

Exploiting Knowledge of Mesenchymal Stromal Cells *In vivo* for Bone Disease Therapy Development

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The candidate confirms that the work submitted is his own, except where work which has formed part of jointly authored publications has been included. The contribution of the candidate and the other authors to this work has been explicitly indicated below. The candidate confirms that appropriate credit has been given within the thesis where reference has been made to the work of others.

List of publications

1. Single-platform quality control assay to quantify multipotential stromal cells in bone marrow aspirates prior to bulk manufacture or direct therapeutic use (chapter 3)

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2. Induced periosteum a complex cellular scaffold for the treatment of large bone defects (chapter 5)

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Abstract

This thesis aims to demonstrate how understanding of the nature of MSCs *in vivo* can be used to guide bone disease therapy development. It first address the unpredictable MSC content of bone marrow (BM) aspirates used for therapy; then examines MSC enrichment using clinical grade immunomagnetic cell selection. Induction of osteogenesis was subsequently explored; by examining an induced membrane (IM) used for bone regeneration. Finally the potential of a janus kinase (JAK) inhibitor to block osteogenesis was assessed, by studying its effect on MSCs *in vitro*.

Flow cytometry was used to enumerate cells expressing a CD45^{-/low} CD271⁺ phenotype in BM aspirate and this was compared to colony forming unit fibroblast (CFU-F) content. MSCs were enriched from BM, enzymatically treated femoral heads (FH) and intramedullary canal aspirates and enumerated. The composition morphology, MSC content and differentiation potential of IM was compared to periosteum. The potential effects of JAK inhibition on MSC colony formation, expansion and differentiation potential were examined.

The concentration of cells expressing a CD45^{-/low} CD271⁺ phenotype strongly correlated with CFU-F concentration (R=0.812, p<0.001). Immunomagnetic cell selection resulted in an increase in the proportion of MSCs in BM, FH and intramedullary canal aspirates by 204, 14.1 and 291-fold respectively. The regenerative potential of periosteum and IM were comparable. JAK inhibition did not affect MSC growth, osteogenesis or chondrogenesis but caused an increase in adipogenesis at concentrations $\geq 100\text{nM}$ compared to controls (1.38 fold, p=0.041).

Flow cytometry may be used to rapidly and accurately predict the MSC content of BM. Clinical grade immunomagnetic selection can substantially increase the purity of MSCs from bone cavities. The similarity, in terms of regenerative potential, of periosteum and IM gives insight into its use for bone regeneration. JAK inhibition did not affect *in vitro* osteogenesis but has potential to affect *in vivo* osteogenesis through stimulation of adipogenesis.

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List of abbreviations

1,25(OH)₂D₃ - 1,25-dihydroxyvitamin D₃

ACDA - anticoagulant citrate dextrose solution A

ALP – Alkaline phosphatase

APC- Allophycocyanin

AS – Ankylosing spondylitis

ATMPs – Advanced therapy medicinal products

BM – Bone marrow

BMP – Bone morphogenic protein

CD – Cluster of differentiation

C/EBPα – CCAAT-enhancer binding protein alpha

Cy - Cyanine

DAPI - 4',6-diamidino-2-phenylindole

DEX - Dexamethasone

DKK-1 – Dickkopf 1

DMSO – Dimethyl sulfoxide

ECM – Extracellular matrix

FACS – Fluorescence activated cell sorting

FCS – Foetal calf serum

FDA – Federal Drug Administration

FH – Femoral head

FITC - Fluorescein isothiocyanate

GAG – Glycosaminoglycan

GH – Growth hormone

GVHD – Graft versus host disease

HC - Haematopoietic cell

HSC – Haematopoietic stem cell

HCT/Ps – Human cellular and tissue based products

HRP – Horseradish peroxidase

HSA – Human serum albumin

IFN – Interferon

IL – Interleukin

IM – Induced membrane

JAK – Janus kinase

LRP – Low density lipoprotein related protein

M-CSF – Macrophage colony stimulating factor

MHC – Major histocompatibility complex

MAPK - Mitogen activated protein kinase

MMP-3 – Matrix metalloproteinase 3

MNC – Mononuclear cell

MSC – Mesenchymal stromal cell

OA – Osteoarthritis

OCT – Optimal cutting temperature

OP – Osteoporosis

OPG – Osteoprotegerin

OSM - Oncostatin M

OSX – Osterix

p – Passage

PO – Periosteum

PBS – Phosphate buffered saline

PBMC – Peripheral blood mononuclear cells

PcP - Peridinin-chlorophyll-protein

PD – Population doubling

PDGF – Platelet derived growth factor

PI3K - Phosphoinositide 3-kinase

PE - R-Phycoerythrin

PMMA - Polymethyl methacrylate

PPAR γ - Peroxisome proliferator-activated receptor gamma

PsA – Psoriatic arthritis

PTFE – Polytetrafluoroethylene

RA – Rheumatoid arthritis RANK – Receptor activator of nuclear factor κ B

RANKL - Receptor activator of nuclear factor κ B ligand

RAR – Retinoic acid receptor

RBC – Red blood cell

RIA – Reamer irrigator aspirator

ROR γ - Retinoid related orphan receptor gamma

RUNX2 – Runt related transcription factor 2

SDF-1 – Stromal derived factor

STAT - Signal transducers and activators of transcription

TGF- β – Transforming growth factor beta

TIMP - Tissue inhibitor of matrix metalloproteinase

TNF- α – Tumour necrosis factor α

TYK – Tyrosine kinase

VEGF – Vascular endothelial growth factor

γ_c – Common gamma chain

1. General introduction

1.1. Regenerative medicine

Regenerative medicine is a field of research that aims to replace or repair diseased or damaged tissue and organs. Regenerative medicine strategies rely on the activation, stimulation or correction of endogenous repair mechanisms (Horwitz et al. 1999; Lee et al. 2010; Homma et al. 2014) or mechanisms involved in natural developmental processes, in order to restore function (Jopling et al. 2011).

Cellular therapeutics is a branch of regenerative medicine that seeks to utilise cells as therapeutic agents. These may be autologous, originating from the patient's own tissue or allogeneic, originating from donor tissue (Galipeau 2013; Homma et al. 2014). They may be administered alone or combined with natural or synthetic scaffolds (Hernigou et al. 2005b; Hattori et al. 2006; Homma et al. 2014). They may also be subject to *in vitro* culture expansion or have undergone genetic manipulation (Okita et al. 2007; Galipeau 2013).

Research into stem cell biology plays a central role in regenerative medicine. Understanding of the nature and behaviour of stem cells *in vivo* is critical for the development of effective treatments.

1.2. Stem cells

The concept of a cell type that is self-renewing as well as capable of giving rise to multiple specialised cell types was first suggested in the 19th century (Ramalho-Santos et al. 2007; Bianco et al. 2008). Stem cells were postulated to account for the observation that certain tissues, such as the blood or skin, were comprised of short-lived cells, yet had the capacity to self-renew for the lifetime of the organism. Alexander Maximow is often credited with coining the term "stem cell", used to describe the common precursor of the blood system in 1909, although uses of the term can be found as early as 1896 (Ramalho-Santos et al. 2007). The eventual identification of stem cells required the development of methods to isolate, as well

as to test the potency of prospective stem cell candidates following *in vivo* transplantation (Bianco et al. 2008).

1.3. Mesenchymal stromal cells

Mesenchymal stromal cells (MSCs), also referred to as mesenchymal stem cells and multipotential stromal cells were first described by Friedenstein et al as a rare population of adherent, highly proliferative, colony forming cells present in the bone marrow (BM) (Friedenstein et al. 1970; Friedenstein et al. 1992). Since then, MSCs have been reported to be resident in numerous tissues including BM (Pittenger et al. 1999; Noth et al. 2002; Quirici et al. 2002; Tormin et al. 2011), umbilical cord (Kouroupis et al. 2013; Kang et al. 2014), periosteum (Arnsdorf et al. 2009; Radtke et al. 2013), adipose tissue (Gronthos et al. 2001; Guilak et al. 2004; Hattori et al. 2004), synovium (Fickert et al. 2003; Jo et al. 2007; Karystinou et al. 2009; Jones et al. 2010a), placenta (in't Anker et al. 2004; Chang et al. 2006; Miao et al. 2006) and endometrium (Schwab et al. 2007; Gargett et al. 2009).

The term mesenchymal stem cell was first coined and described by Arnold Caplan. He theorised the existence of a single cell type capable of differentiating into cells comprising bone, cartilage, tendon, ligament, marrow stroma, adipocyte, dermis, muscle and connective tissue (Caplan 1991). Caplan envisioned a future where a patient's own MSCs could be taken, expanded *in vitro* and reintroduced, in a manner that guaranteed that they would differentiate into a specific tissue.

The potential therapeutic properties of MSCs continue to draw interest. More recent discoveries have further highlighted their therapeutic potential. For example, MSCs have been shown to release potentially regenerative trophic factors (Ponte et al. 2007; Sato et al. 2011), as well as be able to act as immunosuppressive agents (Ren et al. 2009; Galipeau 2013). Table 1 shows current open trials that seek to utilise the therapeutic properties of MSCs.

Many of the properties of MSCs are potentially useful for therapeutic approaches that seek to regenerate damaged bone. However, in some cases bone formation

may not only be undesirable, but actively drive the progression of degenerative diseases. Inappropriate bone formation is seen in some spondioarthropathies and in osteoarthritis (Braem et al. 2012; Zhen et al. 2013).

Table 1: Current open clinical trials using MSCs

Search term: Mesenchymal stem cell AND:	Open studies	Actively recruiting	Mode of action
Osteoarthritis	12	11	Differentiation
Non union	9	4	Differentiation
Graft versus host disease	21	11	Immunosuppression
Ischemia	28	22	Release of trophic factors
Multiple sclerosis	14	10	Immunosuppression
Rheumatoid arthritis	5	4	Immunosuppression
Ankylosing spondylitis	2	1	Immunosuppression
Osteoporosis	1	1	Differentiation
Acute respiratory distress	4	4	Immunosuppression
Systemic Lupus Erythematosus	1	1	Immunosuppression

Data supplied by Clinical trials.gov ([http://Clinical trials.gov](http://Clinical%20trials.gov), accessed on 11/06/2014)

1.4. Differentiation capacity of MSCs

Early *in vitro* and animal implantation studies indicated that BM contained mesenchymal progenitor cells able to contribute to the formation of adipose, cartilage and bone (Gimble et al. 1990; Gundle et al. 1995; Kadiyala et al. 1997; Kuznetsov et al. 1997). However, these studies were not able to discern if tissue progenitors were present as a mixed population of cells or rather as a single cell type capable of multilineage differentiation. Pittenger *et al* expanded an individual colony from BM and demonstrated that these expanded cells could be induced *in*

vitro to differentiate into the adipocytic, chondrocytic or osteocytic lineages (Pittenger et al. 1999).

Although this was undoubtedly a significant step in the search for MSCs, Pittenger failed to identify a single cell phenotype responsible for the formation of colonies. This is important because rates of proliferation, cell morphology and differentiation potential differ widely between individual colonies (Castro-Malaspina et al. 1980; Kuznetsov et al. 1997; Pittenger et al. 1999). Similarly, expression of various adipogenic, chondrogenic and osteogenic markers differ both between individual colonies and as a function of time in culture (Kuznetsov et al. 1997; Wagner et al. 2008). The capacity of BM derived MSCs to consistently form lineages beyond bone, cartilage and fat remains controversial and has never been supported by direct and reproducible experimental evidence (Bianco et al. 2010; Bianco et al. 2013b).

1.5. MSCs and osteogenesis

1.5.1. Bone development

Growth and maintenance of the skeleton occurs by distinct two processes known as endochondral and intramembranous ossification. Endochondral ossification is the process responsible for the replacement of the embryonic cartilaginous skeleton and the growth of long bones until adult height is achieved (Maes et al. 2010; Mackie et al. 2011; Dirckx et al. 2013). This type of bone formation is driven in developing bone initially by chondrocyte proliferation (Maes et al. 2010; Mackie et al. 2011; Dirckx et al. 2013). Proliferating chondrocytes secrete extracellular matrix (ECM) and eventually hypertrophic chondrocytes in the mid shaft are invaded by blood vessels (Maes et al. 2010; Mackie et al. 2011; Dirckx et al. 2013). Blood vessel infiltration begins from the bone collar, which is formed in the perichondrium, and is accompanied by osteoclast and osteoblast precursor migration (Maes et al. 2010; Mackie et al. 2011; Dirckx et al. 2013).

Osteoclasts then remove the cartilage ECM and osteoblasts deposit bone, as a result the cartilage is progressively eroded and replaced by trabecular bone (Maes

et al. 2010; Mackie et al. 2011; Dirckx et al. 2013). Trabecular bone is synthesised by mature osteoblasts that deposit mineralised ECM, a process that is largely mediated by TGF- β superfamily signalling molecules (Maeda et al. 2004; Tsuji et al. 2006; Chen et al. 2012). After birth, the remaining cartilage at the epiphysis also becomes invaded by blood vessels leading to the formation of a growth plate. Further chondrocyte proliferation, at the growth plate, followed by angiogenesis and osteoclast invasion drives longitudinal bone growth (Mackie et al. 2011; Dirckx et al. 2013).

In terms of foetal bone development intramembranous ossification occurs in a relatively small number of bones, those comprising the flat bones of the skull (Mackie et al. 2011; Dirckx et al. 2013; Percival et al. 2013). Here, bone formation occurs directly from pre-osteogenic condensations of mesenchymal progenitors, from which presumptive bone cells rapidly proliferate outward (Percival et al. 2013). Following the initial expansion and mineralisation, bone growth is driven by proliferation and differentiation of pre-osteogenic MSCs at the osteogenic fronts along fibrous sutures (Rice et al. 2003; Percival et al. 2013).

Once formed, mature bone is not static but continually undergoes remodelling (Kular et al. 2012). This is an essential process for the repair of the micro-damage that is a normal consequence of the stress placed on bone during everyday life (Kular et al. 2012). Frost was the first to describe what he termed the basic multicellular unit of bone remodelling comprising osteoclasts, osteoblasts and osteocytes (Frost et al. 1969). Bone is constantly broken down by bone degrading osteoclasts and replaced by bone forming osteoblasts which may then become surrounded by mineralised bone to form osteocytes (Crockett et al. 2011). It has since become clear that bone lining cells also play an important role in the bone remodelling cycle and the complex interaction between all these cell types regulates the balance of bone formation and resorption (Crockett et al. 2011).

The osteoclast is a unique exocrine cell that is capable of dissolving bone as well as degrading ECM (Teitelbaum 2007). It is a large multinucleated cell that forms through fusion of mononuclear precursor cells of the monocyte/macrophage

lineage (Teitelbaum 2007; Crockett et al. 2011). Differentiation is controlled by interactions between stromal cells, osteoblasts and pre-osteoclasts (Teitelbaum 2007). Two important mediators of this are macrophage colony stimulating factor (M-CSF) and receptor activator of nuclear factor- κ B ligand (RANKL). RANKL acts as the key osteoclastogenic cytokine (Teitelbaum 2007; Crockett et al. 2011). Osteoblasts and their precursors are the principle expressers of RANKL which is expressed on their cell surface as a response to parathyroid hormone and the active dihydroxy form of vitamin D₃ (Leibbrandt et al. 2008; Nakashima et al. 2011). Signalling through RANK, the receptor for RANKL, is an important driver of osteoclast differentiation (Teitelbaum 2007; Crockett et al. 2011). Mutations causing the loss of function in either RANKL or RANK result in the development of osteopetrosis, a disease characterised by brittle bones and high bone mass (Sobacchi et al. 2007; Guerrini et al. 2008).

Osteoprotegerin (OPG) is a negative regulator of RANKL activity, OPG competes with RANK as a soluble decoy receptor and is also produced by osteoblast lineage cells (Lacey et al. 1998; Teitelbaum 2007). The balance between RANKL and OPG is critical in controlling osteoclast differentiation. Thus, osteoblasts play an integral role in regulating osteoclast differentiation and this is a pivotal interaction which governs the balance between bone formation and resorption.

1.5.2. Fracture repair

Following fracture, endochondral and intramembranous ossification are recapitulated in a multi-step process with many similarities to foetal skeletal development. Both endochondral and/or intramembranous ossification may occur depending on the type of fracture and the degree of stability (Thompson et al. 2002; Marsell et al. 2011). The most common process by which bone is repaired is conventionally partitioned into four stages, each characterised by a distinct set of cellular and molecular events. These stages are: acute inflammation, soft callus formation, hard callus formation and remodelling (Schindeler et al. 2008; Marsell et al. 2011).

In mice the acute inflammation stage peaks within the first 24 hours after injury (Cho et al. 2002) and involves the secretion of a range of pro-inflammatory cytokines including tumour necrosis factor- α (TNF- α). Bleeding into the fracture site leads to the formation of a haematoma between and around the fracture ends. Degranulating platelets, macrophages and other inflammatory cells infiltrate the haematoma and secrete cytokines and growth factors (Schindeler et al. 2008; Marsell et al. 2011), leading to the recruitment of various cell types into the fracture site including MSCs (Cho et al. 2002; Gerstenfeld et al. 2003; Marsell et al. 2011). Stromal derived factor 1 (SDF-1) signalling via its receptor CXCR4 had been shown to be a critical mediator of MSC migration into fracture sites. Kitaori *et al* showed that SDF-1 increases MSC chemotaxis in a dose dependent manner, it is highly expressed in periosteum and that disruption of SDF-1/CXCR4 signalling resulted in impaired bone formation in a mouse model (Kitaori et al. 2009).

Following the formation of the primary haematoma, MSC differentiate into chondrocytes and their subsequent expansion is responsible for the formation of a soft callus. This typically forms 7-9 days following injury and serves to provide mechanical support as well as a template for the bony callus that will supersede it (Schindeler et al. 2008; Marsell et al. 2011). A hard bony callus is then formed, composed of woven bone (Schindeler et al. 2008; Marsell et al. 2011). This then undergoes remodelling to form laminar bone.

Sometimes fracture healing fails. The US Federal Drug Administration (FDA) council, defines a fracture non-union as a fracture which has not achieved bone union after nine months since the initial injury, and for which a minimum of three months has elapsed with no sign of healing (Fayaz et al. 2011). Several factors are known to be associated with possibility of a fracture progressing to non-union including loss of bone at the fracture site (bone defect), inadequate mechanical stability, extensive soft tissue damage, open fractures, the administration of non-steroidal anti-inflammatory drugs and smoking (Giannoudis et al. 2000). Recently, single nucleotide polymorphisms in noggin, an inhibitor of bone morphogenic proteins (BMP) signalling and SMAD-6, have been shown contribute a genetic component to the development non-union (Dimitriou et al. 2011a).

Radiological observation is commonly used to classify aseptic non-unions into two distinct types. These are hypertrophic, characterised by hyper-vascularisation of the fracture site or atrophic, characterised by poor vascularisation of the fracture site (Megas 2005). The former is often associated with inadequate stabilisation of the fracture site and can be addressed by revision surgery to improve stability (Megas 2005). Whereas the latter is associated with an inadequate healing response (Megas 2005) and is therefore more difficult to treat.

1.5.2.1. MSC therapy for fracture repair

Healey *et al* used percutaneous injection of autologous BM aspirates into the site of failed healing in eight patients treated for primary sarcomas by *en bloc* resection. Union was achieved in five patients, although new bone formation was observed in seven (Healey et al. 1990). These encouraging results were attributed to the presence of “osteoprogenitor” cells (a term used in lieu of MSCs reflecting the uncertainty surrounding MSC identity at the time) within the BM. However, a direct link between the presence of MSCs in BM aspirates and the efficacy of this method of treatment was not proven.

This question was addressed by Hernigou *et al* who demonstrated that the absolute number and concentration of MSCs in a concentrated and injected BM aspirate had a direct impact on the probability of achieving union (Hernigou et al. 2005a). Patients receiving concentrated BM containing 2835 ± 1160 (mean \pm SD) MSCs/ml or a total of 54962 ± 17431 (mean \pm SD) MSCs following percutaneous injection into atrophic non-unions went on to achieve union, whereas patients receiving lower doses of MSCs did not.

It must be noted however, that Hernigou *et al* could not rule out the possibility that other cell types concentrated alongside MSCs were responsible for this effect, nor could the engraftment of injected MSCs into the fracture site be demonstrated. Indeed, it has been established that MSCs are potent inducers of vascularisation (Sato et al. 2011) and that this can be independent of engraftment to the site of action (Preda et al. 2014). Nevertheless this was an important study since it

demonstrated the efficacy of minimally manipulated MSC for fracture repair in the clinical setting. Moreover, it led to the development of several BM concentrator devices that are now actively used in clinical orthopaedics, for treatment of a range of conditions including fracture non-union (Giannoudis et al. 2013), avascular necrosis (Aarvold et al. 2013), chondral defects (Gigante et al. 2012) and critical limb ischemia (Murphy et al. 2011).

One important limitation to the use of BM concentration for fracture repair is the lack of prospective evaluation of the MSC dose delivered to patients. Hernigou *et al* relied on *ex vivo* expansion of individual colonies to determine MSC content, therefore only retrospective analysis was possible (Hernigou et al. 2005a). This meant that the quality of aspirates at the point of use was unknown leading to a proportion of patients receiving far fewer MSCs and failing to achieve union. Methods such as volumetric flow cytometry may offer a means of rapidly evaluating MSC content in BM aspirate. To be a realistic option for use as an intraoperative quality control, it should take no longer than 1 hour to return a result.

1.5.2.2. BMP therapy for fracture repair

BMP-2 is a powerful inducer of bone formation and has been shown to irreversibly induce *in vivo* bone formation after only transient short term expression (Noel et al. 2004). Several clinical studies have demonstrated the efficacy of BMP therapy in accelerating fracture healing and bone regeneration (Govender et al. 2002; McKay et al. 2007; Ristiniemi et al. 2007; Kanakaris et al. 2008). rhBMP-2 was approved by the FDA in 2004 (McKay et al. 2007), this followed a successful clinical trial by Govender *et al* who evaluated the effects of BMP-2 for the treatment of open tibial fracture in 450 patients (Govender et al. 2002). Implantation of 1.5mg of BMP-2 resulted in a 44% reduction in need for secondary intervention due to delayed union and a significantly faster average healing time compared to the standard of care treatment (Govender et al. 2002). BMP-7 has been evaluated for tibial fracture repair with significantly shorter mean time to union reported (Ristiniemi et al. 2007) and BMP-7 therapy with revision of tibial non-union in 67 patients had a reported

success rate of 89.7% (Kanakaris et al. 2008). This rate of success is comparable to the gold standard of care (autologous bone graft) but avoids the associated complications such as donor site morbidity (Kanakaris et al. 2008).

However, while BMP therapy has emerged as a promising addition or alternative to autologous bone grafting, the doses used clinically are significantly higher than physiological concentrations of BMPs (Gautschi et al. 2007). Additionally, several adverse effects associated with BMP therapy have been reported including implant displacement, ectopic bone formation and neurological effects (Carragee et al. 2011). There is also growing concern that BMP-2 therapy may be associated with an increased risk of cancer, although studies have so far have returned inconclusive or conflicting results (Cooper et al. 2013; Fu et al. 2013; Simmonds et al. 2013) This has led several groups to develop novel delivery systems for BMP delivery aimed at providing a more localised, sustained and physiological dose of BMP for bone regeneration (Ben-David et al. 2013; Fan et al. 2014; Shekaran et al. 2014).

In the most severe cases a combination of therapies may be required. The diamond concept of fracture healing, a term coined by Giannoudis *et al*, sets out the four elements that are essential for maximum osteogenic effect (Giannoudis et al. 2007). This approach combines the utilisation of optimal mechanical stability, osteoconductive scaffolds, growth factors such as BMPs and MSCs. (Giannoudis et al. 2007). However, if the MSC element of the diamond takes the form of BM aspirate it is subject to all the before mentioned issues pertaining to unknown and uncontrolled MSC dose. The Diamond concept of fracture healing would be similarly improved by prospective MSC enumeration.

1.5.3. Regulatory networks and osteogenesis

1.5.3.1. The WNT signalling pathway

The WNT family proteins are highly conserved secreted glycoproteins that play essential and diverse roles in development and homeostasis (Baron et al. 2013; Fakhry et al. 2013). Loss of function mutations in the gene encoding for the WNT

co-receptor low density lipoprotein-related protein-5 (LRP5), were found in patients with low bone mass and osteoporosis-pseudoglioma syndrome (Gong et al. 2001). Mutations in the same gene, resulting in gain of function, cause abnormally high bone mass in otherwise healthy patients (Boyden et al. 2002; Little et al. 2002). These findings generated a great deal of interest in WNT signalling and its involvement in bone development and homeostasis.

It has since become clear that the canonical or β -catenin WNT pathway plays a major role in skeletal biology (Baron et al. 2013). β -catenin is the key molecule involved in signal transduction. In the absence of signal, cytoplasmic β -catenin is bound by the multi-protein destruction complex, facilitating proteasomal destruction of β -catenin via sequential phosphorylation and ubiquitination (Clevers 2006; Cadigan et al. 2009; Baron et al. 2013). WNT molecules initiate signalling by binding to a dual receptor complex comprised of the frizzled protein, a seven-pass transmembrane receptor, and either LRP5 or LRP6. This triggers phosphorylation of the cytoplasmic tail of LRP5/6 and binding to Axin, a key constituent protein of the destruction complex (Clevers 2006; Cadigan et al. 2009; Baron et al. 2013). This has the effect of inactivating the destruction complex leading to accumulation of β -catenin and its translocation to the nucleus, where it activates WNT target gene expression (Clevers 2006; Cadigan et al. 2009; Baron et al. 2013).

In general, canonical WNT signalling enhances MSC osteoblastic differentiation (Day et al. 2005; Hu et al. 2005; Bennett et al. 2007) and promotes chondrocyte hypertrophy (Day et al. 2005), whilst inhibiting adipogenesis (Kennell et al. 2005; Kawai et al. 2007; Laudes 2011). Sclerostin and dickkopf-1 (DKK1) are soluble inhibitors of WNT signal transduction, (Bafico et al. 2001; Poole et al. 2005) both inhibit WNT signalling by binding to the LRP5/6 receptor and promoting internalisation (Bafico et al. 2001; Moester et al. 2010). Mutations resulting in a lack of sclerostin were shown to be responsible for the high bone mass observed in Van Buchem disease and sclerosteosis (Balemans et al. 2001; Brunkow et al. 2001). Whereas changes in DKK1 expression have been implicated in the development of fracture non-union (Bajada et al. 2009) and ankylosing spondylitis (Uderhardt et al. 2010).

1.5.3.2. The TGF- β /BMP signalling pathway

The transforming growth factor-beta (TGF- β) superfamily is a diverse group of signalling molecules which control a plethora of cellular responses. TGF- β family cytokines are comprised of over forty members including TGF- β s, Activin and BMPs (Shi et al. 2003). In the canonical signalling pathway, TGF- β cytokines bind to specific type I and type II serine/threonine kinase receptors resulting in the formation of heteromeric complexes of type I and type II receptors and phosphorylation of R-SMAD proteins (Shi et al. 2003; Chen et al. 2012). Phosphorylated R-SMADs form a complex with co-SMADs and SMAD4 which then translocates to the nucleus and brings about expression of target genes (Shi et al. 2003; Chen et al. 2012). TGF- β signalling may also occur through alternative or non-canonical pathway that involves complex interactions between TGF- β receptors and the mitogen activated protein kinase (MAPK), and the phosphoinositide 3-kinase (PI3K) signalling cascades (Moustakas et al. 2009; Massague 2012).

TGF- β 1 increases bone formation by stimulating the proliferation and chemotaxis of osteoblast progenitors and stimulating the production of bone matrix (Janssens et al. 2005). However, TGF- β 1 has been shown to block the late phases of osteoblast differentiation and inhibits mineralisation (Maeda et al. 2004). These later phases are regulated by BMPs. Mice which lack the capacity to produce BMP-2 in their limb bones spontaneously develop fractures that do not heal with time (Tsuji et al. 2006), indicating that BMP-2 is a necessary component of the signalling cascade that governs fracture repair, although BMP-4, 5,6 and 7 all have the ability to induce osteogenesis (Chen et al. 2012).

1.5.3.3. The JAK/STAT signalling pathway

The Janus kinase/signal transducers and activators of transcription (JAK/STAT) signalling pathway is responsible for mediating responses to cytokines and growth factors and is involved in a wide variety of cellular responses (Harrison 2012; Kiu et al. 2012; Stark et al. 2012). Binding of extracellular ligand to the cognate

transmembrane receptors activates JAKs via auto or trans-phosphorylation, leading to phosphorylation of cytoplasmic STAT tyrosine residues (Harrison 2012; Kiu et al. 2012). STAT phosphorylation leads to dimerization followed by translocation to the nucleus, where STATs bind specific sequences and initiate transcription of target genes (Kiu et al. 2012).

In mammals there are a total of four JAKs, JAK1-3 and TYK2 and 7 STATs, STAT1-4, STAT5A, STAT5B and STAT6 (Kiu et al. 2012; Stark et al. 2012). Rodig *et al* showed that JAK1 is required for signalling by cytokines that utilise class II cytokine receptors and those which utilise cytokine receptors that depend on either the common gamma (γ_c) or gp130 subunits (Rodig et al. 1998). Therefore, JAK1 has an indispensable role in mediating biologic responses to interferon (IFN) α/β , IFN γ , Interleukin (IL) 10 (type II receptors), IL-2, IL-4, IL-7, IL-9, IL-15, IL-21 (γ_c subunit), IL-6, IL11, Leukaemia inhibitory factor, Oncostatin M, ciliary neurotrophic factor and cardiotrophin-1 (gp130 subunit) (Rodig et al. 1998; Habib et al. 2002). JAK2 deletion is embryonically lethal due to the absence of definitive erythropoiesis and analysis of JAK2 deficient cells showed that JAK2 has a critical, non-redundant role in mediating responses to IFN γ , thrombopoietin, IL-3, IL-5 and granulocyte/macrophage stimulating factor (Parganas et al. 1998). Signalling via the γ_c subunit is also dependent on JAK3, in humans mutations in the γ_c result in X-linked severe combined immunodeficiency syndrome (Suzuki et al. 2000; Habib et al. 2002). Tyk2 plays a restricted role in IFN α signalling and is required in mediating IL-12 dependent biological response (Shimoda et al. 2000).

The effect of JAK/STAT signalling on MSCs and their differentiated progeny is not well understood. There is substantial evidence for JAK/STAT involvement in adipogenesis (Rosen et al. 2006b; Zhang et al. 2011; Shi et al. 2014); by comparison little is known regarding JAK/STAT activity in MSC proliferation or differentiation into bone and cartilage. However, it has been shown that IL-6 type cytokines, those signalling through the gp130 subunit, are able to stimulate alkaline phosphatase (ALP) and osteocalcin, and promote osteoblast differentiation (Bellido et al. 1997). Additionally, gp-130 null mice died at or before birth with multiple skeletal abnormalities and the number of osteoblasts present in bone was significantly

reduced (Shin et al. 2004). In humans STAT3 mutation is responsible for hyper-immunoglobulin E syndrome, a rare immunodeficiency disorder also known as Job syndrome (Holland et al. 2007; Minegishi et al. 2007). Patients commonly suffer from recurrent fractures, hyper extendable joints and scoliosis (Grimbacher et al. 1999; Grimbacher et al. 2005). Growth hormone (GH) has a well-recognised influence on bone growth, and relies on JAK/STAT signalling for many of its downstream effects (Wang et al. 2004; Hadjidakis et al. 2006; DiGirolamo et al. 2007; Giustina et al. 2008). In BM GH inhibits MSC adipogenesis in favour of osteogenesis and chondrogenesis (Gevers et al. 2002) and also inhibits osteoblast apoptosis (DiGirolamo et al. 2007). Collectively, this suggests an important role for JAK/STAT signalling in osteogenesis, therefore inhibition of this pathway could potentially lead to reduced osteogenesis and/or chondrogenesis.

1.5.3.4. Nuclear receptor signalling

The nuclear receptor superfamily of ligand dependent transcription factors regulate diverse aspects of development and homeostasis, by both positive and negative regulation of gene expression (Glass et al. 2010). Classical steroid hormone receptors comprise the most well characterised subfamily of nuclear receptors and mediate most of the biological effects of mineralocorticoids, oestrogens, progestins and androgens (Glass et al. 2010). They also mediate the effects of glucocorticoids, retinoic acid and vitamin D (Glass et al. 2010).

Glucocorticoid signalling, mediated by the glucocorticoid receptor is of particular importance in osteogenesis (Eijken et al. 2006). The artificial glucocorticoid derivative dexamethasone (DEX) is a common component in *in vitro* MSC differentiation assays (Pittenger et al. 1999; Jones et al. 2010b; Kouroupis et al. 2013). Glucocorticoid signalling stimulates the expression of genes involved in bone formation including Runt-related transcription factor 2 (RUNX2), ALP and osteopontin whilst inhibiting the expression of genes that negatively regulate bone formation (Eijken et al. 2006). Glucocorticoid signalling has also been shown to stimulate osteogenesis by modulating the phosphorylation state of a regulatory

serine residue on RUNX2, in rat primary fibroblasts (Phillips et al. 2006). However, long term and/or high dose glucocorticoid administration, usually for the treatment of oncological disorders, is associated with a range of negative side effect including osteoporosis (Yao et al. 2008; Biddie et al. 2012). This effect was addressed by Yao et al, who showed that in mice glucocorticoid excess drove increased osteoclastogenesis and adipogenesis (Yao et al. 2008).

1,25-dihydroxyvitamin D₃ (1,25(OH)₂D₃) the biologically active metabolite of vitamin D, has been claimed to influence osteogenesis both directly and indirectly. In the intestine vitamin D acts as an essential mediator of calcium homeostasis by stimulating calcium and phosphate absorption (Holick et al. 2011). Additionally, 1,25(OH)₂D₃ has been reported to stimulate osteogenic differentiation *in vitro* and synergise DEX driven osteogenesis, by stimulating expression of genes involved in differentiation and cell cycle regulation (Piek et al. 2010). However, these findings are not supported in animal studies, Tanaka *et al* transplanted bone from vitamin D receptor knockout mice into a wild type mouse, this led to excessive bone formation and mineralisation in the knockout tissue (Tanaka et al. 2004), suggesting that vitamin D is a negative regulator of bone formation *in vivo*. Additionally 1,25(OH)₂D₃ is a powerful inducer of RANKL and strongly promotes osteoclast formation (Suda et al. 2012). Nevertheless 1,25(OH)₂D₃ has been used as a therapeutic agent for the treatment of osteoporosis since 1983 and has been shown to increase bone mineral density and reduce the incidence of bone fracture *in vivo*. (Suda et al. 2012).

Retinoic acid is a small lipophilic molecule derived from retinoids, of which retinol (vitamin A) is the main circulating form in mammals (Rochette-Egly et al. 2009; Rhinn et al. 2012). Retinoid acid signalling is involved in a diverse range of biological processes including limb patterning, brain development and differentiation (Rhinn et al. 2012). Retinoids and retinoic acid can act as powerful teratogens and exposure has been associated with numerous craniofacial defects (Morriss-Kay 1993; Vieux-Rochas et al. 2007). Compound null mutations of the genes encoding retinoid acid receptors (RARs) α and γ in mice resulted in severe craniofacial and

skeletal abnormalities (Lohnes et al. 1994), highlighting the importance of retinoic acid signalling in skeletal development.

The role of retinoic acid in regulating MSC differentiation is not well understood. Early experiments showed that treatment of a mouse mesenchymal cell line with retinoic acid induced expression of ALP and modulated expression TGF- β family proteins (Gazit et al. 1993). Later James et al showed that retinoic acid enhanced osteogenesis in mouse cranial suture derived MSCs (James et al. 2010), suggesting one potential mechanism driving retinoic acid induced craniofacial malformation. Additionally, several studies have suggested a possible synergy between retinoic acid and BMP signalling in potentiating osteogenesis (Cowan et al. 2005; Wan et al. 2006; Zhang et al. 2010), although the precise mechanism underlying this effect remains to be elucidated. Nevertheless this potential synergy has led some to suggest combined retinoic acid and BMP therapy for the treatment of large bone defects (Cowan et al. 2005; Zhang et al. 2010). It must be noted however, that other studies have reported inhibitory effects of retinoic acid on osteogenic induction (Hoffman et al. 2006; Wang et al. 2008). Moreover, in a mouse model of heterotopic bone formation, treatment with a RAR γ agonist almost totally blocked bone formation (Shimono et al. 2011). However, in this model bone is formed by endochondral ossification and it is likely that inhibition of the preceding chondrogenesis rather than inhibition of osteoblast differentiation or activity is the primary mode of action (Kaplan et al. 2011).

1.5.3.5. Transcriptional regulation of bone formation

BMP-2 and TGF- β 1 both stimulate the expression of RUNX2 in a pluripotent mesenchymal precursor cell line (Lee et al. 2000). Targeted disruption of RUNX2 in mice results in total blockage of intramembranous and endochondral ossification owing to arrest of osteoblast maturation (Komori et al. 1997). RUNX2 controls the expression of genes expressed specifically in osteoblasts such as osteocalcin, collagen type I alpha 1, bone sialoprotein and osteopontin through direct binding to the osteoblast-specific cis-acting elements (Ducy et al. 1997). Overexpression of

RUNX2 by adenoviral transfection of adipose derived MSCs resulted in rapid osteoblast differentiation, mineral deposition and inhibition of adipogenesis *in vitro* (Zhang et al. 2006).

Osterix (OSX), also known as Sp7, is a mediator of osteogenesis which acts downstream of RUNX2. OSX null mice develop a cartilage matrix but do not develop bone despite the expression of RUNX2 in pre-osteoblasts (Nakashima et al. 2002). In these mice neither endochondral nor intramembranous ossification occurs despite the invasion of blood vessels into the zone of hypertrophic chondrocytes, suggesting the OSX expression is required for the final stages of osteoblast differentiation.

1.6. The capacity of MSCs to stimulate endogenous bone repair mechanisms

1.6.1. Stimulation of vascularisation

A growing body of evidence suggests that MSCs are able to exert regenerative effects that are independent of differentiation. Recently animal studies have demonstrated that MSCs are able to stimulate angiogenesis (Zhou et al. 2010; Martinez et al. 2013; Kai et al. 2014; Kang et al. 2014). Because of this, MSCs have been investigated as therapeutic agents for the treatment of ischemic heart injury, Hare *et al*, demonstrated that following acute myocardial infarction, patients administered with expanded MSCs showed an improvement in heart function (Hare et al. 2009). Further studies in a porcine model suggested that the favourable effects of MSC infusion are achieved by enhanced neovascularisation as a result of secretion of angiogenic factors such as vascular endothelial growth factor (VEGF) (Sato et al. 2011). Moreover, subcutaneous transplantation of adipose derived MSCs can protect against ischemic injury without any extensive migration from the injection site (Preda et al. 2014).

Several groups are now experimenting with technologies aimed at retaining MSCs at the site of ischemic injury. MSCs seeded onto a variety of materials and applied

as a cardiac patch have shown impressive efficacy in animal models (Zhou et al. 2010; Martinez et al. 2013; Kai et al. 2014; Kang et al. 2014). This suggests that prolonged retention of MSCs, close to the site of injury, augments MSC mediated cardiac repair by increasing the local concentration of angiogenic factors.

The ability of MSCs to stimulate angiogenesis is also of interest in bone disease therapy. Neovascularisation is well recognised as a crucial component to successful fracture healing (Megas 2005). Therefore, in this setting MSCs may provide a dual benefit by stimulating vascularisation and contributing to new bone formation.

1.6.2. Recruitment of cells to sites of tissue damage

The repair of cartilage defects are another area to which MSC therapy is seen to hold promise. In 2002 Wakitani *et al* administered BM derived MSCs to cartilage defects in patients with knee osteoarthritis. Although no significant clinical improvement was observed the authors reported an improvement in arthroscopic and histological grading score (Wakitani et al. 2002). More recently studies in rabbit (Dashtdar et al. 2011) and pig (Nakamura et al. 2012) models have shown significant but incomplete cartilage regeneration upon administration of expanded MSCs. Interestingly, a proof of concept study showed that after excision of proximal humeral condyles in rabbits, implantation of a TGF- β 3 infused scaffold was able to almost totally recapitulate the mechanical properties of native cartilage at the articular surface (Lee et al. 2010). This was done without the addition of any cellular component (Lee et al. 2010). The impressive level of bone and cartilage regeneration seen suggests that stimulating endogenous cells, rather than cell delivery, may be a more successful strategy in treating cartilage defects. A similar strategy is employed with the use of osteoconductive scaffold for the treatment of bone defects (Guda et al. 2013), although in both these cases the exact mechanisms governing MSC recruitment remain unknown.

MSCs are able to stimulate the migration of other MSCs as well as non-MSC cell types (Ponte et al. 2007; Miller et al. 2008). The chemokine SDF-1 has been identified as an important mediator of this property (Ponte et al. 2007; Miller et al.

2008). It is secreted in large amounts by immature osteoblasts and MSCs as well as other cell types including endothelial cells (Ponomaryov et al. 2000; Ma et al. 2011) and expression was found to be increased in the tissue surrounding damaged bone in a murine fracture model (Liu et al. 2013). Human MSCs were also shown to migrate towards an SDF-1 gradient in a dose dependent manner *in vitro* (Ryu et al. 2010). This suggests that SDF-1 release and SDF-1 driven MSC migration plays an important role in recruitment of additional MSCs to the site of healing.

1.6.3. Osteogenesis imperfecta

Perhaps one unintended demonstration of the capacity of MSCs to exert regenerative effects that are independent of differentiation may be seen by attempts to use MSCs for the treatment of osteogenesis imperfecta. Osteogenesis imperfecta (OI) is a genetic disorder most commonly caused by mutations in one of two genes encoding collagen type 1 (Rauch et al. 2004). It is characterised by increased bone fragility, low bone mass and other connective tissue manifestations (Rauch et al. 2004). Horwitz *et al* proposed MSC therapy as a potential treatment for OI. It was hoped that donor MSCs would engraft, expand and differentiate to form normal bone. Horwitz treated six children with severe OI with culture expanded BM derived allogeneic MSCs. Five patients showed accelerated growth velocity during the first six months post infusion but only one patient showed a substantial increase in total body mineral content (Horwitz et al. 2002). However, the observed accelerated growth was not sustained long term leading to the realisation that donor cells had limited regenerative capacity following transplantation (Dominici et al. 2008). Later experiments in murine models of OI suggested that transplanted MSCs do not substantially engraft in bone but rather exert their positive effects via soluble mediators that indirectly stimulate growth (Otsuru et al. 2012).

1.7. Immunomodulation

The immunosuppressive effects of MSCs have been well documented, they have been shown to be capable of suppressing T and B cell proliferation in a dose dependent manner, this is largely mediated by indoleamine 2,3-dioxygenase (Ren et al. 2009; Galipeau 2013). At the same time they do not express major histocompatibility (MHC) class II antigens (Le Blanc et al. 2003). Allogeneic MSCs escape recognition by alloreactive T cells and natural killer cells and do not stimulate a T-cell mediated response (Di Nicola et al. 2002; Sotiropoulou et al. 2006). These properties of MSCs are important, as they allow allogeneic MSCs to be safely administered to patients with intact immune systems (Raynaud et al. 2013).

