minutes. Rinse specimen as described in Step 1.

Step 6: Primary antibody No. 2 or negative control reagent No. 2 Tap off excess wash buffer and wipe slides as before. Apply 200 μ L optimally diluted mouse or rabbit primary antibody or negative control reagent (see section A.3) to cover specimen. Incubate for 10 (\pm 1) minutes. Rinse specimen as described in Step 1.

Step 7: Vial 6, Rabbit/Mouse (LINK) Tap off excess wash buffer and wipe slides as before. Apply 200 μ L Rabbit/Mouse (LINK) to cover the specimen. Incubate for 10 (\pm 1) minutes. Rinse specimen as described in Step 1, twice.

Step 8: Vial 7, Polymer/AP Tap off excess wash buffer and wipe slides as before. Apply 200 μ L Polymer/AP to cover the specimen. Incubate for 10 (\pm 1) minutes. Rinse specimen as described in Step 1, twice.

Step 9: Permanent Red Working Solution Prepare Permanent Red Working Solution as described in section A.2. Prepared Permanent Red Working Solution should be used within 30 minutes. Tap off excess buffer and wipe slides as before. Apply 200 μ L Permanent Red Working Solution to cover the specimen. Incubate for 5-20 minutes. Optimal incubation time may vary and should be determined by each individual laboratory. Rinse slides gently with distilled or deionised water from a wash bottle (do not focus directly on specimen). Collect chromogen-containing waste in a hazardous materials container for proper disposal. Place rinsed slides in a fresh distilled or deionised water bath for 5 minutes.

Step 10: Counterstain (instructions are for hematoxylin) Immerse slides in a bath of hematoxylin. Length of incubation time depends on the strength of hematoxylin used. Follow hematoxylin counterstaining with a thorough rinse in distilled or deionised water. Optional: Immerse tissue slides into a bath of 37 mmol/L ammonia water and rinse gently in distilled or deionised water for 2-5 minutes. Ammonia water (37 mmol/L) is prepared by mixing 2.5 mL of 15 mol/L (concentrated) ammonia hydroxide with 1 litre of distilled or deionised water. Unused 37 mmol/L ammonia water may be stored at room temperature (20-25 °C) in a tightly capped bottle for up to 12 months.

Step 11: Mounting For aqueous mounting, mounting media such as Dako Glycergel™ Mounting Medium, code C0563 or Faramount Aqueous Mounting Medium code S3025 is recommended. (114055-002)

Vial 1

3 x 11 mL **Dual Endogenous Enzyme Block**

Ready-to-use.

Endogenous enzyme block solution, containing 0.5% hydrogen peroxide, detergents, enzyme inhibitors, and preservative, pH 2.

Vial 2

3 x 11 mL **Polymer/HRP**

Ready-to-use.

Dextran polymer conjugated with horseradish peroxidase and affinityisolated immunoglobulins. Supplied in Tris/HCl buffer containing stabilizing protein and preservative.

Vial 3

3 x 11 mL **DAB+ Substrate Buffer**

Substrate buffer solution, pH 7.5, containing <0.1% hydrogen peroxide, stabilizers, enhancers, and an antimicrobial agent.

Vial 4

1 x 1.5 mL **DAB+ Chromogen**

5% 3,3'-diaminobenzidine tetrahydrochloride chromogen solution.

Vial 5

3 x 11 mL **Doublestain Block**

Ready-to-use.

Blocking solution.

Vial 6

3 x 11 mL **Rabbit/Mouse (LINK)**

Ready-to-use.

Dextran polymer coupled with secondary antibodies against mouse and rabbit immunoglobulins. In buffered solution containing stabilizing protein and preservative.

Vial 7

3 x 11 mL **Polymer/AP**

Ready to use

Dextran polymer conjugated with alkaline phosphatase and affinity-isloted immunoglobulins. Supplied in Tris/HCl buffer containing stabilizing protein and a preservative.

Vial 8

3 x 11 mL **Permanent Red**

Substrate Buffer Substrate buffer solution.

Vial 9

1 x 300 µL **Permanent Red Chromogen**

Permanent Red Chromogen solution.

