Optimising Mesenchymal Stromal Cell Harvesting in Orthopaedic Surgery

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List of Abbreviations

ACAN	Aggrecan
ACI	Autologous Chondrocyte Implantation
ACL	Anterior Cruciate Ligament
BGLAP	Bone Gamma-Carboxyglutamic Acid-Containing Protein
BMI	Body Mass Index
BMMC	Bone Marrow Mononuclear Cell
CFU-F	Colony Forming Unit-Fibroblast
cGMP	Current Good Manufacturing Practice
COL2A1	Collagen Type II, alpha 1
CV	Coefficient of Variation

CVA	Cerebro-vascular Accident
DVT	Deep Vein Thrombosis
EDTA	Ethylenediaminetetraacetic acid
ESC	Embryonic Stem Cell
FGF-2	Fibroblast Growth Factor 2
FSC	Foward Scatter
GVHD	Graft Versus Host Disease
ICC	Intra-Class Correlation
IHD	Ischaemic Heart Disease
IMDM	Iscove's Modified Dulbecco's Medium
iPSC	Induced Pluripotent Stem Cell
MACI	Matrix Assisted Chondrocyte Implantation
MeSH	Medical Subject Heading

MFI	Mean Fluorescent Intensity
MHC	Major Histocompatability Complex
MIQE	Minimum Information for Publication of Quantitative Real-Time PCR Experiments
MRI	Magentic Resonance Imaging
MSC	Mesenchymal Stromal Cell
NK cells	Natural Killer cells
NSAID	Non-Steroidal Anti-Inflammatory Drug
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PE	Pulmonary Embolism
PIS	Patient Information Sheet
PRP	Platelet Rich Plasma
qPCR	Quantitative Polymerase Chain Reaction

RCT	Randomised Clinical Trial
RNA	Ribonucleic acid
RPMI	Roswell Park Memorial Institute Media
rt-PCR	Real-Time Polymerase Chain Reaction
RUNX2	Runt-related transcription factor 2
SNR	Signal to Noise Ratio
SOX-9	Sex determining region Y box 9
SPP1	Secreted phosphoprotein I
SSC	Side Scatter
SVF	Stromal Vascular Fraction
TGF-α	Transforming Growth Factor Alpha
VEGF	Vascular Endothelial Growth Factor

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Abstract

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Musculoskeletal tissue is prone to age-related degeneration and to damage which heals poorly. Many current treatments are able to treat only the end stages of these conditions, such as the use of total knee replacements in osteoarthritis. Cellular therapies are seen as a potential source of effective treatments for the earlier stages of these conditions. Orthopaedic surgery has been at the forefront of cellular therapies with treatments such as microfracture and autologous chondrocyte implantation to treat chondral defects.

As the largest area of current cell therapy research, stem cells have become an area of high interest for developing novel treatments. Mesenchymal stromal cells (MSCs) have provided the basis of the majority of orthopaedic treatments because of the relative ease of obtaining them. Despite the development of a number of treatments using both freshly harvested MSCs and culture expanded MSCs there is still a large gap in our knowledge of the mechanisms of actions of these cells and the most appropriate locations for obtaining autologous samples.

Abstract

This thesis seeks to examine the best source of MSCs for surgery around the knee, comparing the pelvis to the femur and tibia. It also seeks to determine if it is possible to improve the yield of MSCs using a simple modification of the standard method of aspiration.

Assessments of the yield of all cells and MSCs showed that the pelvis was the optimum source for MSCs in terms of cell numbers. There was also a large amount of inter-subject variation in the number of cells obtained. There was no difference in the functional abilities of cells from any location. Modification of the aspiration technique did not improve the cell yield.

Future work should focus on improving yields from the pelvis and investigate methods of overcoming the inter-subject variability in yields if standardised treatments are to be successfully developed.

Chapter 1

Introduction

Musculoskeletal tissue, such as articular cartilage, meniscus, tendon, and ligament, is prone to both damage and age-related degeneration. Inevitably this results in a large burden of disease in the population. Information from the Global Burden of Disease Study 2010 suggests that musculoskeletal disorders are the biggest single cause of morbidity as measured by years lived with disability in the United Kingdom[1]; accounting for 30.5% of all disability. Within the category of musculoskeletal disorders there is a wide variety of conditions including back pain, osteoarthritis, rheumatoid arthritis, gout, low bone density, and "other" conditions such as tendinopathy and connective tissue disorders[2]. The development of treatments for these conditions, and for osteoarthritis in particular, is a priority if this burden is to be reduced. This thesis will examine the currently available cellular based therapies for these conditions and seek to address some of the gaps in the basic scientific knowledge regarding the use of stem cells in this field.

Osteoarthritis is defined as:

> "... the most common form of arthritis, involving inflammation and major structural changes of the joint, causing pain and functional disability"[3, p. 1323].

Osteoarthritis is ranked 11th out of 291 conditions in terms of years lived with disability in the Global Burden of Disease Study and with the rise in longevity is a continually increasing problem for society. It affects over half of all individuals aged over 60[4] and was the underlying cause of the majority of the 196,403 total joint arthroplasties that took place in 2012 in the United Kingdom[1][5]. Osteoarthritis and other forms of cartilage damage can be difficult to treat due to the poor ability of articular cartilage to heal. Cartilage is primarily composed of an extra-cellular matrix of collagen (primarily type II), proteoglycans such as aggrecan and decorin, and water[6]. Articular cartilage is avascular and consequently is reliant upon diffusion for the supply of nutrients. It is this avascular nature, combined with the low number of cells present in the tissue, that results in a poor healing ability[7].

Tendon is another component of the musculoskeletal system that is both prone to damage and also has a relatively poor healing capacity. This poor healing capacity is thought to be due to the low metabolic rate of tendon, which, whilst beneficial for the day to day tasks of load bearing and transmission, makes it difficult to transfer cells and nutrients to the site of injury when repair is required[8]. This often leads to inferior healing with the formation of fibrotic scar tissue, rather than new tendon tissue[9].

The poor healing ability of both of these musculoskeletal tissues means that treatments for damage to them are often limited. The treatments that are available often lead to only short term improvements or do not have satisfactory outcomes

[1]Excluding Scotland.

in a significant proportion of patients. For example, damage to tendons of the rotator cuff is most commonly treated by surgical repair if the remaining tissue is physically amenable to it. However, it is well known that a proportion of people treated in this manner will go on to re-tear their tendons. A recent meta-analysis of seven Level I randomised clinical trials (RCTs) studies has shown a re-rupture rate of between one sixth and one quarter depending upon the method of repair[10]. Treatment for damaged cartilage is also affected by variable outcomes. Treatments aimed at trying to repair the damage are discussed in Section 1.A below but, in general, they are able to produce only relatively short-term improvements. Treatments for end-stage cartilage damage, such as partial or total knee replacement, have patient-reported dissatisfaction rates of up to one fifth[11] and are relatively contra-indicated in younger patients with isolated areas of cartilage damage due to the increased rate of wear in more active individuals and the limited options for repeated revision surgery.

The treatment gap between simple measures and end-stage procedures, such as total knee replacement, has been investigated over the last 25 to 30 years using the concepts of cell-based therapies. Most recently the use of stem cells has been explored as a potential treatment for musculoskeletal disease. This large increase in laboratory research has led to a related increase in clinical trials and, to a lesser extent, commercially available treatments.

Despite this, there are still a large number of unanswered questions concerning the basic science behind the use of stem cells in the treatment of orthopaedic conditions. Whilst a variety of treatments have been developed that make immediate use of stem cells that have been harvested in the operating room, the question of which cells should be harvested, and how, has not yet been satisfactorily addressed[12]. The aim of this thesis is to determine where we should

harvest these cells from and how to best achieve this in order to obtain the best yields of high quality stem cells.

A Regenerative Therapy for the Treatment of Musculoskeletal Disorders

A wide range of regenerative therapies has been developed in an attempt to treat cartilage damage, and specifically isolated osteochondral defects. The treatment of osteochondral defects is particularly pressing as they tend to occur in a relatively young population and, if not addressed, can lead to the development of widespread osteoarthritis. The young age of the individuals concerned would then preclude them from being suitable for a joint replacement. The cell-based therapies currently available include:

- Microfracture;
- Autologous Chondrocyte Implantation (ACI);
- Matrix Assisted Chondrocyte Implantation (MACI);
- Osteochondral Transplantation[2];
- Stem Cell Based Treatments.

[2]Also known as Osteochondral Autograft Transplantation or Osteochondral Mosaicplasty.

Despite this wide variety of cellular based treatment options, no treatment has emerged which has demonstrated sustained clinical improvement that would warrant widespread clinical adoption.

A.1 Microfracture

Microfracture of the subchondral bone in order to form a clot from bone marrow material was first described in 1997[13]. The procedure was devised in response to the observed lack of spontaneous healing in defects of articular cartilage. In short, the process involves the identification of the cartilage defect and the débridement of any loose or damaged cartilage from the subchondral bone. An awl is then used to create a number of holes in the subchondral bone approximately three millimetres apart. Bone marrow and blood is then able to enter the cartilage defect from the bone and forms a clot in the defect. The concept behind this treatment is that the clot contains mesenchymal stem cells that are able to differentiate into chondrocytes and so help to form new cartilage tissue. This technique has been used with some success and continues to be popular with surgeons. This may well be due to the fact that the low cost of the treatment, minimal increase in morbidity, and lack of impact on future management make it an attractive first treatment option[14].

However, a recent systematic review of the use of microfracture found that whilst it has good outcomes initially these results do not seem to last past the five year mark when there is a large increase in failures[15]. There are also concerns that the "fracturing" of the subchondral bone can result in a hypertrophic response leading to the formation of bone islands within the cartilage layer[16].

A.2 Autologous Chondrocyte Implantation

ACI has been developed to overcome the problems of microfracture and produce a more natural repair. First described by Brittberg *et al.* in 1994[17], ACI involves harvesting cartilage, extracting the chondrocytes, expanding these in a laboratory for approximately six weeks, and then re-implanting into a cartilage defect. The process is significantly more expensive than microfracture and the cost effectiveness of this procedure relies to a certain extent on assumptions regarding the decreased requirement to carry out joint arthroplasty at a later date due to the prevention of osteoarthritis[18, 19].

A recent report of 10 year outcomes showed that ACI led to a significantly lower failure rate and significantly higher Cincinnati Knee Scores when compared with those who received mosaicplasty[20]. A systematic review of ACI conducted in 2010[21] showed that whilst there was a variation in the outcome of ACI in different trials, there appeared to be a trend towards improved outcomes with ACI treatment compared with the other options, including microfracture and osteochondral transplantation. This review was at odds with other systematic reviews which failed to show a benefit in ACI over other treatment forms[22, 23].

The most recent Cochrane review of ACI was also unable to identify sufficient evidence to recommend its use[24]. The United Kingdom National Institute for Health and Care Excellence (NICE) currently recommends that ACI is used only within the setting of a clinical trial because of the lack of clear evidence for its widespread use[25], although it should be noted that this advice is five years overdue for review.

A.3 Matrix Assisted Chondrocyte Implantation

MACI has developed from the original concept of ACI and combines the use of laboratory expanded chondrocytes with a three dimensional (3D) scaffold in order to enhance the chondrogenic ability of the implanted cells[26]. This procedure is based on the laboratory evidence that chondrocytes perform better when grown in 3D structures rather than in a monolayer. Recent evidence from a trial comparing MACI to microfracture has shown a significant improvement with MACI in Knee Injury and Osteoarthritis Outcome Scores[27]. There is, as yet, no conclusive evidence to support widespread adoption of the technique and MACI is affected by the same problems of cost, and the need for multiple procedures, that affects ACI.

A.4 Osteochondral Transplantation

Osteochondral transplantation was pioneered in the 1990s as a treatment for chondral and osteochondral defects. It involves the harvesting of cylinders of osteochondral tissue from non-weight-bearing areas of a joint and the implantation of these into the defect[28]. Despite promising results in some case series[29], the technique has not been widely adopted. This may be due to concerns regarding the donor site morbidity and the availability of healthy cartilage as well as the fact that a recent comparison of osteochondral transplantation with ACI showed significantly better results for ACI[20]. This study followed 100 patients for a minimum of 10 years and found that failure, defined as a poor clinical outcome, graft failure arthroscopically, or need for revision surgery, was significantly more likely in the osteochondral transplantation group (55% *v.* 17%).

A.5 Tendinopathy Treatment

Whilst not the subject of as much research as cartilage damage, tendinopathy treatment has also been investigated from the point of view of cellular therapies, but with little success to date. The majority of this work has focused on the use of Platelet Rich Plasma (PRP).

PRP is blood plasma in which the platelet concentration has been increased from baseline, usually by a centrifugation-based method. It has been proposed as a treatment for a wide variety of tendon related disorders. A recent randomised controlled trial of PRP in patellar tendinopathy demonstrated improved symptoms (as measured by the Victorian Institute of Sport Assessment) when compared with dry needling alone; although the benefits tended to decrease over time[30]. However, a systematic review of the use of PRP in the treatment of another tendon disorder, lateral epicondylar tendinopathy, could not show any benefit from the use of PRP[31]. PRP use has also been trialled in the treatment of rotator cuff disease. One trial of rotator cuff repair, conducted by Jo *et al,* showed improvement in the structure of tendon following PRP administration (as measured by MRI) but no difference when compared to standard repair in terms of the majority of clinical outcome measures undertaken[32].

Overall, the evidence for the use of PRP in the treatment of tendon disorders is highly equivocal and a recent Cochrane Review concluded:

> "... there is currently insufficient evidence to support the use of PRT [PRP] for treating musculoskeletal soft tissue injuries"[33, p. 2].

It is clear from the above that cellular therapies have, thus far, failed to deliver

a treatment that has proven to be effective enough from both a clinical and cost perspective to gain widespread adoption. This realisation has coincided with the explosion of interest in the use of stem cells to treat a huge variety of disorders in a multitude of medical specialities. Stem cells have, therefore, proven a fertile area for further research into the treatment of these conditions.

B Stem Cells

Stem cells are cells which possess the ability to self renew and are also able to differentiate into a variety of different cell types[34]. They form a vital building-block in the normal development of an embryo and populations of these cells found in most types of tissue are responsible for maintaining the reparative and regenerative capacity of organisms. The supreme stem cell is the fertilised egg which is totipotent and able to form any other tissue and self renew almost indefinitely. As stem cells become more committed to a specific lineage they progressively loose potency and become restricted in their differentiation potential to only certain cell lines. The ability of these cells to repair damaged tissue is what has led to a great deal of interest in their use in the field of regenerative medicine.

Stem cells are seen as providing a potent source of treatment options where PRP, ACI, and similar procedures have failed. Despite the lack of significant progress in the past there is a widespread belief that cellular therapy holds promise for the future[35]. The component fields of cellular therapy, regenerative medicine[36] and tissue engineering[37], are seen as being a huge potential resource of innovation in the treatment of musculoskeletal disorders such as cartilage damage and tendinopathy. Clinicians and businesses have both shown great interest in de-

veloping technologies that could be used to repair damaged tissue in a way that improves on the outcomes already achievable with current treatments.

These treatments have grown mainly from work carried out on stem cells over the last 15 years. Laboratory-based experiments have increased our understanding of the basic science underlying stem cell function and have driven a large amount of translational research and investment from commercial enterprises. In order to understand the developments that have taken place in stem cell therapies it is necessary to understand both the different types of stem cells that can be used, and their function.

Stem cells are divided into a number of different types based upon where they are obtained from and their capacity for self renewal and differentiation. There are three main classes of stem cells:

1. Embryonic Stem Cells (ESCs) - These are undifferentiated, pluripotent stem cells obtained from a pre-implantation embryo. Whilst they are seen as the most "powerful" form of stem cells because of their pluripotent nature[38], they have been little studied in relation to musculoskeletal conditions. This is primarily due to a combination of the practical difficulties involved in obtaining them and, more importantly, the significant ethical concerns regarding their use[39].

2. Induced Pluripotent Stem Cells (iPSCs) - Reprogramming of differentiated adult cells into a more stem-like form potentially holds promise for future treatments. iPSCs were first developed by Shinya Yamanaka in 2006[40]. Whilst it has been established that iPSCs can be differentiated into chondrocyte-like tissue[41], no clinical treatment has yet been

developed based upon iPSCs[42]. There has also been a number of problems with the generation of these cells[43, 44] which has negatively impacted upon the way in which they are perceived[45].

3. Adult Stem Cells - These are the stem cells found in most tissues of an individual. They are characterised by the tissue they are found in and their differentiation potential. A number of different types of adult stem cells have been identified, including haematopoietic stem cells, mesenchymal stem cells, neural stem cells, epithelial stem cells, and skin stem cells[34]. Mesenchymal stem cells are the adult stem cell type found in mesenchymal tissues such as bone, fat, tendon, ligament, muscle, and other associated musculoskeletal tissues. Whilst not as powerful as ESCs, the ease of obtaining them, and the absence of associated ethical concerns, makes them a prime candidate for use in the development of treatments in orthopaedic surgery.

Stem cells have, therefore, become an important research focus in attempts to develop cellular therapies to repair musculoskeletal tissue. For the reasons outlined above, MSCs have become the main focus of research for the treatment of musculoskeletal conditions[46]. Figure 1.1 shows the number of papers indexed in PubMed each year from 1980 to 2013 under the MeSH term "Mesenchymal Stem Cell". As can be seen, the interest in these cells has driven an almost exponential increase in research publications in the last decade.

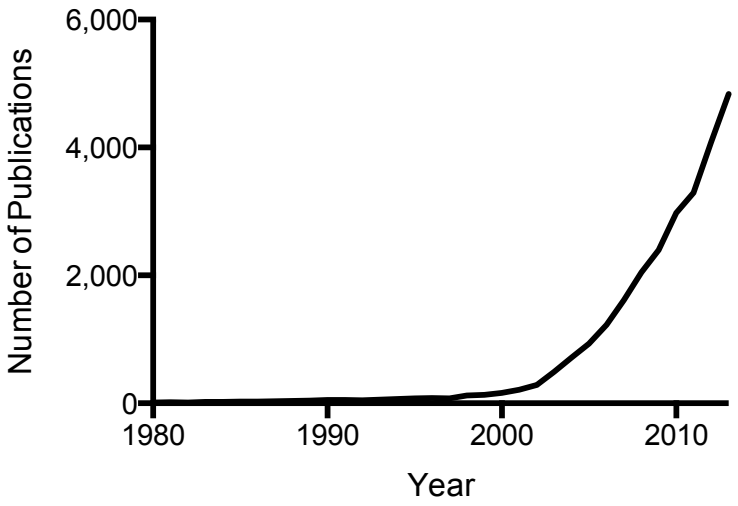

Figure 1.1 – Papers indexed by PubMed on Mesenchymal Stem Cells by Year

B.1 Mesenchymal Stromal Cell Definitions and Terminology

Mesenchymal *stem* cell was the term used by Pittenger *et al.* when first describing these cells in 1999[47]. Since then a wide variety of different terms has been used, including[48]:

- Multipotent mesenchymal stromal cell;
- Bone marrow stem cell;
- Mesenchymal progenitor cell;
- Mesenchymal stromal cell;
- Connective tissue progenitor cell.

The phrase "mesenchymal stem cell", especially when used in relation to the development of clinical treatments, implies that a key feature of these cells is their "stem" qualities, usually the ability to differentiate into other tissues. It also implies that what is being discussed is a homogenous population of cells. As discussed in Section 1.C.1, the majority of single stage procedures currently in use or under development are believed to work via a variety of other effects such as immuno-modulation and paracrine signalling, rather than by relying upon the differentiation of transplanted cells.

It is also apparent that the population of cells in these instances is rarely homogenous. The presence of cells with varying surface antigen phenotypes within the population and the varied function of these cells is evidence of this[49, 50]. It is therefore more appropriate to refer to these cells as mesenchymal stromal cells as this better reflects their origin and does not imply that any particular "stem" action is necessary to bring about their treatment effects.

In this thesis I will, therefore, use the term mesenchymal stromal cell (MSC) to refer to an adult stromal cell obtained from any tissue of mesenchymal origin. This includes:

- Bone marrow-derived MSCs (BM-MSCs);
- Adipose-derived MSCs;
- Tendon-derived MSCs.

It should be remembered, however, that many authors will use the term mesenchymal stem cell when discussing the same heterogenous population of cells as I

am discussing in this thesis. To avoid confusion I use MSC to refer to the group of cells obtained from mesenchymal tissues and the term *stem cells* as an umbrella term for all of the different types of cells mentioned above.

B.2 The Stem Cell Niche

It is important, when considering the functions of these cells, to take into account what is commonly referred to as the *stem cell niche*. This is the microenvironment formed within the bone by the various populations of cells that fall within the MSC umbrella as well as other nearby cells and non-cellular structures. This niche is thought to be vital to the normal functioning of these cells and to impact upon both their differentiation and their homing function[51]. It has also been shown that there is a close interaction between haematopoietic stem cells and mesenchymal stromal cells within their niche in bone marrow[52]. This interaction appears to be important for the normal functioning of these cells but it has yet to be fully understood. As the study of this area continues to develop, it has become apparent that the various components of the system perform vital actions which are not reproduced simply by transplanted cells: for example, the importance of pericytes in contributing to the regenerative process through paracrine signalling[53].

In the absence of a complete understanding of the niche, it is important to remember that it is difficult to fully understand the mechanisms of actions of these cells when using them in a novel treatment.

B.3 Characterisation of MSCs

Despite the large amount of research conducted into mesenchymal stem cells there is still no definitive, easy method of characterising these cells. The most widely accepted definition, proposed by the International Society for Cellular Therapy, defines them as having the ability to[54]:

- adhere to plastic;
- differentiate into adipocytes, chondroblasts, and osteoblasts;
- express specific surface antigens (CD73, 90, 105 positive and CD14, 19, 34, 45, and HLA-DR negative).

However, no single surface marker has been identified as being specific to MSCs. Potentially promising candidates such as CD271 have been proposed[55, 56], but further work has shown that these individual surface antigens appear to identify subsets of MSCs rather than the whole population[57]. This means that they will potentially fail to identify correct cell populations if used in isolation. Mark *et al.* have also shown that the identity of MSCs can change in culture depending upon the culture conditions, with CD105 expression being reduced in the absence of foetal bovine serum[58]. Phenotypic change in culture can cause problems for both the comparison of cell populations and can also potentially lead to a change in their function, making comparisons between *in vitro* and *in vivo* conditions difficult. Figure 1.2 shows the differentiation potential of these cells as it is currently understood. The "core" cell lines of bone, cartilage, and fat are those which are usually used when attempting to confirm the functional abilities of mesenchymal stem cells.

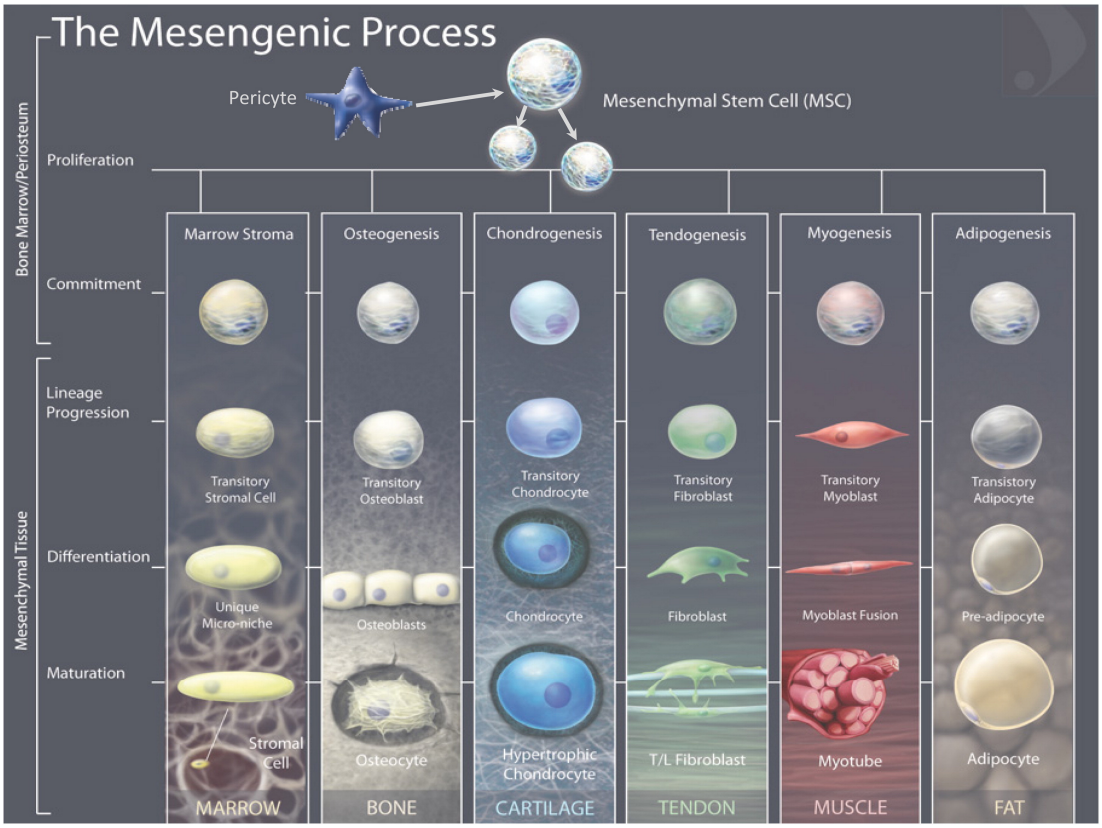

Figure 1.2 – Mesenchymal Cell Differentiation Potential (Reprinted from Caplan and Correa[59] with permission from Elsevier)

B.4 Mechanism of Action

The differentiation potential of MSCs was initially seen as the primary mechanism of their action and underpinned the early development of treatments which relied upon implanting a large number of cells that could transform into the required tissue type.

More recently, a new view of how MSCs may act to repair damaged musculoskeletal tissues has emerged. This takes into account the developing evidence concerning the mechanism of action of MSCs[60, 61]. Research has shown that rather than repairing damaged tissue purely by differentiating into the required cell type, MSCs bring about their reparative effects through a variety of mechanisms, namely:

- Immuno-modulation;
- Paracrine and cell-to-cell interactions;
- Homing to sites of injury.

Immuno-modulation: MSCs have frequently been described as being immune-privileged due to their lack of MHC II antigens[62, 63]. This property is extremely valuable as it would facilitate the use of allogenic MSC treatments without the need for immunosuppression. Further work has shown that apart from having a non-immunogenic profile, MSCs possess an active immunomodulatory function. The exact mechanism by which this function acts is not fully understood. MSCs have been shown to down-regulate B-cells, T-cells, and NK cells[64]. MSCs have also been shown to interact with dendritic cells in order to reduce cell-mediated immune reactions[65, 66]. These abilities may enable MSCs to affect inflamed or damaged tissues to promote healing.

Paracrine and cell-to-cell interactions: MSCs also interact with their local environment via a number of paracrine factors. MSCs secrete a large number of growth factors and other molecules that act locally to promote cell proliferation and angiogenesis, including: TGF-α, TGF-β, FGF-2, and VEGF[67]. Through the actions of these cytokines, MSCs can interact with local tissues to instigate some of the processes required for tissue regeneration.

Homing to sites of injury: A number of studies have also shown that MSCs have the ability to home to areas of damage that require repair. Mokbel *et al.*[68] demonstrated the ability of green fluorescent protein (GFP)-labelled MSCs to target an area of cartilage damage if injected into a joint and to take part in

the repair process as demonstrated by the presence of labelled cells in the newly repaired cartilage tissue. In another study it has been shown that systemically administered MSCs appear to be able to improve the function of damaged muscle tissue in rats, despite the fact that none of the GFP-labelled MSCs remained at the site of injury 21 days after administration[69]. Notably, this study used arterial injection of MSCs as the vascular bed of the lungs is felt to pose a significant barrier to the homing of systemically administered MSCs[70]. Circumventing this filtering effect is thought to be an important hurdle to overcome if systemic treatments are to become practical[71]. A number of animal studies have shown that systemic administration results in the vast majority of MSCs becoming resident in the lungs which would significantly affect their ability to home to injury sites[72, 73].

The variety of immune related functions that MSCs can undertake is demonstrated by the development of treatments for Graft Versus Host Disease (GVHD). GVHD is a condition in which those who have undergone an allogeneic tissue transplant find that their own tissues are attacked by the immune cells of the transplanted tissue, because the transplanted tissue identifies the individual's own tissue as "foreign". This condition is most commonly associated with bone marrow transplants. A number of clinical trials have been undertaken using MSCs to treat the condition, relying upon the immuno-modulatory effects of the transfused cells to dampen down the immune response generated by the graft tissue. Despite the lack of a full understanding of these mechanisms of action, a commercial treatment for GVHD in children has received a license in both Canada and New Zealand[74].

C Stem Cell Use in Orthopaedics

MSCs have been the main focus of stem cell-related research for treating musculoskeletal disorders since they were first described by Pittenger *et al.*[47]. Research into the use of MSCs has attempted to address a number of questions in order to develop an effective clinical treatment, including:

- Where should MSCs be taken from?
- How should they be extracted?
- What musculoskeletal conditions can they effectively treat?

C.1 Types of Treatment with MSCs

Work to develop MSC-based treatments was initially focused upon using MSCs to replace damaged tissue through a process of re-implantation and then presumed differentiation into the required cell type. This process of harvesting, culturing, and re-implanting has been shown to have some success in animal models of both cartilage[75, 76, 77] and tendon damage[78, 79, 80, 81]. A number of human trials have also been conducted using this process of laboratory expansion and re-implantation[82, 83, 84].There are, however, a number of issues surrounding this approach which have not yet been fully addressed.

For example, tissue harvesting, usually bone marrow, requires a separate procedure from the main surgical intervention. This may need to take place some time before the main procedure if laboratory treatment of cells is required. This extra

procedure leads to an increase in both patient morbidity and the total cost of the procedure.

Laboratory procedures to extract and expand MSCs from the harvested tissue are costly and time consuming. The costs associated can be expected to be broadly similar to those associated with other laboratory based procedures such as ACI which is known to cost over £20,000 per treatment[18].

Treatments developed using this paradigm have depended upon the re-implantation of a large number of cultured MSCs that can differentiate into the required tissue type. However, the actual number of cells required for re-implantation has not been established. Yokoya *et al.* used five million MSCs on a scaffold for rotator cuff repair in a rabbit model[85]. McIlwraith *et al.* used 20 million MSCs to treat cartilage defects in horse stifle[3] joints. Interestingly, the authors commented that the consensus in the field appears to be that five million cells are required but that there are no data from studies to demonstrate this[76]. Haleem *et al.* utilised two million culture expanded MSCs per cm^2 to repair cartilage defects in humans[84]. Dutton and colleagues carried out a trial of meniscal tear repair in a porcine model with between one and two million MSCs per treatment[86]. In a case report of the successful treatment of a large cartilage defect in the femoral condyle of an athlete, Kuroda *et al.* used a collagen gel containing MSCs at a concentration of five million cells per millilitre[87]. Uematsu *et al.*, on the other hand, used only one million cells per cm^3 to repair cartilage defects in a rabbit model in combination with a 3D scaffold[88]. Awad *et al.* were unable to demonstrate any difference in the quality of repair of a rabbit patellar tendon injury model using either one, four, or eight million MSCs per millilitre of collagen gel[89]. This wide variation in treatment "dose" makes it difficult to compare

[3]The stifle joint is analogous to the human knee joint.

treatments and also makes it difficult for researchers and clinicians investigating new treatments to know what is the appropriate dose to use.

The concept of MSCs acting via interaction with other tissues, rather than acting via differentiation, has fostered the belief that treatment effects are not necessarily dependent upon the total number of cells that are implanted. This has led to the development of a variety of "single stage" procedures where MSCs are extracted from tissue and implanted into an area of damage in a single procedure. This single stage approach has a number of benefits over a two-stage procedure, not least the reduced morbidity and significantly lower cost due to the lack of a requirement for current Good Manufacturing Practice[4]-level (cGMP) laboratory facilities to process and expand harvested MSCs.

These single stage procedures are, generally, reliant upon the use of either adipose tissue or bone marrow as the source of MSCs due to the relative ease of extracting MSCs from them. The MSCs are extracted as part of a mononuclear cellular fraction which is termed either bone marrow mononuclear cells (BMMCs) or stromal vascular fraction (SVF) depending upon whether it is isolated from bone marrow or adipose tissue. The relatively small amount of processing that these cells require means that they are usually classified as minimally manipulated tissue and thus do not require rigorous safety data prior to implementing a new treatment. This is supported by work by Pak *et al.* which has shown that extraction of MSCs from adipose tissue using liposuction and immediately implanting them into a variety of joints is safe in humans[90]. A pilot study using a caprine model of osteochondral defects has demonstrated that not only can a single stage procedure effectively repair an osteochondral defect, but that at the four month post operative point SVF out-performed MSCs cultured from adipose tissue[91].

[4]As set out by The International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use.

BMMC-based procedures have also been investigated. In a porcine osteochondral defect model, BMMC used in combination with a commercial scaffold (TRUFIT BGS, Smith & Nephew, USA) resulted in a significant improvement in the histological quality of repair tissue compared with the scaffold alone[92]. A number of studies have investigated the use of single stage procedures to treat osteochondral defects in humans with BMMC or un-concentrated bone marrow aspirate[93, 94, 95, 96, 97, 98, 99, 100]. In their review of the majority of these studies, Veronesi *et al.* conclude that the heterogeneity of participants, defects, and treatment methods, along with the lack of control arms in all studies, makes firm conclusions impossible. They do, however, determine that these single stage procedures perform well enough in comparison to procedures based upon MSC culture to be a worthwhile area of further investigation[101].

Commercial interest in the area of single stage procedures has also increased greatly over recent years. Commercial devices to facilitate the isolation of MSCs in a theatre setting for immediate use are available from:

- Biomet MarrowStim™ - a centrifuge based system[102];
- Harvest Terumo SmartPReP 2® BMAC® - a centrifuge based system[103];
- Cesca Therapeutics Res-Q™ 60 BMC System - a centrifuge based system[104];
- Arteriocyte Magellan® MAR0Max™ - a centrifuge based system[105].

All of these systems operate in a similar manner. Bone marrow is extracted from the pelvis using a sterile collection system and placed into a centrifuge. The amount of bone marrow extracted varies but is typically approximately 60 mls.

This is then spun for approximately 10 to 20 minutes to separate the mononuclear cellular layer (containing MSCs) from the plasma and red blood cells. A 5 to 6 ml volume of concentrated mononuclear cells is produced at the end of the process. The mono-nuclear cell fraction can then be injected back into the tissue that requires treatment either on its own or in conjunction with some form of scaffold.

Commercial systems have also been developed to undertake in-theatre cell selection prior to re-implantation. Miltenyi Biotec now produce the CliniMACS® system which is capable of using a magnetic bead separation system in theatre to select cells based on their surface antigens prior to use[106]. This would potentially allow the further purification of bone marrow in order to deliver a more concentrated MSC product.

In addition, Smith & Nephew are currently developing an acoustic/ultrasound assisted filtration device for MSC collection[107, 108]. However, it must be emphasised that these commercial devices have been developed without a full understanding of the basic science underlying these MSC based treatments.

The progress of stem cell therapy in general has lead to an ongoing increase in the number of clinical studies taking place in humans[48]. This may give the impression that the more basic questions regarding the use of MSCs, such as: which tissue to use, where to obtain that tissue from, and how to harvest the tissue concerned, have been answered. However, these questions have *not* been definitively answered and answering them is still an area of significant research interest. Resolving these questions would also enable the single stage procedures currently being trialled to be further optimised in the hope of improving outcomes for patients. It is only by fully identifying these questions, and answering them,

that it will be possible to develop effective treatments using stem cells, and to design robust trials to allow for their assessment[109, 110].

C.2 Which tissue for MSC harvesting?

Although MSCs were first described in bone marrow they have since been isolated from a wide variety of different tissues, including:

- Adipose tissue[111];
- Ligament[112];
- Tendon[113];
- Muscle[114];
- Cartilage[115];
- Synovium[116];
- Periosteum[117];
- Sub-acromial bursa[118].

Currently the majority of stem cell treatments are based on the use of autologous sources rather than allogenic ones. As such, it is important that these autologous cells are harvested from the most appropriate location. When assessing the

location from which to take MSCs there are a number of factors which need to be considered by a clinician. The main issues of concern are the quality and quantity of MSCs that they are able to obtain. This must be weighed against the ease of accessing any particular tissue source and any associated morbidity. As the majority of stem cell based treatments currently in development make use of autologous sources[119] it is also important to bear in mind the health status and associated morbidity of the individual concerned as the reliance upon patients with particular characteristics (for example, low BMI or young age) may significantly limit the utility of the treatment.

