Do Stem Cells Transfected with CXCR4 Enhance Bone Formation in Osteoporosis?

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DECLARATION

I, Anita Sanghani-Kerai, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in this thesis.

ABSTRACT

Osteoporosis affects bone mass and bone micro-architecture, reducing mechanical strength. SDF-1 and its ligand CXCR4 play significant roles in the migration and engraftment of mesenchymal stem cells (MSCs). The aim of this study was to investigate the effects of CXCR4 transfected MSCs on bone formation in osteopenic rats. The hypothesis was that MSCs genetically modified to over-express CXCR4, would enhance migration of stem cells from osteopenic rats and when injected intravenously in ovariectomised (OVX) rats, would improve bone formation.

MSCs were harvested from femora of young, OVX and adult control rats. The differentiation, CXCR4 expression, in vitro migration and phenotypic characteristics of the cells were compared. Although the phenotypic characteristics of cells from all groups of rats were the same, their differentiation capability, CXCR4 expression and migration was significantly different. MSCs were genetically modified to over-express CXCR4 and in vitro migration investigated. It was found that although young MSCs had the highest migration capability (2x more than their uninfected counterparts, p=0.006), the OVX MSCs when transfected with CXCR4 had the most significant migration from their un-transfected counterpart cells(5x more, p=0.025). Additionally, differentiating MSCs to osteoblasts reduced their CXCR4 expression as well as their migration towards SDF1.

The CXCR4 transfected MSCs were administered intravenously in OVX rats. Fluorescent labelled cells were tracked after 1 week and were located in the blood vessels of the femur. 11-weeks post-injection, OVX rats injected with

young-CXCR4 MSCs had significantly higher BMD(694.0±80.1mg/cm^3) ($p<0.05$) in comparison to rats injected with saline. Rats injected with OVX-CXCR4 MSCs(645.4±79.3mg/ccm) had a higher BMD in comparison to those injected with OVX MSCs(631.4±69.5mg/ccm) and saline(563.4±82.9mg/ccm). The L4 vertebral stiffness was also higher in rats treated with young-CXCR4 MSCs in comparison to those treated with saline.

CXCR4 genetically modified MSCs from young and OVX patients may help in boosting bone formation in osteoporosis.

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Abbreviations

AAV – Adeno-associated virus

ADMSCs – Adipose derived Mesenchymal stem cells

ALP – Alkaline phosphatase

APC - Allophycocyanin

BMD – Bone Mineral Density

BMP – Bone Morphogenic proteins

BMSCs – Bone Marrow stem cells

BS – Bone Surface

BSA – Bovine Serum Albumin

BV – Bone Volume

BV/TV – Percentage Bone Volume

Cbfa1- core-binding factor subunit alpha-1

CFU – Colony forming unit

CPC – Cetylpyridium chloride

CXCR4 - chemokine receptor type 4

DBM – Demineralised Bone Matrix

DiI – 1,1′-Dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate

DMEM – Dulbecco's Modified Eagle Medium

DXA – Dual-energy x-ray absorptiometry.

ECM – Extracellular matrix

EDTA – ethylenediamine tetra-acetic acid

EPCs – Endothelial progenitor cells

ES cells – Embryonic stem cells

FGF – Fibroblast Growth Factor

FITC - Fluorescein

FRAX – Fracture-risk assessment

GCSF – granulocyte colony stimulating factor

GFP – Green fluorescent protein

HCL – Hydrochloric acid

HIF-1 – Hypoxia-inducible factor **1**-alpha

HRT – Hormone Replacement Therapy

HSCs – Hematopoietic stem cells.

IGF1 – Insulin-like growth factor 1

IL-1 - Interleukin-1

IOF –International Osteoporosis Foundation

iPS – Induced Pluripotent stem cells.

ITS – Insulin, Transferrin, selenium

MI – Myocardium Infarction

MMP-9 – Matrix metallopeptidase 9

MSC - Mesenchymal stem cells

OPG – Osteoprotegrin

OVX – Ovariectomised

P/S – Penicillin Streptomycin

PBS – Phosphate Buffered Saline

PCR – polymerase chain reaction

PDGF – Platelet-derived growth factor

PE - Phycoerythrin

PLGA - Poly(lactic-co-glycolic acid)

PPARɣ - Peroxisome proliferator-activated receptors

pQCT – Peripheral quantitative computed tomography.