The immunomodulatory effects of MSCs have led to interest in their use for a range of immunological conditions including graft versus host disease, multiple sclerosis and rheumatoid arthritis (Table 1). This property of MSCs may also prove useful in bone disease therapy development, particularly when considered in the light of their ability to stimulate endogenous repair mechanisms. Allogeneic MSCs may be implanted without eliciting an immune response and contribute to new bone formation by releasing chemokines and pro-angiogenic factors.

1.8. MSC as causative agents of bone disease

1.8.1. Osteoarthritis

The causes of primary osteoarthritis (OA) are unknown, but it rarely occurs in people below 40 years old. It is characterised by hyaline cartilage fibrillation and loss, synovial inflammation, muscle weakness, ligament laxity, increased density of the subchondral bone and the development of bony osteophytes (Bailey et al. 1997; Felson et al. 2000; Peat et al. 2001). The involvement of MSCs in OA is poorly understood, but the ability of MSCs isolated from OA patients to undergo chondrogenic differentiation has been shown to be reduced (Murphy et al. 2002). Our laboratory showed that MSCs are present in normal synovial fluid and more numerous in the synovial fluid of patients with early OA (Jones et al. 2008). There is

also a large body of evidence that validates an inverse relationship between OA of the hip and osteoporosis (OP) (Dequeker et al. 1996; Dequeker et al. 2003; Hopwood et al. 2007; Kumarasinghe et al. 2011). WNT signalling has been implicated as a possible mediator of this observation. Velasco *et al* showed differential expression of several WNT related genes in bone samples from OA patients and patients with hip fractures (Velasco et al. 2010). The apparent increase in WNT signalling observed in OA patients may be responsible for both the cartilage catabolism and degradation due to increased matrix metalloproteinase expression, and the formation of osteophytes as well as increased bone mineral density.

A range of molecules from the TGF- β /BMP signalling pathway have been shown to be differentially expressed in OA as compared to healthy controls (Hopwood et al. 2007; Sanchez-Sabate et al. 2009) Recently Zhen *et al* showed that inhibition of TGF- β signalling in MSCs, residing in the subchondral bone, attenuates the progression of OA. Conversely transgenic expression of active TGF- β in osteoblastic cells induced osteoarthritis (Zhen et al. 2013). This implicates enhanced mineralisation, driven by MSCs, as an important event in OA progression, and builds on much earlier studies of OA which indicate that changes in subchondral bone precede cartilage fibrillation (Carlson et al. 1994; Bailey et al. 1997). All these observations taken together make a compelling case for the involvement of MSC osteogenic differentiation in the development and progression of OA.

1.8.2. Ankylosing spondylitis

Spondyloarthritis is a group of heterogeneous diseases which include ankylosing spondylitis (AS) and psoriatic arthritis that share hereditary clinical and pathogenic features, the most frequent of which are sacroiliitis, enthesitis, iritis, oligoarthritis, psoriasis and inflammatory bowel disease. In common with other chronic inflammatory diseases AS affects bone, new aberrant bone formation in the form of syndesmophytes, is seen at the enthesis (Ball 1983). This particularly effects the vertebra of the spine eventually leading to fusion and progressive disability (Braem et al. 2012).

Conversely, AS is associated with the development of features associated with increased bone catabolism such as erosions, generalised osteoporosis and vertebral fractures (Uderhardt et al. 2010; Montala et al. 2011; Klingberg et al. 2012). One of the key mediators of joint remodelling in inflammatory disease is DKK-1. Diarra *et al* showed that in an inflammatory environment DKK-1 blockade stimulates bone formation in the joint both by inhibiting osteoclast formation and simultaneously stimulating osteophyte formation driven by enhanced WNT signalling (Diarra et al. 2007). Blockade of DKK-1 induces fusion of sacroiliac joints in a transgenic mouse that develops bilateral sacroiliitis (Uderhardt et al. 2010). These studies suggest that an increase in osteogenesis, driven by enhanced WNT signalling, may be the underlying cause of aberrant bone formation. However, WNT independent mechanisms influencing MSC differentiation into osteoblasts cannot be ruled out.

Current treatments for AS such as TNF blockade only address the inflammatory element of the disease. Although TNF blockade is very effective in reducing the pain associated with chronic inflammation of the joints, it does not alter the radiographic progression of the disease (van der Heijde et al. 2008a; van der Heijde et al. 2008b). New treatment are required to address the ongoing inappropriate bone formation associated with AS.

1.9. Defining and identifying MSCs

1.9.1. Current perspectives in defining MSCs

A minimum criteria for the definition of MSC was set out in the International Society for Cellular Therapy 2006 position statement (Dominici et al. 2006). MSC are defined as adherent cells that can be maintained in culture and have *in vitro* tri-lineage differentiation capacity (osteogenesis, adipogenesis and chondrogenesis). Cultured cells exhibit positive co-expression of three cell surface antigens: CD105, CD73 and CD90 and negative expression of CD45, CD34, CD14 or CD11b, CD79 α or CD19 and HLA-DR. Although widely recognised and cited these criteria have drawn criticism due to their reliance on artificial conditions and stimuli, and have been shown to poorly reflect the nature of MSCs *in vivo* (Jones et al. 2002; Boxall et al.

2012; Bianco et al. 2013b). One example of this can be taken from our own laboratory, Jones *et al* immunomagnetically selected BM cells based on expression of the D7-FIB antigen. This enriched a population of cells that contained 100% of the colony forming capacity of BM but strongly and consistently expressed HLA-DR (Jones et al. 2002). Some have suggested that the ISCT criteria are simply widely shared properties of connective tissue cells (Bianco et al. 2013b). Observations that MSCs derived from diverse tissues exhibit varying phenotypic characteristics particularly with respect to multi-lineage differentiation capacity (De Ugarte et al. 2003; Baksh et al. 2007; Hayashi et al. 2008), implies some degree of lineage commitment. This has strengthened the notion that MSCs identified by ISCT criteria are more properly referred to as mesenchymal *stromal* cells rather than mesenchymal *stem* cells.

Initial attempts to identify MSCs, based on the expression of cell surface markers, relied on the expression of a host of antigens including CD29, CD44, CD73, CD90 and CD105 (Barry et al. 1999; Pittenger et al. 1999; Deans et al. 2000; Barry et al. 2001; Goodwin et al. 2001). However, these antigens are expressed on a broad range of cell types. For example, CD29 can be expressed on T-cells (Kurachi et al. 2014) as can CD44 (Kurachi et al. 2014), whilst CD73, CD90 and CD105 are all expressed by fibroblasts (Haniffa et al. 2009; Holdsworth-Carson et al. 2014). The monoclonal IgM antibody STRO-1 appears to offer better specificity for MSCs but also cross reacts with erythroblasts (Simmons et al. 1991). Additionally, the target antigen for Stro-1 has yet to be identified despite a more than two decades since its original description (Simmons et al. 1991).

Quirici *et al* first suggested the expression of CD271 on BM MSCs (Quirici et al. 2002). This was Independently confirmed and refined by Jones *et al* by inclusion of CD45 as a negative gating parameter in FACS analysis (Jones et al. 2002). The CD45^{low/-} CD271⁺ population exhibits all the classical characteristics of MSCs, including the uniform expression of other MSC markers such as CD73, CD105 and CD90 (Jones et al. 2002; Boxall et al. 2012). The high level of CD271 expression in BM MSC and the low level of expression of CD271 in all other cell populations (Jones et al. 2002; Jones et al. 2004b), makes CD271 expression a potentially

exploitable characteristic, for antibody based enrichment technology. Further supporting evidence in the utility use of CD271 as a BM MSC marker has been confirmed by several independent studies (Quirici et al. 2002; Jarocha et al. 2006; Buhning et al. 2007).

The CD45⁻ CD146⁺ population in BM transplanted by Sacchetti *et al* and found to have self-renewal potential (Sacchetti et al. 2007), also express CD271 (Tormin et al. 2011). Tormin *et al* showed that colony forming units are present in CD45⁻ CD271⁺ CD146⁻ cells as well as CD45⁻ CD271⁺ CD146⁺ cells and that these population share a similar phenotype (Tormin et al. 2011). CD146 expression on BM MSCs is dependent on localisation specifically their proximity to blood vessels and is up or down regulated in response to normoxia or hypoxia, respectively (Tormin et al. 2011). Table 2 shows positive markers used for identification of BM MSCs.

Table 2: Cell surface markers used to identify MSCs

This table lists positive markers used to identify human MSCs *in vivo*.

Marker	Other names	Function	Reference
D7-FIB	Fibroblast antigen	Unknown	(Jones et al. 2002)
MSCA-1	TNAP	Phosphatase ectoenzyme	(Battula et al. 2009)
STRO-1		Unknown	(Simmons et al. 1991)
SSEA-4	Stage specific antigen 4	Regulation of differentiation	(Battula et al. 2007)
CD10	Neprilysin	Metalloprotease	(Campioni et al. 2003)
CD13	Alanine aminopeptidase	Ectoenzyme	(Campioni et al. 2003)
CD44	H-CAM hyaluronin receptor	Cell adhesion	(Goodwin et al. 2001)
CD56	NCAM	Cell adhesion	(Battula et al. 2009)
CD73	5' nucleotidase	Ectoenzyme, catalysis of purine 5-prime mononucleotides to nucleosides	(Barry et al. 2001)
CD90	Thy-1	Cell adhesion, highly expressed in sites of inflammation	(Jones et al. 2002)
CD105	Endoglin	Receptor for TGF- β I and III	(Barry et al. 1999)
CD106	VCAM-1 INCAM-100	Cell adhesion	(Simmons et al. 1992)
CD146	MUC-18, Mel-CAM, MCAM	Cell adhesion	(Sacchetti et al. 2007)
CD166	ALCAM	Cell adhesion	(Stewart et al. 2003)
CD200	OX-2	Regulation of myeloid cells	(Delorme et al. 2008)
CD271	LNGFR, p75, p75NTR	Neurotrophin receptor	(Jones et al. 2002)

1.9.2. Self-renewal

Self-renewal is widely recognised as one of the key properties of a true stem cell. The ability of stem cells to generate copies of themselves but also give rise to more differentiated progeny is known as asymmetric division (Knoblich 2008; Gomez-Lopez et al. 2014). Sacchetti *et al* demonstrated the presence of BM resident self-renewing sub-endothelial cells in humans (Sacchetti et al. 2007). They were selected from BM based on positive expression of CD146 and negative expression of CD45 and were able to re-capitulate their initial microenvironment after transplantation into an immunodeficient mouse; resulting in the generation of a miniature bone organ (Sacchetti et al. 2007). Importantly, cells expressing the CD45⁻CD146⁺ phenotype were regenerated after transplantation demonstrating asymmetric cell division (Sacchetti et al. 2007). A similar study showed that mouse Nestin⁺ perivascular stromal cells were capable of self-renewal as demonstrated by serial transplantation (Mendez-Ferrer et al. 2010). These cells also expressed high levels of genes that regulate haematopoietic stem cell (HSC) maintenance and retention in the BM in mice (Mendez-Ferrer et al. 2007).

These experiments demonstrated that cells expressing a defined range of cell surface markers are capable of giving rise to terminally differentiated cells and thereby re-constituting the BM microenvironment whilst maintaining their capacity for self-renewal. However, some argue that cells isolated based on expression of the CD45⁻CD146⁺ phenotype represent an enriched population rather than a single cell type (Pittenger 2013).

1.9.3. Maintenance of the haematopoietic niche

Another key role of BM MSCs is maintenance of the haematopoietic niche (Arai et al. 2004; Mendez-Ferrer et al. 2007; Mendez-Ferrer et al. 2010). The CD45⁻CD146⁺ cells isolated by Sacchetti et al were significant producers of angiopoietin-1 a ligand of the tyrosine kinase receptor 2. Tyrosine kinase receptor 2 is expressed specifically on endothelial cells and HSCs (Davis et al. 1996; Hsu et al. 2000) and acts

as a key component of in maintenance of the HSC niche by maintaining HSC quiescence (Arai et al. 2004).

In mouse studies, MSCs co-localise with HSCs in the BM and express genes that regulate HSC maintenance including SDF-1, c-kit ligand, angiopoietin-1, interleukin-7, vascular cell adhesion molecule-1 and osteopontin (Mendez-Ferrer et al. 2010). In addition, selective depletion of MSCs reduces bone marrow homing of HSCs (Mendez-Ferrer et al. 2010). However, it must be noted, that mice and human MSCs can be substantially different (Pittenger 2013), both in terms of surface epitope expression, requirements for optimal growth and rates of propagation *in vitro* (Peister et al. 2004). Although the study of mouse MSC is a useful tool in understanding MSC biology, caution should be exercised when applying findings in mice MSCs to human MSC biology.

1.9.4. *In vitro* differentiation

In vitro osteogenic MSC differentiation can be achieved by the addition of ascorbic acid 2-phosphate, β -glycerophosphate and the synthetic glucocorticoid DEX (Pittenger et al. 1999; Jones et al. 2002; Isaac et al. 2008). Ascorbic acid 2 phosphate is important for extracellular matrix deposition, and acts as a cofactor for prolyl lysyl hydroxylase, a key enzyme in collagen biosynthesis (Temu et al. 2010). β -glycerophosphate acts as a source of phosphate ions, it is required for the later stages of osteoblast differentiation and matrix mineralisation, which is stimulated by DEX (Coelho et al. 2000; Jurutka et al. 2007). MSCs form aggregates or nodules and begin to express ALP, an enzyme required for hydroxyapatite crystallisation (Millan 2013). Calcium, a component of hydroxyapatite, then begins to accumulate. Both ALP and calcium accumulation can be used as indicators of osteogenic induction (Baboolal et al. 2014).

Adipogenic differentiation can be achieved with incubation of MSCs with DEX, isobutylmethylxanthine, indomethacin and insulin. Isobutylmethylxanthine and DEX induce expression of CCAAT-enhancer binding protein alpha (C/EBP α) which is one of two critical transcription factors responsible for regulating the adipogenic

differentiation process (Farmer 2006). Indomethacin is included in the culture media due to its ability to activate the other important transcriptional driver of adipogenesis, peroxisome proliferator-activated receptor γ (PPAR γ) (Lehmann et al. 1997; Farmer 2006). Once adipocyte differentiation has taken place the presence of insulin stimulates glucose transport, eventually resulting in the formation of lipid rich vacuoles. These lipid vacuoles accumulate within cells and eventually combine and can be detected by oil red or Nile red staining after a period of 2-3 weeks (Pittenger et al. 1999; Jones et al. 2002; Aldridge et al. 2013).

MSCs can be induced to undergo chondrogenic differentiation first by centrifugation into a cell pellet, then culturing in the presence of ascorbic acid 2 phosphate, TGF- β_3 and proline. Once again ascorbic acid 2 phosphate is included because of its importance for extracellular matrix deposition. TGF- β_3 stimulates type II collagen gene expression and is essential for optimal synthesis and homeostasis of matrix molecules during chondrogenesis (Brady et al. 2014). The amino acid proline is also included since it is an essential component of mammalian collagen (De Sa Peixoto et al. 2013). Chondrogenesis can be measured by accumulation of glycosaminoglycans (GAGs), which can be detected by staining with toluidine blue (Pittenger et al. 1999; Jones et al. 2002).

1.9.5. The function of CD271

The function of CD271, also known as low affinity nerve growth factor receptor, p75 and p75NTR, in the nervous system has been widely described. It is the common receptor for both neurotrophins and proneurotrophins (Ibanez et al. 2012; Tomellini et al. 2014). Activation of CD271 has been shown to increase the amplitude of excitatory post synaptic currents, affect myelination and neurite outgrowth (Luther et al. 2013; Tomellini et al. 2014). CD271 signal transduction pathways are extremely variable because they are dependent on cell type. CD271 mediated signalling has also been shown to influence apoptosis, cell cycle regulation, cell migration and invasion (Tomellini et al. 2014).

The function of CD271 in BM MSCs is not well understood, although some evidence suggests that it may play a role in mediating differentiation. Akiyama *et al* showed

that overexpression of CD271 in a pre-osteoblast cell line induced alkaline phosphatase activity and mRNA expression of osteoblast related genes, including OSX as well as stimulating proliferation (Akiyama et al. 2014). However, CD271 expression on MSCs is known to decline rapidly during *in vitro* expansion (Jones et al. 2002); this does not correspond with loss of differentiation capacity which is known to persist for several passages (Dominici et al. 2006; Wagner et al. 2009). It is therefore reasonable to assume that CD271 expression is not essential for MSC differentiation, its expression in BM MSCs could be stimulated by cues in the BM microenvironment, but it may have a limited role in governing MSC behaviour.

1.10. Strategies for manipulating MSCs for therapy development

1.10.1. Culture expansion of MSCs prior to therapy

Iliac crest BM aspirates are widely regarded as the gold standard source of MSCs. However, the low concentration of MSCs in BM aspirates (Friedenstein et al. 1992; Pittenger et al. 1999; Jones et al. 2006) limits the number that can be harvested from this source for MSC based therapy. Recently there has been great interest in the application of MSCs for the treatment of GVHD (Tolar et al. 2011). One study undertaken by Ringden *et al* involved the administration of MSCs to patients suffering from therapy resistant GVHD. Patients received up to 2 doses of 1×10^6 MSCs/kg, meaning that a 50kg patient would receive up to 5×10^8 MSC, this number was not achievable from iliac crest aspirates without several rounds of culture expansion (Ringden et al. 2006).

Although culture expansion appears to offer an abundant source of MSCs amenable to therapy, it is well recognised that cultivation history has an important influence on resultant MSC characteristics. The "*in vitro* age" of MSC cultures is critical in determining the proliferative and differentiation potential of the MSC product (Wagner et al. 2008). The number of population doublings undergone by MSC cultures inversely correlates with both their proliferation rate and differentiation

potential, eventually resulting in loss of differentiation potential and culture senescence (Banfi et al. 2000; Banfi et al. 2002; Wagner et al. 2008). Additionally, the chromosomal stability of MSCs subjected to extended culture expansion has been questioned leading to safety concerns surrounding this strategy (Wang et al. 2005; Rosland et al. 2009; Sensebe et al. 2012). Therefore, it is desirable that MSC cultures undergo the minimum number of population doublings required to achieve therapeutic yield. This is dictated by the starting number of MSCs and the required dose for therapy. The highly variable concentration of MSCs in BM aspirates (Friedenstein et al. 1992; Pittenger et al. 1999; Jones et al. 2006) means that the number of population doublings required for therapy may vary considerably. Moreover, initiation of cultures with low numbers of MSCs may result in culture senescence and failure to achieve therapeutic yield. For these reasons it is desirable that the MSC content of aspirates is assessed prior to expansion.

1.10.2. Minimally manipulated MSCs

The use of autologous, minimally-manipulated MSCs that have not been subject to tissue culture expansion is appealing, since it avoids many of the regulatory issues governing human cellular and tissue based products (HCT/Ps). Minimal manipulation is defined by the United States Food and Drug Administration (FDA) as processing that does not alter the relevant biological characteristics of cells or tissues. HCT/P that meet the 1271 criteria under section 361 of the Public Health Service Act are not subject to any premarket review requirements (Deasy et al. 2013). Examples of such products include BM or blood transplants and organ transplants. The FDA has stated that cryopreservation, centrifugation, density gradient separation and cell selection constitute minimal manipulation. Whereas all processes that manipulate the cell or tissue product such as encapsulation, cell activation, gene modification and *ex vivo* expansion are considered more than minimally manipulated (Deasy et al. 2013).

The European union regulatory framework mirrors that of the FDA whereby, stem cell products are defined as advanced therapy medicinal products (ATMPs), and

subject to more rigorous regulation (von Tigerstrom 2008), if they have been manipulated by *ex vivo* expansion or activation. Allogeneic and xenogeneic cells associated with medical devices used *ex vivo* or *in vivo* such as micro-capsules and scaffolds also fall under this definition which may be found in Annex 1 of directive 2001/83/EC (von Tigerstrom 2008).

Minimal manipulation avoids many of the safety issues surrounding cultured cells such as the possibility of spontaneous transformation (Rubio et al. 2005; Wang et al. 2005; Rosland et al. 2009) as well as the potential of animal pathogen contamination resulting from the use of foetal calf serum (FCS) as a culture component (Bernardo et al. 2011). Additionally minimally-manipulated MSCs do not require costly manufacturing facilities and are, therefore, relatively inexpensive to obtain. However the low concentration (Friedenstein et al. 1992; Pittenger et al. 1999; Jones et al. 2006) and high variability of MSC content in BM aspirates (Pittenger et al. 1999; Hernigou et al. 2005a; Veyrat-Masson et al. 2007) makes reliably achieving a therapeutic dose of MSCs problematic.

The use of BM concentrator devices for autologous MSC grafting in orthopaedic surgery is widespread for indications including spine surgery (Oreffo 2005), the treatment of impaired fracture healing (Hernigou et al. 2005a; Hernigou et al. 2005b; Kasten et al. 2008) and femoral head osteonecrosis (Hernigou et al. 2002), with no adverse events reported. Despite the simplicity and strong rationale behind the application of MSCs to these conditions, controlling the number of MSCs administered has remained a problem, with only retrospective analysis possible. A rapid means of evaluating the MSC content in aspirates prior to concentration would give surgeons an opportunity to improve the quality of aspirates and therefore the effectiveness of autologous MSC grafting.

Alternatively, it has been demonstrated that large numbers of MSCs can be recovered from enzymatically treated femoral heads (Jones et al. 2010b) and are present in the liquid waste fraction generated by intramedullary reaming (Porter et al. 2009). MSCs isolated from these tissues have been shown to have phenotypic characteristics similar to those present in BM aspirates (Jones et al. 2010b; Cox et

al. 2012). These tissues may provide an alternative plentiful source of MSCs that could be used either in the orthopaedic setting or in the wider context of MSC therapy. Immunomagnetic cell selection could provide a means to isolate MSCs from a variety of potential sources, if performed using reagents and equipment suitable for clinical application. Additionally, depending of the purity obtained, MSCs may be utilised for allogeneic treatments as well as autologous therapy. CD271 is an ideal candidate surface marker for immunomagnetic cell selection due to its highly specific expression on BM MSCs (Boxall et al. 2012).

1.10.3. Recruitment of endogenous MSCs

Scaffolds that exert a regenerative effect by recruiting endogenous MSCs have been and continue to be of great interest in regenerative medicine. In bone defect repair and fracture non-union, barrier membranes are widely used (Cheung et al. 2007; Behring et al. 2008; Gloria et al. 2010; Dimitriou et al. 2011b). These may be synthetic (Cheung et al. 2007; Gloria et al. 2010) or bio-absorbable natural polymers (Behring et al. 2008; Dimitriou et al. 2012) and often rely on the passive recruitment of MSCs into the site of injury. However, a number of scaffolds are under development that actively recruit, stimulate proliferation or differentiation of endogenous MSCs. Examples of this include a TGF- β 3 infused scaffold for cartilage regeneration (Lee et al. 2010), and several examples of scaffolds aimed at bone regeneration that incorporate growth factors such as BMP-2 and VEGF (Huang et al. 2005; Niu et al. 2009) or chemotactic agents such as SDF-1 (Dashnyam et al. 2014; Jin et al. 2014).

An alternative approach has been developed by Masquelet *et al* who by serendipity discovered that implantation of a bone cement spacer induced the formation of a thick vascularised membrane with impressive regenerative capacity (Giannoudis et al. 2011; Dimitriou et al. 2012; Gruber et al. 2012). A careful examination of this spontaneously generated membrane may elucidate the mechanisms by which it facilitates bone regeneration. Ultimately this could lead to the development of a

new generation of orthopaedic implants that exploit this phenomenon or mimic this induced membrane's characteristics.

1.10.4. Inhibition of aberrant bone formation

In some situations inappropriate or excessive bone formation can have debilitating effects. Heterotopic ossification can occur following surgery or trauma or as a result of the rare genetic disorder fibrodysplasia ossificans progressiva (Yu et al. 2008; Kaplan et al. 2011). Ectopic bone formation occurs within soft tissues including muscles, connective tissue and near blood vessels or nerves (Vanden Bossche et al. 2005; Shimono et al. 2011). It is thought to be as a result of localised inflammation leading to recruitment of MSCs, their differentiation into chondrocytes and eventually endochondral ossification (Shore et al. 2010). Resolving the initial inflammation by treatment with nonsteroidal anti-inflammatory drugs has had limited success, perhaps because by the time the condition has become clinically apparent the induction process is already complete (Kaplan et al. 2011). Inhibition of BMP type I receptor signalling using small molecule inhibitors has shown promising results in preventing heterotopic ossification in a mouse model (Yu et al. 2008). However, inhibition was not sustained after treatment was discontinued (Yu et al. 2008) and concerns regarding long term administration BMP type I receptor antagonists stem from various off target effects and lack of specificity of these compounds, which target multiple BMP type I receptors (Hong et al. 2009). Shimono et al reported potent inhibition of heterotopic ossification by inhibition of chondrogenesis in several mouse models of heterotopic ossification using RAR γ agonists with minimal side effects (Shimono et al. 2011). Moreover, inhibition of heterotopic ossification by RAR γ agonists seemed to be permanent and effective over a wide treatment window (Shimono et al. 2011). However its wider application to treating aberrant bone formation is likely limited to instances where bone formation is driven by endochondral ossification.

The degenerative diseases, such as OA and AS, are also characterised by aberrant bone formation (Braem et al. 2012; Zhen et al. 2013). It has recently been shown

that the inhibition of TGF- β signalling is able to halt disease progression by attenuating the excessive mineralisation of matrix, driven by osteogenic differentiation in OA (Zhen et al. 2013). In AS excessive bone formation at the enthesis causes progressive loss of mobility, this particularly affects the spine. Here aberrant osteogenesis is linked to inflammation and low levels of DKK-1 (Diarra et al. 2007). However, resolution of the inflammatory aspect of AS has little or no effect on aberrant bone formation (van der Heijde et al. 2008a; van der Heijde et al. 2008b). Drugs that modulate the differentiation capacity of MSCs have the potential to transform the lives of patients suffering from AS. However, blocking inflammation remains an important clinical goal since doing so alleviates the pain associated with joint inflammation and significantly improves quality of life (van der Heijde et al. 2008a; van der Heijde et al. 2008b). One drug marketed by Pfizer under the name tofacitinib can inhibit joint inflammation and has clinical application to in rheumatoid arthritis (RA) (Kytтары 2012). It elicits this effect by inhibition of the JAK/STAT signalling cascade and thereby disrupts inflammatory cytokine signalling (Kytтары 2012). This potentially leads to the exciting prospect of simultaneously blocking inflammation as well as aberrant bone formation through inhibition of JAK/STAT signalling.

1.11. Thesis aims and objectives

The aim of this thesis was to test the hypothesis that knowledge of MSCs *in vivo* can be used to improve and develop cellular therapy strategies that utilise minimally manipulated MSCs. This thesis also aims to demonstrate that the understanding of the *in vivo* characteristics of MSCs may be used as a tool for therapy development, by elucidating the mechanisms in which clinical procedures or drugs have beneficial effects.

Objectives:

1. The first objective of this thesis was to test the hypothesis that flow cytometry may be used as a rapid means of assessing the MSC content of BM aspirates and develop a method of standardising BM aspiration with the goal of improving MSC yield.
2. The second objective of this thesis was to evaluate current available technologies aimed at concentrating BM MSCs and test the hypothesis that the expression of CD271 could be used as a means of isolating MSCs from osseous tissues.
3. The third objective of this thesis was to test the hypothesis that the impressive bone formation, observed as a result of the induced membrane (IM) or Masquelet technique, is mediated by MSC recruitment into the IM, and to determine how this drives tissue regeneration.
4. The fourth objective of this thesis was to test the hypothesis that the drug tofacitinib is able to inhibit MSC differentiation into bone and thus provide a new therapeutic approach to the treatment of AS.

2. General materials and methods

2.1. Patient recruitment and sample collection

All patients gave informed consent and research was carried out in compliance with the Helsinki Declaration. Ethics committee approval was obtained from local National Health Service Research & Development Departments, National Research Ethics Service, Leeds East and Leeds West Research Ethic Committees under permit numbers 06/Q1206/127 and 07/Q1205/27 respectively (Appendix 1). In total 61 patients were recruited, 34 male, 27 female, mean age 46 (18-85).

2.2. Harvest of BM aspirate

BM aspirates were obtained from the Iliac crest of acute trauma patients or patients undergoing elective orthopaedic surgery. All samples were collected into K₃EDTA vacuettes (Greiner bio-one) using a syringe/s and trocar (Stryker), both of which were first flushed with 1000U/ml sodium heparin solution (Leo Pharma) to avoid clotting. Heparin solution was drawn into each syringe in turn to coat the surface of the plastic interior, following removal of the central stilet; the trocar (aspiration needle) was also treated with heparin. In the majority of cases samples were taken from the anterior iliac crest, a small incision was made in the skin and the trocar was inserted through the cortical bone. Once the cortical bone was penetrated, the needle was pushed approximately 1cm through the cancellous bone, the stilet was removed and a heparin coated syringe attached, a volume of BM was then aspirated.

2.3. Manual cell counting

The total number of nucleated cells was measured using a haemocytometer and an inverted light microscope. Whole, well mixed BM was diluted 1/20 with Phosphate buffered saline (PBS, invitrogen), then diluted 1/2 with 4% (v/v) acetic acid and incubated at room temperature (RT) for 1 minute to lyse erythrocytes. For cell suspensions that did not contain erythrocytes such as passaged MSCs, an

appropriate dilution was made with PBS and the suspension was subsequently diluted 1:1 with trypan blue (Sigma Aldrich). A 10µl volume of the resulting mixture was then loaded onto a haemocytometer with an improved Neubauer counting chamber which consists of a large square divided into 9 smaller squares each representing a volume of 0.1µl. The following formula was used to calculate the total number of nucleated cells.

$$\text{Nucleated cells/ml} = \frac{\text{Number of cells counted} \times \text{Dilution} \times 10^3}{\text{Volume counted } (\mu\text{l})}$$

In order to maintain accuracy, a minimum number of 100 cells were counted; if this was not possible the procedure was repeated using a lower dilution of PBS.

2.4. Enzymatic digestion of solid tissue

Tissue was broken into small fragments either by shredding with a scalpel or using a bone mill (De Puy) and then weighed. Tissue fragments were incubated with 3000 units of collagenase per gram of tissue at a concentration of 600U/ml (Worthington) in 20%v/v FCS (PAA) and 80%v/v PBS (Gibco) for 4 hours at 37°C with manual agitation every 30 minutes. Following digestion, cell suspension was passed through a 70µM nylon filter (BD) to remove any remaining tissue aggregates. Filtered cell suspension was diluted with 20ml PBS and centrifuged at 400xg, the supernatant was discarded and the cells were resuspended in sterile FACS buffer prior to culture expansion or flow cytometry.

2.5. Volumetric flow cytometry for cell enumeration

Multi-parameter flow cytometry was used to enumerate cell populations in mixed cell suspensions based on the expression of surface antigens (Appendix 4). Accuchecktm counting beads (Invitrogen) were used to enable enumeration on a volumetric basis (number of cells/ml). To perform the staining, 50µl of cell suspension was incubated with directly conjugated mouse monoclonal antibodies for 15 minutes at RT at manufacturer's recommended concentration (Appendix 4).

Erythrocytes were then lysed by the addition of 2ml red blood cell (RBC) lysis buffer (Appendix 2) containing 0.5µg/ml 4',6-diamidino-2-phenylindole (DAPI, Sigma Aldrich) and incubation at RT for 10 minutes. Immediately following red cell lysis, 50µl of Accuchecktm counting beads (Invitrogen) was added and the cell/bead suspension was analysed immediately using a LSR II flow cytometer (BD). Single antibody stained and unstained controls were used before each experiment to ensure accurate spectral compensation and isotype controls (Appendix 4) were used to confirm gating position for all cell populations studied. Centrifugation and re-suspension was avoided since this would result in inaccurate reporting of cell concentration due to loss of cells in the centrifuged supernatant.

2.6. Calculation of total cell number using volumetric counting beads

Flow cytometry data were analysed using FACSDiva Version 5.02 (BD). Beads were identified based on their size and fluorescent properties (Figure 1A-B). As an internal control, the bead preparation is supplied with an equal mix of two types of beads with unique characteristics that allows monitoring of bead sampling homogeneity. Bead 1 is a 6.4µm sphere and presents a low forward and high side scatter signal. Bead 2 is a 6.36µm sphere and presents a high forward and low side scatter signal. To confirm homogeneity, the proportion of bead type A or B with respect to total number of beads must be 45-55%; outside this range the count was regarded as unreliable. A small proportion of counting beads were observed to aggregate despite careful mixing; this was confirmed using doublet discrimination (data not shown). To compensate for this, each doublet bead event was counted as two beads. Cell debris was excluded based on forward and side scatter (Figure 1C). Dead/dying cells were identified by uptake of DAPI (Figure 1D). Once dead cells and debris had been removed from analysis, cells of interest were identified based on the expression of cell surface antigens labelled with specific monoclonal antibodies (Figure 1E). To calculate the absolute count of the cells of interest per ml the following formula was used:

$$C = \left(\frac{N}{T}\right) \times S$$

Where C=Number of cells of interest, N=Number of events of interest, T=Total number of bead events and S=Stock concentration of Beads.

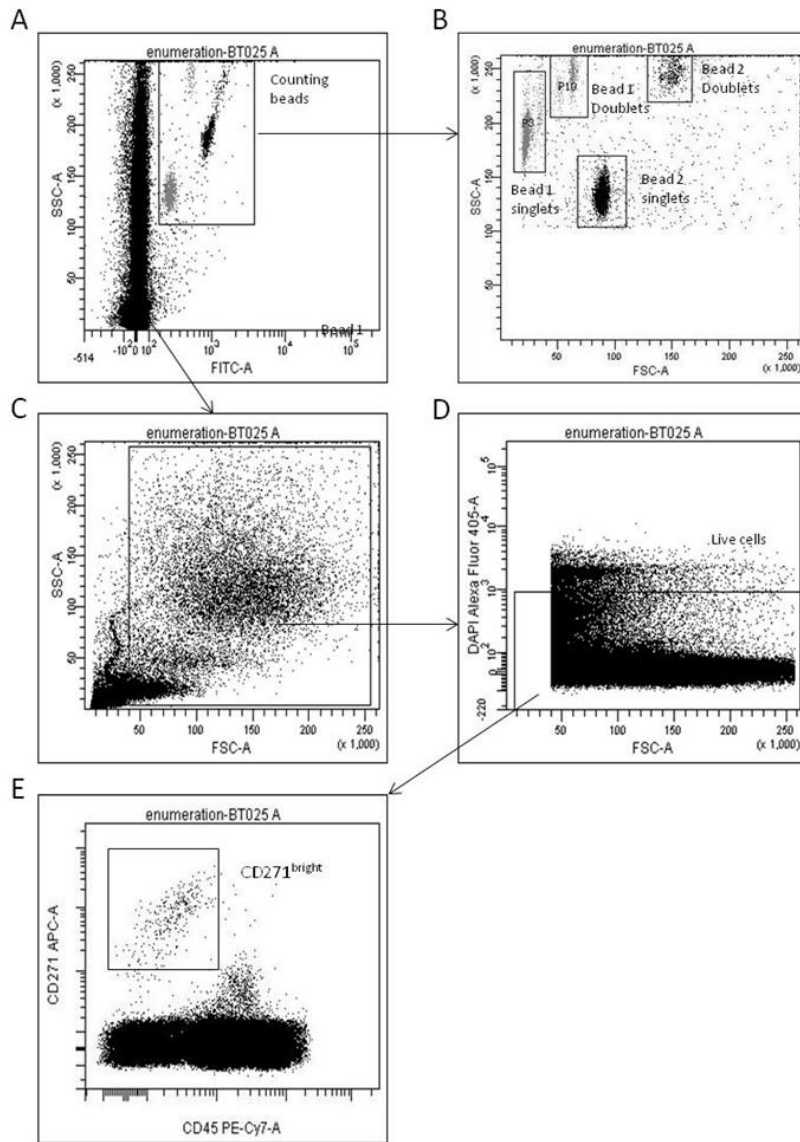


Figure 1: An example of flow cytometry gating strategy for absolute cell quantification using volumetric counting beads

Counting beads were discriminated by fluorescence in the FITC channel (A). Bead types 1 and 2 and bead doublets were discriminated by forward and side scatter profile (B). Cellular debris was removed from analysis using forward and side scatter profile (C). Dead/dying cells were identified and removed by uptake of DAPI (D). Finally cellular populations are identified based on expression of cell surface antigens; in this case BM MSCs are identified by high expression of CD271 and negative to low expression of CD45 (E).

2.7. CFU-F assay

Unless otherwise stated, the CFU-F assay was performed as a modification of the original method described by Galotto *et al* (Galotto et al. 1999) and was used to enumerate MSCs volumetrically. 200µl of BM aspirate was seeded into duplicate 100mm diameter tissue culture dishes (Corning) containing 15ml of Non-hematopoietic stem cell media (NH media, Miltenyi Biotec). The cells were incubated at 37°C, 5% CO₂. After 48 hours non-adherent cells were removed by washing once with 5ml of PBS and 15ml of fresh media was added. Subsequently, half media changes were performed twice a week for 14 days. At the end of the culture period the adherent cells were washed with PBS and fixed with 10ml of 4% v/v formaldehyde (Sigma Aldrich) at RT for 15 minutes. Colonies were stained by a 30 minute RT incubation in 1% w/v methylene blue solution (Appendix 2); a colony was defined as a tight macroscopically-visible cluster of 50 or more cells with a defined focal origin (clusters of less than 50 cells were excluded as were diffuse patches of single cells).

2.8. Measurements of colony area

CFU-F assays were performed on BM aspirates as previously described (2.7). Because manually counting colony number and size is considered fairly subjective (Seebach et al. 2007), digital measurements of total colony area were performed in parallel with manual scoring. For this, CFU-F dishes were scanned using an Epson 3590 digital scanner and digital images were analysed using NIS elements BR 2.20 imaging software (Nikon). The area occupied by colonies was identified based on the intensity of the methylene blue stain. Dish area was classified into un-colonised and colonised regions and the area taken up by each region was calculated. No determination was made of individual colony size or morphology.

2.9. Culture expansion

For expansion of MSCs from mixed cell suspensions such as BM aspirates, nucleated cells were counted manually (2.3), cells were seeded at a density of 8×10^4 cells/cm² in tissue culture grade, flasks (Corning) in an appropriate volume of non-haematopoietic (Appendix 3) stem cell (NH) media (Miltenyi Biotec) and incubated at 37°C, 5% CO₂. After 48 hours the media was removed and the culture vessel was washed with 5ml PBS to remove non-adherent cells. The media was renewed and expansion was allowed to continue until cells achieved approximately 70-80% confluence (denoted passage 0, p0); at this point cells were detached by first washing with 5ml of PBS then incubation with 5ml 0.05%w/v trypsin solution (Invitrogen) at 37°C for up to 5 minutes. Trypsin is a protease used to lift cells from plastic surfaces and works by digesting the proteins responsible for attachment. Flasks were tapped sharply to dislodge any remaining adherent cells and 5ml of NH media was added to the cell suspension. Cells were manually counted using a haemocytometer then centrifuged at 400xg for five minutes. The supernatant was discarded and cells were re-seeded in NH media at 4.5×10^3 /cm² and returned to incubation in the same conditions. Once cells had reached 70-80% confluence (denoted p1) and were again detached, counted and re-seeded as previously described, this was repeated until the desired number of cells was achieved.

2.10. *In vitro* osteogenic differentiation.

Following culture 3×10^4 MSCs were seeded in triplicate into 6 well tissue culture grade plates (Corning), to give 9 replicates in total, with osteogenic media (Appendix 2) and incubated at 37°C, 5% CO₂ (Jaiswal et al. 1997; Jones et al. 2004a). Twice weekly 50% of the culture medium was replaced and cultures were maintained for 14 or 21 days.

2.11. Detection of alkaline phosphatase activity

For determination of ALP activity, osteogenic cultures were terminated on day 14 post-induction. The media was aspirated from the wells, which were washed with 1ml PBS and subsequently fixed with the addition of 2ml of citrate fixative solution (Appendix 2) for 30 seconds at RT. The fixative was then removed and wells were washed twice with dH₂O before the assessment of ALP activity using fast blue salt solution (Jaiswal et al. 1997). After 1ml fast blue dye solution (Appendix 2) was added to each well, the plate was placed in the dark at RT for 30 minutes to allow colour development. Following incubation, the wells were washed twice with 1ml dH₂O and allowed to air dry; dishes were subsequently scanned using an Epson 3590 digital scanner.

2.12. Determination of total calcium accumulation

At termination cells were washed gently with tissue culture grade PBS without calcium (Invitrogen) and subsequently incubated for 5 minutes in 1ml of 600mM HCl to extract Ca²⁺ from mineralized extracellular matrix. Using a disposable cell lifter (Fisher Scientific) HCl and cell debris was removed from each well and transferred into 1.5ml microcentrifuge tubes. Calcium was extracted by incubating for 4 hours at 4°C with constant agitation, and subsequently stored at -20°C. Prior to analysis, samples were brought to RT and centrifuged at 5000xg to remove any remaining cellular debris.

For determination of calcium content, 6µl of supernatant was loaded in triplicate wells of a flat bottomed 96 well plate and the calcium content was measured using a calcium liquid detection kit (Sentinel Diagnostics). 210µl of a buffered 0.3mM cresolphthalein complexone solution was introduced to each sample well or well containing a known concentration of a calcium standard. Cresolphthalein complexone reacts with calcium ions at pH>10 to form a red coloured complex, colour intensity is directly proportional to the concentration of calcium; this was quantified by measuring absorbance at 570nm using an Opsy MR plate reader (Dynex Technologies). Once measured, absorbance can be converted to

concentration of calcium by comparing the absorbance of the calcium standards to the sample absorbance.

2.13. *In vitro* adipogenic differentiation and detection of lipid vacuoles by uptake of oil red

Following culture expansion, 5×10^4 MSCs per well were seeded in triplicate into 24 well tissue culture plates (Corning) containing 1ml adipogenic media (Appendix 2) and incubated at 37°C, 5% CO₂. Twice weekly half of the culture media was replaced and cultures were terminated on day 21 post-induction by washing twice with 1ml PBS followed fixation at RT for 15 minutes with 4% v/v formalin solution, 1ml/well. Oil Red solution (Appendix 2) was then applied at 1ml/well, for 20 min at RT before washing twice with 1ml PBS followed by counterstaining with 1ml of Harris' haematoxylin (Sigma Aldrich) for 2 minutes at RT. This was aspirated and wells were washed with running tap water and subsequently incubated with 1ml of Scott's tap water substitute (Appendix 2) for 1 minute at RT, before a further wash with running tap water. For quantitative assessment of oil red uptake a random low powered field of view (40X magnification) was captured using a C7070 digital camera (Olympus, Tokyo, Japan). These images were then analysed using NIS elements BR 2.20 imaging software (Nikon), the area occupied by lipid vacuoles was identified based on the intensity of oil red staining giving a percentage area occupied by lipid vacuoles on each photomicrograph.