Our lack of understanding of the mechanisms of both action and inter-action of MSCs means that we are only slowly developing an understanding of all the factors that must be considered in the use of MSCs. It has been established that drugs such as busulfan, methotrexate, and cyclophosphamide reduce both the numbers and proliferative capability of MSCs[120]. This is, perhaps, unsurprising given the established methods of action of all three of these drugs, which involve inhibition of DNA synthesis. Fortunately, with the exception of individuals with rheumatoid arthritis who may well be treated with methotrexate, few patients in need of an MSC based treatment for an orthopaedic condition will be on these medications. Given its mechanism of action, it is, perhaps, more surprising that diclofenac has been shown to interfere with the differentiation ability of MSCs in a murine model[121]. This potentially presents more of a problem for the use of autologous MSCs in orthopaedic conditions as it is not unreasonable to expect a large number of individuals with musculoskeletal disorders to be taking diclofenac or some other form of non-steroidal anti-inflammatory drug (NSAID) which may well have a similar effect.

It is therefore necessary when determining which tissue source to use for MSC harvesting to take into account both the general characteristics of the tissue concerned and also the patient-specific factors that may influence the quality and quantity of MSCs available.

C.2.1 Yield

MSCs have been shown to vary greatly in their abundance in different tissues (Table 1.1).

Table 1.1 – Relative Abundance of MSCs in Different Tissues

Tissue	Percentage of MSCs of All Cells	Reference
Bone Marrow	0.001 - 0.01	[122]
Tendon	3 - 4	[122]
Synovium	1.25 - 8	[123]
Adipose Tissue	25 - 30	[124]

Availability of MSCs also varies within tissues. Bone marrow obtained from the pelvis has been viewed as the gold standard against which other bone marrow sources are assessed. Over recent years, work has been carried out demonstrating the feasibility of obtaining MSCs from the humerus[125], femur[126], vertebral body[127], and tibia and calcaneus[128]. This anatomical location variation has also been demonstrated in adipose tissue in a number of different studies[129, 130, 131].

The studies identifying these sources warrant some discussion due to various limitations in their conduct. Fro example, the work of Mazzocca and colleagues[125, 126] has demonstrated the presence of MSCs in a variety of these skeletal locations, notably the proximal humerus and distal femur. However, these studies

established only the ability of cells to differentiate osteogenically and, more importantly, there was no control arm in terms of assessment of MSC yield.

Comparing MSC populations within individuals is vitally important and should be done wherever possible, rather than attempting to compare cell populations between different subjects. This is due to the large amount of variation in MSC populations between individuals[132]. The lack of intra-subject comparison leads to difficulty in interpreting the majority of research looking at MSC yield from different tissues.

It must also be remembered that obtaining tissue from different locations or tissue types can pose varying difficulties. In the treatment of orthopaedic conditions the most obvious choice is bone marrow as, despite the relatively low yield, it is easy to access either during a procedure or as a separate biopsy. Other musculoskeletal tissue sources, such as synovium and tendon, are severely limited in their utility by the difficulty in not just obtaining the tissue due to morbidity concerns, but also in extracting the cells from the tissue which frequently will require some form of tissue digestion or homogenisation.

C.2.2 Function

As well as considering the abundance of MSCs in tissues, it is important to consider the functional abilities of those cells, both in terms of their ability to proliferate and to differentiate into the required tissue.

Utsunomiya *et al.* have shown that MSCs taken from the enthesis of the rotator cuff have a greater chondrogenic potential than those obtained from the synovium

or supraspinatus tendon of the shoulder[133]. MSCs isolated from muscle have been shown to have better tenogenic differentiation abilities than those taken from bone marrow[134]. Rotator cuff derived MSCs have been shown to have better myogenic potential than bone marrow derived cells[135]. As commented on by Jones and Pei, synovium derived MSCs have greater chondrogenic ability than bone marrow derived MSCs[123]. However, the relative inappropriateness of these tissues for large volume harvesting makes them unsuitable for routine use in MSC based treatments.

Strioga *et al.'s*[136] review comparing adipose derived MSCs with bone marrow derived MSCs showed that there is a wide variation in the ability of the different cell types and also that different studies appear to generate different results, with some studies showing no difference in chondrogenic ability between the cell types and others demonstrating a better ability in bone marrow derived cells. It is therefore difficult to choose which tissue to use in terms of the function of the cells obtained.

C.3 How to Obtain MSCs

The method by which bone marrow is harvested has also been shown to be important in terms of its effect upon the yield of MSCs from a source. The most commonly employed method to obtain bone marrow is by aspiration from a medullary canal using an aspiration needle such as a Jamshidi® needle[100, 128, 137, 138].

The Reamer-Irrigator-Aspirator (RIA) System, made by DePuy Synthes, has also been shown to be capable of harvesting large volumes of MSCs. This is,

perhaps, unsurprising given that the system essentially removes most of the bone marrow and trabecular bone from the medullary cavity of a long bone. Cox *et al.* have demonstrated significantly higher yields of MSCs, as demonstrated by colony forming unit-fibroblast (CFU-F) count of RIA aspirate (both liquid and solid phase), from the femur when compared with iliac crest bone marrow[139]. Henrich *et al.* have also shown that the RIA system is able to deliver a higher yield of MSCs than bone marrow samples from the iliac crest and that these cells have the ability to differentiate into osteoblasts[140]. Whilst the Henrich study benefits from having a large number of participants (N=61), it is limited by the fact that iliac crest and RIA samples were obtained from different patients. Whilst the Cox study included only six participants, both samples were obtained from each patient, allowing for intra-subject comparison and so more comparable results for the reasons outlined in Section 1.C.4.

The RIA system requires an entirely separate system for collection and separation of bone graft and is not without risks such as fracture, damage to the knee joint, or disruption of the cortex of the bone[141]. Whilst the rate of complication was found to be lower with the RIA than with bone graft harvesting from the pelvis in a systematic review carried out by Dimitriou *et al.* (6% v 19.4%)[141, 142], this comparison included harvesting via a bone window, rather than simple needle based aspiration or biopsy which is associated with a much lower complication rate of approximately 0.07%[143]. A recent study by Hernigou *et al.,* which looked at almost 1,000 procedures, found a ten-fold decrease in complications from bone marrow aspiration from the pelvis as compared to standard iliac crest bone graft harvesting[144]. They also reported that the types of complications encountered in aspiration were less severe than those found with bone graft harvesting.

Previous work by Hernigou *et al.* has also shown that what might be thought of

as relatively simple factors such as the size of syringe used and the volume of bone marrow aspirated can also have large effects on MSC yield. They showed that better yields could be obtained by using smaller syringes and extracting smaller volumes from each area of bone[145]. This was thought to be due to the fact that, whilst it is necessary to generate some negative pressure to release MSCs from their perivascular attachments, too much negative pressure results in the drawing of blood into the bone from the circulation. This is then drawn into the collection syringe and dilutes the final sample concentration.

Despite the evidence outlined above there is still no definitive method for obtaining MSCs from bone marrow. The importance of seemingly trivial factors such as syringe size and extraction volume has not been fully explored to date. The evidence from the use of the RIA that there are large numbers of MSCs available from the bone marrow means that there must still be room for improvement in the harvesting techniques used to increase MSC yield.

C.4 The Importance of Intra-Subject Comparison

Intra-subject comparison is a vital component of any comparison of MSCs. This is because it has been demonstrated in a number studies that there is wide variation in MSCs numbers between individuals which does not seem to be related to factors such as age, gender, or source of isolation. Siddappa *et al.* showed that in 19 different individuals there was a significant variation in alkaline phosphatase expression following osteogenic differentiation, but that this was not related to age, gender, or source of isolation (acetabulum versus iliac crest)[132]. Scharstuhl *et al.* showed, whilst investigating the variation in chondrogenic ability of MSCs from different sources, that there appeared to be no relationship between either

the age or disease aetiology of an individual and the number of MSCs that it was possible to obtain from bone marrow[146]. They also established that the chondrogenic potential of these cells appears to be independent of age and disease aetiology. For this reason it is important, when investigating the optimum source of MSCs, to ensure that comparisons are, where possible, conducted *within* rather than *between* individuals.

C.5 Sources of MSC Variation in Bone Marrow

Any study seeking to investigate potential sources of MSCs must take into account the fact that there are a number of between-subject factors that are believed to affect the availability of MSCs.

C.5.1 Age

Ageing is thought to have an effect upon both the number and function of MSCs. It is not clear, however, exactly how this relationship operates. Whilst it has long been stated that MSC numbers decrease with age[147], this is not always demonstrated in studies. Stolzing *et al.* found a significant drop in CFU-F numbers in older individuals when comparing donors aged less than 20 years with those aged 21 - 40 years[148]. There was also a decline in the growth kinetics of older individuals after the first five passages in culture. A study of 22 posterior iliac crest aspirates, however, demonstrated no link between age of donor and the cell number and growth kinetics of MSCs[149]. A study of MSCs from the infra-patellar fat pad also failed to demonstrate any relationship between age and cell number or proliferation rates when comparing two groups with mean ages of

57 years and 86 years[150]. However, a study of adipose derived MSCs obtained from lipoaspirate recently showed a decrease in CFU-F count with increasing age as well as increased evidence of senescence as demonstrated by increased senescence-associated beta galactosidase (SA-β-gal) staining [151].

A recent review by Fossett *et al.* stated:

> "... there is a mixed view about the effects of ageing in the current literature. It is important to identify the relationship between ageing and MSCs to find out whether they can be used for autologous transplantation of older patients..."[152, p. 285]

This lack of certainty means that it is currently entirely feasible to attempt to use autologous MSCs to treat patients no matter what their age. However, it is vital that we maximise the yield of MSCs from any given individual to enhance the chance of an MSC based treatment working.

C.5.2 Anatomical Location

As mentioned above, it is possible to isolate MSCs from bone marrow harvested from a wide variety of different anatomical locations. However, the sources can vary quite considerably in the number of MSCs that are available.

Hyer *et al.*[128] compared the availability of osteo-progenitor cells from the iliac crest, distal tibia, and calcaneus. They showed that, when comparing the number of alkaline phosphatase staining colonies (≥2mm in size), the iliac crest provided over 30 times more colonies than the other sites. This study was strengthened by the use of intra-subject comparisons.

Mazzocca *et al.*[125] similarly demonstrated the presence of MSCs in bone marrow taken from the proximal humerus. The study was limited by lack of a comparison collection from the iliac crest. It is worthy of note that the assessment of colony forming units in this study included only those colonies with eight or more cells. This variation in methodology of even a simple assessment such as CFU counts is one of the issues which makes direct comparison between studies difficult. The same group has also successfully demonstrated the presence of MSCs in the distal femur. The same method of CFU counting was used, but as in the previous study, there was no comparison with iliac crest samples[126].

Murphy *et al.* demonstrated reduced chondrogenic and adipogenic activity in bone marrow MSCs taken from either the femur or tibia when compared to the iliac crest. Their study, however, had a relatively old population with a mean age of 71 who were undergoing total hip or knee replacement. More significantly, either femoral or tibial samples were taken rather than both, and the samples were collected after the joint replacement procedure. The large amount of disruption caused to the architecture of the bone by both the instrumentation used in the procedure and the heat effects of the cement which is typically used in joint replacement procedures make it difficult to determine the usefulness of either of these bones as a source of MSCs in individuals who have yet to undergo such a procedure[153].

McLain *et al.* showed that the vertebral body is a better source of MSCs, using CFU assessment, when compared to the iliac crest in matched controls but the difference did not achieve statistical significance[127]. The superior qualities of vertebral MSCs has also been advanced by Barbanti Brodano *et al.* who recently showed improved growth characteristics and osteogenic differentiation abilities when compared with iliac crest MSCs[154]. Their study did, however, use a

variety of different sources, including commercial, for the samples and there was no intra-subject comparison.

This proven variability between different skeletal locations means that it is necessary to formally assess any potential bone marrow harvesting site for MSC yield before using it to develop an MSC based treatment.

C.5.3 Location within Bone

The area within bone that samples are taken from can also lead to significant variation in the yield of MSCs. MSCs obtained from bone marrow have been shown to be closely associated with the microvasculature and to have a close relationship with pericytes. Work by Siclari *et al.* has examined the differences in those MSCs obtained from the trabecular metaphysis and endosteal area and compared it to those obtained from the central part of the bone, relatively distant from the bone surface[155]. It found that, whilst the cells were equivalent in terms of both antigen phenotype and differentiation ability, the endosteal area was superior in terms of both the numbers of MSCs obtained and their proliferative ability.

CD146 positive cells have previously been used to help identify MSCs within bone marrow and have shown that these cells seem to be closely associated with the perivascular area within the bone[156]. This would help to explain the presence of greater numbers of MSCs in the trabecular bone compared with the central part of the bone, given the greater blood supply to the trabecular area[157, p. 89]. Tormin *et al* have shown that the expression of CD146 seems to identify MSCs that occupy a peri-vascular area but fails to identify those obtained from

the remaining bone marrow[158].

These variations from different parts of the bone may help to explain the difference in yield of MSCs from bone marrow depending on the harvesting method used and thus they provide further support for attempting to break down the trabecular bone network prior to harvesting bone marrow.

D The Current State of MSC Use in Clinical Practice

As outlined above, MSCs are now used in a variety of different clinical settings and a wide range of different collection and purification systems are available. These systems and procedures have become increasingly common in clinical practice despite the issues that still surround their use.

The lack of understanding amongst both clinicians and scientists as to the exact mechanisms of action of these cells means that the majority of these treatments are being developed without the knowledge that is required to ensure that they are optimally designed and implemented. In order for the potential of MSCs to be realised it is necessary that their mechanisms of action be fully elucidated. This work, which will require a great degree of effort from the research community, is underway but is still far from complete.

In the absence of this knowledge, and given that these systems are already in clinical practice, it is vital that they are optimised as much as possible. Maximising the availability of these cells to improve the available dose is a potential target for this optimisation. One way of achieving this is to determine the best source for MSCs for use in clinical practice.

D.1 Aims

The aim of this thesis, therefore, is to determine the optimum location for the harvesting of MSCs from bone and to determine if this yield can be improved through simple measures. This will allow the optimisation of single stage procedures until the full mechanism of action of these cells is understood.

E Scope of Thesis

Despite the apparent potential of the treatments described above, and the wide number of clinical trials taking place with MSCs, there are still many unanswered questions. The purpose of this thesis is to attempt to answer two central questions, namely:

1. Which bone marrow location provides the optimum source for obtaining MSCs for use in single stage procedures?

2. Is it possible to increase the yield of MSCs from a bone using simple manoeuvres?

These questions were explored by conducting the studies outlined below.

E.1 Anatomical Location Study

Due to the number of cartilage injuries in the knee, it was decided to determine if bone marrow can be be obtained from a more local site when seeking to treat problems around the knee. Bone marrow obtained from the femur and tibia was compared with the current gold standard of bone marrow from the iliac crest. Bone marrow was collected from the pelvis, femur, and tibia of participants. Samples were then studied to determine:

- The number of cells obtained;
- The number of MSCs obtained;
- The growth kinetics of the MSCs obtained;
- The cell phenotype of MSCs after expansion;
- The osteogenic and chondrogenic differentiation potential of expanded MSCs.

Obtaining samples from all three locations from each individual allowed for intra-subject comparisons to be made to control for the previously mentioned inter-subject variation in stem cell numbers. Differentiation was undertaken in order to allow characterisation of the cells in line with the ISCT criteria[54] and was not designed to reflect any potential regenerative capacity that could be derived from the differentiation of cells. The uncertainty surrounding the exact mechanism of action of MSCs would make it inappropriate to draw conclusions about their clinical effects on the basis of *in vitro* differentiation assays.

E.2 Sampling Method Study

The effect of sampling method upon MSC yield was investigated by comparing two methods of MSC collection from the distal femur. Bone marrow was collected in a standard fashion, with simple insertion of the aspiration needle through the cortex. A modified technique was also used to insert the aspiration needle and then manipulate this in all planes in order to attempt to disrupt the internal bone architecture and so liberate a greater number of MSCs. This was based on knowledge that MSCs are closely associated with the vasculature within the trabecular bone and so disruption of this may free a greater number of MSCs. Samples were analysed in the same manner as for the anatomical location study. Samples obtained in this manner were compared with those from the first study that had been obtained from the femur.

Chapter 2

Methodology

A Clinical Study Design and Conduct

A.1 Introduction

In order to successfully answer the questions posed in Section 1.D it was necessary to design a study that was able to address the questions, whilst at the same time attempting to overcome the limitations of previous research. It was assumed that in the vast majority of situations in which harvesting MSCs would be used to treat cartilage defects the operating surgeon would be an orthopaedic specialist. Given this, it was felt that bone marrow would be the only suitable candidate tissue source given the impracticality of using the majority of other sources (such as periosteum and tendon) and the lack of experience that these surgeons would have in harvesting relatively large amounts of adipose tissue through procedures such as liposuction.

The very large effect that inter-subject variability could have upon results meant

that it was vital to carry out intra-subject comparisons whenever possible. As the aim of the study was to compare the pelvis with the femur and tibia this was addressed by selecting study participants from those individuals undergoing knee replacement surgery. In developing the study design a number of options were considered to attempt to make use of intra-subject comparison for the sampling method study. The two main options considered were:

- Harvesting cells using the standard and modified techniques from the same bone, alternating which was conducted first to control for being the most "fresh" sample. It was felt that this would not be reliable given it would not be possible to harvest using the standard method after the trabecular bone had been disrupted. Whichever method was used second in the sequence may also have suffered from the diluting effects of peripheral blood being drawn into the medullary canal as has been found in previous studies[145].

- The optimum study design would have been to obtain samples from both femurs in each participant, using a different technique on each side. This study design was discounted as it would have proven extremely difficult to obtain ethical approval for such a design and to recruit participants to undergo procedures on both knees simultaneously. The very low numbers of bilateral total knee replacements would have made using such patients equally impractical.

It was decided that it would not be possible to control for intra-subject variation in the second study and that a control arm of different individuals would have to be used instead.

A pragmatic decision was taken to use individuals undergoing joint replacement

of the knee[5]. Cellular therapies and treatments such as ACI are typically aimed at individuals who are younger than those undergoing total joint replacement, with an upper age limit of approximately 55 years[159] and the majority of patients frequently being much younger[24]. However, with a population that is living increasingly longer it will be increasingly desirable to be able to delay end stage treatments such as total joint replacement by making use of regenerative procedures. It would also have proven difficult to obtain access to a younger cohort as the procedures that they would typically undergo, such as anterior cruciate ligament reconstruction, would not afford the necessary surgical access to allow for easy sampling from the femur and tibia. Individuals undergoing total joint replacement of the knee were also chosen due to the relatively minor increase in morbidity that taking part in the study would represent compared with their normal treatment. It was felt that this would be favourable, both in terms of ethical considerations and with respect to ease of participant recruitment.

A study protocol was designed in line with University of Oxford Clinical Trials and Research Governance Department guidelines.

Two groups of patients were required for sufficient sample collection. The first group (Group A) was used to obtain samples from the anterior iliac crest, distal femur, and proximal tibia of the same individual. This group was made up of participants undergoing total or partial knee replacement as this allowed sampling from the distal femur and proximal tibia without the need for separate skin incisions.

The second group (Group B) was used to obtain samples using a modified collection technique. This technique involved attempting to disrupt the trabecular

[5] Although the study protocol included total hip replacement patients as potential participants, only individuals undergoing knee replacement were enrolled.

bone prior to aspiration in order to liberate a greater number of MSCs from their perivascular location. This group was also taken from individuals having a total or partial knee replacement. These individuals had a single sample taken from the distal femur.

The fact that the individuals in both groups were all undergoing joint replacements meant that it was not necessary to institute any extra aftercare treatment or monitoring as they would be receiving sufficient analgesia and monitoring as part of their normal standard of care.

A protocol outlining the conduct of the study was drawn up and the inclusion and exclusion criteria for participation in the study are listed in Table 2.1. Individuals were excluded if there had been previous infection in the limb because of the risk of either sampling quiescent bacteria that may introduce infection into tissue culture samples or re-activating the infection secondary to the sampling process. Individuals with a history of inflammatory arthritis were excluded as previous studies have demonstrated that MSCs from these individuals have reduced proliferative and clonogenic potential[160, 161]. Individuals with inflammatory arthritis are also more likely to have damage affecting the entire joint surface, rather than focal defects, and so would be less likely to be recipients of single stage procedures. Given the relatively small sample sizes being studied, it would have been difficult to control for these changes. The use of medication such as bisphosphonates or oral steroids was also an exclusion criterion. This was, again, because of the interaction between these drugs and MSCs. Bisphosphonates have been shown to increase osteogenesis in human MSCs in *in vitro* experiments[162]. Steroid use has also been shown to interfere with MSCs[163]. Although the effect is not permanent, as this study did not involve the altering of participants' medications, these individuals were excluded as it would not be

Table 2.1 – Study Inclusion and Exclusion Criteria

Inclusion	Exclusion
Able to give informed consent	History of rheumatoid arthritis or other inflammatory arthritis
Either gender	History of septic arthritis in the limb being operated on
Aged 18 years or above	Bisphosphonate use
Undergoing total hip or knee arthroplasty or unicompartmental knee replacement	Oral steroid use

possible to control whether they would still be taking steroids at the time of their surgery.

Potential participants were identified from those listed to undergo an appropriate procedure at the Nuffield Orthopaedic Centre, Oxford. They were approached at the time of their pre-operative assessment and if, after reading the Patient Information Sheet (PIS) and discussing any queries, they wished to take part, a consent form was completed. Data regarding co-morbidities, demographics, and current medication was obtained from participants' medical records. Samples were obtained as outlined in Section 2.B.1 on page 50. All surgical procedures were undertaken by one consultant orthopaedic surgeon or under their direct supervision and were overseen by Benjamin Davies to ensure there was no variability in the methodology of sample collection.

Ethical approval was obtained to include 10 individuals in Group A. Group B had approval for the collection of samples from 20 individuals in order to allow for 10 individuals with standard sample collection and 10 with the alternative collection method. In order to allow for a faster rate of sample collection and processing, it was decided to use the femur samples from Group A as the standard collection method controls for Group B, reducing the number of different samples required. A 20% excess was also approved to allow for any potential problems with samples

such as early infections or failure of expansion of cells.

The study was designed in accordance with the guidance of the NHS National Research Ethics Service[6]. Ethical approval for the study was obtained from the Berkshire Research Ethics Committee (Approval Number: 12/SC/0484). Following ethical approval an application was made to Oxford University Hospitals NHS Trust for NHS approval (Reference: 10253). There was some disagreement between the Ethics Committee and the NHS Research and Development department over the degree of information that was required within the study protocol. This led to a number of revisions having to be made to the study protocol and led to a six month delay in the final delivery of NHS approval. The Patient Information Sheet and consent forms are contained in Appendix E.

A.1.1 Study Power

Previous work by Hyer *et al.*[128] determined that a minimum sample size of 40 patients would be required to achieve a power of 0.80. A power analysis performed using G*Power software[164] determined that 128 participants would be required to achieve a similar power in the second study looking at the modified technique of harvesting.

It was determined that to achieve this power would require a significantly greater amount of time than was available during the course of the study and would also need a much larger budget to facilitate the required experiments. It was therefore decided that it would be most appropriate to use a similar study size to that which had been used in previous similar work.

[6]This service has since been replaced by the NHS Health Research Authority.

Ten participants were therefore chosen for each sub-study as a suitable compromise and this compares well with the number of participants used in a variety of other similar laboratory-based studies. For example, Fernandes *et al.* used an $N = 6$ (two independent groups of 3) in their study of MSCs and chondrocytes in OA patients[165]. Neumann *et al.* used 6 individuals to study the chondrogenic ability of MSCs isolated from subchondral bone[166]. In their study of different commercial collection systems Hegde *et al.* used an $N = 10$ in each independent arm[167].

The power of the anatomical location study was improved by the use of intra-subject comparisons to help control for person-to-person variation.

A.1.2 Human Samples Used in Validation Experiments

In order to establish the correct experimental procedures detailed later in this thesis, and to validate protocols, it was necessary to obtain bone marrow samples outside the scope of the clinical study protocol and ethical approval. These samples were obtained from the Oxford Musculoskeletal Biobank which is regulated by the Human Tissue Authority (Licence Number: 12217) and has received ethical approval from the Oxfordshire Research Ethics Committee C (Approval Number: 09/H0606/11).

A.2 Participant Data Collection

The following information was collected for all individuals who agreed to take part in the study. Information was taken either by direct questioning or by extraction

of the information from the individual's medical record:

- Gender
- Age
- Diagnosis
- Co-morbidities
- Past Medical History
- Current Medication
- Smoking Status
- Body Mass Index
- Activity Level
- Use of Walking Aids
- ASA Score - The ASA system is a score designed to assess the severity of any co-morbidities that an individual undergoing an operation may have. The possible scores are listed in Table 2.2[168].

Medical records were also checked after the procedure to ensure that any complications had been identified at the six week post-operation follow-up appointment

Table 2.2 – ASA Physical Status Classification System

Score	Definition
1	A normal healthy patient
2	A patient with mild systemic disease
3	A patient with severe systemic disease
4	A patient with severe systemic disease that is a constant threat to life
5	A moribund patient who is not expected to survive without the operation
6	A declared brain-dead patient whose organs are being removed for donor purposes

that formed part of the normal standard of care.

A.3 Recruitment

As mentioned in Section 2.A.1, individuals were approached at their pre-operative assessment appointment. Figure 2.1 shows the numbers approached and their final disposition. Two individuals did not wish to take part. All other individuals approached agreed to participate although a small number expressed a preference to be in group B because of issues such as previous surgery to the pelvis. Two individuals were excluded after consenting because between consenting and the time of surgery they had commenced on medication that required exclusion. A number of individuals were excluded for administrative reasons such as being transferred to the care of a surgeon for their operation who was not involved in the study. The study was concluded when sufficient samples had been collected as outlined in the study protocol and so two individuals who had consented were not required to take part in the study.

A.4 Participant Follow Up and Adverse Events

There was only one adverse event affecting a study participant. This individual had a reaction to medication used as part of their normal standard of care and, as such, the adverse event was not related to the study and samples were processed normally. Participants who underwent iliac crest sampling reported that pain was controlled by the analgesia they received as part of their normal care in all cases. No early or late wound problems were reported.

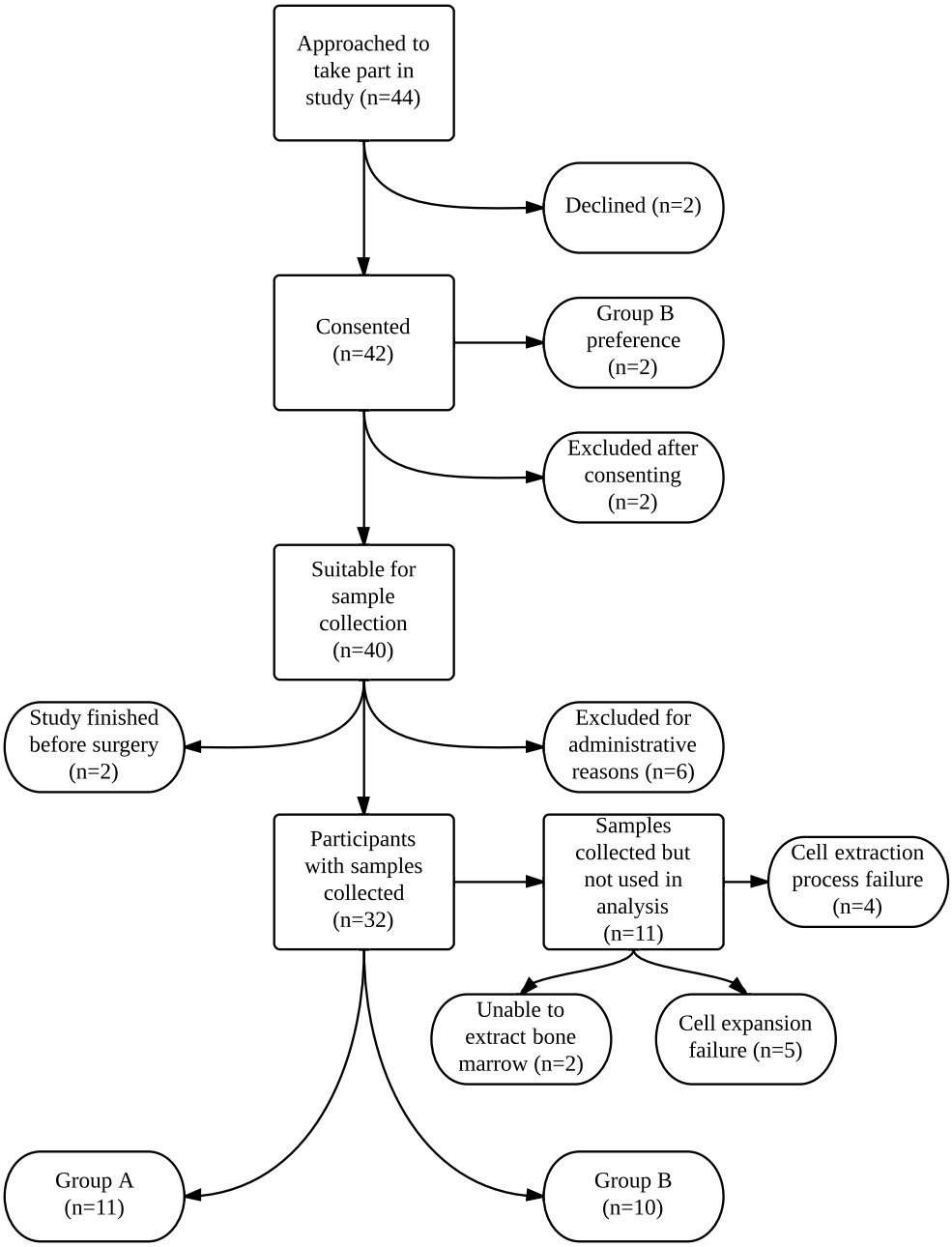

Figure 2.1 – Clinical Study Recruitment Flowchart

B Tissue Harvesting and Culture

In order to carry out the investigative work required for this thesis, it was necessary to design and implement a number of different experimental methodologies and protocols. In this section I will outline the methodologies used for tissue harvesting and culture. These include:

- Harvesting of bone marrow;
- MSC extraction from bone marrow;
- Cell expansion and passaging in culture;
- Differentiation protocols used.

B.1 Bone Marrow Collection

Bone marrow was collected using the method outlined below.

1. An 8G x 15 cm Jamshidi® bone marrow aspiration needle (CareFusion, San Diego, USA) was passed through the cortex of the bone concerned.
2. A 30 ml syringe was attached to the end of the needle and between 5 and 10 mls of bone marrow was aspirated into the syringe.
3. The bone marrow was then immediately placed into a EDTA filled sample collection tube (Becton Dickinson, Franklin Lakes, USA) and inverted three

to five times to ensure mixing of the sample with the EDTA to prevent clotting.

4. Samples were stored at 4°C in an upright position until they were processed.

Five to 10 mls of bone marrow was collected in order to maximise the concentration of MSCs in line with the previously discussed work by Hernigou[145].

Initially, harvesting was attempted using luer-lok syringes. However, these failed to allow for a sufficiently closed seal and thus it was not possible to generate enough negative pressure for sampling. Following a switch to simple hub syringes it was possible to obtain samples as planned. It also proved difficult to achieve sufficient negative pressure with the use of smaller volume syringes when extracting samples from the femur or tibia which is why 30 ml syringes were used for the majority of collections.

When a pelvic sample was taken, this was done either prior to the start of the main procedure, or simultaneously with it. The contra-lateral anterior iliac crest was used for sample collection in order to reduce interference with the main operative site. The area was prepared with 2% chlorhexidine and draped separately. A one to two centimetre incision was made over the anterior iliac crest. Soft tissues were divided using blunt dissection and samples collected as outlined above. This wound was closed with an interrupted 4-0 non-absorbable nylon suture which was removed at the same time as those from the main operative site. The anterior iliac crest was used as this was easily accessible during the planned procedure. The anterior pelvis has also been used in the majority of studies involving human MSCs[169] and so provides the most appropriate standard with which to compare other anatomical sites.

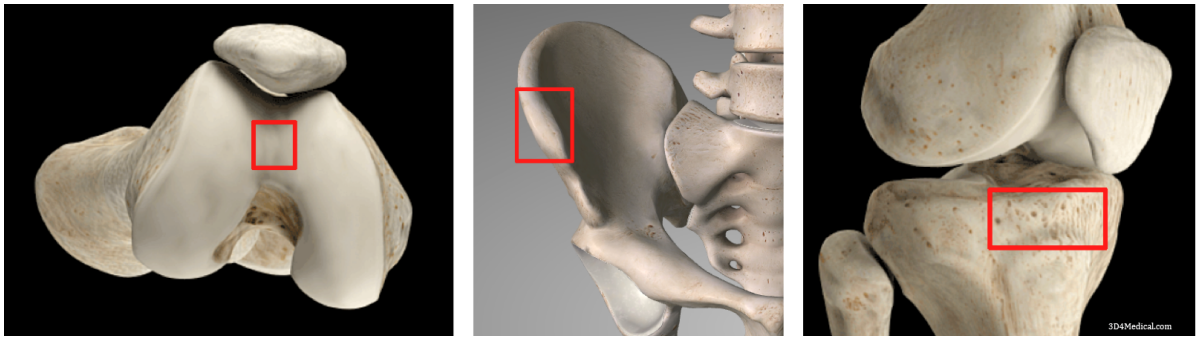

Figure 2.2 – Areas used for Anatomical Location Sampling

As samples were obtained from individuals undergoing knee arthroplasty it was necessary to pause the operating procedure to harvest the femoral and tibial samples. The femoral sample was obtained by inserting the aspiration needle through the trochlear notch in the same area as the intra-medullary rod which would be used for alignment of equipment during the main operation. It was usually necessary to use an awl to help breech the cortex due to the thickness of bone at that point. Tibial samples were obtained by passing the aspiration needle through the anterior aspect of the tibia, approximately one and a half to two centimetres below the tibial plateau surface. Figure 2.2 shows the locations from which each sample was taken, as outlined by the red boxes[7].

For the first two cases the femoral sample was taken once the bone of the femur had been prepared and some osteophytes removed from the femoral condyles. In these cases it was not possible to obtain any femoral samples as it appeared that removal of the osteophytes allowed air to enter the femur and so made it impossible to establish sufficient negative pressure for sampling. In all further cases bone marrow collection was undertaken after exposure of the bone but before any bone preparation had been undertaken.

[7]Images used with permission of 3D4Medical.com.

B.2 Bone Marrow Processing

Mononuclear cells were extracted from the bone marrow using density centrifugation. This technique has been used widely in work on MSCs[55, 154, 170]. Bone marrow was mixed with RPMI media (Life Technologies, UK) and filtered through a 70 μm cell strainer (Fisherbrand, Loughborough, UK). Filtered bone marrow was then layered onto Lymphoprep^{TM} (Axis-Shield, Dundee, UK) and centrifuged for 25 minutes. The mononuclear cell layer was then removed and washed with RPMI media. A cell count was conducted with a Scepter^{TM} cell counter (Merck Millipore, Billerica, MA, USA). Cells were then plated out into tissue culture flasks and 200,000 cells were used to perform a CFU-F count. MesenPRO RS media (Invitrogen) was used to feed and maintain all samples. The media was changed after 48 hours to remove any unattached cells.

B.2.1 Difficulties in Initial Cell Culturing

In four individuals the extracted cells failed to adhere in culture and so no expansion was possible. It was also not possible to obtain CFU-F results for these individuals (P005, P006, P013, and P019). Table 2.3 shows the details of the samples obtained. There was no obvious reason for this failure and there was no obvious differences between this group of individuals and those for whom samples were successfully cultured as shown in Table 2.4. Specifically, there did not seem to be a significant difference in the cell counts between those samples that successfully cultured and those that did not, indicating that low cell number was not a reason for failure to expand. There was a trend towards significance in the gender differences but this may have been due to the fact that there were only four individuals in the unsuccessful group.

