Isolation, Expansion and Characterisation of Mesenchymal Stem Cells from Osteoarthritic and Osteoporotic donors

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Abstract

Osteoporosis and osteoarthritis are common diseases with significant rates of morbidity associated with same. The search continues for effective treatment strategies for both diseases. Mesenchymal stem cells (MSCs) are critical components in the effective formation and repair of healthy bone and cartilage. Understanding the characteristics and behaviour of MSC’s in osteoarthritic and osteoporotic patients can help delineate and characterise the pathogenesis of these diseases further. The first aim of this study involves the creation of a biobank of osteoporotic and osteoarthritic MSC’s. The second aim includes analysing differentiation potential and cell migration/homing capabilities of these diseased MSC’s. Continuing from these results, a third aim is to explore aspects of how diseased MSC’s may interfere with TGF-β1 signalling, given the potent role of this pathway in regulating bone and cartilage metabolism.

Bone marrow aspirates were obtained from osteoarthritic, osteoporotic and healthy donors undergoing scheduled hip surgery in the University of Limerick hospital group. Bone marrow aspirates were extracted through the exposed acetabulum during arthroplasty surgery. MSCs were isolated through standard \textit{in vitro} culture conditions and underwent characterisation and differentiation as per the criteria outlined by the International Society of Cellular Therapy guidelines. Osteoporotic and osteoarthritic MSCs were then analysed for their osteogenic and adipogenic differentiation potential, migratory capacity and interplay with aspects of TGF-β1 signalling.

A total of 10 donors were included in the study with no difficulties or complications arising from obtaining bone marrow aspirates during necessary arthroplasty surgery. MSCs were characterised using flow cytometry and by demonstration of their capacity to differentiate along adipogenic, chondrogenic and osteogenic lineages. Osteoporotic MSCs had an increased propensity to differentiate along the adipogenic lineage in comparison to their osteoarthritic counterparts. Both osteoporotic and osteoarthritic MSCs had altered chemokinetic profiles in comparison to healthy controls. Of particular interest, whilst healthy MSCs migrate towards TGF-β1, osteoporotic MSCs failed to migrate towards this important chemoattractant.

The results of this research detail the successful extraction and isolation of MSCs from patients with osteoarthritis and osteoporosis. The surgical location and technique incorporates a transferable skill that could result in largescale collection of both healthy and diseased MSCs. A critical finding includes the altered migration of MSCs towards TGF-β1. This has implications both in the basic understanding of the pathogenesis of osteoporosis and in the development of future therapeutic targets.
Declaration

This study forms part of original research and I have not previously submitted material to this effect either in published or unpublished form to either University of Limerick or any other institution

Signature

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Siobhan Coyle
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<tr>
<td>ACLT</td>
<td>Anterior cruciate ligament transection</td>
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<td>ASC</td>
<td>Adult stem cell</td>
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<tr>
<td>ASIS</td>
<td>Anterior superior iliac spine</td>
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<tr>
<td>BGP</td>
<td>Beta-glycerophosphate</td>
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<tr>
<td>BM</td>
<td>Bone marrow</td>
</tr>
<tr>
<td>BMP</td>
<td>Bone morphogenetic protein</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
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<tr>
<td>CD</td>
<td>Cluster of differentiation</td>
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<tr>
<td>cDNA</td>
<td>Complementar Deoxyribonucleic acid</td>
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<td>CED</td>
<td>Camurati-Engelmann disease</td>
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<td>COX-2</td>
<td>Cyclo-oxygenase 2</td>
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<td>CXCR</td>
<td>CXC chemokine receptors</td>
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<td>4', 6-diamidino-2-phenylindole</td>
</tr>
<tr>
<td>DEPC</td>
<td>Diethylpyrocarbonate</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco modified eagle medium</td>
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<tr>
<td>DMSO</td>
<td>Dimethylsulfoxide</td>
</tr>
<tr>
<td>dNTP</td>
<td>Deoxynucleotide</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>ERK</td>
<td>Extra-cellular signal related kinases</td>
</tr>
<tr>
<td>ESC</td>
<td>Embryonic stem Cell</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
</tr>
<tr>
<td>GADPH</td>
<td>Glyceraldehyde 3-phosphate dehydrogenase</td>
</tr>
<tr>
<td>GFP</td>
<td>Green fluorescent protein</td>
</tr>
<tr>
<td>HSC</td>
<td>Haematopoietic stem cell</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
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<tr>
<td>IBMX</td>
<td>Isobutylmethylxanthine</td>
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<tr>
<td>ISCT</td>
<td>International Society of Cellular Therapy</td>
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<tr>
<td>IPA</td>
<td>Isopropanol</td>
</tr>
<tr>
<td>iPSC</td>
<td>Induced pluripotent stem cell</td>
</tr>
<tr>
<td>LAP</td>
<td>Latent activated protein</td>
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<tr>
<td>LDS</td>
<td>Loeys-Dietz syndrome</td>
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<tr>
<td>MCP</td>
<td>Monocyte chemoattractant protein</td>
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<td>Mononuclear cell</td>
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<tr>
<td>MSC</td>
<td>Mesenchymal stem cell</td>
</tr>
<tr>
<td>NEAA</td>
<td>Non-essential amino acids</td>
</tr>
<tr>
<td>OA</td>
<td>Osteoarthritis</td>
</tr>
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<td>Osteoporosis</td>
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<tr>
<td>OSX</td>
<td>Osterix</td>
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<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
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<tr>
<td>PC</td>
<td>Polycystein</td>
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<td>PDGF</td>
<td>Platelet derived growth factor</td>
</tr>
<tr>
<td>PPARγ</td>
<td>Peroxisome proliferator-activated receptor gamma</td>
</tr>
<tr>
<td>P/S</td>
<td>Penicillin/streptomycin</td>
</tr>
<tr>
<td>PSIS</td>
<td>Posterior superior iliac spine</td>
</tr>
<tr>
<td>PYH</td>
<td>Pack year history</td>
</tr>
<tr>
<td>RANK-L</td>
<td>Receptor activator of nuclear factor kappa-B ligand</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>rt</td>
<td>Reverse transcriptase</td>
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<tr>
<td>rt-PCR</td>
<td>Reverse transcription polymerase chain reaction</td>
</tr>
<tr>
<td>Acronym</td>
<td>Definition</td>
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<tr>
<td>---------</td>
<td>------------------------------------------------</td>
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<tr>
<td>RUNX2</td>
<td>Runt-related transcription factor 2</td>
</tr>
<tr>
<td>SARA</td>
<td>Smad anchor for receptor activation</td>
</tr>
<tr>
<td>SDF</td>
<td>Stromal derived factor</td>
</tr>
<tr>
<td>SERM</td>
<td>Selective Estrogen Receptor Modulator</td>
</tr>
<tr>
<td>siRNA</td>
<td>Small interfering RNA</td>
</tr>
<tr>
<td>SLC</td>
<td>Secondary lymphoid tissue chemokine</td>
</tr>
<tr>
<td>SOX</td>
<td>Sry related high mobility group box</td>
</tr>
<tr>
<td>TGFβ</td>
<td>Transforming growth factor beta</td>
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<td>UHL</td>
<td>University Hospital Limerick</td>
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</table>
Chapter 1: Literature Review

1.1 Introduction

The purpose of this study is to isolate and characterise mesenchymal stem cells (MSCs) from osteoarthritic and osteoporotic donors. Elements of the extracted MSCs are then further investigated, including the analysis of differentiation capacity, migratory ability and interplay with aspects of the TGF-β1 pathway.

MSCs were originally identified and described in the 1960s [1]. Their initial function was suggested as a supporting role for the more studied and defined haematopoietic stem cell (HSC). A seminal paper in the 1990s from the International Society of Cellular Therapy (ISCT) recommended specific criteria for the accurate identification and characterisation of MSCs [2]. Different nomenclature has been used in recent decades to describe MSCs, including multipotent mesenchymal stem cells, skeletal stem cells and mesenchymal progenitor cells. In this study, we will solely use the term mesenchymal stem cell (MSC) in order to avoid any mis-interpretation of same.

In the past number of decades, interest and research into MSCs has multiplied significantly. There are more than 26,000 entries in PubMed under “mesenchymal stem cells”, more than 100 clinical trials have been registered with MSCs or related cells, and more than six biotechnology companies are in phase II and III clinical trials in efforts to commercialize these cells [3]. However despite these advances in knowledge and research, a significant number of questions remain to be answered. A better understanding of the basic biology of MSCs both in health and disease is vital to the future success of MSCs as therapeutic agents.

The following five chapters represent the core work of this Masters in Surgery (MCh). Chapter 1 outlines the background and rationale for this research and includes a detailed critical appraisal and interpretation of the existing literature. Chapter 2 describes the extraction, isolation and characterisation of MSCs from osteoporotic and osteoarthritic donors. Chapter 3 delineates the differentiation capabilities of the respective MSCs and also
analyses the chemokinetic and chemotactic behaviour of diseased MSCs. Chapter 4 examines initial experiments into the primary cilium and aspects of TGF-β1 signalling in health and disease. This dissertation concludes with a summary of the outcomes of the study and the implications for future research.

Thus the primary aims of this research project are the following:

1. Extraction of MSCs intra-operatively from a novel location.
2. Identification, characterisation and expansion of MSCs from osteoporotic and osteoarthritic donors.
3. Development of a biobank of osteoporotic and osteoarthritic MSCs
4. Analysis of growth characteristics, differentiation capacity and migratory ability of MSCs from osteoporotic and osteoarthritic donors.
5. Analysis of TGFB1 signalling in the primary cilium in MSCs from osteoporotic and osteoarthritic donors.

1.2 Skeletal System

1.2.1 Macroscopic to Microscopic structure

The skeletal system is a highly dynamic organ that is essential for life. Its structural function provides the framework that supports the body, protects many vital organs and allows for effective movement. It is frequently stereotyped as simply a protective and supportive framework for the body and although it provides this function, it is an organ with multiple other functions. These functions include actively maintaining mineral homeostasis and acid-base balance, acting as a reservoir for growth factors and cytokines and ensuring effective haematopoiesis in the marrow cavity [4, 5].

The structure of bone is essential in discerning its relative function in varying parts of the human skeleton. The gross skeleton consists of over 200 bones, which can be structurally divided into axial and appendicular components for descriptive purposes [6]. The axial component comprises of the skull, spine, sternum and the ribs. The appendicular component consists of the long bones and their respective appendices. There are four general categories of bones; long bones, short bones, flat bones and irregular bones. Long bones include the clavicles, humeri, radii, ulnae, metacarpals, femurs, tibiae, fibulae, metatarsals, and phalanges. Flat bones include the skull, mandible, scapulae, sternum and ribs. Flat bones
form by intramembranous bone formation, whereas long bones are formed by a combination of endochondral and intramembranous bone formation with resultant implications on the internal architecture of the respective bones.

The internal architecture of each respective bone can be subdivided into two major types; trabecular and cortical bone. Overall, the human skeleton is composed of 80% cortical bone and 20% trabecular bone [4]. Cortical bone is dense and compact and encompasses the outer component of all skeletal structures (see Figure 1). Its major function is to provide mechanical support and strength to the skeleton. The cortical bone shell can reach a thickness of between several tenths of a millimetre in vertebrae and several millimetres in the mid shaft of long bones [4]. Trabecular bone is composed of a honeycomb like network of trabecular plates and rods interspersed in the bone marrow compartment. In adulthood, trabecular bone is found at the end of the medullary cavities of hollow long bones, throughout the skeleton and in flat bones such as the pelvis. Different bones and skeletal sites within bones have different ratios of cortical to trabecular bone. The vertebra is composed of cortical to trabecular bone in a ratio of 25:75. This ratio is 50:50 in the femoral head and 95:5 in the distal radius [7].

Trabecular bone has historically been described as more metabolically active than cortical bone and consists of active bone marrow in adults [8]. It is frequently the site of bone marrow aspirations facilitating the diagnosis of haematological conditions in clinical medicine. Trabecular bone has a complex, porous spatial arrangement which contributes to the skeletal paradox of combining maximum strength with minimum mass [9]. As previously mentioned, it is composed of a three dimensional lattice work of interconnecting struts. Struts can be present in distinct forms, either rod-like, plate like or an intermediary between these two extremes. This structural appearance has been shown to constantly change throughout life and is different between sites within a single bone. This is reflective of the spatially distributed and temporally changing forces experienced by that bone. The high mineral surface area associated with the arrangement of trabecular bone elements provides a large substrate on which cellular interaction with bone mineral material can occur and therefore a common site for bone marrow aspiration in the clinical setting.

The gross structural elements as outlined above confer important functional elements to bone and these can be further discerned by examination at a microscopic level. The functional unit of cortical bone is known as the osteon. The osteon consists of concentric layers of compact
bone tissue that surround a central canal, known as the haversian canal. The haversian canal contains the main blood and nervous supply to bone. Osteons are connected to each other and the periosteum by oblique channels known as Volkmann’s canals. The functional unit of trabecular bone are termed packets, are semilunar in shape and composed of concentric lamellae [4]. Trabecular packets have a greater surface area than that observed in cortical bone and thus provide an ideal platform for metabolic activity.
**Figure 1: Internal architecture of Trabecular Bone**

**A**

Outline of internal architecture of pelvic bone (A): Gross structure of the pelvic skeleton – Note detailed anatomical diagram of the pelvis included in a later section (Figure 3A) (B): Macroscopic view of the internal pelvic bone architecture with trabecular bone located in the centre. (C): Further magnification of the structure of trabecular bone demonstrating its struts and space for bone marrow and tissue. (D): Microscopic view of trabecular bone with osteocytes, osteoblasts and osteoclasts present.
The skeletal system has a rich vascular supply, receiving 25% of cardiac output at rest [10]. The blood supply varies with different types of bones, but is especially rich in areas that contain haematopoietically active bone marrow such as the vertebrae, ribs, pelvis, sternum, pelvis, scapula, skull and the proximal ends of the femur and humerus. Bone marrow vasculature is composed of arterial vessels entering the marrow through the nutrient foramina and dividing into several arterioles. Small arterioles and capillaries from these vessels span throughout the bone marrow and supply sinusoids, which are interconnected by intersinusoidal capillaries. The sinusoids are radially distributed around the central draining sinus, consist of a single layer of endothelial cells and are devoid of supporting cells [11].

Bone matrix is composed of both organic and inorganic components. At the nanoscopic level, bone consists of approximately 10-30% organic material and 70-90% mineral salts. The inorganic component of this extracellular matrix (ECM) is hydroxyapatite, which is a mineral formed by calcium and phosphate. The organic phase is composed of collagen fibres, mainly type 1 collagen, as well as noncollagenous proteins such as fibronectin, osteocalcin (OCN), osteonectin (ON) and glycosaminoglycans [12, 13]. These basic structural components result in a tough, yet rigid material respectively. The basic building block of bone matrix is the collagen fibril (approx. 100nm in diameter and 5-10 µm in length). Triple helical tropocollagen units self-assemble into fibrillary structures which are then aggregated into fibrils. Fibrils are filled and coated with crystals of apatite.

At a cellular level, cells involved in bone modelling and remodelling include osteoclasts, osteoblasts and osteocytes amongst others. Osteoclasts are large multinucleated cells formed by the fusion of macrophages from the haematopoietic lineage. They are responsible for bone degradation and resorption. Osteoblasts are derived from MSCs and are the bone forming cells responsible for the deposition of ECM and its subsequent mineralisation. Osteoblasts mature to form osteocytes which become entrapped in bone ECM in elliptical cavities known as lacunae. Lacunae are located between concentric layers of lamellae and are connected to each other by canaliculi. Osteocytes are the most abundant cell type in bone (approximately 95%) and form a sensory network throughout the tissue by extending cellular processes enabling cell-cell contact and communication through canaliculi [14, 15].
1.2.2 Bone modelling and remodelling

As mentioned previously, bone is a highly dynamic tissue that undergoes constant regeneration. Bone modelling is a process that works in concert with bone growth and functions to alter the spatial distribution of accumulating bone tissue presented by growth. It involves osteoclast activation and thus resorption of bone or it involves osteoblast activation and bone formation. During bone modelling, bone formation and resorption are not tightly coupled. Bone modelling may be increased in hyperparathyroidism, renal osteodystrophy or treatment with anabolic agents [16-19]. Bone remodelling, in contrast, is a sequence of bone resorption followed by formation at the same location. It is a continuous physiological process with the purpose of maintaining normal bone mass and repairing micro-damage to bone [20]. The process begins before birth and continues until death. The basic multicellular unit (BMU) is the collective term for the main cells responsible for bone remodelling, namely osteoblasts and osteoclasts. Coupling of resorption and deposition is a strictly controlled process in remodelling and alterations in this coupling can lead to systemic diseases such as osteoporosis.

Bone modelling and remodelling relies on complex signalling pathways and mechanisms to allow effective bone formation and resorption. These processes encompass a wide range of hormones’, growth factors and cytokines. Signalling pathways such as wingless-type mouse mammary tumour (Wnt) and transforming growth factor beta (TGF-β) have been linked to the regulation of bone remodelling. Research evaluating Wnt signalling has recently allowed the identification of targets for therapeutic intervention in diseases such as osteoporosis [21]. The transforming growth factor (TGF) family has multiple effects across various organs and evaluating its role during bone remodelling has the potential to lead to new therapeutic agents for common diseases such as osteoporosis and osteoarthritis. Significant progress has been made in manipulating Wnt signalling as a treatment but TGF-β signalling is still relatively poorly understood and therefore has not been exploited as a therapeutic target to date.
1.2.3 Transforming growth factor-β1

Transforming growth factor beta (TGF-β) is a secreted protein that is involved in cell proliferation, differentiation and migration amongst other functions. TGF-β exists in three isoforms, TGF-β1, TGF-β2 and TGF-β3. Recent publications have highlighted the role of TGF-β1 in bone formation and thus has renewed interest in this area of research [22]. TGF-β1 is a ubiquitous, multifunctional growth factor. It regulates a broad range of biological processes including cell proliferation, cell survival, cell differentiation, cell migration and the production of the extracellular matrix [23]. TGF-β1 has a pivotal role in bone remodelling and it has been shown to affect both bone resorption and formation [22]. It is secreted in a latent form by bone cells and is stored in the extracellular matrix. The latency associated protein (LAP) is bound non-covalently to active TGF-β1, masking the receptor binding domains of active TGF-β1 and rendering it inactive as can be seen in Figure 2 [24]. Resorbing osteoclasts are capable of activating TGF-β1 by affecting the latency associated protein. The active TGF-β1 binds to receptors such as TGF β Receptor I and TGF β Receptor II. This results in the downstream activation of proteins including SMAD 2/3 which results in an alteration in gene expression. The effects of TGF-β1 on bone have recently garnered attention with multiple publications on the topic. Tang et al highlighted the significance of TGF-β1 in coupling together both bone resorption and formation [22]. They demonstrated that bone resorption releases active TGF-β1 which induces the migration of MSCs to the site of active bone remodelling and results in bone formation. The chemotactic response of MSCs to TGF-β1 has also been demonstrated in other publications [25-27]. The relationship between TGF-β1, MSCs, osteoporosis and osteoarthritis will be reviewed in the respective relevant sections below.
**Figure 2: TGF-β1 Receptor pathway**

Figure 2: A simplified portion of the complex TGF-β pathway. This section illustrates TGF-β initially in an inactive state bound to the latency activated protein. LAP is cleaved from the complex resulting in active TGF-β. This binds to TGF-β receptor 1 and 2 resulting in an activated TGF-β receptor complex. This results in downstream messengers including SMAD 2/3 becoming phosphorylated and activated. The end result is a targeted gene response. (Note that this is a small representation of a complex pathway.)
1.3 Disease

1.3.1 Osteoarthritis

Osteoarthritis (OA) is a disabling, degenerative joint disease and is the most common joint disorder in the US [28]. It has been predicted to affect 67 million people in the US alone by 2030 [29]. In particular, radiographic hip OA is present in approximately one in four individuals > 45 years of age [30]. Notably, OA has a negative influence on the economy and been reported as the most common condition for people receiving disability living allowance [31]. Its primary symptoms include joint pain, stiffness and loss of function. OA predominately affects weight bearing joints such as the knees, hips and spine. Among adults >60 years of age, the prevalence of symptomatic knee OA has been reported as 10% in men and 13% in women [32].