2.14. *In vitro* chondrogenic differentiation

For chondrogenic differentiation, 2.5×10^5 MSCs suspended in 0.5ml of complete chondrogenic media (Appendix 2) were centrifuged 5 minutes in screw top 1.5ml microcentrifuge tubes at 400xg to create a pellet. Screw caps were fastened loosely in order to allow gas exchange and incubated at 37°C, 5% CO₂; half the media was replaced every 3 days for a total culture period of 21 days. Pellets were either snap-frozen in optimal cutting temperature (OCT) compound (Fisher Scientific)

using the vapour phase of liquid nitrogen prior to cryo-sectioning and staining with toluidine blue or prepared for GAG assay as described in chapter 6 (6.2.6). For toluidine blue staining 7µm sections were prepared using a CM3050S cryostat (Leica) and adhered to superfrost histology slides (Fisher). Slides were allowed to air dry overnight then incubated with methanol (Sigma Aldrich) for 20 minutes at -20°C. Subsequently, slides were removed from methanol and allowed to air dry for 30 minutes. Once dry, slides were incubated in toluidine blue solution (Appendix 2) at 37°C for 30 minutes, slides were then transferred to isopropanol (Sigma Aldrich) to remove excess toluidine blue. Slides were incubated in xylene (Sigma Aldrich) for 5 minutes at RT before coverslips were mounted using DPX mounting media (Sigma Aldrich). Slides were then examined using an Eclipse 1000 (Nikon) microscope and images captured using a C7070 digital camera (Olympus, Tokyo, Japan).

2.15. Statistics

The Shapiro-Wilk normality test was used to assess distribution normality and to determine appropriate correlation and significance testing. For data sets with fewer than 6 data points per group a non-gaussian distribution was assumed. Statistical significance was defined as $p < 0.05$, * indicates $p < 0.05$, ** indicates $P < 0.01$, *** indicates $P < 0.001$. All statistics were calculated using SPSS® Version 19. Graphs were generated using GraphPad Prism® Version 5.04. All box and whisker plots show median (line) interquartile range (box) and extreme values (whiskers). All bar charts show mean (bar height) and standard deviation (whiskers). The Spearman rank correlation coefficient was used to describe statistical dependence.

3. A rapid single platform assay for MSC enumeration in BM aspirates

3.1. Introduction

3.1.1. The CFU-F assay

Following the initial experiments by Friedenstein et al demonstrating the presence of multipotential CFU-Fs in the BM (Friedenstein et al. 1970), the importance of accurately enumerating and determining the biology of MSC *in vivo* has become clear. This is particularly relevant in the context of minimally manipulated MSC therapy (Hernigou et al. 2005a) and the study of aging (Stolzing et al. 2008). The first comprehensive CFU-F assay for determining the MSC content of human BM of was described by Castro-Malaspina *et al* (Castro-Malaspina et al. 1980). This initial technique relied on density gradient sedimentation to separate erythrocytes from mononuclear cells (MNCs) prior to seeding in alpha-MEM supplemented with 20% FCS and culture expansion for 8 to 14 days.

An alternative technique was later described by Galotto *et al* (Galotto et al. 1999) who dispensed with the initial MNC separation step and instead seeded unprocessed whole BM volumetrically prior to culture expansion. This simplification does away with several washing and centrifugation steps reducing the potential for experimental error. In either case following a period of culture expansion, cells were fixed, stained and macroscopically visible colonies counted. By counting the number of these colonies, a determination of the initial starting number of MSCs is therefore possible based on the assumption that one colony is generated from one MSC.

3.1.2. Limitations of the CFU-F assay

Although the development of the CFU-F assay was a critical advance for the measurement of MSC content in a given tissue sample, it has several important limitations. These include dependence on serum source (Castro-Malaspina et al.

1980), plating density (Veyrat-Masson et al. 2007) and subjective and poorly defined scoring criteria (Stolzing et al. 2008). Most importantly, the assay is hindered by the long culture period required (14 days), therefore only retrospective evaluation of MSC content is possible (Hernigou et al. 2005a). In MSC based therapies, regardless of whether MSCs are to be administered with or without expansion, their absolute number in the aspirate is critical in determining efficacy (Hernigou et al. 2005a; Wagner et al. 2008; Wagner et al. 2009).

3.1.3. The importance of prospective MSC enumeration

Although culture expansion appears to offer an abundant source of MSCs amenable to therapy, it is well recognised that cultivation history has an important influence on resultant MSC characteristics. The “*in vitro* age” of MSC cultures is critical in determining the proliferative and differentiation potential of the MSC product (Wagner et al. 2008; Wagner et al. 2009). The number of population doublings (PDs) undergone by MSC cultures inversely correlates with both their proliferation rate and differentiation potential, eventually resulting in loss of differentiation potential and culture senescence (Banfi et al. 2000; Banfi et al. 2002; Wagner et al. 2008). Additionally concerns have been raised as to the safety of MSC therapy after extended expansion due several reports of spontaneous transformation in culture (Wang et al. 2005; Rosland et al. 2009; Sensebe et al. 2012). Therefore it is desirable that MSC cultures undergo the minimum number of PDs required to achieve therapeutic yield. For these reasons it is desirable that the MSC content of aspirates is assessed prior to expansion.

BM derived MSCs that have been obtained by iliac crest aspiration and administered following concentration or cell selection are classed as a minimally manipulated product (Deasy et al. 2013). However, the low concentration (Friedenstein et al. 1992; Pittenger et al. 1999; Jones et al. 2006) and high variability of MSC content in aspirates (Pittenger et al. 1999; Hernigou et al. 2005a; Veyrat-Masson et al. 2007) makes reliably achieving a consistent dose of MSCs problematic; this problem is compounded by the possibility of marrow dilution with

blood (Hernigou et al. 2005a; Veyrat-Masson et al. 2007; Hernigou et al. 2013). A rapid means of evaluating the MSC content in aspirates would give surgeons an opportunity to assess the quality of aspirates prior to administration. This would improve effectiveness of autologous MSC grafting particularly in the context of bone regeneration (Hernigou et al. 2005a).

3.1.4. The CD45^{-/low}CD271⁺ population as a candidate for measuring *in vivo* MSC number

The utility of a cell surface or intracellular marker to identify any cell type is primarily driven by specificity. Previous studies have shown that the CD271 antigen has excellent specificity for human BM MSCs with limited cross reactivity with other cell types (Jones et al. 2002; Kuci et al. 2010; Tormin et al. 2011).

CD271 has been found to have superior specificity for BM MSCs compared to alternative markers (CD13, CD90, CD105, CD10 and STRO-1) (Jones et al. 2002), additionally CD271 is not expressed on uncultured skin fibroblasts (Jones et al. 2002). CD146 and MSCA-1, two other BM MSC markers, are also expressed on CD45^{-/low} CD271⁺ cells (Buhring et al. 2007; Tormin et al. 2011). These studies provide evidence that CD271 could be a useful marker for the enumeration of MSCs in BM aspirates.

Although CD73 is widely expressed on a range of cell types including T-cell, B-cells and fibroblasts (Haniffa et al. 2009; van de Veen et al. 2013; Schuler et al. 2014) making it a less attractive prospect for MSC identification, positive expression of CD73 in conjunction with negative expression of CD45 has been proposed as a means of quantifying BM MSCs (Veyrat-Masson et al. 2007). Although this study did show positive correlation between the percentage of CD45⁻CD73⁺ cells and CFU-F concentration, enumeration relied on density gradient centrifugation and manual cell counting, both potential sources of experimental error. Nevertheless strong correlations observed in this study suggested that CD73 may prove to be a useful addition or even an alternative to CD271 for assessment of MSC number in BM aspirates.

3.1.5. Volumetric flow cytometry as an alternative to CFU-F assay

The International Society for Cell Therapy position statement defines MSCs by cell surface antigen expression based on the positive co-expression of 3 cell surface antigens (CD105, CD73 and CD90) and the negative expression of at least 5 cell surface antigens (CD45, CD34, CD14 or CD11b, CD79 α or CD19 and HLA-DR) (Dominici et al. 2006). Based on these criteria any flow cytometry assay designed to quantify MSCs would be technically challenging and impractical for use in routine clinical laboratories. These issues are further compounded by the high processing speeds and data buffering capacity required for this type of rare cell analysis.

The identification of a BM MSC phenotype based on expression of a single positive marker (CD271) and a single negative marker (CD45) (Jones et al. 2002; Quirici et al. 2002; Jones et al. 2004b), greatly simplifies assay design. This, together with recent advances in flow cytometry, such as the introduction of volumetric counting beads and improved computing technology, allows the possibility of developing a rapid and relatively simple assay.

3.1.6. Chapter aim and objectives

The aim of this chapter was to develop a rapid protocol for the evaluation of the MSC content in BM aspirates. This would for the first time provide a means of assessing total MSC content in aspirates volumetrically without the need for culture expansion and make intra-operative assessment of MSC content possible.

Objectives:

1. To validate a flow cytometry based MSC enumeration assay against the current gold standard CFU-F assay, and to demonstrate how this may be used to predict the MSC content of BM aspirates.
2. To use the above assay coupled with the CFU-F assay in order to provide some simple guidelines to surgeons harvesting MSCs by BM aspiration, in order to improve the quality of aspirates taken for experimental or clinical use.

3.2. Materials and methods

3.2.1. Harvest of BM aspirate

Aspiration of BM was performed from the anterior iliac crest, as previously described (2.2). Patients undergoing elective orthopaedic surgery were selected for this study (n=32, 18 male, 14 female, mean age 43 years). Initially, 20ml of BM was aspirated from a single needle position from each donor (n=10). For later donors (n=15), 5ml of BM aspirate was obtained from a single aspiration site; the position of the needle was then altered by removal and repositioning of the trocar ensuring that the trocar entered the cortical bone at a different angle. This procedure was performed a total of 4 times to give a final volume of 20ml. These two harvesting techniques were performed to address objective 2 and to explore the effect of aspiration technique on BM aspirate quality. Additionally, a subgroup of these samples was collected from BM aspirates used for the treatment of fracture non-union by autologous grafting (n=7). In this case 50ml of BM was aspirated from a single needle position.

3.2.2. Flow cytometry on whole BM aspirates

Since CD271 has been shown to be a highly specific marker for BM MSCs (Jones et al. 2002; Quirici et al. 2002; Buhning et al. 2009; Tormin et al. 2011; Boxall et al. 2012), the utility of measuring the absolute number of cells expressing the CD45⁻/_{low}CD271⁺ phenotype was assessed as a means of predicting the colony forming potential of a BM aspirate. Additionally, because Veyrat-Masson et al have already demonstrated impressive correlation between the colony forming potential of femoral head aspirates and the CD45⁻/_{low} CD73⁺ population (Veyrat-Masson et al. 2007), this population was also investigated in iliac crest aspirates. The proportion of T-cells in BM has been suggested as a potential indicator of BM dilution with blood (Veyrat-Masson et al. 2007). To investigate the utility of using T-cells as an indicator of BM dilution with blood, cells expressing a CD45⁺ CD3⁺ phenotype (T-cells (Gerondakis et al. 2014) were measured. Volumetric flow cytometry was performed as previously described (2.5).

All populations were identified based on binding of antibodies listed in Appendix 4. A minimum of 250,000 events in the live cell gate were collected for each sample. Acquisition time was dependent on the cellularity of the sample; the mean acquisition time was 7 minutes, 23 seconds (range 3:16-14:42), with the event rate being set as approximately 2000 events/second. If more than 5 minutes was required to collect a sufficient number of events, the sample was re-mixed to ensure sample homogeneity. CD45^{-/low}CD271⁺ cells were identified by high expression of CD271 and negative to low expression of CD45 (Figure 1E). In order to investigate variability between different CD271 antibody clones, three BM samples were also stained with CD271-PE (Miltenyi Biotec) and CD271-PE (BD) (Appendix 4). The number of cells expressing the CD45⁻CD73⁺ phenotype was also measured simultaneously, in a subset of samples (n=23) to investigate the utility of CD73 as an alternative or additional marker for MSC enumeration.

3.2.3. Statistics

Friedman's two way analysis of variance by ranks was used to assess differences in cell survival during data acquisition. The Wilcoxon matched-pairs signed rank test was used to detect differences between draw volumes. The Mann Whitney U test was used to detect differences between BM aspiration techniques. The Spearman Rank Correlation Test was used to measure statistical dependence of CFU-F data, flow cytometry and manual counting data as well as to generate a 95% confidence interval of predicted individual values.

3.3. Results

3.3.1. Method validation

3.3.1.1. Linearity and precision of absolute cell counting

A direct linear relationship was observed between automated cell counting using flow cytometry (2.5) with counting beads and manual cell counts using a haemocytometer (2.3) ($R=0.98$, $P<0.001$ (Figure 2)). This high degree of correlation confirmed that automated counting was a reliable means of measuring the number of nucleated cells in BM aspirates, enabling absolute quantification of cell numbers. This also confirmed that the chosen gating strategy for the discrimination of cellular debris from the cells was effective, thus reducing the risk of experimental error as a result of non-specific antibody binding to debris.

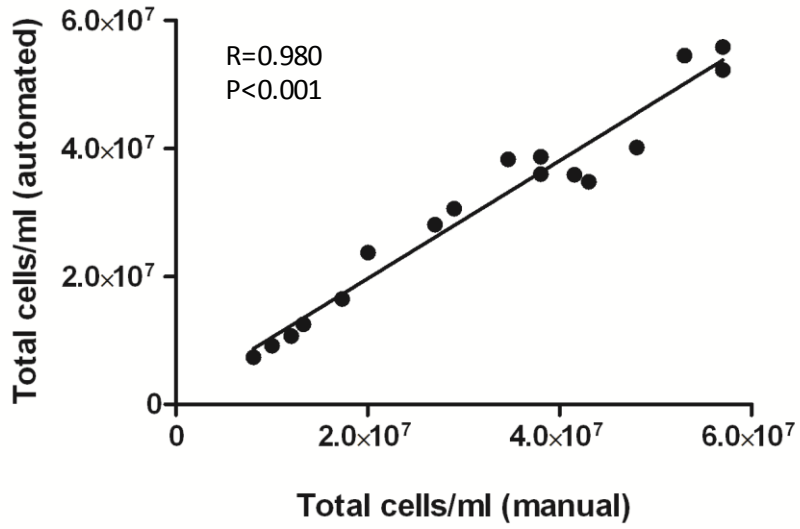


Figure 2: Comparison of manual and automated counting methods

Manual cell counts using a haemocytometer and automated cell counts using volumetric flow cytometry, n=17.

3.3.1.2. Cell viability

Since the CD45^{-/low} CD271⁺ population is extremely rare and the event rate was kept to 2000 events/second to avoid blockage of the cytometer flow cell, up to 10 minutes was required to collect a sufficient number of events for analysis (a minimum of 250,000 events in the live cell gate). It is possible that incubation in RBC lysis buffer could lead to reduced general cell viability and therefore inaccurate reporting. To investigate this, the number of cells that had not taken up DAPI (live cells) was analysed over a 10-minute period following to the initial 10-minute incubation in RBC lysis buffer. This 10-minute period was divided into five 2-minute intervals and the number of cells detected was expressed as a proportion of the total number of similar events; this was repeated using five separate BM aspirates (n=5).

The total number of live cells (Figure 3A) and the number of CD45^{-/low} CD271⁺ cells (Figure 3B) did not change over the analysis period. This indicated that prolonged RBC lysis buffer exposure did not have any detrimental effect on cell viability and detection. The relatively constant cell acquisition rate at the later time points also suggested that samples retained homogeneity over this period. Similarly, detection of CD45^{-/low} CD271⁺ cells was not affected by prolonged incubation in ammonium chloride-containing buffer.

To characterise the intra assay variability of this technique, a single aspirate was split into 6 tubes, which were analysed consecutively. The coefficient of variation for the proportion of CD45^{-/low} CD271⁺ cells was measured at 0.105 indicating a signal to noise ratio of approximately 10:1.

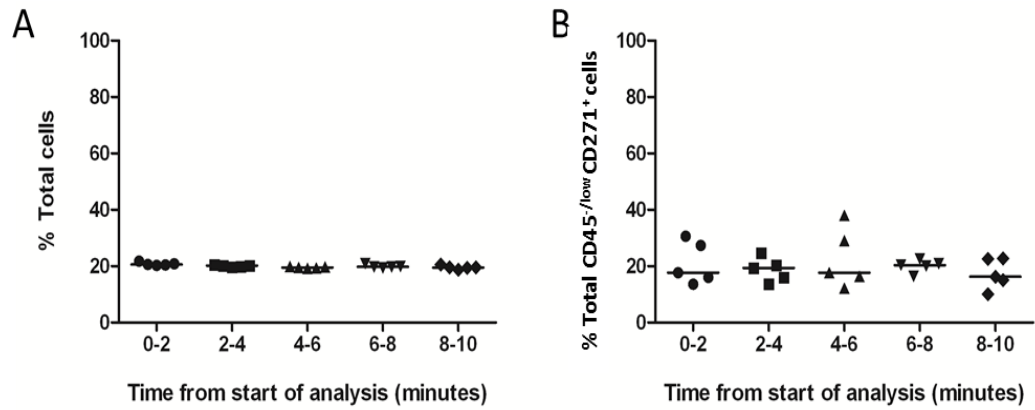


Figure 3: Cell viability in RBC lysis buffer

No change in total cell viability (A) or viability of the CD45^{-/low} CD271⁺ population (B) at any time interval following start of data collection. Data expressed as the proportion of the total events collected between 0 and 10 minutes. All data n=5.

3.3.1.3. Confirmation of antibody selection

Reproducibility is an important quality for any assay. Therefore, the influence of different anti-CD271 antibody conjugates or clones on the detection of CD45^{-/low} CD271⁺ cells was investigated. Enumeration data obtained using anti-CD271 conjugated to APC and anti-CD271 conjugated to PE (both from Miltenyi Biotec, clone ME20.4-1.H4) were compared with each other as well as with data obtained with anti-CD271-PE (clone C40-1457, BD) (n=3, Appendix 4). The representative experiment shown in Figure 4A-C illustrates the very similar results obtained with all three CD271 antibody variants, no statistical significant difference was found between the clones (average values of 4180, 3954 and 4198 CD45^{-/low} CD271⁺ cells/ml for CD271-APC, CD271-PE clone ME20.4-1.H4 and CD271-PE clone C40-1457, respectively). Additionally, since CD73 has been proposed as a useful marker for the assessment of BM MSCs (Veyrat-Masson et al. 2007), CD73-PE was evaluated alongside CD271-APC, with CD45 as a negative gating parameter (Figure 4D). The abundance of the CD45^{-/low}CD73⁺ population as well as the CD45^{-/low} CD271⁺ population were examined in 23 BM aspirates. There was an exceptionally high degree of correlation between these two populations (R=0.985, p<0.001, Figure 4E). This data confirmed the utility of CD73 as an alternative or additional marker to CD271.

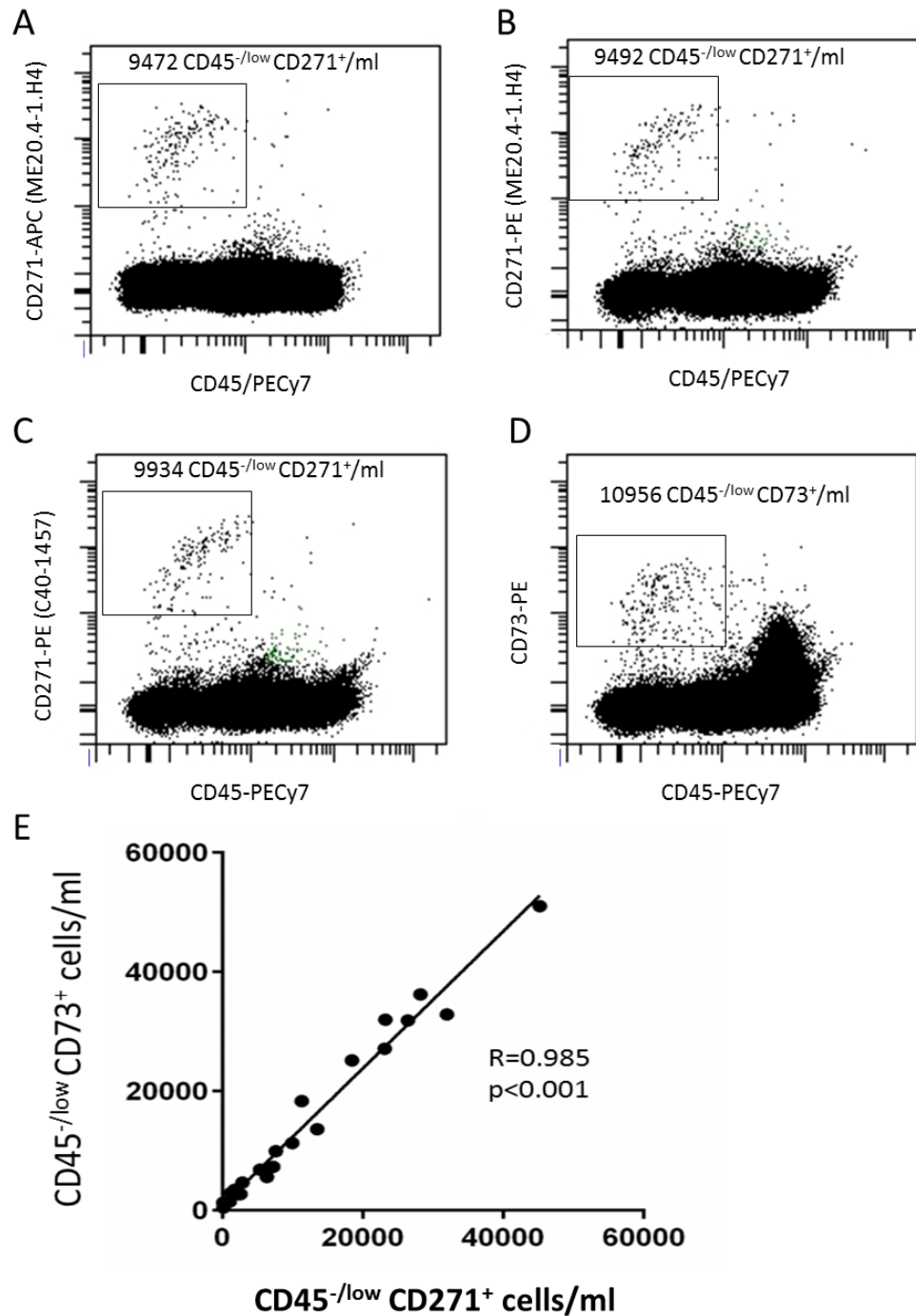


Figure 4: MSC identification and quantification using alternative antibodies

CD45^{-/low} CD271⁺ cells enumerated from a single BM aspirate using anti CD271 antibody clone ME20.4-1.H4 conjugated to APC (A) or PE (B) or using CD271 antibody clone C40-1457 conjugated to PE (C). CD45^{-/low} CD73⁺ cells enumerated from the same sample (D). The correlation between the CD45^{-/low} CD271⁺ and CD45^{-/low} CD73⁺ populations in BM aspirates n=23 (E).

3.3.2. The relationship between the concentration of CD45^{-/low} CD271⁺ cells and CFU-Fs

Examination of 25 BM aspirates showed, a close linear relationship was observed between the number of CFU-F colonies counted manually after 14 days of culture and the number of CD45^{-/low} CD271⁺ cells/ml of aspirate (R=0.812, p<0.001, Figure 5A). The median values of CFU-F/ml and CD45^{-/low} CD271⁺ cells/ml for the studied cohort were 280 and 7230 (ranges 3-1503 and 398-28210), respectively. The line of best fit suggested that approximately 1 in 17 CD45^{-/low} CD271⁺ cells formed a CFU-F colony in these experimental conditions.

The numbers of CD45^{-/low} CD271⁺ cells also correlated strongly with the total area occupied by CFU-F colonies (R=0.66, P<0.001, Figure 5B). The total area occupied by CFU-F colonies and CFU-F colony number correlated closely with each other (R=0.86, P<0.001).

3.3.3. Can enumeration of the CD45^{-/low} CD271⁺ population be used to predict CFU-F concentration

The ability of CD271-based enumeration assay to predict an aspirate's CFU-F content was next explored. A logarithmic data transformation was used to better display the whole range of values (particularly, bottom range) and a 95% confidence interval of predicted individual values was calculated to establish a cut-off point for the exclusion of specimens with potentially poor CFU-F content (<100 CFU-F/ml). As seen in Figure 5C, samples with greater than 10⁴ CD45^{-/low} CD271⁺ cells/ml (or 10 cells/ μ l) were 95% likely to produce good yields of CFU-F colonies (>100 CFU-F/ml). In contrast, the total cellularity of BM samples was a poorer indicator of the CFU-F content (R=0.516, p=0.003, Figure 5D). Representative photographs of duplicate CFU-F cultures with corresponding flow cytometry data of a low yield and high yield aspirate are shown in Figure 5E and F, respectively.

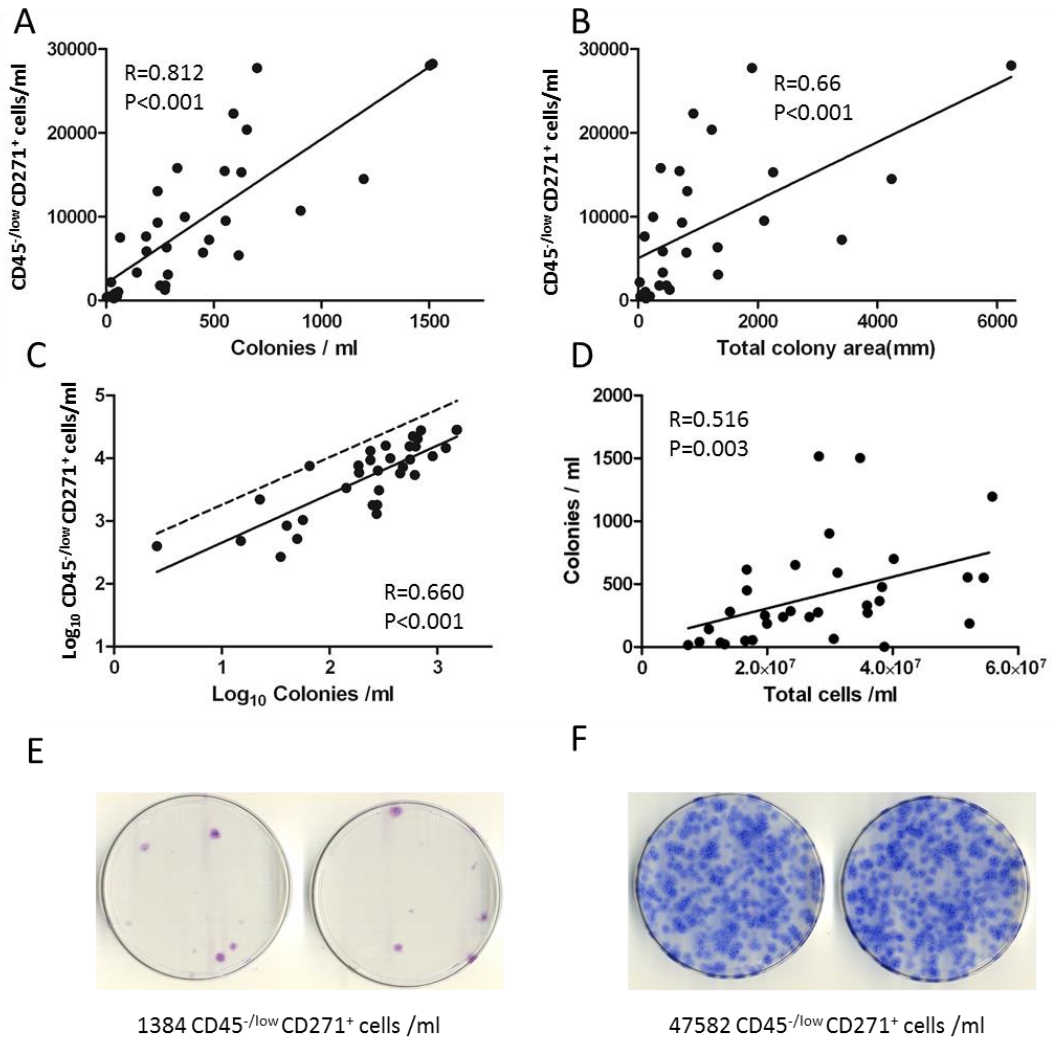


Figure 5: CFU-F colony measurements and correlations with CD45^{-/low} CD271⁺ population measurements.

Correlation between of CD45^{-/low} CD271⁺ population and number of colonies observed following CFU-F assay (A). Correlation between total colony area and the concentration of CD45^{-/low} CD271⁺ cells (B). Data from panel A plotted on logarithmic scale; dotted line represents upper 95% prediction interval (C). Correlation between BM cellularity and number of CFU-F colonies (D). All data A-D n=25. Representative image of duplicate dishes showing a low yield CFU-F sample (E) and high yield CFU-F sample (F), corresponding CD45^{-/low} CD271⁺ cell numbers are shown on the bottom.

3.3.4. Investigation of the CD45⁺CD271^{low} cell population

Aspirated BM consistently contained a population of cells that expressed CD45 and the CD271 antigen at a low level (CD45⁺ CD271^{low}, Figure 6A). In fact, this population was more numerous than CD45^{-/low} CD271⁺ cells (median frequencies of 0.073% and 0.026%, respectively). The absolute number of CD45⁺ CD271^{low} cells positively correlated with total sample cellularity (R=0.681, p<0.001, Figure 6B) suggesting that these cells originated from BM rather than blood, since blood has a much lower concentration of nucleated cells (Hollowell et al. 2005).

When the concentration of CD45⁺ CD271^{low} cells was compared to that of CD45^{-/low} CD271⁺ cells in matched donors, only a weak relationship was found (Figure 6C) and the same was observed for the correlation between CD45⁺ CD271^{low} cells and CFU-F data (Figure 6D). The presence of CD45 and the lack of a strong correlation with CFU-F indicated that CD45⁺ CD271^{low} cells were most likely haematopoietic in origin. A study published by Tormin *et al* came to the same conclusion based on morphology (Tormin et al. 2011).

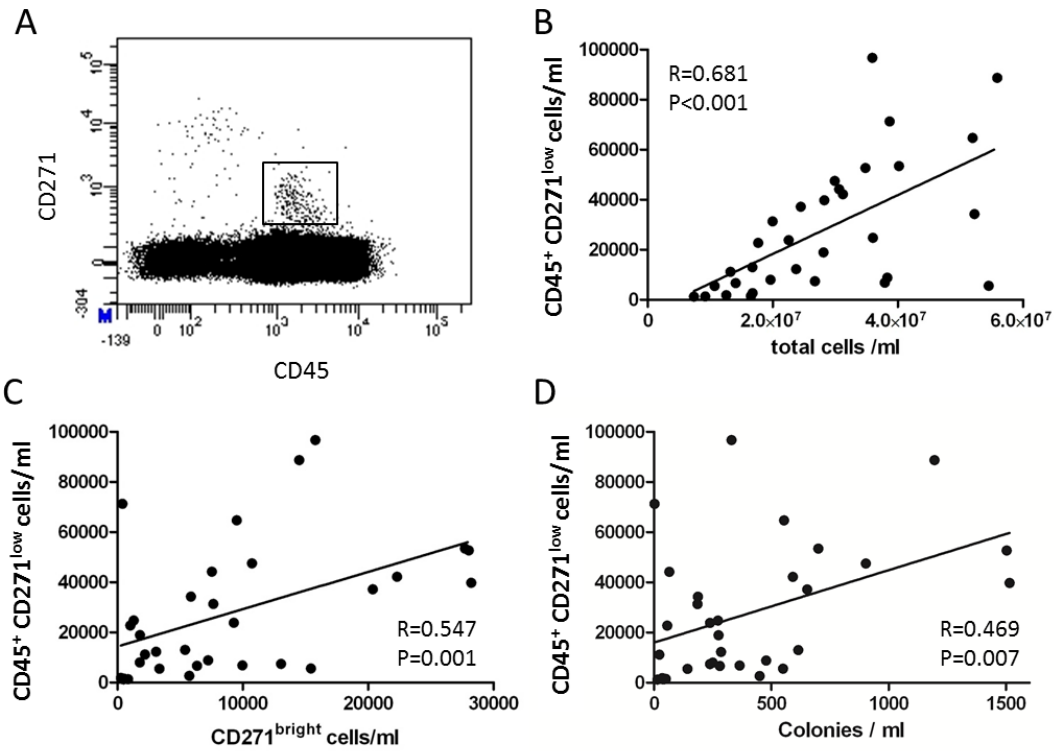


Figure 6: The CD45⁺ CD271^{low} population strongly correlates with sample cellularity but weakly correlates with CFU-F colony concentration.

(A) Representative flow cytometry plot showing gating for the CD45⁺CD271^{low} cell population (boxed). (B) Correlation between BM cellularity and the concentration of the CD45⁺ CD271^{low} population. (C) Weaker correlation between CD45^{-/low} CD271⁺ and CD45⁺ CD271^{low} populations. (D) Weak correlation between number of CFU-F colonies and the concentration of CD45⁺ CD271^{low} cells. All data B-D n=25.

3.3.5. Optimising BM aspiration

As shown on Figure 5, the MSC content of aspirates varies considerably. Although MSC content has been shown to vary in relation to donor age (Stolzing et al. 2008) (cohort's age range was 21-85), dilution of BM with blood may provide an additional source of variability (Veyrat-Masson et al. 2007; Hernigou et al. 2013). This may be the prime reason for the huge (up to 500-fold) variability in the MSC concentration observed.

In order to examine marrow dilution with blood and to optimise the collection of MSCs in BM aspirates (Objective 2), the effect of continuous aspiration was studied. The concentration of CD45^{-/low} CD271⁺ cells in the first 5ml of the aspirate was compared to that in the following 15ml aspirated from the same site, without any change to the insertion angle of the trocar (Figure 7A). The results demonstrated a significant 7-fold drop in the concentration of CD45^{-/low} CD271⁺ cells in the second, 15ml draw fraction (median concentration 2197 cell/ml, range 434-5391, n=7) compared to the first 5ml draw (median concentration 14450 cell/ml, range 3350-43936, p=0.018). The same trends were observed for CFU-F concentration, a 9-fold drop (p=0.028) in the 15ml draw fraction (median concentration 45 colonies/ml, range 23-274) compared to the first 5ml draw fraction (median concentration 410 colonies/ml, range 185-1515, Figure 7B).

Avoiding the dilution of BM with blood is recognised as an important factor in determining the MSC content in BM aspirates (Hernigou et al. 2002; Hernigou et al. 2005a; Hernigou et al. 2013). To examine the overall difference in aspirate quality between a single 20ml draw and 4 separate 5ml draws (n=7 and 13 donors, respectively), the total yield of CD45^{-/low} CD271⁺ cells (Figure 7C) and CFU-F colonies (Figure 7D) was compared. Although both these measures recorded higher median values in the 4x5ml category, these differences failed to reach statistical significance, most likely due to high donor variability, relatively low sample size and the fact that the two groups were not donor-matched. However, these data indicated that small draw volumes may provide higher concentrations of MSCs, this has recently been confirmed independently (Hernigou et al. 2013).

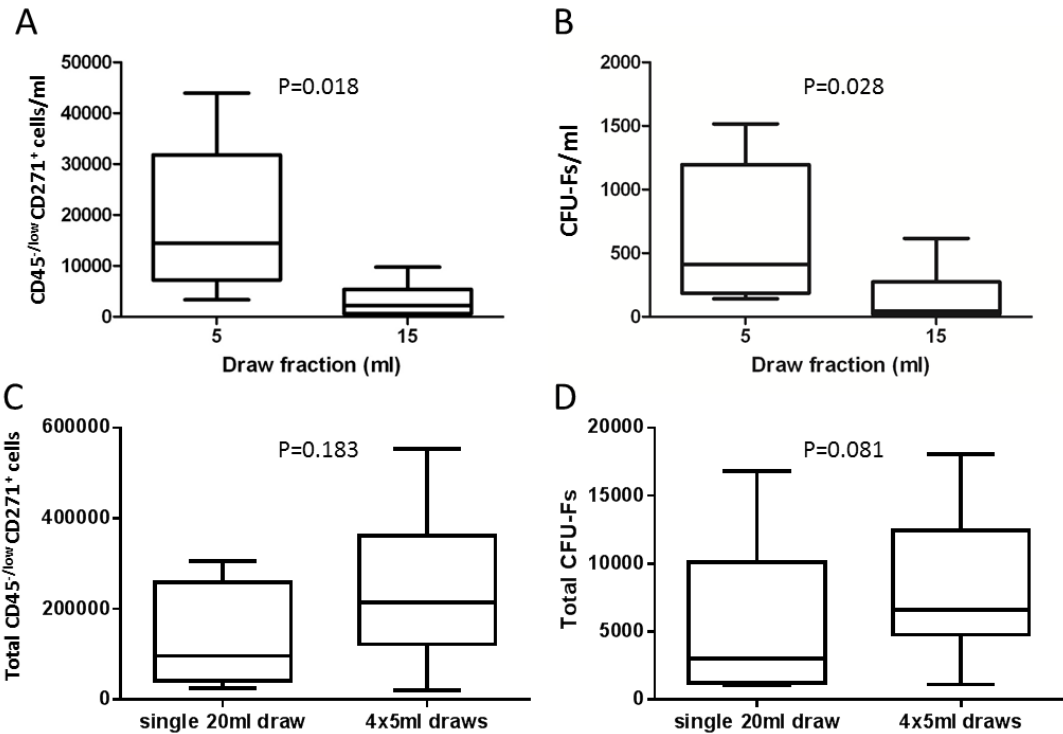


Figure 7: MSC concentration is dependent on BM aspiration technique

A higher number of CD45^{-/low} CD271⁺ cells/ml (A) or CFU-F colonies/ml (B) was observed in the first 5ml of aspirate compared with the following 15ml (n=7). Total number of CD45^{-/low} CD271⁺ cells (C) and CFU-Fs (D) collected by either one 20ml draw (n=7) or 4x5ml draws (n=13).

3.3.6. Further investigation of BM dilution with blood by studying the T-cell content of BM aspirates

The presence of T-cells in aspirates has been suggested to be an indicator of BM dilution with blood (Veyrat-Masson et al. 2007). To investigate the relationship between the MSC content of aspirates and dilution with blood, the absolute numbers and proportions of T-cells in aspirates was measured in a subset of samples (n=15). T-cells were identified by positive expression of CD3 and CD45 (CD3⁺ CD45⁺, Figure 8A). The median number of T-cells was 1.2x10⁵/ml (range 0.8x10⁵ – 2.6x10⁶) representing a median proportion of 7.13% of the total cells detected (range 3.64–9.46). This percentage is significantly below the reported median for CD3 T-cells in blood of 24.5% (Hulstaert et al. 1994). There was a close inverse relationship between the proportion of T-cells in the aspirate and the number of colonies formed in CFU-F assay (R=-0.761, p=0.001, Figure 8B). A much weaker but significant positive correlation was observed between the absolute number of T-cells/ml of aspirate and number of CFU-Fs/ml (R=0.581, p=0.023, Figure 8C). The apparent disparity between these two results can be partly explained by the observation that proportions and absolute numbers of T-cells in aspirates correlated poorly (R=-0.236, p=0.398, Figure 8D), meaning that T-cell proportion is a poor indicator of T-cell concentration in BM.

Furthermore, there was a positive correlation between the concentration of T-cells and CD45^{-/low} CD271⁺ cells (R=0.571, p=0.026, data not shown) as well as a tight positive correlation between T-cell number and the total cellularity of the aspirate (R=0.807, p<0.001, Figure 8E). Additionally, there was an inverse correlation between the proportion of T-cells and the total cellularity of the sample (R=0.729, p=0.002, Figure 8F). Blood is less cellular than BM; in agreement, BM cellularity was found to be in the range of 10-55x10⁶ nucleated cells/ml (Figure 2), this is higher compared to a normal range of 4-12x10⁶ nucleated cell/ml in peripheral blood (Hollowell et al. 2005). Therefore, aspirate cellularity is inversely proportional to inclusion of blood. Data collected on concentration and proportion of T-cells in aspirates indicates that T-cells represent a higher proportion of nucleated cells in

blood making the proportion rather than concentration of T-cells a more appropriate indicator of BM dilution with blood (Figure 9).

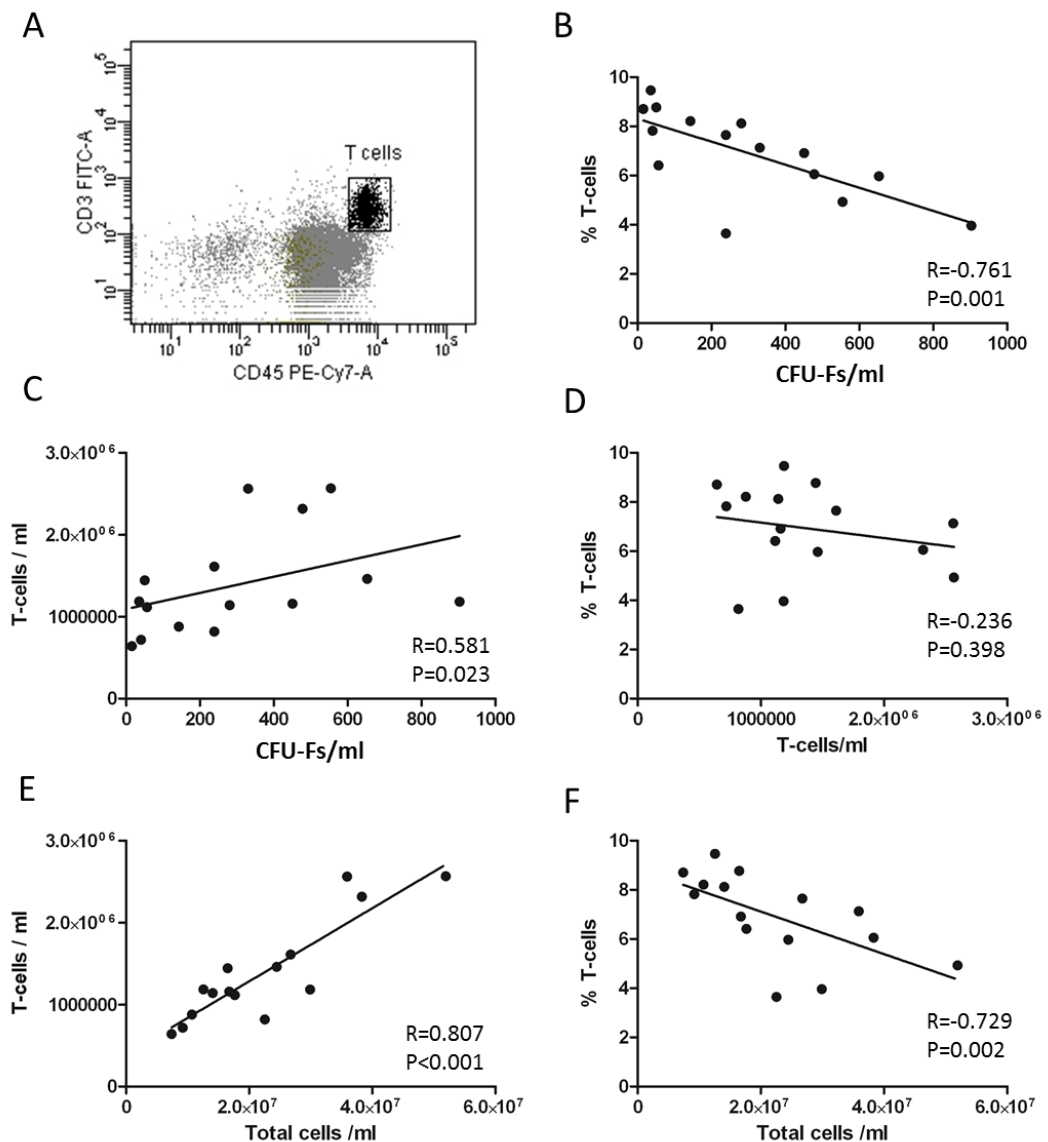


Figure 8: T-cells as an indicator of marrow dilution with blood

Identification of T-cell population (A). Inverse correlation of the proportion of T-cells present in the aspirate and CFU-F colony number (B). Positive correlation between absolute number of T-cells and CFU-F colony number (C). No significant correlation between T-cell proportion and their absolute number (D). Positive correlation between T-cell absolute number and total cellularity (E). Inverse correlation between the proportion of T-cells and the total cellularity of the aspirate (F). All data B-F n=15.