Table 2.3 – Details of Samples Failing to Culture

Participant	Sample Type	Volume Extracted (ml)	Mononuclear Cells per ml (x10^6)
P005	Femur	6	2.960
P006	Femur	5	0.489
P013	Modified	3	1.003
P019	Modified	4	0.773

Table 2.4 – Comparison of Successful to Unsuccessful Initial Culturing

Factor	Successful	Unsuccessful	p value
Gender Ratio (M:F)	12:8	0:4	0.09
Modal ASA	2	2	0.49
Mean Age (*SD*)	65.67 (8.49)	67.11 (10.07)	0.80
Mean BMI (*SD*)	28.72 (4.11)	33.45 (5.26)	0.17
Mean cells per ml (*SD*) (x10^6)	1.316 (1.142)	0.990 (0.728)	0.57
Mean total cells (*SD*) (x10^6)	7.441 (8.818)	6.625 (7.590)	0.94

These individuals were excluded from the experimental analyses presented later.

B.2.2 Automated Cell Counter Accuracy

An automated cell counter (Millipore Scepter) was used to establish the number of cells obtained due to the greater accuracy when compared with a haemocytometer and the significantly reduced variability. In order to confirm this a cell count was performed three times on two different cell suspensions using each method. The haemocytometer produced a mean coefficient of variation of 44.06%, whilst for the Scepter it was 6.09%. The Scepter was therefore used for all study participants to ensure the reproducibility of cell counts between samples.

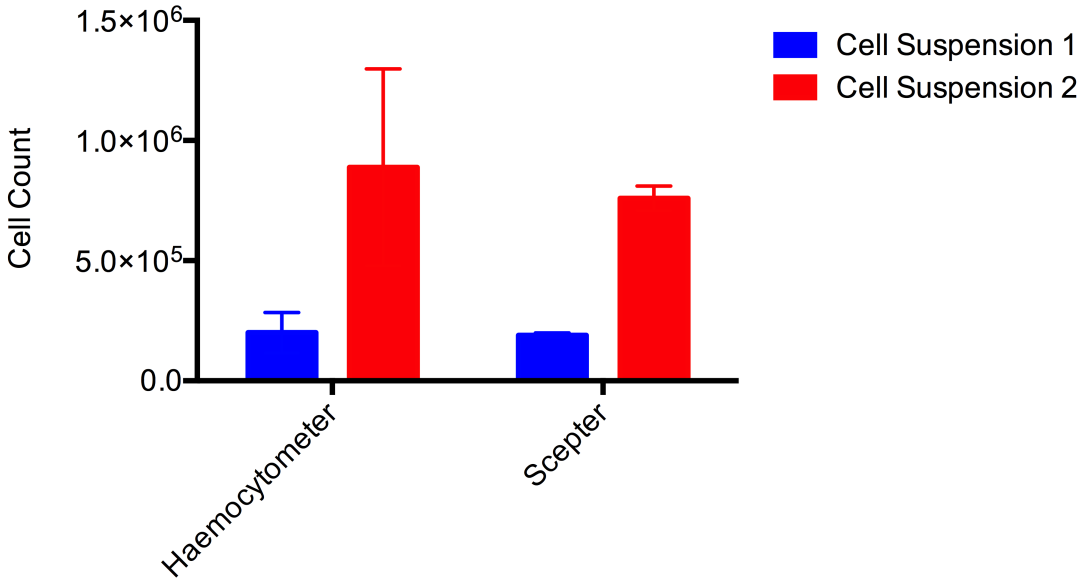

Figure 2.3 – Comparison of Haemocytometer and Scepter Cell Counter Accuracy

B.3 Colony Forming Unit - Fibroblast Count

In order to obtain an estimate of the number of MSCs in each bone marrow sample, a CFU-F count was undertaken on each sample. This is a well established method of estimating the number of MSCs in a sample[125, 171, 172]. Cells from each bone marrow sample were cultured in a six-well tissue culture plate well for 14 days at a density of 100,000 cells per well. They were then fixed with ethanol and stained with Giemsa stain (Merck Millipore, Billerica, MA, USA) to allow for colony visualisation. Colonies were then counted manually if they were at least one millimetre across and stained sufficiently to be visible to the naked eye. Each count was performed in duplicate to minimise sampling errors. Figure 2.4 shows a representative plate after staining and figure 2.5 shows a single CFU-F colony from the P009 femur sample[8].

[8] All microscope images were obtained using a Nikon Eclipse TE300 microscope unless otherwise stated.

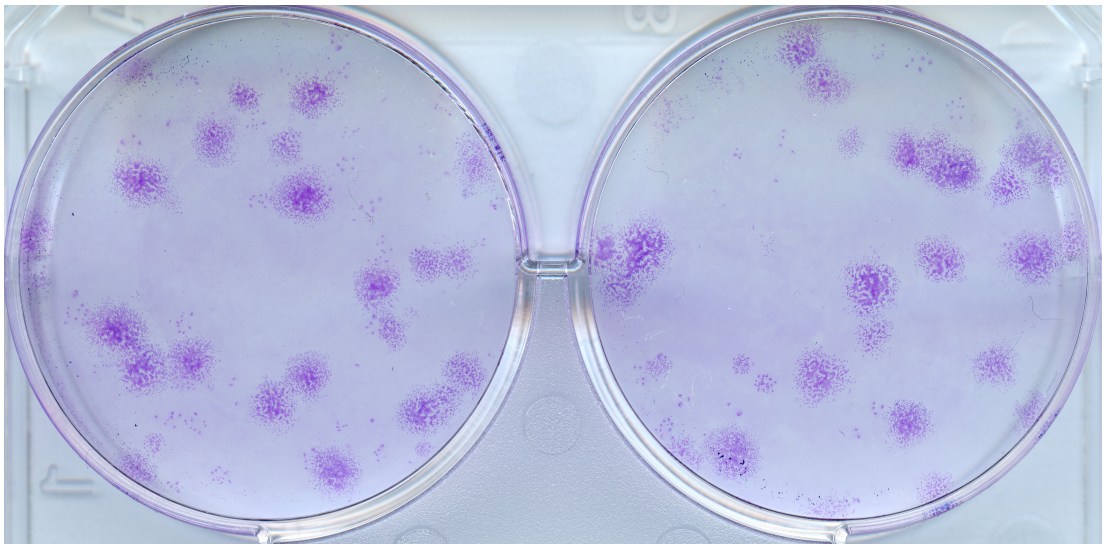

Figure 2.4 – P012 CFU-F Duplicate Wells

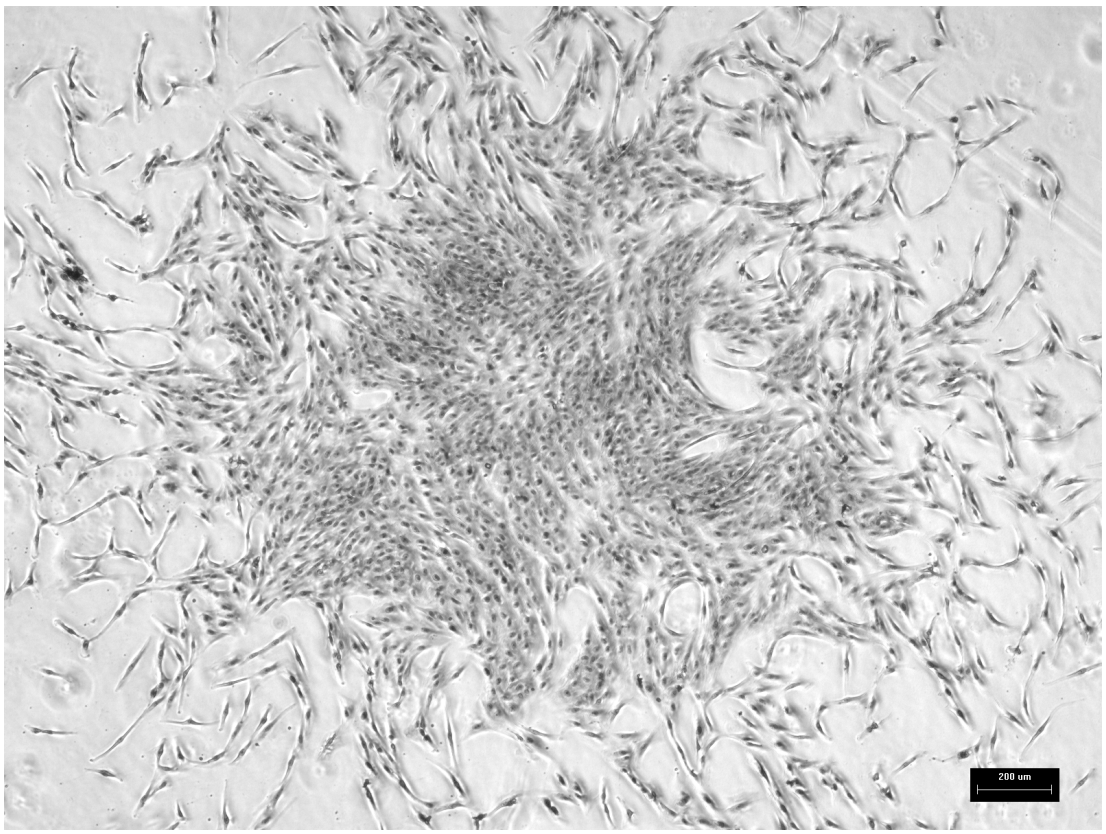

Figure 2.5 – Single CFU-F Colony (x40 magnification)

B.3.1 Validation of CFU-F Count

In order to establish the accuracy of the CFU-F counting a number of inter-and intra-observer comparisons were made. Intra-class correlations (ICC) are well established for assessing the degree of agreement between different observers of the same target[173] and were calculated to ensure that manual counting by one rater was equivalent to that of the other raters. Three independent raters were asked to count the same set of 60 CFU-F samples on two separate occasions. Two of the raters were doctoral students with experience of working with cells and tissue culture experiments. The third rater was a laboratory research assistant with experience in tissue culture and related experiments. The ICC was calculated using STATA for Mac 13 (StataCorp, College Station, Texas). Using a two-way random-effects model, and looking at absolute agreement, the individual observation ICC was 0.90 ($p<0.001$, 95% Confidence Interval: 0.85 - 0.94). An ICC above 0.75 is generally interpreted as indicating good reliability[174, p. 82].

Intra-observer reliability was assessed by determining the ICC for each of the three independent raters between their two sets of observations. The results are shown in Table 2.7.

This, again, indicates a good degree of intra-observer reliability. It was therefore assumed that CFU-F counts undertaken as part of the study were sufficiently robust to allow for comparison between different samples.

Table 2.5 – Comparison of CFU-Counts: Samples 1-30

	Rater 1		Rater 2		Rater 3	
Sample	Count 1	Count 2	Count 1	Count 2	Count 1	Count 2
1	20	16	20	16	10	14
2	19	21	18	23	9	20
3	0	0	0	0	0	2
4	0	0	0	0	1	4
5	3	2	3	3	1	1
6	4	4	5	3	1	3
7	11	12	14	13	10	9
8	13	12	15	15	10	11
9	12	12	12	11	10	12
10	5	6	4	5	5	5
11	3	3	3	3	2	3
12	8	7	8	8	5	7
13	4	4	7	5	2	5
14	3	4	5	3	2	3
15	3	3	4	3	2	2
16	1	3	3	2	1	1
17	0	0	0	0	0	1
18	0	0	1	0	0	1
19	24	26	29	28	25	21
20	21	22	25	27	21	23
21	60	47	81	65	38	52
22	51	39	74	49	35	54
23	4	4	4	4	4	4
24	3	3	3	3	3	3
25	0			0	1	0
26	2			2	1	1
27	0	0	0	0	0	0
28	0	0	0	0	0	0
29	0	0	0	0	0	0
30	0	0	0	0	0	0

Table 2.6 – Comparison of CFU-Counts: Samples 31-60

	Rater 1		Rater 2		Rater 3	
Sample	Count 1	Count 2	Count 1	Count 2	Count 1	Count 2
31	0	0	0	0	0	0
32	0	0	0	0	0	0
33	39	40	65	58	30	35
34	40	36	69	68	32	36
35	0	0	0	0	2	2
36	2	2	2	2	2	2
37	3	3	3	3	2	2
38	3	5	5	5	5	5
39	28	26	30	29	28	24
40	23	18	26	25	18	18
41	1	1	1	1	1	0
42	1	1	1	1	1	1
43	0	0	0	0	0	0
44	3	3	3	3	3	4
45	2			2	1	1
46	2			2	2	1
47	0			0	0	0
48	0			0	0	0
49	30	30	35	31	18	27
50	25	23	33	28	18	25
51	28	30	35	36	24	26
52	36	33	44	46	30	31
53	33	33	39	38	25	29
54	31	31	36	37	28	31
55	35	28	56	48	31	31
56	32	33	57	57	30	34
57	2	3	4	3	3	3
58	8	7	8	9	8	6
59	3	3	3	3	3	3
60	7	6	7	7	7	7

Table 2.7 – Intra-Observer ICC Values for CFU-F Counts

Rater	ICC	95% Confidence Interval
1	0.98	0.97 - 0.99
2	0.98	0.96 - 0.99
3	0.96	0.93 - 0.97

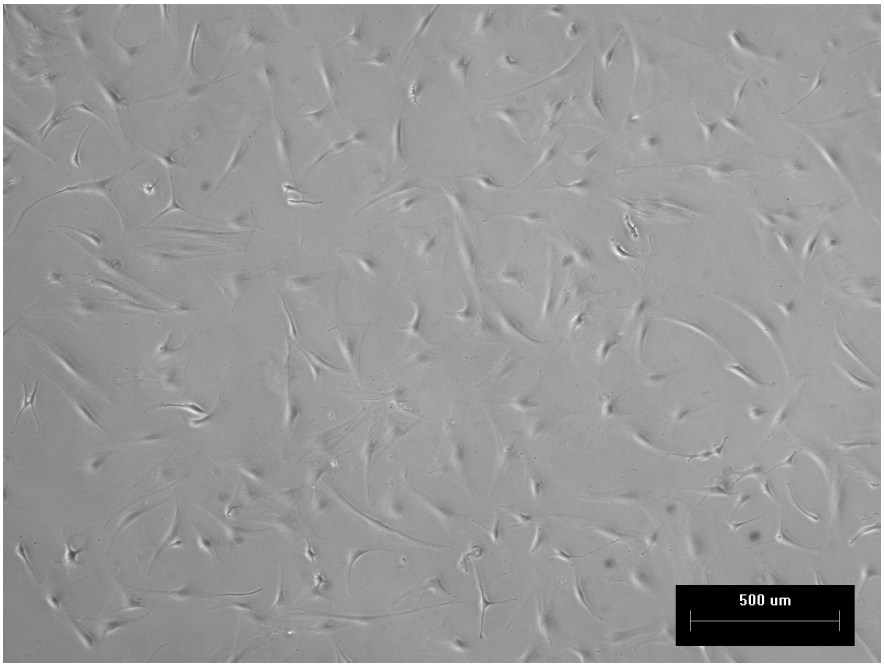

Figure 2.6 – MSCs in Culture (x40 magnification)

B.4 Cell Passaging

Cells were passaged when they reached 70% to 90% confluency. At each passage a cell count was performed to enable calculation of doubling time and number. Cells were re-seeded at a density of 3,000 to 5,000 cells per cm^2 in keeping with the manufacturer's protocol for MesenPRO RS media. Figure 2.6 shows a x40 brightfield microscope image of a representative sample in culture towards the end of passage 3 (P031 Modified Technique Sample). This is typical of the appearance of the cultured cells during expansion and is in keeping with the appearance that would be expected of MSCs in culture.

B.4.1 Population Growth Assessment

Population growth was assessed by determining the number of population doublings for each sample. This was conducted using the formula below, where N

= cell number at the end of passage three and N_0 = cell number at the end of passage zero:

$$\text{Number of Population Doublings} = \frac{log\frac{N}{N_0}}{log2}$$

As not all passages were of the same length, the number of population doublings was divided by the total time from the end of P_0 to the end of P_3. This gave a number of population doublings per day in culture for each sample. In cases where not all cells had been expanded between passages, for example if some were frozen, the cell count was adjusted to take this into account.

B.4.2 Difficulties in Cell Expansion

In five participants (P002, P004, P014, P017, and P025) cells failed to expand in culture despite having been successfully extracted. They were therefore not included in any experiments. These five participants represent a total of 11 samples. P014 was excluded from further study as although the pelvic sample expanded successfully, both the femoral and tibial samples failed to expand sufficiently for further analysis.

The data regarding these individuals is shown in Table 2.8. Reviewing the information regarding these individuals, there did not seem to be any reason for failure such as medication usage or significant co-morbidities. There was no significant difference in demographic factors between the two groups. Whilst it has been mentioned previously that diclofenac has been shown to interfere with MSC

differentiation[121] this drug was not used by any of the study participants. In fact, only four study participants were on any form of NSAID and these participants' samples were all collected and expanded successfully, indicating that NSAID use may not be a problem when trying to obtain MSCs from bone marrow.

Table 2.8 – Comparison of Expansion vs. Failure of Expansion

Factor	Successful Expansion	Expansion Failure	p value
Gender Ratio (M:F)	12:8	4:1	0.62
Modal ASA	2	2	0.68
Mean Age (*SD*)	65.67 (8.49)	51.43 (25.94)	0.29
Mean BMI (*SD*)	28.72 (4.11)	31.03 (6.14)	0.46

In total, across all participants, there were a total of 51 samples from which extracted cells were placed in tissue culture flasks for expansion. Whilst there were no losses due to infection only 40 samples were successfully expanded to the end of P3. Excluded samples typically had either low initial cell counts or had expanded very slowly. In order to allow for all further experiments it was necessary to generate approximately 1.5 million cells by the end of P3. The samples that failed to reach this level could not be used for further experiments.

Samples were not passaged repeatedly until sufficient cells had been obtained. This was because of the evidence that extended culture of stem cells can lead to changes in their differentiation potential and also lead to reduction in proliferative ability[132, 175]. Although there is some more recent evidence from tendon-derived stem cells that proliferative ability may increase with passaging[176] this has not been demonstrated conclusively and so extended culture cells were excluded to avoid this potential source of variation.

The effect of extended culture on cells was demonstrated in the pelvic samples from P004 and P008. These were extended to P5, and as can be seen in Figure 2.7,

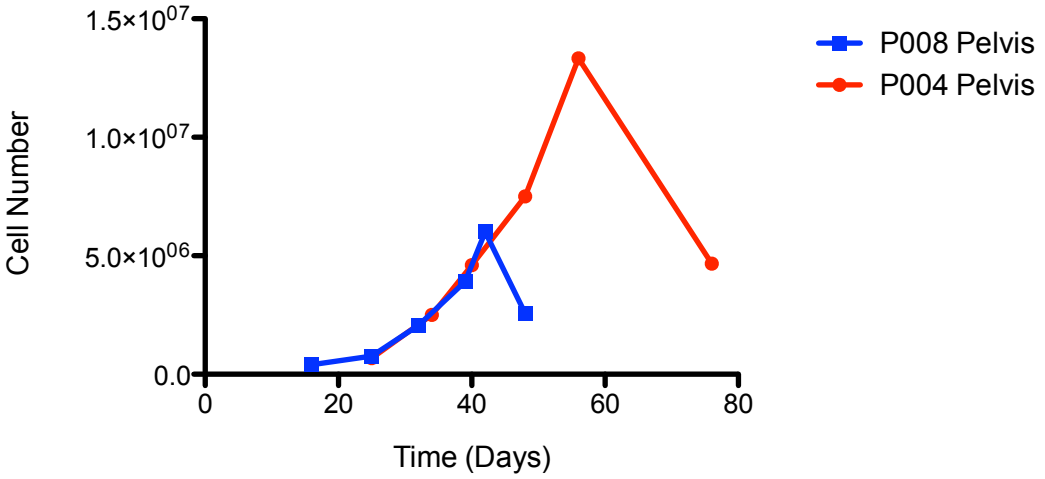

Figure 2.7 – Effect of Extended Culture on Cell Numbers

there was a marked drop off in cell numbers with increased time. These samples were not used for other experiments as their respective femoral and tibial samples had not expanded sufficiently.

B.5 Osteogenic and Chondrogenic Differentiation of MSCs

In order to ensure there were sufficient cell numbers to conduct all experiments, all samples were cultured until the end of passage 3. This provided enough cells for flow cytometry assessment (Section 2.C.2) and differentiation experiments in the majority of samples. Cells were plated into tissue culture plates at the following seeding densities to conduct the differentiation experiments.

- 50,000 cells/6 well plate for control and osteogenic differentiation samples for qPCR;

- 200,000 cells/24 well plate for chondrogenic differentiation samples for qPCR;

- 3,000 cells/96 well plate for control and osteogenic differentiation samples for alizarin red staining;

- 30,000 cells/96 well plate for control and chondrogenic differentiation samples for histological assessment.

Cells were fed with MesenPRO RS media and left for 48 hours to attach. The media was then changed to differentiation media which was replaced every 48 to 72 hours. Differentiation was carried out in duplicate for each sample and with a control that was maintained in MesenPRO RS media. After 14 days those cells undergoing osteogenic differentiation for alizarin red staining were fixed. This was determined to be the optimum time to fix cells for staining as discussed in Section 2.C.3.1. All other cells were continued until day 21 when they were fixed as required for further experiments. Day 21 was chosen as the recommended time for differentiation using the differentiation kits obtained from Lonza[177]. Day 21 has also been used in a number of studies looking at obtaining MSCs from human subjects[146, 178].

Osteogenic differentiation was conducted using the Lonza hMSC Osteogenic BulletKit. This consists of a basal media to which the following components are added: dexamethasone, L-glutamine, ascorbate, penicillin/streptomycin, mesenchymal cell growth supplement, and β-glycerophosphate. This complete media is ready to use and requires no further additions.

Chondrogenic differentiation used the Lonza hMSC Chondrogenic BulletKit. This consists of a basal media with the following additions: dexamethasone, ascorbate, ITS+ (insulin, transferrin, selenium, and linoleic acid), GA-1000 (gentamicin and amphotericin), sodium pyruvate, proline, L-glutamine. This forms an incomplete

media which must be completed immediately prior to use by the addition of 10 ng/ml of TGF-β3.

All wells were checked at each change of media to ensure there was no evidence of infection.

Following expansion and differentiation samples were assessed for phenotype, viability, and differentiation capability as described in the following section.

C Cell Phenotype, Viability, and Differentiation Assessment

In order to determine the viability, phenotype, and differentiation ability of the MSCs cultured from each bone marrow sample the cells were subjected to a number of different experiments during the expansion and differentiation process. These experiments were carried out at specific time points in each sample's expansion and differentiation as detailed below:

- End of P0 - Cell viability assessment;
- End of P3 - Flow cytometry assessment of cell phenotype;
- Day 14 of differentiation - Alizarin red assessment of osteogenic differentiation;
- Day 21 of differentiation - Alcian blue assessment of chondrogenic differentiation;
- Day 21 of differentiation - qPCR analysis of osteogenic and chondrogenic differentiation.

C.1 Cell Viability Assessment

Cell viability assessment was undertaken in order to establish if MSCs from one sample would be better able to survive and proliferate compared with others. Viability was assessed using the PrestoBlue® Cell Viability Reagent (Life Technologies, UK). This test uses a resazurin-based assay to establish the relative viability

of live cells. Resazurin is able to enter cells where it is converted to resorufin. This conversion is accompanied by a colour change from blue to a fluorescent red. Cells which are still viable will carry out this process continuously leading to increasing levels of red fluorescence. PrestoBlue has the advantage of using a shorter incubation time than the longer established alamarBlue® assay. As resazurin-based assays do not damage the cells, it is possible to carry out assays at multiple time points on the same sample.

At the end of passage zero 1 x 10^4 cells were placed in a 12 well plate in biological duplicates and maintained with MesenPRO RS media as outlined above. At regular intervals (typically days 1, 7, and 14) a viability assay was carried out. PrestoBlue was added to each well at a concentration of 10% and incubated at 37°C after which the PrestoBlue was harvested and replaced with fresh media. PrestoBlue assays can be incubated for between 30 minutes and two hours. In order to ensure comparability of samples all assays were carried out with the same incubation period of one hour. Fluorescence was measured on a FLUOstar OPTIMA (BMG Labtech, Aylesbury, UK) with triplicate technical repeats. The mean relative fluorescence for each sample was calculated by establishing the mean fluorescence for each sample and subtracting the fluorescence of a no cell control which was incubated for the same period of time. The relative fluorescence was then plotted against time for each sample.

C.2 Flow Cytometry Analysis of Cell Population

At the end of passage three an aliquot of cells was taken to determine the phenotype of the cell population. In order to determine the percentage of cells that could be phenotypically described as MSCs a panel of antigens/cell surface mark-

ers was determined. Antibodies were selected by examining the literature to determine a suitable panel. Whilst the ISCT position paper[54] lists ten antigens as phenotypic of MSCs, a panel this size would be impractical due to the number of cells required for each experiment and the problems created by spectral overlap of different dyes. Five antibodies were therefore chosen as a core panel based on published studies[179, 180, 181]. CD271 had previously been proposed as a single antibody to identify MSCs[55, 56] and so was also assessed for use in the flow cytometry experiment as a single antibody marker.

Table 2.9 – Antibodies Used in Flow Cytometry Experiments

Antibody	Fluorophore	Volume Used Per Experiment (μl)	Supplier
CD34	PE/Cy5	5	Beckman Coulter
CD45	PE/Cy7	10	Biolegend
CD73	PE	15	Biolegend
CD90	APC/Cy7 (Biotin) & APC	5	eBioscience
CD105	FITC	15	Biolegend
CD271	APC	5	Biolegend

For those antibodies that had not previously been used in the laboratory a titration experiment was carried out in order to determine the amount of antibody to use for the optimum signal to noise ratio (SNR). Each antibody was used to stain BMMCs at four different concentrations. The results obtained from the flow cytometer were gated[9] in order to identify the positive and negative populations. The mean fluorescent intensity (MFI) of each population was then determined and the SNR established by dividing the MFI of the positive population by that of the negative population. The graph below (Figure 2.8) shows the plots of the SNRs for those antibodies not previously used in the laboratory (CD45, CD73,

[9]Gating is the procedure by which a population of cells is selected within the flow cytometry software based on some of its characteristics, such as forward scatter, side scatter, or antigen positivity. Multiple gates can be combined to identify cells which possess a number of different characteristics.

C105, and CD271). The volume of antibody used which had achieved the highest SNR was then used for all further experiments and is shown in Table 2.9.

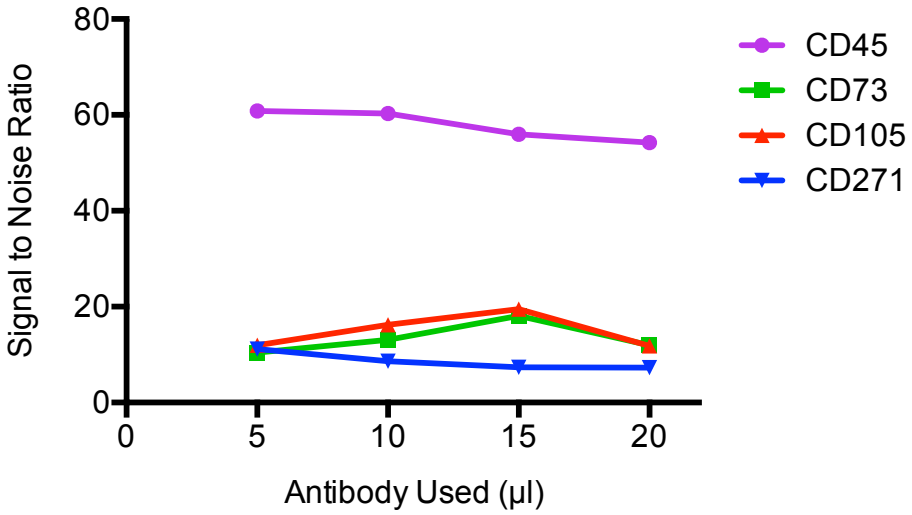

Figure 2.8 – Signal to Noise Ratio for New Flow Cytometry Antibodies

Flow cytometry samples were washed with flow cytometry buffer (see Appendix F) and resuspended at a concentration of one million cells/ml. Aliquots were then prepared as below for each experiment. For experiments where multiple samples were being analysed a mixture of cells from each sample was used as indicated by (mixed):

- One unstained sample (mixed);
- One single stain sample for each antibody (mixed);
- One test sample labelled with CD34, CD45, and CD73;
- One test sample labelled with CD90 and CD105;
- One test sample labelled with all antibodies.

Cells and antibodies were incubated on ice for 30 minutes. After this, they were washed to remove excess antibody. Immediately prior to analysis on the flow cytometer, hoescht dye (Life Technologies, UK) was added to act as a live/dead stain to each test sample (1 µl of 1:100 concentration dye). Samples were then run on a BD LSRFortessa flow cytometer. For BMMCs, fluorescence minus one[10] (FMO) samples were also used to assist with compensation for spectral overlap due to the rare nature of the target population. FMO samples allow for the easy identification of the spectral overlap from other antibodies into the antibody in question so that this can be adjusted for prior to analysis. BD FACSDiva™ software (Version 7.0, BD Biosciences, Oxford, UK) was used to collect data and perform automated compensation. Data was analysed on FlowJo 10.0 (TreeStar Inc., Ashland, OR, USA).

In order to help reduce the number of cells required for flow cytometry analysis, and to allow for easier compensation when dealing with a potentially homogenous population of cultured MSCs from bone marrow samples, compensation beads (BD™ CompBeads) were used for flow cytometry analysis of study participants. The detailed protocol used for flow cytometry is contained in Appendix F on page 207. Samples were analysed to determine the percentage of cells which expressed each antigen of interest.

C.2.1 CD271 Assessment

To assess the suitability of CD271 as a single marker for MSCs, a selection of samples (bone marrow, cells cultured from bone marrow, and commercially sourced MSCs) were tested using all six antibodies. Gating was carried out manu-

[10]For each antibody a sample is run containing all antibodies except the antibody concerned to allow spectral overlaps to be further delineated.

ally. Comparison of the panel of five antibodies (CD34, CD45, CD73, CD90, and CD105) compared to CD271 alone demonstrated that CD271 was unable to accurately identify the same population as that identified by the panel (Table 2.10). It was therefore decided to only use the core panel of five antibodies for analysis of further samples. Assessing the antibodies against commercially sourced MSCs (Lonza, UK) meant that the panel could be validated prior to use on human bone marrow samples. Figure 2.9 shows the flow cytometry results for the commercial cells that were successfully identified by the antibodies chosen. A sequential gating strategy was used for these results meaning that each graph contains only the gated results from the immediately preceding graph. This showed that the panel was able to accurately identify the commercially sourced MSCs.

Table 2.10 – CD271 Antibody Assessment

Sample	Percentage Identified as MSCs by Panel	Percentage CD271 Positive
BMMC	0.02	1.78
Cultured Bone Marrow	39.08	5.70
Commercial MSCs	99.51	45.44

C.2.2 Magnetic Bead Separation of Samples

In parallel to the assessment of CD271 as a single marker for MSCs the use of a magnetic bead system to enrich samples for CD271, was also investigated. Magnetic bead separation works by first binding a target antigen with an antibody. A magnetic microbead is then attached to this antibody. Passing the resultant cell suspension through a separation column housed in a very strong magnet results in the unlabelled cells passing through whilst those that have been labeled with the antibody of interest are retained. The column can then be removed from the magnetic housing and the labelled cells washed out.

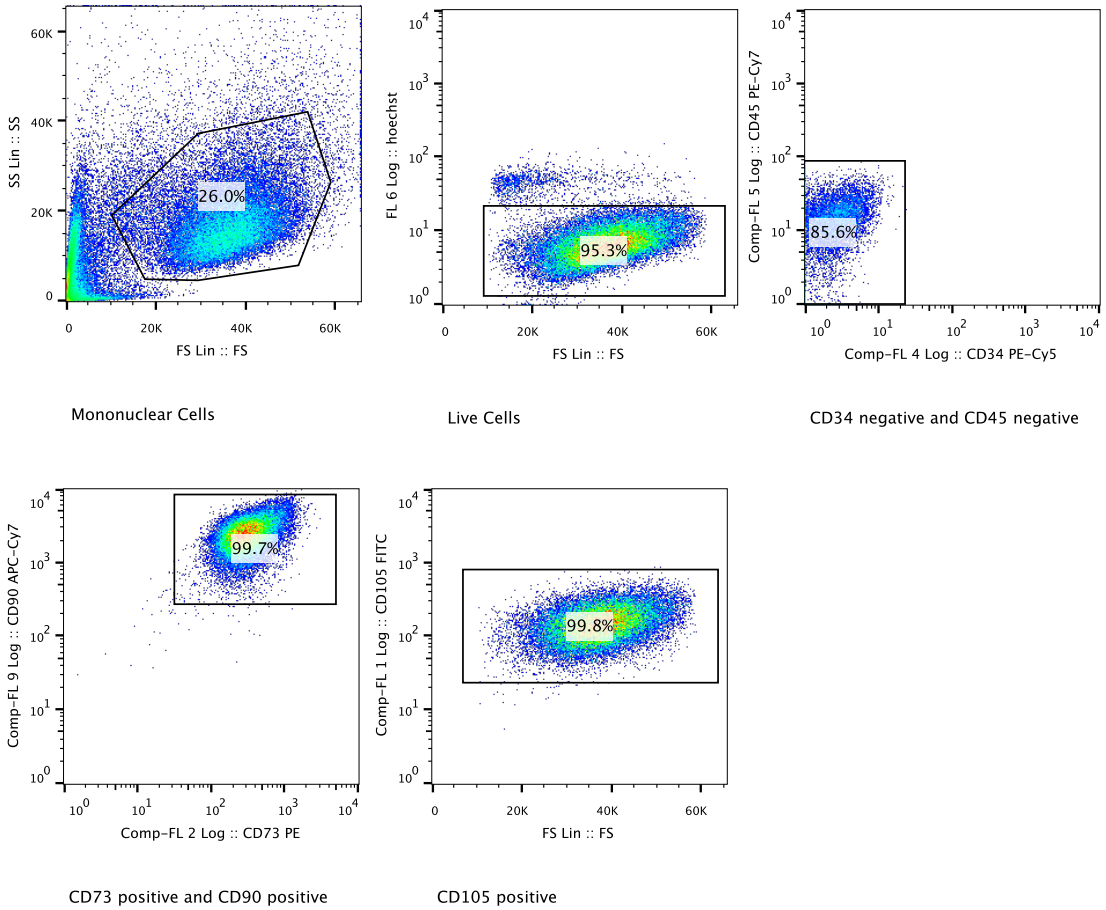

Figure 2.9 – Flow Cytometry Result for Commercial MSCs

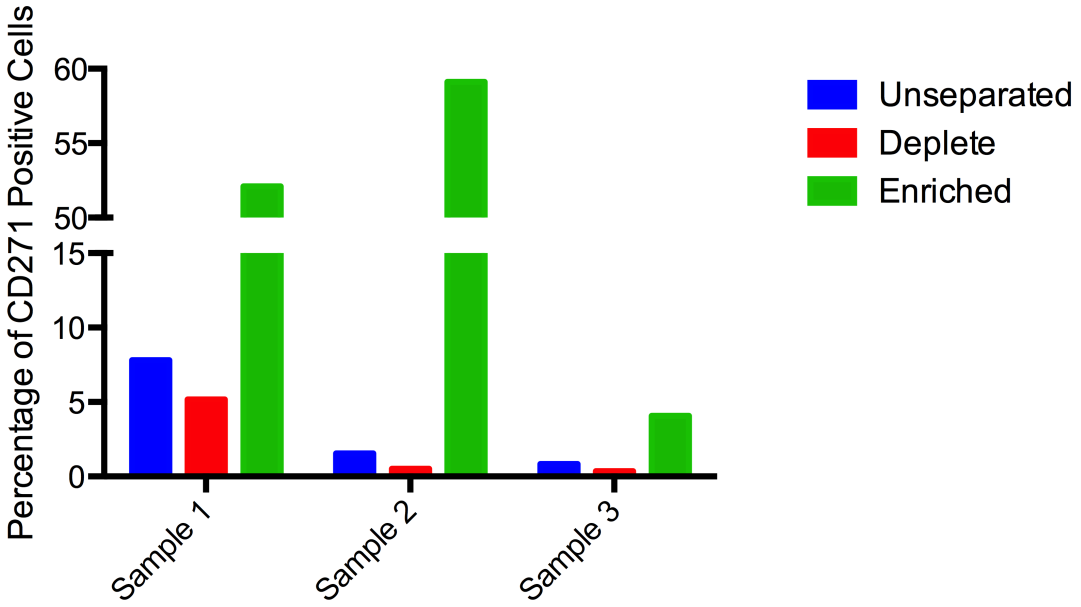

Figure 2.10 – Magnetic Bead Enrichment for CD271

The MACS® system from Miltenyi Biotec was used to enrich the amount of CD271 positive cells in the mononuclear cellular fraction. In a number of preliminary runs using bone marrow obtained from the femur of individuals undergoing either total hip or knee arthroplasty, the enrichment process was successful in increasing the percentage of CD271 positive cells in each sample. However, as CD271 was not able to reliably identify the same population as the five antibody panel, this process was not used for further experiments (Figure 2.10). Magnetic bead enrichment was not used to enrich or deplete for any of the other antibodies as it was felt that, given that four antibodies would still be un-enriched, little would be gained in terms of later flow cytometry analysis and the extra steps may lead to increased cell death in an already rare population.