The traditional view of OA is of a “wear and tear” form of arthritis, assumed to develop as a direct result of an initial biological or mechanical disruption of the joint cartilage. Despite the prevalence and impact of OA, its underlying aetiology has yet to be firmly established [33]. A number of risk factors have been linked with the development of OA and include ageing, obesity, metabolic diseases, mal-alignment of joint surfaces, joint trauma and a family history of OA [34].

Under normal conditions, articular chondrocytes maintain a dynamic equilibrium between the synthesis and degradation of extracellular matrix components. In OA, a disruption of matrix equilibrium leads to the progressive loss of cartilage tissue. With progression, there is frequently an increase in both the degradation and synthesis of extracellular matrix molecules within the joint and an overall shift favouring catabolism over anabolism. An increased synthesis of tissue destructive proteinases (matrix metalloproteinase and aggrecanases) results in an increased death of chondrocytes and an ultimate inability to synthesize components of the extracellular matrix [35, 36].

In this regard, OA has historically been considered a cartilage disorder but recent publications have also shown roles for bone and synovial tissue in the pathogenesis of OA. The homeostasis of articular cartilage relies on its interplay with subchondral bone and synovial tissues [37]. Subchondral bone provides the mechanical support for articular cartilage during the movement of load bearing joints. It undergoes constant adaption in response to mechanical loading through remodelling as shown in figure 2A [38]. Thinning of
subchondral bone has been recently reported to take place not just in the late stages of the disease but also at an early stage [39]. Bone marrow lesions have also been closely associated with pain and have been implicated to predict the severity of cartilage damage in OA [40]. There is now considerable debate as to whether the changes in the underlying subchondral bone may actually precede changes in articular cartilage. However despite extensive work over the past number of decades, a full understanding of the initiators of the disease and the factors that accelerate it remain unknown.

The relationship between TGF-β1 and osteoarthritis is an intense area of scientific research with a number of recent seminal publications in this area. Zhen et al used an anterior cruciate ligament transection (ACLT) mouse model to demonstrate that TGF-β1 is activated in subchondral bone in response to altered mechanical loading. They reported high concentrations of active TGF-β1 in the subchondral bone of the osteoarthritic mouse model and suggested this finding as initiating the pathological changes of osteoarthritis. Knockout of TGF-β-RII in MSCs attenuated the development of osteoarthritis in ACLT mice [41]. Furthermore, mutations in genes involved in the activation of TGF-β, such as Camurati-Engelmann disease (CED) and Loeys-Dietz syndrome (LDS), are associated with high incidences of osteoarthritis [42, 43].
The diagnosis of OA involves clinical examination and radiograph imaging. Clinical symptoms include joint pain, stiffness and loss of function. Clinical examination typically reveals a reduced range of movement. Classical signs on radiography include the presence of subchondral sclerosis, subchondral cysts, narrow joint space and osteophyte formation as shown in figure 2B [44]. However findings on plain film radiography co-relate poorly with the degree of patient’s symptoms [45]. There are currently no biomarkers available to detect OA at an earlier stage [46].
Current treatment for OA relies on symptom prevention and management. Exercise and anti-inflammatory medication are the main components of an initial treatment plan. Trials of disease modifying drugs, intra-articular steroids and growth factor injections have thus far not proven to be beneficial [47]. End stage OA is primarily treated with joint replacement surgery [48]. Greater than 1 million joint replacement surgeries are carried out in the US per year [49]. Thus the market and scope for an earlier intervention in the treatment and diagnosis of OA is immense.

1.3.2 Osteoporosis

Osteoporosis is a systemic skeletal disease characterised by low bone mass, micro-architectural deterioration of bone tissue and a consequent increase in bone fragility with susceptibility to fracture [50]. In Europe, it is estimated that more than 30% of women aged 50 years and older have osteoporosis as defined by the World Health Organisation. An osteoporotic fracture is estimated to occur every 30 seconds in the EU [51]. The lifetime risk for the occurrence of any fragility fracture in Caucasian women at age 50 years approaches 40%. Men have been reported as having a 13% lifetime risk [52]. Common sites for osteoporotic fracture include the hip, spine, distal forearm and the proximal humerae. The remaining lifetime probability in women at menopause of a fracture at any one of these sites exceeds that of breast cancer (approx. 12%) and is also close to the probability of developing coronary artery disease [53]. The morbidity and mortality associated with osteoporotic fractures are considerable. Hip fractures have an overall mortality of 15-30%, with the majority of deaths occurring within the first six months of surgery [54]. In 2010 the number of deaths causally linked to osteoporotic fractures in the European Union was estimated at 43,000 [55]. Osteoporosis is diagnosed by measurement of bone mineral density via dual energy x-ray absorptiometry (DXA). The guidelines of the National Osteoporosis Foundation states that a clinical diagnosis of osteoporosis can be made in at risk individuals who sustain a low trauma fracture [56].

The altered bone profile in advanced stages of life was noted as far back as the early 19th century. Albright initially coined the term osteoporosis in the 1940’s in reference to a postmenopausal state resulting from oestrogen deficiency [57]. As mentioned previously, bone is a dynamic tissue that continuously adapts to its mechanical environment. Bone remodelling is responsible for the removal and repair of damaged bone in order to maintain the integrity of the adult skeleton. This is a tightly regulated event and involves the co-
ordination of osteoblasts, osteoclasts, osteocytes and MSCs in basic multicellular units (BMU). The apparent underlying mechanism in osteoporosis is an imbalance between bone resorption and bone formation [58]. Osteoporosis is an asymptomatic disease until the occurrence of a fragility fracture. Fracture healing is a complex process of bone regeneration, involving a well-orchestrated series of biological events. In recent years, fracture healing in osteoporotic patients has been shown to be impaired [59]. This has also been demonstrated in in-vivo animal models [60, 61].

The role of TGF-β1 in the pathogenesis of osteoporosis is sparsely researched. Gene analysis has proposed an association with single nucleotide polymorphisms in the TGF-β1 gene and alterations in bone mineral density [62]. However multiple studies have resulted in conflicting results in this regard and it is difficult to come to a consistent evidence based conclusion for an association between a given single nucleotide polymorphism and parameters such as bone mineral density, bone mass, bone turnover, and osteoporosis risk [63-65]. However given that TGF-β1 has been shown to be required for coupling bone resorption and formation, then it is my opinion that it is likely to be involved in the disease process [22]. However this requires greater study in order to provide a benefit to our ageing population.

Current therapies for osteoporosis focus mainly on reducing bone resorption. These include bisphosphonates, receptor activator of nuclear factor kappa-B ligand (RANK-L) antibodies and selective oestrogen re-uptake modulators (SERM). Bisphosphonates are the first line therapy used in the treatment of osteoporosis as outlined by multiple clinical guidelines [66]. They are taken up by osteoclasts in bone and have a structural similarity to pyrophosphate, which results in the inhibition of crucial intracellular processes [67]. The RANK-L/RANK/Osteoprotegerin system has garnered recent attention and mediates the production and activity of cells of the osteoclast lineage [68]. This has led to an FDA approved human monoclonal antibody to RANK-L, denosumab, which is currently commonly used in daily clinical practice. Raloxefine is an example of a SERM licenced for the treatment of post-menopausal osteoporosis with benefits in the realm of osteoporotic vertebral fractures [69]. However the spotlight has recently switched to the search for anabolic therapies in the treatment of OP that could promote bone formation. Recombinant parathyroid hormone in the form of a daily subcutaneous injection has been shown to reduce the incidence of vertebral osteoporotic fractures [70]. Numerous studies over the past number of years have provided insights into the role of Wnt signalling in osteoblast differentiation and signalling. The Wnt
signalling pathways are a group of signal transduction pathways made of proteins that pass signals into a cell through cell surface receptors. This work has resulted in the development of romosozumab, a humanized monoclonal antibody directed against the osteocyte derived glycoprotein, sclerostin. Sclerostin functions by inhibiting the Wnt and bone morphogenetic protein (BMP) pathways that are critical for osteoblast proliferation and activity [71]. A sclerostin inhibitor is currently in phase III clinical trials [72]. Following on from the transition of this antibody from the research domain to the clinical field, ongoing efforts are being made to uncover further anabolic therapies for the successful treatment of osteoporosis.

1.4 Mesenchymal stem cells

1.4.1 History of stem cells

The existence of stem cells was first proposed as far back as the 19th century. The ability of tissues to self-renew throughout the lifetime of an organism was attributed to the presence of stem cells. The identification and characterisation of stem cells and their behaviour has enveloped the scientific community with thousands of publications over the past number of decades. The identifying and critical characteristics of stem cells are their ability to self-renew and to differentiate along specialised cell lines. Stem cells are classified according to their origin, either as embryonic stem cells (ESC) or adult stem cells (ASC).

Embryonic stem cells are derived from blastocysts, and have the potential to differentiate into all 3 germ layers, endoderm, ectoderm and mesoderm respectively. A blastocyst is cellular mass that forms early in the embryo development process. It possesses an inner cell mass which subsequently becomes the embryo. The successful culture of mouse ESCs was first achieved in 1981 and the first continuous human ESC line was established in 1998 [73, 74]. ESCs are isolated from human pre-implantation embryos and their extraction and clinical use continues to invoke intense ethical debate [74]. Their use poses a moral dilemma which arises from a duty to protect and alleviate suffering, whilst still respecting the value of human life. Therefore alternative stem cell sources have been sought and these include the use of induced pluripotent stem cells (iPSC) and adult derived stem cells. Pluripotency refers to a stem cell that has the potential to differentiate into any of the three germ layers. iPSCs are generated by reprogramming adult cells to an embryonic like state, gaining back a state of pluripotency. In 2012, Sir Gurdon and Dr Yamanaka won the Nobel Prize for their discovery that adult cells can be reprogrammed to become pluripotent [75]. Clinical trials are currently underway to assess the potential use of iPSCs as therapeutic agents.
Adult stem cells are postnatal cells which are found in fully developed tissues. They repair or replace cells within tissue in response to cell damage or natural cell turnover. They are undifferentiated and unspecialized cells which are capable of self-renewal and multipotent differentiation. Adult stem cells traditionally have been located and extracted from bone marrow tissue. Two main types of stem cells in the bone marrow have been identified, haematopoietic stem cells (HSCs) and mesenchymal stem cells (MSCs).

1.4.2 Hematopoietic stem cells

The original research into the origin and functioning of MSCs arose and heavily relied on analysis of the more well-known and characterised HSC. HSCs mature to form blood cell types from the myeloid or lymphoid lineage [76]. They are primarily located in the bone marrow with only small numbers located in peripheral blood [77]. Research into HSCs came to prominence in the 1950’s with the demonstration that intravenously injected bone marrow cells could rescue irradiated mice from lethality by re-establishing blood cell production [78, 79]. Subsequent experiments utilizing genetic marking have demonstrated that long term engraftment of both the myeloid and lymphoid lineages could be achieved by the progeny of a single cell [80, 81]. The characterisation and isolation of HSCs has advanced to a degree that HSC transplantation has become a recognised standard of care for many patients with defined congenital or acquired disorders of the haematopoietic system or with chemosensitive, radiosensitive or immunosensitive malignancies [82-84]. Thus HSCs have traversed the gap from initial scientific discovery to that of an established therapeutic agent used in daily clinical practice.

1.4.3 Mesenchymal stem cells

Classical experiments demonstrating that transplantation of bone marrow to heterotopic sites results in the de novo generation of ectopic bone can be traced back to the 19th century [85]. However, these and other similar publications were unable to identify any specific stem cell responsible for the generation of ectopic bone. In a series of seminal studies in the 1960’s and 1970’s, Friedenstein et al detected bone of donor origin in bone marrow transplanted animals consistent with cells that could provide non-haematopoietic, mesenchymal tissue [1]. Tavassoli and Crosby reported similar observations when they reported that autologous bone fragments survived after transplantation in extra-medullary sites due to cells that proliferated and gave rise to osteoblasts and trabecular bone [86]. Friedenstein et al subsequently identified a minor subpopulation of bone marrow cells which were capable of osteogenesis
These cells were distinguishable from haematopoietic cells by their adherence to plastic in tissue culture vessels and also their fibroblast like morphology. They further demonstrated that seeding these cells resulted in the formation of discrete colonies potentially initiated by single cells, known as colony forming unit fibroblasts (CFU-F). These stem cells were initially termed an “osteogenic stem cell” or a “bone marrow stromal cell”.

Given the extensive research being undertaken into HSCs from the 1950’s, the role of MSCs was originally examined as a supporting role for the HSC population. Research conducted largely remained within the haematology domain. Further work in the 1980s identified the multipotency of these cells by demonstrating their ability to differentiate into osteoblasts, adipocytes and chondrocytes. The term ‘mesenchymal stem cell’ to describe these cells was proposed by Caplan in 1991 [88].

1.4.4 Identification of MSCs

Given the potential benefits and intense interest in mesenchymal stem cells, accurately identifying MSCs is a premise of multiple publications and an area of ongoing controversy [89]. The assessment and comparison of publications from laboratories becomes extremely difficult if no unified approach to the identification of MSCs exists. With this in mind, in 2006, the MSC committee of the International Society of Cellular Therapy (ISCT) determined three requisite criteria to distinguish MSCs from other cell lines [2]:

1. Cells are plastic adherent
2. Specific cell surface antigen expression
3. Multipotent differentiation potential.

As outlined in the above criteria from the ISCT, the presence of specific cell surface molecules on MSCs is a key component in their accurate identification. The Cluster of Differentiation (CD) nomenclature was originally established for the classification of monoclonal antibodies against epitopes on the surface molecules of leucocytes [90]. The proposed surface molecule is assigned a CD number once two specific monoclonal antibodies are shown to bind to the molecule. Since its original establishment its use has expanded to the identification of many other cell types with more than 320 clusters identified [91]. Flow cytometry is a powerful tool which can be used to determine the phenotype of cells [92].
As per the ISCT criteria, MSCs must be positive for the cell markers CD105, CD73 and CD90. The cell markers SH2 and SH3 were originally proposed by Pittenger et al and have since been shown to correspond to CD105 and CD73 respectively [93-95]. CD105, also known as endoglin, is a membrane glycoprotein and is part of the TGF-β receptor complexes [96]. It is also known to have a crucial role in angiogenesis. Expression of CD105 is not exclusive to MSCs, since it is also expressed on endothelial cells, synctiotrophoblasts, macrophages and connective tissue stromal cells. CD73 is involved in immune regulation, cell migration and cell-cell interactions in the bone marrow environment [97]. CD73 is also not uniquely expressed by MSCs and is expressed by skin fibroblasts [98]. CD90 is known as the thymocyte differentiation antigen. Its exact function is less well known and it has been proposed to be involved in cell-cell interactions [93, 94]. It is also involved in the adhesion of monocytes and leucocytes to endothelial cells and fibroblasts [99, 100]. A further positive marker following in vitro culture of MSCs is CD44. CD44 is an adhesion molecule existing in different isoforms that interact with multiple ligands, such as hyaluronan, selectins, collagen and fibronectin [101]. It has been reported to be highly expressed in in vitro expanded MSCs from both humans and mice [102, 103]. Regardless of their time in culture or passage, MSCs are uniformly positive for CD105, CD73 and CD90 [95].

In order to exclude non MSCs, the selected cells should be negative for markers that identify the HSC population such as CD34 and CD45. CD34 is also known as the haematopoietic progenitor cell antigen. It functions as a cell-cell adhesion factor and as a marker for hematopoietic stem cells [104]. CD45 was originally classified as the leucocyte common antigen. It is present on almost all differentiated haematopoietic cells [105]. CD34 and CD45 can therefore be used to distinguish the haematopoietic population from the MSC population. Further markers which identify the haematopoietic component include CD14, CD11b, CD79α, CD19 and HLA-DR surface molecules [2].

The hunt for a single marker to identify an MSC continues, and has led to a substantial number of publications in this regard. A single marker would allow the rapid isolation of MSCs through prospective isolation and thus negate the need for in vitro culture and expansion. An accurate in vivo marker would also allow the location of MSCs to be more correctly identified. A large number of markers have been proposed and used in publications in recent years [98]. Location, level of confluency and culture conditions may each affect the presence of specific cell surface markers [106]. Recent molecules which have been gaining attention include Stro-1, CD271 and CD146.
Since its first report in 1991, the Stro-1 antibody has played a crucial role in hundreds of studies that have relied on it for the identification and/or the isolation of MSCs from a wide range of tissues [102, 107]. Stro-1 is a cell membrane protein that translocates from the endoplasmic reticulum to the cell membrane in response to the depletion of intracellular calcium [108]. Its function on MSCs remains largely unknown. However, Stro-1 is unsuitable as a sole marker to separate MSCs from bone marrow as greater than 95% of Stro-1 positive cells in the human bone marrow are glycophorin A expressing nucleated erythroid cells [107]. Its use is further compromised by its concurrent \textit{in vivo} expression in the endothelium, with the suggestion that Stro-1 is intrinsically an endothelial antigen and its expression on MSCs may be an induced event [108]. Thus its use as a single marker for the identification of MSCs remains controversial.

CD271 is a receptor for neurotrophins, which stimulate neuronal cells to survive and differentiate. It has been used to pre-select CFU-F from bone marrow aspirates [109]. CD271 has been used as a sole marker in a series of publications defining MSCs from bone marrow aspirates obtained from reaming of the femoral canal intra-operatively [110]. However it is not universally expressed in various MSC types. Its use as a sole marker is further challenged by the finding of a lower tri-lineage differentiation potential in CD105/CD271 +ve expanded cells compared to unsorted bone marrow mononuclear cells (BM-MNC’s) [111].

CD146 has garnered much attention recently following a series of publications from Bianco \textit{et al.} These publications have suggested a perivascular location for the MSC niche. CD146 was shown to label bone marrow adventitial reticular cells that were enriched for CFU-F’s. These cells were able to transfer haematopoietic support in the form of heterotopic ossicles in immune-compromised mice. Ossicles gave rise to CFU-F that could be cultured, thus demonstrating self-renewal ability in vivo. This renders CD146 labelled uncultured cells the first specific MSC population where an in situ counterpart has been identified and has been tested for self-renewal potential both \textit{in vitro} and \textit{in vivo} [112]. Both single labelled and CD271+ve/CD146+ve dual labelled populations were capable of serial xenogenic transplantation [113]. Perisinusoidal MSCs are marked by nestin and leptin receptor in mice and by CD146 in humans [27, 114]. Perisinusoidal MSCs expressing leptin receptor in mice were found to include multipotent, colony forming unit fibroblasts, with osteogenic and adipogenic potential. However the leptin receptor expressing cells failed to develop towards the chondrogenic lineage [115]. The focus and research highlighting the presence of CD146, nestin and leptin receptor outlines a current proposal for a perivascular origin for MSCs. The
bone morphogenetic protein (BMP) antagonist gremlin 1 has also been recently identified as defining a population of stem cells in the bone marrow which generate osteoblasts, chondrocytes but not adipocytes [116]. Chan et al demonstrates that mouse skeletal stem cell (mSSC) niche factors can be potent inducers of differentiation with several specific combinations of recombinant mSSC niche factors resulting in de novo formation of cartilage or bone and bone marrow stroma [117].

The above discussion highlights the inherent difficulty in identifying a sole MSC marker, with CD146 perhaps the most promising avenue of research. However, the ISCT position paper guidelines continue to be essential for the accurate isolation of MSC’s. This allows standardisation and results from different labs to be compared in a more homogenous manner [2]. Thus the isolation of MSCs should be positive for CD90, CD73, and CD105 whilst also being negative for the respective HSC markers.

1.4.5 Differentiation Potential

MSCs are multipotent and thus retain the ability to differentiate into multiple cell types. This property is the final criteria required for their correct identification [2]. MSCs can differentiate into precursors of bone, cartilage and fat tissues; i.e. osteoblasts, chondrocytes and adipocytes respectively. The commitment and differentiation of MSCs along a specific cell line is influenced by the presence of various transcription factors, cytokines, growth factors and extracellular matrix proteins. Beyond their ability to generate osteoblasts, chondrocytes and adipocytes in the in vitro setting, MSCs can give rise to bone and cartilage after ectopic implantation in vivo [118]. This ability to differentiate along multiple pathways identifies MSCs as an ideal candidate for tissue regeneration strategies.