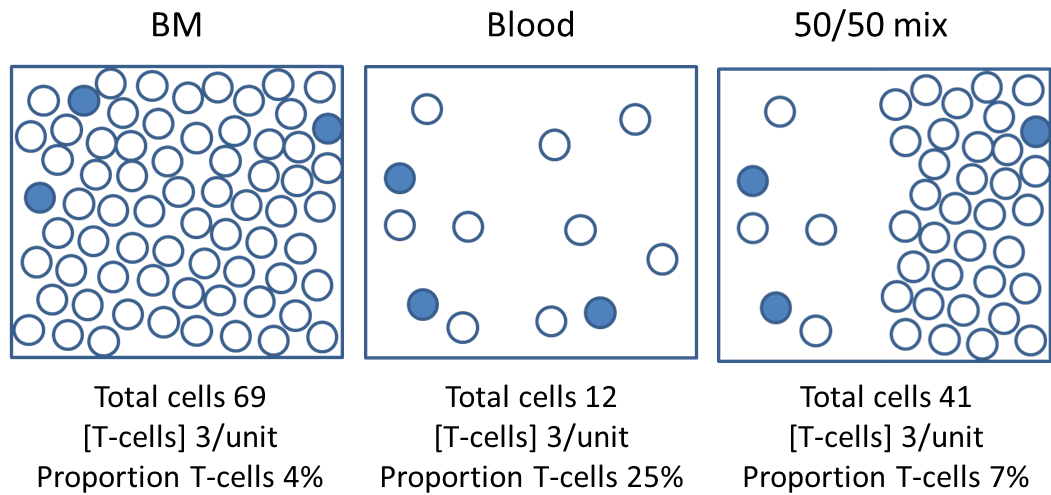


Figure 9: The relationship between total cellularity, T-cell concentration and T-cell proportion.

Figure illustrates BM mixing with blood, T-cells represented by filled circles, all other cells types represented by open circles.

3.4. Discussion

This chapter has demonstrated that a flow cytometry based technique could be used to predict the number of CFU-Fs that will develop from a given volume of aspirate and that the absolute number of the CD45^{-low} CD271⁺ cells per ml of aspirate was a strong indicator of its MSC content. This is supported by the strong correlation between CD45^{-low} CD271⁺ cell concentration and total colony area, indicating that once seeded CD45^{-low} CD271⁺ cells are capable of expansion.

In therapies that involve autologous MSC grafting, for example in the treatment of fracture non-unions, the dose of MSC has been shown to be critical in determining efficacy (Hernigou et al. 2005a). Therefore, the technique described herein may have value as an intra-operative quality control measure of BM aspirate. The utility of this approach is enhanced by the short duration of the assay (<40 minutes). If an aspirate is found to be of poor quality (below 100 CFU-F/ml equivalent to <1720 CD45^{-low} CD271⁺ cells/ml, Figure 5), a second aspirate could be quickly obtained to supplement or replace a poor first sample. It is possible that the application of acoustic focussing technology (Goddard et al. 2007) to this technique may further reduce assay time, perhaps to as little as 15 minutes. This is possible because acoustic focusing enables far faster data acquisition than can be achieved using conventional hydrodynamic focussing, this has the added advantage of not requiring large volumes of sheath fluid, thereby increasing instrument portability (Goddard et al. 2007).

In the bulk manufacture of MSCs the number of PDs needed to achieve a therapeutic yield of MSCs is critical in determining the characteristics of the resulting MSC product (Banfi et al. 2000; Banfi et al. 2002; Wagner et al. 2008). The MSC enumeration technique described in this chapter could provide a means of estimating the number of PDs required to achieve therapeutic yield prior to culture initiation, enabling the production of an MSC product with more predictable and consistent biological properties. Additionally the risk of cultures reaching senescence before achieving the required number of MSCs would be greatly reduced if low quality aspirates could be identified beforehand and excluded.

In order to maximise the absolute number of MSCs the technique used to obtain aspirates was also examined; this was addressed by objective 2 in this chapter. In the treatment of long bone non-unions large volumes of marrow (up to 300ml) are commonly harvested prior to volume reduction and percutaneous injection (Hernigou et al. 2005a; Kasten et al. 2008). This work has shown that following the first 5ml of BM aspirate, the MSC concentration drops dramatically. This may be due to the adherent nature of MSCs and their close association with bone (Jones et al. 2010b; Tormin et al. 2011) as well as gradual dilution of marrow with blood.

In order to maximise the number of MSCs aspirated from the iliac crest for the purpose of MSC expansion or direct therapy, this data suggests that each draw should be limited in volume, ideally to no more than 5ml, at a single site. Furthermore, a precise description of the aspiration technique used to attain BM samples should always be included in any publication seeking to identify changes related to aging or disease. The failure to standardise aspiration volume is a potential source of a large degree of variability in MSC content. Indeed, age related changes to MSC content in BM are a subject of some controversy, with widely disparate results reported (Oreffo et al. 1998; D'Ippolito et al. 1999; Baxter et al. 2004; Stolzing et al. 2008; Zhao et al. 2008). Although this may partly be explained by differences in cohort size and demographics as well as differences in enumeration methodology (Stolzing et al. 2008), it is important to note that in many cases no information is provided with regards to aspiration volume or technique (Oreffo et al. 1998; D'Ippolito et al. 1999; Baxter et al. 2004; Stolzing et al. 2008; Zhao et al. 2008).

The ability to detect the degree of blood dilution in BM would be advantageous for many fields of study. Hernigou et al proposed a method based on the relative haematocrit of BM compared to blood (Hernigou et al. 2013). Haematocrit is defined as the percentage of red cells in the blood, hence Hernigou's method relies on the assumption that BM makes a negligible contribution to haematocrit. Veyrat-Masson et al have proposed another method, namely that the proportion of T-cells may be used as an indicator of marrow dilution with blood; although not explicitly explained by the authors this may have been based on the fact that T-cells mature

outside of the BM (Gerondakis et al. 2014). Therefore, any T-cells detected in BM must originate from the blood. Veyrat-Masson et al did not establish a correlation between T-cell proportion and aspirate quality in femoral head aspirates (Veyrat-Masson et al. 2007). However, I observed a tight inverse correlation between the proportion of CD3⁺ cells and the MSC content of iliac crest aspirates. This disparity may be explained by the different anatomical regions aspirated as well as differences in the enumeration protocols used in each study. The percentage of cell expressing CD3 might be a useful indicator of blood dilution in iliac crest aspirates. This could easily be tested by deliberate mixing of matched blood and BM samples. It is possible that by taking matched peripheral blood at the same time as BM aspirates, a protocol for the quantitative measurement of BM dilution with blood could be developed.

The variability of MSC content in BM aspirates remains a challenge for research aimed at describing the *in vivo* characteristics of MSCs. Whilst dilution with blood is undoubtedly partly responsible, clotting of BM following aspiration can also influence MSC concentration. I have often observed clotting of BM aspirates even in the presence of anticoagulant, and associate clotting with poor aspirate quality. Also MSCs have been also shown to form aggregates with haematopoietic cells (Miao et al. 2004; Ahmadbeigi et al. 2012), and it is possible that MSCs are not distributed evenly throughout BM but are instead clustered into aggregates consisting of perhaps several hundred MSCs and other cell types. Indeed, non-homogenous distribution of MSCs is supported by the observations that total cellularity correlated only weakly with CFU-F concentration. These aggregates may themselves have a specific distribution being more or less common in a particular region; if this were the case, it would also contribute to sampling variability, particularly when comparing aspirate samples from disparate sites such as the posterior and anterior iliac crest. To address these issues investigators should aspirate from multiple sites, carefully report aspiration location and discard any clotted samples, in order to accurately report MSC numbers.

In summary, this chapter has described work to validate a robust, rapid and relatively straightforward assay for the determination of the absolute number of

MSC in a BM aspirate. This assay has the potential to improve bulk manufacturing of MSCs by enabling the production of a more standardised and therefore more predictable MSC product. As an intra-operative quality control it would allow clinicians to determine the number of autologous MSCs administered to patients or loaded onto scaffolds prior to implantation and therefore has potential to improve the efficacy of minimally-manipulated MSC therapy.

4. Optimising strategies for minimally manipulated MSC therapy

4.1. Introduction

4.1.1. Current clinical applications of culture expanded MSCs

The potential use of cultured MSCs as therapeutic agents has been recognised since their original description (Caplan 1991). To date clinical trials for a range of conditions including those of the skeletal system and non-skeletal system applications including cardiovascular, gastroenterological, and neurologic have been proposed (Table 1). Skeletal applications have included cartilage and bone repair in both the experimental and clinical settings (Hernigou et al. 2002; Wakitani et al. 2002; Hernigou et al. 2005a; Hernigou et al. 2005b; Dashtdar et al. 2011; Nakamura et al. 2012) and rarer indications including the therapy of osteogenesis imperfecta (Horwitz et al. 1999; Horwitz et al. 2002). Prominent examples of non-skeletal applications of cultured MSCs as cell therapy include acute myocardial infarction (Hare et al. 2009) and steroid resistant graft versus host disease (Le Blanc et al. 2004). Unfortunately many of these trials have produced variable outcomes (Trounson et al. 2011; Galipeau 2013; Raynaud et al. 2013). This may be due to differences in culture conditions, inadequate control of the number of cell divisions required to achieve a therapeutic yield and issues relating to cryopreservation leading to a loss of desirable phenotypic properties (Francois et al. 2012; Bianco et al. 2013a; Bianco et al. 2013b; Galipeau 2013).

4.1.2. The potential of minimally-manipulated MSCs for cell therapy

Non-expanded, minimally-manipulated cell therapies are subject to far less regulatory burden compared to culture expanded cell therapies (von Tigerstrom 2008; Deasy et al. 2013). Haematopoietic-cell (HC) transplantation is one example of a minimally manipulated cell therapy and represents the most successful and widely used cell based therapy after blood transfusion. In 2006 50,000 people in

500 or more centres were treated with HC transfusion worldwide for a range of haematological conditions (Appelbaum 2007). The development of similar technologies for homologous use of minimally-manipulated MSCs is therefore an attractive prospect.

However, several technical challenges have so far hampered the development of uncultured MSC based therapies, the most problematic of which is the low and variable concentration of MSCs in BM aspirates (Friedenstein et al. 1992; Pittenger et al. 1999; Jones et al. 2006). This was noted and addressed in the previous chapter as was the notion that the number of CD45^{-/low} CD271⁺ cells/ml could be used to predict the MSC content of BM aspirates. In this chapter a clinically-applicable MSC selection technology based on positive selection of CD271⁺ cells able to obtain uncultured BM MSCs is explored.

4.1.3. Autologous BM implantation in clinical orthopaedics

One strategy currently being utilized in clinical orthopaedics for the treatment of large bone defects and fracture non-union is to take a large amount of BM aspirate and reduce its volume. Concentrated BM can then be administered directly to the site of deficient bone healing, either by percutaneous injection or by mixing with an appropriate bone scaffold prior to implantation (Hernigou et al. 2005a; Hernigou et al. 2005b; Homma et al. 2014). Several systems have been developed that use filtration (Dawson et al. 2013; Otsuru et al. 2013), but most available systems utilise centrifugation to select nucleated cells based on their relative density (Hernigou et al. 2005a; Kasten et al. 2008; Giannini et al. 2009; Betsch et al. 2013; Lee et al. 2014). This approach has been employed by the MarrowStim (Biomet) and SmartPrep 2 (Harvest) concentrator devices (Appendix 6). Although several studies have confirmed the safety of this strategy and examined its clinical effectiveness (Hernigou et al. 2005a; Hendrich et al. 2009; Lee et al. 2014), relatively few studies have examined the effectiveness of individual concentrators in terms of their ability to concentrate MSCs.

4.1.4. Limitations of BM concentration approaches

The use of BM concentrates has limitations. Simply reducing the volume of the aspirate by removing erythrocytes and plasma is unlikely to substantially increase the proportion of MSCs in the concentrate. Since the majority of cells in a BM aspirate are of hematopoietic lineage (Jones et al. 2002; Jones et al. 2006; Veyrat-Masson et al. 2007), there is no reason to suggest that these cells do not constitute the overwhelming majority of the cellular content of BM concentrates. If considering allogeneic use, the administration of large numbers of unmatched hematopoietic cells to patients with an intact immune system is bound to result in dangerous immune reactions. Consequently, BM concentrators have not been considered for any allogeneic MSC therapy. Additionally, the reliance on centrifugation makes dealing with large volumes of liquid problematic, hence most concentrators are designed to concentrate no more than 60ml of BM (Kasten et al. 2008; Giannini et al. 2009; Lee et al. 2014). Also this strategy relies on the separation of a “buffy coat” of intermediate density (Hernigou et al. 2005a; Kasten et al. 2008; Giannini et al. 2009; Betsch et al. 2013; Lee et al. 2014), which may be difficult to identify and isolate in solutions other than blood and BM. These issues potentially limit the effectiveness of centrifugation for obtaining MSCs from sources other than BM aspirates.

4.1.5. CD271 expression as a means of MSC isolation

An alternative strategy is the use of a clinical grade cell separation system such as CliniMACS (Miltenyi Biotec) to positively select for uncultured MSCs (Figure 10A). Here, a cell suspension such as a BM aspirate (Figure 10B), is incubated with a magnetic microbead conjugated to a monoclonal antibody specific to an antigen of interest. Incubation of a cell suspension with magnetic beads results in specific antibody/bead binding to cells expressing a chosen antigen. Cells are then passed through a column in the presence of a magnetic field, bead bound cells are retained, whereas unlabelled cells are eluted. The magnetic field is then released and bead labelled cells are eluted.

This technology has already been used in clinical practice and has been confirmed to be safe for positive selection and administration of CD34⁺ HSCs (Ringhoffer et al. 2004), CD133⁺ HSCs and endothelial progenitor cells (Stamm et al. 2007), CD14⁺ monocytes (Holtl et al. 2005) and CD4⁺ CD25⁺ regulatory T-cells (Di Ianni et al. 2011), but has not been applied to MSCs. CD271 expression offers an attractive target for magnetic isolation of human BM MSCs due to its specificity for MSCs and relatively low cross-reactivity with hematopoietic lineage cells, as stated in previous chapters (Jones et al. 2002; Jones et al. 2006; Tormin et al. 2011; Bianco et al. 2013b).

A research grade bead has already been used for the selection of BM MSCs in the laboratory setting and has been proven to be effective (Jarocha et al. 2006; Jones et al. 2006; Jarocha et al. 2008). Recently, the development of a clinical grade CD271 microbead has opened the possibility of using this technology as the basis of a new non-expanded MSC therapy.

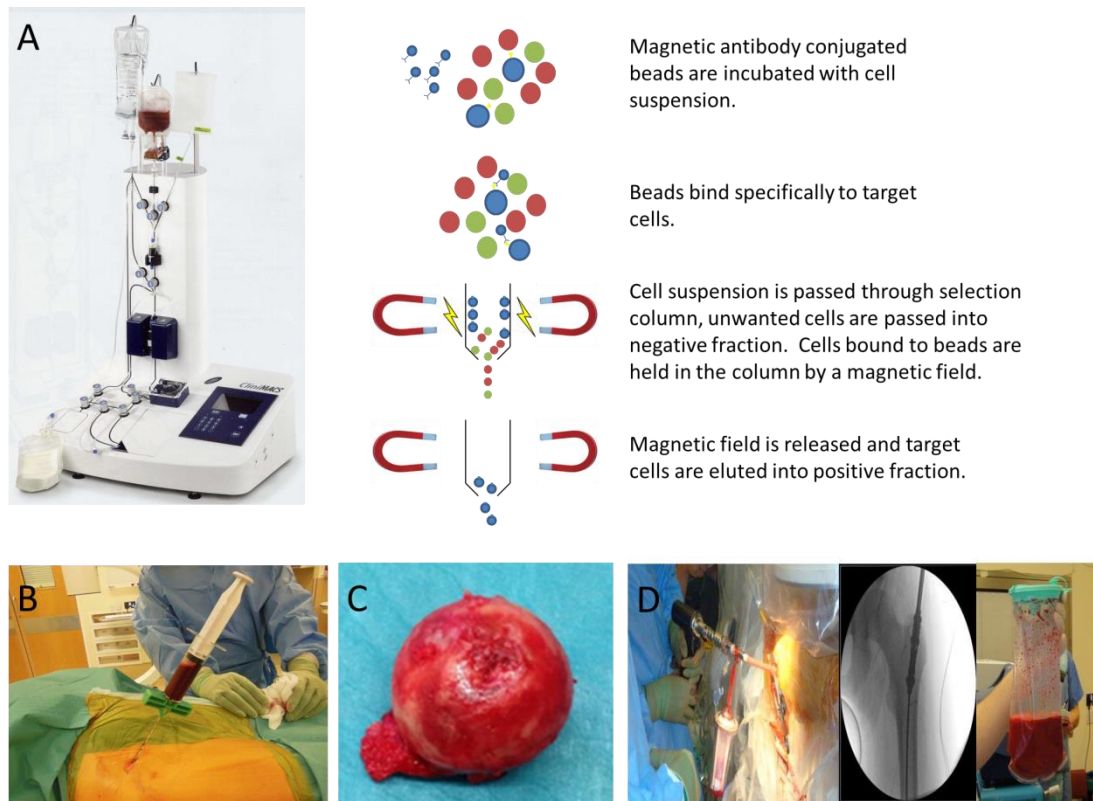


Figure 10: The CliniMACS system and three intra-osseous sources of MSCs used on this study

The CliniMACS device and a cartoon explanation of the microbead technology used (A) (adapted from Miltenyi Biotec). A freshly drawn BM aspirate (B). A femoral head prior to processing (C). A reaming procedure and a bag of RIA waste fluid (D) (Adapted from Churchman *et al* 2013). Operating theatre images were kindly provided by P.V. Gainnoudis.

4.1.6. Alternative sources of BM MSCs

Several investigations have shown that large numbers of MSCs can be recovered from trabecular bone with enzymatic extraction (Noth et al. 2002; Tuli et al. 2003; Sakaguchi et al. 2004), this led to the hypothesis that aspiration is a relatively inefficient means of collecting MSCs from the marrow. Recently, Jones *et al* demonstrated that upon digestion with collagenase, MSCs could be recovered as a far greater proportion of total cellularity from pelvic bone fragments compared to BM aspirates (Jones et al. 2010b). Cell sorting based on the negative or low expression of CD45 and positive expression of CD271 isolated cells with equivalent proliferative, osteogenic, and phenotypic properties to BM MSCs (Jones et al. 2010b). This dispels the dogma that BM MSCs are an extremely rare population and gives a potential source of large numbers of clinically useful MSCs. Additionally the high incidence of total hip replacement (National Joint Registry for et al. 2011), provides a plentiful source of otherwise discarded femoral heads (FHs), that could serve as the starting material for MSC extraction (Figure 10C). Similarly, Porter et al showed that both the solid and liquid waste fractions of intramedullary reaming waste following use of the reamer irrigator aspirator (RIA) device contained large numbers of MSCs (Porter et al. 2009). Cells sorted based on CD271 expression from the liquid waste fraction (Figure 10D) were later shown to have similar phenotypic and transcriptional profiles to BM MSCs (Churchman et al. 2013), making this another source of large numbers of CD271⁺ MSCs.

Fluorescence activated cell sorting has been an invaluable tool for phenotypic identification of MSCs. However, this technique is not suitable for clinical isolation of MSCs, due to lengthy sort times and high potential for pathogen carry over. Therefore, cell selection using a single antigen (CD271) using a fully clinical grade system could offer a viable alternative to cell sorting.

4.1.7. Chapter aim and objectives

The aim of this chapter is to test the currently available technology for MSC concentration and enrichment and to assess their potential for therapy development.

Objectives:

1. To evaluate and compare two commercially available BM concentrators, the MarrowStim and SmartPrep 2, with respect to their ability to concentrate MSCs.
2. To develop a protocol for the positive selection of MSCs from iliac crest aspirates, using clinical-grade CD271 microbeads and the CliniMACS cell separator, using equipment and reagents which comply with good manufacturing practice standards throughout.
3. Given the low numbers of MSCs in aspirates, to investigate enzymatically digested femoral heads and the liquid waste component generated by the RIA device as potential sources of MSCs suitable for clinical-grade CD271 based cell selection.

4.2. Materials and methods

4.2.1. Patient recruitment

For BM aspirate samples, patients undergoing elective orthopaedic surgery for metalwork removal or non-union revision were invited to participate. For the comparison of BM concentrator devices, aspirates were collected for concentration using the SmartPrep 2 device (Harvest) (n=8, mean age 52 years, 2 male 6 female) or the MarrowStim BM concentrator (Biomet) (n=10, mean age 57 years, 6 male 4 female). For CliniMACS experiments, BM samples were selected based on high MSC content using the enumeration assay described in chapter 3 (n=3, 1 male 2 female with a mean age of 32). Patients with any underlying disease for example osteoporosis, osteosarcoma and osteogenesis imperfecta were excluded.

For the collection of FH samples, patients admitted for total hip arthroplasty for the relief of osteoarthritis pain were invited to participate (n=3, all male mean age 72; patients that underwent arthroplasty for any other reason for example as a result of hip fracture or rheumatoid arthritis were excluded. For RIA waste fluid samples, patients admitted for the treatment of fracture non-union involving autologous grafting of cortical bone were invited to participate (n=3, 1 male 2 female mean age 47).

4.2.2. Concentration of BM aspirates using the MarrowStim™ and SmartPrep 2® BM devices

A concentrated BM aspirate was prepared by the operating surgeon, using either MarrowStim or SmartPrep 2 device as a part of the elective procedure undertaken for non-union revision surgery, according to the manufacturer's instructions (Appendix 6). For both devices, a trocar was inserted in the anterior iliac crest and 50ml of BM was then drawn from a single insertion. This was loaded into the devices in the presence of 5ml anticoagulant citrate dextrose solution A (ACDA) and subsequently centrifuged. In the case of MarrowStim, this procedure results in BM being separated into three compartments containing serum, erythrocytes or

nucleated cells; the latter were then drawn off using a syringe and had a mean volume of 7.5ml (n=10). In the case of SmartPrep 2, BM is separated into two compartments containing erythrocytes or nucleated cells below a layer of serum. Serum is removed prior to collection of the nucleated cells which occupied a mean final volume of 6.7ml (n=8). A small sample of the BM before concentration (1ml, denoted Pre) as well as the concentrated sample (1ml, Post) were taken for analysis of their MSC content by the CFU-F assay (2.7) and flow cytometry (2.5).

4.2.3. Femoral head processing using bone mill and collagenase digestion

Removed, FHs were bisected by the operating surgeon and stored immersed in sterile saline, overnight at 4°C in a specimen pot. The following morning FHs were removed from saline, and broken into small fragments (<1g) using a sterile Noviomagus bone mill (De Puy). The wet weight of fragments was recorded and the sample was digested with collagenase to release single cells, as previously described (2.4). Once filtered to remove larger aggregates, the cell suspension was taken for MSC isolation as described (4.2.5) without washing or centrifugation.

4.2.4. Reamer irrigator aspirator waste fluid processing

The RIA device (Synthes Inc) was operated by the surgical team during the revision of long bone non-unions (Giannoudis et al. 2009; Porter et al. 2009; Cox et al. 2011a; Cox et al. 2011b). It is a one-pass reamer that allows simultaneous irrigation and aspiration of tibial or femoral contents disrupted during reaming (Giannoudis et al. 2009; Porter et al. 2009; Cox et al. 2011a; Cox et al. 2011b). The system was developed in order to eliminate the risk of systemic embolism caused by increased inter-medullary temperature and pressure during conventional reaming (Muller et al. 2006; Giannoudis et al. 2009). After its adoption into clinical practice both the solid and liquid phases of the RIA aspirate were shown to contain substantial

amounts of bioactive and osteogenic factors and cells including MSCs (Porter et al. 2009; Cox et al. 2011b; Cox et al. 2012).

For this study, only patients undergoing femoral reaming were selected in order to maximise the amount of cellular material collected. Briefly, a drill was used to open the tip of the greater trochanter and a guide wire was inserted down to the distal metaphyseal region of the femur (Giannoudis et al. 2009). The RIA device was mounted onto a guide wire and reaming began; a constant flow of sterile saline was directed toward the reamer head where it mixed with disrupted femoral canal contents. The saline/tissue mixture was then drawn away by suction and the solid phase was retained in a collection filter; this material was used by the surgeon as autologous graft (Giannoudis et al. 2009). The liquid phase passed to a waste bag; it is this material that has been utilized in these experiments. The volumes of fluid collected were 859, 958 and 783ml.

4.2.5. MSC isolation from BM aspirate, FH cellular fractions and RIA fluid using the CliniMACS system

A small volume (1ml) of BM aspirate, digested FH cell suspension and RIA liquid phase waste was retained for analysis by CFU-F assay and flow cytometry; this will be referred to as the pre enrichment fraction (Pre).

The remaining sample was transferred into a sterile silicone transfer bag (Miltenyi) and transported to the Seacroft NHS blood and tissue facility. The volume of liquid in the bag was adjusted to 500ml by addition of GMP-grade PBS, with EDTA (Miltenyi) containing 5% w/v GMP-grade human serum albumin (HSA) (Bio Products Laboratory). This was done by attaching a separate bag containing the PBS/EDTA/HSA solution to the transfer bag using a TSCDI sterile tube welder (Terumo) and allowing buffer to flow into the transfer bag. Once the correct volume had been achieved, the buffer bag was detached using a sterile tube fuser (Terumo) and the transfer bag was centrifuged at 400xg for 20 minutes. The supernatant was subsequently removed, into a second transfer bag, attached using a sterile tube welder, by gently squeezing the bag using a plasma expresser (Fenwal) until a

minimal volume (<50ml) of supernatant remained. The second transfer bag was then detached using a sterile tube fuser (this will be referred to as the 1st wash fraction) and the cell pellet was re-suspended in the remaining supernatant. If more than one transfer bag was required due to the large initial volume of the cell suspension, the contents of both transfer bags were combined in a single transfer bag at this point.

The volume in the final transfer bag was adjusted to 95ml by addition of PBS/EDTA/HSA solution as above, to which the contents of one vial of anti-CD271 clinical-grade microbeads were added using a syringe and needle. The cell suspension was then incubated for 30 minutes on a tilting shaker (Stuart) at RT. Subsequently, the volume was adjusted to 500ml with addition of PBS/EDTA/HSA, followed by centrifugation, removal of supernatant (referred to the 2nd wash fraction), and volume adjustment to 95ml, as detailed above. At this stage, 1ml of the cell suspension was taken using a sterile syringe for CFU-F/flow cytometry analysis; this will be referred to as the before separation (BS) fraction. The transfer bag was loaded onto the CliniMACS device and attached to a CliniMACS tubing set (Miltenyi). An automated protocol originally intended for CD34 isolation (Dvorak et al. 2013) was initiated resulting in separation of the cell suspension into two fractions: a positive fraction containing CD271 expressing cells (Post) and a negative fraction containing all other cell types (Negative fraction).

4.3. Results

4.3.1. Comparison of MarrowStim and SmartPrep 2 concentrator devices for their ability to concentrate MSCs

Total nucleated cells were concentrated 5.0-fold using the MarrowStim device ($p=0.028$, Figure 11A) and 6.3-fold using the SmartPrep 2 device ($p=0.012$, Figure 11B). In terms of concentrating MSCs, the MarrowStim was successful on 9 of 10 trials resulting in a median 7.3-fold increase in the numbers of MSCs/ml, as measured by the CFU-F assay ($p=0.007$, Figure 11C). The SmartPrep 2 concentrator was successful on 7 of 8 trials resulting in a median of 2.8-fold increase in MSCs, but this was short of significance due to one failed trial ($p=0.093$, Figure 11D).

To assess whether MSCs were enriched or simply concentrated in a smaller volume, the proportion of MSCs in the Pre and Post-concentration fractions was analysed in relation to total nucleated cells. No enrichment in MSCs was observed following the use of either device (Figure 11E). The proportions of CD45⁺ leukocytes remained unchanged following processing with either device (Figure 11F).

The MSC contents of BM aspirates Pre-concentration using the MarrowStim device were particularly low (median of 11 CFU-F/ml), this may be related to the requirement of collecting an aspirate as a single large-volume (50ml) draw, leading to dilution of marrow with blood (Hernigou et al. 2013). Aspirates collected, using a single 50ml draw, prior to SmartPrep 2 concentration had higher concentrations of MSCs (median of 258 CFU-F/ml), but were still poorer compared to those harvested using an optimised low volume draw technique (4x5ml) as detailed (2.2) (a median of 455 CFU-F/ml, Figure 11G).

These results clearly showed that both devices were able to concentrate MSCs (and other cells) into a smaller volume, but they did increase the purity of MSCs.

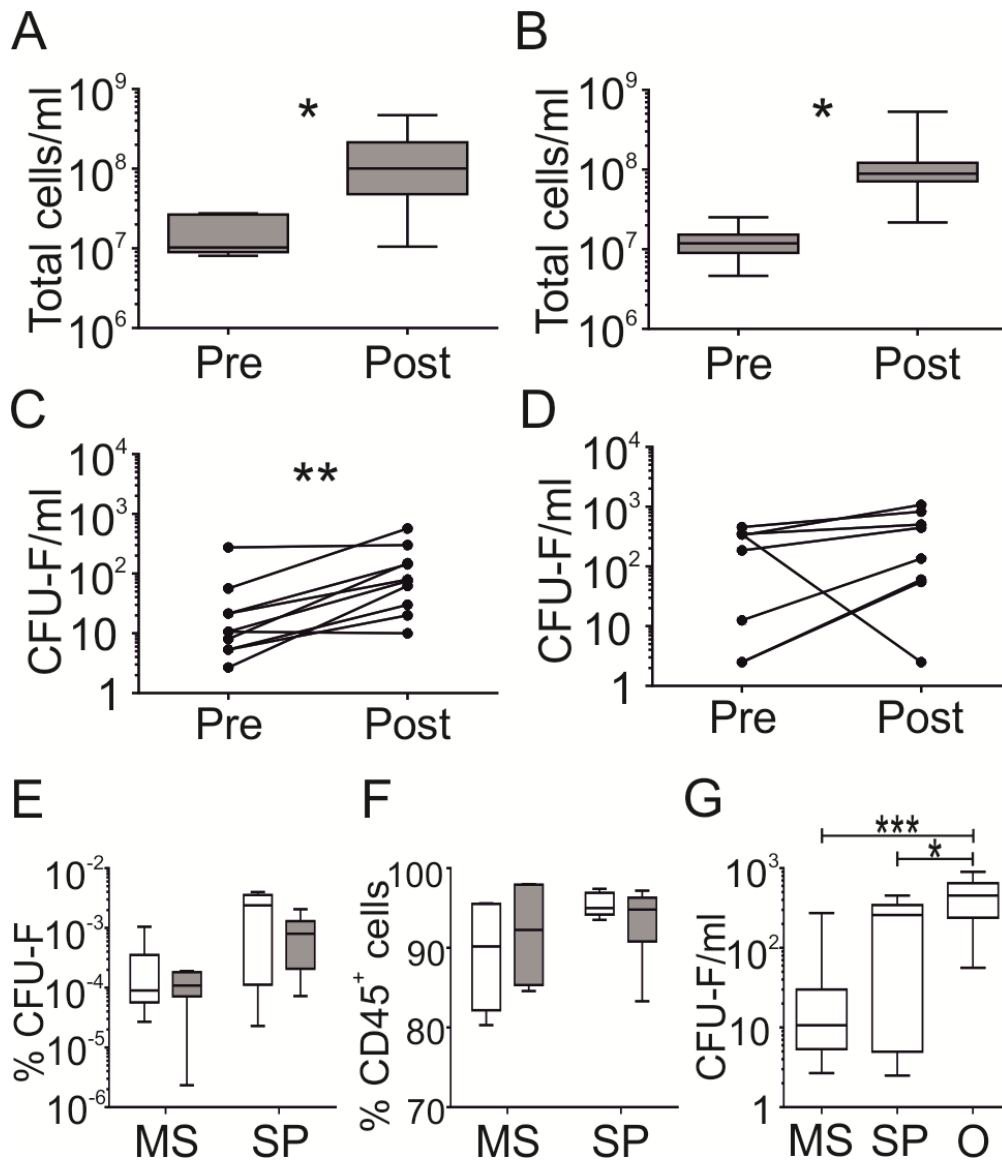


Figure 11: Comparison of MarrowStim and SmartPrep 2 concentrator devices

The total nucleated cell content of the Pre and Post concentration fractions processed using the MarrowStim (A) and SmartPrep 2 (B) devices. MSC content Pre and Post concentration using MarrowStim (C) and SmartPrep 2 (D) devices. The percentage of MSCs (E) and CD45⁺ cells (F) in Pre (white box) and Post (grey box) fractions following the use of MarrowStim (MS) and SmartPrep 2 (SP) devices. MSC content of BM aspirates prior to concentration with MS or SP compared to optimised (O) aspiration technique n=13 (G). All MS data n=8 donors, all SP data n=10 donors, box plots show interquartile range (box) median (line) and extreme values (whiskers), *p<0.05, **p<0.01, ***p<0.001.

4.3.2. Enrichment of MSCs using the CliniMACS System

Given that magnetic bead technology relies on binding of microbeads to surface CD271 molecules, there is a possibility that this could compromise further binding of 'detection' anti-CD271 antibodies. Furthermore, transient binding of microbeads could also result in internalisation of the CD271 molecule (Carcenac et al. 2001); both factors could therefore affect MSC purity determination following selection if relying on detection of CD271 expression. In order to ensure accurate determination of MSC number and purity by flow cytometry following anti-CD271 CliniMACS separation, CD73 was utilized as an alternative marker for quantification of MSCs, since this has already been demonstrated as a useful alternative marker to CD271 as detailed in (Figure 4E).

Following CliniMACS cell separation, the proportion of cells expressing a CD45^{-/low} CD73⁺ phenotype was measured in the Pre (Figure 12A) and Post-enrichment fractions (Figure 12B). The proportion of cells expressing a CD45^{-/low} CD73⁺ CD271⁺ phenotype was also measured Pre and Post enrichment (Figure 12C-D).

In the Pre enriched BM, FH and RIA the mean proportion of cells expressing a CD45^{-/low} CD73⁺ phenotype was 0.078%, 7.31% and 0.25%, respectively. This was consistent with previously-published data from Leeds and elsewhere (Jones et al. 2006; Jones et al. 2010b; Alvarez-Viejo et al. 2013; Churchman et al. 2013). These proportions were increased in the Post fraction to 14.5%, 60.0% and 40.8% for BM, FH and RIA samples, respectively (Figure 13A). Similarly, the proportion of cells expressing a CD45^{-/low} CD73⁺ CD271⁺ phenotype was also increased in BM, FH and RIA from a mean of 0.066%, 3.72% and 0.12% to 13.5%, 52.4 and 34.9%, respectively (Figure 13B). This data suggests that the potential effect of antigen masking or internalisation as a result of microbead binding was minimal.

However, some disparity was observed between the proportion of CD45^{-/low}CD73⁺ and CD45^{-/low}CD73⁺CD271⁺ cells after enzymatic digestion of FH. This could have led to over estimation of the number of MSCs as defined by the CD45^{-/low}CD73⁺ phenotype in the Pre enrichment fraction. Inclusion of CD271 as a gating parameter using the same antibody clone used for selection resulted in only a minor alteration

to the proportion of MSCs detected in the Post enrichment fraction, one possible explanation for these observations is that microbeads are not present in excess allowing further binding of detection antibody.

Unfortunately no data was collected for the mean proportion of CFU-Fs Pre and Post enrichment in BM aspirates, this was due to the low overall numbers of cells isolated from this source, but in FH and RIA these rose from 0.56% and 0.0049% to 5.57% and 0.85% respectively (Figure 13C).

In summary CliniMACS technology was successful in enriching MSCs from all 3 tissues tested, proving the concept of clinical grade CD271 based MSC enrichment.

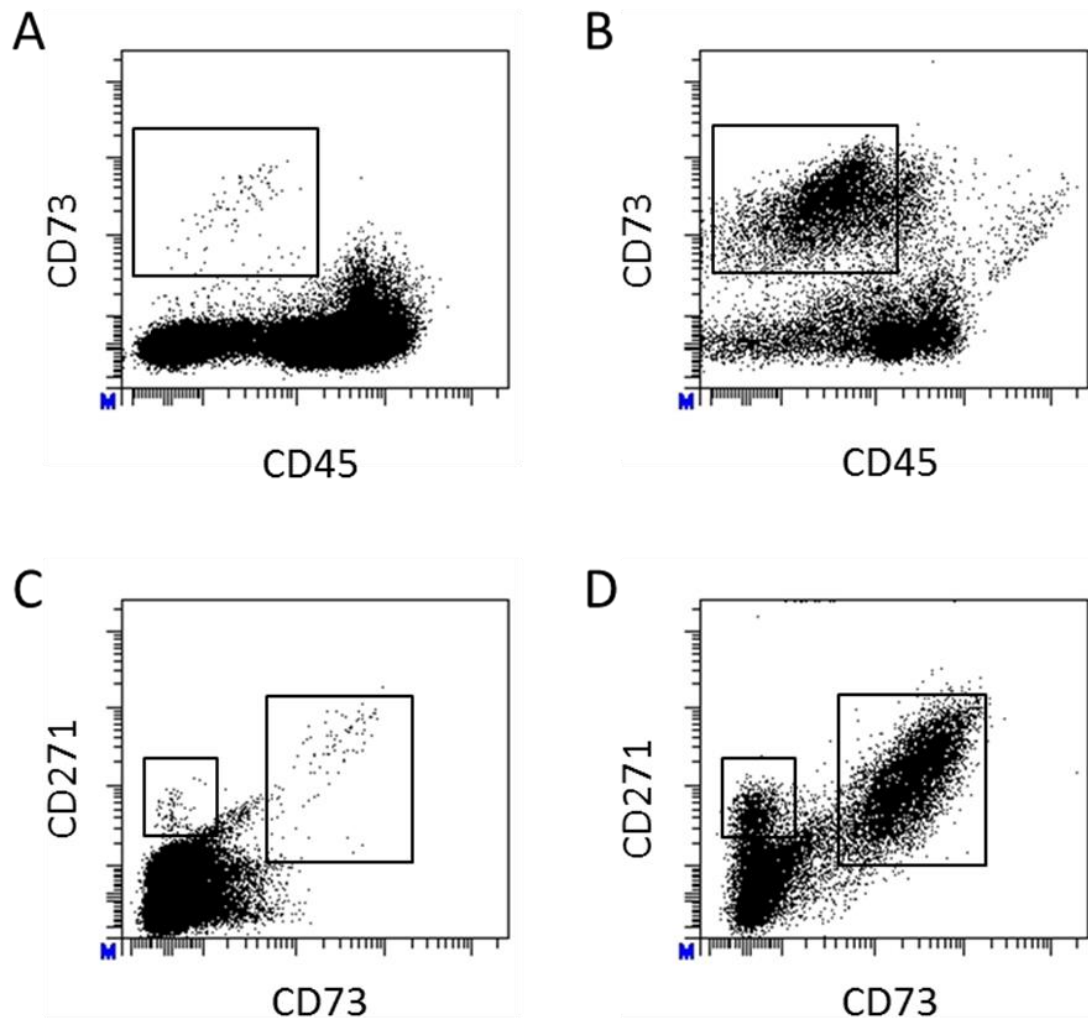


Figure 12: An example of MSC gating strategy Pre and Post CliniMACS enrichment

Representative matched dot plots showing the $CD45^{-/low} CD73^{+}$ population in unprocessed BM (Pre) (A) and the positive (Post) fraction (B). Representative matched dot plots showing the $CD45^{-/low} CD73^{+} CD271^{+}$ population (double-positive, large square) and $CD45^{+} CD271^{low} CD73^{-}$ population (single positive, small box) in unprocessed BM (C) and the processed positive fraction (D).