C.3 Alizarin Red Quantification of Osteogenic Differentiation

Alizarin red staining is well established as a technique to assess calcium deposition in cell culture systems[182]. It has been used to qualitatively assess osteogenic differentiation in a number of studies[150, 183, 184, 185, 186] and by releasing the stain from deposited calcium it can also be used for semi-quantitative assessments[176]. Quantification was carried out by seeding 3,000 cells into 96 well plates in duplicate. A control was included for each sample. Cells were fixed at day 14 with cold ethanol. The functional activity of alizarin red stain is pH dependent. The pH of the stain was therefore checked prior to each use to ensure it was within the optimum working range of 4.1 - 4.3 using a SevenEasy pH meter (Mettler Toledo, OH, US). pH was adjusted as required with hydrochloric acid. Adequate functioning of the stain was confirmed by staining cultured osteoblast cells to ensure staining of the deposited calcium. After removing the excess stain and washing with PBS the samples were allowed to dry at room temperature.

Stained samples were then imaged and quantification undertaken. Alizarin red staining was released by adding 500 µl of acetic acid and agitating the samples on an orbital shaker until all stain had been released. This was then quantified by measuring absorbance at 405 nm on a SpectraMax® Plus^{384} absorbance microplate reader (Molecular Devices, Wokingham, UK). Each sample was measured in triplicate technical repeats. Differentiated samples were plotted relative to controls.

C.3.1 Optimisation of Alizarin Red Staining Protocol

In order to ascertain the optimum number of cells that should be seeded in each 96 well plate and the amount of time for which differentiation should be undertaken, cultured MSCs were seeded into 96 well plates in triplicate at densities of 1,000, 2,000, and 3,000 cells per well and differentiated for 7, 14, and 21 days. Alizarin red staining was then assessed as outlined above. Mean absorbances were then plotted and it was determined that the optimum combination of density and time would be 3,000 cells for 14 days to ensure that enough calcium deposition could occur to enable a result to be obtained (Figure 2.11).

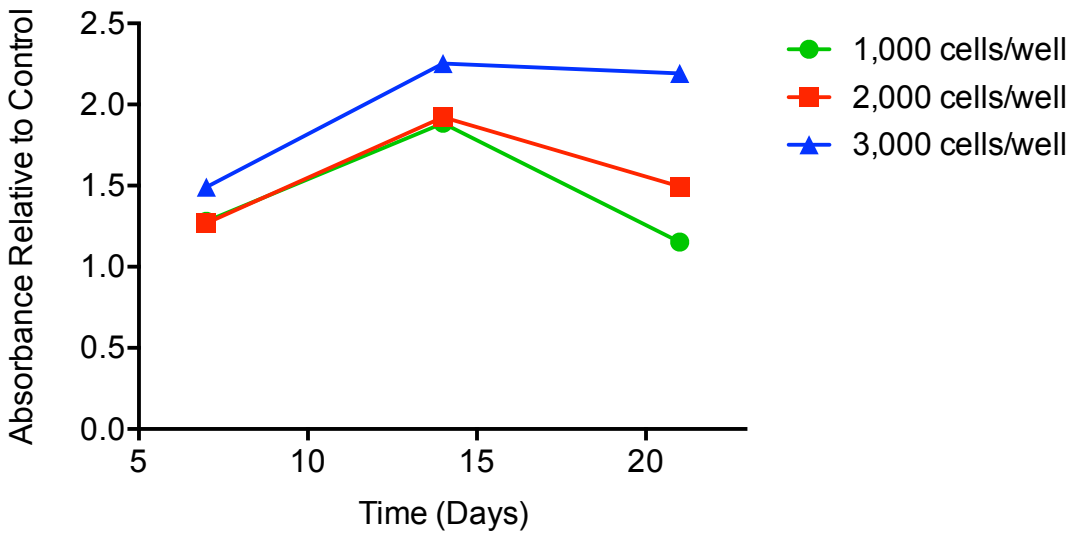

Figure 2.11 – Alizarin Red Quantification at Different Seeding Densities and Time Points

C.4 Alcian Blue Assessment of Chondrogenic Differentiation

Alcian blue staining was used to assess chondrogenic differentiation. The dye stains glycosaminoglycans found in cartilage and so is frequently used to stain chondrogenically differentiated cells[155, 187, 188]. Samples were chondrogenically differentiated in 96 well plates in duplicate with a control maintained in MesenPRO RS media. After 21 days they were fixed with 10% formalin for two to three hours, washed with PBS, and then stored at 4°C. To stain the cells the PBS was removed and alcian blue added. The cells were incubated at 4°C overnight and the dye was then removed. Images were then taken of the samples. The tendency of samples that had undergone chondrogenic differentiation to undergo spontaneous micromass formation meant that it was not possible to obtain detailed microscope images of the differentiated cells.

C.5 RNA Extraction and cDNA Synthesis from Differentiated Cells

In order to facilitate later PCR based experiments it was necessary to extract RNA from differentiated samples and convert these samples to cDNA. A number of different methods of RNA extraction were trialled to determine the most efficient method for use with the samples that had undergone differentiation for 21 days. After obtaining RNA using the different methods outlined below, the yield and quality of the RNA was assessed using a NanoDrop 1000 Spectrophotometer (Thermo Scientific, MA, USA). This provided information on both the yield of RNA in the sample (in ng/µl) and the quality of the RNA. RNA quality was assessed by the 260/280 ratio as advised by the MIQE guidelines (The Minimum Information for Publication of Quantitative Real-Time PCR Experiments)[189]. This compares the absorbance at 260 nm and 280 nm. A "pure" RNA sample will give a ratio of approximately 2.0. Contamination with proteins, phenol, or DNA will lead to a lowering of this ratio. In order to be acceptable for use with clinical study samples it was necessary to ensure the 260/280 ratio was reliably in the range of 1.8 - 2.2.

Initially, at the end of differentiation, cells were fixed with RNA*later* (Qiagen, Hilden, Germany) and stored at -20°C. The cells were then thawed and RNA was extracted using the Qiagen RNeasy Mini kit as per the manufacturer's instructions. The RNeasy Mini kit is a column based system in which RNA is bound to a filter in the column whilst other material is washed off. It is then eluted in pure water which frees it from the filter. Analysis of the RNA obtained using the RNeasy kit showed that the yield was extremely low and the quality, as assessed by the 280/260 ratio, was highly variable. This was possibly due to the presence of a large amount of extra-cellular matrix which interfered with the RNeasy system.

Table 2.11 – Representative RNA Yield using RNeasy Mini Kit

Sample	RNA (ng/μl)	260/280 Ratio
1	0	0.24
2	0	0
3	2.4	12.52
4	5.21	1.82
5	0.44	-0.98
6	0.2	0.36

C.5.1 TRIzol/Chloroform RNA Extraction Protocol

Following these poor results the method of extraction was changed to a TRIzol® (Life Technologies, UK) method as outlined below. This extraction method is based on the ability of TRIzol to stabilise RNA whilst homogenising other tissue[190].

1. At the end of differentiation, fix cells with 1 ml of TRIzol and stored at -80°C.

2. Thaw cells and add 200 μl of chloroform, vortex for 15 seconds.

3. Centrifuge at 12,000 rpm for 15 minutes at 4°C.

4. Transfer upper aqueous phase to new tube.

5. Add an equal volume of isopropranolol and incubate at room temperature for 10 minutes.

6. Centrifuge at 12,000 rpm for 10 minutes at 4°C.

7. Remove supernatant and wash RNA pellet with 1 ml of 70% ethanol.

8. Re-suspend RNA in 30 µl of RNase-free water.

This method of extraction led to an improvement in the quality of the RNA obtained as well as the yield, but the results were still sub-optimal with a mean 280/260 ratio of below 1.8.

Table 2.12 – Representative RNA Yield using TRIzol/Chloroform RNA Extraction

Sample	RNA (ng/µl)	260/280 Ratio
1	994.25	1.7
2	508.35	1.53
3	623.01	1.41
4	619.10	1.58
5	678.41	1.53
6	285.86	1.69

C.5.2 TRIzol and Direct-zol Spin Column RNA Extraction

In order to further improve the quality of the RNA obtained a hybrid RNA extraction method was trialled. The Direct-zol^{TM} RNA MiniPrep system (Zymo Research, CA, USA) uses a spin column system to obtain RNA from cells already fixed in TRIzol . The samples were processed as per the manufacturer's instructions including an on-column DNase digestion step to aid in the reduction of DNA contamination of samples. RNA was eluted into 25 µl of water. This system resulted in a much improved quality of RNA which was consistently within the range of 1.8 to 2.2.

Table 2.13 – Representative RNA Yield using TRIzol and Direct-zol Spin Column

Sample	RNA (ng/µl)	260/280 Ratio
1	156.02	1.81
2	97.47	1.82
3	238.89	2.04
4	163.63	2.05
5	39.31	2.06
6	51.36	2.04

The figure below shows the comparison of the ratios obtained from the three different methods. The red lines represent the acceptable range of 260/280 ratio of 1.8 to 2.2.

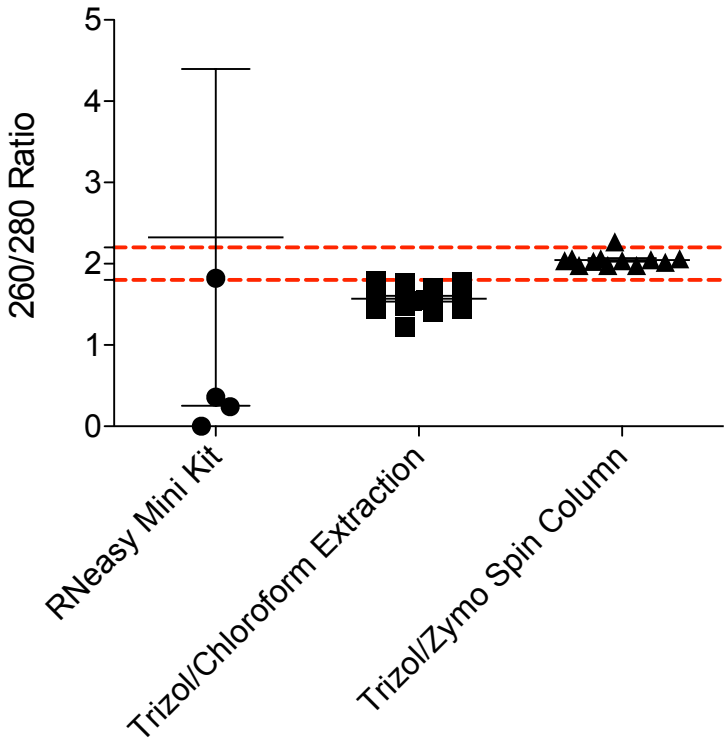

Figure 2.12 – Comparison of Different RNA Extraction Methods

The coefficients of variation for the three different methods were 218.1%, 9.4%,

and 3.8% respectively. It was therefore decided to use the Direct-zol extraction method for all study participant samples. Once extracted, the RNA was stored at -80°C until required for cDNA synthesis.

C.5.3 cDNA Synthesis

Having successfully obtained RNA from the differentiated samples, these were then converted into cDNA using a High Capacity RNA-to-cDNA^{TM} Kit (Life Technologies) according to the manufacturer's instructions. This kit uses up to 9 µl of RNA and so for all samples 1000 ng of RNA was converted to cDNA, or as much as could be obtained from 9 µl of RNA if this was a smaller amount. cDNA was then stored at -20°C until required for qPCR. In some cases, despite the successful extraction of RNA, the relatively low concentration obtained meant that it was not possible to carry out any PCR analysis on the sample. This was due to a combination of facts: creating the cDNA effectively diluted the sample by 2.22 times due to the 9 µl starting volume of RNA and 20 µl reaction volume; and the need for at least 180 ng of cDNA in total to allow for all the qPCR reactions required.

C.6 Quantitative PCR

Quantitative PCR (qPCR)[11] is a technique that is extremely popular for determining the relative expression of genes from cells. The use of specific gene primer sequences in combination with fluorescent reporter probes allows for the detection of different amounts of gene cDNA. In order to determine the relative levels

[11] Also known as real-time PCR (rt-PCR)

of gene expression in samples that have been treated differently, it is necessary to compare the expression of genes of interest to the expression of genes that are expressed at a constant level in different cells (endogenous control genes). Having done this, it is possible to show whether genes involved in specific processes, such as osteogenic and chondrogenic differentiation, have been up-regulated. All qPCR experiments were undertaken using TaqMan reagents and primers according to the manufacturer's protocols (Tables 2.14 and 2.15)[191]. Experiments were carried out on a ViiA 7 Real-Time PCR System (Applied Biosystems). Data was analysed using the ViiA 7 Software v1.2.2 and Expression Suite Software v1.0.3 (both Life Technologies).

Table 2.14 – qPCR Reaction Set Up

	96 Well Reaction (20 µl)	384 Well Reaction (10 µl)
TaqMan Fast Advanced Master Mix	10.0	5.0
TaqMan Gene Expression Assay	1.0	0.5
Nuclease-free Water	7.0	2.5
cDNA	2.0	2.0

Table 2.15 – Thermal-Cycling Conditions for qPCR Reactions

	UNG incubation	Polymerase activation	40 cycles of PCR	
	Hold	Hold	Denature	Anneal/ Extend
Time (m:ss)	2:00	0:20	0:01	0:20

For each experiment cDNA was diluted to provide 4 ng per reaction volume. Samples were initially mixed into 96-well plates and then aliquoted into a 384-well plate to create quadruplicate technical replicates. In each experiment a no template control was run for each gene to ensure that there had not been any contamination of either the master mix, expression assay, or water. Where pos-

sible, samples were also run as duplicate biological replicates, provided sufficient cDNA had been created from each sample. The analysis software was able to combine both the technical and biological replicates to determine average gene expression for the sample. The control used in each case was a sample from the same anatomical location that had been maintained in MesenPRO RS media. In cases where it had not been possible to obtain enough cDNA from the relevant anatomical location control, a control was used from a different anatomical location of the same individual.

Results are presented as the relative expression of the gene of interest to the anatomical location control.

C.7 Gene Selection

In order to assess the extent of chondrogenic and osteogenic differentiation, genes were selected that are expressed both early and late in each pathway.

C.7.1 Osteogenic Differentiation Genes

Osteogenic differentiation involves the up-regulation of a vast number of genes[192]. To monitor early signs of differentiation *RUNX2* was selected as it has previously been widely used to monitor osteogenic differentiation[193, 194]. *RUNX2* is also expressed in the hypertrophic stages of chondrogenesis and so would also provide an indication of chondrogenic hypertrophy in those cells undergoing chondrogenic differentiation[195]. Initially, *BGLAP* (osteocalcin) was selected for detection of later osteogenic differentiation[135, 196, 197]. However, whilst trying to optim-

ise the qPCR experiments it was not possible to obtain reliable results from *BGLAP*. Therefore *BGLAP* was exchanged for *SPP1* (osteopontin) which is also a late marker of osteogenic differentiation[196, 198, 199].

C.7.2 Chondrogenic Differentiation Genes

Early chondrogenic differentiation was initially monitored with *COL2A1* (collagen 2A1)[200, 201, 202]. Measurement of *COL2A1* gene expression proved extremely difficult in early optimisation experiments. It was therefore decided to switch to *SOX-9* which, whilst also a marker of early chondrogenic differentiation, is expressed at an even earlier stage than *COL2A1*[75, 202, 203]. Later chondrogenic differentiation was measured by *ACAN* (aggrecan)[155, 166, 176].

C.7.3 Endogenous Control Gene Selection

The MIQE guidelines[189] specify that a minimum of two endogenous control genes should be used for the majority of qPCR experiments. Previous work has shown that selecting an appropriate endogenous control for qPCR work involving differentiated MSCs can be difficult and prone to error[178, 204]. Therefore, cDNA samples of control, osteogenically differentiated, and chondrogenically differentiated cells from two different individuals were selected to run against a panel of potential controls. The TaqMan Array Human Endogenous Control plate (Life Technologies) containing 32 different candidate reference genes was used. Experiments were run using 2 ng of cDNA and according to the manufacturer's protocol. Genes were assessed on the basis of both their stability of expression across different samples, and the point at which they were expressed. ExpressionSuite

software[205] assigned a score to each candidate control on the basis of the stability of its expression across multiple samples with a lower score representing a more stable gene. Endogenous controls were also selected based on their relative abundance as demonstrated by a low C_T value. Candidates with mean C_T values above 30 were not considered as potential controls due to the possible issues with low expression in experiments.

On the basis of this experiment *GAPDH* and *PGK1* were chosen as the optimum genes for use as endogenous controls.

The details of all the primers used in qPCR experiments can be found in Table C.1 on page 213.

D Statistical Analysis

Statistical analysis of all results was carried out using Prism 6 (GraphPad Software Inc., La Jolla, CA, USA) unless otherwise stated[206]. Statistical analysis which could not be undertaken in Prism 6, such as the imputation of missing values and point biserial correlations, was carried out using STATA 13[207].

D'Agostino-Pearson omnibus normality tests were carried out on all results. Normally distributed results were analysed using parametric tests (t tests and ANOVA tests). Non-normally distributed results were analysed with the appropriate non-parametric test (Mann-Whitney U tests, Wilcoxon matched-pairs signed rank tests, and Friedman tests)[208]. The significance level was set at 0.05 for all tests.

Table 2.16 – Endogenous Control Gene Selection

Gene	Stability Score	Mean C_T Value
18S	1.384	11.663
ABL1	0.787	28.098
ACTB	1.295	22.923
B2M	1.189	23.118
CASC3	0.867	28.610
CDKN1A	0.923	25.313
CDKN1B	0.730	29.962
EIF2B1	0.693	29.399
ELF1	0.805	29.083
GADD45A	0.976	29.756
GAPDH	0.810	22.031
GUSB	1.645	30.727
HMBS	0.891	30.790
HPRT1	0.792	29.431
IPO8	0.693	29.976
MRPL19	0.650	28.616
MT-ATP6	1.571	22.077
PES1	0.688	28.781
PGK1	0.752	25.873
POLR2A	0.731	29.573
POP4	0.687	29.223
PPIA	0.745	22.992
PSMC4	0.734	28.306
PUM1	0.659	28.416
RPL30	1.082	24.846
RPL37A	1.020	23.473
RPLP0	0.983	24.063
RPS17	0.929	24.155
TBP	0.757	30.292
TFRC	0.738	28.282
UBC	0.831	24.137
YWHAZ	0.887	31.336

Pearson's r was calculated to determine correlations between continuous variables with a normal distribution. Spearman's rank order correlation (r_s) was used in the case of non-normally distributed variables[209, p. 210-215]. Point biserial correlations were calculated in STATA 13 to compare binary and continuous variables[210]. Comparison of categorical variables to continuous variables was undertaken by graphical assessment and one-way ANOVA if required.

Where relevant, all graphs are presented as the mean with error bars representing standard deviations.

Chapter 3

Results

A MSC Yield from Different Anatomical Bone Marrow Locations

A.1 Introduction

Cartilage defects, and in particular those of the knee, have been one of the longest investigated conditions for cellular therapy treatments. As such, they are a prime candidate for treatment with a single stage MSC treatment. As discussed in Chapter 1 on page 24, the optimum source of bone marrow has still not been determined for this kind of procedure.

When considering the treatment of conditions around the knee, the obvious sites from which to obtain bone marrow are the distal femur and proximal tibia given their closeness to the primary operative site. The option to rationalise the number of discrete operative sites from two to one, and so reduce the opportunity for post-operative complications and pain, is extremely attractive to surgeons. To

establish whether either of these sites is suitable they were compared to the currently accepted gold standard of bone marrow from the pelvis.

A.2 Participants

Ten study participants were included in this arm for analysis purposes (Participant IDs P008, P009, P011, P012, P015, P016, P021, P023, P030, and P034). The mean age was 65.7 years (SD=6.2 yrs; range=55.9-77.2 yrs). There were six males and four females. The mean BMI was 28.3 (SD=4.5; range=19.9-36.5)[12]. All individuals underwent unicompartmental or total knee replacement for osteoarthritis. Demographic and clinical data for all participants can be found in Appendix A.

A.3 Sample Collection Information

Bone marrow was collected from the pelvis, femur, and tibia as previously described. In two individuals (P005 and P006) it was not possible to obtain a sample from the pelvis and so these individuals were not included in the study.

The samples were all analysed on the day of collection and Table 3.1 shows the characteristics of the samples collected. It was noted intra-operatively that in the majority of cases it was much easier to obtain the desired five to ten mls of bone marrow from the pelvis than from either the femur or tibia. This is reflected in the mean volumes of aspirate obtained from each location of 10.35 mls (SD = 2.68), 4.60 mls (SD = 2.05), and 4.95 mls (SD = 1.50) from the pelvis, femur,

[12]All continuously measured demographic data satisfied the D'Agostino & Pearson omnibus test for normality.

Table 3.1 – Anatomical Site Sample Characteristics

Sample	Pelvis		Femur		Tibia	
	Volume (mls)	Cells/ml ($x10^6$)	Volume (mls)	Cells/ml ($x10^6$)	Volume (mls)	Cells/ml ($x10^6$)
P008	10.0	1.108	5.0	0.377	5.0	0.166
P009	9.0	1.174	3.5	0.545	4.0	0.627
P011	12.0	2.074	3.0	0.996	5.0	0.787
P012	11.5	3.628	5.5	0.883	2.0	1.177
P015	10.0	0.874	4.0	0.684	5.0	0.387
P016	13.0	0.718	8.0	0.432	8.0	0.237
P021	9.5	0.764	8.0	0.750	4.5	1.304
P023	5.5	2.347	4.0	0.686	6.0	0.735
P030	8.0	2.063	3.0	0.610	5.0	0.545
P034	15.0	1.351	2.0	1.272	5.0	0.313
Mean (*SD*)	10.35 (2.68)	1.61 (0.95)	4.60 (2.05)	0.724 (0.53)	4.95 (1.50)	0.628 (0.35)

and tibia respectively. The results regarding aspiration volumes and initial cell counts were normally distributed.

Two-way ANOVA testing of these results showed that there was a significant difference in both the volume of bone marrow aspirated [$F\ (2, 18) = 24.55$, $p < 0.0001$] and the number of mononuclear cells per ml of aspirate [$F\ (2, 18) = 11.19$, $p = 0.0007$]. The pelvis was superior to the femur and the tibia in both respects.

In order to determine if there was any relationship between either the volume of aspirate or the number of cells per ml and any of the demographic data collected, a number of correlation tests were performed. For continuous variables a Pearson's r was calculated. For binary variables (such as gender) the point-biserial correlation coefficient was used (r_{pb}). In order to examine any association between ASA grade and the outcomes of volume of bone marrow aspirate and cell count per ml the means and standard deviations were plotted graphically. No association was identified and one-way ANOVA testing revealed no significant differences between

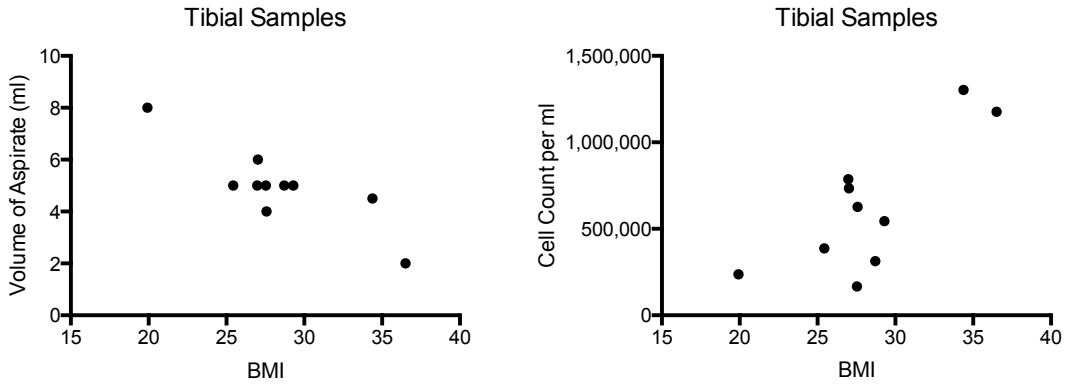

Figure 3.1 – Correlation Effects in Sample Collection

groups. Detailed results of all correlation tests are contained in Appendix D.

The significant correlations that were identified were:

- Volume of aspirate against BMI for tibial samples (r = -0.864, p = 0.001)
- Cell count per ml against BMI for tibial samples (r = 0.783, p = 0.007)

The graphs in Figure 3.1 show these correlations.

The number of correlations identified was relatively small and did not seem to produce any particular pattern which a clinician would need to take into account when deciding whether a site may provide a good yield of MSCs or not.

A.4 CFU-F Assessment of MSC Numbers

CFU-F counts were conducted in duplicate on all samples obtained. This gave an approximation of the number of MSCs per 100,000 cells which were seeded into each 6-well plate. These results are presented in Table 3.2 as the mean of

the duplicate wells. The results were not normally distributed. There was a significant difference between the different anatomical sites [Friedman statistic = 7.2, $p = 0.0303$].

Table 3.2 – CFU-F Count by Anatomical Location

Sample	Pelvis	Femur	Tibia
P008	19.0	4.0	0.0
P009	14.5	8.0	5.5
P011	6.0	3.5	0.5
P012	27.0	77.5	3.5
P015	67.0	4.0	1.0
P016	28.0	1.0	1.5
P021	34.0	39.5	37.5
P023	56.5	6.0	5.0
P030	7.5	4.5	47.5
P034	80.5	3.5	0.0
Mean (*SD*)	34.0 (25.7)	15.2 (24.6)	10.2 (17.3)

Number is CFU-F per 100,000 cells

As the aim of this study was to determine the most suitable site for obtaining bone marrow for single stage procedures, the CFU-F count data were combined with the number of mononuclear cells per millilitre of bone marrow aspirate in order to determine the CFU-F count per millilitre of bone marrow. These results are presented in Figure 3.2. There was still a significant difference between the different anatomical sites [Friedman statistic = 78.6, $p = 0.012$] following adjustment for the number of mononuclear cells per ml.

These data indicate that the pelvis is superior to both the femur and the tibia in terms of both the amount of aspirate that can be obtained using a simple aspiration technique and the CFU-F count that can be obtained from each millilitre of aspirate which is a reasonable estimate of the likely MSC yield.

As for the sample collection data, correlation tests were performed. These showed

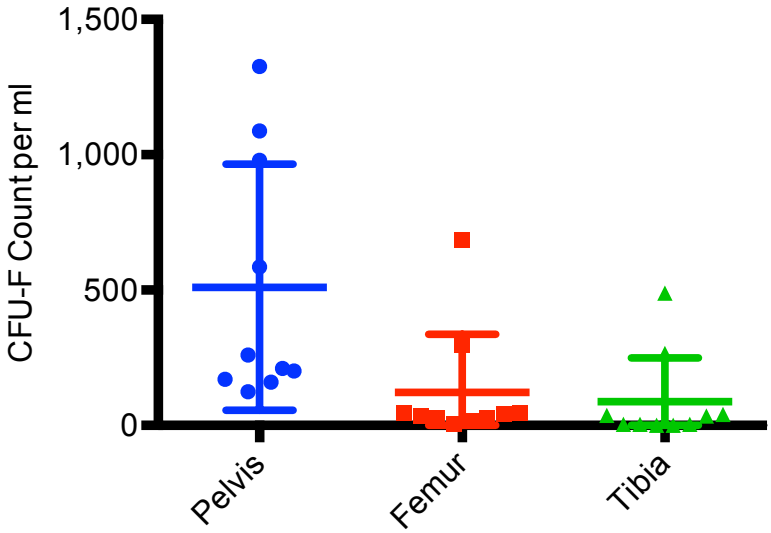

Figure 3.2 – CFU-F Count per ml of Bone Marrow Aspirate

a significant correlation for CFU-F per ml against BMI in the femoral and tibial samples ($r_s = 0.782$, $p = 0.01$ and $r_s = 0.905$, $p = 0.002$ respectively).

A.5 Sample Viability and Proliferative Assessment

In order to assess the ability of extracted cells to both expand in culture and remain viable over time, the plastic adherent cells were expanded in culture as detailed above. Adherent cells from each sample were expanded until the end of P3. At the end of P0 an aliquot of cells was taken to assess viability by PrestoBlue. The failure of some samples to expand at this stage meant that it was not possible to obtain this information in all cases

The number of cells present at each passage was recorded and used to determine the number of population doublings. Table 3.3 shows the number of population doublings per day between the end of P0 and P3 for each sample (with imputed values in red). The average time that samples took to reach the end of P3 from

Table 3.3 – Population Doublings per Day by Anatomical Location

Sample	Pelvis	Femur	Tibia
P008	0.17	0.17	0.24
P009	0.29	0.17	0.17
P011	0.21	0.05	0.32
P012	0.21	0.19	0.34
P015	0.26	0.17	0.20
P016	0.31	0.07	0.06
P021	0.23	0.22	0.21
P023	0.21	0.24	0.24
P030	0.17	0.04	0.18
P034	0.18	0.19	0.24
Mean (*SD*)	0.22 (0.05)	0.18 (0.08)	0.18 (0.09)

Table 3.4 – Time in Culture from P0 to P3

Sample	Pelvis	Femur	Tibia
P008	23	23	24.5
P009	25	25	25
P011	17	84	14
P012	19	19	13
P015	14	53.4	26
P016	16	61	69
P021	18	18	18
P023	18	18	18
P030	21	84	21
P034	22	21	24.3
Mean (*SD*)	19.3 (3.4)	39.2 (28.7)	25.5 (18.2)

Time measured in days.

the end of P0 was 19.30 days ($SD = 3.401$) for the pelvis, 39.22 days ($SD = 28.70$) for the femur, and 25.50 days ($SD = 18.18$) for the tibia (Table 3.4).

A.5.1 Imputation of Missing Values

In order to allow statistical analysis of the doubling number and time from P0 to P3 data, it was necessary to address the missing values in the data set (P008 Tibia, P015 Femur, and P034 Tibia). This was done using the imputation command[211]

in STATA 13[207]. This method can be used when up to 50% of values are missing[212]. Values were imputed 1,000 times and the average taken. This gave values of P008 Tibia = 0.24, P015 Femur = 0.17, and P034 Tibia = 0.24. The population doublings per day data were normally distributed. Using these data there was no significant difference between the different anatomical locations in terms of the number of population doublings per day [Two-way ANOVA, F (2, 18) = 0.890, $p = 0.43$]. Serial t testing confirmed the lack of any significant difference between the groups.

Imputation was also used to calculate the matching missing information for time from P0 to P3. This gave values of P008 Tibia = 24.5, P015 Femur = 53.4, and P034 Tibia = 24.3. The tibial location results were not normally distributed and so a Friedman test was used to compare time spent in culture. This showed no significant difference [Friedman statistic = 1.680, $p = 0.474$].

A.5.2 PrestoBlue Proliferation Assessment

PrestoBlue analysis allowed a growth curve to be constructed for each sample. Growth curves were constructed from the three days on which measurements were taken (D1, D7, and D14). The exponential growth equation function was used in Prism 6 to calculate the growth curves. A non-linear regression was then carried out to compare the growth curves of each sample from a given individual. The growth curves for the samples from six of the study participants are shown in Figure 3.3. The dotted lines represent the modelled non-linear regression curves for each sample.

It was not possible to use a single curve to fit each anatomical location in any of

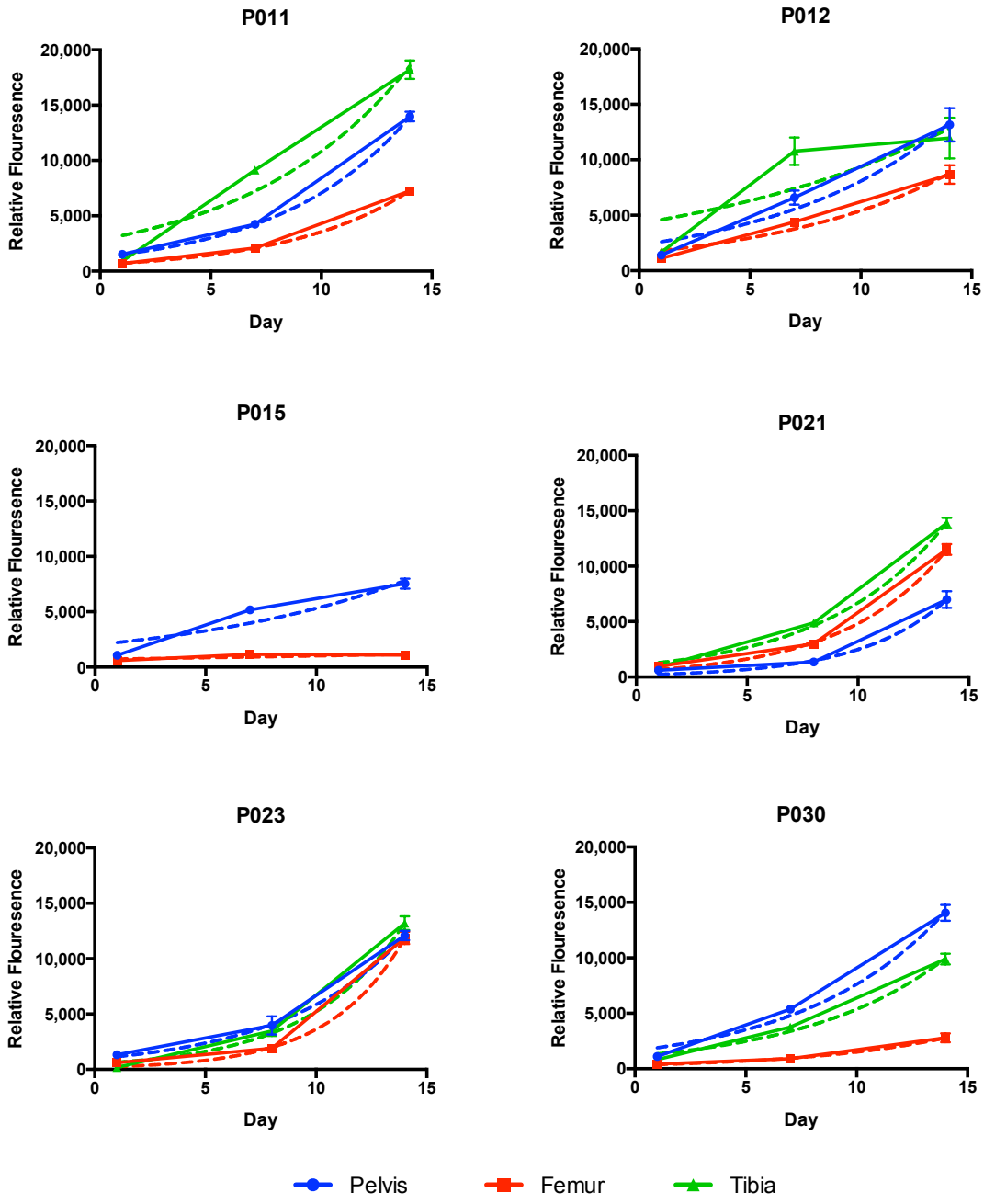

Figure 3.3 – PrestoBlue Viability of Anatomical Location Samples

the samples shown in Figure 3.3. Whilst this shows that the proliferative abilities of the samples does vary by anatomical location this effect appears to dissipate over time given the lack of difference in doubling rate over the growth period from the end of P0 to the end of P3.

A.6 Flow Cytometry Analysis of P3 Samples

There were sufficient cells to allow for flow cytometry analysis of the following samples at the end of P3:

- P009 Pelvis, Femur, and Tibia;
- P012 Pelvis, Femur, and Tibia;
- P021 Pelvis, Femur, and Tibia;
- P023 Pelvis, Femur, and Tibia;
- P030 Pelvis and Tibia;
- P034 Pelvis and Femur.

Flow cytometry was carried out as previously detailed. Each test sample was run in technical triplicates and the average result recorded. The results for each of the samples are shown in Figure 3.4 below. Results are expressed as the percentage of live cells that were positive for the specified antigen.