In addition to the traditional differentiation pathways, MSCs have also been reported to differentiate into multiple other cell types of both mesodermal and non-mesodermal origin. This includes the ability to differentiate into neural cells, endothelial cells, hepatic cells and cardiomyocytes [119-122]. However, there are significant concerns regarding the lack of globally standardised methods of isolation, identification and expansion of MSCs with such multipotent capabilities not universally accepted to date. This remains a controversial area of research with numerous reports contradicting the ability of MSCs to differentiate along these respective lineages [123, 124]. Claims for in vivo differentiation into other cell types are also controversial, as bone marrow derived MSC cultures have been shown to contribute to many tissues upon transplantation through fusion with endogenous cells and not through
differentiation into mature cell types [125, 126]. Thus the ISCT position paper defines MSCs as having the ability to differentiate along osteogenic, adipogenic and chondrogenic lineages.

1.5 MSC location and extraction

1.5.1 Tissue source of MSCs

In attempting to isolate MSCs, the first question posed is in relation to the type of tissue used for extraction. The seminal publications on MSCs referred to cells that were primarily derived from bone marrow tissue [87, 88]. However, MSCs have subsequently been successfully isolated from multiple tissue sources including synovial tissue, adipose tissue, dental pulp and muscle. They have been isolated from almost every postnatal connective tissue [127-130]. Controversy remains as to the standardisation of isolation and the basic biological features of MSCs from different tissues.

Adipose derived MSCs have become an attractive alternative to bone marrow derived MSCs due to the ease of tissue collection, high initial cell yields and robust in vitro proliferative capacity. Adipose tissue derived MSCs are mainly extracted from tissue removed during liposuction, lipoplasty or lipectomy procedures [131]. Alternatively adipose tissue can also be sampled from fat pads during knee or hip surgeries [132]. MSCs can also be isolated from birth related tissues such as the umbilical cord, amniotic fluid and the placenta. MSCs have been isolated from four different compartments of the umbilical cord including Wharton’s jelly, tissue surrounding the umbilical vessels, umbilical cord blood and from the subendothelium of the umbilical vein [133]. Synovial MSCs are obtained during arthroscopic surgery with minimal invasiveness or risk of complications [134, 135].

However, despite the isolation of MSCs from multiple tissues, controversy continues regarding the characteristics of MSCs from the respective tissues. A number of differences have been reported in MSCs from different origins. The isolation yield varies considerably among the respective host tissue. The amounts of MSCs that can be obtained from bone marrow in comparison to adipose tissue vary considerably [136, 137]. From 1 gram of adipose tissue, 5 X 10^3 MSCs can be isolated, which is 500 times more than from the equivalent amount of bone marrow tissue [138]. This can be seen as a considerable advantage for the use of adipose tissue derived MSCs rather than bone marrow derived MSCs in bulk for clinical therapy. Peripheral blood exhibit a low colony unit forming ability ranging from 1.2 to 13 per million mononuclear cells [139]. Notably, isolation success rates from adipose
and bone marrow derived MSCs approaches 100%, but ranges from 10% to 63% in those derived from the umbilical cord [140-142].

The characteristics of MSCs during standard in vitro culture conditions also show some degree of variability. MSCs derived from foetal tissues have been shown to proliferate faster than those derived from adult tissues [22, 143-147]. The proliferation rates of adipose derived MSCs has been shown to vary according to the region of body the tissue is isolated from [148, 149]. Sagakuchi et al obtained MSCs from bone marrow, synovium, adipose tissue and skeletal muscle during anterior cruciate ligament reconstruction surgery and directly compared the proliferative capacity of each tissue source. They concluded that there were significant differences in the proliferative capacities of MSCs isolated beyond donor and experimental variability. Muscle derived MSCs and adipose derived MSCs had a lower proliferation potential than other MSCs, whilst synovial derived cells had a greater expansive ability than bone marrow derived MSCs [150].

The ability to differentiate into osteoblasts, adipocytes and chondrocytes is a defining feature of MSCs, as per the International Society of Cellular therapy [2]. It has been reported that MSCs derived from varying locations show bias in their differentiation capabilities. For example, adipose derived MSCs had inferior osteogenic and chondrogenic potential when compared to bone marrow derived MSCs [119, 151, 152]. Further, there is evidence that the regenerative capacities of adipose derived MSCs vary according to the regional source of the adipose tissue in question. Adipose derived MSCs from the infrapatellar fat pad appear to be specifically pre-programmed for chondrogenic differentiation [153].

Several studies have found differences in protein structure, differences in messenger RNA molecules and differences in the transcriptional potential of MSCs from different tissues which may account for the source dependent lineage preferences [154-156]. This also has future implications in the use of MSCs in therapeutic endeavours.

The isolation of MSCs from different tissue sources is promising and the use of adipose derived MSCs in clinical therapeutics is currently undergoing clinical trials. However the above discussion highlights the presence of basic differences amongst MSCs according to their source. Therefore it is imperative that researchers choose a tissue source that is relevant to the area of study. Almost all MSC based products in clinical trials prior to 2008 were sourced from allogenic donor bone marrow. Since then, MSC donor and tissue source diversity has substantially increased. However the majority of clinical trials continue to use
bone marrow as the source for MSC derived products (>55% in 2012) [157]. In this study we are attempting to investigate primarily bone related disorders and therefore the most appropriate source of MSC in this study is bone marrow tissue.

1.5.2 Location of MSCs

In order to extract and isolate MSCs from skeletal tissue, knowledge of their precise location would be of immense benefit. However, in the absence of a specific or unique markers that would allow the precise identification of MSCs \textit{in vivo}, histological localisation of MSCs has proven unattainable. To this end, multiple publications have attempted to uncover a “stem cell niche” for a more accurate location. The “niche” hypothesis was proposed by Schofield in 1978 to describe the physiological microenvironment that supports haematopoietic stem cells [158]. A complete definition of the niche concept was given by Scadden \textit{et al}; “specific anatomic locations that regulate how stem-cells participate in tissue generation, maintenance and repair. The interplay between stem cells and their niche creates the dynamic system necessary for sustaining tissues. The simple location of stem cells is not sufficient to define a niche. The niche must have both anatomic and functional dimensions.” [159]

HSCs replenish myeloid and lymphoid lineage cells daily while maintaining themselves over decades through a carefully orchestrated set of intrinsic and extrinsic processes. The distinct niches in bone marrow that support survival and control proliferation and differentiation of HSCs are well described [160]. One niche has been described at the endosteal surface of the trabecular bone. The endosteum is a membranous structure covering the inner surface of cortical, trabecular and Volkman’s canals in bone. In live transplanted animals, HSCs were found to accumulate closer to endosteal surfaces [161]. A second perivascular niche is found at the site of bone marrow sinusoids, the endothelial cells of blood vessels. Initial studies in live animals indicated that cells injected into non-irradiated recipients localised to perivascular regions, where they proliferated [162]. Subsequently, in situ HSCs were found to be in a perivascular location on bone sections [163]. The arterioles penetrating bone are most prominent in the periendosteal region and thus this may reflect the association of HSCs with subtypes of vasculature. It may also reflect the ability of osteolineage cells of the endosteal surface to affect and support the perivascular cells. The exact location of the HSC niche remains unclear, but putative HSC niches have been located near the endosteum, or in association with sinusoidal endothelium (perivascular niche).
The relationship between MSCs and the haematopoietic stem cell niche may advance our knowledge regarding the in vivo location of MSCs. Bone marrow resident MSCs have long been proposed to provide modulatory signals to haematopoietic progenitors based on the fact that mixed cultures derived from the adherent fraction of BM stroma promote survival and proliferation of HSCs ex vivo [164]. Depletion of OSX (a transcription factor for osteoblast differentiation) in postnatal animals prevented osteolineage differentiation and, with it, the loss of haematopoiesis in bone marrow [165]. The existence of a “dual stem cell niche” in which MSCs and HSCs directly interact in perivascular spaces of the bone marrow has been proposed in two recent publications. A landmark study from Mendez-Ferrer et al identified a nestin positive MSC population that are closely associated with putative HSCs. Nestin is a perivascular marker for MSCs in mice. Mice expressing nestin tagged with green fluorescent protein (nestin-GFP) were used to visualise the association between the respective stem cells. Nestin-GFP cells were capable of producing bone and other mesenchymal lineage cells in vitro and in vivo, consistent with MSCs. They reported that nestin-GFP cells were perivascular in the bone marrow, were adjacent to HSCs and expressed high levels of HSC maintenance genes. Selective deletion of nestin-GFP cells had a direct impact in HSC numbers and homeostasis [114, 166].

Efforts to track the identity of tissue resident MSCs in humans have suggested that they also lie adjacent to blood vessels [167]. Crissan et al reported that cultured perivascular cells from a variety of tissues exhibit a phenotype that is strikingly similar to that of MSCs derived from bone marrow. Single cultured perivascular cells seeded in vitro developed at a high frequency into clones, which all yielded osteoblasts, chondrocytes and adipocytes when seeded in the appropriated differentiation conditions [168]. This multi-lineage differentiation potential has been demonstrated in other publications [169-171]. Collectively, these studies outline evidence for the perivascular location of MSCs, however considerable further research is required before the location of the MSC niche is accurately defined and outlined.

As outlined from the above discussion regarding the location of MSCs, indicators would support a perivascular location for same. Thus easily accessible highly vascular structures from a single tissue source could be proposed as a suitable source for the development of a biobank investigating diseases such as osteoporosis and osteoarthritis. The pelvic bones are highly vascularised structures as outlined in the following section and thus suitable as a source of bone marrow aspirates for the purpose of isolating mesenchymal stem cells.
1.5.3 Bone marrow aspiration

The traditional location for bone marrow aspiration is from the iliac crest and this procedure is carried out on a daily basis in clinical practice. It involves obtaining an aspirate from the pelvic bone. An aim of this project is to obtain a bone marrow aspirate from the pelvic bone but with a vastly alternative entry point. The pelvic skeleton itself is formed posteriorly by the sacrum and coccyx and anteriorly by the ilium, ischium and pubis as outlined in Figure 5A. The principle function of the pelvic bony complex is to transmit forces between the axial skeleton and the lower limbs [172]. The adult hip bone is formed by the fused orientation of the ilium, ischium and pubis which diverge in different directions from the focus of the acetabulum. The ilium is a large flattened area of bone which projects superiorly from its acetabular portion into a large wing like expanse termed the ala. The iliac crest is S-shaped and terminates anteriorly in the anterior superior iliac spine (ASIS), and posteriorly in the posterior superior iliac spine (PSIS). The acetabulum is a deep cup-shaped hemispherical depression that articulates with the femoral head. It is formed internaly by the pubic bone, above by the ilium, behind and below by the ischium. Approximately 2/5 are formed by the ilium, 2/5 by the ischium and the remaining 1/5 by the pubic bone. Around most of the acetabular perimeter, a bony lip causes a dense incomplete rim of bone which is open inferiorly manifesting as the acetabular notch leading into the acetabular fossa in the central aspect of the acetabulum. The centre of the fossa is characterised by a roughened non-articular appearance, which is surrounded by the articular portion of the joint presenting as a smooth lunate surface. The pelvic skeleton is classified as a flat bone and is mainly composed of trabecular bone. It contains active bone marrow in both youth and adulthood.

The vasculature of the pelvic bone has been described in fresh cadaveric as well as radiological studies. The pelvic bones have multiple sources of blood supply arising mainly from the gluteal, obturator, iliolumbar and internal pudendal arteries as seen in Figure 4. The predominant blood supply to the ilium is from the nutrient arteries. The nutrient arteries of the ilium are reported to arise from the iliac branches of the obturator and iliolumbar arteries. These anastomose with each other and with branches of the superior gluteal artery to give rise to numerous small vessels to supply the pelvic bones [173]. The acetabulum itself is reported to be supplied by the artery of the roof of the acetabulum which arises from the inferior gluteal artery, the artery of the ischium arising from the internal pudendal artery and the acetabular branch of the obturator artery [174, 175]. Its blood supply encompasses a
superficial blood supply from the periosteum and from large nutrient arteries that penetrate directly into the bone.

**Figure 4: Blood supply to the pelvic bones**

Bone marrow aspiration is a common clinical procedure, mainly undertaken in order to diagnose haematological malignancies. In order to obtain a sample with sufficient cellularity, the anterior superior iliac spine (ASIS) and the posterior superior iliac spine (PSIS) are frequently used in daily clinical practice. These locations are also easily accessible for the clinician and provide a source of bone marrow with a high cellular content. However bone marrow aspiration and graft harvest are procedures that are associated with risks and complications. These include pain at the site of aspiration, infection and minor bleeding [176]. Chronic pain at the donor site has been reported in up to 39% of cases [177]. Serious adverse events are rare with a reported rate of 0.07% [178]. There have been case reports of
retroperitoneal haemorrhage, gluteal artery laceration and internal iliac artery pseudoaneurysm [179, 180].

The technique and clinical expertise of bone marrow aspiration from the haematological field were used in the initial experiments attempting to extract MSCs [95]. However, bone marrow aspiration from the iliac crest for the purposes of research necessitates the recruitment of patients for a procedure which would not otherwise be undertaken by the donor. Thus, there has been an intense interest in attempting to obtain bone marrow aspirates from alternative sites without the necessity for a separate procedure and therefore minimizing any risk to the donor. The vertebral body has been used to obtain MSCs with a similar concentration to that obtained via aspiration at the iliac crest during spinal operations [181]. Mazzocca et al reported the isolation of bone marrow MSCs from the humeral head during arthroscopic rotator cuff repair, with comparable results to those obtained from the vertebral body and iliac crest [182]. Bone marrow aspirates have been obtained from the distal femur during arthroscopic surgery, and also the proximal femur [183, 184]. Porter et al have evaluated the use of the aspirate obtained following intramedullary nailing. This involved the use of the Reamer-Irrigator-Aspirator (RIA) system. This system irrigates the intramedullary canal and aspirates the contents during the reaming process prior to insertion of an intramedullary nail for fracture repair [185]. However to date, no publication has utilised bone marrow aspirate from the exposed acetabulum intra-operatively.

Hip arthroplasty surgery involves the removal of the defective femoral component and replacement with a suitable prosthesis as shown in Figure 5B. It is a common procedure with over 300,000 hip replacement procedures carried in the US in 2010 [186]. During the procedure the femoral head is detached from the femur and removed from the acetabular socket. This allows the acetabulum to be exposed. It is at this interval that a bone marrow aspirate can be obtained and the operation can then continue without any further interruptions. In a total hip arthroplasty, the next stage involves reaming of the acetabulum in preparation for the new prosthesis. In the case of a hemi arthroplasty, the next stage involves preparation of the femoral component for the insertion of the femoral prosthesis.

Thus an aim of this study involves the acquisition of bone marrow samples from the pelvic bone intra-operatively. This will allow the isolation of MSCs from a highly cellular and vascularised network of trabecular bone.
5A: Normal Pelvic anatomy

Figure 5A: Anatomy of the pelvic bone in a female. The acetabulum is composed of the pubic bones, the ilium and the ischium. The traditional location of bone marrow aspirations in haematology practice is the anterior superior iliac spine (ASIS).

5B: Steps of a total hip arthroplasty

Figure 5B: Steps of a total hip arthroplasty including 1. Removal of the femoral head; 2. Insertion of the acetabular and femoral components, 3. Placement of the femoral component into the acetabular socket, 4. Articulation of the prosthetic joint
1.6 MSCs in bone disease

1.6.1 Osteoarthritis and MSCs

As described earlier, MSCs have the potential to play important roles in cartilage and bone based disorders such as OA. Understanding how MSCs behave in the osteoarthritic patient could be critical to uncovering the pathophysiology of this disease, and hence potentially lead to improved treatment strategies. A number of publications have analysed differences in the characteristics and behaviour of osteoarthritic MSCs with varying results. Li et al reported that a significantly greater number of MSCs can be recovered from the affected joints of OA patients, compared with healthy joints. Furthermore, the number of MSCs recovered were also reported to increase with the severity of the disease [187]. Murphy et al reported that the proliferative rates of MSCs derived from osteoarthritic bone marrow were significantly reduced with respect to healthy controls [188]. They also documented alterations in the differentiation profile with reduced adipogenesis and chondrogenesis noted in the osteoarthritic cohort. No difference was detected in osteogenic capability when compared to normal control MSCs [188]. Of note these trends were present in osteoarthritic MSCs isolated from multiple sources including the iliac crest, femur and tibia. An analysis of retropatellar fat pad derived MSCs suggested a decrease in adipogenic, chondrogenic and also osteogenic differentiation potentials in osteoarthritic donors in comparison to healthy donors [189]. Scharstuhl et al reported that the proliferative capability and chondrogenic differentiation ability were independent of age and OA aetiology [190]. Thus the literature presents somewhat conflicting reports in respect to both proliferative profiles and differentiation capabilities. Trends suggest a decreased adipogenic and chondrogenic ability with conflicting reports regarding osteogenic ability.

1.6.2 Osteoporosis and MSCs

Further understanding the role of MSCs in osteoporosis will allow greater clarity as to the pathogenesis of this disease and potentially uncover new therapeutic avenues. Growing evidence suggests that the structural abnormalities associated with osteoporotic bones are a consequence of dysfunctional MSCs during bone cell differentiation. Benisch et al performed a micro-array analysis of MSCs in osteoporotic patients and age matched controls. They detected distinct differences in MSCs from elderly donors with and without osteoporosis indicating that intrinsic alterations in MSC biology are involved in the pathophysiology of osteoporosis [191]. In a series of publications, Rodriguez et al reported that MSCs from
osteoporotic donors have a similar ability to form CFU but have decreased proliferation rates, in comparison to healthy samples. Osteoporotic MSCs had diminished alkaline phosphatase activity and a reduced calcium concentration compared with cells from mature donors [192, 193]. Conversely Stenderup et al reported that the number of MSCs, proliferation potential and osteogenic commitment were maintained in osteoporotic patients with no difference seen on comparison to young healthy donors [194]. Conflicting results also arise from osteoporotic rat models which suggest elevated osteogenic potentials as compared to controls [195, 196]. Circulating MSCs had increased numbers in osteoporotic donors and had a reduced expression of osteogenic transcription factors in comparison to healthy controls [197]. Haaster’s et al reported a reduction in the ability of osteoporotic MSCs to migrate towards the chemoattractants BMP2 and BMP7 [198].

Significant efforts have been made to enhance the altered osteogenic differentiation capabilities of osteoporotic MSCs. Zheng et al demonstrated that leptin can be transfected successfully into osteoporotic rat MSCs and the subsequent overexpression of leptin enhances the capacity of MSCs to undergo osteoblast differentiation. Further experiments are needed to validate this method but it highlights the efforts being undertaken to stimulate osteogenesis in osteoporotic MSCs [199].

1.6.3 MSCs and smoking

Tobacco use has been casually linked to a number of diseases including lung cancer and chronic obstructive lung disease as well as being identified as a risk factor for other diseases [200]. Cigarette smoking was first identified as a risk factor for osteoporosis decades ago [201]. The strongest evidence outlining the effects of smoking on bone mineral density arises from a meta-analyses of 29 studies. This study proposed that approximately one in eight hip fractures was attributable to cigarette smoking. Post-menopausal bone loss was greater in current smokers than in non-smokers, with bone density diminishing by approximately an additional 2% for every 10 year increase in age with a difference of 6% at age 80 [202, 203].

Data for the association between smoking and osteoarthritis is less clear and frequently contradictory. The first Health and Nutrition Examination Survey (HANES-1) in the US found a modest, inverse relationship between smoking and the presence of radiologically diagnosed knee OA [204]. A few further population based studies have also outlined a similar relationship between OA and smoking [205, 206]. However in a meta-analysis of 48 studies, the inverse association between smoking and the development of OA was observed only in
case-control studies but did not reach statistical significance in cohort and cross-sectional studies [207, 208]. They concluded that smoking did not appear to reduce the incidence or progression of OA [208].

The direct influence of nicotine on MSCs is not fully characterised or defined. Applying nicotine treatment to umbilical MSCs results in the inhibition of cell proliferation in a dose-dependent manner [209]. An analysis of cigarette smoking and fertility suggested that smoking inhibits the recruitment of MSCs to the uterus [210]. The influence of cigarette smoke was analysed by Liu et al with the chemotactic response of bone marrow derived osteoprogenitor cells inhibited by cigarette smoke in a concentration dependent manner [211]. Cigarette smoke extract (CSE) inhibited the differentiation of osteoprogenitor cells toward osteoblast like cells both in monolayer and in three dimensional gel culture [211]. These studies outline the potential direct effects of cigarette smoke on MSCs and their behaviour with the implications of same yet to be fully understood.