Primary Antibodies used:

DAKO CD45 (monoclonal mouse) Leucocytes Common Antigen-Human
Ref: Mo701 (1:50)

RabMAb® Anti-CD73 EPR6114 (ab133582) (1:100)

Appendix 2



Health Research Authority

NRES Committee Yorkshire & The Humber - South Yorkshire

North East REC Centre
Unit 002, TEDCO Business Centre
Rolling Mill Road
Jarrow
Tyne and Wear
NE32 3DT

Telephone: 0191 428 3561

14 April 2014

Professor Dennis McGonagle
Professor of Investigative Rheumatology
Division of Musculoskeletal Disease
Leeds Institute of Molecular Medicine
Chapel Allerton Hospital
Chapelton Road
Leeds
LS7 4SA

Dear Professor McGonagle

Study title: **Collection of joint Mesenchymal Stem Cells by aspiration, biopsy, joint retrieval at arthroplasty or by synovium agitation during arthroscopy.**
REC reference: **14/YH/0087**
IRAS project ID: **100077**

Thank you for your letter of 11 April 2014, responding to the Committee's request for further information on the above research and submitting revised documentation.

The further information has been considered on behalf of the Committee by the Chair.

We plan to publish your research summary wording for the above study on the HRA website, together with your contact details, unless you expressly withhold permission to do so. Publication will be no earlier than three months from the date of this favourable opinion letter. Should you wish to provide a substitute contact point, require further information, or wish to withhold permission to publish, please contact the REC Manager Joan Brown, nrescommittee.yorkandhumber-southyorks@nhs.net

Confirmation of ethical opinion

On behalf of the Committee, I am pleased to confirm a favourable ethical opinion for the above research on the basis described in the application form, protocol and supporting documentation as revised, subject to the conditions specified below.

Mobeen Fazal

03/12/2015

Research & Innovation Department

Professor Dennis McGonagle
Professor Of Investigative Rheumatology /
Hon Consultant Rheumatologist
Chapel Allerton Hospital
Chapelton Road
Leeds
LS7 4SA

34 Hyde Terrace
Leeds
LS2 9LN

Tel: 0113 392 0162
Fax: 0113 392 0146

<http://www.leedsth.nhs.uk/research/>

Dear Professor Dennis McGonagle

Re: LTHT R&I Number: RR14/11102 (100077/WY): Collection of joint Mesenchymal Stem Cells by aspiration, biopsy, joint retrieval at arthroplasty or by synovium agitation during arthroscopy.
REC: 14/YH/0087

Thank you for your email regarding an amendment (**Amendment date: 06 November 2015**) to the above research study.

The amendment may be implemented with immediate effect in the Leeds Teaching Hospitals NHS Trust under the existing NHS Permission. Please note that you may only implement the changes described in the amendment notice or letter

Continued NHS Permission for the project is subject to the following conditions:

- Research Ethics Committee approval/regulatory approval for the amendment, if required, has been obtained
- Any contractual arrangements relating to this change have been addressed
- The Research Lead/Clinical Director for the Clinical Support Unit has approved any resource implications for the Directorate
- Implications for support departments working on the project have been assessed and approved by the relevant support department.

If you have any queries about this acknowledgement please do not hesitate to contact the R&I Department on telephone (0113) 392 0162.

With kind regards

Yours sincerely



Anne Gowing
Research Governance Manager

PATIENT INFORMATION SHEET

Re:

Collection of joint mesenchymal stem cells (MSC)

PI: Professor D McGonagle

Collection of Knee Tissues after Total Knee Replacement

You are being invited to take part in a RESEARCH study. Before you decide, it is important for you to understand why the research is being done and what it will involve. Please take the time to read the following information carefully and discuss it with friends and relatives if you wish. Please ask us if there is anything that is not clear, or if you would like more information. Take time to decide whether or not you wish to take part.

Thank you for reading this.

1. What is the purpose of the study?

Special cells in the body termed Mesenchymal Stem Cells (MSC) can make cartilage, bone, muscle, tendon and ligament. MSC have been found in many tissues to date but just how they work is still not understood. There is interest in the use of MSC as a way of repairing damaged joints and bones. Our research is aimed at understanding how these cells work in health and in disease.

2. Why have I been chosen?

Osteoarthritis has been estimated to affect approximately 20 per cent of population over 60 and so are of great importance. Osteoarthritis is a painful illness causing inflammation (or soreness) in the joints. The main cause is the alternations in joint movements, the eventual loss of the articular cartilage and the excessive bone formation which cause narrowing in the joint space and remodelling of bone. This **may eventually** require joint replacement.

As part of you normal arthroscopic, open knee surgery or joint replacement procedure any removed joint tissue or joint fluid is normally discarded. We would like to use these diseased **tissue and fluid** to study the function of MSC. We will grow these cells in the laboratory for a short time and then study their ability to make cartilage and bone.