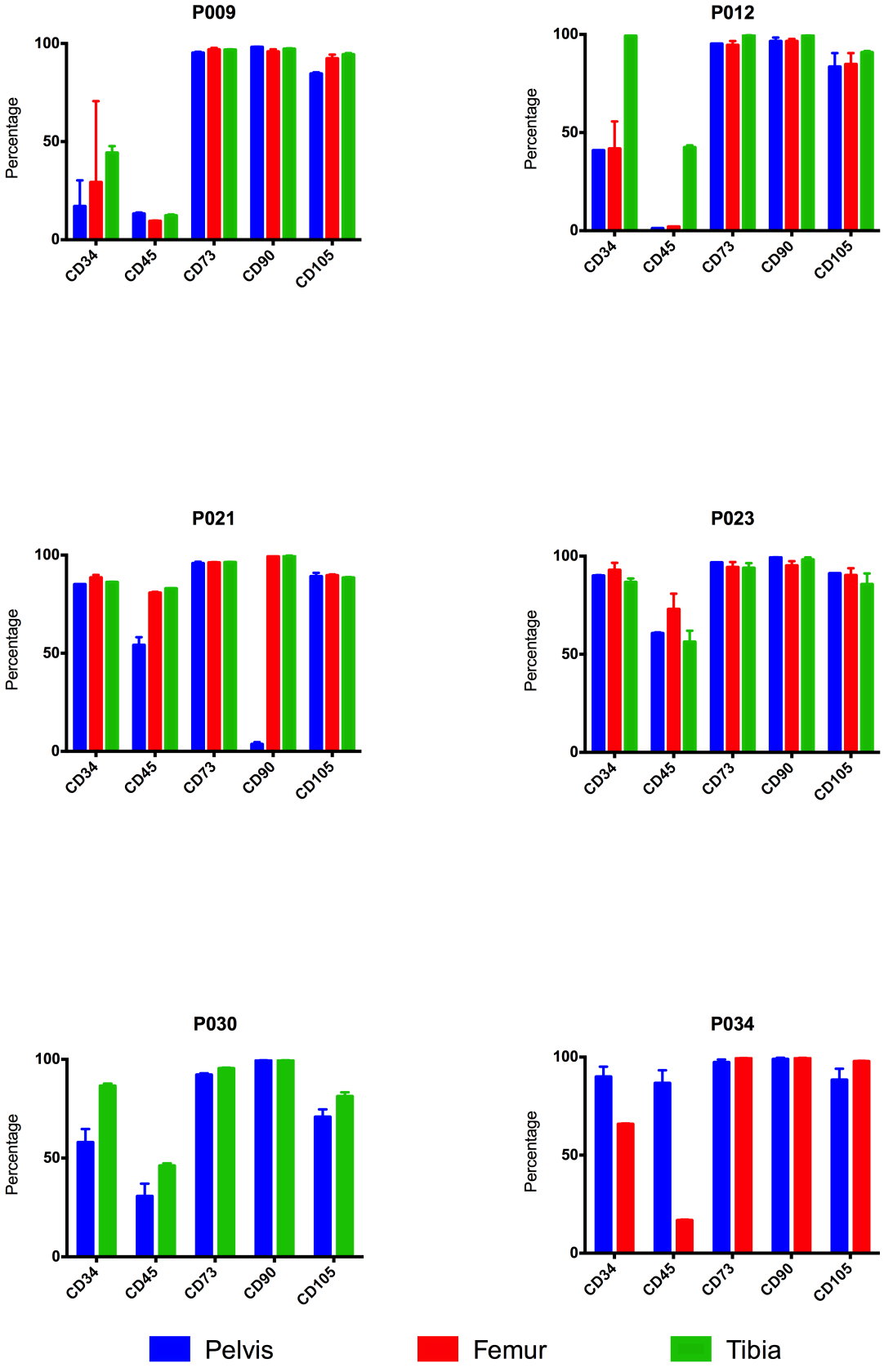

Figure 3.4 – Antigen Expression by Anatomical Location at P3

These results show that at the end of passage 3 there was some variation in the antigen expression found, compared with the expected levels. Whilst MSCs would be expected to lack CD34 and CD45 whilst maintaining high levels of CD73, CD90, and CD105 expression, this is not what was found in all samples. The high levels of expression of the CD73, CD90, and CD105, were relatively well maintained in nearly all samples with the exception of a loss of CD90 expression in the pelvic sample from P021.

CD34 and CD45 expression was, however, much more prone to variation. Despite this, within study participants antigen expression levels were usually reasonably similar. This suggests that in terms of the antigen profile of extracted cells, the location from which the bone marrow is harvested has little impact. The failure of the cells to maintain an archetypal expression profile may well be due to the effect of repeated passage. The fact that these cells were all adherent to plastic, displayed MSC morphology on microscopy, and produced colony forming units, suggests that at least the majority of the cells in culture had been MSCs.

A.7 Comparison of Differentiation by Location

For those samples, where enough cells were obtained at the end of P3, osteogenic and chondrogenic differentiation was undertaken as outlined in Section 2.B.5. In situations where only a limited number of cells were available only some of the differentiation assessment experiments were undertaken.

A.7.1 Alizarin Red Quantification

There were sufficient cells to carry out alizarin red quantification on the following samples:

- P009 Pelvis, Femur, Tibia;
- P011 Pelvis, Tibia;
- P012 Pelvis, Femur, Tibia;
- P015 Pelvis, Tibia;
- P021 Pelvis, Femur, Tibia;
- P023 Pelvis, Femur, Tibia;
- P030 Pelvis, Tibia;
- P034 Pelvis, Femur.

Alizarin red staining was undertaken as outlined above and images taken for reference. A sample image from P012 is shown in Figure 3.5. Quantification was then undertaken and the absorption calculated relative to controls which had been maintained in MesenPRO RS media. These results are shown in Figure 3.6.

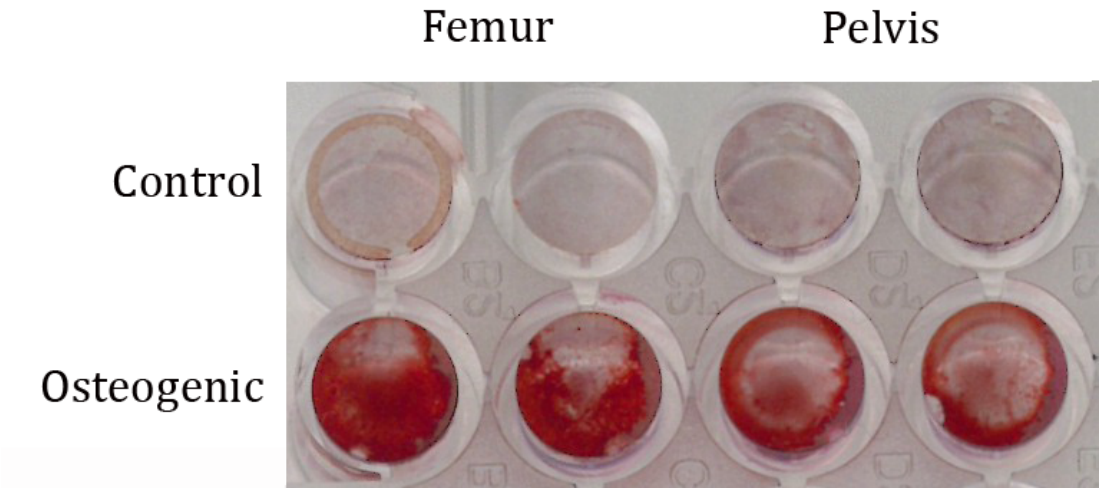

Figure 3.5 – Alizarin Red Staining of P012 Samples

The results were not normally distributed and so the locations were compared in sequence using Wilcoxon matched-pairs signed rank tests. This showed that there was no significant difference in the alizarin red quantification between the different anatomical sites (Pelvis vs. Femur $W = 7.0$, $p = 0.438$, Femur vs. Tibia $W = 10.0$, $p = 0.125$, Pelvis vs. Tibia $W =$ -10.0, $p = 0.469$).

A.7.2 Alcian Blue Staining for Chondrogenic Differentiation

Chondrogenic differentiation and alcian blue staining was carried out on the following samples:

- P008 Pelvis, Femur;
- P009 Pelvis, Femur, Tibia;
- P011 Pelvis, Tibia;

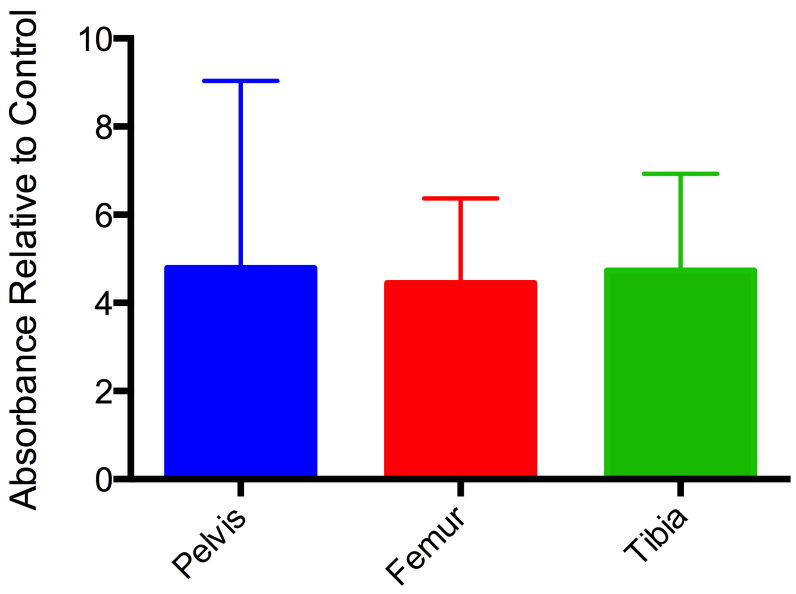

Figure 3.6 – Alizarin Red Quantification by Anatomical Location

- P012 Pelvis, Femur, Tibia;
- P015 Pelvis, Tibia;
- P021 Pelvis, Femur, Tibia;
- P023 Pelvis, Femur, Tibia;
- P034 Pelvis.

In all cases those cells treated with chondrogenic differentiation media formed into spherical masses whilst those maintained in MesenPRO RS remained in monolayer. Alcian blue staining was carried out on all samples. Control samples retained minimal amounts of stain whilst the spherical micromasses all stained blue and retained stain even after washing with PBS. Representative images are shown in Figure 3.7 and Figure 3.8[13].

[13]All alcian blue stained images were obtained using a Zeiss AX10 microscope.

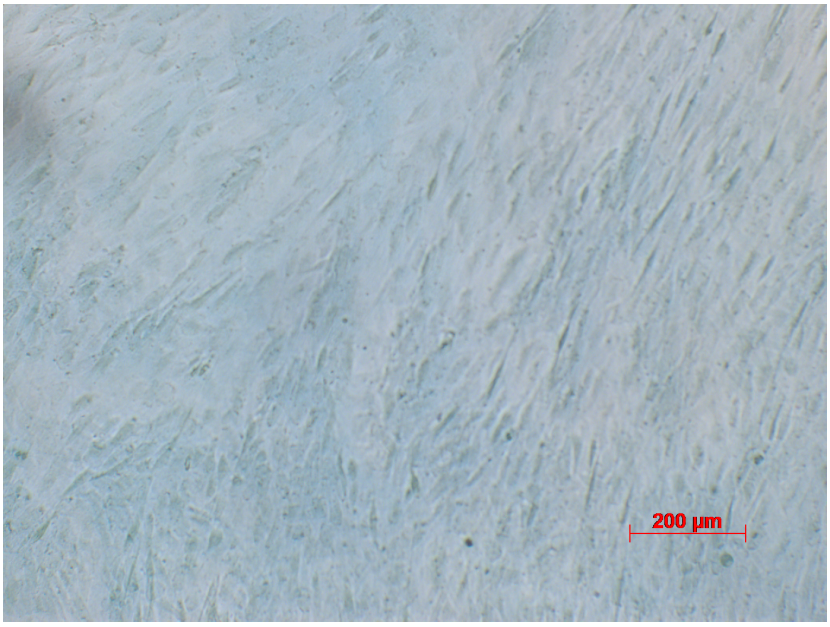

Figure 3.7 – P023 Femur Control Sample stained with Alcian Blue (x100)

A.7.3 qPCR

Following differentiation RNA was extracted from samples and assessed for quantity and purity using a NanoDrop 1000 Spectrophotometer as outlined above. RNA of sufficient purity and quantity was obtained from the following samples:

- P009 Pelvis, Femur, Tibia;
- P012 Pelvis, Femur, Tibia;
- P021 Pelvis, Femur, Tibia;
- P023 Pelvis, Femur, Tibia;
- P034 Pelvis, Femur.

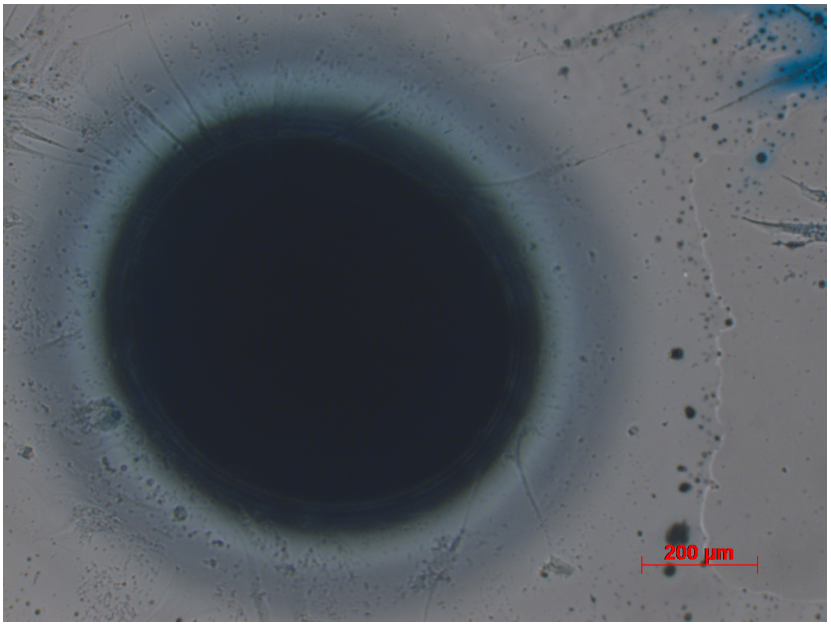

Figure 3.8 – P023 Femur Chondrogenic Sample stained with Alcian Blue (x100)

Osteogenically differentiated samples were assessed for up-regulation of *RUNX2* and *SPP1*. The results of this are shown in Figure 3.9 and presented for each study participant individually. Figure 3.10 shows the results for chondrogenic differentiation where *ACAN* and *SOX9* were assessed. There was no clear pattern of differentiation capability across the different individuals. For example, in P021 the femur seemed the most osteogenically active sample but in P023 the tibia was the most osteogenically active.

RUNX2 analysis of chondrogenically differentiated samples showed up-regulation, potentially indicating hypertrophy in three samples as shown in Figure 3.11.

A.8 Conclusions

The results from these experiments highlight a number of important facts that need to be considered in the development of any treatment that seeks to make use of MSCs. In terms of the yield of MSCs that can be obtained from bone

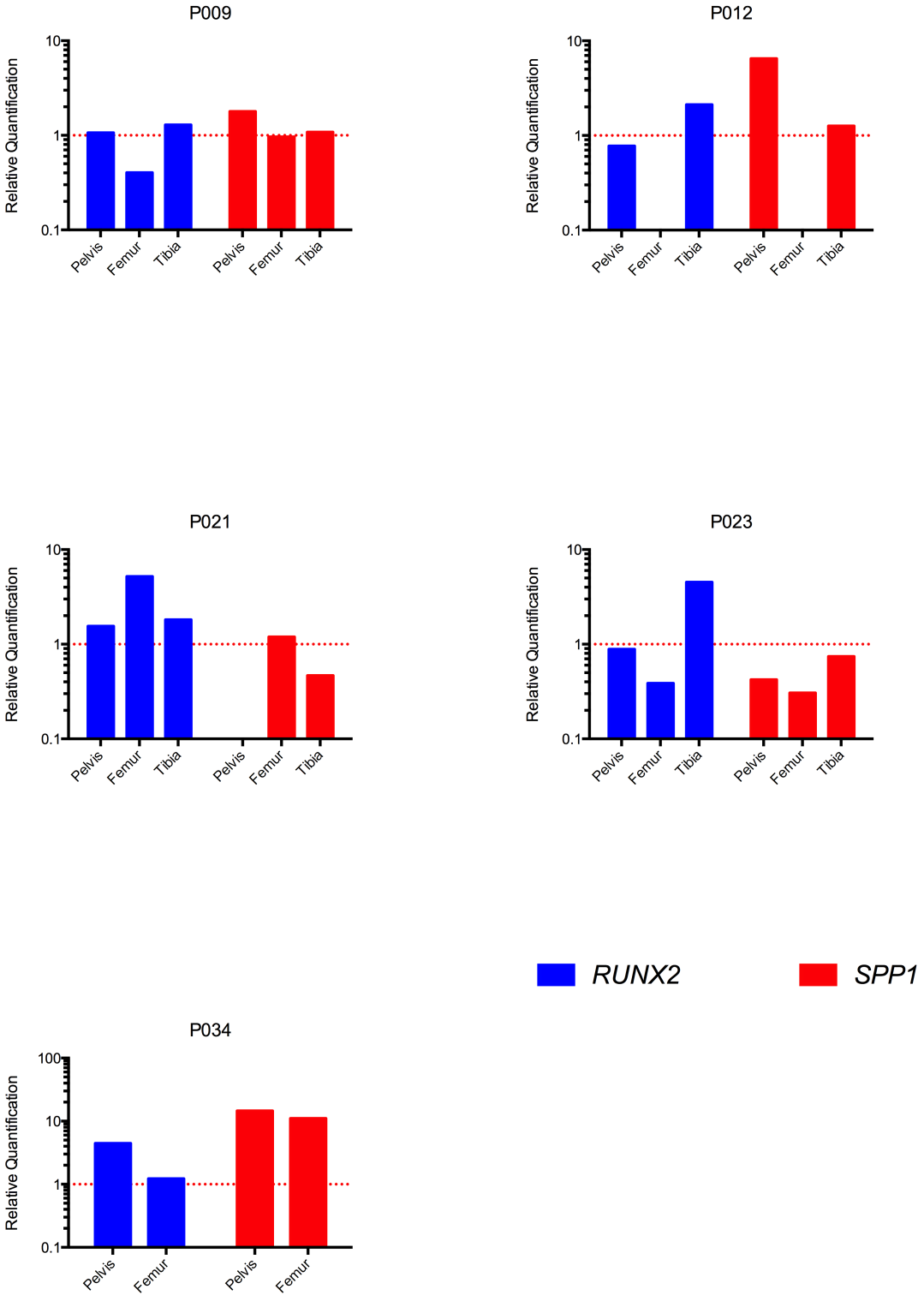

Figure 3.9 – Relative Quantification of Osteogenic Differentiation

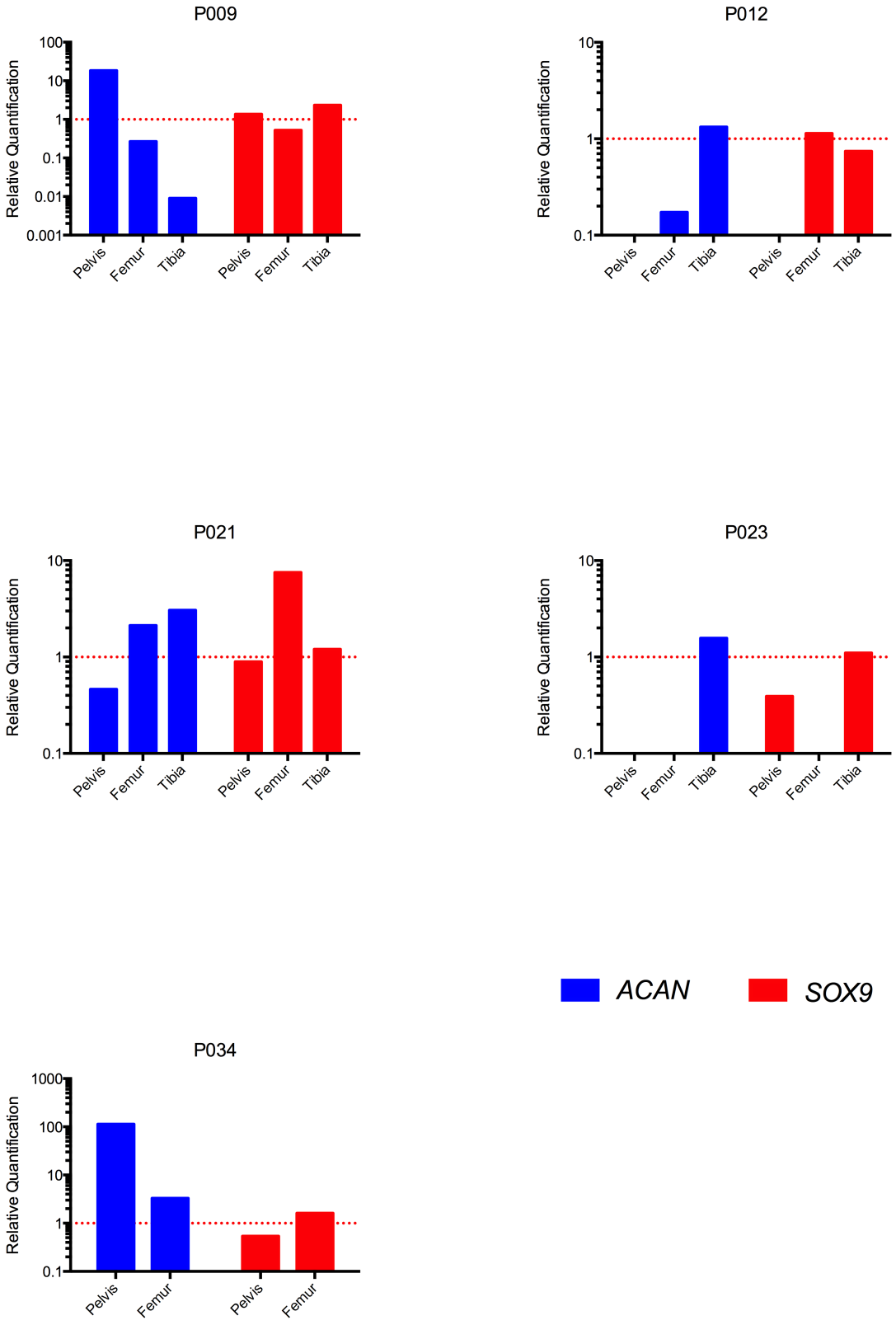

Figure 3.10 – Relative Quantification of Chondrogenic Differentiation

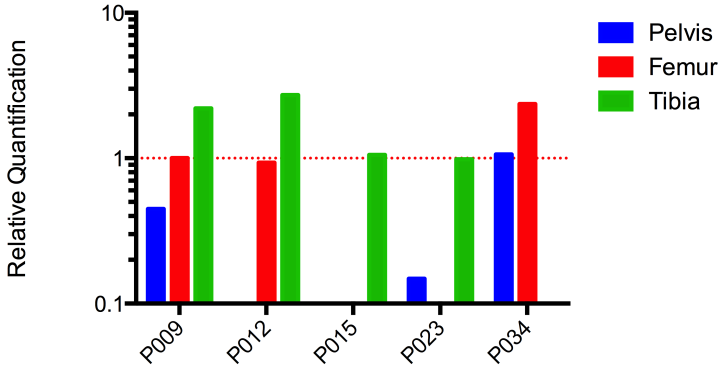

Figure 3.11 – *RUNX2* Expression in Chondrogenic Samples

marrow it is clear from these results that the pelvis is the optimum source within any one individual. However, it should be noted that the wide variation in MSC yields between individuals means that the femur or tibia of one individual may provide a better source than the pelvis of a different individual.

It should also be noted that in two cases it was not possible to obtain bone marrow from the pelvis. Difficulty with obtaining samples could perhaps have been overcome with a more invasive approach but as all participants were volunteers it was not appropriate to carry out a more invasive harvesting procedure than they had consented to.

Antigen phenotype analysis of the samples that had undergone expansion to P3 provides evidence that although cells may behave in the typical manner of MSCs in terms of their properties in culture, this may not necessarily be reflected in terms of their antigen phenotype. The results from the flow cytometry analysis also show that it is difficult to maintain a pure population of cells, even after three passages.

Although it was not possible to obtain sufficient cells to carry out differentiation on all study samples, the results obtained from those samples that did undergo

differentiation make it possible to draw a number of conclusions in regard to the function of these cells. The most obvious conclusion is that there is no clear pattern in the differentiation capabilities of MSCs harvested from different locations. Histological assessments of differentiation, both alizarin red and alcian blue, did not demonstrate any real difference in osteogenic or chondrogenic ability between different sites. qPCR results were highly variable between individuals and no clear pattern was discernible.

B MSC Yield from Different Sampling Techniques

B.1 Introduction

As has been discussed previously, the exact location of the MSC niche within bone marrow has not been fully determined. However, over time it has become apparent that MSCs are not uniformly distributed within the intra-medullary canal and that the majority are located in proximity to trabecular bone. This fact may well underpin the previously mentioned relative success of the RIA system in achieving high yields due to the fact that it removes such a large quantity of the trabecular bone from the intra-medullary canal.

The aim of this study was to determine if it was possible to harness some of that improved yield obtained by disrupting the trabecular bone network, without having to resort to large amounts of extra equipment or separate surgical sites. The modified collection method was designed to require no extra equipment over the normal collection method. To that end the Jamshidi aspiration needle was inserted into the distal femur as described above (Section 2.B.1). Prior to aspiration of the bone marrow the needle was rotated in a circular motion as well as being moved back and forth in anterior-posterior and medial-lateral planes. The bone marrow was then aspirated from this potential void as previously described.

As discussed previously, the femoral samples collected from individuals who took part in the anatomical location study were used as the control arm for this study as they represented the normal method of collection and provided the only practical control.

B.2 Participants

Ten study participants were included in this arm for analysis purposes (Participant IDs P031, P032, P035, P036, P037, P038, P039, P040, P041, and P042). The mean age was 65.6 years (SD=10.6 yrs; range=45.6-79.5 yrs). There were six males and four females. The mean BMI was 29.1 (SD=3.8; range=23.1-35.3).

The demographics of this group were compared to those from the anatomical site study to ensure the two groups were comparable. All demographic data was normally distributed. There was no significant difference in ages of the groups [Unpaired two tailed t test with Welch's correction, t (14) = 0.018, p = 0.99] or in the variances of the groups [F (9) = 2.95, p = 0.123]. The ratio of males to females was the same for each group. There was no difference in ASA scores between the two groups [Unpaired two tailed t test with Welch's correction, t (18) = 1.309, p = 0.207]. There was no significant difference in BMI between the two groups [Unpaired two tailed t test with Welch's correction, t (17) = 0.408, p = 0.688].

B.3 Sample Collection Information

Samples were collected and analysed as before. All sample collection data was normally distributed. There was no significant difference between the modified collection method and standard collection with respect to the number of cells per ml [Unpaired two tailed t test with Welch's correction, t (15) = 1.373, p = 0.19]. However, there was a significant difference in the volume of bone marrow aspirated [Unpaired two tailed t test with Welch's correction, t (14) = 2.231, p = 0.036] with a larger volume collected using the normal method. The significant

Table 3.5 – Modified Collection Method Sample Characteristics

Sample	Modified Method	
	Volume (mls)	Cells/ml (x10^6)
P031	2	0.567
P032	2	0.336
P035	2	0.075
P036	4	0.370
P037	4.5	0.401
P038	2	0.083
P039	2	1.496
P040	3	0.097
P041	3	0.982
P042	4.5	0.561
Mean (*SD*)	2.9 (1.08)	0.497 (0.447)

findings when comparing collection data to demographic information were that being an ex-smoker (as opposed to never having smoked) was associated with an increase in cell count per ml of bone marrow aspirate [$r_{pb} = 0.652$, $p = 0.041$] and there was a positive correlation between BMI and cell count per ml [r =0.818, $p = 0.004$] . The correlation between smoking and cell count was confirmed by carrying out an unpaired two tailed t test with Welch's correction, t (5) = 2.672, $p = 0.044$.

B.4 CFU-F Assessment of MSC Numbers

CFU-F assessment was carried out as in the anatomical location study. The average CFU-F count for each sample is shown in Table 3.6.

Adjusting the CFU-F count for the number of cells per ml of bone marrow allowed the modified technique to be compared to the femur samples collected in the anatomical location study. The data were not normally distributed. There was

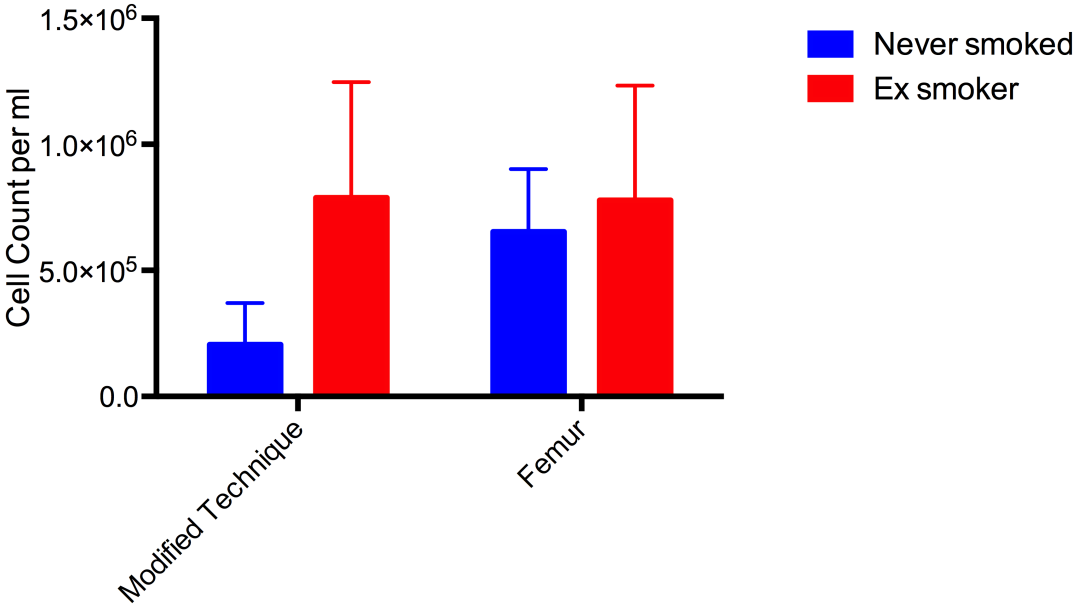

Figure 3.12 – Cell Counts per ml Comparison by Smoking Status

Table 3.6 – CFU-F Count For Modified Collection Method

Sample	Average CFU-F Count
P031	2.5
P032	62
P035	7.5
P036	0
P037	8
P038	0
P039	24
P040	31.5
P041	1
P042	3.5

Number is CFU-F per 100,000 cells

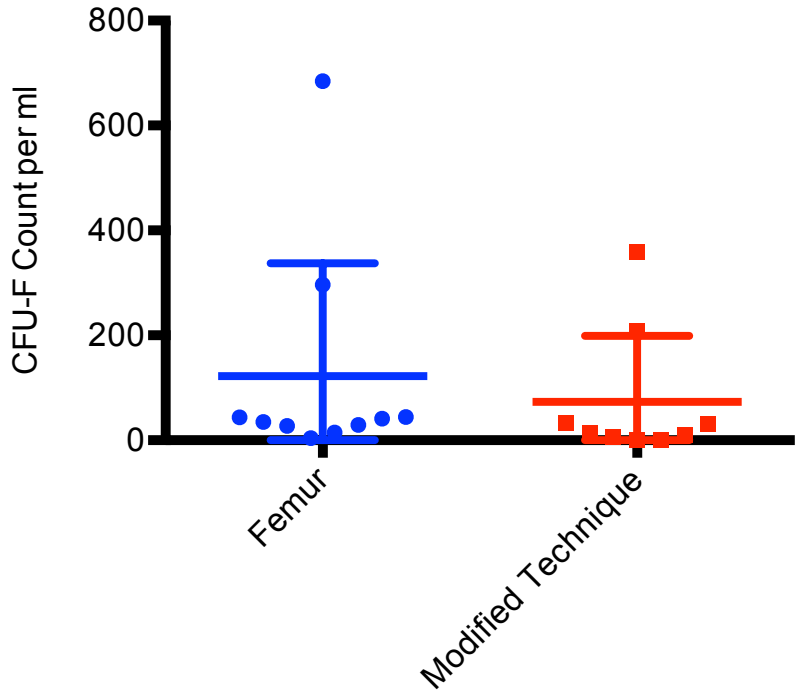

Figure 3.13 – CFU-F Count per ml of Bone Marrow Aspirate using Modified Collection Method

no significant difference in the number of CFU-Fs per ml using the two different collection methods [Mann-Whitney U test, $U = 28$, $p = 0.176$].

No significant correlations were identified between the CFU-F results and the demographic information.

B.5 Sample Viability and Proliferative Assessment

Viability and proliferative capability were assessed as before. Population doublings were calculated on a per day basis. Three of the samples had not yielded enough cells to successfully expand and so were used for CFU-F counts only (P035, P038, and P040). Two samples failed to expand in culture and so doubling times could not be calculated (P036 and P042). It was not possible to establish

that the results were normally distributed due to the small sample size and so non-parametric tests were used for analysis. A Mann-Whitney U test showed that there was no significant difference between the modified collection technique and the standard technique in terms of population doubling rate [$U = 19.5$, $p = 0.725$]. The average time taken to reach the end of P3 from P0 was 34 days ($SD = 20.02$). There was no significant difference between this and the mean time taken for femoral samples from the anatomical study [Mann-Whitney U test, $U = 22$, $p = 0.746$].

Table 3.7 – Population Doublings per Day using Modified Technique

Sample	Doublings Per Day
P031	0.06
P032	0.24
P035	Insufficient
P036	Failed
P037	0.23
P038	Insufficient
P039	0.16
P040	Insufficient
P041	0.12
P042	Failed

PrestoBlue viability assessments (carried out on P031, P032, P037, and P039) showed a variation in the viability of the samples obtained using the modified technique in a similar manner to that found during the anatomical location study. It was not possible to determine if this variability was due to the individuals or the new collection method, but given that this variability had been seen in the anatomical location study, it would be reasonable to assume that it was due to the shared feature between the two studies, namely inter-person variability, rather than a function of the harvesting method.

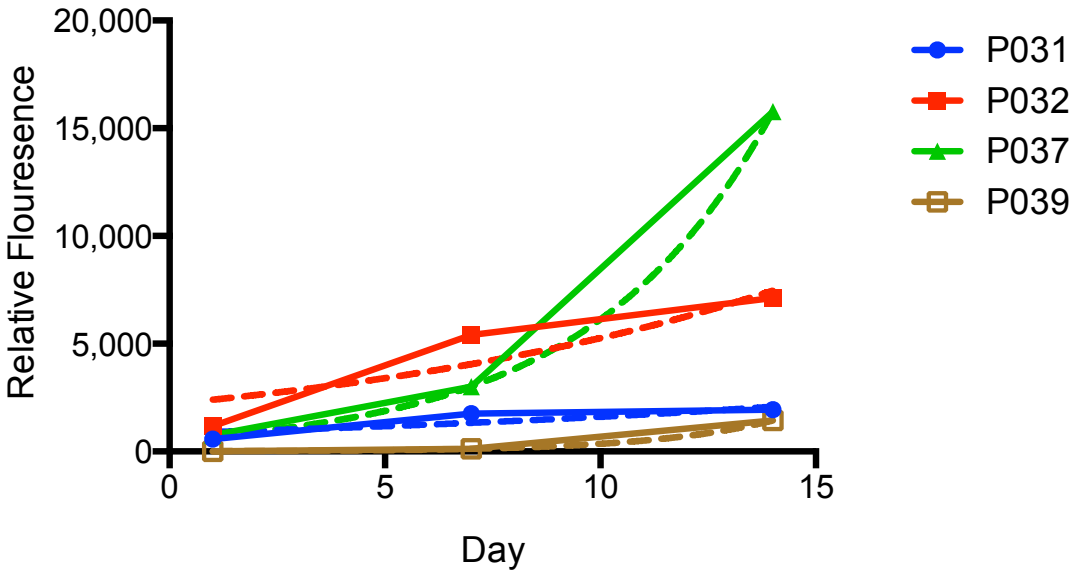

Figure 3.14 – PrestoBlue Viability Curves for Modified Collection Technique

Table 3.8 – Comparison of Antigen Expression between Normal and Modified Technique

Antigen	U	p
CD34	4	0.393
CD45	6	0.786
CD73	3	0.250
CD90	4	0.393
CD105	5	0.500

B.6 Flow Cytometry Analysis of P3 Samples

Flow cytometry was conducted as before on samples from P032, P037, and P039. These samples underwent flow cytometry which showed variable antigen expression in a similar manner to that found for the anatomical location study (Figure 3.15), with no clear MSC antigen profile being established. Figure 3.16 shows the comparison of the average antigen expression found using the modified technique with that produced by the normal harvesting technique. Mann Whitney U tests showed that there was no significant difference for any of the antigens (Table 3.8).