1.7 Primary Cilia

Primary cilia are organelles that were originally discovered over a century ago [212, 213]. Initially, they were believed to exist as vestigial organisms with little influence on cellular behaviour [214]. They received much less attention than the motile cilia found in the respiratory and reproductive tracts. However in the past decade, a significant number of publications have identified the primary cilia to be capable of both sensing and responding to its mechanical and chemical environment [215]. The emerging picture is that the primary cilium can act as a versatile nexus, whereby extracellular signals are sensed and processed to initiate a cellular response. The relationship between the primary cilia and bone formation has been a recent intense area of research with numerous publications in this domain. Tummala et al demonstrated the existence of primary cilia on bone marrow derived MSCs. Upon staining with alpha-acetylated tubulin, >90% of cells imaged possessed a primary cilium projecting from the peri-nuclear region of the cell [216]. Using small interfering RNA (siRNA) targeting, the formation of the primary cilium was inhibited and the baseline expression of transcription factors runt related transcription factor 2 (RUNX2) in bone, sry related high mobility group box transcription factor 9 (SOX9) in cartilage, and peroxisome proliferator activated receptor gamma (PPARγ) in fat were quantified. By simply inhibiting the ability of MSCs to form primary cilia, the expression levels of all three transcription factors was reduced. Polycystin 1 (PC1) is a large transmembrane protein that co-localises to the cilia,
and acts to control proper tubular diameter. Xiao et al reported that a mouse model which contains a mutation in the Pkd1 gene which encodes the ciliary protein PC1, results in an osteopenic phenotype [217].

The pathophysiology of osteoporosis, as mentioned previously, describes an imbalance in bone remodelling. It is well documented that the prolonged absence of mechanical loading can lead to altered bone mineral density and osteoporosis [218, 219]. Studies have recently outlined a role for primary cilia in effective cellular mechanotransduction [220, 221]. Evidence has accumulated and demonstrated that deflection of the primary cilium under fluid flow results in an increase in the gene expression of the osteogenic marker Cox-2 [222]. In addition to sensing fluid flow, primary cilia have been shown to sense and respond to other mechanical signals such as pressure, touch and vibration [223, 224]. Hoey et al explored the response of MSCs to fluid flow, following transfection which rendered the MSCs unable to form primary cilia. They demonstrated that without the primary cilium, osteogenic markers such as COX-2 and BMP-2 did not upregulate in response to fluid flow [225]. It has further been suggested that changes in the structural mechanics of primary cilia greatly affect the ability of the cell to respond to its mechanical environment. For example, studies have demonstrated that the primary cilium decreases in length corresponding with a reduction in amiloride sensitive sodium current [226-228]. Therefore, it has been hypothesised that alterations in the length of primary cilia may be a central mechanism for regulating cellular mechanosensitivity.

The relationship between TGF-β signalling and the primary cilium has been a recent area of exciting research. Clement et al reported that mice defective in the formation of primary cilia had cardiac anomalies characteristic of defective TGF-β signalling [229, 230]. It has also been more recently suggested that activation of TGF-β signalling occurs at the ciliary pocket in fibroblasts and in stem cells differentiating into cardiomyocytes [231]. Thus the role of the primary cilium and TGF-β signalling in mechanobiology is an evolving area of research with the significant implications for diseases such as osteoporosis and osteoporosis.

1.8 Potential treatment strategies

MSCs have generated a huge amount of excitement in the past number of years with numerous clinical trials eager to harness their potential clinical benefits. The unique in vivo capabilities of isolated MSCs suggest a critical role in maintaining bone’s innate capacity both for remodelling in response to mechanical stimuli and regeneration upon damage.
Currently in August 2015, MSC based clinical trials have been conducted for at least 12 different pathological conditions. The first trial using culture expanded MSCs was carried out in 1995 when 15 patients became the recipients of autologous cells [232]. Autologous MSCs involve the removal of MSCs from a patient, and re-insertion of these cells to a specific target region in the same patient. Currently in August 2015 there are 529 clinical trials using MSCs for a wide variety of clinical applications. Most of these trials are in Phase I (safety studies), Phase II (proof of concept for efficacy in human patients), or a mixture of Phase I/II studies. A small number of trials are in Phase III (comparing a newer treatment to the standard or best known treatment). In general, MSCs appear to be well tolerated, with most trials reporting lack of adverse effects in the medium term, although a few showed mild and transient peri injection effects [233].

MSCs have also been trialled for the treatment of cardiovascular diseases, graft versus host disease (GVHD), autoimmune disorders such as systemic lupus erythematosus (SLE) and liver cirrhosis [234-237]. MSCs have been shown to be beneficial in treating bone disorders such as osteogenesis imperfecta (OI). OI is a disorder of bone fragility chiefly caused by mutations in the genes that encode type 1 procollagen. Paediatric patients with OI underwent allogenic haematopoietic stem cell transplantation. The transplanted bone marrow cells engrafted and generated functional osteoblasts leading to improvement in bone structure and function [238]. A follow up study showed continued improvement for patients 18 to 36 months post bone marrow transplantation [239]. However, these patients were treated with whole bone marrow transplantations rather than MSC transplantation alone. Therefore it is difficult to attribute the observed effects to MSCs alone. Although the number of clinical trials is limited, bone marrow transplantation in paediatric patients for the treatment of OI appears to result in some clinical improvement. [240].

MSCs have also been proposed as a therapy for the treatment of OA. A search of trials registered with the US National Institute of Health lists 37 trials involving MSCs and OA. Of the listed trials, 21 involve the use of autologous MSCs [241]. Results from initial case studies suggest that the use of autologous MSCs in the treatment of OA may improve cartilage growth and patient’s symptoms [242-248]. The use of autologous treatments negates the response of the host immune system, which is frequently a difficult barrier to overcome when using allogenic transplants. However, these studies analyse the results of case reports and it remains unclear as to the underlying mechanism resulting in cartilage regeneration in
these case reports. A phase III trial regarding the use of MSCs as treatment for OA has yet to be published.

The results of clinical trials across different clinical fields have been promising but also highlight the critical challenges that must be addressed in future research. At present the use of MSCs as a form of therapy poses significant challenges as the underlying mechanisms of action of the transplanted cells are largely unknown.
Chapter 2:
Generation of a Biobank of Human Osteoporotic and Osteoarthritic MSCs

2.1 Introduction

The primary aim of this research was to develop a biobank of osteoporotic and osteoarthritic MSCs. This incorporates the process of identifying suitable donors to participate in effective research, obtaining consent for bone marrow aspiration and thus enabling the extraction of MSCs from the respective aspirates in the laboratory. This chapter outlines the details of the biobank created including the methodology involved, the characteristics, demographics and co-morbidities of the donors involved. MSCs are isolated from bone marrow aspirates in this study, but the location and timing of aspirate sampling in this research is novel. Aspirates are obtained intra-operatively during necessary hip operations. This chapter progresses to outline the results of the surgical technique employed. Identification of MSCs is carried out using ISCT criteria which includes the use of flow cytometry to identify cell surface antigens. Following on from the identification of MSCs, growth characteristics are outlined using the respective population doubling times of donor samples according to disease state.
2.2 Materials and Methods

2.2.1 Selection of patients

Ethical approval for this study was approved by the University of Limerick and the Health Service Executive and adhered to throughout the duration of the study (see appendix). The cohort of patients came from the University Hospital Limerick Group, which encompasses both University Hospital Limerick (UHL) and the Mid-Western orthopaedic hospital in Croom, Co. Limerick. Bone marrow samples were obtained from 10 patients undergoing necessary hip surgery.

Osteoarthritic patients were identified prior to elective hip replacement surgery (total hip arthroplasty). Each patient had clinical manifestations of osteoarthritis necessitating joint replacement surgery. Patients were identified through theatre schedule lists identifying their upcoming joint replacement surgery. Osteoarthritic patients were approached prior to surgery and following a discussion indicating a willingness to participate in the study, informed consent was obtained from each donor.

Osteoporotic patients were identified following the diagnosis of a femoral neck fragility fracture. Osteoporosis was clinically diagnosed as per the National Osteoporosis Foundation Guidelines which states that “a clinical diagnosis can be made in at risk individuals who sustain a low trauma fracture” [56]. Osteoporotic patients with a fracture necessitating a hemiarthroplasty or a total hip arthroplasty were included. Patients were identified on admission and following listing for surgery, consent was obtained.

Healthy patients were identified following the diagnosis of a femoral neck fracture requiring a hemiarthroplasty or a total hip arthroplasty to repair same. Healthy patients did not have a history of osteoporosis. The mechanism of injury must be significant to result in this injury (i.e. not a fragility fracture).

Exclusion criteria included patients unable to consent to the procedure, active malignancy or patients receiving osteoporotic medication prior to admission.

2.2.2 Bone marrow aspiration

Bone marrow aspirates were taken from the pelvis intraoperatively. During a total hip replacement or a hemiarthroplasty, the femoral head is removed in preparation for the prosthesis [249]. Thus the acetabulum is exposed prior to insertion of the prosthesis intra-
operatively. A Jamshidi needle (Stryker) was then used for bone marrow aspiration. A Jamshidi needle is a trephine needle, which is cylindrical and has a tapered cutting tip (see Figure 6). The tapered end reduces the potential of crush artefact. A T-handle attached to the needle allows a leur lock syringe to be applied and withdraw a specific volume of bone marrow into the respective syringe.

On exposure of the acetabulum, a Jamshidi needle was inserted in the superior rim of the acetabulum. This was inserted 3-4cm in depth, resulting in bone marrow aspirate being withdrawn from the ilium. The insert of the Jamshidi needle was then removed. A 50ml syringe was used to withdraw 30mls of bone marrow aspirate. Each procedure was carried out by an experienced surgeon. The bone marrow aspirate was transferred to sterile containers and transported to the laboratory. The sterile containers were transported at room temperature. Time from extraction to arrival in laboratory was 20 minutes in duration. The average frequency of sampling was one aspirate per fortnight.
Figure 6: (A): Pre-operative anatomy showing the femoral head articulating with the acetabulum. (B): Femoral head is removed from the femoral shaft to allow a prosthesis to be inserted (C): The acetabulum is now exposed and under direct vision of the surgeon. Prior to the next stage of acetabular reaming, 30mls of bone marrow aspirate is obtained from the superior rim of the acetabulum. The point of needle entry is marked by an X on the diagram. Red arrow highlights the anterior superior iliac spine, the point of entry during a routine bone marrow aspiration (D): A Jamshidi needle and a 50ml Leur lock syringe is used to withdraw the bone marrow aspirate.
2.3 Isolation and expansion of MSCs

2.3.1 Isolation of MSCs

As mentioned previously 30mls of bone marrow aspirate was obtained from each donor. This was separated into 5ml aliquots in sterile 50ml tubes. 35ml of sterile phosphate buffered saline (PBS) was added to each aliquot. This diluted suspension underwent centrifugation at 900g for 10 minutes and the supernatant aspirated from same. The cells were resuspended in complete growth medium. Complete growth medium consisted of Dulbecco Modified Eagle Medium (DMEM) low glucose, 10% Fetal Bovine Serum (FBS), 1% Penicillin/Streptomycin (P/S) and 1% Non-essential amino acids (NEAA). A 50µl sample of the cell suspension was removed and further diluted with 450µl of PBS. 50µl of this solution was further added to 50µl of 4% acetic acid to lyse red blood cells and the total number of mononuclear cells was then counted using a haemocytometer. The mononuclear cell fraction, containing the MSCs was seeded at a density of 250,000 cells/cm² onto tissue culture treated flasks. MSCs were expanded in optimal growth conditions by incubation at 37°C, 5% CO₂ and 90% humidity. A volume of 30mls of complete growth medium per T-175 flask was used. Medium was aspirated from the flasks at 72 hour intervals and replaced with fresh medium. Sub-culture was performed when cells became 70% confluent (between 10 and 14 days). Cells were subcultured to passage 3 and were then used for experimentation at passage 3 or cryopreserved in liquid nitrogen for future experiments.

2.3.2 Sub-culturing of MSCs

MSCs were subcultured according to the level of confluency observed under magnification. Level of confluency was assessed daily under a magnification of 4X (Olympus IX53). At a level of 70% confluency, cells were sub-cultured. Medium was aspirated and the adherent cells were washed with PBS. 4mls of Trypsin-EDTA was added and the cells were incubated for 5 minutes. The cells were viewed under the microscope at 4X magnification to ensure cellular detachment. 30mls of complete medium was then added and the cellular suspension was centrifuged at 400g for 5 minutes. The supernatant was then aspirated and the cells suspended in 10ml complete MSC medium. The cells were counted and plated at 2 X 10³/cm² in complete MSC medium.
2.3.3 Freezing and thawing of MSCs

MSCs were expanded to passage 3 (P3), and then either used for experimentation or cryopreserved for future use. Freezing medium consisted of 50% DMEM low glucose and 45% FBS. Following sub-culturing as outlined above, MSCs were suspended in the appropriate volume of freezing media. MSCs were frozen at a concentration of $1 \times 10^6$ per vial. 5% of dimethylsulfoxide (DMSO) was added to each vial. Vials were stored in -80°C overnight and transferred to a liquid nitrogen freezer.

MSCs were thawed when required for experimentation. The appropriate vial was removed from the liquid nitrogen freezer. Following a brief immersion in 37°C, the thawed cell suspension was added to complete MSC medium. A further 1ml of medium was used to rinse the excess cells from the vials and added to the cell suspension. The cells were then cultured in conditions relevant to the experiment being undertaken.

2.3.4 Determination of proliferation kinetics

In order to assess proliferation rate, the number of cells in each flask were counted at each time of sub-culturing using a haemocytometer. An excel sheet was used to record the number of cells present against the timing of sub-culturing. The population doubling time (PDT) was also calculated for each passage [250]. This calculation is based on the number of cells harvested and the number of cells initially seeded, versus the duration of time in culture.

\[
PDT = \frac{t \cdot (\log 2)}{\log N_h - \log N_i}
\]

$N_h$ = Number of cells harvested

$N_i$ = Number of cells initially plated

$t$ = duration of culture in days

2.3.5 Colony forming unit assay

Bone marrow aspirates were observed for their ability to form colony forming units (CFU). Following initial centrifugation and re-suspension, $2 \times 10^5$ cells in complete medium were seeded in a T-75 flask. 15ml of complete medium was then added. Medium was aspirated at 72 hour intervals and replaced with fresh medium. On day 14, medium was aspirated and cells were washed with PBS. 15ml of 4% Crystal violet staining solution was added. An
incubation period of 15 minutes was observed. Distilled water was then used to wash the cells and this step was repeated for a total of three times. The flask was divided into quadrants and colonies were counted manually based on size greater than 0.5mm.

2.3.6 Flow cytometry

Flow cytometry was used to analyse the expression of cell surface molecules. Flow cytometry is a biophysical technology employed in cell counting and biomark detection. It is conducted by suspending cells in a stream of fluid and passing them by an electronic detection apparatus. At P3, cells underwent trypsinisation and were resuspended in staining buffer (PBS, 2% FBS and 1mM EDTA). The cells were centrifuged at 400g for 5 minutes and resuspended in rinsing solution (PBS and 0.5mM EDTA) at a density of $1 \times 10^5$ per microtube. The cells were incubated on ice for 30 minutes before centrifugation at 400g for 5 minutes. The supernatant was removed and the cells were resuspended in rinsing solution. This centrifugation and re-suspension step were repeated twice more. Following the final removal of supernatant, the cells were incubated for 30 minutes on ice with the antibody of interest. The cells were then centrifuged as previously and resuspended in staining buffer. This step was repeated twice. Following the final centrifugation, the cells were suspended in serum free medium. 100µL of the suspension was transferred to 96 well flat bottomed plate in triplicate. Control wells included cells alone without an antibody and cells incubated with immunoglobulin (IgG) isotype control.
The same analysis strategy using Guava software was carried out on each sample. In addition to fluorescence, two types of light scatter were measured. Forward scatter is roughly proportional to the diameter of the cell and side scatter is proportional to its granularity. Cells were gated on forward scatter and side scatter to exclude cell debris and cell aggregates. Unstained cells in the form of cells alone with PBS were analysed prior to the labelled samples. Regions were thus set for auto-fluorescence for subsequent cell surface marker analysis. A minimum of 5,000 events per sample were analysed during data acquisition. Two parameter plots can show four distinct populations as can be seen in Figure 7. The upper left quadrant represents the population that is negative for the x axis fluorochrome and positive for the y-axis fluorochrome. The upper right quadrant represents the population positive for both fluorochromes. The lower left quadrant represents the population negative for both fluorochromes. The lower right quadrant represents the population positive for the x axis fluorochrome and negative for the y axis fluorochrome. Each antibody was analysed in triplicate for each sample. The mean and standard deviation of positive events was thus calculated for each antibody incubated with the cells under assessment.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Dilution</th>
<th>Manufacturer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse anti-human CD105 Phycoerythrin</td>
<td>1:10</td>
<td>Abcam (ab91238)</td>
</tr>
<tr>
<td>Mouse anti-human CD73 Phycoerythrin</td>
<td>1:20</td>
<td>Abcam (ab157335)</td>
</tr>
<tr>
<td>Mouse anti-human CD90 Phycoerythrin</td>
<td>1:40</td>
<td>Abcam (ab95700)</td>
</tr>
<tr>
<td>Mouse anti-human CD44 Allophycocyanin</td>
<td>1:20</td>
<td>Abcam (ab81424)</td>
</tr>
<tr>
<td>Mouse anti-human CD34 Phycoerythrin</td>
<td>1:20</td>
<td>Abcam (ab46970)</td>
</tr>
<tr>
<td>Mouse anti-human CD45 Phycoerythrin</td>
<td>1:20</td>
<td>Abcam (ab1176)</td>
</tr>
<tr>
<td>Mouse anti-human IgG Isotype control Phycoerythrin</td>
<td>1:20</td>
<td>Abcam (ab81200)</td>
</tr>
</tbody>
</table>

**Table 1: List of antibodies and dilution for cell surface antigen detection**
2.4 Statistical methods

Values are displayed as the mean +/- standard deviation of the mean (SD) or mean +/- standard error of the mean (SE). Significance of datasets were analysed using one way or two way ANOVA and Bonferroni’s multiple comparison post test. A value of <0.05 was considered statistically significant and marked with a “*” symbol.
2.5 Results

2.5.1 Patient selection

A total of 10 patients were selected for inclusion in this study. The following section outlines the demographics and characteristics of the participants. A total of 4 osteoporotic samples, 5 osteoarthritic and 1 healthy sample were obtained as displayed in table 3. All 4 osteoporotic samples were obtained from donors who had suffered a fragility hip fracture and were subsequently undergoing a hip hemiarthroplasty. All osteoarthritic patients had clinically significant hip osteoarthritis necessitating a total hip arthroplasty for same. A single healthy aspirate was obtained following a high trauma femoral neck fracture in the form of an equestrian accident. This patient underwent a total hip arthroplasty as a result of same.

Table 2: Outline of bone marrow aspirates obtained according to disease state and operation

<table>
<thead>
<tr>
<th>Age</th>
<th>Gender</th>
<th>Disease</th>
<th>Operation</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>59</td>
<td>F</td>
<td>Traumatic Fracture</td>
</tr>
<tr>
<td>2</td>
<td>64</td>
<td>M</td>
<td>Osteoporosis</td>
</tr>
<tr>
<td>3</td>
<td>86</td>
<td>F</td>
<td>Osteoporosis</td>
</tr>
<tr>
<td>4</td>
<td>80</td>
<td>F</td>
<td>Osteoporosis</td>
</tr>
<tr>
<td>5</td>
<td>89</td>
<td>F</td>
<td>Osteoporosis</td>
</tr>
<tr>
<td>6</td>
<td>60</td>
<td>M</td>
<td>Osteoarthritis</td>
</tr>
<tr>
<td>7</td>
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<td>75</td>
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<td>70</td>
<td>F</td>
<td>Osteoarthritis</td>
</tr>
<tr>
<td>10</td>
<td>70</td>
<td>F</td>
<td>Osteoarthritis</td>
</tr>
</tbody>
</table>

2.5.2 Donor Co-morbidities
Age is a significant risk factor for both of the diseases under investigation in this study [251, 252]. A high incidence of co-morbidities is seen in elderly populations. Thus each of the donor co-morbidities are outlined in Table 3. The most common co-morbidity was hypertension, with one third of patients receiving medication for hypertension. In all cases, blood pressure was adequately controlled at the time of operation. In the osteoporotic cohort, one patient suffered from Parkinson’s disease. Osteoporosis and osteopenia are very common findings in patients with Parkinson’s disease, affecting up to 91% of men and 61% of women [253]. The only concurrent musculoskeletal disorder was a single case of gout. In this instance, symptoms had been limited to hands and feet, with no recent exacerbations of same. Of significance, one patient in the osteoarthritic cohort had been diagnosed with hyperthyroidism. Thyroid disease has widespread systemic manifestations and this includes an impact on bone metabolism [254]. Evaluation of thyroid function tests in this donor routinely carried out prior to the scheduled operation were within the normal range, and there had been no recent change in thyroid mediation. Of note, no patient in any of the groups had been diagnosed with osteoporosis, or were receiving treatment for osteoporosis prior to bone marrow aspiration.