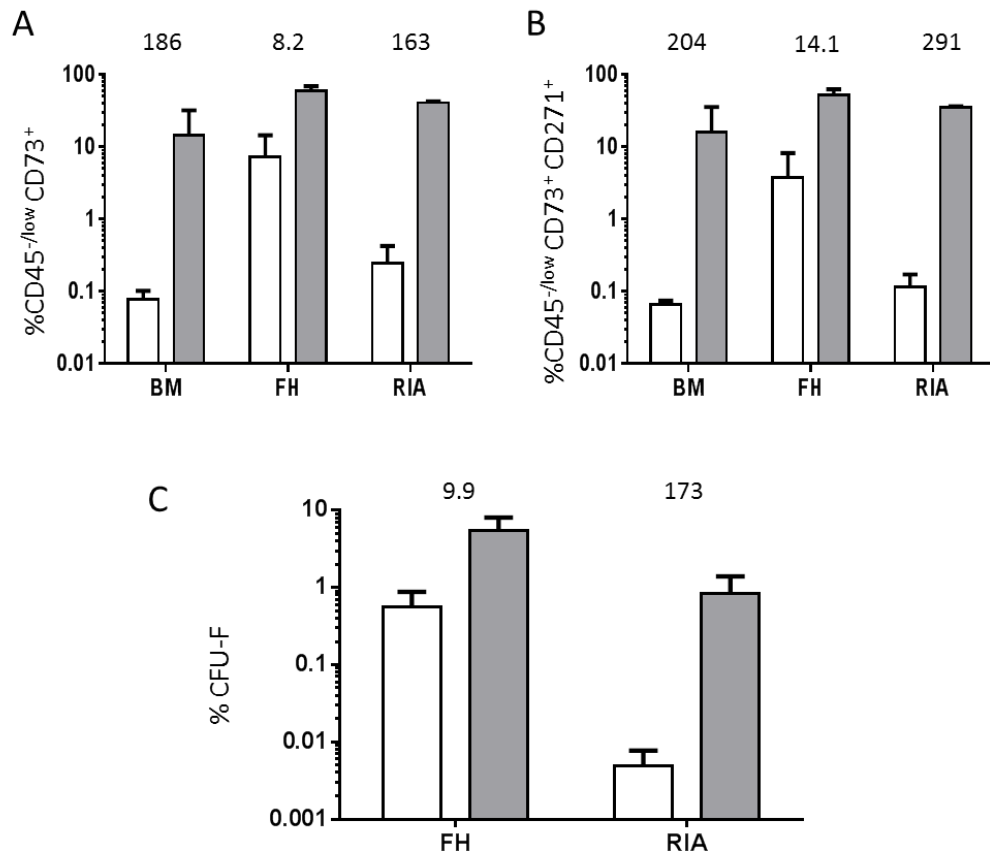


Figure 13: Enrichment of MSCs using the CliniMACS system

The mean percentage of the CD45^{-/low} CD73⁺ population (A) and the double-positive CD45^{-/low} CD73⁺ CD271⁺ population (B) measured by flow cytometry Pre (white bar) and Post (grey bar). The mean proportion of CFU-Fs Pre (white bar) and Post (grey bar) CliniMACS enrichment (C). Values above each box pair show mean fold increase. All data n=3 displayed as mean and standard deviation.

4.3.3. Depletion of unwanted cells and assessment of total cell viability following CliniMACS enrichment

The contamination of the Post enrichment, positive fraction with other cell types (non-MSCs) is a critical factor when considering this technology for clinical therapy development. This is of particular importance when considering allogeneic use. Although MSCs do not illicit an immune response (Ren et al. 2009; Francois et al. 2012; Krampera et al. 2013) other cell types would, potentially with serious consequences if administered systemically.

CD45⁺ cells, leukocytes (Mason et al. 1987) represent the majority of non-MSC cells in a BM aspirate. Before enrichment, the mean proportion of cells expressing a CD45⁺ CD271⁻ CD73⁻ phenotype in BM, FH and RIA samples were 80.3%, 67.8% and 86.1%, respectively. These cells were depleted in each case to 18.7%, 20.3% and 35%, respectively, in the Post enriched fraction (Figure 14A). Although CD45⁺ cells were depleted substantially there was still a considerable proportion remaining in the Post enrichment fraction.

There was also a significant population of CD45⁻ cells in BM aspirates, cells expressing a CD45⁻ CD271⁻ CD73⁻ phenotype are possibly erythroid progenitor cells and RBCs (Della Porta et al. 2006), They initially represented a mean proportion of 18.2%, 23.5 and 13.4% of the cellular content of the Pre fraction of BM, FH and RIA, respectively; this was substantially increased following enrichment to 59.2% in BM, but was reduced in FH and RIA to 14.2% and 5.1%, respectively (Figure 14B).

Additionally as detailed in Chapter 3, BM contains a significant portion of cells that express CD271 at a low level as well as the CD45 antigen (CD45⁺ CD271^{low} CD73⁻) shown in Figure 12C. Since the microbeads used in this CliniMACS-based procedure are specific to CD271 only, contamination of the Post enrichment positive fraction with this 'passenger' cell type was anticipated. There was however, a relatively modest increase in this population in BM and FH from a mean of 0.48% and 1.42% to 7.10% and 5.46%. When RIA samples were analysed, a more substantial increase in the proportion of these cells was detected, from 0.24% to 18.2% (Figure 14C).

Lastly, it is important to note that total cell viability was not substantially compromised by the enrichment process. Mean cell viability, measured by exclusion of DAPI, dropped slightly after enrichment in BM and RIA from 92.6% and 97.7% to 89.2% and 91.4%. There was a more substantial drop in viability in FH samples, from 89.4% to 74.6% (Figure 14D), possibly due to increased sample storage, and enzyme treatment.

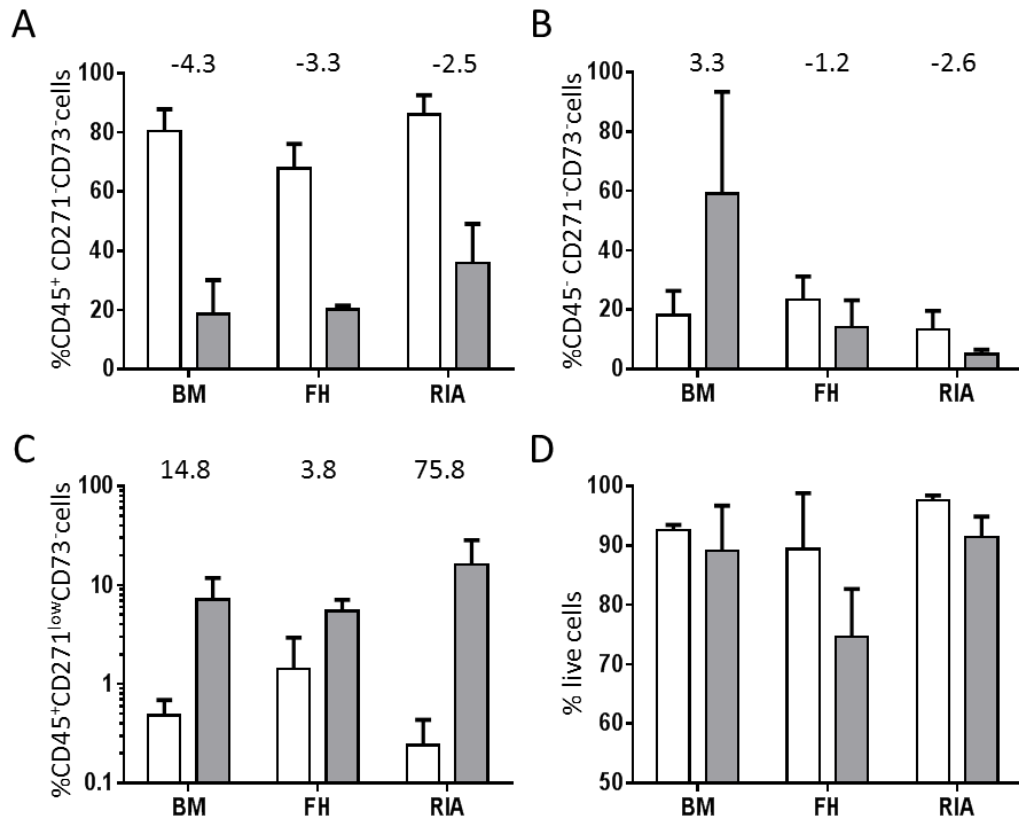


Figure 14: Unwanted cell content and total cell viability Pre and Post CliniMACS enrichment

The proportion of cells expressing a CD45⁺CD271⁻CD73⁻ (A), CD45⁻CD271⁻CD73⁻ (B) and CD45⁺CD271^{low}CD73⁻ (C) phenotype Pre (white bar) and Post (grey bar) CliniMACS enrichment procedure. Values above each box pair show mean fold change. The proportion of viable cells Pre (white bar) and Post (grey bar) CliniMACS enrichment (D). All data n=3 displayed as mean and standard deviation.

4.3.4. Examining the MSC enrichment efficiency of the CliniMACS system

Volumetric flow cytometry and CFU-F assays were used to assess the absolute number of MSCs present in the Pre enriched samples and in the Post enriched positive fractions. The total number of CD45⁻CD73⁺ cells in BM, FH and RIA dropped from 6.4×10^5 , 5.0×10^7 and 1.1×10^7 to 2.9×10^4 , 1.88×10^6 and 3.08×10^5 , respectively (Figure 15A). A similar drop was observed in the number of double-positive (CD45⁻CD73⁺CD271⁺) cells in BM, FH and RIA, from 5.1×10^5 , 2.6×10^7 and 5.8×10^6 , to 2.9×10^4 , 1.6×10^6 and 2.7×10^5 , respectively (Figure 15B).

Further confirmation of substantial losses in MSC numbers was given by analysis of the total number of CFU-Fs in the Pre and Post enrichment fractions. No data was collected for BM aspirates, but the mean total number of colonies in FH dropped from 7.5×10^6 to 2.4×10^5 and from 1.8×10^5 to 1.2×10^4 in the RIA (Figure 15C).

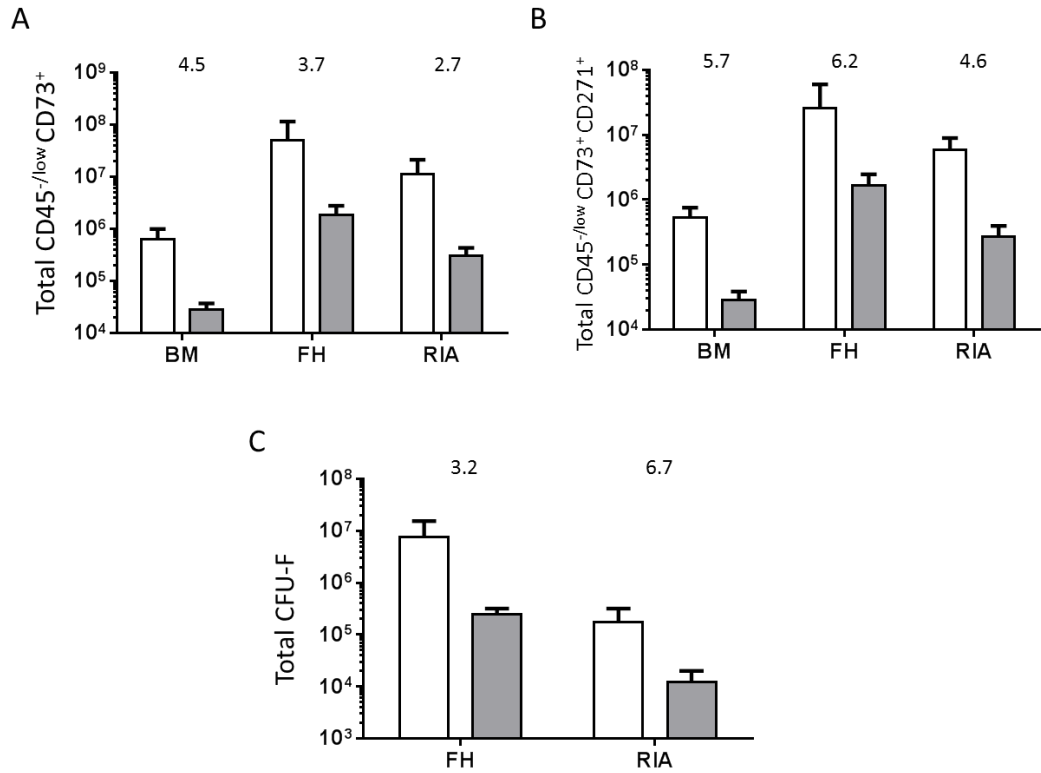


Figure 15: Analysis of the MSc isolation Efficiency of the CliniMACS system

The total number of cells expressing a CD45^{-/low} CD73⁺ (A) and CD45^{-/low} CD73⁺ CD271⁺ (B) phenotype and the total number of CFU-Fs (C) present in sample Pre (white bar) and Post (grey bar) CliniMACS enrichment. Values above each box pair show the proportion of cells recovered in the Post fraction, as percentage of Pre fraction. All data n=3 displayed as mean and standard deviation.

4.3.5. Where are MSCs lost during the CliniMACS enrichment procedure

During the enrichment process there are several possible stages where MSCs could be lost. To assess the possible cause of MSC loss I analysed all cell fractions generated throughout the enrichment process from BM aspirate (n=1) by flow cytometry, in this case by examining the CD45^{-/low} CD271⁺ population. These were: the Pre fraction (the BM immediately following collection), the cell suspension immediately prior to cell separation (before separation, BS) fraction, the 1st and 2nd wash fractions, the cells discarded in the supernatant following washing, the cells discarded in the negative fraction and the cells collected in the Post fraction. This experiment is shown in Figure 16. Of the initial 4.9×10^5 CD45^{-/low} CD271⁺ cells present in the unprocessed BM (Figure 16A) roughly half were lost prior to initiation of the automated portion of the separation procedure (Figure 16B). These losses could not be accounted for by loss from the discarded supernatant (Figure 16C-D). Of the remaining MSCs, 12% were discarded in the negative fraction (Figure 16E) and only 3.1×10^4 MSCs representing 12% of the 2.6×10^5 loaded into the CliniMACS device, were recovered in the positive fraction, although at relatively high purity (Figure 16F).

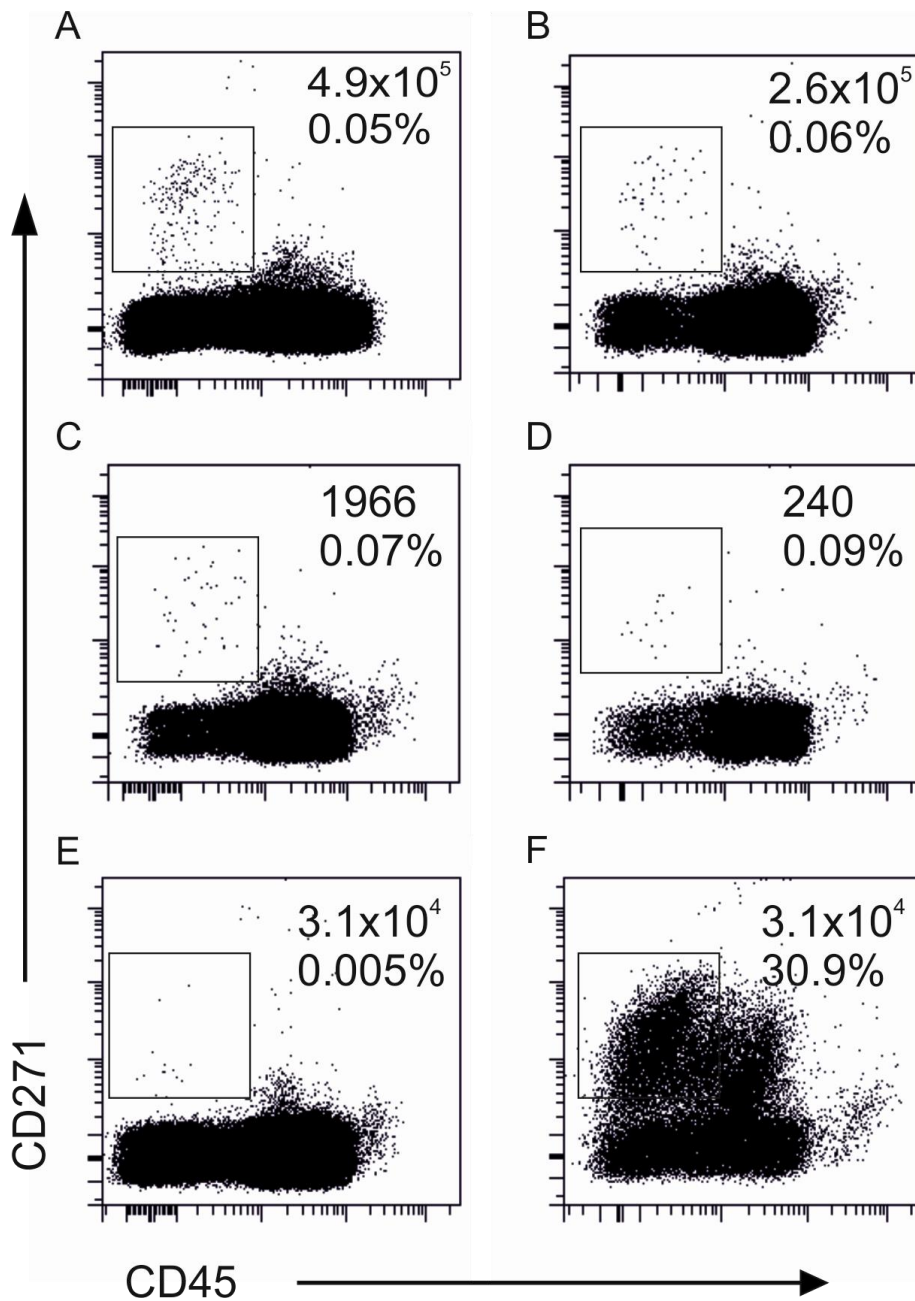


Figure 16: MSC content in process fractions generated by CliniMACS enrichment.

Representative dot plots showing total MSC number and percentage purity in 6 fractions generated as a result of the CliniMACS purification procedure. Unprocessed (Pre) BM (A), BM immediately prior to bead selection (B), 1st wash fraction (C), 2nd wash fraction (D), negative fraction (E), positive fraction (Post) (F). Values in top left corners show total number and proportion of MSCs in each fraction.

4.4. Discussion

This work demonstrates that BM MSCs can be effectively concentrated using both the MarrowStim and SmartPrep 2 concentrator devices. Whereas both significantly increased the total cellularity of BM aspirates, the MarrowStim device was more effective at increasing MSC concentration relative to its initial value. Despite good performance at concentrating the total cellular content of BM aspirates, the SmartPrep 2 device was not as effective at concentrating MSCs specifically. The discrepancy indicates that efficient concentration of total cellular content does not guarantee efficient MSC concentration. The fact that the MarrowStim device suffered less from this issue suggests that careful optimisation of the centrifugation technique to accommodate the specific characteristics of MSCs could improve the efficiency of these devices.

The number of MSCs in the Pre concentrated BM was far greater in samples concentrated with the SmartPrep 2 device, this could be due to the normally high variability of MSCs observed in aspirates (D'Ippolito et al. 1999; Pittenger et al. 1999; Hernigou et al. 2005a). However, since both devices are supplied with the reagents and equipment used to obtain aspirates including trocar, subtle differences between the equipment used for BM aspiration could also influence aspirate quality.

The failure of either of these devices to increase the purity of MSCs in BM reinforces the notion that this type of device is not suitable for allogeneic therapy. This is likely the case for other types of concentrator devices based on filtration since cell size is the only selection criteria (Dawson et al. 2013; Otsuru et al. 2013). Additionally the inclusion of other cell types, particularly large numbers of erythrocytes, may be a problem in autologous therapy. Although largely marketed as being capable of removing erythrocytes significant numbers can persist, neither are erythrocytes removed in the case of filtration devices (Dawson et al. 2013; Otsuru et al. 2013). This is important, since *in vitro* evidence suggests that colony formation is more efficient in the absence of erythrocytes (Horn et al. 2008; Horn et al. 2011). Conversely it is likely that platelets are also carried over into the Post fraction, this may represent an added benefit since MSCs proliferation is known to

be stimulated by platelet derived growth factor (PDGF) (Doucet et al. 2005; Zaky et al. 2008; Dhillon et al. 2012). Indeed, platelet rich plasma alone and in combination with concentrated BM has been investigated for its potential to promote bone and cartilage regeneration, with some success (Betsch et al. 2013; Lee et al. 2014). However, it has been suggested that metabolising, contaminating cells may limit MSC survival by competing for oxygen and nutrients (Hernigou et al. 2005a). Therefore with the exception of platelets, it is desirable to remove as many contaminating cells as possible from MSC preparations.

A high level of enrichment can only be achieved clinically by immunomagnetic cell selection. CD271 is an ideal candidate for immunomagnetic cell selection due to its ubiquitous expression on BM MSCs and low cross-reactivity with other cell types (Jones et al. 2002; Jones et al. 2006; Tormin et al. 2011; Boxall et al. 2012; Bianco et al. 2013b). In every case CD271 selection resulted in a substantial increase in the proportion of MSCs. Aspirated BM aspirates had the lowest starting proportion of MSCs out of all the tissues tested but enrichment resulted in the highest fold increase in purity (186 or 204 fold depending on analysis method used). However, MSCs enriched from this source had the lowest final purity. Indeed, the initial purity of samples processed with this system seems to largely determine the final purity of the product, BM has the lowest initial (0.066%, determined with the CD45⁻/lowCD73⁺CD271⁺ phenotype) and final purity (13.5%), RIA was intermediate initial (0.12%) and final purity (34.9%) and FH had the highest initial (3.72%) and final purity (52.4%).

Importantly, leukocytes were not fully depleted remaining at around 20% of the enriched product in FH, 30% in RIA and 19% in BM aspirate. CD271 enrichment did not cause substantial loss of cell viability in the majority of cases, although some loss of cell viability was observed after MSC enrichment from FH. However, this tissue was stored for a prolonged period and exposed to aggressive enzyme treatment, factors that are likely to negatively impact cell survival. Although some enrichment of the CD45⁺CD271^{low} CD73⁻ non-MSC population was observed, in all cases these cells were enriched to a lesser degree than that seen with MSCs,

suggesting that CD271 microbeads preferentially select MSCs possibly because they express high levels of the CD271 antigen (Jones et al. 2002; Tormin et al. 2011).

The substantial losses of MSCs observed during enrichment demonstrate that this magnetic enrichment procedure remains sub-optimal. In one experiment, in which this was investigated in detail, approximately 50% of MSCs were lost prior to the selection process, most likely as a result of clotting. This may be prevented in the future by the inclusion of a more stringent anticoagulation regime such as inclusion of ACDA. A substantial proportion of MSCs were not eluted in either the positive or negative fractions indicating that these may have been retained within the tubing; this could be tested in the future by removing the tubing and flushing with trypsin solution followed by flow cytometry. Furthermore, although the proportion of MSCs in the negative fraction was substantially lower, they accounted for approximately 50% of the eluted MSCs, this may be due to an insufficient number of beads present at the labelling stage leading to incomplete or sub-optimal bead loading. Although the concentration of beads may be increased, this may also increase the level of contamination with CD45⁺ CD271^{low} CD73⁻ 'passenger' cells. Although cell recovery may be improved, some cell loss is inevitable and has been reported with other rare cell selections using the CliniMACS system. Di Ianni et al reported an average recovery of 33.6% for regulatory T-cell selection (Di Ianni et al. 2011), and Stamm et al reported an average recovery of 19.6% for CD133⁺ HSCs (Stamm et al. 2007).

There were over 71000 total hip replacements in England and Wales in 2011 (National Joint Registry for et al. 2011), the ready availability of raw material makes isolation from FHs without culture expansion an attractive prospect especially for allogeneic transplantation. Furthermore, there are three important issues that must be addressed before this approach can be adopted. Firstly, the purity of the end product and related to this the composition of the non-MSC contingent of the end product. Although allograft containing a limited proportion of leukocytes (~6%) (Baboolal et al. 2014) has already been shown to be safe and effective (Hollawell 2012; Tohmeh et al. 2012; Ammerman et al. 2013; Neman et al. 2013), cells enriched from FH, despite being the purest with respect to MSC content, still

contained roughly 20% leukocytes and this raises serious doubts regarding its suitability for allogeneic therapy. Further optimisation of CD271 enrichment or a second purification step to deplete leukocytes could address this issue. Secondly, although poorly understood, aberrant MSC activity has been implicated in the progression of OA (Murphy et al. 2002; Zhen et al. 2013). The abnormalities described included inappropriate subchondral bone formation driven by TGF- β in MSCs (Zhen et al. 2013). For these reasons further research is required to ascertain that MSCs isolated from the osteoarthritic tissue do not possess undesirable phenotypic characteristics. Lastly, although allogeneic culture expanded MSCs have been shown not to illicit an immune response and are considered safe for transplantation (Ringden et al. 2006; Le Blanc et al. 2008; Galipeau 2013), this has not been unequivocally demonstrated for uncultured MSCs.

Intramedullary reaming is also a common procedure often performed prior to insertion of intramedullary nails (Porter et al. 2009), and could also provide a plentiful source of MSCs for allogeneic therapy. However, collection of MSCs using the RIA system has a number of advantages over FHs. Since the tissue that is collected is not associated with any underlying disease there is less risk of transferring MSCs with undesirable characteristics. Crucially, since no enzymatic digestion is required as is the case with FHs (Jones et al. 2010b) therefore, processing time is reduced. This makes enrichment from RIA a credible option for intraoperative autologous MSC therapy. However, the minimum processing time for enrichment from RIA (>4 hours) still remains far greater than the typical processing time associated with the BM concentrator devices tested (~15 minutes).

The absolute number of MSCs required for any given therapy is difficult to determine. Hernigou *et al* showed that successful treatment of fracture non-union, by percutaneous injection of concentrated BM, was associated with an average total dose of 30,000 MSCs, measured by CFU-F assay (Hernigou et al. 2005a). For successful treatment of avascular necrosis of the femoral head, by the same method an average of 147,000 MSCs are required (Hernigou et al. 2005b). However, Hernigou could not determine if the total dose or the concentration of MSCs was the critical factor in determining outcome. Nakamura *et al* showed in a

pig cartilage defect model that implantation of between 2.2×10^7 and 3.8×10^7 MSCs could effect some degree of cartilage regeneration (Nakamura et al. 2012). However, this study used culture expanded MSCs and is therefore not directly applicable to minimally manipulated MSC therapy. One human study, again using culture expanded cells for full thickness cartilage repair used 1.3×10^7 autologous cells on average, but did not report any significant clinical improvement (Wakitani et al. 2002).

In the case of non-union and avascular necrosis the numbers of MSCs required are attainable from iliac crest aspirates. However, this requires a lengthy and technically demanding harvesting technique (Hernigou et al. 2005a; Hernigou et al. 2013). Additionally, cell losses experienced with CliniMACS enrichment (Stamm et al. 2007; Di Ianni et al. 2011) further add to the limitations of using iliac crest aspirates in conjunction with this technology. Bone digests have a limited appeal for autologous therapy, due to donor site morbidity. RIA aspirates in conjunction with CD271-based cell selection have genuine promise as a source of MSCs that may be used for autologous therapy in bone and may be a credible alternative to concentrated BM. Intramedullary reaming is a relatively simple technique (Giannoudis et al. 2009; Cox et al. 2011a; Cox et al. 2011b) and although the protocol described in this chapter was sub-optimal, it recovered an average of 14,000 MSCs, measured by CFU-F assay. Even so, this compared favourably to the most successful concentrated BM tested which had a total of 6450 MSCs, measured by CFU-F assay. If efficiency could be improved to a level comparable with other rare cell enrichments using the CliniMACS (Stamm et al. 2007; Di Ianni et al. 2011), the number of MSCs recovered may be substantially improved.

5. Characterisation of the membrane generated as a result of the Masquelet technique

5.1. Introduction

5.1.1. Critical size defects

Large bone defects represent a particular challenge in the field of clinical orthopaedics. A critical size defect is defined as a bone defect that cannot be spontaneously bridged and is therefore, beyond the capacity of the bone to self-heal leading to non-union (Gugala 2007; Dimitriou et al. 2011b; Dimitriou et al. 2012). It is generally accepted that a defect ≥ 1.5 to 2 times the diameter of the long bone diaphysis is of a critical size (Gugala 2007; Dimitriou et al. 2012). Several factors influence the likelihood that a defect will progress to non-union including the mechanical environment, the location of the defect, the condition of the surrounding soft tissue and patient related factors such as age and related co-morbidities (Dimitriou et al. 2005; Talbot et al. 2008; Reichert et al. 2009). They may result from trauma, tumour resection, infection, deformity correction or aseptic loosening around implants (Gugala 2007; Dimitriou et al. 2011b; Dimitriou et al. 2012).

Several methods for bone reconstruction exist but all have specific limitations. Autologous cancellous bone grafting is the most common approach but this has major shortcomings. These include poor structural integrity, limited availability of graft material and donor site morbidity also, this approach is not suitable for defects larger than 6cm due to rapid graft resorption (Gugala 2007). Distraction osteogenesis and bone transport is capable of regenerating larger bone defects. Here following mechanical stabilisation, closure of the defect is achieved by bone transport, simplistically this means pushing the remaining bone together to close the defect (Tsuchiya et al. 1997). A fracture is then made in an area of healthy bone whilst keeping the periosteum intact. Bone is then regenerated by gradual mechanical stretching of the newly formed callus allowing constant vascularisation and remodelling (Tsuchiya et al. 1997), a process that mimics longitudinal bone

growth in adolescence. However, this process is technically demanding and has several disadvantages, the most important of which is the lengthy treatment period required, both in the distraction phase (1mm per day) and the consolidation period, which is usually twice that of the distraction phase (Gugala 2007; Dimitriou et al. 2011b). Quarto *et al* suggested an alternative which relies on *in vitro* expansion of autologous MSCs followed by seeding onto a porous ceramic scaffold (Quarto et al. 2001). This scaffold was then implanted into >4cm defects and integration into the host bone was observed after two months (Quarto et al. 2001). The speed of healing demonstrated was impressive however, this technique has not been adopted widely, perhaps due to technical, regulatory and logistical difficulties associated with the use of *in vitro* expanded MSCs in this setting.

5.1.2. Guided bone regeneration and the use of barrier membranes

The concept of guided bone regeneration, to treat bone defects, was initially developed based on the hypothesis that a non-cell permeable membrane can exclude the invasion of inhibiting cells to the defect site; whilst simultaneously allowing angiogenic and osteogenic cell ingress from the BM (Greenstein et al. 2009; Dimitriou et al. 2012; Dahlin et al. 2014). Initial experiments using a Teflon membrane for repair of a mandibular defect in rats demonstrated the efficacy of non-absorbable membranes for this approach (Dahlin et al. 1988). Non bio-absorbable membranes are available as Polytetrafluoroethylene (PTFE, Teflon), titanium reinforced PTFE and titanium mesh (McAllister et al. 2007; Francois et al. 2012). Bone regeneration involves angiogenesis and ingress of osteogenic cells from the periphery towards the centre of the defect followed by woven bone deposition and finally remodelling and lamellar bone formation (McAllister et al. 2007; Dimitriou et al. 2012).

Despite the success demonstrated with PTFE, the outcome of therapy using this type of membrane was limited due to complications of soft tissue dehiscence leading to membrane exposure and infection (McAllister et al. 2007). This led to the

development of bio-absorbable scaffolds and these are now available as synthetic or natural polymers. Aliphatic poly-esters such as polyglycolic acid are the most widely used synthetic polymers (Cheung et al. 2007; Gloria et al. 2010); whereas, cross-linked bovine collagen is the most commonly used natural bio-absorbable polymer (Behring et al. 2008; Dimitriou et al. 2012).

5.1.3. The induced membrane technique

The Masquelet or induced membrane (IM) technique is a two stage procedure which involves the implantation of a cement spacer into the defect site in the first stage, resulting in the induction of a biological membrane around the implant (Giannoudis et al. 2011; Dimitriou et al. 2012; Gruber et al. 2012). At the second stage (6-8 weeks later) the cement is removed and the IM serves as a conduit to contain autologous cancellous bone graft which may be augmented by BMPs and BM aspirate (Giannoudis et al. 2011; Dimitriou et al. 2012; Cuthbert et al. 2013). Using this procedure Karger *et al* treated patients with defects larger than 10cm, using the IM technique and union was achieved in an average of 9 months with a success rate of 94% (Karger et al. 2012). Moreover, time to union was not dependent on defect size (Karger et al. 2012); this is important given that defects of up to 25cm have been repaired using the IM technique (Masquelet et al. 2000).

These impressive results have generated interest in the properties of the IM itself. Animal studies have shown the IM to have osteogenic, osteoinductive and angiogenic properties, (Pelissier et al. 2004; Masquelet et al. 2010), but to date there have been no studies addressing the functional properties and characteristics of the IM in man. The anatomical location of IM in relation to the cement spacer implant closely resembles that of the diaphyseal periosteum in relation to underlying bone, suggesting that these tissues may be analogous.

The periosteum is widely recognized to be of critical importance in bone formation and regeneration (Young 1962; Arnsdorf et al. 2009; Chan et al. 2012). Structurally it is divided into two distinct layers: an inner cambium layer and outer fibrous layer (Squier et al. 1990; Fan et al. 2008). The cambium layer has been shown to be a

reservoir of progenitor cells with an osteogenic potential comparable to BM derived MSCs and superior to synovial MSCs (Ng et al. 2005; De Bari et al. 2006; De Bari et al. 2008; Arnsdorf et al. 2009). The presence of intact periosteum has been shown to be an important factor in successful fracture healing (Knothe et al. 2005; Yu et al. 2010), it is also highly vascularized and provides the cortical blood supply (Squier et al. 1990; De Bari et al. 2006; Fan et al. 2008). Following injury, an acute inflammatory reaction can be seen at the periosteum, this leads to periosteal cell proliferation and thickening of the periosteum itself, a process known as periosteal activation (Lin et al. 2014). Periosteal cells are critical for callus formation and induce robust chondrogenesis and osteogenesis accompanied by marked vascularization (Colnot et al. 2012).

Given the excellent bone repair achievable by the IM technique and given the ability of MSCs to heal bone, a simple yet attractive hypothesis is that the IM technique leads to large scale recruitment of native MSCs to effect bone repair. This concept has been demonstrated for cartilage repair using a hydroxyapatite scaffold coated with TGF- β 3 infused collagen type 1 (Lee et al. 2010). For bone regeneration an electrospun barrier membrane composed of poly-caprolactone and β -gelatin loaded with SDF-1, a potent MSC chemokine (Kitaori et al. 2009), was six times more effective than membrane alone in a rat model (Ji et al. 2013). These studies demonstrate the utility of recruiting endogenous MSCs to sites of tissue injury and thereby maximising the body's own regenerative capacity.

5.1.4. Chapter aim and objectives

The aim of this chapter was to investigate the regenerative properties of the IM harvested from a series of patients undergoing treatment for large diaphyseal defects, in order to identify characteristics that may facilitate bone repair.

Objectives:

1. To use histology and immunohistochemistry techniques to build understanding of the morphology and localization of cells, growth factors and chemokines involved in bone regeneration.
2. Use flow cytometry to investigate the cellular composition of IM and periosteum, in order to identify potentially regenerative cells present in IM.
3. To confirm the presence of MSCs by examining the differentiation capacity of enzymatically released cells, in order to gain an understanding of the regenerative potential of IM.

5.2. Materials and methods

5.2.1. Surgical procedures and patient selection

All procedures in sections 5.2.2 to 5.2.4 were carried out by clinical staff at the Academic unit of Trauma and Orthopaedics, Clarendon Wing, Leeds Teaching Hospitals NHS Trust.

5.2.2. Patient inclusion criteria

Patients were selected from those admitted for treatment of either upper or lower extremity bone loss using the IM technique and invited to participate in this study. Patients that underwent treatment of bone defects by other methods of bone regeneration (i.e. distraction osteogenesis, allograft implantation following tumour excision) were excluded.

5.2.3. Patient characteristics

Eight patients (7 male, 1 female) with a mean age 60 years (18-80) gave their consent to participate in this study. All defects were of post-traumatic nature. The distribution of the anatomical site of the defect is shown in Appendix 5. The mean size of the defect was 36.25mm (range 25-50mm). Six cases (numbers 1,2,3,4,6,7) were infected non-unions, most commonly with staphylococcus aureus, whereas 2 cases (numbers 5,8), were aseptic bone defects.

5.2.4. Induced membrane technique and tissue harvesting

An incision was made adjacent to the defect area of the affected limb. Once skin and muscle tissue had been withdrawn dead damaged or infected tissue was removed (debridement) from the defect area and a polymethyl methacrylate (PMMA) cement spacer (Heraeus Medical GmbH) was implanted. The affected limb was then stabilised with external fixation, by six to eight week following the initial stage an IM had formed around the cement spacer. The spacer was then removed,

during the second stage of the procedure by re-opening the defect site as before and incising the IM then removing the cement spacer. An area of at least of 1cm² of IM tissue was harvested at the centre of the bone loss area (Figure 17). Subsequently, a 1cm² sample of periosteum was harvested from the normal diaphyseal bone, this served as a control tissue for IM due to its similar localisation and the recognised importance of periosteum to bone regeneration (Young 1962; Arnsdorf et al. 2009; Chan et al. 2012).

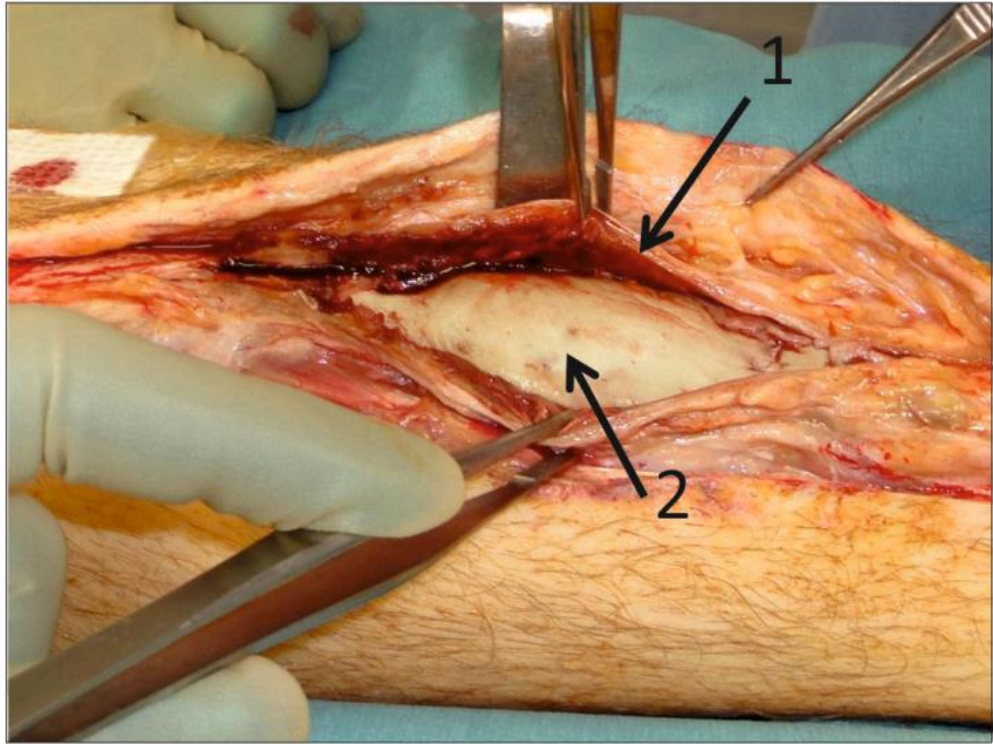


Figure 17: Intra operative photograph showing the induced membrane

Induced membrane (1) separated and elevated off the cement spacer (2) (tibial defect). Photograph kindly provided by P. Giannoudis.

5.2.5. Digestion of IM and periosteum to release resident cells

Tissue samples were bisected; 50% of each sample was retained for histological processing. Under sterile conditions the remaining tissue was first shredded using a scalpel, once transferred to a 50ml centrifuge tube, its total wet weight was recorded. The tissue was then enzymatically digested with collagenase as previously described (2.4). The resulting single cell suspension was then washed by dilution with 10ml of PBS, centrifugation at 400xg and re-suspension. Subsequently cells were manually counted and aliquots of 1×10^5 cells were taken for volumetric flow cytometry, as previously described (2.5) or frozen using standard freezing media (Appendix 2) at 5×10^5 cells/vial. Freezing was used to ensure functional assay reproducibility owing to the rarity of these samples and the long intervals between collections.

5.2.6. Processing of IM and periosteum and preparation of histological slides

To preserve tissue architecture and prevent mortification prior to the preparation of histological tissue sections, the collected tissue was embedded into paraffin wax blocks. The tissue was first incubated overnight at RT in fixing buffer (Appendix 2), once fixed samples were stored in 70% ethanol. Subsequently, tissue was loaded into an ASP300 (Leica Biosystems) tissue processor for graded alcohol dehydration followed by xylene and wax impregnation. Once processing was complete, the tissue was mounted into wax blocks and $4 \mu\text{m}$ sections were prepared using a RM225 rotary microtome (Leica Biosystems).

5.2.7. Recovery of frozen cells from digested tissue

Cryopreserved samples were defrosted and transferred to 10ml fluorescence activated cell sorting (FACS) buffer (Appendix 2) containing 20 U/ml DNase I (Sigma Aldrich). Cells were centrifuged at 400xg and re-suspended in $150 \mu\text{l}$ of the same solution and counted manually. Two $45 \mu\text{l}$ aliquots were put aside for flow

cytometry, 2.4×10^5 cells were put aside for CFU-F assay and the remaining cells were expanded to generate MSC cultures as previously described (2.9).

5.2.8. CFU-F assay

CFU-F assay was performed to enumerate the MSCs recovered from the digested tissue. Recovered cell were seeded into duplicate 35mm diameter tissue culture dishes (Corning) at a density of 1.2×10^5 cells/dish, the assay then proceeded as previously described (2.7).

5.2.9. Flow cytometry

Flow cytometry was performed to examine the cellular composition of IM and periosteum. Two aliquots of cells were incubated in FACS buffer with 10% v/v FcR blocking reagent (Miltenyi) for 20 minutes at RT. Each aliquot was then incubated for 20 minutes at RT with one of 2 preparations of monoclonal antibody cocktails: Mix 1: CD271-APC, CD45-PECy-7, CD146-PE, CD34-PcP, CD90-FITC. Mix 2: CD271-APC, CD45-PECy-7, CD146-PE, CD73-PcPCy-5.5, CD31-FITC (Appendix 4). Cells were subsequently centrifuged at $400 \times g$ for 5 minutes and re-suspended in $500 \mu\text{l}$ FACS buffer (Appendix 2) the cell suspension was analysed using a LSR II flow cytometer (BD). Single antibody stained and unstained controls were used before each experiment to ensure accurate spectral compensation.

5.2.10. Differentiation assays

Enzymatically recovered MSCs were expanded as previously described (2.9) to provide a minimum of 5×10^5 total cells; this required up to 2 passages. Cells were then seeded for osteogenic differentiation, as previously described (2.10). For chondrogenic differentiation, cells were seeded into ChondroPrime SF media (PAA laboratories), otherwise differentiation proceeded as previously described (2.14).

For adipogenic induction cells were seeded into NH adipoDiff medium (Miltenyi), otherwise differentiation proceeded as previously described (2.13).

5.2.11. Haematoxylin and eosin staining of sectioned IM and periosteum tissue

Paraffin-embedded tissue sections (4µm thick) were de-waxed by 3 serial incubations in xylene for 5 minutes at RT and rehydrated through a graded ethanol series to water 100% ethanol 3x1 minute, 90% ethanol 1x1 minute, 70% ethanol 1x1 minute, dH₂O 1x3 minutes. Sections were then incubated in Mayer's haematoxylin solution (Sigma Aldrich) for 5 minutes, washed under running water briefly and transferred to Scott's Tap-water substitute (Appendix 2). After a second brief wash in running water, sections were transferred to eosin Y solution (Sigma Aldrich) and incubated at RT for 1 minute. Sections were then washed in running water again before being de-hydrated by reversing the re-hydration schedule above and returned to xylene. Finally sections were mounted using DPX mountant (Sigma Aldrich). Photomicrographs were taken using a C7070 digital camera (Olympus, Tokyo, Japan) attached to an Eclipse E1000 microscope (Nikon). These images were then analysed using NIS elements BR 2.20 imaging software (Nikon, Tokyo, Japan), for measurements of IM and periosteum thickness.