In addition suitable patient will be chosen to have their stem cells retrieved by sampling of the joint lining during arthroscopic procedures using a cytology/PAP brush, This will be undertaken with sterile, single use instrumentation already approved for use in man. The collection of cells in this way will be minimally invasive, would only add a few minutes on to the operative time.

3. Do I have to take part?

It is up to you to decide whether or not to take part. If you decide to take part, you will be given this information sheet to keep and be asked to sign a consent form. If you decide to take part, you are still free to withdraw at any time and without giving a reason. This will not affect the standard of care that you receive.

4. What will happen to me if I take part?

If you decide to take part you will be asked to sign an informed consent form, and you will be given a copy of the information sheet and the signed consent form to keep. You will be asked to donate your joint tissues normally thrown after your operation.

Some suitable patient will be chosen to have their stem cells retrieved by sampling of the joint lining during arthroscopic procedures using a cytology/PAP brush, This will be undertaken with sterile, single use instrumentation already approved for use in man. The collection of cells in this way will be minimally invasive, would only add a few minutes on to the operative time.

5. What do I have to do?

There are no other requirements/tests needed beyond the surgical procedure.

It is very important, when giving your medical history to the doctor, that you tell him/her whether or not you are regularly taking any medicine.

6. What are the side effects of taking part?

For patient that are not undergoing brushing of the joint lining there are no side effects as knee tissues are normally thrown after the operation. The collection of MSC's with the cytology/PAP brush is not harmful to the patient, and uses a brush that already is commonly used within humans.

7. What are the possible benefits of taking part?

You will not benefit directly from taking part in this research and all other aspects of your care will be the same as if you did not take part.

8. What if something goes wrong?

If you are harmed by taking part in this research, there are no special compensation arrangements. If you are harmed due to someone's negligence, then you may have grounds for a legal action but you may have to pay for it. Regardless of this, if you wish to complain about any aspect of the way you have been approached or treated during the course of this study, the normal NHS complaints mechanism may be available to you.

9. Will my taking part in this study be kept confidential?

As soon as tissue sample is taken from you, all information that identifies you will be removed so that you cannot be recognised.

10. What will happen to the results of the study?

At the end of the study, the results will be written into a scientific paper for publication in a scientific journal.

11. Who is organising the research?

This project is being organized by Doctors of St. James and Chapel Allerton Hospital under the supervision and support of Leeds University.

12. Who has reviewed this study?

This study has been reviewed by the independent ethics committee called the Leeds (West) ethics committee. This committee is appointed to determine that research studies are ethical and do not impair the rights or well-being of patients. We have received approval by this committee to be able to do this research study.

13. Contact for further information

For further information on the study please contact:

Professor D McGonagle, Telephone 0113 3924474.

Consent Form

Re:

Collection of joint mesenchymal stem cells (MSC)

PI: Professor D McGonagle

Please initial box

1. I confirm that I have read and understand the patient information sheet dated for the above study. I have had an opportunity to consider the information, ask questions and have had these answered satisfactorily.

2. I understand that my participation is voluntary and that I am free to withdraw at any time, without giving any reason, without my medical care or legal rights being affected.

3. I understand that relevant sections of any of my medical notes will be looked at by responsible individuals from the NHS Trust, where it is relevant to my taking part in this research. I give permission for these individuals to have access to my records.

4. I agree to take part in this research study

..... Date:Signature.....
Name of patient:

..... Date:Signature.....
Name of person taking consent
(if different from researcher)

..... Date:Signature.....
Researcher



Health Research Authority
Yorkshire & The Humber - Leeds West Research Ethics Committee

Room 001, Jarrow Business Centre
Rolling Mill Road
Jarrow
Tyne and Wear
NE32 3DT

Telephone: 0191 428 3444

18 February 2016

Professor Dennis McGonagle
Professor of Investigative Rheumatology
University of Leeds
Leeds Institute of Rheumatic and Musculoskeletal Medicine
Chapel Allerton Hospital
Chapeltown Road
Leeds
LS7 4SA

Dear Professor McGonagle

Study title: **A proof-of-concept study to examine the safety and efficacy of a novel joint stem cell brushing method for endogenous mesenchymal stem cells mobilisation during knee joint microfracture for cartilage repair.**

REC reference: **15/YH/0512**

IRAS project ID: **180298**

Thank you for your letter of 12 February 2016, responding to the Committee's request for further information on the above research and submitting revised documentation.