Table 3: Outline of donor co-morbidities and medication
<table>
<thead>
<tr>
<th>Age</th>
<th>Co-morbidities</th>
<th>Medication</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Healthy</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Osteoporosis</td>
<td>Rasagiline 1mg OD Carbidopa-Levodopa 50/200mg TDS Quetiapine 25mg nocte</td>
</tr>
<tr>
<td>3</td>
<td>Hypertension</td>
<td>Mirtazapine 30mg daily Paracetamol 1 gram PRN Esomeprazole 20mg PRN</td>
</tr>
<tr>
<td>4</td>
<td>Hypertension</td>
<td>Bendroflumethiazide 5mg OD</td>
</tr>
<tr>
<td>5</td>
<td>Gout</td>
<td>Esomeprazole 40mg BD Cymbalta 30mg BD Allopurinol 300mg OD Pregabalin 25mg OD Aspirin 75mg OD Calcichew D3 Forte 500/400iu daily</td>
</tr>
<tr>
<td>6</td>
<td>Osteoarthritis</td>
<td>Paracetamol/Codeine Phosphate 30/500mg PRN</td>
</tr>
<tr>
<td>7</td>
<td>Hyperthyroidism</td>
<td>Eltroxin 100mcg daily Etoricoxib 60mg daily Diclofenac 75mg PRN Salbutamol inhaler PRN</td>
</tr>
<tr>
<td>8</td>
<td>Hypertension</td>
<td>Hydrochlorothiazide/Olmesartan/Amlodipine 40mg/5mg/12.5mg OD</td>
</tr>
<tr>
<td>9</td>
<td>Nil</td>
<td>Diclofenac 75mg PRN Tramadol 50mg PRN</td>
</tr>
<tr>
<td>10</td>
<td>Asthma</td>
<td>Salbutamol inhaler PRN</td>
</tr>
</tbody>
</table>

**2.5.3 Donor age**
The age of the donors varied from a minimum age of 59 to a maximum age of 89 years old. The individual ages of donors can be discerned from table 6. The mean age across all donors was 72.8 years, with a mean of 79.5 and 70 years in the osteoporotic and osteoarthritic groups respectively. The higher mean age in the osteoporotic cohort is consistent with an increasing osteoporotic fracture incidence with age [255]. However, there was no statistically significant difference between the osteoporotic and osteoarthritic groups in mean age as can be seen in figure 5.

Table 4: Table of donors and disease state including age and smoking history

<table>
<thead>
<tr>
<th></th>
<th>Age</th>
<th>Gender</th>
<th>Disease</th>
<th>Smoking</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>59</td>
<td>F</td>
<td>Traumatic Fracture</td>
<td>Nil</td>
</tr>
<tr>
<td>2</td>
<td>64</td>
<td>M</td>
<td>Osteoporosis</td>
<td>Nil</td>
</tr>
<tr>
<td>3</td>
<td>86</td>
<td>F</td>
<td>Osteoporosis</td>
<td>60pyh</td>
</tr>
<tr>
<td>4</td>
<td>80</td>
<td>F</td>
<td>Osteoporosis</td>
<td>60pyh</td>
</tr>
<tr>
<td>5</td>
<td>89</td>
<td>F</td>
<td>Osteoporosis</td>
<td>Nil</td>
</tr>
<tr>
<td>6</td>
<td>60</td>
<td>M</td>
<td>Osteoarthritis</td>
<td>20pyh</td>
</tr>
<tr>
<td>7</td>
<td>75</td>
<td>F</td>
<td>Osteoarthritis</td>
<td>Nil</td>
</tr>
<tr>
<td>8</td>
<td>75</td>
<td>M</td>
<td>Osteoarthritis</td>
<td>25pyh</td>
</tr>
<tr>
<td>9</td>
<td>70</td>
<td>F</td>
<td>Osteoarthritis</td>
<td>50pyh</td>
</tr>
<tr>
<td>10</td>
<td>70</td>
<td>F</td>
<td>Osteoarthritis</td>
<td>Nil</td>
</tr>
</tbody>
</table>

Figure 8: Graphical representation of the mean age of each disease group (Mean +/- SD). No statistical significance identified between osteoporotic and osteoarthritic donors. (p=0.1365)
2.5.4 Gender of donors

There was a greater number of female donors in this study as depicted in figure 6. The osteoarthritic cohort had a ratio of 3:2 female to male donors. The discrepancy in gender balance arises from the osteoporotic cohort, which had a 3:1 ratio of female to male donors. This is reflective of the increased prevalence of osteoporosis in the female population and the female gender is a prominent risk factor for the development of osteoporosis [256, 257].

**Figure 9:** Number of female to male donors

![Figure 9: Number of female and male donors across all groups (Female n=7; Male n=3)](image)

2.5.5 Donor smoking history

It has been well demonstrated that smoking can have a significant impact on bone metabolism. Smokers are at an increased risk of osteoporosis and the risk rises with increased tobacco consumption [258, 259]. A pack year is a quantification of a patient’s smoking history and is frequently used in both the clinical and research setting [260]. It is calculated by multiplying the number of packs of cigarettes smoked per day by the number of years that the person has smoked. There was a greater pack year history (PYH) of smoking in the osteoporotic group although no statistical significance was observed (p=0.57). The individual smoking history of each donor is outlined in table 6.
2.5.6 Surgical technique

This research employed a novel technique in obtaining bone marrow aspirates from human patients. This technique can have implications for the development and expansion of further biobanks of MSCs in the future. Hip arthroplasty surgery involves the removal of the defective femoral component and replacement with a suitable prosthesis. It is a common procedure with over 300,000 hip replacement procedures carried in the US in 2010 [186]. During each procedure in this study, the femoral head was detached from the femur and removed from the acetabular socket. This allowed the acetabulum to become exposed as shown in figure 8. It is at this interval that a bone marrow aspirate was obtained using a Jamshidi needle. The Jamshidi needle was inserted into the superior rim of the acetabulum to a depth of 3-4cm. The inner sleeve of the needle was removed and a 50ml Leur Lock syringe attached to the needle. 30mls of bone marrow aspirate was withdrawn on each occasion with no dry aspirates recorded. All aspirates were obtained with a single insertion of the Jamshidi needle and no second attempts were required. The bone marrow aspirate was then placed in sterile pots for transportation to the lab. The Jamshidi needle was removed from the acetabulum and the operation continued as normal for the respective procedure being undertaken. In a total hip arthroplasty, the next stage involved reaming of the exposed acetabulum in preparation for the new prosthesis. In the case of a hemi arthroplasty, the next stage involved preparation of the femur for the insertion of the femoral prosthesis. No complications were observed during the procedures and there were no adverse outcomes for the patients involved.

Figure 10: Smoking status as measured in pack year history per disease state. (Mean +/- SD) A greater PYH is noted in the osteoporotic group, however this lacks statistical significance (p=0.57). PYH = Pack year history
Figure 11: (A) Following removal of the femoral head, the acetabulum is exposed. The superior acetabular rim is identified under direct vision. (B) A Jamshidi needle is inserted into this point to a depth of 5-6cm. (C) 30mls of bone marrow aspirate is withdrawn and transferred to sterile containers.
2.6 Isolation and characterisation of MSCs

2.6.1 Adherence to plastic

Following the successful aspiration of bone marrow, the next step is to successfully isolate and identify the presence of MSCs. The first criteria as per the ISCT in identifying MSCs, involves demonstrating the ability of cells to adhere to plastic in standard culture conditions [2]. Adherent cells of a fibroblast morphology were visualised in each sample at a mean of day 8 (+/-1.2 days), with a representative sample depicted in figure 12.

![Adherent cells visualised under microscopy at day 8 of cell culture from donor 2 (osteoporotic donor) Scale bar = 50µm](image)

**Figure 12:** Adherent cells visualised under microscopy at day 8 of cell culture from donor 2 (osteoporotic donor) Scale bar = 50µm
2.6.2 Population growth and Population doubling time

The growth characteristics of each aspirate was analysed and the number of cells plotted against time in culture. This data was obtained from counting MSCs at each time of sub-culture. Osteoporotic samples trended towards a higher yield of MSCs but without statistical significance as seen in Figure 13. There was no difference in the respective growth curve obtained from either disease state. Population doubling time was calculated for each aspirate as per the equation in section 3.2.4. The mean PDT between osteoporotic and osteoarthritic samples displayed no difference. No significant difference in PDT was noted between respective passage numbers. Subgroup analysis for gender and smoking history also failed to reveal any difference between the osteoarthritic and osteoporotic cohort.
Figure 13: Population growth per disease state (A): Mean number of cells per donor plotted from day 12-20 per disease state. (Mean +/- SD) (B): Box and whisker plots outlining the minimum, 25th, 50th, 75th, and maximum population doubling times per disease state from passage 1 to passage 2. (C): Population doubling time from passage 2 to passage 3 (PDT = population doubling time). No statistical difference noted in PDT for either graph.
2.6.3 Colony Forming Unit Assay

Crystal violet staining solution was used to visualise the formation of colonies following 14 days of culture in complete MSC medium. An example of colonies macroscopically and under magnification at 4X can be seen in figure 14. A greater number of colonies was present in the osteoporotic cohort but without statistical significance. The assay was also analysed for differences between gender and smoking status with no statistical difference demonstrated.
Figure 14: Colony Forming unit Assay

A Number of Colony Forming Units per disease state

B Picture of colony forming units stained by crystal violet in a section of a T-75 flask.

C Colony forming units stained with crystal violet under microscopic visualisation

D Smoking History

E Gender

Figure 14: Colony forming unit assay (A): number of colony forming units per disease state (Mean +/- SD; p=0.0874) (B): Picture of colony forming units stained by crystal violet in a section of a T-75 flask. (C) Colony forming units stained with crystal violet under microscopic visualisation (D): Representation of the number of colony forming units per smoking status (Mean +/- SD p=0.6507) (E): Number of colony forming units per gender in the osteoarthritic cohort (Mean +/- SD p=0.6727) Scale bar = 50µm
2.6.4 Cell surface markers

Adherent cells were analysed for the presence of specific cell surface markers, in order to identify the presence of MSCs. Each bone marrow aspirate at P3 underwent flow cytometry to analyse the presence of cell surface markers CD34, CD45, CD73, CD90, CD105 and CD44. All samples were analysed for each antibody in triplicate. A representative sample of flow cytometry results from an osteoporotic donor can be seen in Figure 15. IgG and cells alone were used as controls to account for cell auto fluorescence. It can clearly be seen that this representative sample is positive for the CD antigens, CD73, CD105, CD90 and CD44 and negative for CD45 and CD34.
**Figure 15:** Flow cytometry results of donor 2 (A): Cells alone with no antibody present representing autofluorescence of cells alone. (B): Cells incubated with CD34 and CD45 antibodies respectively. No change in fluorescence indicating a negative result. (C): Cells incubated with CD44, CD73, CD105, CD90 with a marked change in fluorescence indicating positive results. (Percentages detail number of events positive for antibody under investigation in each respective quadrant)
Following replication for each marker in triplicate, the mean and standard deviation of positive events are recorded as outlined in Table 5. Each sample was negative for the cell surface antigens CD34 and CD45 as indicated by the minimal number of positive events for these markers. Each sample reported a significant positive number of events for the cell surface antigens CD90, CD73, CD105 and CD44.

**Table 5**: Percentage positive events of cell surface markers per donor (mean +/- SD)

<table>
<thead>
<tr>
<th></th>
<th>CD34</th>
<th>CD45</th>
<th>CD90</th>
<th>CD73</th>
<th>CD105</th>
<th>CD44</th>
</tr>
</thead>
<tbody>
<tr>
<td>Healthy</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>1.98% (+/- 0.3%)</td>
<td>1.68% (+/- 0.25%)</td>
<td>96.27% (+/- 0.78%)</td>
<td>99.82% (+/- 0.03%)</td>
<td>91.3% (+/- 0.43%)</td>
<td>97.92% (+/- 0.23%)</td>
</tr>
<tr>
<td>Osteoporosis</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>1.4% (+/- 0.24%)</td>
<td>0.26% (+/- 0.32%)</td>
<td>99.01% (+/- 0.22%)</td>
<td>99.7% (+/- 0.15%)</td>
<td>92.1% (+/- 0.45%)</td>
<td>98.9% (+/- 0.34%)</td>
</tr>
<tr>
<td>3</td>
<td>1.34% (+/- 0.23%)</td>
<td>2.11% (+/- 0.31%)</td>
<td>99.27% (+/- 0.15%)</td>
<td>99.41% (+/- 0.36%)</td>
<td>93.2% (+/- 0.45%)</td>
<td>98.82% (+/- 0.11%)</td>
</tr>
<tr>
<td>04</td>
<td>1.50% (+/- 0.12%)</td>
<td>1.12% (+/- 0.22%)</td>
<td>98.5% (+/- 0.14%)</td>
<td>99.34% (+/- 0.33%)</td>
<td>92.50% (+/- 0.53%)</td>
<td>97.71% (+/- 0.44%)</td>
</tr>
<tr>
<td>Osteoarthritis</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>2.28% (+/- 0.12%)</td>
<td>1.75% (+/- 0.30%)</td>
<td>94.4% (+/- 0.24%)</td>
<td>99.74% (+/- 0.04%)</td>
<td>91.34% (+/- 0.44%)</td>
<td>98.02% (+/- 0.03%)</td>
</tr>
<tr>
<td>6</td>
<td>1.9% (+/- 0.09%)</td>
<td>1% (+/- 0.21%)</td>
<td>96.92% (+/- 0.32%)</td>
<td>99.47% (+/- 0.22%)</td>
<td>96.4% (+/- 0.34%)</td>
<td>96.2% (+/- 0.42%)</td>
</tr>
<tr>
<td>7</td>
<td>1.43% (+/- 0.23%)</td>
<td>2.11% (+/- 0.17%)</td>
<td>99.49% (+/- 0.03%)</td>
<td>96.8% (+/- 0.23%)</td>
<td>94.03% (+/- 0.30%)</td>
<td>98.46% (+/- 0.20%)</td>
</tr>
<tr>
<td>8</td>
<td>0.7% (+/- 0.12%)</td>
<td>1.42% (+/- 0.32%)</td>
<td>99.59% (+/- 0.03%)</td>
<td>99.56% (+/- 0.02%)</td>
<td>96.05% (+/- 0.23%)</td>
<td>99.38% (+/- 0.12%)</td>
</tr>
<tr>
<td>9</td>
<td>1.58% (+/- 0.32%)</td>
<td>1.65% (+/- 0.21%)</td>
<td>99.50% (+/- 0.12%)</td>
<td>98.78% (+/- 0.15%)</td>
<td>95.20% (+/- 0.34%)</td>
<td>98.50% (+/- 0.12%)</td>
</tr>
<tr>
<td>10</td>
<td>1.21% (+/- 0.13%)</td>
<td>1.43% (+/- 0.21%)</td>
<td>98.20% (+/- 0.16%)</td>
<td>97.98% (+/- 0.34%)</td>
<td>94.56% (+/- 0.25%)</td>
<td>97.98% (+/- 0.12%)</td>
</tr>
</tbody>
</table>
There was no statistical difference between disease state and the presence of the respective cell surface markers in the cases of CD34, CD45, CD90, CD73 and CD44. However, of note a difference in the presence of cell surface antigen CD105 was noted. A statistically significant lower percentage of positive events was noted in the osteoporotic cohort in comparison to the osteoarthritic cohort as seen in Figure 16.

**Figure 16**: Percentage of positive events during flow cytometry per cell surface marker and disease state. (A): Percentages indicating a negative result for CD34 and CD45 and a positive result for CD90, CD73, CD105 and CD44. (B): Percentage positive for CD105 in osteoporotic and osteoarthritic donors indicating a statistically significant difference in positive events recorded. (Mean + SD; \( p=0.0018 \))
2.6.5 Cryopreserved MSCs

Following identification as outlined above, MSCs were cryopreserved for future use. As can be seen in Table 6 a total of 109 vials of MSCs were cryopreserved.

Table 6: Number of vials frozen per sample

<table>
<thead>
<tr>
<th>Sample</th>
<th>Disease state</th>
<th>Passage Number</th>
<th>Number of Vials Frozen</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Healthy</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>2</td>
<td>Osteoporosis</td>
<td>3</td>
<td>17</td>
</tr>
<tr>
<td>3</td>
<td>Osteoarthritis</td>
<td>3</td>
<td>15</td>
</tr>
<tr>
<td>4</td>
<td>Osteoarthritis</td>
<td>3</td>
<td>6</td>
</tr>
<tr>
<td>5</td>
<td>Osteoarthritis</td>
<td>3</td>
<td>17</td>
</tr>
<tr>
<td>6</td>
<td>Osteoporosis</td>
<td>3</td>
<td>8</td>
</tr>
<tr>
<td>7</td>
<td>Osteoarthritis</td>
<td>3</td>
<td>5</td>
</tr>
<tr>
<td>8</td>
<td>Osteoporosis</td>
<td>3</td>
<td>10</td>
</tr>
<tr>
<td>9</td>
<td>Osteoporosis</td>
<td>3</td>
<td>20</td>
</tr>
<tr>
<td>10</td>
<td>Osteoarthritis</td>
<td>3</td>
<td>7</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td></td>
<td><strong>109</strong></td>
</tr>
</tbody>
</table>
2.7 Discussion

The primary aim of this study was to successfully extract and isolate osteoarthritic and osteoporotic MSCs with the creation of a functional biobank. Research into the behaviour and application of MSCs has come to the forefront over recent decades. In conjunction with this, the search for reliable and reproducible sources of MSCs for both research and clinical purposes continues. This study involved using a surgical technique in a novel location to extract bone marrow aspirates from donors already undergoing necessary hip surgery. MSCs were then extracted from the aspirates and identified according to flow cytometry. They were cultured to passage 3 with a large number of vials cryopreserved for future use.

MSCs have been isolated from multiple tissues including bone marrow, adipose, umbilical cord and placental tissues amongst others. Although they appear to exist ubiquitously throughout a variety of tissues, current literature suggests that significant differences exist in the differential and proliferative capacities of MSCs obtained from different tissue sources [150, 261]. Human bone marrow and adipose tissue derived MSCs currently account for the vast majority of publications investigating MSC behaviour and application [157]. In order to compare results between laboratories and to produce results relevant to the disease under investigation, it is necessary to choose the most appropriate type of MSCs suited to the respective research goals and objectives. The primary goal of this study was to isolate MSCs from osteoarthritic and osteoporotic patients. Abnormalities in bone remodelling are fundamental corner stones in our current understanding of the pathophysiology of osteoporosis [262]. The role of subchondral bone remodelling in osteoarthritis has been a recent focus of research with new insights into the pathophysiology of this highly prevalent disease [263]. The creation of an MSC biobank from these cohorts of patients has the potential to delineate the respective underlying pathophysiology further. As outlined previously in the literature review, differences in the behaviour of MSCs have been demonstrated according to the tissue source of extraction [150]. Thus, when using bone marrow as a tissue source is relevant in investigating MSC behaviour in bone related diseases.