PRP – Platelet Rich Plasma.

PTH – Parathyroid hormone

RANK - Receptor Activator of Nuclear Factor κ B

RANKL - Receptor activator of nuclear factor kappa-B ligand

rBMCs – Rat Bone marrow mesenchymal stem cells

ROI – Region of Interest

RUNX2 - Runt-related transcription factor 2

SCF – Stem Cell Factor

SDF1- Stromal cell-derived factor 1

Tb. N – Trabecular number

Tb. Sp – Trabecular separation

Tb. Th - Trabecular thickness

TGF-B - Transforming growth factor beta

UBC-MSCs – Umbilical cord derived mesenchymal stem cells

VEGF – Vascular endothelial growth factor

WNT –Wingless pathway

Chapter 1. Literature Review

1.1 Introduction to the Thesis

Osteoporosis is a debilitating condition of the bone that results in fragility fractures, which are then difficult to treat. Fragility fractures also cause a socio-economic burden to the patient and the society. There have been problems with current treatment therapies as they are anti-catabolic, and the focus is therefore moving towards using anabolic treatments such as the use of stem cells. The aim of this work is to use genetically modified stem cells that over-express CXCR4, to help improve bone formation in osteopenic rats.

The main purpose of this work is look at the role of CXCR4 in osteoporosis. Therefore, the main introduction of the thesis is a general overview of osteoporosis and bone and current treatments of osteoporosis will be described. There is also a detailed description of the role of stem cells in bone formation, the signalling pathways in bone formation and how ageing and osteoporosis affects stem cells. The mobilization mechanism of stem cells will also be explained and how it influences formation of bone and a significant focus will be on the SDF1-CXCR4 pathway and how this pathway influences the homing of stem cells. Finally, the last section of the introduction will explain the current animal models used to emulate osteoporosis in humans.

1.2 Osteoporosis

Osteoporosis is a disease that causes a decrease in bone mass (osteopenia) and deterioration in bone micro-architecture which leads to an enhanced fragility of the skeleton and therefore a greater risk of fracture, otherwise known as fragility fractures. It results in a significant psychological and financial burden on the affected individual, their family and the society (Jee and Yao, 2001). A reduction in bone density causes increased chances of vertebral fractures, Colles' fracture of the distal forearm, and hip fractures, as well as fractures at other sites in the body (Kanis et al., 1994).

Worldwide, approximately 200 million women are affected by osteoporosis and in developed countries 1 in 3 women and 1 in 5 men over the age of 50 years will suffer from an osteoporotic-related fracture. Annually, there are approximately 1.6 million reported cases of osteoporotic hip fractures worldwide, with a projected rise of 4.5 to 6.3 million cases by 2050 (Johnell and Kanis, 2006, Gullberg et al., 1997). With the world's ageing population increasing, the future economic burden of osteoporotic fractures is also expected to dramatically increase. The International Osteoporosis Foundation (IOF) has estimated the direct medical costs of treating 2.3 million fractures in Europe and United States as $27 billion (Burge et al., 2007).

Bone mineral density (BMD) can be assessed using dual x-ray (DXA), and osteoporosis is defined by a T score of less than 2.5 standard deviations below the average of a young adult. More recent advancements to predict an individual's 10 year risk of sustaining an osteoporotic hip fracture, include using a decision-making method of fracture-risk assessment tool (FRAX), integrated clinical risk factors with DXA-based BMD as well as a 10 year probability of incurring a major osteoporotic fracture (Rachner et al., 2011). However, problems with DXA include its inability to distinguish between cortical bone, trabecular bone, the outer shell and the spongy inner part of bone, which play significant roles in determining the bone strength and loss at different rates and it does not give a true volumetric density of bone. DXA is also incapable of determining the morphology of the trabecular organisation or the actual porosity of cortical bone that is the bone microarchitecture which plays a significant role in bone strength (Rachner et al., 2011, Kanis, 1994).