5.2.12. Immunohistochemistry

Immunohistochemistry was used to determine the tissue localisation of specific cell types and proteins and was carried out on formalin-fixed paraffin embedded histological tissue sections. Immunohistochemistry staining was performed using a REAL peroxidase/DAB+ detection system (Dako) and specific mouse and rabbit primary antibodies to detect CD271, CD31, CD146, BMP-2, SDF-1 and VEGF (Appendix 4). Paraffin-embedded tissue sections (4µm thick) were de-waxed in xylene and rehydrated through a graded ethanol series to water (5.2.11). Endogenous peroxidase activity was quenched by incubation with peroxidase block

kit component; antigen retrieval was performed in a 900W microwave oven for 10min with 10mM citrate buffer (pH 6.0). Non-specific binding sites were blocked by 30 minute RT incubation with antibody diluent solution (Life Technologies).

All primary antibodies were diluted with antibody diluent (Dako, refer to Appendix 4 for working concentrations). Secondary antibody labelling and colour development were carried out according to Dako REAL peroxidase kit instructions. Sections were counterstained by a 5 minute RT incubation with Mayer's haematoxylin solution (Sigma Aldrich), followed by a 1 minute RT incubation in Scott's tap-water substitute (Appendix 2). The slides were dehydrated by incubation in a graded ethanol series (5.2.11) and 'cleared' with xylene before being mounted with coverslips with DPX mountant (Sigma Aldrich). Positive control tissue (umbilical cord liver and kidney) was used to assess antibody specificity, negative controls (omission of primary antibody) were included in each assay. Photomicrographs were taken using a C7070 digital camera (Olympus).

5.3. Results

5.3.1. Histological comparison

In order to understand the morphology of IM and how this may be related to its functional characteristics, a histological and immunohistochemical examination of IM was carried out using periosteum as a comparator. The gross morphology as well as the localisation of cells and extracellular factors known to be associated with bone and blood vessel formation was examined. The cell markers CD31 (endothelial cells) (Garlanda et al. 1997), CD271 (MSCs) (Jones et al. 2002; Tormin et al. 2011) and CD146 (Pericytes) (Chen et al. 2009) were chosen as well as the extracellular factors VEGF (blood vessel formation) (Gerber et al. 1999), BMP-2 (osteogenesis induction) (Noel et al. 2004) and SDF-1 (MSC migration) (Ponte et al. 2007). All antibodies performed well with strong positive staining in control tissues, no staining was observed in negative controls (Figure 18).

The IM shared a number of morphological features with periosteum including the presence of layers analogous to the inner cellular cambrial layer and outer fibroblastic/collagenous layer observed in periosteum (Squier et al. 1990; Chan et al. 2012). However, in IM the total median thickness was significantly greater: 1422 μ m (range: 981-2126) compared to 860 μ m (range: 468-1019) in periosteum ($p=0.032$) (Figure 19A). The presence of blood vessels was confirmed by CD31 expression at the luminal border of vessels (Figure 19B). Perivascular CD146 positive staining was indicative of the presence of pericytes (Chen et al. 2009) in both periosteum and IM (Figure 19C). The presence of CD271, a marker of BM MSCs (Jones et al. 2002; Quirici et al. 2002) was observed in high abundance in both tissues; staining was always closely associated, but not limited, to blood vessels (Figure 19D).

Tissues were also stained for molecules known to be important in osteogenesis and vessel formation (Pelissier et al. 2004). BMP-2 was predominantly observed on the luminal edges and the outer border of blood vessels in both tissues (Figure 19E). VEGF and SDF-1 expression was found throughout the structure of blood vessels in both tissues (Figure 19F-G). Semi-quantitative scoring of the intensity of staining

(Smith et al. 2006) for these molecules did not reveal any significant differences (Figure 20).

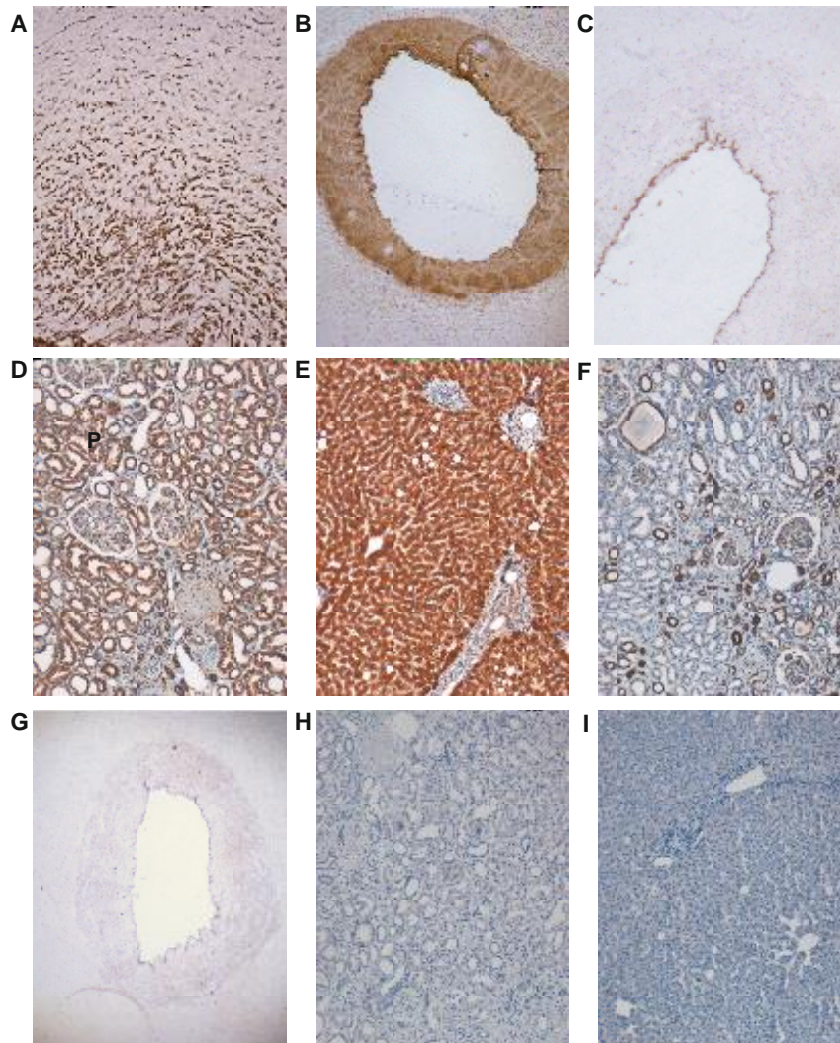


Figure 18: Immunohistochemistry controls.

Figure shows example positive control staining for CD271 in umbilical cord (x200 magnification) (A), CD146 in umbilical cord (x100 magnification) (B), CD31 in umbilical cord (x200 magnification) (C), BMP-2 in kidney (x100 magnification) (D), VEGF in liver (x200 magnification) (E), and SDF-1 in kidney (x200 magnification) (F). G, H and I show negative controls (omission of primary antibody) in umbilical cord (x100 magnification), kidney (x100 magnification) and liver (x100 magnification) respectively. Umbilical cord, kidney and liver sections were kindly donated by D. Kouroupis and M Shires.

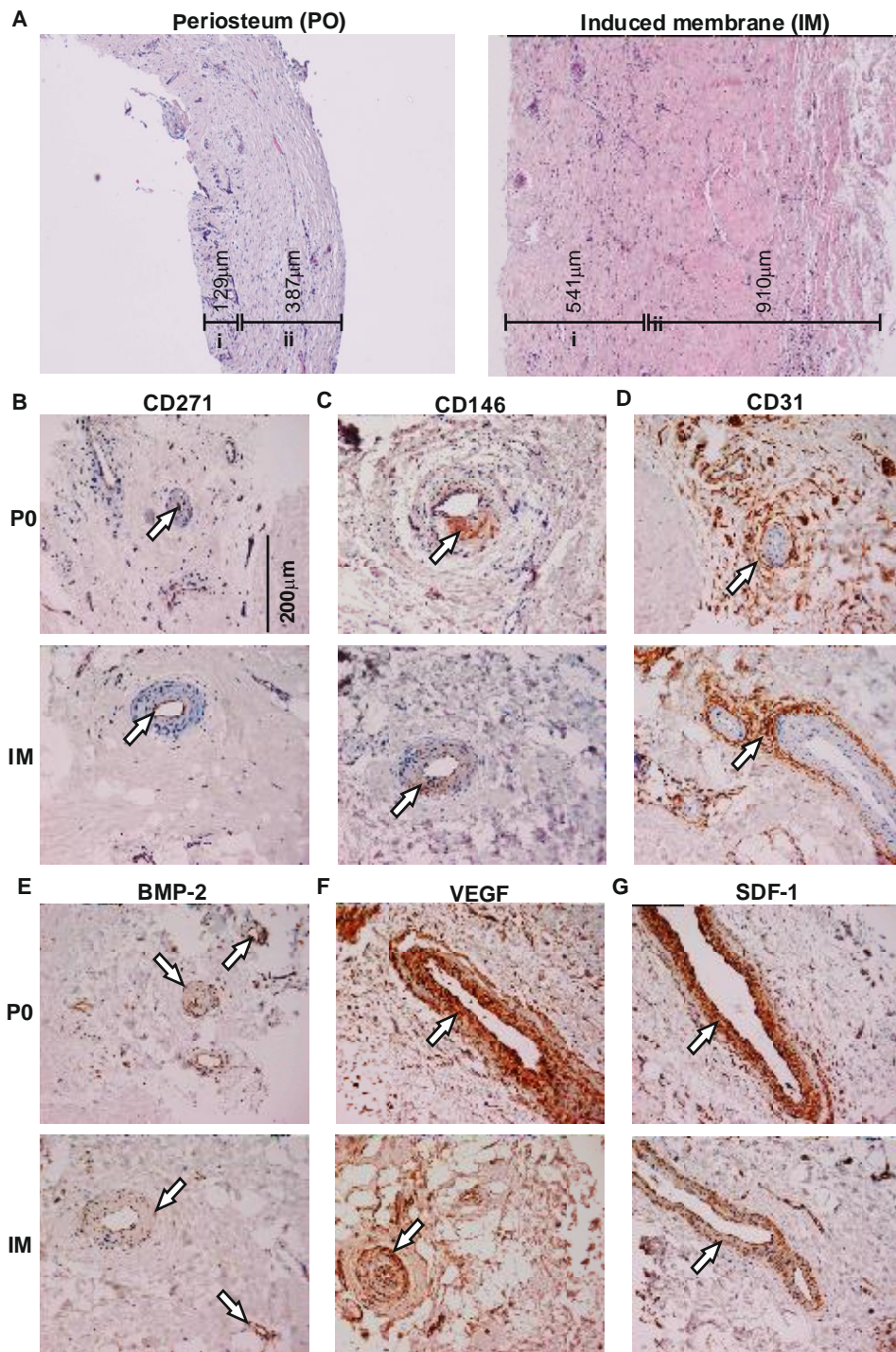


Figure 19: Tissue architecture cell and growth factor localisation in IM and control periosteum

Histological sections stained with haematoxylin and eosin, i: inner cellular layer, ii: outer fibrous layer (x40 magnification) (A). Sections labelled with specific antibodies to CD31 (B), CD146 (C), CD271 (D) BMP-2 (E), VEGF (F) and SDF-1 (G) (all x200 magnification), white arrows indicate regions of positive staining.

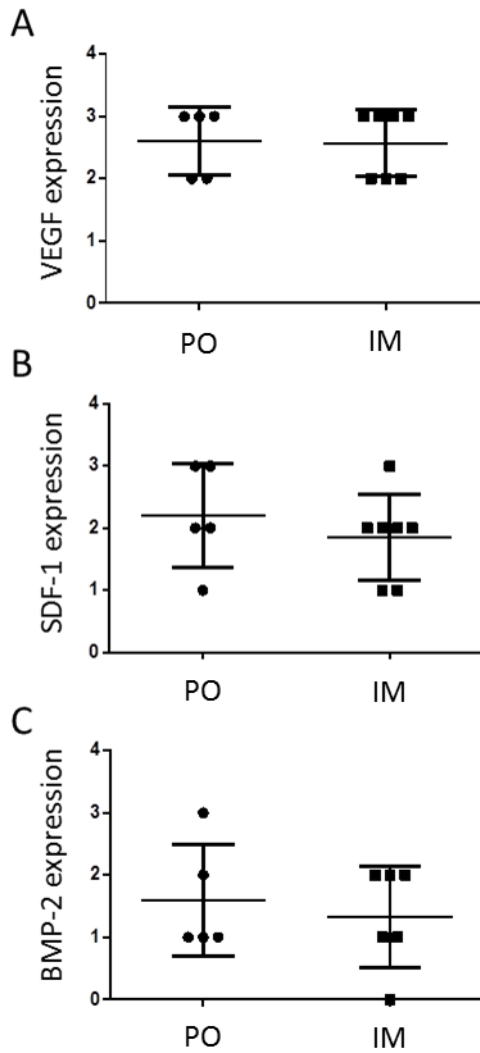


Figure 20: Semi-quantitative scoring of extracellular protein expression

Manual histological scoring of VEGF (A), SDF-1 (B) and BMP-2 (C) expression in periosteum (PO) n=5 and induced membrane (IM) n=7. Total labelled protein expression ranked 0-3 where 0=no staining, 1=weak staining, 2=moderate staining and 3=strong staining. Slides were kindly scored blind by Thomas Baboolal.

5.3.2. Quantitative comparison of cellular composition following enzymatic release

The total number of viable cells per gram of tissue measured by flow cytometry immediately following enzymatic digestion was 11-fold higher in IM ($p=0.043$), compared to matched periosteum (Figure 21A). In IM the number of infiltrating lymphocytes identified as $CD45^+$ with a low side scatter profile (Jones et al. 2010a) per gram of tissue was greatly increased, with IM having 31-fold more lymphocytes (median 9.9×10^5) than periosteum (median 3.2×10^4 , Figure 21B); this possibly reflects a more active process of immune cell recruitment. Similarly, the number of $CD45^{-/low}CD271^+$ cells/gram was greater by a factor of 15.5 in IM (median 1.4×10^6) compared to matched periosteum (median 8.7×10^4 , $p=0.043$, Figure 21C). Leading on from this, a more detailed phenotypic analysis was performed on cryogenically preserved cells (5.2.7). The relative proportion of endothelial cells, identified as $CD45^-CD31^+$ (Jones et al. 2010a) was comparable in IM and periosteum (Figure 22A). However, the proportion of cells expressing markers of pericytes ($CD45^-CD34^-CD146^+$) (Chen et al. 2009) was 3 fold greater in IM ($p=0.043$, Figure 22B).

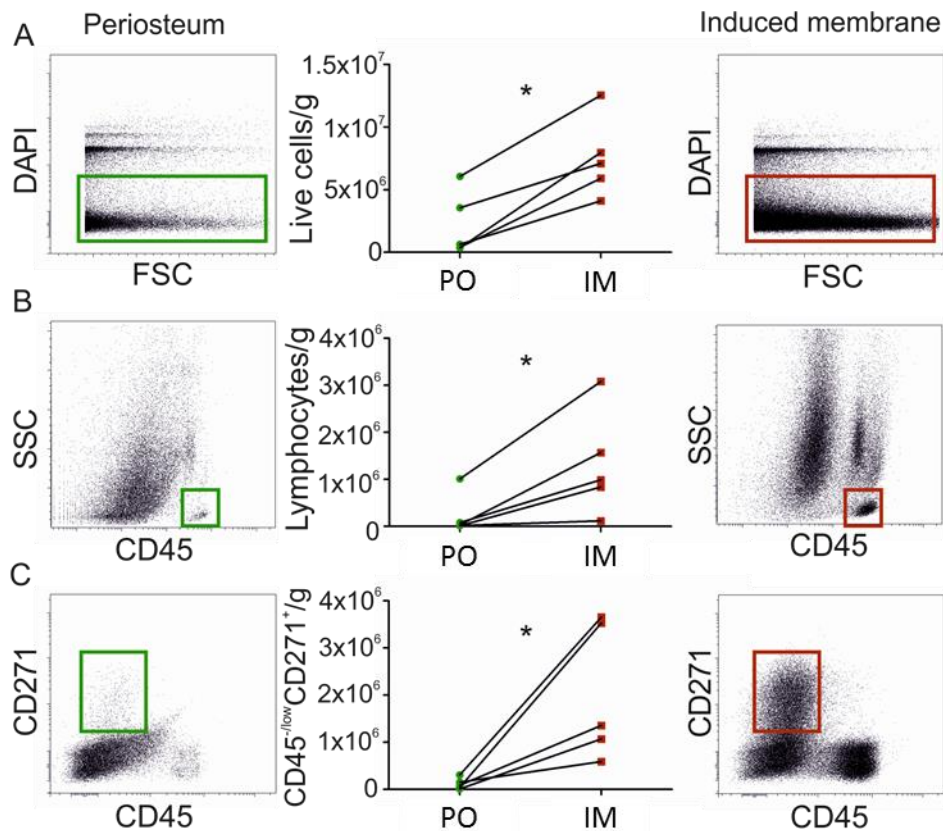


Figure 21: Cell composition measured by volumetric flow cytometry immediately following enzymatic digestion of matched periosteum (PO) and induced membrane (IM).

Total viable cells (A), infiltrating lymphocytes (B), and CD45⁻CD271⁺ cells (C), expressed as cells per gram of digested tissue. For (B) and (C) events were gated on DAPI⁻ (live cells) prior to gating shown. Absolute cell numbers were calculated by inclusion of a volumetric counting bead. Cell populations of interest are highlighted in coloured boxes green: Periosteum (left), red: Induced membrane (right). All data n=5, * denotes p<0.05.

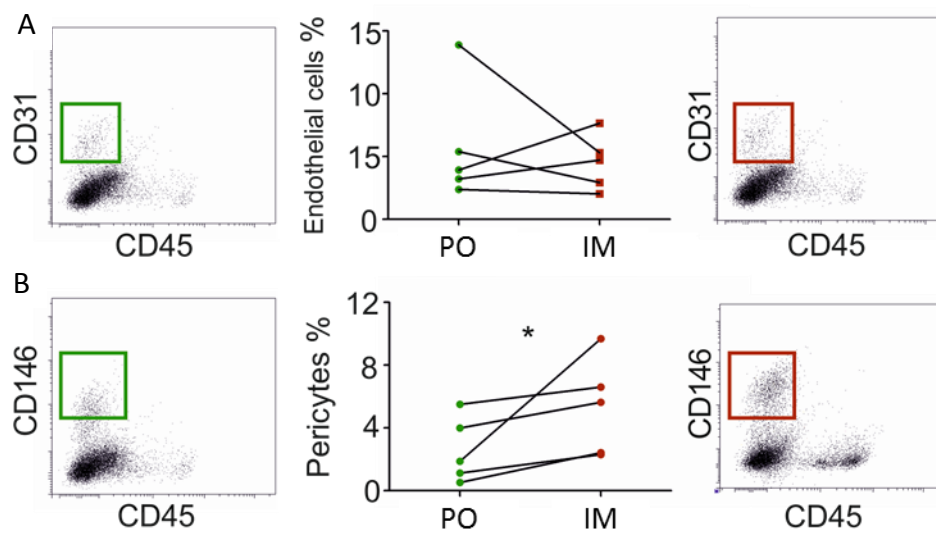


Figure 22: Cell composition measured by flow cytometry following recovery of frozen enzymatically released cells from matched periosteum (PO) and induced membrane (IM).

Proportion of $CD45^-CD31^+$ endothelial cells (A), events were gated on $DAPI^-$ (live cells) prior to gating shown. $CD45^-CD34^-CD146^{bright}$ pericytes (B), events were gated on $DAPI^-$ and $CD34^-$ prior to gating shown to exclude endothelial cells. Cell populations of interest are highlighted in coloured boxes-green: Periosteum (left), red: Induced membrane (right). All data $n=5$, * denotes $p<0.05$.

5.3.3. Colony forming potential

To investigate the proportion of MSCs present in IM and periosteum able to initiate MSC colonies, the colony forming potential of enzymatically released cells was determined. Following enzymatic digestion, adherent colonies grow readily from both tissues; colonies were composed of fibroblastoid cells which were highly proliferative and formed discrete radial colonies (Figure 23A). There was no significant difference between the proportion of MSCs between matched periosteum and IM (n=6, Figure 23B). This was surprising given the highly elevated number of CD45^{-low}CD271⁺ cells detected in IM by flow cytometry.

5.3.4. Differentiation potential

Adherent, culture expanded cells (passage 2) derived from enzymatically digested matched tissues (n=5) were subjected to conditions promoting adipogenesis, chondrogenesis and osteogenesis. In adipogenic conditions, accumulation of lipid vacuoles was observed and highlighted by uptake of Oil Red (Figure 24A). Cell pellets obtained from cultures derived from both tissues exposed to chondrogenic conditions displayed cartilage specific metachromasia when stained with toluidine blue (Figure 24B). Osteogenic culture conditions led to induction of alkaline phosphatase after 14 days to similar degrees in both tissues. The degree of calcium deposition at 21 days post induction was also similar in both periosteum and IM (Figure 24C). Altogether, these data demonstrated that enzymatically released, plastic adherent cells from both tissues possessed tri-lineage differentiation potential upon expansion, thus confirming that they were true MSCs with the capacity to participate in tissue regeneration.

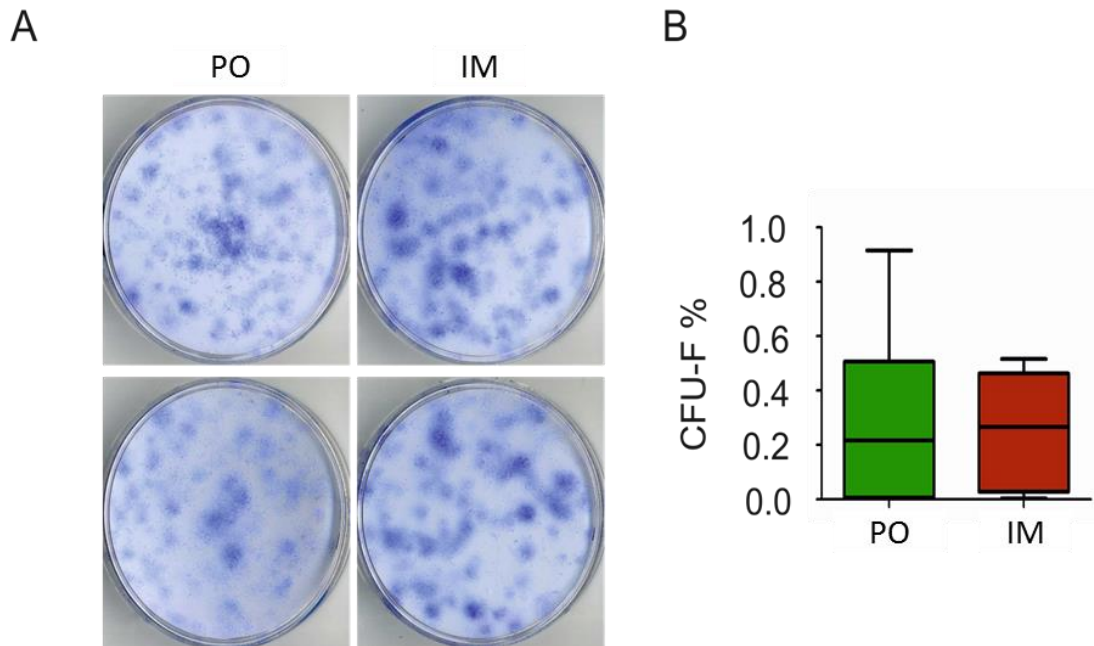


Figure 23: Colony forming potential following recovery of frozen enzymatically released cells for matched periosteum (PO) and induced membrane (IM).

Representative duplicate donor-matched CFU-F plates generated by cells released from digested periosteum and induced membrane, stained with methylene blue (1 colony >50 tightly clustered cells) (A). The Proportion of the 1.2×10^5 cells seeded into each dish that resulted in the formation of a colony $n=6$ (B). Box plot shows interquartile range median and extreme values.

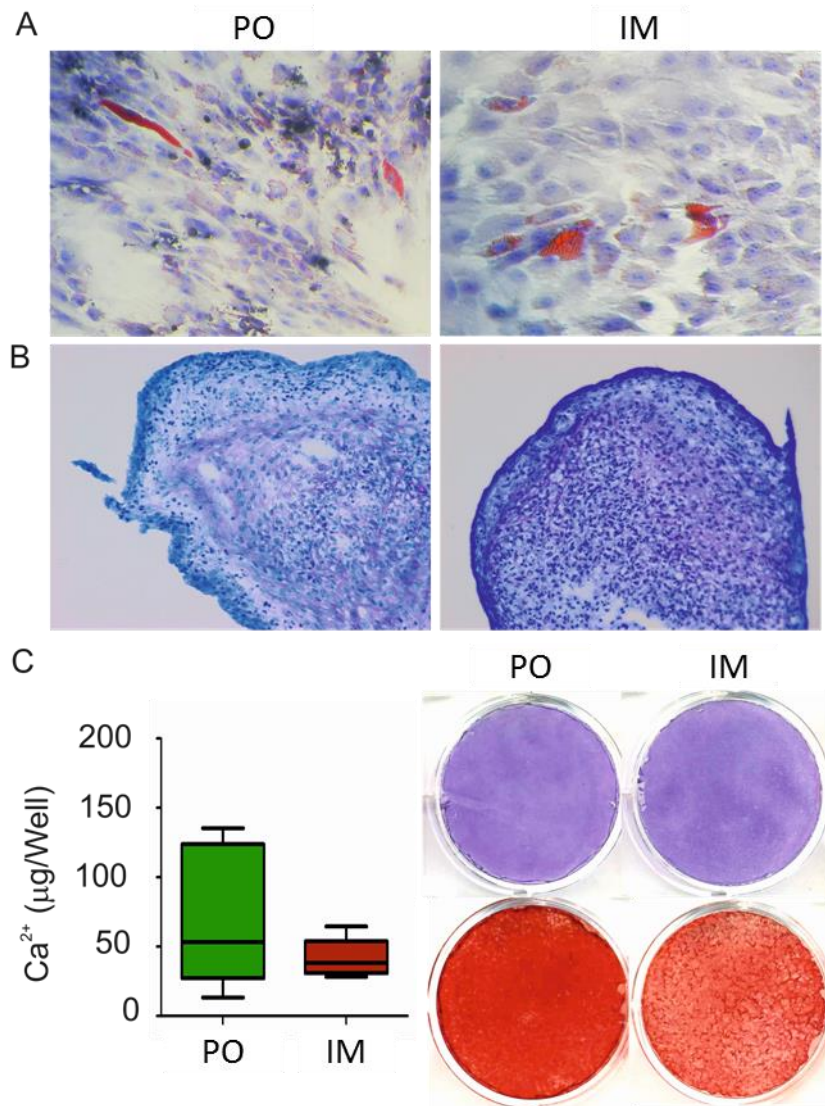


Figure 24: Differentiation potential following recovery of frozen enzymatic digested tissue: matched periosteum (PO) and induced membrane (IM).

Representative donor matched wells following adipogenic induction, adipocytes shown by uptake of oil red (x400 magnification) (A). Chondrogenic pellets showing purple metachromasia (cartilage) after toluidine blue staining of cell pellets (x200 magnification) (B). Calcium generated per well from osteogenic cultures with matched alkaline phosphatase (upper, blue) and alizarin red (lower, red) stain n=5 (C). Box plot shows interquartile range median and extreme values.

5.3.5. Examination of MSC transcriptional profile

Matched IM and periosteum samples (n=5) were cultured up to passage 2 RNA was isolated and cDNA was produced for analysis on a Taqman low density array (Life Technologies) [34]. Five donor matched IMs and periosteum were analysed, together with control unmatched, passage 2 iliac crest BM-MSCs (n=4) and skin fibroblast cell lines (n=3, Lonza and ATCC). All gene expression was normalised to HPRT. Open-source clustering software utilising clustering methods described by Eisen *et al* was used to perform cluster analysis.

The expression level of genes known to influence MSC proliferation, osteogenesis, chondrogenesis, migration and stimulation of angiogenesis were measured. These included receptors for fibroblast growth factor (FGF) and PDGF (proliferation) (Gronthos et al. 1995; Zaragosi et al. 2006), proteins associated with TGF- β /BMP and WNT signalling (osteogenesis) (Bennett et al. 2007; Chen et al. 2012), aggrecan and sex determining region Y box 9, (chondrogenesis) (Yano et al. 2005), SDF-1 (migration) (Miller et al. 2008) and VEGF (angiogenesis) (Sato et al. 2011). Analysis revealed close clustering between periosteum and IM MSCs. MSCs from both these tissues clustered more closely to iliac crest MSCs than negative control fibroblasts (Figure 25A). When the molecular profile of IM MSCs was compared to those originating from the periosteum the gene encoding SDF-1 was significantly up-regulated (p=0.043, Figure 25B). All other transcripts showed no significant change. This data further confirms that MSCs are present in the IM, the increase in SDF-1 transcript may suggest a potential mechanism for regenerative cell recruitment to IM.

Analysis of the transcriptional profile of expanded MSCs from periosteum and IM was undertaken by Sarah Churchman.

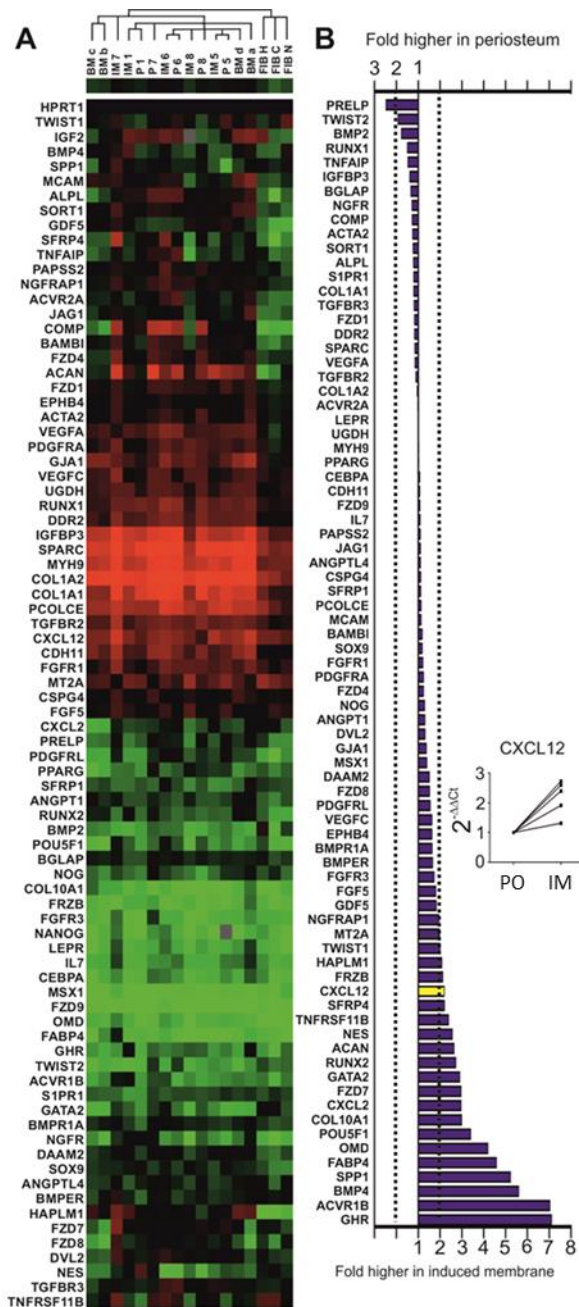


Figure 25: Molecular profile of induced membrane and periosteum derived MSCs

Cluster analysis of log₂ transformed relative expression data: 2^{-ΔΔCt} normalised to *HPRT* (A). IM - induced membrane, PO - matched periosteum, BM - control bone marrow (a-d) and FIB – fibroblasts (C: CRL-2063, H: HFF1 and N-NHDF). Green < *HPRT*, red > *HPRT*, black = *HPRT*, grey is missing data n=17. (B), mean fold change of relative expression data; comparative 2^{-ΔΔCt} method normalising to *HPRT* and periosteum n=5. Yellow indicates p < 0.05, inlayed: difference in expression (2^{-ΔΔCt}) of *CXCL12* (SDF-1) between matched periosteum and induced membrane. Data provided by Sarah Churchman

5.4. Discussion

The purpose of this chapter was to investigate the cellular and molecular basis for the excellent fracture healing noted in man following the use of the Masquelet or IM technique (Masquelet 2003; Retzepi et al. 2010). Experiments in a rabbit model have shown some important characteristics of IM, such as vascularisation and production of VEGF, TGF β 1 and BMP-2, (Pelissier et al. 2004). Additionally the presence of MSCs capable of differentiation into bone and cartilage has been demonstrated in a rat model (Gruber et al. 2012). However no human studies detailing the characteristics of IM had been reported. The IM and periosteum shared strong architectural similarities including, vascular features and growth factor expression. This suggests that IM is actively participating in new bone formation and these tissues share common functional roles.

The colony forming potential and tri-lineage differentiation capacity of enzymatically released cells confirmed that IM is a rich source of MSCs. Furthermore, the number of CFU-Fs generated from IM and periosteum was similar and both far greater than the observed frequency in BM aspirates. Chapter 3 of this thesis demonstrated that the absolute number of CD45^{-/low}CD271⁺ cells to closely correlate with the number of CFU-F colonies generated from BM aspirates. This was not replicated in periosteum or IM digests, suggesting that CD271 expression is not limited to MSCs in this tissue. This is interesting due to the significantly increased number of cells expressing CD271 in the IM and warrants further investigation. The histological composition of the IM showed that it was well vascularised and comparable to periosteum, but the membrane was much thicker. In keeping with this, flow cytometry revealed an increased number of cells with a pericyte phenotype suggesting more active vessel formation and/or maturation (Chen et al. 2009; Stratman et al. 2012).

The molecular analysis of expanded cells from IM and periosteum revealed a broadly similar RNA profile to BM-MSCs, but significantly different from skin fibroblasts with respect to osteoprogenitor and chondroprogenitor transcripts. However, the relative abundance of SDF-1 transcript (*CXCL12*) was greater in expanded cells from IM compared to periosteum. SDF-1 is a chemokine released by

MSCs which acts on a number of cell types including MSCs themselves (Ponte et al. 2007), endothelial progenitor cells, haematopoietic lineage cells and haematopoietic stem cells (Ponte et al. 2007; Miller et al. 2008; Maksym et al. 2009). The expression of this molecule has been validated by staining for SDF-1 protein on tissue biopsies, and was shown to be present in the areas surrounding vessels. It is possible that SDF-1 is involved in autocrine and paracrine regulation of MSC trafficking into the newly-formed tissue. This property of IM derived MSCs suggests an enhanced ability to recruit cells into the defect area capable of directly or indirectly participating in tissue repair. The increased numbers of lymphocytes found in IM also supports this hypothesis.

Additionally, expanded MSCs from both tissues showed similar osteogenic potential. Therefore, the regenerative capacity of these tissues *in vivo* is likely to be comparable, an important finding given the recognised importance of periosteum in both endochondral and intramembranous ossification during fracture healing (Chan et al. 2012). In order to achieve the number of cells required to demonstrate tri-lineage differentiation some culture expansion was carried out. The effect of culture expansion on MSC tri-lineage potential has been well documented, as the number of population doublings increases differentiation capacity declines (Banfi et al. 2002; Wagner et al. 2008; Wagner et al. 2009) this particularly affects adipogenic differentiation, which has been shown to decline more rapidly with culture expansion than osteogenic and chondrogenic differentiation potentials (Wagner et al. 2008) and may explain the low level of adipogenesis observed. Similarly it has been shown that phenotypic properties of MSCs such as their immunosuppressive capacity can be transiently suppressed following freezing (Francois et al. 2012). For these reasons it is possible that *in vivo* MSCs in both periosteum and IM have greater differentiation capacity than observed *in vitro*.

The implantation of a scaffold that replicates the ability of a cement spacer to induce membrane formation, without the need for removal at a later stage could result in improved clinical outcomes. Therapy could be carried out as a one-step procedure, minimising economic impact and patient recovery time. This would represent a radically simple and inexpensive approach compared to alternative

strategies that have been suggested such as composite scaffolds (Wang et al. 2007), decellularised bone scaffolds (Grayson et al. 2010) and bioactive scaffolds incorporating growth factors such as BMP-2 and VEGF (Huang et al. 2005; Niu et al. 2009). Such approaches normally incorporate extensive MSC culture expansion and are therefore subject to regulation under GMP guidelines further increasing their cost (Couto et al. 2012).

Although the work presented here gives insight into the mechanism by which the IM promotes bone regeneration, it does not address the underlying mechanisms that are responsible for the generation of the IM itself. Nor does it address the role played by the nature and application of the cement spacer. Future work should be directed at understanding the mechanisms by which the IM is generated. The process of IM formation may be similar to that which occurs as part of the foreign body response; here acute inflammation is followed by local vascularisation and fibrous capsule formation (Luttikhuisen et al. 2006; Anderson et al. 2008). Certainly, there is clearly an increased number of CD45⁺ leukocytes present in IM, compared to periosteum and the presence of fibrous tissue. However, the similarity of IM to periosteum may indicate a unique reaction, driven in part by the proximity of intact periosteum.

6. The effect of tofacitinib on MSC proliferation and differentiation

6.1. Introduction

In the last chapter it was seen that native MSCs were very abundant in the IM, a surgically induced periosteum like tissue. Some human arthropathies such as ankylosing spondylitis (AS) and psoriatic arthritis (PsA) are characterised by spontaneous new bone formation at the entheses (tendon and ligament insertions). In AS this particularly effects the spine and culminates in joint fusion (Ball 1983; Braem et al. 2012).

Whilst new bone formation is highly desirable in major bone defects, in the context of AS and PsA it may be desirable to inhibit bone formation, thus avoiding the associated, progressive disability (Braem et al. 2012). Unfortunately, the most effective drugs currently available to treat AS and PsA, the anti-TNF agents, although very effective at resolving inflammation, do not stop ongoing new bone formation (van der Heijde et al. 2008a; van der Heijde et al. 2008b). Whilst tumour necrosis factor α blockade often halts joint erosion in RA (Lipsky et al. 2000; Feldmann 2002), radiographic progression, in this case bone formation, to a variable degree continues in AS (van der Heijde et al. 2008a; van der Heijde et al. 2008b). The failure of TNF blockade to address radiographic progression, has served as an impetus for continued research and has stimulated the search for alternative therapies to treat AS and PsA.

Developed under drug development code CP-690,550 and first noted for its ability to prevent allograft rejection (Changelian et al. 2003), tofacitinib is one of several JAK inhibitors in clinical use for the treatment of RA (Kyttaris 2012). It is a small molecule inhibitor which competitively binds the ATP binding site of janus kinase 1 (JAK1), JAK2, JAK3 and to a lesser extent tyrosine kinase 2 (TYK2) (Karaman et al. 2008; Meyer et al. 2010). Administered orally, tofacitinib has shown promising results in phase III trials with patients showing rapid and clinically meaningful improvements in signs and symptoms of RA over six months, with an acceptable safety profile (Burmester et al. 2013). It is now licenced in many countries, but not

the UK. The interest in using tofacitinib to treat PsA and AS is building especially with data showing efficacy for psoriasis (Papp et al. 2012), an important clinical manifestation of PsA (Busquets-Perez et al. 2012). Given the important role of MSC biology in bone homeostasis, this offers a novel opportunity to investigate the effect of tofacitinib on MSC biology using a drug that is destined for the clinic.

6.1.1. Chapter aim and objectives

The aim of this chapter was to examine the effect of tofacitinib on MSC growth and differentiation, as a pre-clinical *in vitro* model, to look for effects on new bone formation.

1. Objectives: To confirm the ability of tofacitinib to inhibit STAT phosphorylation following JAK activation.
2. To characterise the effect of pharmacological doses of tofacitinib on MSC proliferation and differentiation.

6.2. Materials and methods

6.2.1. JAK activation in peripheral blood mononuclear cells

In order to confirm the ability of tofacitinib to inhibit JAK activity, STAT-3 phosphorylation following cytokine stimulation was assessed in peripheral blood mononuclear cells (PBMCs). Blood was taken from a single donor (Female, age 22) and PBMCs were isolated by density gradient centrifugation; 20ml of whole blood was mixed in a 1:1 ratio with PBS, this was then divided into two equal volumes and carefully layered onto 15ml lymphoprep solution (Axis Shield) in two 50ml centrifuge tubes. These tubes were then centrifuged at 1150 xg for 20 minutes with moderate acceleration and no brake. Following centrifugation the PBMCs had formed a distinct turbid band, which was harvested using a Pasteur pipette. Following addition of 30ml of PBS a further centrifugation at 650 xg was used to remove the lymphoprep solution and the PBMCs were re-suspended in 10ml of DMEM (Invitrogen).

PBMC were counted using a haemocytometer (2.3) and divided into 8 separate aliquots each containing 3×10^6 cells suspended in 3ml DMEM. The cells were subsequently exposed to tofacitinib and/or cytokines as seen in Table 3. IL-6 (10ng/ml) and IL-21 (50ng/ml) (Cocco et al. 2012) were chosen to stimulate STAT-3 phosphorylation since signalling by these cytokines is known to be mediated by STAT-3 (Rodig et al. 1998; Habib et al. 2002). Cells were incubated in DMEM or DMEM plus drug for 30 minutes at 37°C prior to any cytokine stimulation. Cytokines were added directly to the cell suspensions which were then incubated at 37°C for 30 minutes, cells were subsequently pelleted by centrifugation and the supernatant was removed. Cells were then lysed by addition of sample buffer (Appendix 2), and heated to 90°C for 10 minutes to prevent further enzyme activity and denature cellular proteins. Cell lysates were then frozen and stored at -20°C.

6.2.2. Detection of phosphorylated STAT-3 by western blotting

Frozen PBMC lysates generated in section 6.2.1 were probed for the presence of phosphorylated STAT-3 (pSTAT-3) using western blotting with anti-human pSTAT-3 (Millipore). The BioRad Protein 3 mini electrophoresis system was used for casting gels and all electrophoresis steps. A 10% acrylamide gel was prepared as described in Appendix 2, with a thickness of 1mm using a western blot gel mould (Bio-Rad) and allowed to fully set, a stacking layer was added to improve band resolution (Appendix 2). The gel was subsequently loaded into an electrophoresis chamber with running buffer (Appendix 2). Following a 5 minute incubation at 90°C, 20µl of each protein lysate generated in section 6.2.1 were loaded into separate wells; 10µl of a visible protein ladder Precision Plus Protein™ Dual Color Standards (BioRad) was also loaded into one well in order to determine the molecular weight of labelled proteins. A constant 125V was applied to the gel for 1 hour, after which it was removed and from the gel tank and taken out of its mould.