Traditionally bone marrow aspiration of MSCs is carried out as an isolated procedure, i.e. the donor presents solely for extraction of a bone marrow aspirate from the iliac crest. This involves donors undergoing a procedure that would not otherwise be undertaken. This isolated procedure has associated complications and donor morbidity [176]. This study is
successful in obtaining bone marrow aspirates from patients whilst they are undergoing a scheduled operation. Previous studies obtaining bone marrow aspirates during such scheduled surgeries have used a separate site such as the iliac crest for bone marrow aspiration [264]. This still has the risk of donor site morbidity as it involves a separate puncture site with an associated risk of bleeding and infection. However the site of needle entry in our study is from the superior rim of the exposed acetabulum, during a necessary hip operation. The entry site is in the same location as the surgery being undertaken with the resultant risk to the patient is minimised. Therefore, the inconvenience to a donor is minimal, which has the potential to increase donor participation. This technique of bone marrow aspiration could potentially allow the creation of a large and continuously updateable functioning biobank of MSCs. The surgical technique is also quick and efficient with no impact on the length of surgery. This could be carried out by any orthopaedic surgeon or appropriate trainee. Involving clinicians and hospitals in this area of research could potentially be more attractive with little inconvenience or learning curve required for the operating surgeon.

The bone marrow aspirate in this study is obtained from a similar area to that of the traditional iliac crest aspiration. Thus a highly vascular, cellular region is aspirated in order to obtain a useful number of MSCs for analysis. There was no difficulty in obtaining the predetermined 30mls of bone marrow aspirate from any donor in this study. No complications were recorded and each operation continued in a standard manner post bone marrow aspiration.

MSCs were characterised according to the recommendations outlined by the ISCT in their seminal position paper [2]. All aspirates contained cells that were adherent to plastic under standard culture conditions. Following analysis by flow cytometry, all aspirates demonstrated the positive cell surface markers CD90, CD73, CD44 and CD105, whilst being negative for CD45 and CD34. The final criteria was fulfilled by the demonstration of trilineage differentiation capacity from each donor as outlined in Chapter 5.

The CFU-F assay is used by many investigators as a method to quantify the frequency of MSCs in a bone marrow aspirates. As originally indicated by Friendenstein et al, one of the most prominent properties of MSCs is their ability to generate colonies following plating at low density [1]. Indeed, many publications have used the CFU-F assay alone as representative of the numbers of MSCs per aspirate without characterisation or differentiation of the respective MSCs [265, 266]. There was no significant difference in the CFU-F ability
between osteoarthritic and osteoporotic donors. There was a trend for osteoporotic donors to have a greater ability to form colonies but without any statistical significance. These results indicate a similar frequency of MSC in both osteoporotic and osteoarthritic donors. This is relevant to support a similar extraction method for each aspiration. In a similar vein, analysis of growth characteristics revealed that osteoporotic donors produced a higher number of MSCs but these results again lacked any statistical significance. There was no statistically significant difference in direct comparison of mean PDT’s between the respective disease states. Thus from our study the population growth kinetics appear to be unaltered with osteoporosis and osteoarthritis.

Whilst PDT was unaltered amongst disease state, there was a wide variability in the number of adherent cells identified at P1. This is indicated in the wide margins of standard deviation in Figure 13. Thus the PDT for each sample remained similar but different quantities of MSCs were obtained due to the varying number of adherent cells identified initially.

The primary aim of this research was to develop a biobank of characterised osteoporotic and osteoarthritic MSCs for use to study these respective disease states. A novel surgical technique has been employed imparting minimum morbidity and inconvenience to the patient. This surgical technique is transferrable to orthopaedic surgeons and their trainees and thus employable in other institutions. Bone marrow from patients suffering from osteoporosis and osteoarthritis has thus been extracted from a novel location with the successful isolation and characterisation of MSCs for further research.
Chapter 3: 
Characterisation of Human Osteoporotic and Osteoarthritic MSCs

3.1 Introduction

The content of this chapter outlines the methods and results for the trilineage differentiation of isolated MSCs. The ISCT criteria require MSCs to display the ability to differentiate along the osteogenic, adipogenic and chondrogenic lineages [2]. As previously outlined in the literature review, MSCs from different sources and different disease states have been shown to differentiate along lineages to different degrees. However conflict continues in the published literature as to the altered differentiation capabilities of osteoporotic and osteoarthritic MSCs. This chapter also involves quantification of the ability of the extracted MSCs to differentiate along the adipogenic and osteogenic lineages.

The ability of MSCs to migrate to the necessary site of action and subsequently differentiate into the required tissue is an important, yet understudied, area of research. In the adult skeleton, bone is continuously being formed and resorbed, which is accomplished by a precise co-ordination of the relevant cells [267]. This occurs at specific anatomical sites and follows a well-defined sequence of events [268]. In order to participate in repair and regeneration, MSCs have to be mobilised and then migrate to the target sites and integrate with the local tissues. MSC research has highlighted the signalling pathways that control migration of MSCs including chemoattractant receptor axes and intracellular signalling pathways [269]. Some of the most potent chemoattractants for MSCs belong to the TGF-β
superfamilies [270]. Previous studies have shown that members of the TGF-β family, namely bone morphogenetic protein (BMP) –2 and BMP -7 are known to induce migration in hMSCs [271, 272]. Tang et al. highlighted the significance of TGF-β1 in coupling together both bone resorption and formation [22]. They demonstrated that bone resorption releases active TGF-β1 which induces the migration of MSCs to the site of active bone remodelling and results in bone formation. The chemotactic response of MSCs to TGF-β1 has also been demonstrated in other publications [25-27]. The chemotactic response in diseases such as osteoporosis and osteoarthritis is less well known with a limited number of publications on same. Haasters et al. demonstrated a reduced chemotactic response of osteoporotic MSCs in response to BMP-2 and BMP-7. Thus this chapter progresses to investigate both the chemotaxis and the chemokinetic response of healthy, osteoporotic and osteoarthritic MSCs to factors including TGF-β1.

3.2 Materials and Methods

3.2.1 Osteogenic differentiation

A sample from each patient was used for differentiation measurement. At P3, cells were seeded in a 6 well flat bottomed plate at a density of 2 X 10^5 cells per well. Once 80% confluency was reached, 3 wells were induced along the osteogenic lineage and 3 wells were cultured in complete MSC medium as controls. Osteogenic medium consisted of DMEM low glucose, 10% FBS, 1% Penicillin/Streptomycin (P/S), 100nM dexamethasone, 0.05mM Ascorbic acid and 10mM Beta-Glycerophosphate (BGP). Medium was changed every 72 hours and cells were cultured for 21 days in these conditions.

At day 21, the medium was removed and the cells were washed with PBS. The cells were then fixed in 10% formalin for 15 minutes. The cells were rinsed in PBS and incubated with 2% alizarin red solution for 20 minutes at room temperature with gentle shaking. The pH of the alizarin red solution was maintained between 4.1 and 4.3 prior to staining. The cells were then washed 4 times with distilled water with a gentle rocking motion. The 6 well plates were imaged without magnification ie without a microscope. Calcium forms an alizarin red-S calcium complex in a chelation process and the end product is a bright red stain.
3.2.2 Osteogenic differentiation quantification

Extraction of the alizarin red stain was performed post imaging of same. The cell layer was incubated with 10% acetic acid for 30 minutes with a gentle rocking motion. The monolayer, now loosely attached to the plate, was detached using a cell scraper. The monolayer and 10% acetic acid were then transferred to micro-centrifuge tubes and vortexed for 30 seconds. Each tube was sealed and placed in an 85°C water bath for a 10 minute period. Following this, the tubes were immediately transferred to ice for 5 minutes. Each tube was centrifuged at 20,000g for 15 minutes. 500µL of the supernatant was removed and transferred to a new micro-centrifuge tube. 200µL of 10% (v/v) ammonium hydroxide was added to each tube. 100µL of each sample was then pipetted in triplicate into a flat bottomed 96 well plate. The absorbance at 405 nm was recorded using a spectrophotometer. Standards were created by serial dilution of alizarin red solution and also measured at 405nm in order to plot a standard curve for each sample. Calcium concentration was calculated using the absorbance measured for each sample against its respective standard curve [273].

Osteogenic quantification was also carried out on 2 samples of healthy MSCs obtained from ATCC. The ATCC samples were bone marrow derived human MSCs. They were purchased at passage 2. Both samples were male in gender. The age of samples were 24 and 30. These were cultured in complete MSC medium until P3. At P3, MSCs were seeded as above for osteogenic differentiation and evaluated at day 21. Thus these 2 donor samples were used alongside the extracted healthy sample to complete a healthy donor cohort.

3.2.3 Adipogenic differentiation

The potential of MSCs to differentiate along the adipogenic lineage was measured by the formation of lipid droplets. At P3, cells were seeded in 6-well flat bottomed plates at a density of 2 X 10^5 cells per well. Cells were expanded until reaching a confluency of 80 % using complete MSC medium. 3 wells were then induced along the adipogenic lineage and a further 3 wells were cultured in complete MSC medium. The experimental wells were induced for 72 hours in induction medium and then cultured in maintenance medium for 24 hours. Induction medium consisted of DMEM low glucose, 10% FBS, 1% P/S, 10µg/ml insulin, 1µM dexamethasone, 200µM Indomethacin and 0.5mM isobutylmethylxanthine (IBMX). Maintenance medium consisted of DMEM low glucose, 10% FBS, 1% P/S and 10µg/ml insulin. The induction and maintenance cycle was repeated until the completion of 3 full cycles. Following completion of the 3rd cycle, the medium was removed and the cells
were washed in PBS. The cells were then fixed with 10% formalin for 30 minutes. The fixation buffer was aspirated and the cells were incubated with 60% isopropanolol (IPA) for 5 minutes. A stock solution of Oil Red O (0.3 grams in 100ml of 100% IPA) was diluted in a ratio of 3:2 with distilled water. The cells were then incubated with the Oil Red O working solution at room temperature for 15 minutes. The cell layer was then washed with distilled water 3 times. The cells were imaged using a light microscope with imaging software.

3.2.4 Adipogenic differentiation quantification

Following microscopy and imaging, the Oil Red O stain used to detect lipid droplets was extracted for analysis. The cells were incubated with 100% IPA for 15 minutes with a gentle rocking motion. The solution was then transferred to a cuvette for analysis. The absorbance of the extracted stain was quantified using a spectrophotometer at a wavelength of 500nm. All extracted samples were standardised to a control consisting of 100% IPA alone. Two healthy samples of MSC were obtained from ATCC and adipogenic differentiation and quantification was carried out on same alongside extracted MSC’s.

3.2.5 Chondrogenic differentiation

At P3, 1 x 10^5 cells per well were seeded in a 96 well U bottom suspension plate. After 24-48 hours the formation of spheroids was noted and chondrogenic medium added. 3 wells continued to be cultured in complete MSC medium as controls. Chondrogenic medium consisted of DMEM low glucose, 10% FBS, 1% penicillin/streptomycin, 0.1 µL dexamethasone, 1µM ascorbic acid, 1% sodium pyruvate, 10ng/ml TGF-β1 and 10% ITS supplement. ITS supplement is a mixture of human insulin, human transferrin and sodium selenite. Medium was changed every 72 hours for 21 days. At day 21, the cartilage spheroids were washed twice in PBS. They were then incubated in 10% formalin for 60 minutes. The spheroids were then washed in distilled water. Alcian blue staining solution was prepared by mixing 10mg alcian blue 8 GX in 60mls of ethanol and 40mls of acetic acid. The spheroids were incubated in the dark with alcian blue staining solution overnight at room temperature. The spheroids were then washed with destaining solution (3mls of Ethanol: 2mls of Acetic Acid) for 20 minutes and this was repeated three times. The spheroids were observed and imaged with and without magnification.

Chondrogenic differentiation was verified visually by a positive blue staining pellet. To verify that this positive stain represented chondrogenesis throughout the pellet, the pellet from the healthy sample (sample 1) was sent to the pathology department at University
Hospital Limerick for histological analysis. At day 21 of culture in chondrogenic medium, the pellets from sample 1 were placed in 10% Formalin. They were transported to UHL and embedded in paraffin. The pellets were then sectioned using a microtome and mounted onto glass slides. The glass slides were then stained with alcian blue as per the methodology outlined above. Imaging was carried out under the microscope at 4X and 10X magnifications.

3.2.6 Cell migration

For analysis of cell migration, transwell inserts (Millicell, 8.0µm, polyethylene terephthalate membrane) were placed in 24 well flat bottomed plates. One ml of serum free medium was placed in each well. 1 X 10^5 MSC’s were suspended in serum free media at P4 and placed in the upper compartment of each insert. An incubation period of 4 hours was observed to allow for cell adhesion. Following this, the inserts were transferred to a new 24 well plate containing the chemotactic factor under investigation. Three osteoporotic donor samples were used for analysis in their response to serum free media and 10% FBS respectively. Each sample was carried out in triplicate. This experiment was repeated on a second occasion with three osteoporotic samples. 2 osteoarthritic and 2 healthy samples obtained from ATCC (see previous details of same), were used to analyse their response to serum free media, 10% FBS. The cells were incubated with their respective chemokine for 18 hours to allow for migration. The cells were then washed in PBS, and fixed in 10% formalin for a 10 minute period. Haematoxylin was used to stain the cells. Cells which did not migrate were removed from the upper portion of the insert with a cotton bud. The inserts were washed a minimum of four times with distilled water. The membrane was cut from the insert and fixed to a glass slide with DPX mountant. The slides were viewed at 20X magnification under microscopy (Olympus CH2 model). Cells were counted in nine different fields. Three fields were chosen from the top portion of the slide, three from the middle and three from the bottom portion of the slide. Each analysis was performed in triplicate. Migratory patterns were represented by the number of cells migrating per field of view and the chemotactic index. Chemotactic index was calculated by the number of cells migrating towards the chemoattractant divided by the number of cells migrating spontaneously.
3.3 Results

3.3.1 Osteogenic differentiation

Osteogenic differentiation was detected using alizarin red staining solution. Calcium ions form an alizarin red S-calcium complex in a chelation process. Alizarin red thus stains the calcium deposits that occur when differentiated MSC’s mineralise the secreted extracellular matrix [274]. Each bone marrow aspirate demonstrated the presence of calcium deposition following the application of alizarin Red staining solution. An example of this from an osteoarthritic and an osteoarthritic donor can be seen in Figure 17.

Figure 17: Alizarin staining demonstrating the presence of calcium deposition in a well of a 6 well plate (A) Osteoporotic donor, (B) Osteoarthritic donor, (C): Healthy donor, and (D): example of control well with nil evidence of calcium deposition following alizarin red staining
3.3.2 Osteogenic differentiation quantification

The ability to differentiate along the osteogenic lineage was quantified for each bone marrow aspirate. Calcium deposition was quantified using an alizarin red based colorimetric assay. Healthy MSCs included the single traumatic sample along with two healthy samples purchased from ATCC. No significant difference in mineralisation was determined between the osteoporotic and osteoarthritic groups as outlined in Figure 18. A significant difference was observed between the osteoarthritic group and healthy group. The healthy cohort had a much higher mineralisation ability ($p=0.0381$).

![Figure 18: Calcium concentration following osteogenic differentiation for each disease state. * indicates that calcium concentration is significantly increased in healthy MSCs versus osteoarthritic MSCs ($p=0.0383$)](image)

Further analysis in the context of smoking status suggested a higher calcium concentration level in the non-smoking group but without statistical significance. This can be seen in figure 19. No statistical significance was identified in the level of calcium concentration and gender.

![Figure 19: Calcium concentration following Alizaran Red stain extraction (A): Calcium concentration per smoking status ($p=0.4866$). (B): Calcium concentration per gender Calcium concentration following extraction per smoking status ($p=0.2825$).](image)
3.3.3 Adipogenic differentiation

Oil Red O staining solution was used to detect the presence of adipocytes following incubation with inducing agents. Oil Red O is a fat soluble diazol dye which stains neutral lipids and cholesteryl esters but not biological membranes [275]. Each bone marrow aspirate demonstrated the presence of adipocytes following staining. No adipocytes were noted in control wells cultured in complete MSC medium without inducing agents. An example of adipocytes observed at 4X magnification is seen in Figure 20.

![Figure 20: Adipogenic differentiation as demonstrated by staining with Oil Red O (A): Osteoporotic donor (B): Osteoarthritic donor (C): Healthy donor and (D): Control well with MSCs cultured without adipogenic induction medium. Scale bar = 50µm](image-url)
3.3.4 Adipogenic differentiation quantification

The ability to differentiate along the adipogenic lineage was quantified following extraction of oil red O staining solution. Osteoporotic donors had a greater ability to differentiate into adipocytes as outlined in Figure 21. Sub-group analysis displayed a higher level of adipogenesis in the current smoker cohort but without statistical significance. There was no apparent relationship between adipogenic differentiation ability and gender.

Figure 21: Adipogenic differentiation measured by spectrometer absorbance at 500nm. (A) Difference in absorbance at 500nm between osteoporotic and osteoarthritic cohorts. * indicates that difference in absorbance at 500nm is statistically significant for the osteoporotic cohort in comparison to the osteoarthritic cohort ($p=0.0245$) (B): Difference in absorbance per gender ($p=0.848$) (C): Difference in absorbance per smoking status ($p=0.3243$).
3.3.5 Chondrogenic differentiation

The presence of chondrocytes was assessed using alcian blue staining. Chondrogenic differentiation was verified visually by a positive blue staining pellet. Alcian blue stains sulphated proteoglycans that are indicative of functional chondrocytes. To verify that this positive stain represented chondrogenesis throughout the pellet, the pellet from sample 1 was sent to the pathology department at University Hospital Limerick for histological analysis. All samples stained positively for the presence of chondrocytes. An example of staining with Alcian blue post histological processing can be seen in Figure 22. Alcian blue staining of spheroids cultured in 96 well suspension plates are also shown.

Figure 22: Chondrogenic differentiation following the application of Alcian Blue Staining solution. (A): Example of Alcian Blue staining of MSC pellets cultured in normal MSC medium with no evidence of chondrogenic differentiation. (B): Evidence of chondrogenic staining with uptake of Alcian blue staining in MSC’s cultured in chondrogenic medium. Top well osteoarthritic sample, bottom well osteoporotic sample. (C) Sample 1 following histological sectioning and Alcian Blue staining indicating chondrogenic differentiation throughout the pellet. (D): Histological sectioning of Sample 1 Control pellet cultured in normal MSC medium without evidence of chondrogenic differentiation.
3.3.6 Cell migration - Chemokinesis

The migratory patterns of healthy, osteoporotic and osteoarthritic MSCs were analysed using transwell inserts. Chemokinesis is defined as random cell movement in the absence of chemoattractant gradients. Figure 23 outlines the chemokinetic behaviour of healthy, osteoporotic and osteoarthritic MSCs. There was a significant decrease in the number of osteoporotic MSCs migrating per field of view in comparison to healthy controls. In comparing osteoarthritic MSCs and healthy MSCs, no difference was discerned in regard to chemokinesis. Comparing osteoarthritic and osteoporotic MSCs reveals a statistically significant alteration in chemokinetic behaviour. As mentioned previously, the healthy cohort in this study have a significantly lower mean age. However, a difference in chemokinetic behaviour is noted between osteoporotic and osteoarthritic groups, where age is similar.

![Figure 23](image.png)

**Figure 23**: Number of cells migrating per field of view for healthy, osteoporotic and osteoarthritic samples without the presence of any chemoattractant. ** indicates a statistically significant greater number of cells migrating per field of view in healthy MSCs versus osteoporotic MSCs ($p=0.00027$). *** indicates a statistically significant greater number of cells migrating per field of view in osteoarthritic MSCs versus osteoporotic MSCs ($p<0.0018$). One way ANOVA for graph $p<0.0001$. 
3.3.7 Chemotaxis

Chemotaxis is the guided movement of cells towards a chemical gradient. To calculate the chemotactic index, the number of cells migrating in response to a specific chemoattractant is divided by the number of cells spontaneously migrating. 10% FBS was used as a positive chemoattractant to analyses chemotaxis per disease state. Healthy cells, as expected, migrated in a statistically significant manner towards 10% FBS. Osteoporotic cells also migrated towards 10% FBS, however osteoarthritic cells failed to migrate in a similar manner.