The further information has been considered on behalf of the Committee by the Vice-Chair.

We plan to publish your research summary wording for the above study on the HRA website, together with your contact details. Publication will be no earlier than three months from the date of this opinion letter. Should you wish to provide a substitute contact point, require further information, or wish to make a request to postpone publication, please contact the REC Manager, Miss Christie Ord at nrescommittee.yorkandhumber-leedswest@nhs.net.

Confirmation of ethical opinion

On behalf of the Committee, I am pleased to confirm a **favourable** ethical opinion for the above



Dr Ann Morgan
Senior Lecturer and Honorary Consultant
Rheumatologist
Molecular Medicine Unit
Clinical Sciences Building
St James's University Hospital
Beckett Street
Leeds
LS9 7TF

Leeds (East) Research Ethics Committee
Room 5.2, Clinical Sciences Building
St James's University Hospital
Beckett Street
Leeds
LS9 7TF

Enquiries to Ann Prothero
Ethics Secretary
Direct Line/Ext: 0113 (20) 65652

22 October 2004

Dear Dr Morgan,

***Full title of study: Functional characterisation of the genes and proteins involved in the development and severity of autoimmune and (auto)inflammatory diseases
REC reference number: 04/Q1206/107***

Thank you for your letter of 08 October 2004, responding to the Committee's request for further information on the above research.

The further information has been considered on behalf of the Committee by the Chairman

Confirmation of ethical opinion

On behalf of the Committee, I am pleased to confirm a favourable ethical opinion for the above research on the basis described in the application form, protocol and supporting documentation.

The favourable opinion applies to the following research site:

Site: The Leeds Teaching Hospitals NHS Trust
Principal Investigator: Dr Ann Morgan

Conditions of approval

The favourable opinion is given provided that you comply with the conditions set out in the attached document. You are advised to study the conditions carefully.

Approved documents

The final list of documents reviewed and approved by the Committee is as follows:

Application dated 15/08/2004
Investigator CV dated 15/08/2004
Protocol for 'Investigation of the molecular mechanisms involved in Tumour Necrosis Factor Receptor associated periodic syndrome (TRAPS) dated 16/08/2004

Protocol for The Role of Fc gamma receptor polymorphisms in the pathogenesis of rheumatoid arthritis dated 16/08/2004
Peer Review dated 16/08/2004
Participant Information Sheet for patients with a specific autoimmune or inflammatory disease dated 15/08/2004
Participant Information Sheet for patients with a periodic fever or with relatives with an autoimmune or inflammatory disease dated 15/08/2004
Participant Information Sheet for the generation of human autoantibody producing B-cell lines dated 15/08/2004
Participant Consent Form dated 15/08/2004
Response to Request for Further Information dated 08/10/2004
Information sheet for patients under 16 with a periodic fever or with relatives that may have an autoimmune or inflammatory condition dated 08/10/2004
Information sheet for parents and guardians of children with periodic fever or who have a relative who may have autoimmune or inflammatory condition dated 08/10/2004
Consent form for parents/guardians dated 08/10/2004
Assent form for patients under 16 dated 08/10/2004

Management approval

The study may not commence until final management approval has been confirmed by the organisation hosting the research.

All researchers and research collaborators who will be participating in the research must obtain management approval from the relevant host organisation before commencing any research procedures. Where a substantive contract is not held with the host organisation, it may be necessary for an honorary contract to be issued before approval for the research can be given.

Notification of other bodies

We shall notify the University of Leeds The Leeds Teaching Hospitals NHS Trust and the Medicines and Health-Care Products Regulatory Agency that the study has a favourable ethical opinion.

Statement of compliance (from 1 May 2004)

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees (July 2001) and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

REC reference number: 04/Q1206/107	Please quote this number on all correspondence
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Yours sincerely,


Dr P R F Dear
Chairman

Cc: Claire Skinner, University of Leeds

Research and Development Department, LTHT

Enclosures Standard approval conditions SL-AC2

PATIENT INFORMATION SHEET (HEALTHY CONTROLS)

Functional characterisation of the genes and proteins involved in the development and severity of autoimmune and (auto) inflammatory diseases.

PART 1

1. Invitation

You are being invited to take part in a research study. Before you decide whether or not to take part, it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully, and discuss it with others if you wish.

PART 1 tells you the purpose of this study and what will happen to you if you take part.