Next, the gel was loaded into a transfer cassette in contact with a nitrocellulose membrane (Amersham). The transfer cassette was loaded into a second electrophoresis tank containing transfer buffer (Appendix 2). A constant 100V was applied for 1 hour at 4°C. Once transfer was complete, the nitrocellulose membrane was taken out of the cassette and incubated in 15ml of blocking buffer containing 5% w/v marvel milk powder (Appendix 2) for 3 hours at RT to block non-specific protein binding. Using the visible protein ladder as a guide the membrane was then cut into a high molecular weight (>50kDa) and a low molecular weight (<50kDa) portion.

The high molecular weight portion was incubated overnight at 4°C in 15ml of blocking buffer containing a monoclonal rabbit anti human pSTAT-3 (Tyr705) antibody (Appendix 4). The membrane was then washed 4 x 20 minutes in 15ml of washing buffer and incubated in 15ml of blocking buffer containing goat anti-rabbit IgGs conjugated to horseradish peroxidase (HRP) enzyme (Appendix 4) for 1 hour at RT. Finally, the membrane was washed again, as previously described and incubated with SuperSignal West Pico Chemiluminescent Substrate (Thermo) for 5 minutes, enzymatic conversion of the substrate by HRP results in the generation of

a chemiluminescent product. The excess reagent was discarded and the membrane was wrapped in Saran wrap (Dow). In the dark, Amersham hyperfilm ECL film (GE healthcare) was exposed to the now chemiluminescent membrane for 1 minute and then developed using a SRX-101A developer (Photon). pSTAT-3 was expected to appear at 84Kda (pSTAT-3 α) or 76DKa (pSTAT-3 β) (Chakraborty et al. 1996).

The low molecular weight portion was incubated overnight at 4°C in 15ml of blocking buffer containing mouse anti human β -Actin monoclonal antibody (Appendix 4), this was done to demonstrate equal total protein loading into each well. The membrane was then washed 4 x 20 minutes in washing buffer and incubated in 15ml of blocking buffer containing HRP conjugated goat anti mouse IgGs (Appendix 4) for 1 hour at RT. The membrane was washed as previously described and subsequently incubated with Pierce ECL Western Blotting Substrate (Thermo) for 1 minute. The excess reagent was discarded and the membrane was wrapped in Saran wrap. In the dark, Amersham hyperfilm ECL film was exposed to the membrane for 5 seconds and then developed as previously described. B-actin was detected as a 42kDa band (Hu et al. 1993).

Table 3: Activation conditions used to stimulate STAT-3 phosphorylation in PBMCs

Aliquot / well number	Cytokine	Tofacitinib
1	IL-6 10ng/ml	-
2	IL-21 50ng/ml	-
3	IL-6 10ng/ml	500nM
4	IL-21 50ng/ml	500nM
5	IL-6 10ng/ml	100nM
6	IL-21 50ng/ml	100nM
7	-	-
8	-	500nM

6.2.3. CFU-F assay

The effect of tofacitinib on MSC colony initiation and growth was determined. Iliac crest BM aspirates were taken from 8 donors (mean age 46, 5 male 3 female). The

assay was performed using whole BM aspirates and proceeded as described (2.7) with some modifications. 100µl of BM aspirate was seeded in duplicate 10cm culture dishes containing 15ml of NH media. Tofacitinib was added to the culture media at a range of concentrations, 100nM, 500nM, 1000nM and 10000nM. These concentrations were decided in consultation with Pfizer, with 1000nM the expected pharmacological concentration. A carrier control was also included containing dimethyl sulfoxide (DMSO) at a concentration equivalent to that required to constitute the 10µM dose, this was achieved by adding 1.5µl DMSO per dish. A no drug/no DMSO control was also included. The number of colonies was scored as previously described (2.7) and the total colony area was measured as previously described (2.8).

6.2.4. Tri-lineage differentiation assays

To assess the effect of tofacitinib on MSC differentiation, osteogenic, adipogenic and chondrogenic differentiation assays were performed as described (2.14). For these assays passage 3 MSC cultures from 6 BM donors were used (mean age 47, 3 male, 3 female). Osteogenic cultures were terminated after 14 days for assessment of ALP activity (2.11), and after 21 days for analysis of calcium production (2.12). Chondrogenic cultures were terminated after 21 days and glycosaminoglycan (GAG) accumulation was assessed by toluidine blue staining (2.14) and alcian blue assay (6.2.6). Adipogenic induction was terminated at 14 or 21 days and assessed by oil red (2.13) and Nile red (6.2.5) staining.

Tofacitinib was added to each differentiation media (Appendix 2) at a concentration of 500nM, 1000nM and 10000nM and as mentioned above, a DMSO control was included. Media changes proceeded as described previously for all assays with tofacitinib addition as above at each media change. This assay assumed no stability associated decrease in drug concentration in culture.

6.2.5. Quantitative assessment of adipogenesis

Quantitative measurement of adipogenic induction was assessed by spectral analysis of photomicrographs taken following oil red staining as described (2.13), and by the measurement of fluorescence following Nile red staining.

For Nile red staining, culture expanded MSCs were seeded in 0.5ml/well of adipogenic media (Appendix 2) in triplicate at a density of 4×10^4 cells/well into 48 well tissue culture grade plates (Corning), differentiation then proceeded as for oil red assay previously described (2.13). At the end of the culture period, following formalin fixation in fixing buffer (Appendix 2, 0.5ml/well), cells were washed with 0.5ml PBS; which was aspirated fully and further 200 μ l of PBS was added to each well. A Mithras LB 940 plate reader (Berthold) was then used to measure emission at 460nm and 535nm following excitation at 355nm and 485nm respectively, this was taken as a background reading. The PBS was then replaced with PBS supplemented with 0.2% saponin (Sigma Aldrich), 1 μ g/ml DAPI (Sigma Aldrich) and 1 μ g/ml Nile red (Sigma Aldrich), saponin forms pores in lipid bilayers enabling the entry of Nile red which is strongly fluorescent in the presence of lipid. Cells are then incubated for 15 minutes at RT before washing with 1ml PBS. As before, wells were filled with exactly 200 μ l of PBS and read at 460nm and 535nm following excitation at 355nm and 460nm respectively. To calculate fold induction of adipogenesis, the background reading for each individual well was subtracted from the second reading; the mean emission at 535nm for each triplicate well was divided by the mean reading of the control (DMSO only) triplicate to give fold change in adipogenic induction. This was repeated to calculate the relative difference in DAPI fluorescence at 460nm. Fluorescent images were capture using an Eclipse Ti microscope (Nikon).

Following initial experiments using tofacitinib at concentrations between 500nM and 10000nM the assay was repeated at concentrations of tofacitinib of 10, 50, 100 and 500nM. The induction time was lengthened to 21 days and the assay was performed on passage 3 expanded BM MSCs from 7 donors (mean age 51, 5 male 2 female). This was to further explore the effect of tofacitinib on adipogenic induction

at lower concentrations of drug, a longer induction time was used in order to increase assay sensitivity.

6.2.6. Detection of glycosaminoglycans

Glycosaminoglycan production was measured in order to quantitatively measure chondrogenic induction. Day-21 chondrogenic pellets generated in section (2.14) were washed with 1ml of PBS and digested by incubation overnight at 65°C overnight in 1ml of papain buffer (Appendix 2). The resulting solution was mixed by vortexing and then centrifuged at 5000xg for 5 minutes to remove the residual debris. Subsequently a, sGAG quantitative kit (Wieslab) was used to measure the produced sGAG according to manufacturer's instructions. This assay utilises alcian blue binding to GAGs, which were then detected by measuring absorbance at 600nm using an Opsys MR plate reader (Dynex Technologies).

6.2.7. Statistics

Friedman's two-way analysis of variance by ranks was used to detect statistical difference in all data sets, with pairwise post-hoc comparison used to analyse significance between variables. The significance level was set at 95%. *denotes statistical significance * $p < 0.05$, ** $p > 0.01$, *** $p > 0.001$.

6.3. Results

6.3.1. Effect of tofacitinib on cytokine induced STAT-3 phosphorylation in PBMCs

In order to confirm the ability of tofacitinib to inhibit JAK mediated STAT phosphorylation, PBMS were exposed to cytokine stimulus known to be mediated by STAT-3 phosphorylation (Kiu et al. 2012). PBMCs were chosen due to the presence of cells known to be responsive to IL-6 and IL21 (Cocco et al. 2012). Pre-incubation with 500nM tofacitinib has a clear inhibitory effect on IL-6 and IL-21 mediated STAT-3 phosphorylation in PBMCs (Figure 26). Lysates from cytokine-stimulated PBMCs showed a high level of pSTAT-3 which was clearly reduced with pre-incubation of 500nM tofacitinib to a level equal to or below the unstimulated control. Phosphorylation of the heavier α -isoform in particular was inhibited leading to loss of detection at the 500nM concentration of tofacitinib. Tofacitinib pre-incubation at a concentration of 100nM also had a reduced but clear inhibitory effect on STAT-3 phosphorylation, especially with IL-21 stimulation. Tofacitinib was not able to inhibit baseline STAT-3 phosphorylation. In summary this data shows that tofacitinib is able to inhibit IL-6/IL-21 induced JAK mediated STAT-3 phosphorylation, this experiment provides a positive control for subsequent CFU-F and differentiation assays.

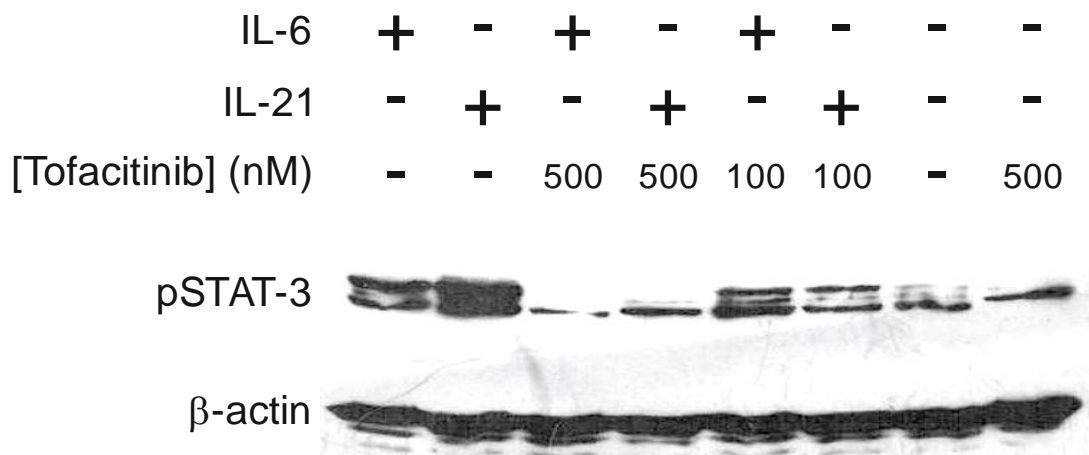


Figure 26: Western blot of pSTAT-3 / β -Actin in IL-6 and IL-21 stimulated PBMCs

Scanned image of combined western blot films showing pSTAT-3 α (84kDa), pSTAT-3 β (76kDa) and β -Actin (loading control, 42kDa) from lysates of activated PBMCs (stimulated with IL-6 and IL-21, pre incubated with 500nM or 100nM tofacitinib).

6.3.2. Effect of tofacitinib on BM CFU-F potential

Once the inhibitory effect of tofacitinib on JAK activity had been confirmed, its influence on MSC colony formation and differentiation was investigated. The addition of tofacitinib did not have any detectable effect on BM MSC colony forming potential at any of the concentrations tested (Figure 27A). Tofacitinib did not have any discernible effect on overall colony area (Figure 27B) or the average size of individual colonies (Figure 27C). Overall there was no evidence to suggest that tofacitinib has any effect on MSC attachment or expansion *in vitro* (all data n=8 donors).

A well-known mediator of MSC self-renewal is FGF (Tsutsumi et al. 2001; Bianchi et al. 2003; Zaragosi et al. 2006), this elicits its response independently to the JAK/STAT pathway. Other mediators of MSC proliferation include PDGF and epidermal growth factor (EGF) (Gronthos et al. 1995). Although both these factors are known to activate the STAT pathway, their effect on MSC proliferation is thought to be mediated by activation of the MAPK and PI3K pathways (Kratchmarova et al. 2005; Rodrigues et al. 2010). This negative result supports the notion that JAK/STAT signalling is not important for *in vitro* MSC expansion.

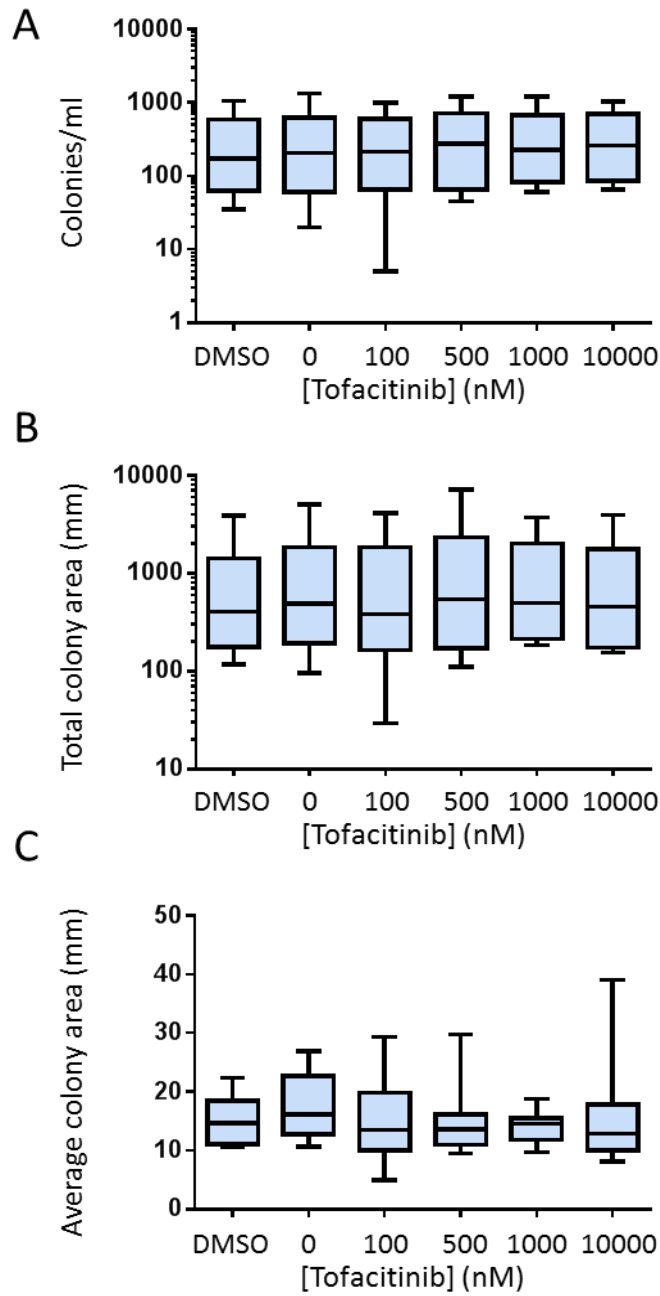


Figure 27: The effect of tofacitinib on MSC colony forming potential.

The effect of Tofacitinib on the ability of BM MSCs to initiate adherent colonies and expand *in vitro* (A). The effect of Tofacitinib on the rate of MSC expansion measured as the total area occupied by colonies (B), or as the mean area occupied by a single colony (C). All data n=8.

6.3.3. The Effect of tofacitinib on *in vitro* MSC osteogenesis

The inclusion of tofacitinib in culture medium during osteogenic induction of MSCs did not have a noticeable effect on alkaline phosphatase activity following 14 days of induction (Figure 28A). The accumulation of calcium was similarly unaffected by tofacitinib at any of the concentrations tested (Figure 28B). Overall there is no evidence to suggest that tofacitinib had any effect on *in vitro* osteogenesis of MSCs at the concentrations of tofacitinib tested (all data n=6 donors). This result indicates that JAK/STAT signalling is not required for *in vitro* osteogenesis in this particular model system despite the documented stimulatory effect of GH on osteogenesis mediated by JAK2 (Wang et al. 2004; Hadjidakis et al. 2006; DiGirolamo et al. 2007; Giustina et al. 2008).

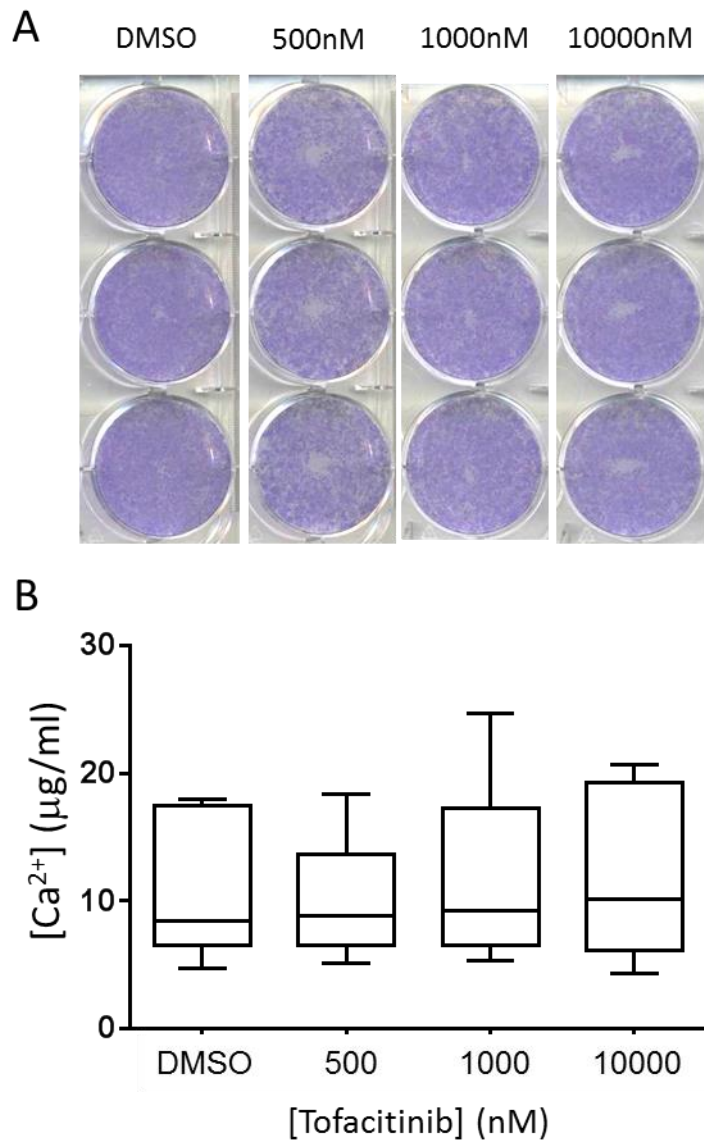


Figure 28: Effect of tofacitinib on MSC osteogenesis.

Fast blue staining for ALP activity following osteogenic induction of MSC differentiation in the presence or absence of tofacitinib. Figure shows a single representative donor sample 14 days post induction, shown in triplicate wells (A). The Effect of tofacitinib on osteogenic differentiation assessed by accumulation of calcium day-21 post osteogenic induction n=6 (B).

6.3.4. Effect of tofacitinib on *in vitro* MSC chondrogenesis.

The inclusion of tofacitinib in culture medium during chondrogenic induction of MSCs did not affect the accumulation of GAGs in chondrogenic pellets (all data n=6 donors, Figure 29). Tofacitinib had no effect on pellet formation or GAG content as shown in Figure 29A. Quantitative measurement of GAG production following chondrogenic induction confirmed this observation (Figure 29B). This result is encouraging for the use of tofacitinib in RA especially, where cartilage preservation is a critical concern. It also further weakens the hypothesis that tofacitinib can inhibit *in vivo* osteogenesis since endochondrial ossification is preceded by chondrocyte proliferation (Maes et al. 2010; Mackie et al. 2011; Dirckx et al. 2013).

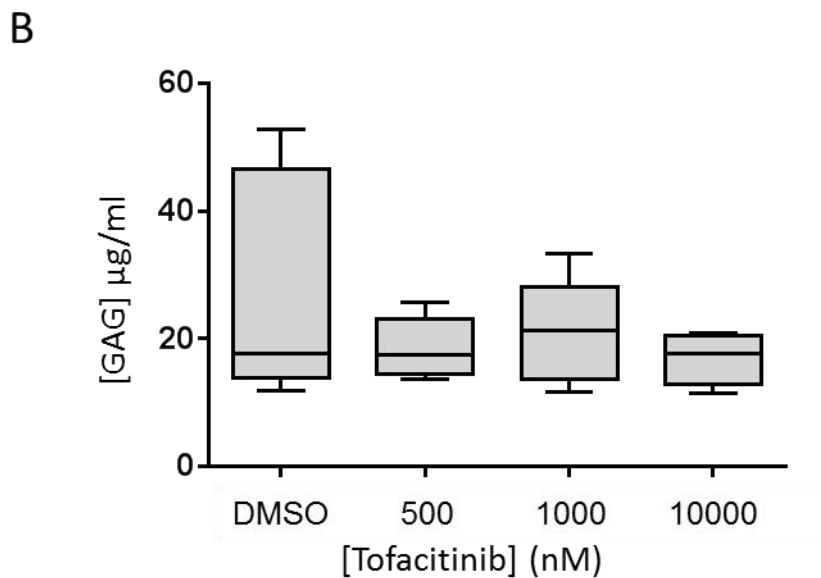
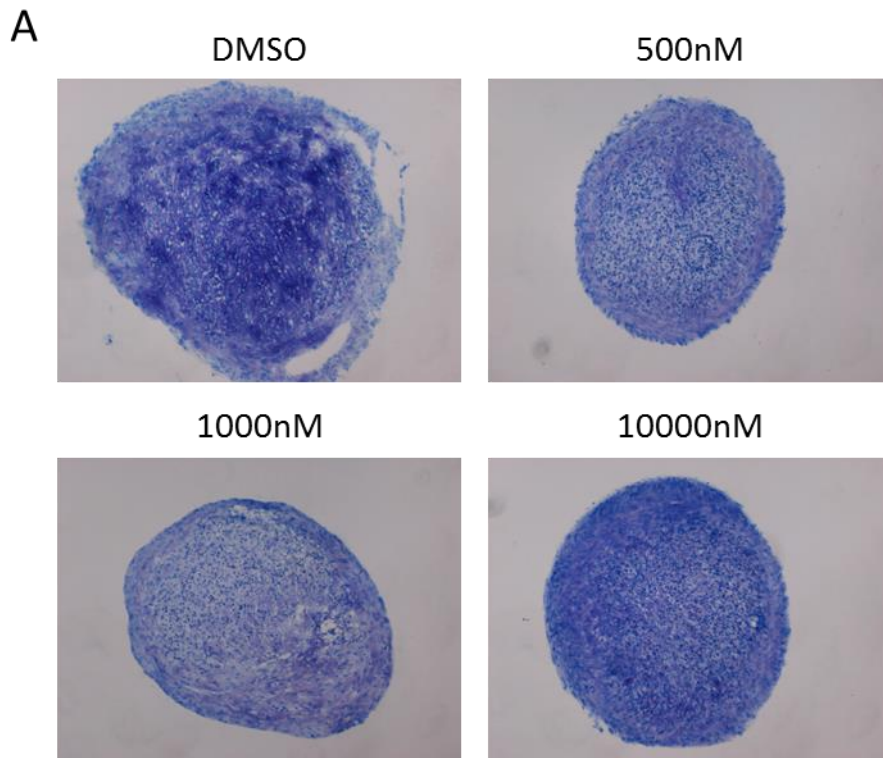


Figure 29: Effect of tofacitinib on MSC chondrogenic differentiation.

The effect of tofacitinib on MSC chondrogenic differentiation, assessed by accumulation of GAGs following 21-day chondrogenic induction. Toluidine blue staining of chondrogenic pellets (A). Assessment of total GAG content in digested chondrogenic pellets n=6 (B).

6.3.5. The effect of tofacitinib on *in vitro* MSC adipogenesis.

When MSCs were cultured in adipogenic conditions the presence of tofacitinib had a clear effect. The rate of adipogenesis was noticeably increased in cultures exposed to high concentrations of tofacitinib (Figure 30A). The median proportion of area occupied by lipid vacuoles stained with oil red, was increased by incubation with 1000nM tofacitinib to 8.21% (range 4.14-15.69) compared to 5.35% (range 2.08-11.20) in the DMSO control; although this change was short of statistical significance. Statistical significance was achieved at a tofacitinib concentration of 10000nM (10.67%, range 4.15-22.06, $p=0.022$, Figure 30B).

Further confirmation of the stimulatory effect of tofacitinib on *in vitro* MSC adipogenesis was observed following the analysis of fluorescence after Nile red staining (Figure 31A). As with oil red measurement, there was a significant increase in the extent of adipogenesis measured by median fluorescence emission of Nile red, detected between the DMSO control and 10000nM tofacitinib concentration (2.02 fold, range 1.34-4.44, $p=0.022$). (1.99 fold, $n=6$ donors, range 0.91-3.16, $p=0.075$).

Further investigation of this phenomenon using lower concentrations of tofacitinib and a longer adipogenic induction (21 days), revealed a clear dose-dependent increase in adipogenesis. There were significant increases in the median fluorescence emission after Nile red staining with a 100nM dose (1.38 fold, range 1.01-1.54, $p=0.041$) and a 500nM dose (2.14 fold, range 1.26-2.57, $p<0.001$) relative to the carrier control (Figure 31C). A significant difference was also observed between the 10nM dose compared to 100nM and 500nM doses ($p=0.041$ and $p<0.001$ respectively). These data provided compelling evidence that JAK inhibition in adipogenically differentiating MSCs by tofacitinib leads to an increase in adipogenesis. In order to determine if this effect was a result of proliferation of increased adipogenic induction the ratio of Nile red to DAPI fluorescence was compared at each concentration of tofacitinib. When standardised to DAPI signal tofacitinib induction of adipogenesis was only significant compared to the carrier control at the 500nM dose ($p=0.001$, Figure 31D), although a significant change was also observed between the 10nM and 50nM doses compared to the 500nM dose

($p=0.004$ and 0.041 respectively). The drop in sensitivity observed suggests that proliferation as well as enhanced adipogenic induction

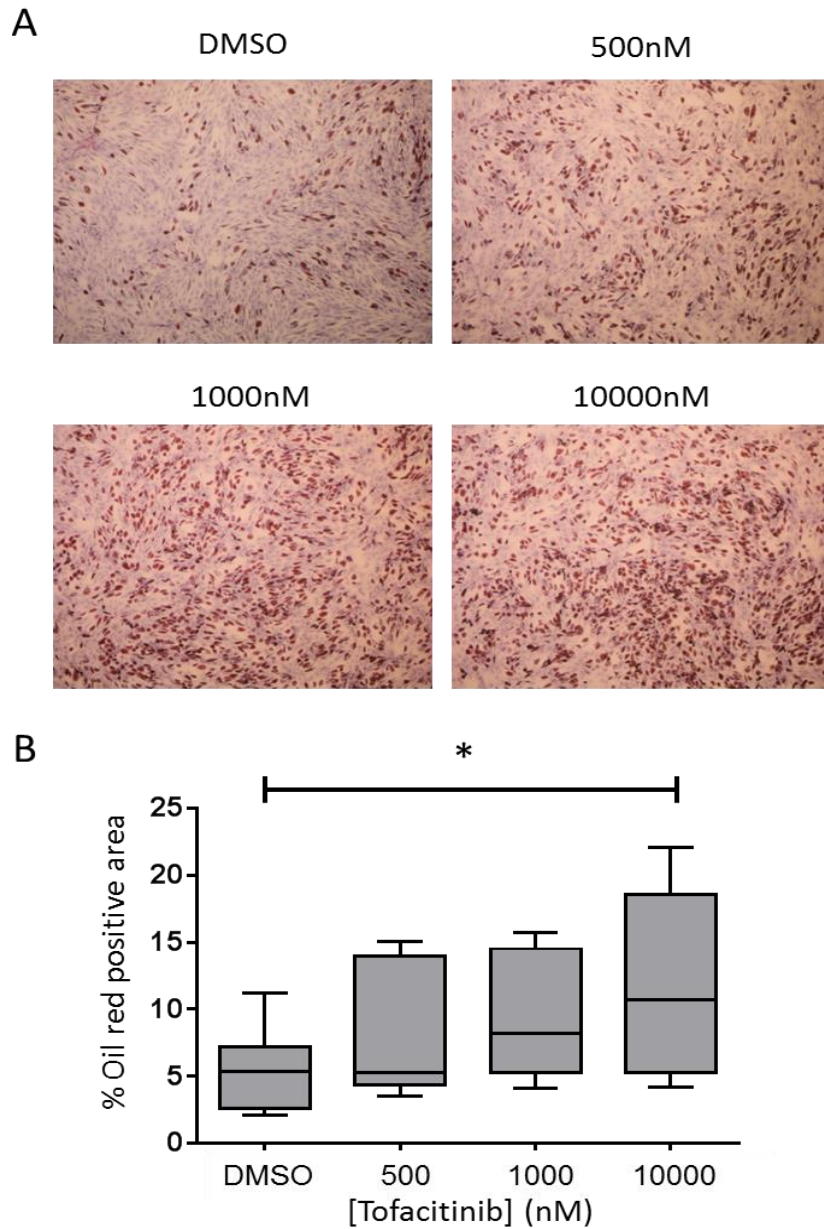


Figure 30: Oil red staining on day 14 of MSC adipogenic differentiation in the presence of tofacitinib.

Representative photomicrographs showing the effect of different concentrations of Tofacitinib or DMSO carrier control on MSC adipogenesis, shown by uptake of oil red into lipid vacuoles (red) counterstained with haematoxylin (A). Measurement of oil red stained area in photomicrographs of adipogenic cultures supplemented with tofacitinib n=6 (B). Box plot shows interquartile, range median and extreme values, *denotes $p < 0.05$.

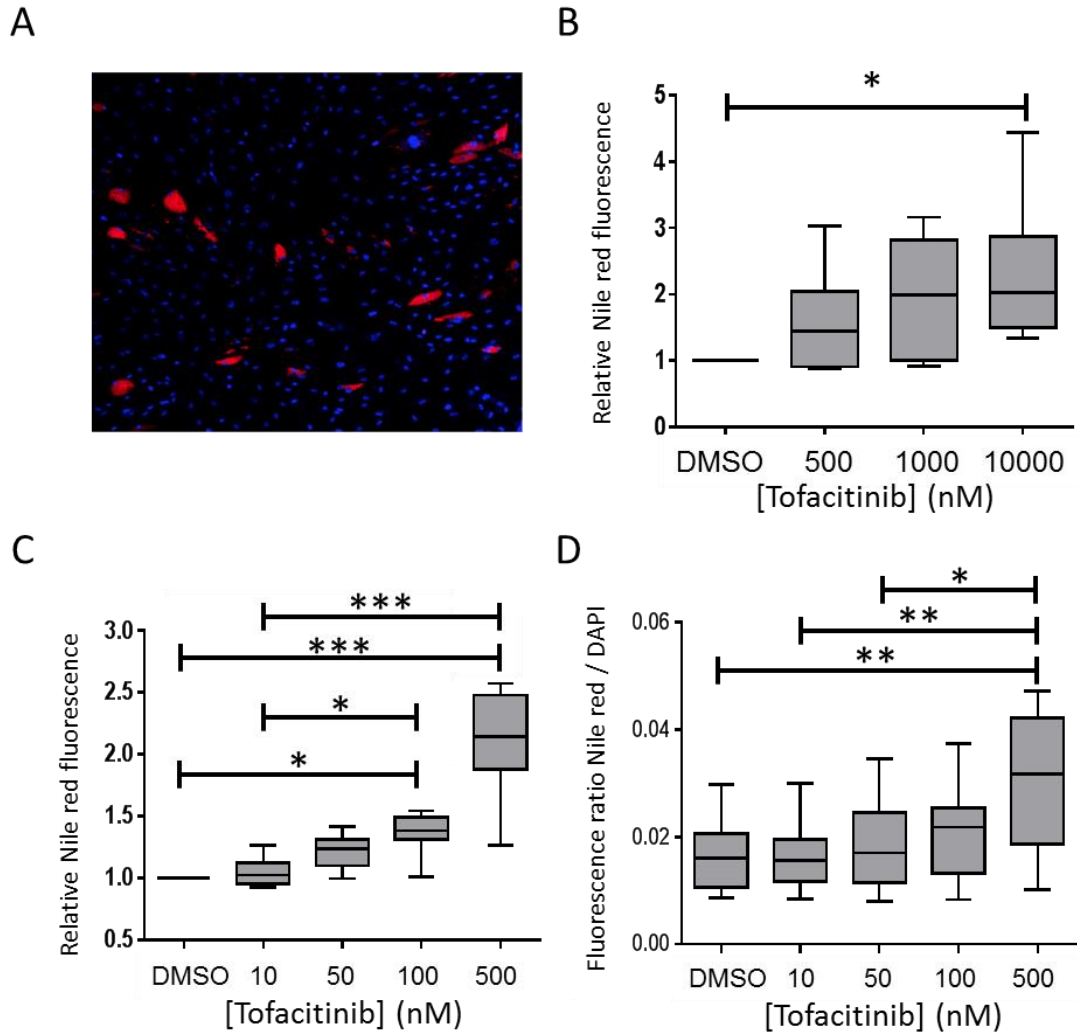


Figure 31: Adipogenic differentiation of MSCs in the presence of tofacitinib measured by Nile red fluorescence.

Representative photomicrograph showing Nile red uptake into lipid vacuoles (red), counterstained with a nuclear DAPI stain (blue) (A). MSC adipogenesis in the presence of tofacitinib or carrier control shown by Nile red fluorescence following 14 day induction n=6 (B) and 21 day induction n=7 (C) Adipogenesis shown as a Nile red fluorescence normalised to DAPI fluorescence following a 21 day adipogenic induction in the presence of tofacitinib. Box plots show interquartile range, median and extreme values, * denotes $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

6.3.6. The effect of tofacitinib on *in vitro* adipocyte proliferation

The measurement of DAPI fluorescence has previously been proposed as a means of standardising Nile red signal in quantitative adipogenic assays (Aldridge et al. 2013). However, this assumes that the proportion of MSCs that successfully differentiate into adipocytes is the only factor governing the number of adipocytes present. Increased proliferation of MSCs or pre-adipocytes could increase the DAPI signal. Therefore, it is not appropriate to use DAPI fluorescence as a means of standardising this assay. Nevertheless DAPI fluorescence is a useful indicator of the relative number of cells in adipogenic cultures since DAPI forms highly fluorescent complexes with DNA (Wang et al. 2000).

Induction of adipogenesis for 14 days resulted in a significant increase in DAPI fluorescence over the DMSO control at 500nM (1.44-fold, range: 1.33-2.11), and 10000nM (1.69-fold, range: 1.30-2.25) concentrations of tofacitinib (n=6, p=0.044 and 0.005 respectively, Figure 32A). A 21 day adipogenic induction resulted in a significant increase in DAPI signal at the 500nM (1.10-fold, range: 1.03-1.21) concentration of tofacitinib (n=7, all p=0.001, Figure 32B). All together this data shows that the total number of cells following *in vitro* MSC adipogenesis is increased by the presence of tofacitinib. Tofacitinib has been shown not to affect MSC proliferation *in vitro* (Figure 27), therefore this increase is likely partly a result of increased pre-adipocyte or adipocyte proliferation.

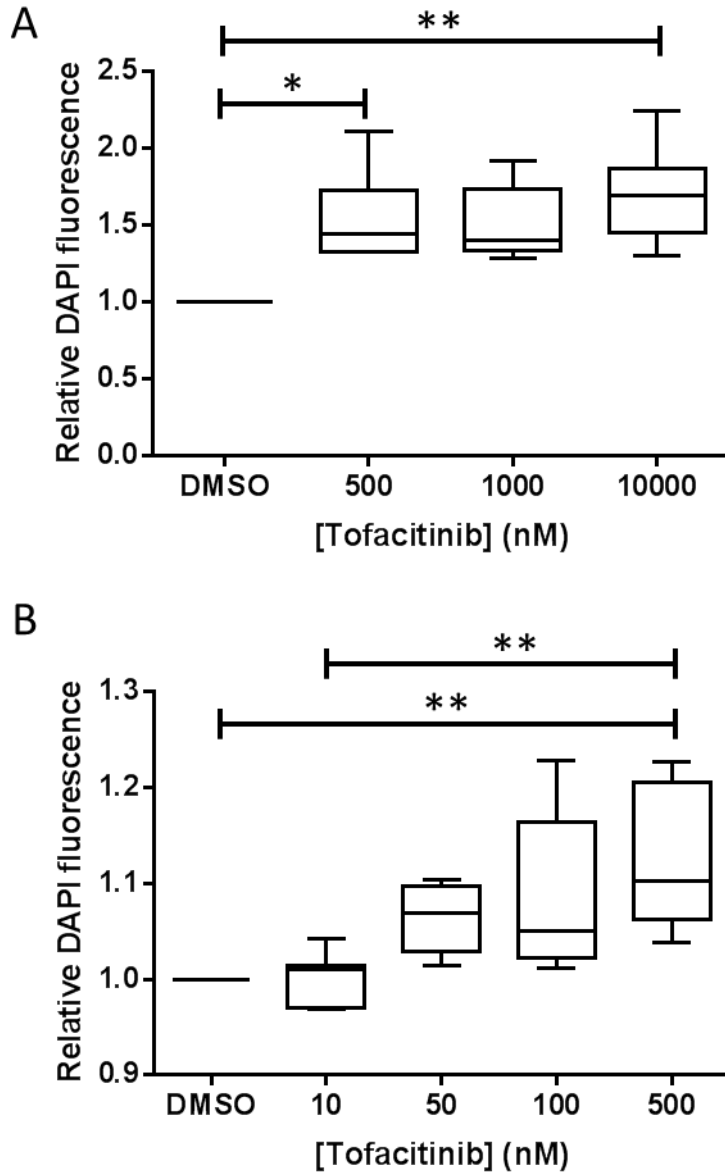


Figure 32: Adipocyte hyperplasia during adipogenic differentiation of MSCs in the presence of tofacitinib.

Relative cell number measured by DAPI fluorescence after 14 day n=6 (A) and 21 day n=7 (B) induction of MSC adipogenesis in the presence of tofacitinib. Box plots show interquartile range median and extreme values, * denotes $p < 0.05$, ** $p < 0.01$.

6.4. Discussion

Aberrant enthesal new bone formation in AS and PsA has been tentatively associated with dysregulated osteoprogenitor activity (Lories et al. 2005). Existing therapies fail to control this abnormal post inflammatory reaction (van der Heijde et al. 2008a; van der Heijde et al. 2008b). This chapter examined the effect of tofacitinib, a novel JAK inhibitor in pre-clinical development for AS and PsA, on MSC proliferation, osteogenic, chondrogenic and adipogenic differentiation. Tofacitinib clearly inhibited JAK mediated STAT3 phosphorylation following the stimulation of PBMCs with both IL-6 and IL-21. However, JAK inhibition did not have a detectable effect on MSC colony forming potential or proliferation assessed by measurement of colony area. Neither did JAK inhibition effect osteogenic or chondrogenic differentiation.

Tofacitinib had a clear effect during adipogenic differentiation. Concentrations as low as 50nM were able to significantly increase the lipid content measured by Nile red assay in comparison to control cultures. DAPI fluorescence indicates that total cellularity is also significantly increased with tofacitinib stimulation. This is surprising given that studies examining adipogenesis in 3T3-L1 cells, a cell line widely used as an *in vitro* adipogenic differentiation model (Deng et al. 2000; Floyd et al. 2003; Zhang et al. 2011) suggested that STAT5A and STAT5B promoted adipogenesis particularly in the early stages of differentiation, and one might expect that a JAK inhibitor would antagonise this process (Nanbu-Wakao et al. 2002; Floyd et al. 2003). Moreover, Deng *et al* showed that STAT3 was activated and bound to DNA during proliferative phases of adipogenesis and suggested that STAT-3 regulates preadipocyte proliferation (Deng et al. 2000). This was later confirmed and JAK2 was found to act upstream of STAT-3 as demonstrated by small interfering RNA silencing (Wang et al. 2010; Zhang et al. 2011). However, pre-adipocytes are responsive to several cytokines that have been shown to inhibit adipogenesis including IFN- γ (Gregoire et al. 1992), oncostatin M (OSM) (Song et al. 2007) and neuropoietin (White et al. 2008), all of which are potent activators of JAK kinases.

Adipose-specific disruption of JAK2 or STAT3 signalling *in vivo* results in increased adipose mass associated with hypertrophy in mice, adipocytes became enlarged with increased lipid content (Cernkovich et al. 2008; Shi et al. 2014). Shi *et al* found that adipocyte-specific JAK2 deficiency led to increased weight gain, impaired lipolysis and insulin resistance with aging. This led to the proposition that JAK2 inhibition causes excessive lipid accumulation in mature adipocytes (Shi et al. 2014). This is interesting given that hyperlipidaemia has been reported as a side effect of tofacitinib treatment of rheumatoid arthritis (Kremer et al. 2012; van Vollenhoven et al. 2012; O'Shea et al. 2013).

Measurement of DAPI fluorescence following adipogenic induction in the presence of tofacitinib indicated increased cellularity and likely reflects enhanced pre-adipocyte proliferation. Careful measurement of adipocyte size during adipogenesis is required to determine if adipocyte hypertrophy also occurs in this context, or if the enhanced lipid content observed was solely driven by pre-adipocyte differentiation. Further investigation is required to elucidate the mechanism by which tofacitinib stimulates adipogenesis.

The ability of tofacitinib to induce adipogenesis *in vitro* may also have implications for *in vivo* osteogenesis and chondrogenesis. There is a significant body of evidence that suggests that osteogenesis and adipogenesis are antagonistic in the context of WNT signalling (Kennell et al. 2005; Kawai et al. 2007; Laudes 2011). A growing body of evidence suggests that this dichotomy is also relevant to the JAK/STAT pathway. Indeed, OSM a member of the IL-6 family of cytokines, has been shown to promote osteogenesis and suppress adipogenesis in MSCs, an effect for which JAK2 is a crucial mediator (Song et al. 2007; Nawa et al. 2013). Leptin, secreted by adipocytes, is another cytokine known to signal via the gp130 family of cytokine receptors (Vaisse et al. 1996). Obese mice deficient in leptin or the signalling form of its receptor have increased vertebral trabecular bone volume due to increased bone formation (Ducy et al. 2000). Leptin has been shown to have a direct inhibitory effect on MSC adipogenesis, which is lost in MSCs derived from osteoporotic donors (Astudillo et al. 2008). The data presented in this thesis demonstrates that inhibition of JAK/STAT signalling has a direct stimulatory effect

on MSC adipogenesis. Although no decrease in osteogenesis was observed, this may be because artificial, chemical induction of osteogenesis is not sensitive to the more subtle cues that govern *in vivo* cell fate.