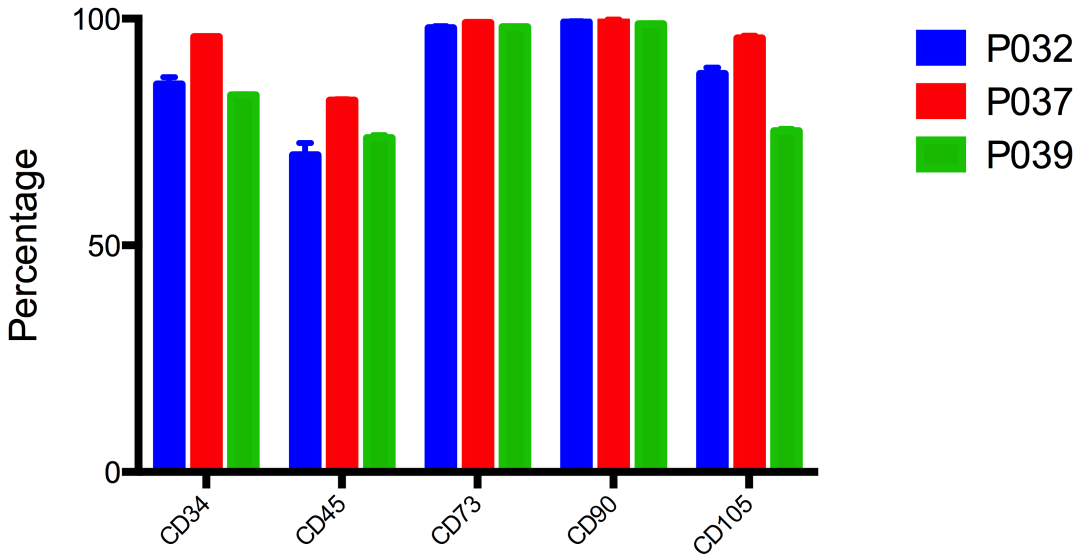

Figure 3.15 – Antigen Expression for Modified Technique Samples

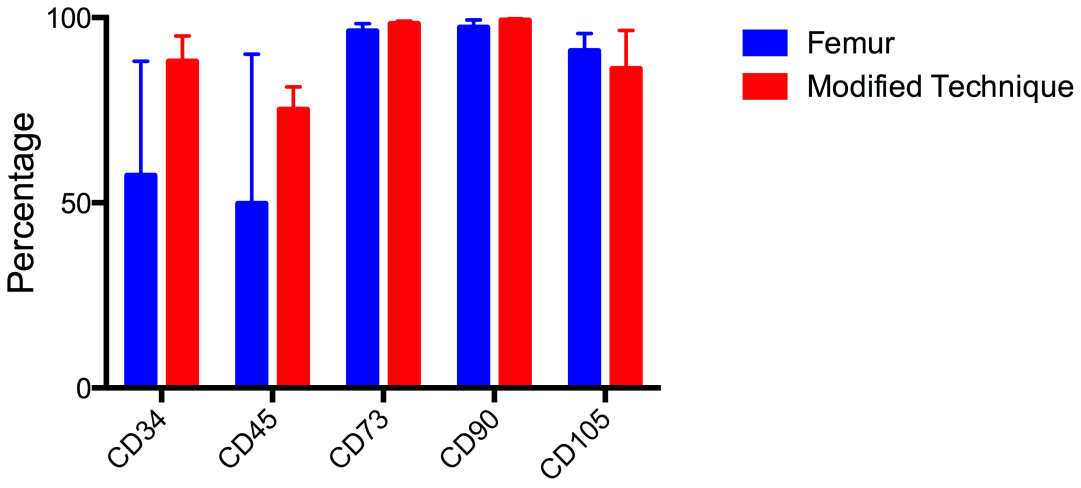

Figure 3.16 – Comparison of Antigen Expression between Normal and Modified Technique

B.7 Differentiation of Cells Extracted Using Modified Technique

The poor expansion of cells obtained using the modified technique meant that only a small number of samples were available for differentiation (P032, P037 and P039).

B.7.1 Alizarin Red Assessment of Osteogenic Differentiation

Alizarin Red quantification is shown in Figure 3.17. A Mann-Whitney U test showed that there was no significant difference between the quantification in this study as compared with that achieved for femoral samples in the anatomical study [$U = 2$, $p = 0.143$].

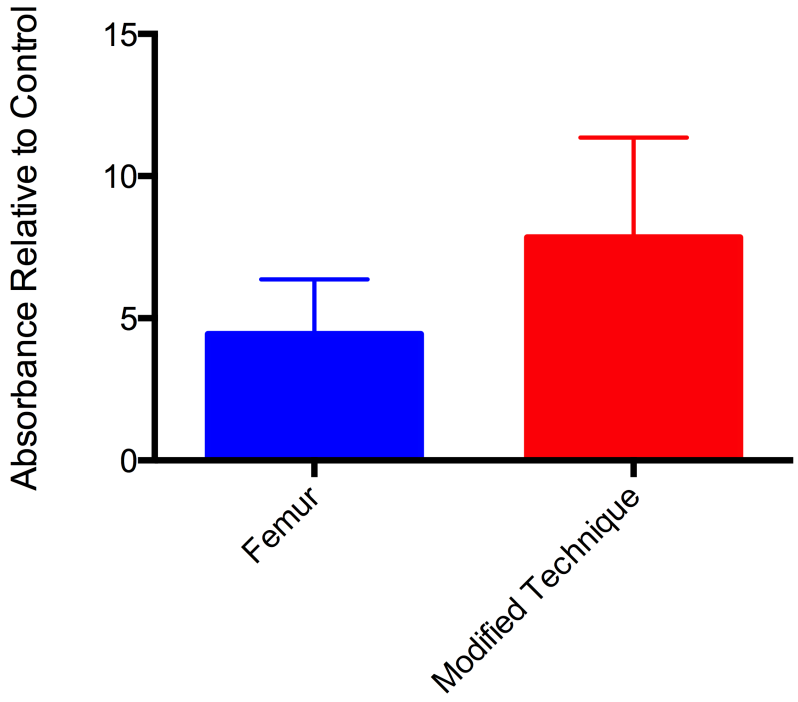

Figure 3.17 – Alizarin Red Quantification of Modified Technique

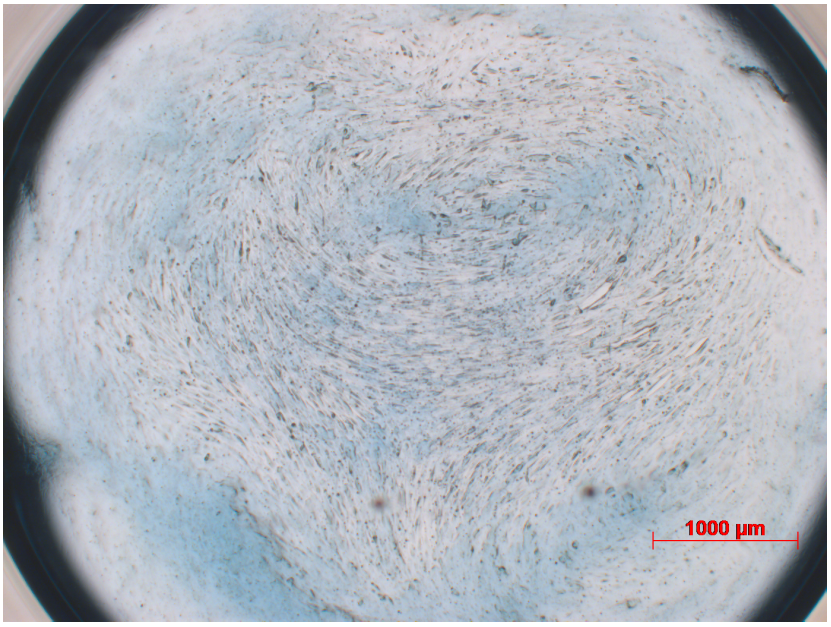

Figure 3.18 – P037 Control Sample stained with Alcian Blue (x25)

B.7.2 Alcian Blue Assessment of Chondrogenic Differentiation

Alcian blue staining was carried out on differentiated cells from samples P032, P037, and P039. As in the anatomical location study, all differentiated samples formed spherical masses and retained stain on addition of alcian blue. Figures 3.18 and 3.19 show the control and differentiated samples from P037.

B.7.3 qPCR

The difficulties in obtaining RNA of sufficient quality and quantity to undertake qPCR meant that it was only possible to carry out qPCR on cDNA from one sample extracted using the modified technique. The samples that underwent chondrogenic differentiation produced very low levels of RNA and it was not possible to assess any gene expression in these samples. Macroscopically these samples did appear to successfully undergo chondrogenic differentiation as

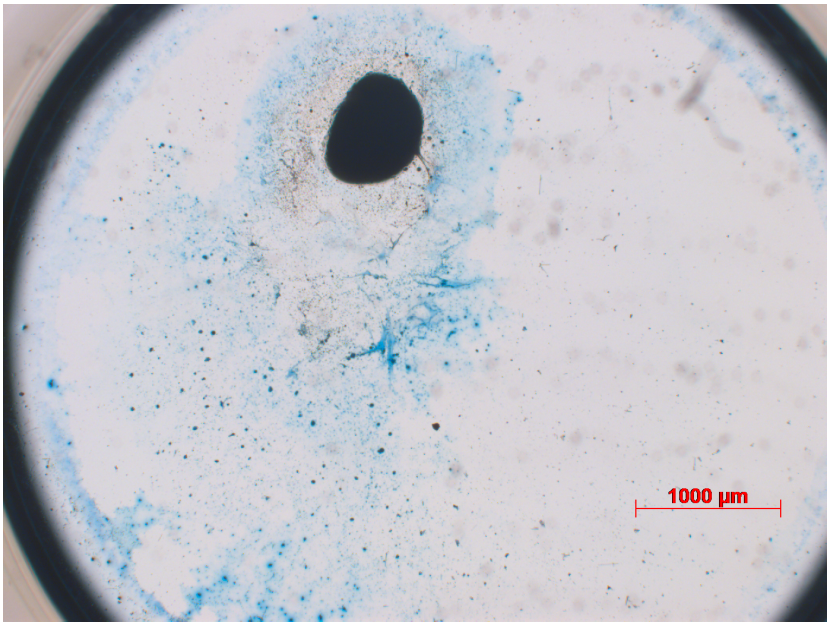

Figure 3.19 – P037 Chondrogenic Sample stained with Alcian Blue (x25)

evidenced by their formation of micromasses after initial plating in monolayer. Figure 3.20 shows that the femoral samples appeared to produce more osteogenically active MSCs, although this is difficult to confirm given the single sample studied.

B.8 Conclusion

These results show that the modified technique does not appear to lend any advantage over standard aspiration methods in the harvesting of MSCs. Although, millilitre for millilitre, the two techniques produce similar numbers of BMMCs, the modified technique actually results in smaller volumes being harvested easily from the bone marrow. The large number of samples harvested in this manner, that failed to expand as desired and did not undergo differentiation with enough success to allow qPCR analysis, does raise the question as to whether the modified technique may damage the cells in a way which interferes with their survival ability and proliferative capacity.

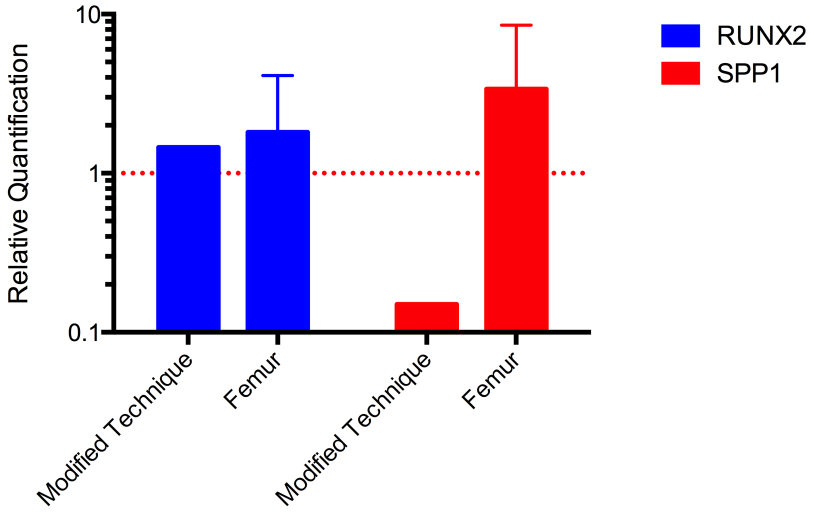

Figure 3.20 – Modified Technique Osteogenic Gene Expression

Chapter 4

Discussion

For more than 20 years, regenerative medicine has been seen as the next great advance in the treatment of a wide variety of diseases. Within this field, cell-based therapies have been explored extensively as a treatment method for conditions where early treatment has either proven elusive, or has only been available for end-stage disease. The discovery of MSCs and their potential therapeutic effects have spurred an explosion in both basic science and clinical treatment development designed for these conditions. However, this leap for clinical treatments has occurred without a full understanding of the basic science behind MSCs, such as their relative differentiation and proliferation abilities, and without sufficiently robust clinical trials to demonstrate the effectiveness of these treatments.

In Section 1.C.1 I detailed how these treatments can be broadly divided into laboratory-based and single stage procedures. These different approaches have been underpinned by two different, yet not mutually exclusive, paradigms for the methods by which MSCs are able to bring about repair and regeneration of previously damaged tissues. The laboratory based approach is under extensive investigation but does suffer from a variety of problems. Some of these are inherent

to any laboratory process and difficult to fully mitigate (such as the costs associated with the provision of cGMP facilities). Although the use of allogenic MSCs in an off-the-shelf model may well enable these costs to be reduced, such as in the use of Trinity Evolution® for the treatment of bone defects and non-union,[14] this is not without difficulties due to the developing information regarding the extent to which MSCs are immune privileged due to their lack of MHC Class II antigens. Recent work by Schnabel *et al.* in the equine model has shown that the extent to which MSCs can be relied upon to not elicit an immune reaction can be highly variable[138].

These difficulties partially underlie the development of single stage procedures using autologous MSCs that are harvested and immediately used in the theatre environment. This thesis aimed to explore a number of the basic science questions regarding these cells that have remained unanswered, despite the widespread development of these techniques. As knee surgery has been an early adopter of other cellular therapies, such as ACI, the thesis focused on answering these questions with specific respect to surgery around the knee where they have, to date, gone unanswered. The questions that it sought to answer were:

1. Which bone marrow location provides the optimum source for obtaining MSCs for use in single stage procedures?

2. Is it possible to increase the yield of MSCs from a bone using simple manoeuvres?

The results of this thesis may well provoke more questions than they answer, which further underscores the requirement for a greater understanding of some

[14]Trinity Evolution is an allograft of cancellous bone and allogenic MSCs, combined with demineralised cortical bone. It is sold by Orthofix, TX, USA.[213]

of the fundamental questions surrounding MSCs before they can, or should, be subjected to clinical trials.

A Sample Collection and Expansion Failures

Concerns that participant recruitment would be adversely affected by the need to undergo a separate procedure secondary to their knee replacement proved unfounded as demonstrated in Figure 2.1, with only two individuals declining to take part.

Once the study had started a number of issues came to light which had both an effect upon the samples collected as part of the study and would also potentially affect any treatment designed around the aspiration of bone marrow for immediate use.

The most significant findings from those samples that were not studied further were the number of individuals in whom it was not possible to obtain a sample and the difficulty in getting some samples to expand. It proved impossible to obtain samples from two individuals out of the twelve in whom pelvic sampling was undertaken, despite a number of attempts in each case. This failure rate of 16.7%, in the hands of an orthopaedic surgeon with a full time clinical practice, indicates that it should not be assumed that obtaining these samples in a minimally invasive way is always possible and without the risk of complications. This potential failure rate must be taken into consideration when examining any single stage procedure which is going to be reliant upon successful bone marrow harvesting. Failure with minimally invasive bone marrow aspiration in such a situation would make it necessary to either abandon the procedure, or potentially proceed with

a more invasive "open" type of procedure.

The next practical hurdle was that, even in situations where samples were obtained, there was a high degree of variability in the number of cells obtained per ml of bone marrow aspirate. The mean number of cells per ml extracted from all 40 samples was 0.865 x10^6 (SD = 0.698 x10^6). There were no identified correlations between cell counts and demographic information which would be clinically relevant. It was noted that the aspirates did not have uniform appearances, with some appearing to contain more fat than others. Figure 4.1 shows the bone marrow samples from P040, P041, and P042 just prior to processing (femoral samples collected using the modified technique). As can be seen, there were highly variable amounts of fat in the different samples and the amount of separation observed occurred despite identical storage and carriage conditions from the operating theatre to the laboratory. The degree of apparent fat in the samples did seem to bear some relation to the cell count per ml in these samples with P040 having a cell count per ml a factor 10 below the other two samples. However, this relationship was reversed in the CFU-F count where P040 produced an average of 31.5 CFU-Fs compared to 3.5 and 1 from P042 and P041 respectively.

This variability in the gross constituents of the samples was not possible to quantify but may have had an impact on the MSC yield from all samples studied. As has previously been mentioned, adipose tissue has a much higher proportion of MSCs than bone marrow[124]. Bone marrow aspirates containing high fat levels may therefore yield a greater quantity of MSCs, although the functional ability of these cells may not be what is desired in orthopaedic applications due to their stronger adipogenic differentiation capability[214]. The difficulty in assessing the degree of fat in any bone marrow aspirate, especially if it is destined for immedi-

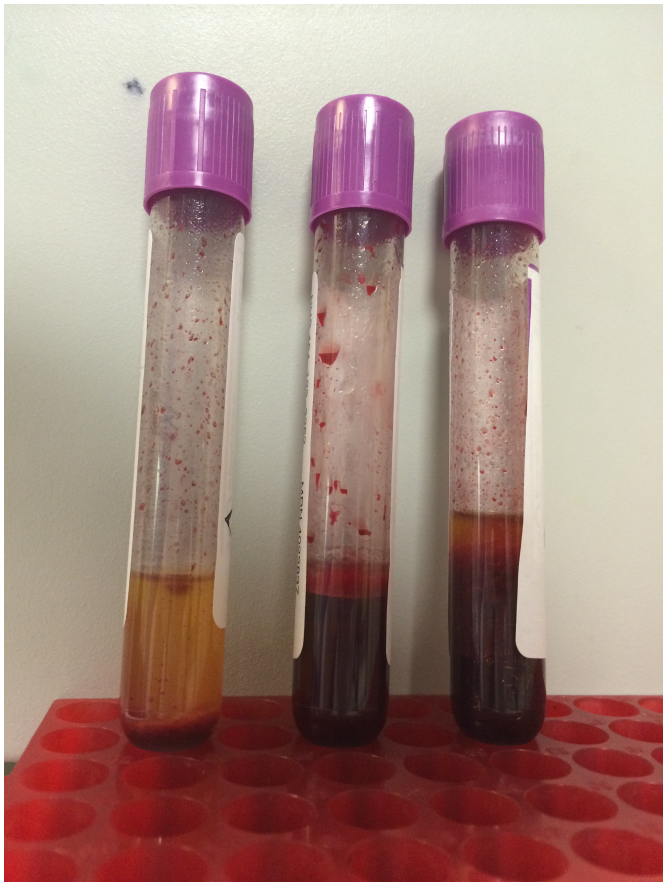

Figure 4.1 – Sample Separation in Collection Bottles

ate use in a single stage procedure, makes it extremely difficult to determine the effect that this may have upon any procedure and to control for it in such a way as to allow for standardisation of treatment between patients.

In a number of samples where cells were successfully extracted they then failed to adhere and proliferate in tissue culture and so could not be studied further. As discussed in Section 2.B.2.1, there was no obvious reason for this failure. There was no evidence of infection in the tissue culture flasks and no problems with other samples being cultured at the same time which would indicate a problem with tissue culture media or incubator function. This failure occurred in four samples out of a total of 56 that were placed into tissue culture flasks, a rate of 7.1%. This unexplained failure may have been due to an unrecognised technical problem or may have resulted from an issue with the cells following harvesting, which would represent a further potential difficulty with harvesting these cells for immediate use. If the process is likely to render the cells unviable in over one in 20 samples it may not be suitable for widespread use without some form of intra-operative viability confirmation. This would be technically very challenging and potentially be complex enough to render a single stage procedure unfeasible.

The final problem related to the processing of samples was the failure of some samples to expand sufficiently, despite initially adhering appropriately in tissue culture. As discussed in Section 2.B.4.2, there was a failure of adequate expansion in samples taken from five individuals in the location study and two in the method of harvesting study. There was no obvious demographic difference between those samples that failed to expand and those that expanded normally. In terms of individual samples studied, this represented a total of 12 samples that failed to expand sufficiently out of a total of 52 that successfully adhered in culture. This 23.1% failure rate must also be taken into account when considering the

use of procedures involving these cells for single stage procedures. No specific reason for the failure of expansion was identified. It is, however, important to bear this information in mind when considering treatments based on this type of MSC harvesting. Whilst single stage procedures are founded upon the principle of the paracrine and cell-to-cell interactions of MSCs, rather than their ability to expand and differentiate into target tissue types, this expansion failure may indicate an underlying problem with the harvested cells, in much the same way as those samples that failed to adhere in tissue culture.

Taken together, these problems with either obtaining samples, extracting sufficient cells, or carrying out expansion in tissue culture, occurred in 13 out of the 33 individuals from which samples were taken during the study. This equates to a problem in obtaining sufficient cells due to one reason or another of 39.39%. With no practical and immediate way of assessing bone marrow samples to determine presence of adequate numbers of functional MSCs in a sample, this error rate would have a potentially significant impact upon any treatment based on these techniques.

The most obvious criticism of this conclusion is that a number of papers have been published on harvesting bone marrow using similar techniques without any report of similar levels of problems with obtaining viable cells in sufficient numbers. However, in many papers the complete outcome for each sample is often not reported. For example, in their paper comparing the anterior to posterior iliac crests as sources of MSCs, Pierini *et al.* obtained samples from 22 individuals who were all included in initial cell counts. Later experiments, however, used an n of 3, 4, and 17, with no explanation as to whether these smaller numbers had been used for practical reasons or because of problems with cell viability and expansion in some samples[172]. Povsic *et al.* reported a 11.2% rate of failure

to obtain bone marrow when sampling from the femur during hip replacement surgery[215]. Hyer *et al.'s* study looking at the pelvis, distal tibia, and calcaneus reported mononuclear cell counts as low as 100,000/ml and MSC counts as low as zero/ml of bone marrow. These extremely low yields, which are similar to some found in this study, can be easily lost in the midst of the reported mean cell yields and would not have provided enough cells to conduct further experiments in the manner carried out in this thesis[128]. The real world effect of this error rate should therefore not be dismissed as simple operator error and must be addressed in future work.

B MSCs from Different Locations

The aim of the first part of this thesis was to establish the most appropriate site for harvesting bone marrow. It has already been established that, whilst bone marrow may not provide the highest yield of MSCs, its accessibility to the orthopaedic surgeon and relative ease of harvesting make it the most appropriate source for single stage procedures. As discussed in Section 1.C.2.1, previous work has demonstrated the feasibility of harvesting MSCs from a number of different skeletal locations. However, a number of these studies were relatively limited in scope and so it is difficult to draw firm conclusions from their results.

In their respective studies both Mazzocca *et al.*[125] and Beitzel *et al.*[126] demonstrated the presence of MSCs in the humerus and femur. They did not, however, compare these locations to the pelvis. Differentiation capability was also only assessed with respect to the osteogenic lineage. The primary purpose of both papers was to compare different methods of isolating MSCs from bone marrow and so, whilst they must not be judged too critically with respect to their mes-

sage regarding MSC yields, the faults mentioned above must not be forgotten. The femur has also been demonstrated to hold MSCs in the presence of disease. Lee *et al.*[216] showed the presence of MSCs around areas of osteonecrosis in the proximal femurs of three individuals. Again, this study did not have any other location for comparison and primarily was designed to demonstrate the presence of potentially regenerative cells close to areas of necrosis. García-Álvarez *et al.*[217] have also shown the similarity of MSC yields from individuals undergoing joint arthroplasty to those being treated for femoral fractures. This study did not carry out any comparison to other anatomical locations but it is of particular relevance to the work carried out in this thesis as it shows that there is no reason to believe that the presence of OA per se should have impacted upon the relevance of the results obtained.

McLain *et al.*[127] did include a comparison to the iliac crest when assessing the vertebra as a suitable source of MSCs to assist in spinal fusion. They were able to obtain more MSCs from a vertebra than from the iliac crest of the same individual. However they failed to carry out any assessment of differentiation making it difficult to comment on the functional ability of the cells obtained.

Hyer *et al.*[128] sought to determine the best source of MSCs for augmentation of non-union or fusion procedures. Their study demonstrated the superiority of the pelvis to either the distal tibia or calcaneus in terms of yield of MSCs, but again there was no assessment of the differentiation capability of the cells obtained.

It is against this background that the results of the location study must be assessed. In order to overcome the biggest problems with previous work in this area, intra-subject comparison was used for all assessments where practical. This allowed for the exclusion of inter-subject variability which is, potentially, the

greatest source of variability in results in this type of study.

B.1 Aspirate and MSC Yields

Perhaps the most striking feature of the results obtained from the anatomical location study is the high degree of variability of MSC yields between individuals, irrespective of the location sampled. This can be demonstrated by looking at the coefficient of variation of the results obtained. The coefficient of variation (CV) can be used to determine the dispersal of a continuous variable. It is calculated as shown below and allows one to compare the variability between one set of results and another:

$$CV = \frac{standard\ deviation}{mean}$$

Table 4.1 shows the coefficient of variation in both BMMC and CFU-F numbers per ml of bone marrow from both this study and four similar published studies. As can be seen, there is a wide variation in the coefficient of variation. Such variation poses a fundamental problem for any procedure based on the immediate use of harvested MSCs. Whilst point-of-care systems, such as those listed on page 22, are designed to concentrate the BMMC proportion of the bone marrow aspirate, the highly variable nature of the starting material means that it would be extremely difficult to standardise the yield that is produced by the device. Currently, none of these point-of-care devices are able to provide a real time quantification of the number of cells in the processed bone marrow aspirate.

Table 4.1 – Variation in Bone Marrow Yields in a Selection of Studies

Study	Sample	CV (%)	
		BMMC/ml	CFU-F/ml
Current Study	Pelvis	57.06	89.06
	Femur	37.27	176.13
	Tibia	61.10	184.65
	Modified technique	90.10	171.36
Hyer *et al.*[128]	Pelvis	248.08	104.03
	Tibia	201.72	211.84
	Calcaneus	221.13	237.74
Pierini *et al.*[172]	Posterior iliac crest	49.46	68.73
	Anterior iliac crest	62.90	80.41
Gobbi *et al.*[218]	Iliac crest		31.56
Hernigou *et al.*[145]	Small volume of BM	7.14	
	Large volume of BM	80.34	

Leaving aside the inherent variability apparent in the yield of MSCs from different individuals, the results of the anatomical location study provides a clear indication that the pelvis is the optimum source for MSCs in a normal clinical setting when seeking to obtain tissue for surgery around the knee. A decision was made to limit the volume of bone marrow aspirated to approximately 10 mls in order to limit the effect of dilution by peripheral blood. However, it proved much easier to obtain this level of aspirate from the pelvis than from the other two locations, as shown by the significantly higher volumes of aspirate achieved from the pelvis when compared with the femur and tibia [10.35 ($SD = 2.68$) mls vs. 4.60 ($SD = 2.05$) mls vs. 4.95 ($SD = 1.50$) mls respectively]. This difference was consistent in nearly all individuals despite the same technique being used to obtain bone marrow from each location. It should be remembered, however, that for two individuals it was not possible to extract bone marrow from the pelvis. This did not occur in the femur or tibia, with the exception of those femoral cases where osteophytes were removed prior to aspiration.

Independent of the volume of aspiration, the pelvis also provided the highest yield of BMMCs of all three sites. There was no correlation between the total volume

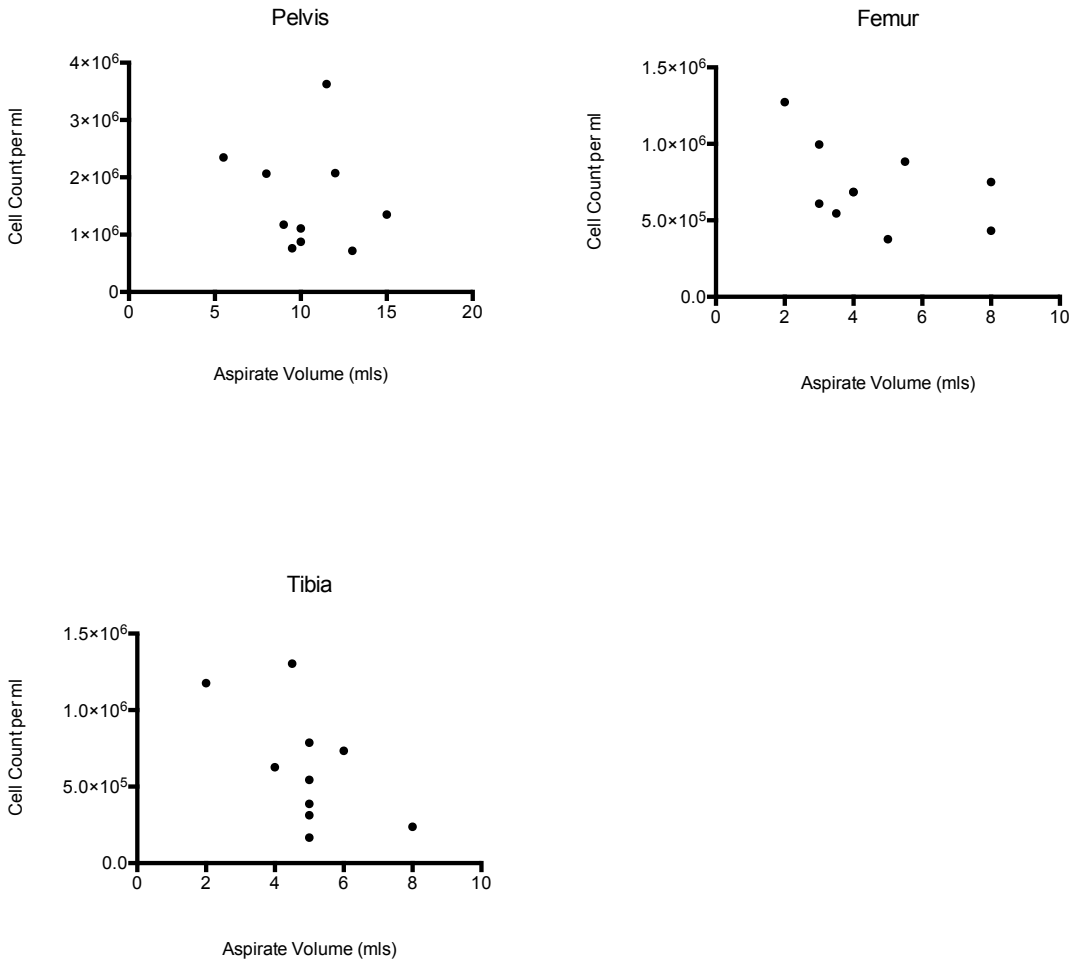

Figure 4.2 – Relationship of Aspirate Volume to Cell Count per ml

of aspirate and the number of BMMCs per ml of aspirate for the pelvis, femur, or tibia [Pearson's r = -0.153, p = 0.674 vs. r = -0.468, p = 0.219 vs. r = -0.589, p = 0.347 respectively]. This implies that even if one were to repeatedly aspirate from the femur and tibia until volumes similar to those obtained from the pelvis were reached, it would not be possible to achieve the same yield of MSCs. The higher cell count, in combination with the greater aspirate volumes, means that the pelvis is the clear first choice when seeking to maximise the yield of BMMCs.

Taking the CFU-F count results as an indicator of the MSC yield from each sample, it is clear that the pelvis is also the best source in terms of the number

Table 4.2 – Wilcoxon Matched-Pairs Signed Rank Assessment of CFU-F Yield per ml by Location

Comparison	W	p
Pelvis vs. Femur	53.0	0.004
Femur vs. Tibia	21.0	0.322
Pelvis vs. Tibia	41.0	0.037

of MSCs per 100,000 cells. As shown in Figure 3.2, this effect is even more pronounced when the greater number of cells obtained from the pelvis is taken into account. In order to confirm this, serial paired Wilcoxon matched-pairs signed rank tests were carried out (Table 4.2). These confirmed that the pelvis was superior to both the femur and tibia as a source of MSCs when using simple aspiration techniques.

The information regarding correlations between the results obtained and demographic information provides a number of important insights. The most obvious is that there are no correlations that are consistent across all anatomical locations - making it difficult to claim that any of the factors examined (age, gender, BMI, ASA grade, smoking status, and use of walking aids) have a significant impact upon the yield of cells or the CFU-F count.

The lack of any association between age and cell yield or CFU-F count is worth emphasising. As discussed on page 31, the effect of the age of an individual upon their autologous MSC population has been investigated in a number of studies with somewhat conflicting results. The long-held belief has been that the number of MSCs that an individual possesses declines with age. This is exemplified in Caplan's review article of 2007 which describes a 200-fold fall in MSC numbers during an individual's lifetime[147]. Whilst this large decrease may well be true, it is of limited applicability to orthopaedic surgery as the major part of the decline occurs during childhood.

A number of the studies that have demonstrated an age related decline in MSC yields are worthy of comment. Nishida *et al.* showed a decrease in CFU-F in females with ages ranging from four to 88 years split into 10 year wide age groups. The majority of their age groups were affected by small size and the majority of the decrease occurred in the first two decades of life[219]. In 2004, Baxter *et al.* showed a similar decrease in CFU-F numbers when comparing a group aged one to 15 years to a group aged 59-75 years[220]. Both these papers demonstrated a significant decline with age with reference to a skeletally immature group who would be unlikely to be the recipients of the majority of the cell-based therapies being investigated and developed in orthopaedics. Stolzing *et al.* demonstrated a statistically significant difference in CFU-F numbers between a group aged 0-20 and one aged 21-40. However, all but one of the high CFU-F readings were obtained from individuals aged 16 or younger indicating that the 0-20 years age group was mainly representative of a skeletally immature population[148].

Concerns about the use of MSC based therapies in older individuals may well, therefore, be predicated upon an extrapolation of the difference between the skeletally immature and older individuals to a perceived difference between two cohorts of different age. When comparisons of the MSC yield from bone marrow are undertaken in study populations that do not include skeletally immature individuals, it is much more difficult to demonstrate a significant relationship between age and MSC yield. In a group of forty individuals aged from 23 to 80 years Hyer *et al.* found no association between increasing age and declining MSC numbers[128]. This mirrors the findings of Povsic *et al.*, who found no association between age and MSCs numbers in a group of 81 individuals with an age range of 18 to 85 years[215].