PART 2 gives you more detailed information about the conduct of the study.

Ask us if there is anything that is not clear, or if you would like more information. Take time to decide whether or not you wish to take part.

2. What is the purpose of the study?

Many autoimmune and (auto)inflammatory diseases, such as rheumatoid arthritis, systemic lupus erythematosus, vasculitis and the connective tissue diseases are associated with the presence of specific changes in either an individual's genetic makeup or their immune system. This can lead to alterations in the different components of the immune system or in the proteins that are produced by these genes. We feel these changes may be important for the development of either the disease itself, specific antibodies/ complications or that they may even predispose to or even help us predict more severe disease. We would like to perform some further research to gain a better understanding of their biology and how they may contribute to these various diseases.

3. Why have I been chosen?

In studies of this type we need to compare the genes, proteins and components of the immune system in people with the diseases we are interested in compared to those people of the same age who do not have the disease ("controls"). You have been identified as someone who is unlikely to have one of these conditions and we would like to store some of your blood, such as the white blood cells, proteins and sample of your DNA, and/ or a urine sample to form part of a "control" sample bank. These samples will only be used in future studies that continue with this agreed line of research.

4. Do I have to take part?

It is up to you to decide whether or not to take part. If you decide to take part, you will be given this information sheet to keep and be asked to sign a consent form. If you decide to take part, you are still free to withdraw at any time and without giving a reason.

5. What will happen to me if I take part?

If you decide to take part you will be asked to sign an informed consent sheet, and you will be given a copy of the information sheet to keep. You will be asked to donate a sample of blood (up to 50mls). Alternatively, we may ask for a sample of your urine.

The blood sample will be used for genetic testing, but this is purely for research purposes and you will not be told the results of the tests on your samples. Insurance companies, however, may ask you whether you have previously had genetic tests. Should this situation arise, we advise you to answer "no" in your insurance policy application form. This is because the genetic tests we are doing are purely for research purposes and have no bearing whatsoever on your current or future insurance policies.

6. What do I have to do?

Apart from donating a small volume of blood for research, there are no other requirements/ tests.

7. What are the side effects of any treatment received when taking part?

You may develop a bruise at the site of the needle but, as stated above, taking part in this study does not involve any additional blood tests.

8. What are the possible benefits of taking part?

You will not benefit directly from taking part in this research, and all other aspects of your care will be the same as if you did not take part.

9. What if there is a problem?

If you have a concern about any aspect of this study, you should ask to speak with the researchers who will do their best to answer your question. **If you remain unhappy and wish to complain formally, you can do this through the NHS Complaints Procedure. Details can be obtained from the hospital.**

In the event that something does go wrong and you are harmed during the research study there are no special compensation arrangements. If you are harmed and this is due to someone's negligence then you may have grounds for a legal action for compensation but you may have to pay your legal costs. The normal National Health Service complaints mechanisms will still be available to you.

10. Will my taking part in this study be kept confidential?

Yes. All the information about your participation in this study will be kept confidential. The details are included in Part 2.

11. Contact Details

Please do not hesitate to contact your GP or any other independent person if you need advice. For further information on the study please contact Dr Ann W Morgan (0113 3438414) or Dr Dawn Cooper or Dr Sarah Mackie (0113 3438413).

This completes Part 1 of the Information Sheet.

If the information in Part 1 has interested you and you are considering participation, please continue to read the additional information in Part 2 before making any decision.

PART 2

12. Will my part in this study be kept confidential?

If you consent to take part in this study, the records obtained while you are in this study as well as related health records will remain strictly confidential at all times. The information will be held securely on paper and electronically at your treating hospital and the University of Leeds, the main team managing this research under the provisions of the 1998 Data Protection Act. Your name will not be passed to anyone else outside the research team or the sponsor, who is not involved in the research.

Information will be transferred from your hospital site to the team at the University of Leeds organizing the research, to enable processing of blood samples, this will be done by mail, however your name will only appear on your consent form and blood sample until it is separated and stored. All other records will have your name removed and will only feature your initials and date of birth.

Your records will be available to people authorised to work on the trial but may also need to be made available to people authorised by the Research Sponsor, which is the organisation responsible for ensuring that the study is carried out correctly. A copy of your consent form may be sent to the Research Sponsor during the course of the study. By signing the consent form you agree to this access for the current study and any further research that may be conducted in relation to it, even if you withdraw from the current study.