In summary, the data presented in this chapter clearly shows that JAK/STAT signal inhibition has a direct effect on MSC adipogenesis *in vitro*. The effect of tofacitinib on lipid metabolism warrants further investigation and has potential important implications to insulin sensitivity and type II diabetes. Finally, the possibility that stimulating adipogenesis may lead to inhibitions of osteogenesis *in vivo* has important implications to bone metabolic disorders including OP, OA and AS.

7. General discussion

This thesis has sought to demonstrate that insights into the nature of human MSCs *in vivo* can be used to develop novel therapeutic approaches for the treatment of bone disease. The development of MSC based therapies in general has been hampered by a number of factors; these include the limited utility of animal models, due to interspecies differences (Peister et al. 2004; Pittenger 2013), the difficulty of obtaining large numbers of MSCs from human subjects (Hernigou et al. 2005b; Jones et al. 2010b; Hernigou et al. 2013), the historical lack of unique and consistent phenotypic markers (Buhring et al. 2009; Nombela-Arrieta et al. 2011; Boxall et al. 2012) and culture expansion induced changes to MSCs (Wagner et al. 2008; Wagner et al. 2009; Boxall et al. 2012). The concept articulated by Caplan in 1991 of isolation, expansion and re-introduction of MSCs leading to differentiation in order to replace damaged tissue (Caplan 1991), has not been translated into widespread clinical applications. Early hopes that haploidentical MSCs may be transplanted, leading to engraftment and differentiation also were frustrated (Horwitz et al. 2001; Otsuru et al. 2012).

The notion of using autologous MSCs that have not been subject to culture expansion has produced some encouraging successes in the field of bone regeneration (Hernigou et al. 2002; Hernigou et al. 2005a; Hernigou et al. 2005b). The use of autologous MSCs in the form of BM aspirates has become fairly common practice in orthopaedic medicine (Dimitriou et al. 2011b; Giannoudis et al. 2011; Giannoudis et al. 2013; Kassem 2013), despite the relatively low and highly variable MSC content in BM aspirates (Hernigou et al. 2005b; Jones et al. 2010b; Hernigou et al. 2013). This is important, because MSC content has been shown to dictate treatment outcome (Hernigou et al. 2005a) and cannot be assessed easily at the point of use. This issue was addressed in chapter 3, which detailed a rapid MSC enumeration technique that can be performed in <40 minutes. Although this technique as it stands could be used as an intraoperative quality control, its reliance on specialist expertise limits its value. However, as a proof of concept it does have significance, as it demonstrates that enumeration of the CD45^{-/low} CD271⁺ population can be used to accurately predict the MSC content of aspirates. Further

refinement of this assay, using more advanced equipment, such as acoustic focussing flow cytometers and the development of simplified protocols could cut assay time significantly. This would be achieved by faster sample preparation time and more rapid data acquisition. A total assay time of <20 minutes, with a protocol simplified such that it may be performed by non-specialist theatre staff is a realistic goal for the development of this technique, and would greatly enhance its utility for MSC therapy in the orthopaedic setting. Careful control of the number of MSCs administered could have an immediate impact in therapeutic approaches that seek to stimulate bone regeneration by the application of concentrated BM.

The dogma that MSCs are rare in the bone has been challenged both by this thesis and by others, who have shown that trabecular bone contains large numbers of MSCs (Noth et al. 2002; Sakaguchi et al. 2004; Jones et al. 2010b). FHs removed as part of total hip replacements offer a potential abundant source of MSCs that may be used for therapy (Noth et al. 2002; Sakaguchi et al. 2004; Jones et al. 2010b). Yet the safety and efficacy of cell therapy utilising MSCs recovered from osteoarthritic hips remains unproven, an issue highlighted by the discovery of a MSC driven component to OA (Zhen et al. 2013).

The liquid waste product of intramedullary reaming is also a potential source of large numbers of MSCs (Porter et al. 2009; Cox et al. 2012; Churchman et al. 2013). Unlike FHs they are not associated with diseased tissue and do not require enzymatic recovery, their primary disadvantage to BM MSCs for therapy lies with the large volume of liquid that they are collected in. The enrichment of these cells using the CliniMACS device together with an anti-CD271 microbead, as demonstrated in chapter 4 goes some way to solving this problem. However the long processing time required (>4 hours) will inevitably limit clinical uptake of the CliniMACS system for immediate use. The Prodigy cell sorter (Miltenyi Biotec) represents the next generation of magnetic bead cell sorters, is a completely automated enclosed system. This has the potential to reduce the time required for sample processing by eliminating the need for manual handling during enrichment.

The utility of MSC therapy is dependent on several factors the most salient of these are safety, purity, time required for processing, preservation of desirable phenotypic properties and cost. The usefulness of CD271 microbead enrichment of MSCs will ultimately be determined by the application. For autologous therapy, MSCs enrichment from RIA waste fluid offers an attractive alternative to concentrated BM, giving far higher numbers of MSCs at superior purity. However, further optimisation of the technique used in these experiments is required to maximise MSC yield. The addition of an anticoagulant at the point of harvesting should greatly improve MSC yield by reducing the number of cells lost prior to selection. Optimisation of the harvesting technique itself may also improve yields, currently harvesting is optimised only for collection of the solid retentate with no consideration to the liquid filtrate. A standardised procedure for collection of this fraction could increase the initial number of MSCs harvested prior to processing.

In chapter 4 the proportion of MSC as well as the proportion of leukocytes prior to and following enrichment was measured. A detailed assessment of the cell types, other than MSCs, that are carried over into the enriched fraction was not performed. This is important because the identity of these cells, particularly those expressing the CD271^{low} CD45⁺ CD73⁻ phenotype may facilitate their removal; a necessary step if this technology were ever considered for allogeneic use. Nor was the differentiation capacity of cells isolated from these tissues confirmed. However, confirmation of the tri-lineage potential of CD271 selected cells from BM has already been reported by others using a research grade bead (Jarocho et al. 2008). Additionally, the presence of MSCs expressing CD271 has already been demonstrated in FH digests and the intramedullary cavity of long bones (Jones et al. 2010b; Cox et al. 2012). Therefore, it is likely that CD271 selection from these tissues will yield MSCs capable of tri-lineage differentiation, although for completion this should be demonstrated.

There is an increasing interest in the use of “smart” scaffolds that rather than relying on MSCs extracted from a remote location such as the iliac crest, seek to stimulate migration of endogenous MSCs into the scaffold matrix (Dashnyam et al. 2014; Jin et al. 2014). Chapter 5 dealt with the examination of an induced

membrane that is formed as a reaction to the implantation of a bone cement spacer, that could be considered as a natural analogue of synthetic smart scaffolds.

The tissue formed was rich in MSCs, the fact that these cells expressed significantly more SDF-1 transcript than MSCs isolated from the periosteum even following culture expansion is significant. SDF-1 transcript expression in MSCs declines rapidly with culture expansion (Churchman et al. 2012), implying that the *in vivo* expression of IM MSC may be considerably greater than that observed in these experiments. In light of evidence suggesting perivascular localisation of MSCs *in vivo* (Sacchetti et al. 2007; Tormin et al. 2011), the observed perivascular expression of SDF-1 protein strengthens this conclusion. Data also suggests that IM is an active site of vascularisation, with a higher proportion of pericytes than in normal periosteum. This is an important quality due to the well-recognised importance of neovascularisation in resolving fractures (Megas 2005; Frolke et al. 2007).

Greater understanding of the mechanism that drives IM formation could potentially lead to a new generation of orthopaedic implants that actively participate in stimulating new bone formation by driving the recruitment of regenerative cells and vascularisation. In many respects, IM formation mirrors the foreign body reaction, where a fibrous capsule is formed around an implant following an initial inflammatory stage (Anderson et al. 2008). However, the high degree of vascularisation seen in IM is not a typically observed feature of the foreign body reaction (Zhang et al. 2013). This suggests that IM formation may be a unique event, driven in part by the proximity of the periosteum. The periosteum contains a developed vasculature and a reservoir of MSCs (Chan et al. 2012; Colnot et al. 2012); its close proximity may influence MSC recruitment and vascularisation of the defect site.

This begs the question, what role does the implant itself play in IM formation? Is it merely a flat surface that provides support for outgrowth of the periosteum or do certain chemical properties of PMMA bone cement itself induce membrane formation? The experiments presented in this thesis do not address these questions, but the mechanism of IM formation and the effect of the cement spacer

are important when considering therapy development. Currently, bone cement must be carefully removed at the second stage of the procedure, whilst ensuring the membrane is left intact (Giannoudis et al. 2011). If a bio-absorbable alternative could be used, this would at least simplify the second stage of the IM procedure, and at best eliminate the need for a second surgical stage altogether.

Although animal models are by no means an ideal tool in investigating human MSCs *in vivo*, investigations into the potential use of other materials in the IM technique necessitates *in vivo* testing. A rabbit model may be most suitable, since this has already been shown to be a useful tool for study of IM (Pelissier et al. 2004). Substituting the material and location of the implant, as well as investigating the timing of implant removal could answer many of the open questions relating to the influence of the periosteum and the composition of the implant on membrane formation.

Understanding the factors that drive MSC migration and differentiation is also important when considering diseases that have a MSC driven component. AS and OA are two examples where inappropriate or abnormal bone formation negatively effects the progression of the disease (Braem et al. 2012; Zhen et al. 2013). In these cases, inhibition of bone formation may be desirable. Chapter 6 showed that tofacitinib does not have any effect on MSC proliferation or differentiation into bone or cartilage. Whilst this is an advantage when considering tofacitinib for the treatment of RA where preservation of bone and cartilage is paramount, it is less encouraging for the treatment of AS which shares the inflammatory aspect of RA but is associated with excessive bone formation at the enthesis rather than bone and cartilage erosion at the joints (Ball 1983; Braem et al. 2012).

There is the possibility that although tofacitinib does not affect *in vitro* osteogenesis, promotion of adipogenesis seen with tofacitinib treatment may be sufficient to inhibit chondrogenesis and osteogenesis *in vivo*. Indeed, a large body of evidence suggests an inverse relationship between BM adiposity and bone formation in the progression of OP (Rosen et al. 2006a; Astudillo et al. 2008; Duque et al. 2008). The experiments presented in this thesis cannot fully address the effect

of tofacitinib on MSCs, since *in vitro* differentiation cannot replicate the subtle cues that influence *in vivo* cell fate. Further experiments *in vivo* must be used to address this issue. Currently, collaborative experiments are being carried out using a DBA/1 mouse model of spontaneous arthritis; these DBA/1 mice develop ankylosis as a result of enthesal cell proliferation and chondrogenic differentiation (Braem et al. 2012). If administration of tofacitinib to these animals does prevent the progression of ankylosis, the effect of tofacitinib on MSC adipogenesis is one possible mechanism for this affect.

Tofacitinib was found to strongly enhance *in vitro* adipogenesis, a result that runs contrary to much of the published literature describing JAK/STAT signalling in adipogenesis (Deng et al. 2000; Nanbu-Wakao et al. 2002; Floyd et al. 2003). Interestingly, the adipocyte hyperplasia observed with tofacitinib stimulation is also seen in a mouse model of adipocyte specific knockout of retinoid related orphan receptor γ (ROR γ) (Meissburger et al. 2011b). In this study ROR γ disruption resulted in reduced adipocyte size, enhanced adipogenesis and protected against loss of insulin sensitivity in mice fed with a high fat diet. Meissburger *et al* suggested that this effect was mediated by loss of matrix metalloproteinase 3 (MMP-3) expression (Meissburger et al. 2011b). A separate study also showed that a related protein, tissue inhibitor of matrix metalloproteinase 1 (TIMP1) negatively regulates adipogenesis (Meissburger et al. 2011a). This is significant in the light of a study by Li *et al* who showed that JAK3 inhibition blocked OSM induced MMP and TIMP expression (Li et al. 2001). I believe this provides a testable hypothesis that would explain the pro-adipogenic action of tofacitinib, which could be tested by monitoring adipocyte size and the expression of MMP-3 and TIMP1 during tofacitinib stimulated adipogenesis.

Further evidence of a link between drug induced adipogenesis and the reduction and bone mass is provided by the use of thiazolidinediones to treat of type II diabetes (Ahmadian et al. 2013). These drugs are powerful insulin sensitizers but have the serious side effects of increasing fracture rates caused by reduced bone mass, weight gain, edema and congestive heart failure in humans (Ahmadian et al. 2013). Although this does support the idea that stimulating adipogenesis can lead

to reduced bone formation it also raises serious concerns, particularly for long term tofacitinib treatment.

In summary, understanding the *in vivo* nature of MSCs is crucial for bone disease therapy approaches that seek to harvest and reapply MSCs to sites of bone damage or loss. Understanding the phenotypic characteristics of *in vivo* MSCs can lead to improved clinical practice by providing the basis of a quality control procedure and can also be utilised to enrich MSCs to high levels of purity. Appreciation of the factors that govern MSC recruitment towards implants such as bone cement may lead to the development of new orthopaedic implants that actively induce bone formation. Finally, evidence that tofacitinib induces adipogenesis *in vitro* coupled with understanding of the *in vivo* dichotomy between adipogenesis and osteogenesis may provide important insights into the potential use of tofacitinib to treat spondyloarthropathies.

8. Future work plan

There are several ways that the work contained in this thesis could be expanded upon. The use of BM aspirates for MSC therapy is hampered by their extremely variable quality in relation to MSC content. For this reason I developed a rapid means to quantify the MSCs in BM aspirates based on CD271 expression that could be used as an intra-operative quality control. Further experiments should focus on optimising the technique to make it faster, this will largely be achieved by exploiting the latest flow cytometry technology which relies on acoustic rather than hydrodynamic focussing (Goddard et al. 2007). The purpose would be to streamline and simplify the protocol, to a point where it could be routinely used by non-specialist theatre staff. A training program for the use of this technology by non-research staff should also be developed. I believe this approach represents an important innovation that could revolutionise the practice of autologous BM grafting.

Next I hope to develop protocols for the rapid seeding of MSCs onto biological scaffolds. I plan to select MSCs from BM aspirates using clinical-grade anti-CD271 microbeads in order to produce a highly-enriched MSC fraction; this will be used to explore cellular interactions governing MSC attachment to biological scaffolds. By combining rapid MSC enumeration for quality control of aspirates, with a rapid and efficient seeding technique, I could develop intraoperative scaffold loading protocols applicable to a broad range of therapeutic applications. However, due to the recognised utility of MSCs in bone repair my chief focus will be scaffolds that are applicable to bone regeneration.

Lastly, I plan to demonstrate the broad utility of this approach to the field of musculoskeletal tissue regeneration as a whole. This will be achieved by demonstrating that MSCs are retained and proliferate on scaffolds, and that they are able to act as initiators of endogenous repair by the release of tropic factors, in addition to their already well characterised differentiation potential. The focus of these experiments will be to detect the stimulation of angiogenesis and cell migration towards seeded scaffolds *in vitro*. If successful, this will demonstrate the

benefit of providing an autologous MSC-driven kick-start to enhance endogenous colonisation.

Further investigation of the induced membrane formed as a result to the IM procedure also merits further investigation. Understanding of the mechanism driving IM formation is key to advancing clinical practice. Research should focus on determining if the chemical nature of the cement spacer itself is critical in inducing membrane formation, whether IM formation is possible in other locations other than long bone defect sites and how the properties of the IM are modulated over time. It is also important to determine the net contribution of each of the components used in the second stage of the procedure, what is the benefit of BMP use over autologous bone graft alone. Indeed, is it possible that membrane induction without autologous grafting is sufficient for defect repair?

These questions are important because although this procedure has clear advantages over other techniques used to reconstruct large bone defects, it relies on a second surgical procedure after membrane induction (Giannoudis et al. 2011). If this could be avoided it would represent a substantial improvement in patient care as well as a significant reduction in the cost associated with this treatment. Experiments using an animal model are the most appropriate means of addressing these questions. The experiment carried out in this thesis may form the basis for assessing membrane induction. However, inter-species differences in terms of antibody specificity and tri-lineage differentiation induction must be considered. The ultimate goal of this investigation would be the development of a means of stimulating IM formation that facilitates treatment as a one stage procedure.

Finally, the effect of tofacitinib on attenuating aberrant bone formation is already under investigation by a collaborative effort, using a mouse model of AS. Aside from this, I propose a more detailed investigation of the effects of tofacitinib on adipogenic differentiation. My hypothesis is that tofacitinib drives enhanced adipogenesis by inhibition of MMP-3 and TIMP1 expression; this may be tested by western blotting for expression of these proteins during adipogenic induction in the presence and absence of tofacitinib. An accurate means of determining adipocyte size following adipogenic induction should also be developed. If MMP-3 and TIMP1 expression are found to be reduced and this is accompanied by a reduction in

adipocyte size this would indicate that tofacitinib treatment may mimic the phenotype observed in mice following homozygous deletion of Ror γ (Meissburger et al. 2011b). If this proves to be the case, the next step should be to examine the effect of tofacitinib administration to obese mice, since Ror γ deletion was shown to recover insulin sensitivity in obese mice (Meissburger et al. 2011b). It should also be determined if other JAK/STAT inhibitors have similar effects. In summary it is possible that JAK/STAT inhibition may provide a novel means of controlling obesity-associated insulin resistance.

Appendix 1: Ethics



National Research Ethics Service

Leeds (East) Research Ethics Committee

Room 5.2, Clinical Sciences Building
St James's University Hospital
Beckett Street
Leeds
LS9 7TF

Telephone: 0113 2065652
Facsimile: 0113 2066772

31 August 2006

Prof Peter Giannoudis
Department of Trauma and Orthopaedics
St James's University Hospital
Beckett Street
Leeds
LS9 7TF

Dear Prof Giannoudis

Full title of study: Biological properties of Mesenchymal Stem Cells in Fracture Healing
REC reference number: 06/Q1206/127

Thank you for your letter of 22 August 2006, 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.

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.

Ethical review of research sites

The Committee has designated this study as exempt from site-specific assessment (SSA). There is no requirement for other Local Research Ethics Committees to be informed or for site-specific assessment to be carried out at each site.

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:

Document	Version	Date
Application		14 July 2006
Investigator CV		20 December 2005



Health Research Authority
NRES Committee Yorkshire & The Humber - Leeds East

North East REC Centre
Room 002
TEDCO Business Centre
Viking Industrial Park
Rolling Mill Road
Jarrow
NE32 3DT

Tel: 0191 4283545

12 December 2013

Prof Peter Giannoudis
Consultant
University of Leeds
Department of Trauma and Orthopedics
St James's University Hospital
LS9 7TF

Dear Prof Giannoudis

Study title: Biological properties of Mesenchymal Stem Cells in Fracture Healing
REC reference: 06/Q1206/127
Amendment number: 1
Amendment date: 15 June 2010
IRAS project ID:

Thank you for your letter of 15 June 2010, notifying the Committee of the above amendment.

The Committee does not consider this to be a "substantial amendment" as defined in the Standard Operating Procedures for Research Ethics Committees. The amendment does not therefore require an ethical opinion from the Committee and may be implemented immediately, provided that it does not affect the approval for the research given by the R&D office for the relevant NHS care organisation.

Documents received

The documents received were as follows:

Document	Version	Date
Notification of a Minor Amendment – Extension to study (January 2017)	1	15 June 2010

Statement of compliance

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

A Research Ethics Committee established by the Health Research Authority

06/Q1206/127:

Please quote this number on all correspondence

Yours sincerely



Hayley Jeffries
REC Manager

E-mail: nrescommittee.yorkandhumber-leedseast@nhs.net

30/04/2007

Research & Development Directorate
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www.leedsteachinghospitals.com

Dear Dr Elena Jones

Re: LTHT R&D Approval of RR07/8115: Biology of mesenchymal stem cells and their use in tissue engineering

I write with reference to the above research study. I can now confirm that this study has R&D approval and the study may proceed at The Leeds Teaching Hospitals NHS Trust (LTHT). This organisational level approval is given based on the information provided in the documents listed below.

As principal investigator you have responsibility for the design, management and reporting of the study. In undertaking this research you must comply with the requirements of the *Research Governance Framework for Health and Social Care* which is mandatory for all NHS employees. This document may be accessed on the Department of Health website at <http://www.dh.gov.uk/research>

R&D approval is therefore given on the understanding that you comply with the requirements of the *Framework* as listed in the attached sheet "Conditions of Approval".

If you have any queries about this approval please do not hesitate to contact the R&D Department on telephone 0113 392 2878.

Indemnity Arrangements

The Leeds Teaching Hospitals NHS Trust participates in the NHS risk pooling scheme administered by the NHS Litigation Authority 'Clinical Negligence Scheme for NHS Trusts' for: (i) medical professional and/or medical malpractice liability; and (ii) general liability. NHS Indemnity for negligent harm is extended to researchers with an employment contract (substantive or honorary) with the Trust. The Trust

Chairman Martin Buckley Chief Executive Neil McKay CB

The Leeds Teaching Hospitals incorporating: Chapel Allerton Hospital Cookridge Hospital Leeds Chest Clinic
Leeds Dental Institute Seacroft Hospital St James's University Hospital The General Infirmary at Leeds
Wharfedale Hospital

WTA280

only accepts liability for research activity that has been managerially approved by the R&D Department.

The Trust therefore accepts liability for the above research project and extends indemnity for negligent harm to cover you as principal investigator and the researchers listed on the R&D approval form provided that each member of the research team has an employment contract (substantive or honorary) with the Trust. Should there be any changes to the research team please ensure that you inform the R&D Department and that s/he obtains an employment contract with the Trust if required.

Yours sincerely



Dr D R Norfolk
Associate Director of R&D

Approved documents

The documents reviewed and approved are listed as follows

<i>Document</i>	<i>Version</i>	<i>Date of document</i>
Protocol		Received 25/04/07
SSI Form	5.3	16/02/07
CMT Approval		15/02/07
NHS REC Application Form	5.3	16/02/07



Health Research Authority

NRES Committee Yorkshire & The Humber - Leeds West

Jarrow REC Centre
Room 002, Jarrow Business Centre
Rolling Mill Road
Jarrow
NE32 3DT

Tel: 0191 4283548

21 March 2014

Dr Elena Jones
Lecturer in Rheumatology
Leeds University
Room 5.24
Clinical Sciences Building
St James's University Hospital
Leeds
LS9 7TF

Dear Dr Jones

Study title: Biology of mesenchymal stem cells and their use in tissue engineering
REC reference: 07/Q1205/27
Protocol number: NA
Amendment number: Last Approved Amendment 2, Approved 26 Oct 2010
Amendment date: 06 March 2014

The above amendment was reviewed by the Sub-Committee in correspondence.

Ethical opinion

The members of the Committee taking part in the review gave a favourable ethical opinion of the amendment on the basis described in the notice of amendment form and supporting documentation.

Approved documents

The documents reviewed and approved at the meeting were:

Document	Version	Date
Notice of Substantial Amendment (non-CTIMPs)		06 March 2014
Protocol	2	26 October 2010

Membership of the Committee

The members of the Committee who took part in the review are listed on the attached sheet.

R&D approval

Appendix 2: Standard Solutions

Acrylamide gel 10% (Western blotting)

dH₂O – 2.95ml, 30% (w/v) acrylamide/methylene bisacrylamide solution (37.5:1 ratio) (National Diagnostics) – 2.5ml, 1.5M Tris base (Fisher) solution (pH8.8) – 1.88ml, 10% Sodium dodecyl sulfate (SDS, Sigma Aldrich) - 75µl, 10% ammonium persulfate (Sigma Aldrich) - 100µl, N,N,N',N'-Tetramethylethylenediamine (Sigma Aldrich) - 5µl.

Acrylamide gel stacking (Western blotting)

dH₂O – 3.56ml, 30% (w/v) acrylamide/methylene bisacrylamide solution (37.5:1 ratio) (National Diagnostics)– 0.75ml, 0.5M Tris base (Fisher) solution (pH6.8) – 625µl, 10% SDS (Sigma Aldrich) - 50µl, 10% ammonium persulfate (Sigma Aldrich) - 25µl, N,N,N',N'-Tetramethylethylenediamine (Sigma Aldrich) -7µl.

Adipogenic media

DMEM (Gibco/Life Tech) – 22320-022) contains 1g/l D-Glucose, L Glutamine, HEPES, Pyruvate. with additional supplements: 10% foetal calf serum (PAA), 10% horse serum (Stem Cell Technologies), 0.5 mM isobutylmethylxantine (Sigma Aldrich), 60 µM indomethacine (Sigma Aldrich), 0.5mM hydrocortisone (Sigma Aldrich), Penicillin/streptomycin (Sigma Aldrich).

Blocking buffer (western blotting)

PBS with 5% marvel w/v milk powder (Premier International Foods), 0.1% v/v Tween 20 (Sigma Aldrich).

Cell freezing media

90% FCS (PAA), 10% DMSO (Sigma Aldrich).

Chondrogenic Media

DMEM (2.5g/L glucose) with additional supplements: Ascorbic acid 2 phosphate 200mM (Sigma Aldrich), Sodium Pyruvate (Sigma Aldrich), Proline (Sigma Aldrich), BSA (PAA laboratories) and Penicillin/streptomycin.

With additional supplements added prior to use: Insulin transferrin sodium selenite (ITS+3) cell culture supplement 1:100, (Sigma Aldrich), TGFβ3 10μg/ml (R&D Systems) and Dexamethasone 100nM.

Citrate fixative (ALP assay)

Two volumes of citrate working solution: 1:100 dilution of citrate concentrate (Sigma Aldrich) with 3 volumes of acetone (Sigma Aldrich).

FACS Buffer

PBS with 1% w/v bovine serum albumin (BSA) (Sigma Aldrich).

Fast blue solution (ALP assay)

One capsule of fast blue RR salt (Sigma Aldrich) dissolved in 48ml of distilled water, with the addition of 2ml Naphthol AS-MX phosphate alkaline solution (Sigma Aldrich).

Fixing buffer (histology)

4% formalin solution (v/v) (Sigma Aldrich) in PBS.

Methylene blue solution (CFU-F assay)

1% w/v methylene blue, dissolved in 10mM disodium tetraborate (pH 8.0).

Oil red solution (adipogenesis assay)

0.5% w/v oil red in isopropanol solution is diluted 3:2 in dH₂O. The solution is then passed through 0.8μm and 0.22μm filters respectively to remove precipitate.

Osteogenic Media

DMEM (Gibco/Life Tech) containing 1g/l D-Glucose, L Glutamine, HEPES, Pyruvate with additional supplements: Foetal calf serum 10% (PAA), Ascorbic-2-phosphate (As2P) 100 μ M (Sigma Aldrich), β glycerophosphate 10mM (Sigma Aldrich), Dexamethasone 100nM (Sigma Aldrich), Penicillin/streptomycin (Sigma Aldrich).

Papain buffer (GAG assay)

50mM sodium phosphate buffer pH 6.5 (15mM Na₂HPO₄, 35mM NaH₂PO₄), containing 2mM Acetyl cysteine (Sigma Aldrich), 2mM EDTA (Sigma Aldrich), and 1mg/ml Papain (Sigma Aldrich).

Red blood cell lysis solution

168mM NH₂Cl, 10mM KHCO₃, 1mM EDTA (pH8.0).

Running buffer (western blotting)

dH₂O – 10L, Tris base (Fisher) – 30.3g, glycine 144.2g, SDS (Sigma Aldrich) – 10g.

Sample buffer (western blotting)

50mM Tris pH 6.8 (Fisher), 5% β -mercaptoethanol (Sigma Aldrich), 2% w/v SDS (Sigma Aldrich), 0.1% w/v bromophenyle blue (Sigma Aldrich), 10% glycerol (Sigma Aldrich).

Scott's tap-water substitute (histology)

Sodium bicarbonate 42mM (Sigma Aldrich), magnesium sulphate (heptahydrate) 8mM (Sigma Aldrich) dissolved in dH₂O.

Toluidine blue solution (chondrogenesis assay)

1% Toluidine Blue (Sigma Aldrich) in 50% v/v isopropanol (Sigma Aldrich) 50% v/v dH₂O.

Transfer buffer (western blotting)

H₂O – 3.5L, Glycine (Sigma Aldrich) – 50g, Tris base (Fisher) - 10.6g, methanol (Sigma Aldrich) - 700ml.

Washing buffer (western blotting)

PBS with 0.1% v/v Tween 20 (Sigma Aldrich).

Appendix 3: Tissue culture plastic use

Throughout this report, unless otherwise stated, the volume of media added to each type of culture vessel was as follows. All tissue culture plastic was supplied by Corning unless otherwise stated.

Type of vessel	Volume of media applied
T150	30ml
T75	20ml
T25	8ml
10cm dish	15ml
6 well plate	4ml/well
12 well plate	2ml/well
24 well plate	1ml/well
48 well plate	0.5ml/well

Appendix 4: Antibodies used

Specificity	Application	Conjugate	Clone	Host	Dilution	Manufacturer
CD31	IHC	None	JC70A	Mouse	1:50	Dako
CD31	FC	FITC	WM59	Mouse	1:10	AbD Serotech
CD34	FC	PcP	8G12	Mouse	1:5	BD Pharmingen
CD34	FC	PE	563	Mouse	1:5	BD Pharmingen
CD45	FC	PECy-7	HI30	Mouse	1:20	BD Pharmingen
CD73	FC	PE	AD2	Mouse	1:5	BD Pharmingen
CD73	FC	PECy-5.5	AD2	Mouse	1:20	BD Pharmingen
CD90	FC	FITC	5E10	Mouse	1:5	BD Pharmingen
CD146	IHC	None	P1H12	Mouse	1:50	Chemicon Int.
CD146	FC	PE	3A6	Mouse	1:5	BD Pharmingen
CD271	IHC	None	NGFRS	Mouse	1:50	Abcam
CD271	FC	APC	ME20.4-1H4	Mouse	1:11	Miltenyi Biotec
CD271	FC	PE	ME20.4-1H4	Mouse	1:11	Miltenyi Biotec
CD271	FC	PE	C40-1457	Mouse	1:5	BD Pharmingen
BMP-2	IHC	None	Polyclonal	Rabbit	1:100	AbD Serotech
SDF-1	IHC	None	79018	Mouse	1:20	R&D Systems
VEGF	IHC	None	Polyclonal	Rabbit	1:20	Santa Cruz
Isotype	FC	FITC	MOPC-31C	Mouse	1:5	BD Pharmingen
Isotype	FC	PE	MOPC-21	Mouse	1:5	BD Pharmingen
Isotype	FC	APC	IS5-21F5	Mouse	1:11	Miltenyi Biotec
pSTAT-3	WB	None	EP2147Y	Rabbit	1:20000	Millipore
β-Actin	WB	None	AC-15	Mouse	1:20000	Sigma Aldrich
Rabbit IgGs	WB	HRP	Polyclonal	Goat	1:20000	Jackson Labs
Mouse IgGs	WB	HRP	Polyclonal	Goat	1:10000	Dako

Appendix 5: Patient Demographics

Patient	Sex	Age	Size of defect	Location of defect
1	M	46	45mm	Femur
2	M	72	40mm	Femur
3	M	64	40mm	Tibia
4	M	66	25mm	Radius
5	M	27	25mm	Radius
6	M	18	25mm	Ulna
7	F	80	40mm	Femur
8	M	72	50mm	Radius

Table shows patient sex, age at time of procedure (second stage), the size of the defect and its anatomical location.

Appendix 6: Concentrator product inserts

SmartPrep2 concentrator product insert (re-produced from literature supplied with the SmartPrep2 device).

INSTRUCTIONS FOR USE:

Draw Bone Marrow Aspirate

NOTE: Bone Marrow should be collected only by qualified medical professionals using proper anticoagulation (i.e., ACD-A) and sterile techniques. Dilution with peripheral blood can be minimized and bone marrow-derived cell concentration maximized by limiting the aspiration volume per puncture. For best results, bone marrow should be collected in heparin-coated syringes. **Heparin / sodium chloride bath consists of a minimum of 25 ml of heparin / sodium chloride in a concentration of 2000 µ/ml.** Refer to the Instructions for Use accompanying the aspiration needles for additional instructions and information.

Prepare Aspiration Syringes and MarrowPREP Filter Bag

Pass contents of Bone Marrow Aspiration Pack to sterile field (Circulator Nurse).

1. Pass BMA Aspiration Tray & Sterile Field Wrapped MarrowPREP Filter Bag, BMA Aspiration Kit, Transfer Syringes, Blunt Plastic Cannulas and BMA Aspiration Needle(s) to sterile field.
2. Transfer minimum of 25 ml Heparin/sodium chloride solution (2000 µ/ml) to heparin well in BMA aspiration tray or suitable container.
3. Rinse aspiration syringes with Heparin / sodium chloride solution (2000 µ/ml). Ensure heparin solution has coated the entire marrow contacting surfaces.
4. Rinse Aspiration Needles (Sharp & Blunt) with heparin solution so that all marrow-contacting (including needle lumen) surfaces are coated.
5. Return any remaining heparin solution from the syringe to heparin well in BMA aspiration tray.
6. Transfer the ACD-A solution (2 ml for each 30 ml marrow collected) to the scrub tech/nurse.
7. Close the "white" slide clamp between the filter and the marrow bag.
8. Remove & discard red cap from in-port.
9. Place 2 ml of ACD-A into BMA filter bag in-port for every 30 ml Bone Marrow to be collected. Ensure ACD-A coats entire inside surface of the BMA Filter Bag.



Fig. 1

Collect and Inspect Aspirate (Recommendations)

1. Using BMA Sharp Aspiration Needle, gain initial entry into bone space (approximately 2 cm).
2. Replace Sharp Stylet with Blunt Stylet (11g Needle only) and advance the needle further into the bone marrow space.
3. Aspirate bone marrow into each heparin rinsed aspiration syringe.
 - a. Draw up small volume with one pull on the syringe. Rotating VacLok plunger allows small vacuum steps to ease aspiration steps.
 - b. Rotate ($\pm 90^\circ$) and withdraw aspiration needle several millimeters and draw an additional small volume with one pull on the syringe.
 - c. Repeat step until syringe is filled.
 - d. Re-direct needle as necessary; re-rinse needle and stylet with heparin solution for each puncture and re-entry attempt.

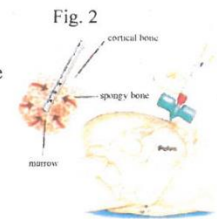


Fig. 2

BMA Filter Bag

1. Transfer Bone Marrow Aspirate to in-port of BMA Filter Bag containing 2 ml ACD-A for every 30 ml marrow collected.
2. Mix contents thoroughly and inspect for large aggregates or clots; if seen, record on run sheet.
3. Repeat steps until desired volume of BMA is collected.
4. Open "white" slide clamp and gently squeeze filter chamber to allow marrow to enter filter chamber.
5. Mix bag contents thoroughly, attach BMA transfer syringe to MarrowPREP Filter Bag out-port.
6. Fill syringe; attach blunt plastic cannula to marrow filled BMA syringe(s), repeat as necessary. Pass transfer syringe(s) out of sterile field for processing.



Fig. 3

Processing Disposable (PD)

7. Remove Processing Disposable(s) (PD) from packaging and place on an appropriate workspace.
8. Slowly dispense BMA into the marrow chamber of the PD through the RED access site (Fig.5 & 6)

Note: Do not overfill PD.

Precaution: Always place the blunt Cannula in the "center" of the injection access site port to avoid dislodging the port. Use proper syringe technique when dispensing BMA.

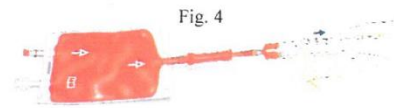


Fig. 4

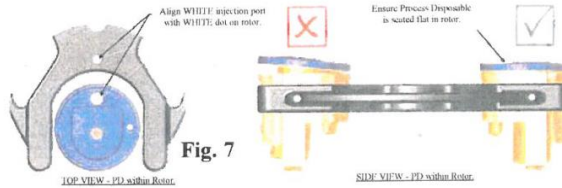


FIG. 5 & 6

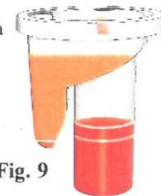
4003170 / 09

Bone Marrow Aspirate Processing

1. Load Centrifuge: Place the PD (or PD's) into the SmartPreP2 System. Insert the appropriate reusable Balance Weight (BW) into the opposite bucket. Note: Align the white dots on the PD and balance weight to the white dot on the rotor for proper alignment.
Precaution: Use appropriate Balance Weight when running one PD. Without Balance Weight, the resulting imbalance will shutdown centrifuge.
Precaution: Do not force the PD or BW into Rotor Trunnion. The PD and BW should fit snugly but should not require excessive force to install.
2. Close lid on machine, the AMBER "LID OPEN" light must be off. Press GREEN "START" button to start the process. Total processing time is approximately 14 minutes.
3. Carefully remove PD (s) when cycle is complete, keeping PDs leveled when handling in up-right position

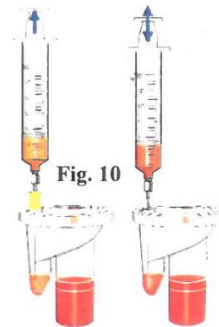


4. Cell Resuspension: Using the alcohol pad moving in a concentric spiral, wipe the red port of the PD prior to entry. The plasma volume used for cell resuspension is based on user discretion. The syringe with blunt cannula and spacers is used to withdraw supernatant from the plasma chamber of PD by being careful not to disturb the lower cell layer and drawing until bubbles are observed.
 Note for 60 mL PD: Using both spacers will leave approximately 10 ml in the plasma chamber while removing one spacer will leave approximately 7 ml.
 Note for 30 mL PD: Using all 3 spacers will leave approximately 4 mL in the plasma chamber while removing the first small spacer (leaving 2 spacers) will leave approximately 3 mL.



It is not advised to remove any more spacers as removal of the plasma will then tend to disturb the cell layer at the base of the plasma chamber.) Using the gradations on the syringe a known volume of plasma may be dispensed back into the plasma chamber to adjust the final volume of desired concentrate.

5. To resuspend the bone marrow cells into the plasma:
 - Withdraw remaining plasma into BMAC syringe with blunt cannula (NO SPACER) and gently inject back and forth into the plasma chamber.
 - Repeat above step 2-3 times (until cells are visibly resuspended in the plasma) and withdraw total volume into syringe.
 - Observe base and walls of plasma chamber to confirm all cells have been withdrawn into BMAC syringe.
6. Transfer back into sterile field by connecting BMAC syringe to sterile luer lock connector on BMAC Receiving syringe held by the scrub nurse in the sterile field. (Fig.11)



MarrowStim product insert (reproduced from literature supplied with MarrowStim device).

MarrowStim™ Concentration System-Bone Marrow Aspiration⁴

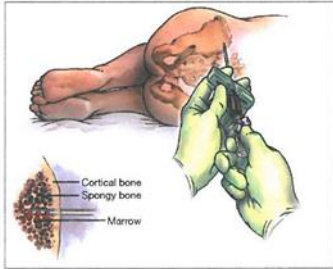


Figure 1

1. Prepare Patient

After suitable anesthesia is achieved, place the patient in the lateral decubitus position. Using sterile technique, prepare the skin with antiseptic and drape (Figure 1).



Figure 2

2. Rinse and Prepare Syringes and Needle

Rinse MarrowStim™ bone marrow aspirate needle and two 30ml syringes with ACD-A anticoagulant to ensure inner surfaces are coated. Following rinsing, load both 30ml syringes with 5ml of ACD-A. This will prevent clotting of bone marrow during aspiration.

Heparin anticoagulation: If heparin (not supplied in kit) is to be used as the anticoagulant, rinse MarrowStim™ bone marrow aspirate needle and two 30ml syringes with heparin solution (1000U/ml) to ensure inner surfaces are coated. Following rinsing, load both 30ml syringes with 3ml of heparin.

MarrowStim™ Mini System: Only one 30ml syringe is necessary.

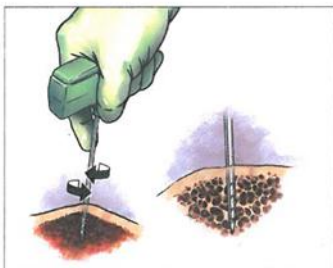


Figure 3

3. Position and Advance Needle

Hold the needle with proximal end in palm and the index finger against the shaft toward the tip (Figure 2).

Using gentle but firm pressure, advance the needle, rotating it in an alternating clockwise /counterclockwise motion. Entrance into the marrow cavity is generally detected by decreased resistance. All of the side holes at the distal end of the needle must be introduced into the marrow cavity beyond the cortical bone, otherwise air with extra bony soft tissue may appear with the aspirated marrow (Figure 3).

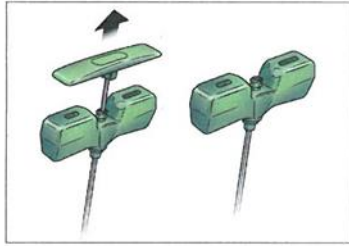


Figure 4

4. Remove Stylet

Once needle is in place, remove the stylet by pulling straight out (Figure 4).



Figure 5

5. Aspirate Marrow

Attach ACD-A loaded 30ml syringe with a luertaper to the hub of the bone marrow harvest needle using a firm push and twist motion. Apply suction by withdrawing the syringe plunger. Remove the syringe with the harvested marrow. Repeat the harvest procedure until an appropriate amount of marrow is obtained to satisfy the clinical requirement (Figure 5).

MarrowStim™ System: Two of the 30ml syringes from the MarrowStim™ standard kit are pre-filled with 5ml of ACD-A, for a total of 60ml of anticoagulated marrow. In the case of the MarrowStim™ Mini System, only one 30ml syringe is pre-filled with 5ml of ACD-A, for a total of 30ml of anticoagulated marrow.



Figure 4

2. Balance Centrifuge

Press red button to release lid of centrifuge. Open and place the tube into the centrifuge (Figure 4).

MarrowStim™ Mini System: If using the mini kit, the purple mini buckets must be inserted into the centrifuge.



Figure 5

Fill Biomet Biologics™ counterbalance with 60ml of sterile saline into opposite side of centrifuge (Figure 5).

MarrowStim™ Mini System: Fill the Biomet Biologics™ Mini counterbalance with 30ml of sterile saline and place into opposite side of centrifuge.



Figure 6

3. Spin MarrowStim™ Disposable

Close lid. Set speed at 3200 RPM and time to 15 minutes. Press green button to start spin. Once spin is completed, press red button to release lid and open (Figure 6).



Figure 7

4. Extract Cell Poor Plasma

Remove MarrowStim™ tube from centrifuge and ensure BMA has separated into three distinct layers.

Remove yellow cap on side port No. 2 and connect a sterile 30ml syringe. Invert the tube and withdraw the cell poor plasma (Figure 7).

Reattach yellow cap to side port No. 2.



Figure 8

5. Suspend Nucleated Cell Concentrate

While holding the tube in the upright position, shake vigorously for 30 seconds to suspend the nucleated cells (Figure 8).

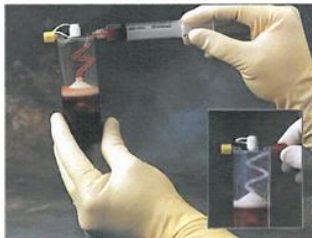


Figure 9

6. Extract Nucleated Cell Concentrate

Remove red cap from side port No.3 and connect a sterile 10ml syringe to extract the nucleated cell concentrate (Figure 9).

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