Reports suggest that between the ages of 12 and 18 years, there is a maximum rate of bone mass acquisition, due to a combination of hormonal and environmental factors interacting with the genetic makeup of an individual, to stimulate bone formation. Conditions such as anorexia nervosa, growth-hormone insufficiency, delayed puberty and amenorrhea change the normal hormonal balance which results

in lower peak bone mass. In women bone mass reduces after menopause and the level of peak bone mass which is a consequence of bone formation at puberty, is important. Reduced or impaired bone formation during puberty, results in a greater risk of fracture later in life. By the age of 50, on average, both in men and women, bone resorption outpaces bone formation, setting into pace a process of inevitable bone loss. This condition is further escalated by the decline of osteoblast function late in life, further exaggerating the imbalance between bone resorption and formation (Rosen and Bouxsein, 2006)

Ageing is one of the risk factors for the development of osteoporosis. Age-related bone loss is characterised by the uncoupling of the bone remodelling network whereby there is a reduction in bone formation and an increase in bone resorption (Kawai et al., 2012). Bone remodelling in a healthy skeleton entails removal of the older bone and replacing it with newer bone, and in osteoporosis this balance is interrupted, resulting in greater bone removal than replacement. This imbalance occurs during menopause and advancing age. During menopause the rate of bone remodelling increases which amplifies the impact of the remodelling imbalance. The loss of bone tissue leads to a disordered skeletal architecture and an increase risk of fracture (Cosman et al., 2014).

There are two types of osteoporosis; primary osteoporosis and secondary osteoporosis. Primary osteoporosis is the deterioration of bone mass caused by either a decrease in sex hormone, ageing or both. In women, menopause or premenopausal oestrogen deficiencies can increase the development of osteoporosis. Secondary osteoporosis results due to chronic conditions such as excess endogenous and exogenous thyroxin, malignancies, gastrointestinal diseases, hyperparathyroidism, connective tissue diseases, renal failure and medications such as glucocorticoids. Additionally prolonged periods of inactivity or immobilization, inadequate calcium

intake, alcohol and tobacco abuse can also contribute to osteoporosis (Downey and Siegel, 2006).

Osteoporosis does not only affect women. Men are also affected by osteoporosis due to secondary causes mentioned above. Some of the secondary causes include corticosteroid use, excessive alcohol use, hypogonadism, and vitamin D deficiency, as well as testosterone deficiency which act in synergy with oestrogen in men. Studies have suggested that in men bone loss accelerates after the age of 70 years. However unlike women who lose trabeculae connectivity due to bone resorption with age, in men the bone loss due to trabeculae thinning is as a result of reduced bone formation (Ebeling, 2008, Gennari and Bilezikian, 2007).

1.3 Bone

1.3.1 Function of bone and the skeleton

Bone is a dynamic and a highly vascularised tissue. It has a unique capacity to heal and remodel itself without leaving a scar, therefore making it an important structural support for the body. Additionally, the skeleton also serves as a mineral reservoir, supports muscular contraction resulting in motion, withstands load bearing and protects internal organs (Rodan, 1992, Sommerfeldt and Rubin, 2001, Knight and Hankenson, 2013). Structurally, bone functions as a load bearing tissue to support and protect the human body for daily physical activities. Bone is composed of organic and mineralised components mainly made up of a matrix of cross-linked type I collagen mineralised with nano-crystalline, carbonated apatite (Olszta et al., 2007, Ruppel et al., 2008).

The skeleton is composed of two parts: the axial skeleton consists of the bones of the head and trunk and the appendicular skeleton which is made up of bones of the limbs and pelvic girdle. A long bone is usually used as a standard example to discuss the macroscale structure of whole bones in the appendicular skeleton. Long bones such

as the tibia, femur, and humerus are divided into three parts: the epiphysis, metaphysis and diaphysis. The physis is a layer of growth cartilage that separates the epiphysis from the metaphysis. The epiphysis is found at either end of the bones while the metaphysis is a region between the physis and central portion or shaft of a long bone, the diaphysis (Morgan E F, 2008).

1.3.2 Gross structure of bone

1.3.2.1 Cortical and cancellous bone structure

Cortical bone is a dense tissue and has a relatively low surface area. 80% of the mass of the skeleton is made up of cortical bone. It is found in the shaft of long bones, the outer shell at the end of joints, the vertebrae and ribs. Mineralised collagen fibres are arranged to form lamellae. The lamellae surround a central canal containing blood vessels and this forms the Haversian canal (Ralston, 2005, Rho et al., 1998b).

Trabecular or cancellous bone has a lower density and a much higher surface area as it is a porous structure. The medullary cavities at the end of long bones and the interior of short bones such as ribs and vertebrae are normally composed of trabeculae bone. It is made up of an interconnecting meshwork of bony trabeculae separated by spaces filled with bone marrow and it therefore fills the middle of long bones, flat bones and the vertebrae (Ralston, 2005).