The information collected about you may also be shown to authorised people from the UK Regulatory Authority and Independent Ethics Committee; this is to ensure that the study is carried out to the highest possible scientific standards. All will have a duty of confidentiality to you as a research participant.

In line with Good Clinical Practice guidelines, at the end of the study, your data will be securely archived for a minimum of 15 years. Arrangements for confidential destruction will then be made.

20. What will happen to any samples I give?

Once it is sent to the University of Leeds, your blood sample will be anonymised and separated into its component parts so that some of your DNA, serum and cells will be stored to form part of our "disease sample repository" so that we can use it in current and future genetic and immunological studies. Any additional studies will be subject to additional independent ethical committee review.

21. Will any Genetic testing be done?

Yes, once anonymised your DNA will be used in genetic testing aimed at finding out which genes are important in both the development and severity of a number of autoimmune and (auto)inflammatory diseases. Your DNA will only be used in future studies which continue the research themes outlined above.

22. What will happen to the results of this research?

The results of the studies using the "disease sample repository" will usually be published in a medical journal or be presented at a scientific conference. The data will be anonymous and none of the patients involved in the study will be identified in any report or publication.

23. Who is organising and funding this research?

This project is partly being funded by grants from the National Institute for Health Research, Arthritis Research Campaign, Research into Ageing and the University of Leeds.

24. Who has reviewed the study?

This study was given favourable ethical opinion for conduct in the NHS by Leeds (East) Research Ethics Committee. This committee is appointed to determine that research studies are ethical and do not impair the rights or well-being of patients. We have received approval by this committee to be able to do this research study.

25. Contact for further information

You are encouraged to ask any questions you wish, before, during or after your treatment. If you have any questions about the study, please speak to your study nurse or doctor, who will be able to provide you with up to date information about the procedure(s) involved. If you wish to read the research on which this study is based, please ask your study nurse or doctor. If you require any further information or have any concerns while taking part in the study please contact one of the following people:

Dr Ann W Morgan 0113 2065117

Alternatively if you or your relatives have any questions about this study you may wish to contact your GP or an organisation that is independent of the hospital at which you are being treated:

Arthritis Research Campaign (*arc*) is a registered charity providing information about all aspects of arthritis for patients and their families. They can provide useful booklets. You can contact them on 0870 850 5000, or access their web site at <http://www.arc.org.uk>

If you decide you would like to take part then please read and sign the consent form. You will be given a copy of this information sheet and the consent form to keep. A copy of the consent form will be filed in your patient notes, one will be filed with the study records and one may be sent to the Research Sponsor.

You can have more time to think this over if you are at all unsure.

Thank you for taking the time to read this information sheet and to consider this study.

PATIENT CONSENT FORM

Functional characterisation of the genes and proteins involved in the development and severity of autoimmune and (auto) inflammatory diseases.

Patient ID Initials: Date of Birth:

Patient Initial each point

- 1 I confirm that I have read and understand the information sheet dated 23/09/2011(version 5.0) for the above study, and have had the opportunity to ask questions. I understand that my participation is voluntary and that I am free to withdraw at any time without my medical care or legal rights being affected. I agree to take part in the study. _____
- 2 I understand that my medical records may be looked at by authorised individuals from the Sponsor for the study, the UK Regulatory Authority or the Independent Research Ethics Committee in order to check that the study is being carried out correctly. I give permission, provided that strict confidentiality is maintained, for these bodies to have access to my medical records for the above study and any further research that may be conducted in relation to it. I also give permission for a copy of my consent form to be sent to the sponsor for the study. _____
- 3. I understand that even if I withdraw from the above study, the data and samples collected from me will be used in analysing the results of the study, unless I specifically withdraw consent for this. I understand that my identity will remain anonymous. _____
- 4. I consent to the storage including electronic, of personal information for the purposes of this study and that it may be transferred in anonymised form only to third parties in the UK, the EU, Switzerland and/or USA together with my sample or parts thereof. I understand that any information that could identify me will be kept strictly confidential and that no personal information will be included in the study report or other publication. _____
- 5. I agree to the samples and cell lines being stored for future research. _____
- 6. I agree to have genetic tests done on samples for research purposes. _____

Name of the patient Patient's signature and the date the patient signed the Consent form

Name of the Investigator the taking written consent Investigator's signature and date the Investigator signed consent form

Original to be retained and filed in the site file. 1 copy to patient, 1 copy to be filed in patient's notes, 1 copy for Sponsor