**Figure 24:** Migration represented by the chemotactic index. Chemotactic index is calculated by the number of cells migrating towards the chemoattractant divided by the number of cells migrating spontaneously. Number of cells migrating per field of view in 10% FBS is normalised to serum free media for each respective disease state. **Chemotactic index is significantly greater in healthy MSCs migrating towards 10% FBS as opposed to migration towards serum free media (p=0.0082). Of note osteoarthritic MSCs did not migrate towards 10% FBS (p=0.0083). ***Chemotactic index from osteoporotic MSCs migrating in 10% FBS is statistically greater than that in serum free medium (<0.0001). One way ANOVA p<0.0001.***
**3.6 Discussion**

Extracted bone marrow MSCs from each donor demonstrated the ability to differentiate along the osteogenic, adipogenic and chondrogenic lineages. Our study suggests that bone marrow derived osteoarthritic MSCs have a reduced osteogenic and adipogenic capability in comparison to healthy controls. A statistically significant increase in adipogenesis is noted in osteoporotic MSCs. A critical aspect of this chapter involves examining the migratory capacity of MSCs. An altered chemokinetic profile was noted when osteoporotic and osteoarthritic MSCs were analysed. Significant differences were highlighted in the chemotactic response of diseased MSCs to 10% FBS.

Determining underlying differences in the ability of MSCs to differentiate along a certain lineage could potentially explain the macroscopic changes in bone structure and function seen in diseases such as osteoporosis and osteoarthritis. As outlined in the literature review, conflicting reports have been published regarding the trilineage capabilities of osteoarthritic and osteoporotic MSCs. Bone marrow derived osteoarthritic MSCs have been suggested to have a reduced adipogenic and chondrogenic potential, with no difference in osteogenic potential in comparison to MSCs derived from healthy donors [188]. Adipose derived osteoarthritic MSCs have been reported as having an impaired differentiation capacity across all three lineages [189]. Our study suggests that bone marrow derived osteoarthritic MSCs have a reduced osteogenic and adipogenic capability in comparison to healthy controls.

Osteoarthritis has historically been considered a cartilage disorder, but more recent publication have emphasised the role of bone in its pathogenesis and in particular the role of subchondral bone. Subchondral bone provides the mechanical support for articular cartilage during the movement of load bearing joints. Thinning of subchondral bone has recently been reported to take place in not just the late stages of the disease but also at an early stage [39]. A reduced ability to differentiate along the osteogenic lineage, as demonstrated in this study, is thus relevant to current research in this field.

Current research into the trilineage capabilities of osteoporotic MSCs also reports conflicting results. Rodriguez et al reports a diminished osteogenic capability whilst Stenderup et al reports that osteogenic commitment is maintained in osteoporotic patients [192-194]. Our study demonstrates a reduced osteogenic capability although not statistically significant. A statistically significant increase in adipogenesis is noted when osteoporotic MSCs are compared to their osteoarthritic counterparts. Cross sectional studies in humans using
different methods to assess bone density and marrow fat have found that lower bone density is accompanied by higher marrow fat [276, 277]. Non-invasive image methods have also outlined an association between higher bone marrow fat and lower bone mineral density [278]. Thus, our finding of an increased adipogenic capability from osteoporotic MSCs is consistent with imaging and histomorphometrical studies. The altered capacity to differentiate along the adipogenic and osteogenic lineage highlight’s the relevance of MSCs in the pathogenesis of osteoporosis.

Subgroup analysis in this study investigates the effect of smoking and gender on the behaviour of MSCs. It has been well demonstrated that smoking can have a significant impact on bone metabolism. Smokers are at an increased risk of osteoporosis and the risk rises with increased tobacco consumption [258, 259]. There was no statistical difference in the smoking habits of each group; osteoporotic, osteoarthritic and healthy respectively. A trend was noted for non-smokers to have a greater ability to differentiate along the osteogenic lineage although lacking statistical significance. The cohort of non-smokers also displayed a lower adipogenic profile. Thus a trend in this study showed that MSCs derived from non-smokers had a higher osteogenic and a lower adipogenic ability in comparison to donors who currently smoked. However the number of donors in this study is underpowered, and therefore inadequate to confirm significant differences amongst these sub-groups. A further evaluation with higher number of samples is required to confirm the trends identified in this study. An interesting aspect of this study would be to evaluate the differences in trilineage potential according to gender. However there are insufficient numbers of each gender per disease state in this study and it would be inappropriate to draw conclusions from same.

The migratory capacity of MSCs is critical to the basic understanding of their biology in health and disease. Migration towards sites of tissue injury is the first step of stem cell mediated tissue regeneration. Homing of MSCs towards injured tissues relies upon their ability to migrate to and interact with the local micro-environment in a manner that secures their anchorage at sites where their effector functions are required. Chemotaxis is defined as the directional movement of cells towards concentration gradients of solubilized attractants whereas chemokinesis is defined as random cell movements in the absence of chemoattractants [279]. Osteoporotic MSCs had a significantly reduced cell movement in the absence of any chemoattractants in comparison to healthy control samples. Osteoarthritic MSCs did not show any statistical significant difference in chemokinesis in comparison to healthy controls.
Healthy cells migrated towards 10% FBS. Osteoporotic MSCs also migrated similarly manner towards 10% FBS. However, osteoarthritic MSCs displayed an altered chemotactic profile in comparison to both osteoporotic and healthy controls. Osteoarthritic MSCs failed to migrate towards a positive control of 10% FBS. This is in contrast to the increased migratory response of osteoarthritic MSCs towards platelet derived growth factor demonstrated by Rollin et al [280]. As discussed earlier, recent publications have highlighted the involvement of subchondral bone in the pathogenesis of OA. An inability of osteoarthritic MSCs to migrate to the site of initial damage could potentially be of relevance to the resultant development of OA. The migratory capacity of osteoarthritic MSCs also has implications in the consideration of autologous MSC therapy. Numerous clinical trials currently use autologous bone marrow or adipose derived MSCs as treatment for osteoarthritis, commonly in the form of intra-articular injections [281-283]. Investigating in more detail the reduced migratory capacity of osteoarthritic MSC’s to chemoattractants may provide a new avenue of improved therapeutic agents whilst also delineating the pathogenesis of osteoarthritis more accurately.

The confounding factor of age must be taken into account when analysing the above results. The healthy cohort as outlined previously in Chapter 2 is mainly comprised of MSCs obtained from ATCC. Thus there is a significant mean lower age in the healthy cohort. The migratory capacity of aged MSCs is unknown and this may influence the findings observed in our results. Bustos et al evaluated the migratory response of murine MSCs in the context of acute lung injury and identified lower migration rates in aged MSCs. They also identified significant down regulation of multiple chemokine receptors and other genes involved in BM-MSC migration [284] However despite having a different mean age, osteoporotic MSCs had a similar fold increase in migration towards 10% FBS as healthy MSCs, suggesting that the chemokinetic differences identified may be disease related.

As mentioned previously, osteoporotic MSCs displayed a reduction in cell migration in the absence of any chemoattractants. Regardless of age, an altered chemokinetic profile could have significant implications on the use of autologous MSCs from osteoporotic patients in the clinical setting. The use of autologous MSCs is considered favourable as it negates the host immune response from allogenic transplants. Thus a clinical scenario can present whereby an osteoporotic patient requires autologous MSCs for the treatment of a specified condition. The altered chemokinetic profile suggested by this study needs to be considered in the dosing and administration of such therapeutic agents in order for the desired effect to be obtained.
Analysis of osteoporotic and osteoarthritic MSCs in the context of trilineage differentiation highlights suspected disease related differences. Osteoarthritic MSCs had a reduced osteogenic and adipogenic capacity in comparison to healthy controls. Osteoporotic MSCs had a reduced osteogenic capacity but an increased ability to differentiate along the adipogenic lineage, in keeping with current literature. Of interest is the altered migratory capabilities of the respective MSCs. This has implications in both the basic biology of the respective diseases as well as in the field of autologous therapy.
Chapter 4: TGFβ signalling in Human MSCs

4.1 Introduction

Differences in the trilineage potentials of extracted MSCs were identified in Chapter 3 along with altered migratory capacities. Chapter 4 aims to investigate the altered response of osteoporotic and osteoarthritic MSCs in relation to a chemokine known to be important in regulating MSC activity in bone remodelling. As mentioned previously, Tang et al highlighted the significance of TGF-β1 in coupling together both bone resorption and formation [22]. They demonstrated that bone resorption releases active TGF-β1 which induces the migration of MSCs to the site of active bone remodelling and results in bone formation. The chemotactic response of MSCs to TGF-β1 has also been demonstrated in other publications [25-27]. Research from our laboratory has demonstrated that TGF-β1 is a positive chemoattractant when evaluating the migratory abilities of healthy MSCs. This chapter explores the chemotactic response of osteoporotic and osteoarthritic MSCs towards TGF-β1.

As outlined in the literature review, the primary cilium is an antenna like, non-motile structure that extends from the surface of most mammalian cell types into the extracellular space. Early research suggested that the primary cilium was a vestigial structure, however several labs have demonstrated that primary cilia are critical for chemosensing and mechanosensing in a variety of tissues including cartilage, bone and kidney [285-288]. Mechanical aspects of cilia such as cilia length have come under investigation for their respective influence on cell function. Knight et al reported that adipogenic differentiation requires primary cilia elongation associated with the recruitment of IGF-1Rβ onto the cilium [289]. To date, no studies have measured cilia length in MSCs from diseases such as osteoporosis and osteoarthritis.
As mentioned previously, TGF-β1 signalling and its influence on bone remodelling is increasingly emerging as critical for healthy bone. Christensen et al reported that TGF-β receptors localise to the ciliary tip and endocytic vessels at the ciliary base in fibroblasts and that TGF-β stimulation increases localisation and activation of downstream effectors at the ciliary base [231]. Recent work in our lab have located elements of the TGF-β1 signalling pathway in the primary cilium of healthy MSCs. Data awaiting publication has suggested that significant aspects of the TGF-β1 signalling machinery localises to the primary cilium in healthy cells. TGF-βRI and TGF-βRII have been demonstrated to localise to the primary cilium at percentages of 14% and 89.6% respectively. To date, no publication has evaluated this in diseases such as osteoporosis or osteoarthritis.

4.2 Materials and Methods

4.2.1 TGF-β1 migration analysis

Cell migration was carried out as previously outlined in Section 3.2.6. The samples outlined were analysed for their response to serum free media, 10% FBS and also 0.1pg of TGF-β1. The methods and samples used are as outlined in Section 3.2.7.

4.2.2 Cilia length (Immunocytochemistry)

Immunocytochemistry was carried out to analyse the length of the primary cilium in the respective disease states. Three osteoporotic and three osteoarthritic samples were used in this experiment. Each sample was completed in triplicate. To generate control samples, an osteoporotic sample was used along with a healthy sample obtained from ATCC. MSCs were seeded onto fibronectin coated cover slips at 20,000 cells per slide in a 12 well plate. Each sample was plated in triplicate. The seeded cells were maintained in DMEM low glucose, 10% FBS and 1 P/S for 24 hours. They were then serum starved for a further 48 hours by exchanging the medium to DMEM low glucose, 0.5% FBS and 1% P/S. Following 48 hours of serum starvation, the cells were fixed with 10% formalin for 10 minutes. Fixed cells were then rinsed three times with PBS. 0.1% Triton X-100 was used to permeabilize the cells over a 10 minute period. 1% Bovine serum albumin (BSA) was used to wash the cells over a 2 hour period. The cells were then incubated in monoclonal mouse anti-acetylated α tubulin diluted 1:1,500 in 1% BSA overnight at 4°C. The fixed cells were then washed in 1% BSA for a five minute period and two further 10 minute periods. This was followed by centrosomal staining which involved the application of an anti-pericentrin antibody at a dilution of 1/1500. This incubation period was one hour at room temperature. The fixed cells
were further washed in 1% BSA for a five minute period and two ten minute periods prior to the application of secondary antibodies. Secondary antibodies of Alexa 594 anti-mouse IgG and Alexa 488 anti-rabbit IgG at a dilution of 1:500 respectively were applied for one hour at room temperature. The fixed cells were washed with 1% BSA for five, ten and a further ten minutes. Glass slides were cleaned with ethanol and 4', 6-diamidino-2-phenylindole (DAPI) applied. The coverslips with fixed cells were mounted onto the glass slides with application of nail polish the following day as a sealant. The slides were then observed at the Olympus inverted fluorescent microscope (Olympus IX53). Cilia length were measured using 60x oil immersion objective. Cilia length were further analysed using a confocal microscope (Zeiss LSM 710 META). This involved imaging at 630x magnification. Z stack images were taken of the cell monolayer. The Z stacks were then reconstructed using image J. Each primary cilium was measured at its maximum projection. Greater than 100 cilia were measured for each coverslip.

4.2.3 TGF-β Receptor Analysis - Immunocytochemistry

TGF-βRII staining was carried out on MSCs from 3 different osteoporotic donors. ICC was carried out as outlined in Section 4.2.2. Primary antibodies used were acetylated α tubulin and TGF-β-RII antibody (gift from Dr Sorenson’s lab). Secondary antibodies included Alexa 594 anti-mouse IgG and Alexa 488 anti-rabbit IgG. DAPI was again used for nuclear staining. Analysis was carried out using the Confocal microscope (Zeiss LSM 710 META). Three coverslips were prepared per sample and this experiment was repeated twice. A minimum of 100 cilia were analysed per coverslip.

4.2.4 Gene expression

TGF-βRII gene expression was analysed in MSCs from 3 different osteoporotic donors. Cells were seeded at a density of 20,000 cells per well in a 6 well flat bottomed plate. Cells were grown in complete MSC medium until a confluency of 80% was reached. The cells were then lysed for PCR analysis. Ribonucleic acid (RNA) isolation was performed using Trizol reagent, as per the manufacturer’s instructions. Following the removal of culture medium, 1 ml of Trizol reagent was added to each well and pipetted over the cells several times. Cell scrapers were then used to scrape the monolayer of cells from the culture dish and the cell lysate was transferred to a microcentrifuge tube. The cell lysate was homogenized by pipetting a minimum 20 times. The microcentrifuge tubes were incubated for seven minutes at room temperature followed by the addition of 0.2ml of chloroform per tube. Each tube was
vortexed for 15 seconds followed by a further incubation period of 15 minutes at room temperature. The microcentrifuge tubes were then centrifuged at 12,000g for 15 minutes at 4°C. The lysate was now separated into a red organic phase (protein) at the bottom of the tube, an interphase pellet (DNA) in the middle and an upper clear aqueous phase (RNA) at the top. The aqueous phase was transferred to a new microcentrifuge tube, with care taken to avoid the interphase material. The RNA was precipitated from this solution by the addition of 0.5ml of isopropyl alcohol (IPA). The samples were allowed to stand at room temperature for 10 minutes. The RNA pellet was isolated by centrifugation at 14,000g for 10 minutes at 4°C. The supernatant was removed and the RNA pellet was washed with 1ml of 75% ethanol. After a further centrifugation at 7,500g for a five minute period, the supernatant was carefully removed. The RNA pellet was allowed to air dry with any remaining ethanol removed with Kimwipes. The RNA was dissolved in 20µL of RNase free water.

The concentration and purity of the RNA was measured using the nanodrop 1000 (Thermo Scientific). Samples with a high level of contamination were not included in the analysis. Reverse transcription was carried out using the “High capacity complement deoxyribonucleic acid (cDNA) reverse transcription kit” (Applied Biosystems). Mastermix solution consisted of 10x RT (reverse transcriptase) buffer, 25x dNTP mix, 10x RT Random primers, multiscribe reverse transcriptase and RNase inhibitor. A total of 6.8µL of Mastermix solution was added to 13.2µL of RNA diluted in RNase free water. The thermal cycle consisted of 10 minutes at 25°C to equilibrate, 120 minutes at 37°C for reverse transcription and 5 minutes at 85°C for denaturation. The resultant cDNA was diluted with RNase-free water to 5ng/2.25µL RNase free water and stored at -80°C until required for analysis.

The samples were then assessed for relative transcript levels using rt-PCR. cDNA (5ng in 2.5µL volume) was combined with Taqman universal PCR mix and the respective gene assay as outlined in Table 7. Gene assay includes forward primer, reverse primer and Taqman probe. Each sample was analysed in triplicate.
Table 7: rt-PCR components

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>TaqMan Universal PCR Mix</td>
<td>2.5 ul</td>
</tr>
<tr>
<td>Gene assay</td>
<td>0.25 ul</td>
</tr>
<tr>
<td>cDNA template</td>
<td>0.5 ul</td>
</tr>
<tr>
<td>Water (Diethylpyrocarbonate)</td>
<td>1.75 ul</td>
</tr>
<tr>
<td>Total</td>
<td>5 ul</td>
</tr>
</tbody>
</table>

The amplification conditions were as follows: 10 minutes at 95°C and 40 cycles of: 95°C for 15 seconds and 60°C for 60 seconds. A standard curve was run to verify there were no contaminating products present in the reaction.

Relative gene expression was analysed using the $2^{-\Delta\Delta Ct}$ method [290, 291]. Glyceraldehyde 3-phosphate dehydrogenase (GADPH) was used as the housekeeping gene in all scenarios [292]. The mean Ct value was calculated for the gene of interest. The $\Delta$Ct (Mean Ct gene of interest – Mean Ct housekeeping gene) was subsequently determined. From this $2^{-\Delta\Delta Ct}$ could be calculated and the levels of gene expression compared to the respective control cells.
4.3 Results

4.3.1 TGF-β1 migration analysis

Healthy, osteoporotic and osteoarthritic MSCs were incubated with 0.1pg TGF-β1 to analyse migratory capabilities. As outlined in Figure 25, healthy MSCs towards the chemoattractant 0.1pg TGF-β1. Both osteoporotic and osteoarthritic MSCs failed to migrate towards 0.1pg TGF-β1.

![Figure 25: Migration of MSCs incubated for 18 hours in serum free media and 0.1pg TGF-β1, * indicates that the chemotactic index of healthy MSCs migrating towards 0.1pg TGF-β1 is statistically greater than the chemotactic index in serum free media (p=0.0162).](image-url)
### 4.3.2 Primary cilia incidence and length

As mentioned previously the primary cilia is emerging as an integral component in mechanotransduction and bone remodelling. Cilia length was investigated to determine if alterations in cilia length may account for the altered migratory pattern identified in earlier sections. Cilia length was measured using both the Olympus and the Confocal microscope. A representative image from the Confocal microscope is seen in Figure 26. There was no significant difference in cilia length in healthy, osteoporotic or osteoarthritic patients. A mean length of 2.04µm, 1.97µm, 1.96µm, was noted in the healthy, osteoarthritic and osteoporotic groups respectively. There was also no change in change in the number of ciliated cells following exposure to 0.1pg of TGF-β1 at periods of 0, 30 minutes and 2 hours respectively.

![Graph A: Percentage of ciliated cells](image1)

![Graph B: Cilia Length](image2)

**Figure 26:** (A): Percentage of cells with primary cilium. (B): Length of primary cilia of healthy, osteoporotic and osteoarthritic MSCs. (C+D): Maximum intensity image of primary cilia following Z stack reconstruction on Confocal microscopy. Blue = Dapi Green = centrosomal staining Red = α tubulin
4.3.3 TGF-βRII localisation in osteoporotic samples

Data awaiting publication from our laboratory has suggested that significant aspects of the TGF-β1 signalling machinery localises to the primary cilium in healthy cells. TGF-βRI and TGF-βRII have been demonstrated to localise to the primary cilium at incidences of 14% and 89.6% respectively. Clement et al previously identified the ciliary pocket as the location for elements of the TGF-β1 machinery in fibroblasts and MSCs undergoing cardiomyocyte differentiation [231]. We analysed the localisation of TGF-βRII in 3 osteoporotic samples. TGF-βRII localised to the primary cilium at a percentage of 16% +/- 3% (SD). This is lower in comparison to the co-localisation seen with healthy MSCs which have been reported to be as high as 89% [231].

Figure 27: Confocal imaging of a ciliated nucleus with TGF-βRII co-localisation (A): DAPI staining of nucleus, (B): acetylated α tubulin staining of primary cilia, (C): TGF-βRII staining, (D): Combined image demonstrating co-localisation. Blue = DAPI; Green = TGF-βRII; Red = acetylated α tubulin
4.3.4 TGF-βRII gene expression

Following from an altered localisation of TGF-βRII in osteoporotic MSCs as opposed to healthy MSCs, the level of gene expression of TGF-βRII gene was subsequently analysed. This was in order to determine if the change in migration in osteoporotic MSCs observed was due to an alteration in gene expression. There was no significant difference in TGF-βRII gene expression between healthy and osteoporotic MSCs.