The hierarchical structure of human cortical bone is approximately 95% solid and 5% porous. A cross section of human long bone is composed of osteons and interstitial tissue between periosteal and endosteal lamellae. Lamellae are layered sheet-like structures and are the basic building blocks of the human cortical bone. Osteons (also called Haversian systems) are tube-like structures of multiple concentric lamellae with a canal in the centre for blood vessels to transport. Trabecular bone is also composed of lamellae but these are usually organised in flattened sheets (Figure 1.1) (Reznikov et al., 2014).

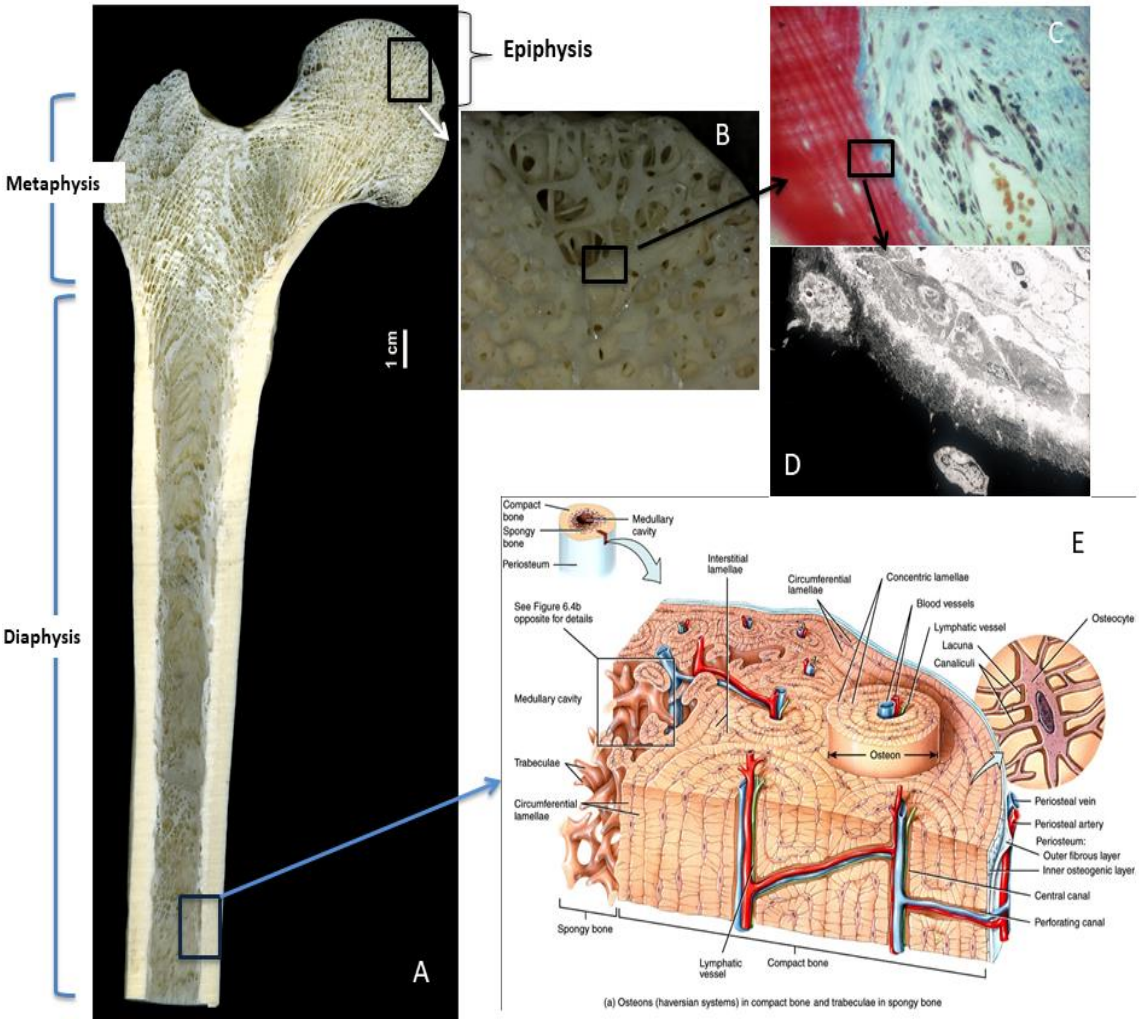

Figure 1.1:The trabecular and cortical structure of bone (A). The rods and plates in the trabecular bone (B). Martius Scarlet Blue stain demonstrating collagen stained in red and bone stained in blue, separated by the osteoid. A scanning electron microscopy image of osteoblasts and osteocytes embedded in bone. The cortical structure of bone consists of the Haversian system that is made up of a central canal of blood vessels surrounded by the concentric collagen lamellae (E) (Adapted from (Tortora and Derrickson, 2013).