The failure of the current study to demonstrate an age-related decline in MSC

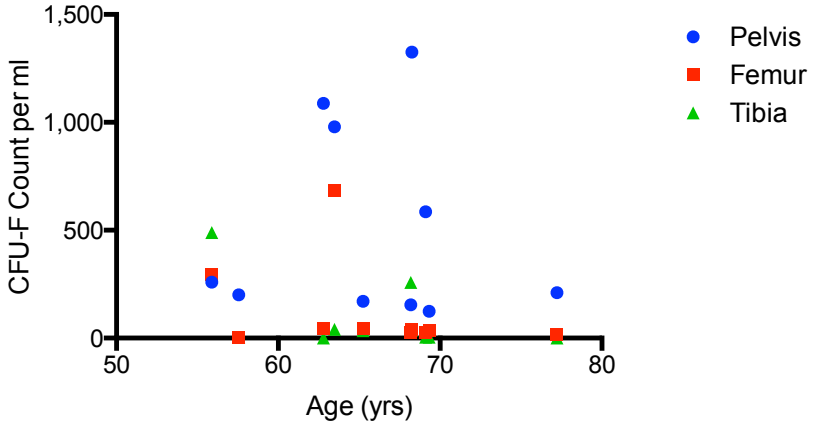

Figure 4.3 – Relationship between Age and CFU-F Count per ml of Bone Marrow Aspirate

numbers in a cohort aged from 55.9 to 77.2 years, in combination with the previously discussed results, would indicate that concerns regarding the yield of MSCs from a bone marrow aspirate of an individual in their seventies could be no more significant than for an individual in their forties. Determining the true effect of age upon MSC numbers is fraught with difficulty due to the problems caused by the large inter-subject variation that exists, combined with the potentially confounding effects of co-morbidities, smoking, medication, gender, and activity levels, as well as any number of unrecognised potential confounders. It would be necessary to conduct an extremely large study in order to account for all of these effects and as such it may not be feasible to determine the answer to this question within the current structure of clinical research where the cost involved in this type of basic research would be beyond the scope of most charitable concerns and unlikely to attract funding from a commercial source.

Overall, it is fair to conclude from this study that, allowing for the effects of inter-subject variation, the pelvis remains the optimum source for MSC harvesting irrespective of any other factors such as age, gender, medication, and co-morbidities. Again, this matches well with the previously mentioned studies looking at the use of bone marrow to obtain MSCs, which have not identified any significant re-

lationships between MSC yields and participant related factors[127, 128, 215].

B.2 Functional Comparisons

B.2.1 Growth Kinetics

Having established that the pelvis is the optimum source for harvesting MSCs in terms of yield, it was important that the functional ability of these cells also be assessed. The ability of the harvested MSCs to proliferate was the first assessment carried out, both by assessing the number of population doublings and by PrestoBlue viability assessment of the cells.

As detailed in Section 3.A.5 on page 92, there was no significant difference in the rate of population doublings between the three different anatomical locations. In order to check that converting the number of population doublings to a rate had not altered the results, the number of population doublings were also assessed. This, again, did not reveal any significant difference between the different locations [Wilcoxon matched-pairs signed rank tests: Pelvis vs. Femur W = -14.00, p = 0.383, Femur vs. Tibia W = 8.0, p = 0.578, Pelvis vs. Tibia W = 3.00, p = 0.910].

Analysis of the time spent in culture between P0 and P3 did not demonstrate any difference [Friedman test = 1.680, p = 0.474]. Figure 4.5 shows the time spent in culture plotted graphically. As can be seen, the majority of samples took around 20 days to reach the end of P3 with only a small number of outliers taking longer.

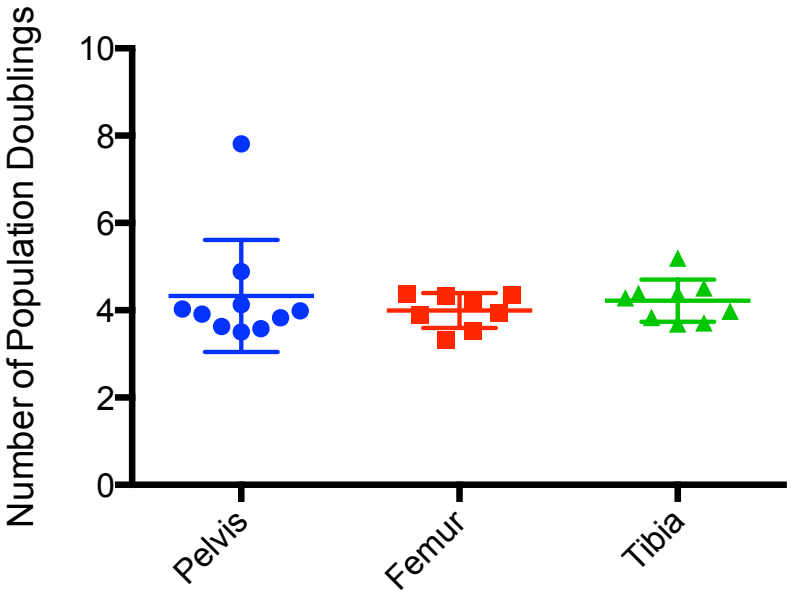

Figure 4.4 – Number of Population Doublings by Anatomical Location

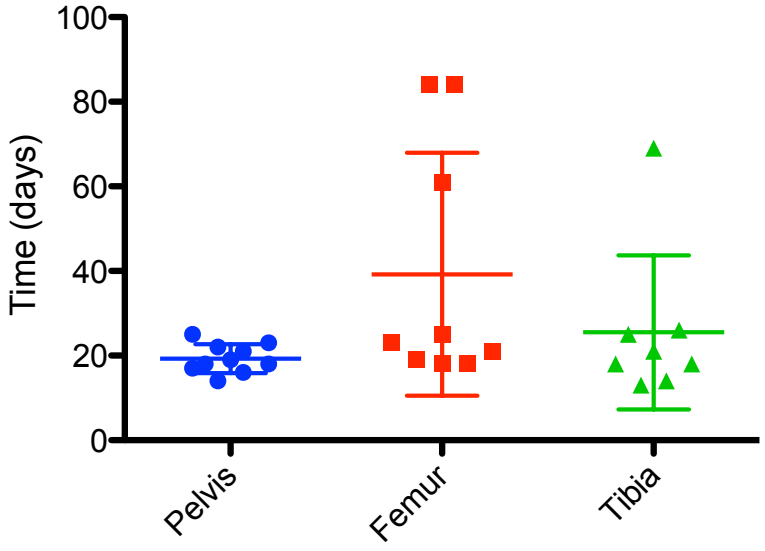

Figure 4.5 – Time from P0 to P3 by Anatomical Location

The results of the PrestoBlue assays (Figure 3.3) produced growth curves that did vary between the different anatomical locations in the case of every sample that was analysed. Whilst this is contrary to the information obtained from the population doublings results, it may be explained by the fact that the samples would in general not spend enough time in culture for the differences in growth curves to exert that great an effect. It should also be noted that there was no clear anatomical location which produced the "fastest" growth curve in every sample.

Age has previously been shown by Stolzing *et al.* to result in a plateauing of growth curves over time for MSCs harvested from bone marrow[148]. Their paper does show, however, that in the early phase of expansion in culture up to the five week point age did not appear to have an effect upon cell proliferation. In order to confirm this a Pearson's correlation (or, where appropriate, a Spearman's correlation) was carried out to compare both the population doubling rate and the time from P0 to P3 with age. These results (Table 4.3 and Figure 4.6) show that age did not have any effect upon the growth kinetics of the samples studied.

Table 4.3 – Correlation of Growth Kinetics to Age

Sample	Doubling Rate		Time from P0 to P3	
	r or r_s	p	r or r_s	p
Pelvis	-0.560	0.092	0.247	0.491
Femur	0.149	0.701	0.113	0.773
Tibia	0.027	0.949	-0.108	0.800

The population doubling rate and time from P0 to P3 results were also correlated with gender, smoking status, operation, and use of walking aids as previously carried out for the aspiration volume and CFU-F results. The only significant findings were that BMI was negatively correlated with time from P0 to P3 (r = -0.749, p = 0.033) and positively correlated with the population doubling rate (r = 0.747, p = 0.033) in the tibial samples. These results were not mirrored in

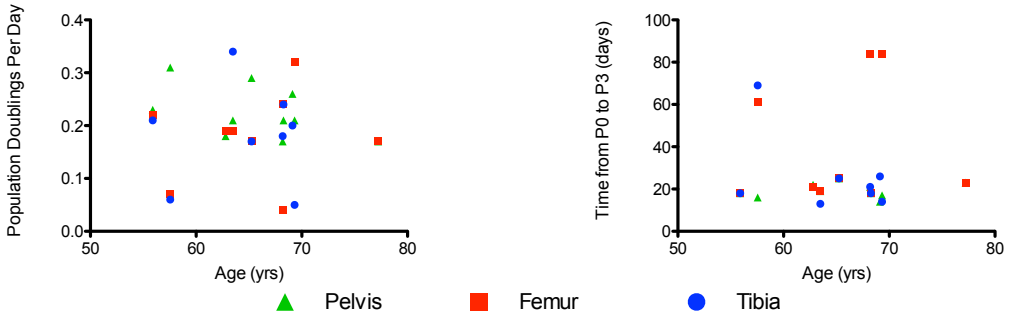

Figure 4.6 – Comparisons of Growth Kinetics against Age

either the pelvic or femoral samples. Given that there was no significant difference in the growth kinetics of the different anatomical locations, and that the tibia is a relatively poor source of MSCs when compared to the pelvis, the association between higher BMI and improved growth kinetics is of little clinical relevance.

From these results it can be concluded that the growth kinetics of harvested MSCs are similar, irrespective of the location from which they are taken. This reinforces the superiority of the pelvis as the optimum source of MSCs, since the alternative locations are unable to compensate for low initial yields of MSCs by proliferating at a faster rate. This result matches that obtained by Barbanti Brodano *et al.* who showed no difference in population doublings between iliac crest and vertebral MSCs up to approximately the 40 day mark (after which vertebral MSCs perform better)[154].

The lack of association with age is in line with work carried out by Lund *et al* on bone marrow obtained from 40 individuals ranging from 0.8 to 58 years old[221]. They showed that there was no significant difference in the proliferative abilities of the harvested MSCs up to 28 days in culture (approximately the end of P3). A more recent study by Siegel *et al.* also found no association between age and population doubling times, although they did find that females had a

shorter doubling time than males which was not demonstrated in the current study[222].

B.2.2 Cell Surface Phenotype and Differentiation Ability

Flow cytometry showed that across the samples studied (P009, P012, P012, P023, P030, and P034) expression of the positive markers of MSCs, namely CD73, CD90, and CD105 remained relatively constant. CD34 and CD45 expression was much more variable, with low levels of expression in most of the samples taken from P009 and high levels of expression in the samples taken from P034. The most relevant finding regarding the cell surface phenotype in relationship to this study is that in each individual the expression profile was relatively constant across the different sites. This would indicate that the MSCs from each location have similar cell surface phenotypes and react to being placed in a tissue culture environment in a similar manner.

It is not surprising that the antigen expression profiles identified did not match up completely with the archetypal MSC pattern of CD34-, CD45-, CD73+, CD90+, and CD105+. A number of factors can come into play to alter the identified expression pattern from what is expected. Firstly, it has previously been established that a small proportion of MSCs may be CD34+ and CD45+[223]. More recently, it has been postulated that MSCs may be to a great degree CD34+, but that the process of culturing them leads to alterations in expression levels[224]. This CD34+ population may well have contributed to some of our samples as the only method of MSC selection undertaken was adherence to tissue culture plastic. It has also been shown that the act of placing MSCs in a culture environment can lead to changes in other antigen expression levels. Mark *et al.* have shown that

the use of certain serum-free media can lead to a change in CD105 expression from 95.8% in serum containing media to only 51.7% in serum free media[58]. An analysis of bone marrow from the iliac crest of 18 individuals identified significant changes in antigen expression during the course of up to 10 passages[225]. CD73 and CD105 expression was found to be stable throughout culture but CD90 developed a small weakly positive population, having been strongly positive initially. This study also identified that even CD34- cells contained CD34 mRNA intra-cellularly which may play a role in the apparent CD34+ nature of some MSC populations. It is now believed that CD34 may act as a marker for a diverse range of progenitor cells including BM-MSCs, haematopoietic stem cells, and muscle MSCs[226]. The fact that antigen expression appears to be affected by a number of external factors and that the presence or absence of certain antigens no longer excludes a cell from being defined as an MSC means that the flow cytometry results obtained in this study should not be taken as evidence of lack of stem cell characteristics in the cultured populations. The fact that these cells were able to adhere easily to plastic and possessed clonal potential has already indicated that they possess the characteristics of MSCs.

A final step in assessing the MSC nature of the cultured cells was to undertake differentiation experiments on the P3 cells. Differentiation was carried out on all samples that produced enough cells by the end of P3 for the required experiments. Chondrogenic and osteogenic differentiation were undertaken as these are of most relevance when it comes to the use of MSCs in the treatment of orthopaedic conditions. Differentiation was assessed by histological and qPCR methods in order to allow for dual confirmation of results.

It was possible to detect chondrogenic differentiation macroscopically as samples placed in chondrogenic differentiation media converted from monolayer into a

spherical mass after approximately seven days in culture. This process usually took a few days and could be observed as a gradual rolling up from the edges of the cell monolayer. This occurred in both the 96 well plates used for histological analysis and the 24 well plates used for qPCR. This rolling up occurred in all samples placed in chondrogenic differentiation media. It did not take place in any of the control samples maintained in MesenPRO RS media. This would indicate that at least at the macroscopic level all samples were able to undergo differentiation, although it was not possible to determine the nature of the change that had taken place in this way. Alcian blue staining confirmed the presence of large quantities of GAGs in all of the chondrogenically differentiated samples, irrespective of the site from which they had been harvested. There was minimal alcian staining in the control samples. Unfortunately, it was not possible to carry out any quantification of the alcian staining as the differentiated masses would not fully release the absorbed alcian stain, even when placed in guanidine HCL for 48 hours. Sectioning of the masses was not possible due to their small size.

Osteogenic differentiation could not be seen macroscopically, but it was possible to detect areas of calcium deposition in a standard brightfield microscope image. Alizarin red staining revealed marked differences in staining between control and osteogenically differentiated samples (Figure 3.5 on page 100). The semi-quantitative results showed that there was no statistical difference between the three anatomical locations. Combined with the chondrogenic results this shows that, histologically, all cultured MSCs are able to differentiate into chondrocytes and osteoblasts irrespective of the site of harvesting.

qPCR analysis proved more challenging and as a result was carried out on fewer samples. The reasons for this were mainly related to the requirement for a sufficiently large amount of high quality RNA with which to undertake cDNA

conversion and later qPCR. Approximately 32% of the samples that underwent differentiation for qPCR analysis could not be analysed further as the extracted RNA was either of an unacceptable quality (as measured by the 280/260 ratio), or was of too low a yield to enable further experiments.

qPCR results demonstrated highly variable degrees of gene expression of the relevant genes in both the osteogenically (*RUNX2* and *SPP1*) and chondrogenically (*ACAN* and *SOX9*) differentiated samples. This variation was present both between locations and between individuals, so that it is not possible to conclude that one location differentiated better than another across samples, or that any particular location provides MSCs more prone to one form of differentiation than the other.

It is difficult to determine the reason for the relatively high number of samples that failed to produce adequate RNA. Whilst some samples were of poor quality, these tended to be combined with poor yields. It was difficult to identify a source for the poor yields, but it may have been that in some samples the cells had become inactive, especially in the chondrogenic samples where the formation of spherical masses may have starved centrally located cells of nutrients. There was evidence of chondrogenic hypertrophy in three samples (P009 Tibia, P012 Tibia, and P034 Femur) as demonstrated by up-regulation of *RUNX2* which may indicate that in some samples differentiation had already been completed. The use of biological duplicates did mean that for a number of samples there was at least one sample of RNA for further analysis.

Although differentiation capacity may not be vitally important in the setting of a single stage procedure using MSCs, it is an important assessment to carry out as it enables us to confirm that the cells being studied are MSCs - as they have proven

differentiation capability on top of the previously established plastic adherence and proliferative abilities. This assessment is also important as it is one of the few ways in which we can carry out any form of functional assessment on this type of cell. Assessment of the paracrine functions of MSCs *in vitro* has been undertaken recently, mainly to assess the immunomodulatory effects of MSCs[222, 227]. However, there is no defined standard for paracrine effects in MSCs as yet. It is also unclear as to the relevance of these effects to orthopaedics, although it has been postulated that the anti-inflammatory effects of MSCs, combined with their ability to recruit native MSC populations, may allow them to be used in the treatment of joint wide osteoarthritis[228].

In summary, all of the samples studied underwent differentiation into both osteoblasts and chondrocytes, although it was not possible to identify one anatomical location as being a superior source of MSCs for either form of differentiation.

The differentiation ability of MSCs from different sources has been compared in many studies; although it should be remembered that in a number of studies that have compared MSCs from one location to another only yield has been assessed[127, 128]. These studies can be divided into two groups: those comparing MSCs harvested from the same type of tissue in different locations (in a similar manner to the current study) and those comparing MSCs from different types of tissue such as bone marrow and adipose tissue.

Comparing different tissues, Pleumeekers *et al.* recently showed that bone marrow derived MSCs were superior to adipose tissue derived MSCs in terms of chondrogenic ability, although both sources performed relatively poorly in *in vitro* alginate bead culture when compared to chondrocytes harvested from cartilage[229]. These results are similar to those obtained by Danisovic *et al.* when using a

micromass culture system and assessing chondrogenic differentiation by immunohistochemical labelling for collagen type II[230]. The weight of evidence would now seem to support the idea that MSCs taken from bone marrow undergo chondrogenic differentiation more successfully than adipose derived ones despite the findings of a few papers such as Kern *et al.*[231] which could not demonstrate any difference using histological staining for comparison. Osteogenic differentiation has previously been compared by Vishnubalaji *et al.* who showed that bone marrow derived MSCs produce greater amounts of calcium deposition when undergoing osteogenic differentiation than those obtained from adipose tissue[232]. This result is at odds with that found by Pachón-Peña *et al.* who could not find any difference in osteogenic potential between adipose and bone marrow derived MSCs[214]. It is notable that of all the studies comparing different tissue types, only Danisovic *et al.* used different tissues from the same individuals to avoid inter-subject variations and their study had a sample size of two.

Comparison of the differentiation ability of MSCs taken from bone marrow in different locations has been carried out in relatively few studies. Using the RIA system to harvest MSCs from the femur, Uppal *et al.* found no difference between femoral and iliac crest MSCs in terms of osteogenic potential, but used different individuals in each group[186]. Barbanti Brodano *et al.*, again using unmatched samples, demonstrated improved osteogenic and chondrogenic differentiation of vertebral body MSCs when compared to iliac crest MSCs[154]. Assessment of differentiation was, however, only undertaken by assessing the percentage of staining with alizarin red, safranin O, or alcian blue. Pierini *et al.*'s comparison of the anterior and posterior iliac crests as a source of MSCs demonstrated no difference in osteogenic or chondrogenic differentiation as assessed by quantitative alizarin red staining and glycosaminoglycan content respectively[172]. All other studies previously discussed which have demonstrated the presence of bone marrow MSCs in

different anatomical locations have not included an assessment of differentiation which compared them to the gold standard of the iliac crest[125, 126, 127, 128].

It is difficult, given the small number of studies involved and the variable results, to draw firm conclusions regarding the effect of different locations on differentiation ability of MSCs in previous work. Therefore, the results of this study, which demonstrated no difference between the different locations should be seen as a likely reflection of reality.

When all the results above are taken together it is possible to say that the pelvis is a superior source of MSCs for use in orthopaedic procedures. Whilst the functional abilities of MSCs appear to be similar in all locations, the improved yield obtained from the pelvis means that it should remain the gold standard source of MSCs. It is not appropriate to attempt to use the femur or the tibia, despite the temptation of the reduced cost and reduced morbidity associated with a less complicated procedure.

C Obtaining MSCs Using a Modified Technique

The second part of this study was designed to establish if it was possible to enhance the yield of MSCs from bone marrow using simple techniques. Whilst it is well established that MSC yields from bone marrow can be greatly improved by using devices such as the RIA system[139, 154], these systems require significant amounts of extra equipment and potentially much greater levels of morbidity. In contrast, a simple bone marrow aspiration requires only a relatively inexpensive aspiration needle[15] and a syringe and it has already been demonstrated that bone

[15]The Jamshidi needles used in this study cost under £30 per unit.

marrow aspiration is associated with a very low complication rate[144].

It was hypothesised that, given the close relationship of MSCs to the trabecular bone[155], a technique to increase disruption of the trabecular bone matrix would lead to an increase in the yield of MSCs from bone marrow. The technique devised, involving movement of the aspiration needle back and forth in anterior-posterior and medial-lateral planes as well as rotating around the point of entry, was designed to disrupt a cone of trabecular bone within the metaphysis.

The control group for this study was provided by the femur location specimens from the anatomical location study and, as detailed in Section 3.B.2 on page 109, there were no significant differences between the two groups. Sample collection was carried out successfully in all cases. Having previously gained experience during the anatomical location study extreme care was taken to ensure that no osteophytes were removed prior to sampling and the aspiration needle was introduced carefully to ensure the entry hole was not larger than the needle itself.

C.1 Aspirate and MSC Yields

The modified technique produced smaller amounts of bone marrow aspirate than those obtained using the normal method. This was despite the surgeon attempting to obtain the same 10 mls of aspirate on each occasion. This result was somewhat surprising as it was believed that the disrupted trabecular bone would result in a greater volume of aspirate. The cause of this reduced volume was not clear, but it is hypothesised that the action of manipulating the aspiration needle may have widened the entry point in the cortex enough to reduce the seal around

the needle and so prevent adequate suction into the collection syringe. The modified technique did not result in any improvement in the number of cells per ml of aspirate and so overall resulted in fewer BMMCs being obtained from the bone marrow. The association of being a non-smoker with decreased cell yields in the modified technique group is likely to have little clinical relevance as this effect was avoided by using the standard collection method where smoking status had no effect. It should be noted that none of the participants were current smokers so it is not possible to determine whether current tobacco use would effect yields.

The lack of difference between the modified technique and normal collection in terms of the CFU-F count, in combination with the superior BMMC yield using the normal collection method indicates that, unfortunately, the modified technique is not able to produce an improved MSC yield.

C.2 Functional Comparison

C.2.1 Growth Kinetics

Assessment of growth kinetics demonstrated no significant difference in either population doubling rate or time from P0 to P3 between the modified technique and normal collection methods. PrestoBlue viability assessment was, again, similar to that obtained from the femoral samples in the anatomical location study. As mentioned previously, the inability of five of the samples to expand sufficiently to allow a calculation of the doubling number or carry out a PrestoBlue viability assessment may well provide some indication of the problems with using this technique to obtain MSCs. This failure of expansion rate of 50% did pose significant questions regarding the usability of any cells obtained with this method. Further

assessment was therefore carried out to determine if there were any functional differences in these cells.

C.2.2 Cell Surface Phenotype and Differentiation Ability

Cell surface phenotypes were similar between all three samples that underwent flow cytometry analysis (P032, P037, and P039). As in the anatomical location study, high levels of CD73, CD90, and CD105 expression were well maintained. There were also reasonably high levels of CD34 and CD45 expression. Whilst not fulfilling the classical definition of MSCs[54], this expression profile is not necessarily incompatible with MSCs as discussed in Section 4.B.2.2.

The small number of samples that produced sufficient cells for differentiation made a complete assessment of functional ability difficult. Macroscopically all samples appeared to undergo chondrogenic differentiation as evidenced by formation of spherical masses in culture. They also became stained with alcian blue appropriately, indicating deposition of glycosaminoglycans. Quantification of alizarin red staining did not demonstrate any difference in osteogenic ability between the modified technique and normal collection methods. qPCR results were only available for one sample, and only for osteogenic differentiation, making any conclusions difficult despite the apparent evidence of improved osteogenic properties in the normal collection method samples.

The overall findings regarding the use of the modified collection method are that, on the basis of the limited evidence available, there is likely to be no functional difference between the MSCs collected using this method and the normal method. The modified technique is, however, inferior to normal collection in terms of both

the yield of MSCs and the survivability of those cells in laboratory conditions. This survivability does not directly affect the use of samples obtained in this way in a single stage procedure, but it would be imprudent to continue with this method of collection without further investigation to identify the cause of this issue and to determine if it would have an effect upon the clinical use of these cells.

Methods of collecting MSCs are subject to a wide degree of variation due to the lack of any standardised system in most situations. The impact that changes in collection methodology can have is clearly demonstrated in the study by Hernigou *et al.* which identified the large effect that differences in syringe size and percentage of syringe filled can have on MSC yields from bone marrow. It is notable that they commented on the importance of maintaining a vacuum when seeking to extract MSCs:

> "The vacuum pressure exerted in harvesting bone marrow is one of the factors that regulates bone marrow aspiration since MSCs are attached to bone and some vacuum pressure is necessary to release them."[145, p. 2284]

A lack of vacuum may have played an important role following manipulation of the cortex although there were no obvious air leaks detected during the procedure. Previous studies have also suggested that more than eight mls should be collected in order to avoid low yields and this is one of the reasons that the target of 10 mls was used when aspirating bone marrow, although it was difficult to achieve this in any sample taken from the femur or tibia[233].

Further modification of the collection technique to try and improve the yield obtained may well be possible. It would, however, probably require either a

modification of the collection equipment used (with a consequent effect upon cost) or a significant modification of the collection method. Neither of these were done as part of this study as the aim was to attempt to improve yields with minimal implications for both cost and morbidity.

In summary, the results of this thesis show that the pelvis remains the optimum source of MSCs when compared to the femur and tibia and that it is not possible to improve this yield using simple techniques to try and break down the trabecular bone of the metaphysis.

D Study Limitations

The main limitations of this study are related to the problems caused with working with primary cells and the associated problems that were encountered with culturing and analysing samples in the laboratory.

The study was designed to have 10 subjects in both the anatomical location and modified technique sections. This number was chosen as it represented a pragmatic choice with respect to the time available, the resources required, and the likely number of individuals that could be recruited. *Post hoc* power analysis demonstrated that the study would have required 16 subjects in the anatomical location study to achieve a power of 0.8[16]. The modified collection technique would have required 206 participants in each arm, due to the independent nature of the two arms, to achieve a similar power. This should be taken into account when interpreting the results. The data from this study will allow future studies to be appropriately powered where possible.

[16] All *post hoc* power analysis was carried out using STATA 13.

The large variation in results between individuals is an underlying reason for the large sample sizes required to achieve statistical power and also makes it difficult to draw firm conclusions regarding the results obtained due to the frequent overlap in standard deviations.

The sample size and power calculations were all carried out with respect to the CFU-F per ml of bone marrow results. The problems encountered with culturing samples to provide cells for growth kinetics and differentiation studies means that achieving a similar level of power with respect to these results would be even more difficult. The small number of samples available for some experiments make it difficult to draw conclusions as to the functional abilities of harvested cells.

All samples were collected by one surgeon in this study and so if seeking to evaluate a new collection method it would be beneficial to include a variety of different surgeons to ensure the technique used is robust enough to withstand inter-operator variations. The use of a single surgeon whilst developing a new technique does offer the benefit of controlling for these errors and so should continue to be used in the initial stages of technique design.

It was not possible to avoid the formation of spherical masses in those samples undergoing chondrogenic differentiation. As this prevented quantification of the degree of alcian blue staining it would be beneficial to use a different form of quantification in future experiments. It would be possible to carry out an assessment of GAG deposition[172] but, as these assays require specialist forms of fixation, it was not possible to carry this out on samples that had been collected as part of this study.

The results obtained from the qPCR experiments were based on maintaining

samples in culture for the optimum time (21 days) as advised by the manufacturer's protocol for the differentiation media[177]. It would be possible in future work to investigate this by collecting RNA over a time course such as day 7, day 14, and day 21. This would, of course, require a greater number of cells and would therefore only be possible on high yield samples. It would then be possible to construct expression curves for each of the genes of interest over time.

Overall, future studies should be guided by the need to ensure that a large enough number of subjects are recruited to allow for the difficulties in culturing sufficient primary cells to allow for downstream experiments.

Chapter 5

Implications for Future Work

The implications of this research cover a number of different areas including research, clinical applications, and the commercial development of treatments.

A Research

As demonstrated by Figure 1.1, there has been a huge increase in MSC related research over the last 20 years. Despite this, we are still in the position of needing to find answers to basic questions such as those addressed in this thesis. This lack of answers to some of the fundamental questions concerning MSCs has not stopped research from jumping ahead to answer more downstream questions such as whether we can influence differentiation with biologically active molecules[234, 235], or physical cues such as surface topography[236, 237].

Whilst these and similar questions are important, the experiments upon which they are based may turn out to be flawed as we come to learn more about the basic mechanisms of action and the fundamental properties of MSCs in tissue

culture.

A number of questions have already been identified as important when considering studies involving MSCs, but no firm answers to these have yet been forthcoming:

- Culture media has been identified in at least one study as having an effect upon antigen expression in MSCs. Mark *et al.* showed that CD105 expression dropped in serum-free media, but without any apparent functional difference[58]. The lack of functional difference is encouraging, as serum free culture is a requirement of translational product development, but the functional abilities of these cells will need to be investigated thoroughly and any effect of antigen expression changes on cells in an *in vivo* setting will need detailed assessment.

- Confluence does not appear to effect MSC phenotype[238], despite many commercial laboratory protocols advising cells be passaged at a maximum of 70-80% confluence. It may therefore be possible to allow culturing to continue to higher densities and so reduce the total number of passages required to produce a given amount of cells.

- Time in culture has been shown to effect MSCs as shown in a number of studies which have demonstrated reduced growth kinetics with prolonged culture[154, 221, 239]. This effect needs to be fully elucidated so that culture times can be limited to a level that maintains MSC function. It is also important that any relationship between these maximum culture times and subject age be determined so that laboratory experiments can be designed to control for this.

If autologous MSCs are to be used in any clinical treatments it is vital that all of the factors affecting how they function are fully understood and controlled for in experiments. They must also be controlled for in a way that will eventually be translatable to a clinical setting. For example, there would seem to be little point in standardising all laboratory-based MSC studies using media that contained an ingredient that would not be suitable for use in a clinical application such as bovine serum. As stated in a recent review by Bara *et al.*:

> "We suggest that the global standardisation of culture parameters, although practically a challenge, would improve consistency between research groups and ultimately enhance the quality and impact of MSC research."[240, p. 1720]

Without standardisation of these parameters the inherent variability caused by the factors mentioned above and by inter-subject variation will make the drawing of meaningful conclusions regarding the clinical applicability of basic science extremely difficult[241].

Having standardised these protocols, the next task of research should be to attempt to further determine the mechanisms of action of MSCs. This is important as the qualities of a "good" MSC and a "good" source of MSCs will need to be determined by parameters drawn up around these mechanisms of action. For example, if it is determined that differentiation into specific tissue types and formation of regenerated tissue is important then differentiation ability will be of paramount importance. If, as appears more likely, paracrine effects and cell-to-cell interactions are the predominant mechanism of action, then some form of assessment of these parameters will be required to identify the best cells. This research will be able to help inform the design and implementation of translational studies aimed at introducing MSCs into widespread clinical use.

The most important implication of this study, which has been identified in some previous work[132] but is still not factored into research to a sufficient degree, is the extent to which there is wide variation in the numbers of MSCs that can be obtained from different locations. It is vital that this is factored into any laboratory based work looking at the functioning of MSCs as well as the factors mentioned above related to the effects of culture parameters on MSCs. The demonstration of the superiority of the pelvis to other sites means that laboratory studies should continue to use these as the gold standard against which to measure other sources. This is especially important given the number of studies in which there is no standard against which to measure a potential new source[125, 126, 242].

B Clinical

In a similar vein, a wide variety of clinical applications have been developed using MSCs without answers to the questions posed above. Without knowing the mechanism of action of these cells, the location of the most effective cells, and the effect of donor demographics, it is difficult to effectively design new treatments or conduct trials that are not subject to numerous confounders. These unknowns have not stopped the development and trialling of a number of clinical treatments.

The variability in yields between different individuals identified in this study means that clinical treatments must be designed and assessed in a way which can minimise this variation. Treatments must be developed in the knowledge that the pelvis continues to be the optimum site for MSC harvest. This has frequently not been the case in the studies that have been conducted to date.

A recent review of the clinical reports of MSC use in the treatment of cartilage damage identified 25 publications[243]. These ranged from single case reports to one study with 339 participants[244]. These trials have covered a range of cell types including BMMC, bone marrow derived MSCs, and adipose derived MSCs, as well as the use of PRP to supplement treatments. Studies have used immediate use protocols and cell expansion in a laboratory.

It is possible to identify a number of weaknesses that can be found in many of these studies and as stated by Grässel and Lorenz regarding their review:

> "There are several common limitations, of which at least one applies to each clinical study listed."[243, p. 9]

The lack of any form of comparator or control arm in most clinical studies makes drawing reliable conclusions about the clinical effects of a treatment very difficult. Nineteen of the 25 trials mentioned contained no form of control or comparison. As a number of studies have shown that surgery can have a large placebo effect[245] it is vital that future clinical studies involving MSCs include a control arm or comparator.

A control arm or comparator is also essential if we are to determine whether these new treatments are likely to be cost effective. The availability of relatively cheap and effective treatments such as microfracture mean that new, significantly more costly, treatments need to show that they are of sufficient clinical value to justify their implementation.

The uncertainty regarding the appropriate dose of MSCs for treating musculoskeletal conditions must also be addressed. This is partially predicated upon

a determination of whether cells are differentiating or having a paracrine effect. Some animal studies using cell tracking have already established that repair tissue is not made up solely of implanted cells[68, 246, 247], lending some support to the paracrine effect theory. Further, similar studies may help to confirm this. Having established this, the dose required for effective treatment in humans can be established. Knowledge of this is vital to help establish the feasibility of single stage procedures as very high doses of MSCs may not be available in any way other than with laboratory-based expansion. The variability of MSC yields between individuals is likely to have an impact at this point as it will be necessary to ensure that treatments would be effective across the large range of doses that could be achieved from different individuals. With no immediate assessment of dose available, clinicians must be confident that dose variation plays no role in treatment efficacy.

It is also very important that the effect of dose is examined in relation to the source of the cells being used. A number of MSC treatments are being developed using allogeneic MSCs (such as Cartistem®) which seek to avoid low yields by making use of allogeneic sources which are cultured in a laboratory. The multifactorial nature of the mechanism of action of MSCs and the other components of the stem cell niche may make a lower dose of autologous cells more effective than allogeneic ones. This requires further study.

The functional similarity of MSCs from different locations means that any location could potentially be used for the purposes of obtaining MSCs for culture expansion. It would be necessary, however, for studies to establish that the extended time spent in culture to expand to the necessary population from a low yield source, such as the tibia, would not impact upon the function of those cells once implanted.

It should also be mentioned that the methods used to assess clinical studies require development and standardisation. The selection of appropriate outcomes in clinical trials is potentially difficult given the wide variety of assessments that can be conducted. Given that the aim of any clinical treatment is to improve patients' symptoms, it may well be that the most appropriate outcome measure would be a patient reported outcome measure. It must also be remembered, however, that much of this treatment is designed to prevent long term deterioration in an individual's function. To that end, it is appropriate to objectively assess the outcome via some form of standardised clinical assessment as well as to assess repaired tissue, ideally via histological analysis and arthroscopy or MRI. Obtaining samples for histological analysis is difficult in human subjects but is vital when operating on the assumption that hyaline cartilage repair is required to prevent early deterioration and so justify these procedures.

Perhaps the most important recommendation for future clinical work is that, given the uncertainties surrounding the true identity of the cells involved, their mechanism of action, and what is required for an effective treatment, *all* patients receiving stem cell-based treatments should be enrolled in a clinical trial. This would enable the collection of as much evidence as possible to determine the true value of these treatments. This advice is similar to that given for ACI by NICE in 2005 when that treatment faced a similar lack of clear evidence as to its benefit[25].

The enrolling of all patients in clinical trials would also enable a better picture of the safety profile of these treatments to be established. A number of studies[244, 90] and a systematic review[248] have not demonstrated any significant concerns with these treatments. However, the lack of long term follow-up means that it is not possible to be completely confident about their safety, especially given their

known ability to transform into different cell types. The removal of these cells from their normal niche and reimplantation at a different location means that their ongoing behaviour is not entirely predictable.

In summary, all future stem cell treatments should be enrolled in clinical studies that need to be informed by laboratory research to determine the precise mechanisms of action of MSCs. This information must then be used to develop treatments using appropriate cell types and numbers. The outcomes of these trials will require assessment using robust measures that can be used across multiple studies in different centres.

C Commercial

The market for stem cell treatments is projected to exceed US $5 billion in 2014[249]. This has driven a large degree of innovation with a variety of products being developed for both single stage procedures (as detailed on page 22) and using laboratory culture to produce large numbers of MSCs[213, 250].