![Gene expression analysis](image)

**Figure 28**: Gene expression analysis of osteoporotic and healthy MSCs to assess TGF-βRII gene expression with results normalised to the expression of housekeeping gene GADPH ($p=0.9109$).
4.4 Discussion

Tang et al recently outlined the critical role that TGF-β1 plays in bone formation and bone remodelling. Following the resorption of bone tissue by osteoclasts, TGF-β1 is released and directs the migration of MSCs towards the site of bone resorption. TGF-β1 induced cellular migration is being proposed as a crucial step in this coupling process [22]. MSCs have been consistently shown to migrate towards TGF-β1 in numerous publications [25-27]. The response of osteoporotic MSCs to TGF-β1 was significantly different from that observed with healthy cells. Haasters et al previously reported a reduced migratory response of osteoporotic MSCs to BMP 2 and BMP 7 [198]. A failure of MSCs to migrate to fracture sites has been postulated as a mechanism for the reduced healing rates in osteoporotic fractures [198, 293]. Healthy MSCs in our study migrate in a statistically significant manner towards 0.1pg of TGF-β1 over an 18 hour incubation period. However osteoporotic MSCs did not migrate towards 0.1pg of TGF-β1 over the same time period. This experiment was repeated in triplicate with 3 different osteoporotic donors. Both healthy and osteoporotic MSCs migrated towards the positive chemoattractant, 10% FBS as demonstrated in the previous chapter. The critical step of TGF-β1 induced migration of MSCs, linking bone resorption and bone formation thus appears to be impaired in osteoporotic MSCs.

The role of TGF-β1 is becoming increasingly important and the modality of this alteration in function is under much investigation. A recent publication by Clement et al reported that TGF-β receptors localise to the ciliary tip and endocytic vessels at the ciliary base in fibroblasts. They also demonstrated that TGF-β stimulation increases receptor localisation and activation of SMAD2/3 at the ciliary base. Similarly TGF-β signalling during cardiomyogenesis correlated with the accumulation of TGF-β receptors and activation of the SMAD 2/3 pathway at the ciliary base [231]. Following on from the current literature, our laboratory has localised components of the TGF-β1 machinery to the primary cilium in healthy MSCs (results in preparation for publication). These include the TGF-β1 receptors, TGF-βRI and TGF-βRII. Data in preparation for publication have determined the localisation of TGF-βRII to the primary cilium of healthy MSCs at a mean of 89.6%. In contrast TGF-βRII co-localises to the primary cilium of osteoporotic MSCs at a mean of 16%. A reduction in the localisation of TGF-βRII to the primary cilium may be an element in the altered migratory response observed in osteoporotic MSCs when incubated with TGF–β1. However as mentioned in previous sections, the confounding factor of age difference will need to be explored prior to definite conclusions being extrapolated. Nevertheless in conjunction with
previous work from Tang et al regarding MSC behaviour and osteoporosis, the co-localisation of TGF-βRII to the primary cilium in healthy MSCs and the reduction in co-localisation in osteoporotic MSCs offers a further avenue for investigation [22].

Follow on work from these findings include attempting to elicit the presence and activity of downstream effectors of the TGF-β1 pathway in both healthy and diseased MSCs. There are multiple different ways to approach this research question and we proposed to initially analyse the gene expression of TGF-βRII, given the preceding findings of altered co-localisation patterns in healthy and osteoporotic MSCs. However, we uncovered no significant differences in the gene expression of TGF-βRII when comparing osteoporotic and healthy donors. There was also no significant difference in gene expression following treatment with TGF-β1 over an 18 hour period. TGF-β1 is present in large quantities in an inactive state in the cell, where it is bound to the latency activated protein (LAP). Therefore gene expression of TGF-β receptors may not be crucial in the activation pathway, and rather a switch from inactive components of the TGF-β1 machinery to active of more relevance. However this requires further experimentation to decipher in more detail.

The importance of the primary cilium in the field of bone mechanobiology is becoming more defined with recent significant publications highlighting its critical role in bone remodelling [14, 220, 294]. The primary cilium is a rod-like, microtubule based structure that projects from the cell surface. Recent evidence suggests that the length of primary cilia can influence cilia signalling pathways including mechanotransduction, hedgehog, and wnt signalling [295-297]. Interestingly the length of the primary cilium differs between various differentiated cell types. For example epithelial cells typically have longer cilia than articular chondrocytes. Therefore the hypothesis that the length of primary cilia may differ between diseases such as osteoporosis and osteoarthritis is of immense interest. However no difference was detected in the length of the primary cilium, either between disease states or in comparison to healthy controls. Given this result and the importance of the primary cilium in mechanotransduction, a follow on experiment from this study could include analysing the change in length of the primary cilium in response to processes such as adipogenic differentiation. Indeed a publication from Lin et al has demonstrated that adipogenic differentiation requires primary cilia elongation and is associated with the recruitment of insulin like growth factor-1 receptor β into the primary cilium [289]. However our results suggest that prior to differentiation there does not appear to be any significant difference in the length of primary cilium in osteoarthritic and osteoporotic MSCs.
Thus this chapter outlines an alteration in the ability of osteoporotic MSCs to migrate towards TGF-β1. It also suggests a reduced co-localisation pattern of TGF-βRII to the primary cilia in osteoporotic MSCs in comparison to healthy controls. This has significant implications given recent publications highlighting the importance of TGF-β1 in coupling together effective bone resorption and formation. Further research could delve further into gene expression analysis to uncover the underlying differences affecting osteoporotic MSCs as opposed to their healthy counterparts.
Chapter 5:

Concluding discussion

With an ever increasing elderly population, diseases such as osteoporosis and osteoarthritis will become more and more prevalent and topical [30, 298]. Each of these diseases have significant morbidity and mortality as outlined in the literature review. Delineating and uncovering the underlying pathogenesis of the relevant disease has the potential to transform the treatment options available to millions of patients worldwide. MSCs are critical to the process of healthy bone modelling and remodelling. Alterations in the behaviour of MSCs has the potential to lead to significant bone disease, with manipulation of their basic behaviour offering therapeutic avenues for exploration.

MSCs have been trialled as a form of therapy for immune disorders such as systemic lupus erythematosus and Crohn’s disease [299-302]. They have also been considered in scenarios requiring tissue repair such as the treatment of myocardial infarction and cornea damage amongst others [303, 304]. The use of autologous MSCs is considered favourable as it negates the host immune response from allogenic transplants. Thus a clinical scenario can present whereby an osteoporotic patient requires autologous MSCs for the treatment of a specified condition. Implanting diseased MSCs, such as MSCs from osteoporotic or osteoarthritic patients, may not be optimal or achieve the desired effect.

The primary aim of this research was to develop a biobank of osteoarthritic and osteoporoticMSCs. This involved the extraction of MSCs from a novel location during scheduled hip surgery. MSCs have traditionally been isolated from bone marrow and adipose tissue with some institutions availing of MSCs isolated from placental tissue, umbilical tissue and Wharton’s Jelly amongst others. However, differences in the behaviour of MSCs from tissue sources has been highlighted in current literature. In order to evaluate bone based disorders such as osteoporosis, MSCs were extracted from the pelvic bone in this body of research.

Donor recruitment for the provision of bone marrow samples targets a niche population of people who are willing to undergo an invasive procedure with complications attached to
same. Our research involves bone marrow aspirates which are obtained from patients already undergoing necessary surgery. There is no separate puncture wound with the procedure taking place during the operation and hence when a spinal anaesthetic is in place. It uses commonly stocked equipment for any orthopaedic theatre and the skill is easily transferrable amongst orthopaedic consultants and trainees. This has the potential to increase participation in bone marrow aspirate donation and also facilitate obtaining MSCs from osteoporotic and osteoarthritic patients.

The ISCT guidelines were followed in order to characterise the extracted MSCs from each bone marrow aspirate. Thus isolated cells adhered to plastic, underwent flow cytometry for relevant positive and negative surface markers, and were also capable of differentiating along the osteogenic, adipogenic and chondrogenic lineages. A more thorough characterisation was also conducted as the migratory ability of MSCs was evaluated.

The ability of diseased MSCs to differentiate along the respective lineages has been suggested to be altered in varying publications. This study suggests that osteoarthritic MSCs have a reduced ability to differentiate along the osteogenic and adipogenic lineages when compared to healthy control samples. However the age of the healthy control samples in these experiments are significantly younger than the osteoarthritic donors under investigation. Previous studies addressing the effect of ageing on MSCs have produced conflicting results [305]. Some studies have shown an age-dependent decrease in osteogenic or chondrogenic potential of MSCs from aged bone marrow while others show no age associated relationship at all [306-310]. Thus it still remains unclear as to the effect of ageing on the differentiation capabilities of MSCs. Further control samples in the same age cohort as the diseased osteoarthritic MSCs will need to be collected to confirm the results of a reduced ability of osteoarthritic MSCs to differentiate along the osteogenic and adipogenic lineages. Directly comparing osteoarthritic and osteoporotic MSCs and thus negating the effect of age, reveals an increased propensity of osteoporotic MSCs to differentiate along the adipogenic lineage.

Thus MSCs have been successfully extracted, isolated, and respective differentiation capabilities further dissected. A crucial aspect of MSC behaviour critical to its cellular function in an organ system is cell migration. Chemokinesis analysis demonstrates that healthy cells and osteoarthritic MSCs migrate in the absence of any chemoattractants. However osteoporotic MSCs failed to migrate in a similar manner. This highlights a potential altered function of osteoporotic MSCs with implications for its pathogenesis and also for
their use in autologous therapy. Healthy MSCs and osteoporotic MSCs migrated in a statistically significant manner towards 10% FBS. However osteoarthritic cells failed to migrate towards this positive chemoattractant. Current clinical trials involve the use of autologous MSCs as therapy for osteoarthritis. The failure of osteoarthritic MSCs to migrate towards a positive chemoattractant suggests an alteration in its migratory ability with implications on their effectiveness as a clinical therapy.

The role of TGF-β1 in coupling bone resorption and deposition has become a recent area of intense research. Previous work from Tang et al have outlined the role of osteoclasts in resorbing bone and thus releasing TGF-β1 to act as a chemoattractant for MSCs, resulting in effective bone formation [22]. Thus in order to investigate the altered migratory capacity of osteoporotic MSCs, the role of TGF-β1 is sought in this study. Both healthy and osteoarthritic MSCs migrated towards 0.1pg TGF-β1. However, osteoporotic MSCs failed to migrate in a statistically significant manner towards 10% FBS. This has important implications as a failure to migrate towards TGF-β1 could result in ineffective bone formation. Eliciting this response to TGF-β1 as an element in the pathophysiology of osteoporosis has implications both for future research in this area and also highlights an avenue for persual in the search for therapeutic agents.

Upon identifying an altered migratory capacity, the location of TGF-β1 receptors and their relationship to the primary cilium is of further interest. Localising the effect of TGF-β1 and its downstream effectors on healthy and diseased MSCs could further outline the pathogenesis and involvement of TGF-β1 in the respective disease states. The primary cilium is no longer considered a vestigial organism, and plays a significant role in the mechanobiology of effective bone formation. Our laboratory has localised aspects of the TGF-β1 machinery to the primary cilium in healthy MSC’s. Applying the same experiments to osteoporotic counterparts unveils a reduced co-localisation of TGF-βRII to the cilium in osteoporotic MSCs. This implicates the primary cilium as a component in the altered response to TGF-β1. Research involving the primary cilium and the field of mechanobiology is ever expanding. Highlighting a reduction in localisation of receptors to the primary cilium in diseases such as osteoporosis indicates that further research needs to be invested into this critical organism.

Thus this work is successful in its aims of developing a biobank of osteoporotic and osteoarthritic MSCs. It outlines an altered differentiation capacity of both osteoporotic and
osteoarthritic MSCs. The length of the primary cilium was found to be similar in both disease states but altered co-localisation patterns of TGF-βRII was identified in osteoporotic MSCs. A critical finding is the reduced ability of osteoporotic MSCs to migrate towards TGF-β1.

A number of limitations are identifiable in this study. The donor numbers are low and result in difficulty creating the power required to determine statistical significance between relevant groups. However now that the surgical technique employed has been verified as adequate, the collection of donor samples can vastly increase creating a large biobank. A second limitation is the age of the donors involved in the recruitment process. The donors involved in the study had established osteoarthritis and osteoporosis and thus were in the elderly age range. One donor was as a result of a traumatic hip fracture and thus younger. In order to directly compare healthy MSCs to osteoarthritic and osteoporotic MSCs, it would be ideal to have similar age profiles involved. However a larger scale collection of bone marrow aspirates has the potential to incorporate higher numbers of traumatic samples without osteoporosis or osteoarthritis. This would therefore give the adequate numbers for direct comparison amongst groups.

Implications from this research include the demonstration and feasibility of a technique to obtain bone marrow aspirates from healthy, osteoporotic and osteoarthritic patients. This can allow institutions to develop large biobanks of diseased cells for further research with minimal complications or inconvenience imparted to the donor. Osteoporotic and osteoarthritic MSCs displayed an altered trilineage potential in comparison to healthy MSCs as previously documented in current literature. The underlying reasons for this altered differentiation capacity was further investigated with diseased MSCs displaying altered chemokinetic behaviour. TGF-β1 is a member of a superfamily of chemokines with significant roles in bone biology. Interestingly, osteoporotic MSCs failed to migrate towards TGF-β1 and also displayed a reduced ciliary co-localisation of TGF-βRII in comparison to healthy controls.

Ongoing investigation is required to uncover in more detail the causation for the altered migratory patterns in diseased MSCs as this can have significant implications for the application of future therapeutic agents in the fight against these common conditions. Future research could delineate further the causation of a reduced migratory pattern towards TGF-β1. This could involve analysing content of downstream effectors of the TGF-β1 pathway in health and disease. Gene expression analysis of all the TGF-β1 receptors may highlight some
causative factors. Further research into these aspects of common diseases such as osteoporosis has the potential for incalculable gains to both scientists, clinicians and patients.
Chapter 6:
References


269. Li, L., et al., **Signaling pathways involved in migration of mesenchymal stem cells.** Trends Biopharm Ind, 2010. 6: p. 29-33.


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### Appendix A: Additional Tables

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Table outlining antibodies and dilutions used for staining of the primary cilium
Osteoprogenitor Regulation in Loading-Induced Bone Formation.

PATIENT INFORMATION SHEET-RESEARCH TEAM COPY
**Osteoprogenitor Regulation in Loading-Induced Bone Formation.**

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Signed (Clinician):
Principal Investigator: Dr. David Hoey
Co-investigator: Mr. Brian Lenehan
Research Team: Dr. Marie-Noëlle Labour, Ms. Elena Stavenschi and Ms. Michele Corrigan

Many thanks for taking the time to consider your participation in this research study and for reading this patient information leaflet.

Purpose of the study:
Osteoporosis is a common debilitating bone disease where the bones of the skeleton become weak and therefore more prone to fractures. In a healthy individual there are cells that replace old damaged bone with new healthy bone. In osteoporosis this repair technique is faulty. Therefore our research project aims to gain a greater understanding of how cells originating in the bone marrow (stem cells) form new healthy bone. We hope this will lead to the development of new ways to ways to activate these cells resulting in the formation of new bone. This represents a potential novel treatment for osteoporosis.

We wish to collect samples of bone marrow from healthy patients and those suffering from osteoporosis. The bone marrow samples are an ideal source of stem cells for our study. Following isolation of stem cells from the marrow samples we can model the conditions they would be under in bone and perform experiments to monitor how they behave under different conditions.

Duration of the study
We would expect that the entire project will run for 12 months to collect samples from a number of patients.

For you, during one of your visits to Mr. Lenehan’s clinics one of his staff will speak with you about our project. Another brief meeting will be required prior to your surgery where you will be informed of the details of the study and then be given the option to complete a consent form.

The study is not expected to add any additional time to your surgical procedure or pre/post-procedure care.

How participants are chosen
All patients who are scheduled to undergo a surgical procedure where the bone marrow will be exposed and who are under the care of Mr. Brian Lenehan will be asked to consider taking part in the study.

Voluntary participation
Taking part in this study is entirely up to you. There is no obligation to take part and your treatment will not be affected if you refuse to take part. Similarly, if you change your mind and wish to withdraw consent at a later date, it will be facilitated and again, your treatment will not be affected in any way.

What the research study entails
Your consultant orthopaedic surgeon has scheduled you for an operative procedure and during the course of this the surgeon will expose a section of your bone marrow cavity. We wish to take a sample of the bone marrow from this cavity for use in our research study. There will be no change to how you are prepared for the procedure. Similarly, there will be no difference in how the operation will be performed. What is different is that we will take a sample of the bone marrow exposed during the surgical procedure. Stem cells will be isolated from the sample and we will test them with specially designed laboratory equipment in the University of Limerick. The cells will be frozen until we have completed this testing before being destroyed.
Disadvantages in taking part in the research study
Taking part in this study will not present any disadvantages to your care plan.

Risks of taking part in the research study
There are risks associated with the surgical procedure, which will be explained to you by your consultant orthopaedic surgeon.
You will not be exposed to additional risks by agreeing to take part in this study.

Benefits of taking part in the research study
There will be no specific benefits to yourself for taking part in this study; your contribution, however, will allow us to understand better how stem cells are recruited in the process of bone formation.

Permission for access to medical records
For the purposes of this research study, we would like to collect data relating to your previous medical history. We would greatly appreciate if you would allow us access to the information contained within your medical records.

Data confidentiality
Your medical records will only be accessed by the principal investigator (Dr. David Hoey) and his research team for the purposes of this research study. All information will be coded to provide anonymity and will be accessed only by the named investigators.

Data storage
All data collected will be coded anonymously and will be stored in password-protected files on secure networks in a locked office only accessible by the research team. All paper documentation will be shredded on completion of the study.

Access to results of the research study
The results of the research study will only be accessible by the research team for analysis. Before publication outside of our research team all data will be coded to protect anonymity.

This research study, patient information leaflet and consent documentation have all been approved by the Medical Ethics committee for the University Hospitals Limerick Group.
I have read the foregoing information, or it has been read to me. I have had the opportunity to ask questions about it and any questions that I have asked have been answered to my satisfaction. I consent voluntarily to participate in this research.

Print Name of Participant __________________

Signature of Participant ____________________

Date ___________________________ Day/month/year

Statement by the researcher/person taking consent
I have accurately read out the information sheet to the potential participant, and to the best of my ability made sure that the participant understands the procedure that will be followed.
I confirm that the participant was given an opportunity to ask questions about the study, and all the questions asked by the participant have been answered correctly and to the best of my ability. I confirm that the individual has not been coerced into giving consent, and the consent has been given freely and voluntarily.

A copy of this Informed Consent Form has been provided to the participant.

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There will be no specific benefits to yourself for taking part in this study; your contribution, however, will allow us to understand better how stem cells are recruited in the process of bone formation.

**Permission for access to medical records**
For the purposes of this research study, we would like to collect data relating to your previous medical history. We would greatly appreciate if you would allow us access to the information contained within your medical records.

**Data confidentiality**
Your medical records will only be accessed by the principal investigator (Dr. David Hoey) and his research team for the purposes of this research study. All information will be coded to provide anonymity and will be accessed only by the named investigators.

**Data storage**
All data collected will be coded anonymously and will be stored in password-protected files on secure networks in a locked office only accessible by the research team. All paper documentation will be shredded on completion of the study.

**Access to results of the research study**
The results of the research study will only be accessible by the research team for analysis. Before publication outside of our research team all data will be coded to protect anonymity.

This research study, patient information leaflet and consent documentation have all been approved by the Medical Ethics committee for the University Hospitals Limerick Group.
I have read the foregoing information, or it has been read to me. I have had the opportunity to ask questions about it and any questions that I have asked have been answered to my satisfaction. I consent voluntarily to participate in this research.

Print Name of Participant __________________

Signature of Participant ___________________

Date ___________________________  
Day/month/year

Statement by the researcher/person taking consent
I have accurately read out the information sheet to the potential participant, and to the best of my ability made sure that the participant understands the procedure that will be followed.
I confirm that the participant was given an opportunity to ask questions about the study, and all the questions asked by the participant have been answered correctly and to the best of my ability. I confirm that the individual has not been coerced into giving consent, and the consent has been given freely and voluntarily.

A copy of this Informed Consent Form has been provided to the participant.

Print Name of Researcher/person taking the consent________________________

Signature of Researcher/person taking the consent_________________________

Date ___________________________  
Day/month/year

This research study, patient information leaflet and consent documentation have all been approved by the Medical Ethics committee for the University Hospitals Limerick Group.