The single stage device market is especially attractive as the use of minimally-manipulated autologous cells provides a relatively easy regulatory pathway that must be traversed compared with laboratory-based treatments[251]. This has a consequent impact upon development costs and even the extent to which regulated clinical trials need to be undertaken. The results from this study show that it is vital that any such single stage treatment is designed to ensure that the large variability in the number of cells that can be obtained from any individual is factored in. Failure to account for this in product development and Phase II and III trials is likely to result in study results that are unlikely to be applicable

to the general population of patients.

The appropriate development of these commercial products can be improved by the development of collaborations between commercial entities, clinicians, and researchers to enable the findings of studies such as this one to be more easily incorporated into product development and also allow basic research to be conducted in a way which enables easy transfer into the clinical environment.

Chapter 6

Conclusion

Musculoskeletal conditions represent a large burden of disease. We currently have difficulty in effectively treating the early stages of a number of these conditions such as focal cartilage damage, osteoarthritis, and tendon damage. Orthopaedic surgery has been at the forefront of the development of cellular therapies since the introduction of procedures such as microfracture and ACI for the treatment of chondral defects in the early 1990s. As part of this work, stem cells, and most commonly MSCs, have been investigated in an attempt to develop treatments for these conditions.

MSC based treatments have developed along two separate paths:

- Single stage procedures designed for the processing and immediate use of MSCs from tissues such as bone marrow and fat;

- Laboratory-based treatments in which harvested MSCs are expanded in the laboratory prior to reimplantation in an area of tissue damage.

The development of clinical applications and the use of these techniques has grown rapidly over recent years. This is despite the lack of answers to some of the more basic questions surrounding these cells, such as their exact mechanism of action and how can they most successfully be harvested and cultured.

The aim of this thesis was to address some of these questions with particular reference to the use of MSCs in single stage procedures to treat cartilage damage around the knee. To that end it was decided that bone marrow was the most practical source due to the fact that it could be readily obtained by an orthopaedic surgeon and the potential sites of harvesting are close to the area of operation (in this case the knee).

The pelvis has previously been the main source of bone marrow for obtaining MSCs and as such is currently considered the gold standard. It was therefore decided to compare this source to bone marrow obtained from the femur and tibia. A modified collection technique using standard bone marrow aspiration equipment was also investigated to see if it was possible to improve the number of MSCs that could be obtained from bone marrow.

Samples were obtained from individuals undergoing knee replacement and analysed to obtain the following information:

- Aspiration volume;
- Mononuclear cell yield;
- CFU-F count;

- Growth kinetics;

- Cell surface phenotype after passage to P3;

- Osteogenic and chondrogenic differentiation potential as assessed by histological staining and qPCR.

Analysis of these results showed that the pelvis was superior to both the femur and tibia in terms of aspiration volumes, yield of BMMCs per ml, and CFU-F counts per ml. There was no significant functional difference between the cells obtained from different locations.

Modifying the collection technique to try and disrupt more of the trabecular bone matrix prior to aspiration was not successful in improving MSC yields and had a detrimental effect upon the total volume of bone marrow aspirated.

The other significant finding from this study was a confirmation of the high degree of variability in MSC yields between different individuals. This variation was not found to be related to any one factor such as age, BMI, or smoking status.

The inter-subject variability did have an effect upon the ability to draw definitive conclusions from the data. Future studies should bear this in mind and may well require relatively large sample numbers if they are to ensure that they are sufficiently powered. This is especially true given the number of samples that failed to expand in culture or provide results in experiments in this work.

Overall, these results show that treatments that seek to use MSCs in single stage procedures should use the pelvis as their source of bone marrow. Whilst proced-

ures relying on laboratory expansion may be able to achieve the desired number of cells from other locations, other work has demonstrated the possible loss of expansion potential with extended culture. Simple modifications of the collection technique are not useful in improving sample yields.

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Appendix A

Participant Information

The tables on the following four pages detail the demographic and operative information for the participants involved in the study.

Table A.1 – Participant Baseline Information and Operation Details

ID Number	Gender	Smoking Status	Walking Aids	BMI	ASA Grade	Operation	Age at Operation
P008	Male	Ex-smoker	1 stick	27.53	2	Right TKR	77.22
P009	Male	Non-smoker	1 stick	27.58	3	Right Medial UKR	65.25
P011	Male	Non-smoker	None	26.98	1	Right Lateral UKR	69.32
P012	Female		None	36.51	2	Right Medial UKR	63.48
P015	Male	Non-smoker	1 stick	25.44	3	Right Medial UKR	69.11
P016	Female	Non-smoker	None	19.92	2	Right Medial UKR	57.55
P021	Female	Non-smoker	None	34.38	1	Left Medial UKR	55.89
P023	Male	Ex-smoker	None	27.02	2	Left Medial UKR	68.26
P030	Female	Non-smoker	None	29.30	2	Left TKR	68.19
P031	Male	Ex-smoker	None	26.82	2	Left Medial UKR	68.11
P032	Female	Ex-smoker	1 stick	25.82	2	Right TKR	77.05
P034	Male	Ex-smoker	None	28.72	2	Right Medial UKR	62.79
P035	Male	Non-smoker	None	23.11	1	Right Lateral UKR	65.32
P036	Female	Non-smoker	None	31.63	2	Right TKR	69.98
P037	Male	Non-smoker	None	29.67	1	Left Lateral UKR	45.57
P038	Male	Non-smoker	None	27.41	1	Left Medial UKR	61.96
P039	Female	Ex-smoker	None	35.34	1	Left Medial UKR	54.44
P040	Female	Non-smoker	None	27.85	2	Left Medial UKR	75.16
P041	Male	Ex-smoker	1 stick	34.42	3	Right Medial UKR	79.54
P042	Male	Ex-smoker	None	28.99	1	Right Medial UKR	59.21

Table A.2 – Participant Co-morbidities and Medication

ID Number	Co-morbidities	Medication
P008	Asthma, hypothyroidism,glaucoma	Symbicort, levothyroxine,lansoprazole, aspirin
P009	Angina, IHD, hypertension, NIDDM	Losartan, metformin, paracetamol, aspirin, bendroflumethiazide
P011	Nil Sig	None
P012	Hypertension, asthma, thyroidectomy	Salbutamol, levothyroxine, lisinopril
P015	Angina, hypertension	amlodipine, atenolol, aspirin, oxynorm, nitrazepam, pregabalin
P016	Hypertension	amlodipine, co-codamol
P021	Previous DVT	ibuprofen, paracetamol, codeine
P023	Tinitus, back pain	tramadol
P030	Hypertension	amlodipine, bendroflumethiazide, naproxen
P031	Previous DVT, CVA	clopidogrel
P032	Hypothyroidism	levothryoxine, paracetamol, ibuprofen gel
P034	Hypertension	candesartan, bendrofluazide,
P035	Previous spinal fusion	omeprazole
P036	Anxiety, back pain, hypertension	losartan
P037	Previous ACL reconstruction	co-codamol
P038	Nil sig	ibuprofen
P039	Back pain, depression	co-dydramol
P040	Hypertension, hypothyroidism, NIDDM	bisoprolol, levothyroxine, metformin, paracetamol, ramipril, spironolactone
P041	Hypertension, myocardial infarction	aspirin, doxazosin, paracetamol, propranolol
P042	Hiatus hernia	paracetamol, codeine

Table A.3 – Excluded Sample Participant Baseline Information and Operation Details

ID Number	Gender	Smoking Status	Walking Aids	BMI	ASA Grade	Operation	Age at Operation
P002	Male	Non-smoker	None	25.38	2	Right Medial UKR	66.92
P004	Female	Non-smoker	None	25.48	1	Left Lateral UKR	52.30
P005	Female	Ex-smoker	None	26.29	2	Right Medial UKR	66.00
P006	Female	Current	None	34.82	2	Left Medial UKR	53.36
P013	Female	Non-smoker	1 stick	33.78	2	Right Medial UKR	75.94
P014	Male	Non-smoker	None	34.11	3	Left Medial UKR	64.01
P017	Male	Ex-smoker	None	39.87	2	Left TKR	61.67
P019	Female	Non-smoker	1 stick	38.91	2	Right Medial UKR	73.12
P025	Male	Ex-smoker	None	30.30	2	Right Medial UKR	59.23

Table A.4 – Excluded Sample Participant Co-morbidities and Medication

ID Number	Co-morbidities	Medication
P002	Previous DVT & PE, Pelvic fracture	lisinopril, amlodipine
P004	Migraines	None
P005	Migraines	None
P006	Hypertension	amlodipine
P013	Hypertension, breast cancer	lisinopril, bendroflumethiazide, co-codamol
P014	Hypertension, IHD	aspirin, amlodipine, naproxen, co-codamol
P017	Hypertension, aortic valve disease, NIDDM, anaemia	lisinopril, aspirin, furosemide, spironolactone, tramadol, paracetamol, oramorph, metformin
P019	Hypertension, back pain	Adcal D3, bendroflumethiazide, co-codamol, piroxicam gel
P025	Hypertension	lisinopril, lercanidipine

Appendix B

Detailed Methodology

A Bone Marrow Processing

1. Bone marrow was transferred from the EDTA collection tube to a 15 ml falcon tube and then mixed with RPMI media (Life Technologies, Carlsbad, USA[17]) in a 1:1 ratio.

2. Diluted bone marrow was then passed through a 70 μm cell strainer (Fisherbrand).

3. 10 mls of filtered bone marrow was then layered onto 5 mls of Lymphoprep^{TM} (Alere, UK).

4. This was centrifuged at 400 x g for 25 minutes with no brake.

5. The mononuclear cell layer was then removed and washed twice with MesenPRO RS media.

[17]All cell culture media was obtained from Life Technologies unless otherwise stated.

6. After the second wash a cell count was undertaken with a Scepter^{TM} cell counter (Merck Millipore, Billerica, MA, USA).

7. Cells were then placed in an appropriately sized tissue culture flask in MesenPRO RS media and left for 48 hours before non-adherent cells were removed.

8. 200,000 cells from each sample were used to perform a CFU-F count experiment in duplicate.

B PrestoBlue Viability Assay

1. PrestoBlue reagent was mixed with MesenPRO RS media to form a 10% solution.

2. Well media was exchanged for the reagent solution.

3. Cells were incubated for 60 minutes in a 37°C incubator with 5% carbon dioxide.

4. Media was then removed and 100 µl was placed in triplicate into a 96 well plate.

5. Fluorescence was then measured using a FLUOstar OPTIMA (BMG Labtech, Aylesbury, UK) with an excitation wavelength of 535 nm and an emission wavelength of 615 nm.

6. No-cell controls were subtracted from the fluorescence value of each experimental well and relative fluorescence was then plotted over time.

C CFU-F Protocol

1. 100,000 cells were placed into a well of a 6-well tissue culture plate and fed with MesenPRO RS media.

2. Cells were cultured for 14 days with media being changed every two or three days.

3. On day 14 cells were fixed with methanol for 10 minutes and then washed with distilled water.

4. Giemsa stain was applied for 10 minutes and then washed with distilled water.

5. Plates were allowed to dry and colonies were then counted. A colony was included if it was at least 1mm across and stained sufficiently to be visible to the naked eye.

D Cell Passage

1. Media was removed and tissue flasks washed with PBS.

2. TrypLE^{TM}dissociation reagent (Life Technologies) was added and the cells placed in an incubator at 37°C for five to seven minutes.

3. Dissociation was confirmed by microscopy.

4. TrypLE^{TM}was inactivated by addition of an equal volume of MesenPRO RS media.

5. Tissue culture flasks were washed 3 times and the cell suspension was then placed in a falcon tube.

6. The suspension was centrifuged at 1,400 rpm for 5 minutes.

7. The resultant cell pellet was re-suspended in MesenPRO RS media and a cell count performed using a haemocytometer.

8. Cells were placed in tissue culture flasks at a seeding density of 3 to 5,000 cells per cm^2.

D.1 Long Term Cell Storage

Cells that were found to be surplus to requirements were stored at -80°C using the following protocol:

1. Cells were resuspended in MesenPRO RS media at 2 x 10^7cells per ml.

2. Freezing media was made as below:

a) MesenPRO RS Media 60%;

b) Foetal Bovine Serum 20%;

c) DMSO[18] 20%.

3. 1 x10^7cells were mixed with 500 µl of freezing media and placed in a -80°C freezer.

E Alizarin Red Quantification

1. Wash with 200 µl ddH_2O twice.
2. Fix in 100 µl of 70% cold ethanol.
3. Wash with 200 µl ddH_2O twice.
4. Allow to dry.
5. Stain with 100 µl Alizarin Red for 10 minutes at room temperature.
6. Wash with 200 µl ddH_2O twice.
7. Store at -20°C until dye extraction.

The following process was used to quantify alizarin staining:

[18]Dimethyl sulfoxide

1. Add 200 µl of 10% acetic acid to each well.

2. Incubate for 10 minutes on an orbital shaker, making sure all the staining is dissolved in acid.

3. Collect all the volume of acetic acid into 1.5ml eppendorfs.

4. Add 80 µl of 10% ammonium hydroxide to neutralise, vortex.

5. Load 80 µl into 96 well plate in triplicates and read absorbance at 405 nm on SpectraMax® Plus^{384} absorbance microplate reader (Molecular Devices, Wokingham, UK).

6. Plot treatments relative to control.

F Flow Cytometry Protocol

Flow cytometry was carried out on an BD LSRFortessa Flow Cytometer. The antibodies used are detailed in Table 2.9. The protocol below includes the optional steps for magnetic bead depletion and FMO samples which were not used in the analysis of study participants' samples.

Flow cytometry buffer which was used as the media for all flow cytometry steps contained the following:

- 500 ml IMDM (Life Technologies, UK)

- 50 mls FBS (LabTech, UK)

- 500 µl DNase (Roche, UK)

1. Wash aliquot of cells with flow cytometry media (at least 500,000 cells).

2. Take aliquot of cells from each sample and mix together to form combined unstained sample for calibration of flow cytometer.

3. Magnetically label and separate cells (optional).

    a) Place cells in a 15 ml conical tube (up to 10^7cells).

    b) Centrifuge at 1,200 rpm for 10 minutes and then resuspend in 80 µl of media.

    c) Add 10 µl of FcR blocking reagent and 10 µl of CD271 antibody.

    d) Mix well and refrigerate for 10 minutes.

    e) Wash and resuspend in 70 µl of media.

    f) Add 10 µl of FcR blocking reagent and 20 µl of microbeads.

    g) Refrigerate for 15 minutes.

    h) Wash.

i) Place magnetic bead column in separator.

j) Wash with media.

k) Add cell suspension to column.

l) Wash with media three times.

m) Total effluent is unlabelled fraction (CD271 deplete).

n) Remove column from separator.

o) Wash with media using plunger to force labelled cells to dissociate from beads.

p) Label this sample as CD271 enriched.

4. Add antibodies to tubes.

a) One unstained sample.

b) One single antibody tube for each antibody (to act as compensation controls).

c) One FMO sample containing every other antibody except for the target antibody (optional).

d) One test tube containing CD34, CD45, and CD73 antibodies.

e) One test tube containing CD90 and CD105 antibodies.

f) One test tube containing all antibodies.

5. Add at least 100,000 cells to each test tube and unstained sample.

6. Add BD compensation beads to each single antibody tube as per manufacturer's protocol and to a separate unstained tube.

7. Leave on ice for 30 minutes.

8. Add 3 ml of PBS.

9. Centrifuge at 1,200 rpm for 5 minutes.

10. Decant off excess supernatant.

11. Vortex to resuspend cells.

12. Add 1 µl of 1:100 hoechst to each test tube to act as Live:Dead stain.

13. Run samples on Flow Cytometer.

The unstained sample was used to establish the correct voltages to use to ensure that the forward scatter (FSC) and side scatter (SSC) values obtained from the sample were within the recordable range. It was necessary to determine separate FSC and SSC values for the cells and the beads as they were not identical and

so voltages had to be adjusted between using the beads for compensation and running the actual samples.

Having established the correct FSC and SSC values, the single antibody tubes were run through the flow cytometer. It was then possible to use the FACSDiva software to compensate for any overlap in spectra between the different antibodies used. The test samples could then be run and results stored for later analysis.

Appendix C

Real Time PCR

A TaqMan Primer Details

Primers were all obtained from Life Technologies. With the exception of *PGK1* the primer used was the one suggested by the manufacturer to provide best results for gene expression. A different *PGK1* primer was used in order to maintain consistency with the primer included in the Endogenous Control plate that was used to select endogenous controls.

Table C.1 – TaqMan Primer Information

Gene Name	Symbol	Use	Assay ID
Glyceraldehyde 3-phosphate dehydrogenase	*GAPDH*	Endogenous Control	Hs02758991_g1
Phosphoglycerate kinase 1	*PGK1*	Endogenous Control	Hs99999906_m1
Osteopontin / Secreted phosphoprotein 1	*SPP1*	Osteogenesis	Hs00959010_m1
Runt-related transcription factor 2	*RUNX2*	Osteogenesis	Hs00231692_m1
Osteocalcin / Bone gamma- carboxyglutamate (gla) protein	*BGLAP*	Osteogenesis	Hs01587814_g1
SRY (sex determining region Y)-box 9	*SOX9*	Chondrogenesis	Hs01001343_g1
Aggrecan	*ACAN*	Chondrogenesis	Hs00153936_m1
Collagen 2A1	*COL2A1*	Chondrogenesis	Hs00264051_m1

Appendix D

Correlation Results

The following tables contain the full correlation results discussed in Chapters 3 and 4. Table D.1 details how dichotomous data were coded for statistical analysis.

Table D.1 – Dichotomous Data Coding For Analysis

Variable	Coded as "0"	Coded as "1"
Operation	UKR	TKR
Gender	Male	Female
Smoking Status	Non-smoker	Ex-smoker
Walking status	Independent	Uses 1 stick

Table D.2 – Correlation Results - Pelvis

Comparison	Test Used	Test Statistic	p value
Aspirate vol. vs. age	Pearson	-0.310	0.384
Aspirate vol. vs. BMI	Pearson	-0.110	0.765
Aspirate vol. vs. Gender	Point Biserial	0.046	0.900
Aspirate vol. vs. Smoking Status	Point Biserial	-0.014	0.972
Aspirate vol. vs. ASA grade	One-way ANOVA	0.110	0.901
Aspirate vol. vs. Walking status	Point Biserial	-0.167	0.645
Aspirate vol. vs. Operation	Point Biserial	-0.252	0.482
Cell Count per ml vs. age	Pearson	0.164	0.650
Cell Count per ml vs. BMI	Pearson	0.542	0.106
Cell Count per ml vs. Gender	Point Biserial	0.163	0.653
Cell Count per ml vs. Smoking Status	Point Biserial	0.246	0.523
Cell Count per ml vs. ASA grade	One-way ANOVA	0.630	0.559
Cell Count per ml vs. Walking status	Point Biserial	-0.398	0.255
Cell Count per ml. vs. Operation	Point Biserial	-0.013	0.971
CFU-F per ml vs. Age	Pearson	-0.039	0.916
CFU-F per ml vs. BMI	Pearson	0.242	0.501
CFU-F per ml vs. Gender	Point Biserial	-0.200	0.580
CFU-F per ml vs. Smoking Status	Point Biserial	0.655	0.055
CFU-F per ml vs. ASA Grade	One-way ANOVA	0.870	0.459
CFU-F per ml vs. Walking Status	Point Biserial	-0.270	0.450
CFU-F per ml. vs. Operation	Point Biserial	-0.360	0.307
Doubling Rate vs. Age	Pearson	-0.560	0.092
Doubling Rate vs. BMI	Pearson	-0.369	0.294
Doubling Rate vs. Gender	Point Biserial	-0.141	0.699
Doubling Rate vs. Smoking Status	Point Biserial	-0.040	0.921
Doubling Rate vs. ASA Grade	One-way ANOVA	0.440	0.660
Doubling Rate vs. Walking Status	Point Biserial	n/a	n/a
Doubling Rate vs. Operation	Point Biserial	-0.602	0.065
Time from P0 to P3 vs. Age	Pearson	0.247	0.491
Time from P0 to P3 vs. BMI	Pearson	0.236	0.512
Time from P0 to P3 vs. Gender	Point Biserial	-0.192	0.595
Time from P0 to P3 vs. Smoking Status	Point Biserial	0.327	0.391
Time from P0 to P3 vs. ASA Grade	One-way ANOVA	0.300	0.749
Time from P0 to P3 vs. Walking Status	Point Biserial	0.263	0.463
Time from P0 to P3 vs. Operation	Point Biserial	0.397	0.256

Table D.3 – Correlation Results - Femur

Comparison	Test Used	Test Statistic	p value
Aspirate vol. vs. age	Pearson	-0.556	0.950
Aspirate vol. vs. BMI	Pearson	0.023	0.949
Aspirate vol. vs. Gender	Point Biserial	0.607	0.063
Aspirate vol. vs. Smoking Status	Point Biserial	-0.274	0.476
Aspirate vol. vs. ASA grade	One-way ANOVA	0.310	0.744
Aspirate vol. vs. Walking status	Point Biserial	-0.138	0.703
Aspirate vol. vs. Operation	Point Biserial	-0.146	0.687
Cell Count per ml vs. age	Pearson	-0.218	0.545
Cell Count per ml vs. BMI	Pearson	0.381	0.277
Cell Count per ml vs. Gender	Point Biserial	-0.166	0.648
Cell Count per ml vs. Smoking Status	Point Biserial	0.183	0.637
Cell Count per ml vs. ASA grade	One-way ANOVA	0.420	0.675
Cell Count per ml vs. Walking status	Point Biserial	-0.457	0.184
Cell Count per ml. vs. Operation	Point Biserial	-0.427	0.218
CFU-F per ml vs. Age	Spearman	-0.417	0.265
CFU-F per ml vs. BMI	Spearman	0.782	0.010
CFU-F per ml vs. Gender	Point Biserial	0.498	0.143
CFU-F per ml vs. Smoking Status	Point Biserial	-0.203	0.600
CFU-F per ml vs. ASA Grade	One-way ANOVA	0.180	0.842
CFU-F per ml vs. Walking Status	Point Biserial	-0.284	0.427
CFU-F per ml. vs. Operation	Point Biserial	-0.234	0.515
Doubling Rate vs. Age	Pearson	0.149	0.701
Doubling Rate vs. BMI	Pearson	0.272	0.479
Doubling Rate vs. Gender	Point Biserial	-0.519	0.153
Doubling Rate vs. Smoking Status	Point Biserial	0.194	0.646
Doubling Rate vs. ASA Grade	One-way ANOVA	1.850	0.237
Doubling Rate vs. Walking Status	Point Biserial	-0.056	0.886
Doubling Rate vs. Operation	Point Biserial	-0.468	0.204
Time from P0 to P3 vs. Age	Pearson	0.113	0.773
Time from P0 to P3 vs. BMI	Pearson	-0.414	0.268
Time from P0 to P3 vs. Gender	Point Biserial	0.196	0.614
Time from P0 to P3 vs. Smoking Status	Point Biserial	-0.552	0.156
Time from P0 to P3 vs. ASA Grade	One-way ANOVA	0.240	0.792
Time from P0 to P3 vs. Walking Status	Point Biserial	-0.284	0.460
Time from P0 to P3 vs. Operation	Point Biserial	0.266	0.489

Table D.4 – Correlation Results - Tibia

Comparison	Test Used	Test Statistic	p value
Aspirate vol. vs. age	Pearson	-0.118	0.746
Aspirate vol. vs. BMI	Pearson	-0.864	0.001
Aspirate vol. vs. Gender	Point Biserial	-0.041	0.911
Aspirate vol. vs. Smoking Status	Point Biserial	0.034	0.930
Aspirate vol. vs. ASA grade	One-way ANOVA	0.140	0.874
Aspirate vol. vs. Walking status	Point Biserial	-0.124	0.733
Aspirate vol. vs. Operation	Point Biserial	0.017	0.964
Cell Count per ml vs. age	Pearson	-0.432	0.213
Cell Count per ml vs. BMI	Pearson	0.783	0.007
Cell Count per ml vs. Gender	Point Biserial	0.340	0.252
Cell Count per ml vs. Smoking Status	Point Biserial	-0.326	0.392
Cell Count per ml vs. ASA grade	One-way ANOVA	1.720	0.247
Cell Count per ml vs. Walking status	Point Biserial	-0.400	0.253
Cell Count per ml. vs. Operation	Point Biserial	-0.355	0.314
CFU-F per ml vs. Age	Spearman	-0.357	0.385
CFU-F per ml vs. BMI	Spearman	0.905	0.002
CFU-F per ml vs. Gender	Point Biserial	0.562	0.091
CFU-F per ml vs. Smoking Status	Point Biserial	-0.332	0.382
CFU-F per ml vs. ASA Grade	One-way ANOVA	1.360	0.317
CFU-F per ml vs. Walking Status	Point Biserial	-0.302	0.397
CFU-F per ml. vs. Operation	Point Biserial	0.131	0.719
Doubling Rate vs. Age	Pearson	0.027	0.949
Doubling Rate vs. BMI	Pearson	0.747	0.033
Doubling Rate vs. Gender	Point Biserial	0.173	0.682
Doubling Rate vs. Smoking Status	Point Biserial	0.448	0.314
Doubling Rate vs. ASA Grade	One-way ANOVA	0.350	0.723
Doubling Rate vs. Walking Status	Point Biserial	0.023	0.957
Doubling Rate vs. Operation	Point Biserial	-0.005	0.991
Time from P0 to P3 vs. Age	Spearman	-0.108	0.800
Time from P0 to P3 vs. BMI	Spearman	-0.659	0.076
Time from P0 to P3 vs. Gender	Point Biserial	0.261	0.532
Time from P0 to P3 vs. Smoking Status	Point Biserial	-0.201	0.666
Time from P0 to P3 vs. ASA Grade	One-way ANOVA	0.330	0.733
Time from P0 to P3 vs. Walking Status	Point Biserial	n/a	n/a
Time from P0 to P3 vs. Operation	Point Biserial	-0.094	0.826

Table D.5 – Correlation Results - Modified Technique

Comparison	Test Used	Test Statistic	p value
Aspirate vol. vs. age	Pearson	-0.331	0.351
Aspirate vol. vs. BMI	Pearson	0.278	0.437
Aspirate vol. vs. Gender	Point Biserial	-0.114	0.754
Aspirate vol. vs. Smoking Status	Point Biserial	-0.186	0.607
Aspirate vol. vs. ASA grade	One-way ANOVA	0.050	0.950
Aspirate vol. vs. Walking status	Point Biserial	-0.186	0.607
Aspirate vol. vs. Operation	Point Biserial	0.047	0.899
Cell Count per ml vs. age	Pearson	-0.170	0.639
Cell Count per ml vs. BMI	Pearson	0.818	0.004
Cell Count per ml vs. Gender	Point Biserial	0.142	0.406
Cell Count per ml vs. Smoking Status	Point Biserial	0.652	0.041
Cell Count per ml vs. ASA grade	One-way ANOVA	0.800	0.488
Cell Count per ml vs. Walking status	Point Biserial	0.181	0.616
Cell Count per ml. vs. Operation	Point Biserial	-0.161	0.657
CFU-F per ml vs. Age	Spearman	-0.500	0.391
CFU-F per ml vs. BMI	Spearman	0.100	0.873
CFU-F per ml vs. Gender	Point Biserial	0.557	0.095
CFU-F per ml vs. Smoking Status	Point Biserial	0.454	0.188
CFU-F per ml vs. ASA Grade	One-way ANOVA	0.130	0.880
CFU-F per ml vs. Walking Status	Point Biserial	0.172	0.635
CFU-F per ml. vs. Operation	Point Biserial	0.151	0.677
Doubling Rate vs. Age	Spearman	-0.200	0.747
Doubling Rate vs. BMI	Spearman	-0.300	0.624
Doubling Rate vs. Gender	Point Biserial	0.410	0.493
Doubling Rate vs. Smoking Status	Point Biserial	-0.450	0.448
Doubling Rate vs. ASA Grade	One-way ANOVA	0.230	0.815
Doubling Rate vs. Walking Status	Point Biserial	0.194	0.754
Doubling Rate vs. Operation	Point Biserial	0.516	0.374
Time from P0 to P3 vs. Age	Spearman	0.154	0.805
Time from P0 to P3 vs. BMI	Spearman	0.205	0.741
Time from P0 to P3 vs. Gender	Point Biserial	-0.591	0.294
Time from P0 to P3 vs. Smoking Status	Point Biserial	0.325	0.594
Time from P0 to P3 vs. ASA Grade	One-way ANOVA	0.580	0.631
Time from P0 to P3 vs. Walking Status	Point Biserial	-0.061	0.922
Time from P0 to P3 vs. Operation	Point Biserial	-0.400	0.505

Appendix E

Study Paperwork

A Patient Information Sheet: Mesenchymal Stem Cell Research

We are inviting you to take part in a research project. Before you decide whether to take part it is important for you to know why the research is being done, and what it will involve for you. Please take time to read the following information carefully to decide whether you wish to take part. Please feel free to talk to others about the study if you wish.

What is the Research Project about?

We are investigating the use of a type of cell called a mesenchymal stem cell (MSC) in the treatment of orthopaedic conditions affecting cartilage and tendon. These are cells that are present in tissue such as bone marrow and fat. They are thought to be able to help repair damaged tissue.

These cells are currently being used in systems designed to concentrate them into small volumes that can be used to help heal damaged cartilage, tendon and bone.

The aim of this project is to determine what is the best way of obtaining these cells from patients and if we are able to obtain large numbers of cells from the currently available technology which works by spinning bone marrow samples rapidly whilst a patient is in theatre undergoing their operation.

This project is a pilot study and forms part of the DPhil of Benjamin Davies who is the chief investigator.

Why have I been chosen to take part?

You have been chosen to consider taking part in this study as you are due to have a replacement of either your hip or your knee joint at the Nuffield Orthopaedic Centre.

What will it involve if I decide to take part?

You will be allocated into 1 of 2 groups depending on the operation that you are having.

Group 1 (total or unicompartmental knee replacement participants) – during your operation, after your anaesthetic, you will have 3 samples of bone marrow taken via a needle, these sample(s) would not normally be taken as part of your routine

surgery. There will be one sample taken from each of the bones being operated on (the femur (thigh bone) and tibia (lower leg bone)) and one sample taken from your iliac crest (pelvis). You will have a small extra dressing on the iliac crest site. Ten participants will be recruited into this group. These will be the first ten participants in the study. Each sample will be approximately 10 to 15 mls.

Group 2 (total or unicompartmental knee or total hip replacement) – during your operation, after your anaesthetic, you will have 2 samples of bone marrow taken, these sample(s) would not normally be taken as part of your routine surgery. These will be from either the femur or tibia. One will be taken with a needle and the other after the needle has been used to loosen the material within the bone or when the bone has been opened as part of your normal operation. Twenty participants will be recruited into this group. These will be the next twenty participants. Each sample will be approximately 10 to 15 mls.

We do not anticipate any additional significant side effects from the donation of bone marrow in participants undergoing total or unicompartmental joint replacement.

We will also collect and store information, possibly including x-rays and scans, from your health care records for research. This information will be held in an anonymous form.

What are the advantages and disadvantages of taking part?

There are no significant disadvantages in contributing to this study. If bone marrow is taken from your pelvis you will have an extra dressing. There is

a small risk of some local pain in the pelvis but if present should respond to the painkillers you are given as part of your normal treatment. There is also a very small risk of infection but this is no higher than that associated with your operation and minimised by taking samples in theatre.

There are no advantages to you, but the results of research using samples taken from you and others may help patients in the future.

What will happen to any samples that I give?

The samples that you give will be used to help evaluate the currently available techniques and to help determine where is best to obtain these cells from. You are asked to donate your tissue freely for research and you will not receive a financial reward either now or in the future. Your samples will not be sold for profit to other researchers. Your samples may be used for research that may lead to the development of new drugs or therapies, which may eventually be marketed, and companies may sell these drugs for profit.

What will happen to my samples and data when this research project ends?

With your permission, any remaining tissue samples can be transferred to the Oxford Musculoskeletal Biobank (OMB) when no longer required by the project. The OMB is an ethically approved and regulated licensed tissue and data bank for existing and prospective research projects that study diseases of bone, joint and other soft tissues. Samples stored by the OMB will only be released to ethically

approved studies. These studies may include commercial and/or genetic research in the UK and the rest of the world. Many of these studies are ongoing and require the collection and storage of tissue and data over many years to provide a useful research resource.

Will my General Practitioner be informed of my involvement in the study?

We will not routinely inform your GP that you are taking part as you are only being requested to donate tissue.

What will happen if I don't want to carry on?

If you change your mind later about taking part please contact us using the details below to withdraw permission. You need not give any reason for your decision. If you withdraw from the study, we will destroy all your identifiable samples and any un-analysed material, but we will need to use the data collected up to your withdrawal.

What if there is a problem?

The University has arrangements in place to provide for harm arising from participation in the study for which the University is the Research Sponsor. NHS indemnity operates in respect of the clinical treatment with which you are provided. If you wish to complain about any aspect of the way in which you have been ap-

proached or treated during the course of this study, you should contact Benjamin Davies on the details below or you may contact the University of Oxford Clinical Trials and Research Governance (CTRG) office on 01865 572224 or the head of CTRG, email heather.house@admin.ox.ac.uk

Who is organising and funding the research?

This project is being funded by the National Institute for Health Research's Musculoskeletal Biomedical Research Unit which is based within the Nuffield Department of Orthopaedics, Rheumatology and Musculoskeletal Sciences at the University of Oxford. The University of Oxford Stem Cell Institute is also providing funding. All of the research is taking place at the Nuffield Orthopaedic Centre and the Botnar Research Centre.

Who has reviewed this study?

Research in the NHS is reviewed by ethics committees; this study was reviewed by NRES Committee, South Central – Berkshire and given a favourable opinion.

Will my taking part in the project be kept confidential?

Yes, all the information about your participation in this project will be kept confidential. If you consent to take part in this project we would need to record some details of your medical history for the purpose of analysing the research results. You would also be anonymous if we need to publish results, or share data

or samples with other laboratories for research purposes. Responsible members of the University of Oxford or the Oxford University Hospitals NHS Trust may be given access to data for monitoring and/or audit of the study to ensure we are complying with regulations. Anonymised data collected during the course of the study may be passed on to other organisations which may include commercial organisations.

Research enquiries:

Mr Benjamin Davies, Botnar Research Centre, University of Oxford, Windmill Road, Oxford, OX3 7HE

Phone: 01865 227644

Email: benjamin.davies@ndorms.ox.ac.uk

You can speak to our research team for further information about giving tissue samples or the research.

B Consent Form

Consent form Final V2.2 dated 05/03/2013

Oxford University Hospitals NHS Trust

Nuffield Department of Orthopaedics, Rheumatology & Musculoskeletal Sciences.
Botnar Research Centre, Windmill Road, Headington
Oxford, OX3 7HE United Kingdom
Telephone: 01865 227644
E-mail: benjamin.davies@ndorms.ox.ac.uk

Title of Project: Mesenchymal Stem Cell Research
Chief Investigator: **Mr Benjamin Davies**
Berkshire REC Reference: **12/SC/0484**

[Patient sticky here]

MESENCHYMAL STEM CELL RESEARCH
CONSENT FORM

If you would like to take part in this research, please initial the boxes and sign the form.

1	I confirm that I have read, understood and have had time to consider the information sheet dated 05/03/2013 (Version 2.2) and have been given a copy to keep. I have had the opportunity to ask questions about this project and have had these answered satisfactorily.	Initial
2	I agree to give samples of bone marrow for research as detailed in the patient information sheet.	Initial
3	I understand how the samples will be taken, that participation is voluntary and that I am free at any time to withdraw my permission for the storage and distribution of my samples providing that they have not already been used in research.	Initial
4	I agree that Research staff can collect and store information, including x-rays and scans, from my health care records for research. I understand the Research staff will keep my information confidential. Information will only be passed to other researchers in an anonymous way that protects my identity.	Initial
5	I agree that the sample(s) I have given and the information gathered about me can be stored by the Nuffield Department of Orthopaedics, Rheumatology, and Musculoskeletal Sciences.	Initial
6	I understand that data collected during the study may be looked at by authorized individuals from the University of Oxford and the Oxford University Hospitals NHS Trust where it is relevant to my taking part in this research. I permit these individuals access to my research records.	Initial
7	During the study, I agree that any surplus samples and data I have given can be stored by the Oxford Musculoskeletal BioBank for other research projects. This may include genetic and/or commercial research in the UK and the rest of the world.	Initial
8	I agree to gift samples/data taken for the purpose of the research study to the University of Oxford. If a commercial product were developed as a result of this study, I will not profit financially from such a product.	Initial

Name of patient	Signature	Date

I have discussed the study with this patient who has agreed to give informed consent.

Name of person taking consent	Signature	Date