1.3.3 Bone cells

Three distinctly different cell types can be found within bone; osteoblasts, osteocytes and osteoclasts (Rodan, 1992, Sommerfeldt and Rubin, 2001, Knight and

Hankenson, 2013). The human skeleton is composed of approximately 20% trabecular bone and 80% cortical bone. Trabecular bone has a higher remodelling level compared to cortical bone as its 3D microarchitecture is directly controlled by mechanical strains exerted on it (Chappard et al., 2008). Bone is divided into hierarchical structural levels; the macrostructure which is composed of the cancellous and cortical bone; the microstructure composed of the Haversian systems, osteons and single trabeculae and the sub-microstructure (Rho et al., 1998a).

Mesenchymal stem cells or osteoprogenitor cells migrate from the marrow, endosteum, periosteum and bone canals, and they differentiate to osteoblasts and osteocytes. Osteoblasts are cuboidal cells and they are responsible for depositing the bone matrix and they regulate the differentiation and activity of the osteoclasts, therefore making them important role players in calcium homeostasis. Osteoblasts contain characteristic rough endoplasmic reticulum, golgi apparatus and various secretory vesicles therefore making them protein synthesising cells. As polarised cells, the osteoblasts secrete osteoid toward the bone surface. The commitment of MSCs towards osteoblasts is influenced by certain genes and pathways such as Bone Morphogenetic proteins (BMPs) and Wingless (WNT) pathway (Mackie, 2003, Downey and Siegel, 2006, Florencio-Silva et al., 2015).

Some of the osteoblasts producing bone eventually become trapped in the bone matrix and differentiate into osteocytes and form the mechano-sensor cells of the bone tissue. Osteocytes are encapsulated in the bone matrix and form long processes that extend through the lacunocanalicular system of the bone. They create an interconnected network in bone that allows intercellular communications between both neighbouring osteocytes and the surface-lining osteoblasts and which allows the transmission of mechanical and chemical signals across a network created by mechanical forces (Morgan E F, 2008).

Osteoclasts are multinucleated cells that are involved in the resorption of fully mineralised bone at sites called Howship's Lacunae. They originate from the hematopoietic stem cell lineage (Sommerfeldt and Rubin, 2001, Teitelbaum, 2000, Ralston, 2005). Osteoclasts dissolve the inorganic fraction of bone matrix by acidification and lysosomal enzymes. The mineralised bone surface in contact with the osteoclast is broken down by acidification while lysosomal enzymes digest the organic components. The acid and the enzyme capable of operating under acidic conditions (cathespin K) is formed in intracellular vesicles and released by fusion of the vesicles with the plasma membrane. This causes the plasma membrane in contact with the bone surface to form a ruffled border. The osteoclasts are adherent to the bone surface around their periphery producing a sealing zone rich in alpha 5-beta 3-integrins which are vitronectin receptors. In this way a resorption pit is formed under the central portion of the osteoclast and this is called a Howship Lacunae (Stenbeck, 2002, Florencio-Silva et al., 2015).

Aging bone is steadily replaced by new bone through a process called bone remodelling or turnover and this process occurs through the co-ordinated action of osteoblasts and osteoclasts, in a defined anatomical space called a basic multicellular unit (BMU). A remodelling cycle begins when bone-lining cells disappear and osteoclasts invade the region. The osteoclasts generate a Howships resorption lacunae over a 2-week interval. After this osteoclasts are inactivated, and osteoblasts are recruited to the site, to fill the resorption cavity with new bone (Weitzmann and Pacifici, 2006, Riggs et al., 2002).

1.3.4 Stem cells and bone

Stem cells have the capability to self-renew and to differentiate into at least one or more mature cell types (Krause, 2002). If provided with certain environmental stimuli,

they are capable of generating cells belonging to other tissues, and this process is known as stem cell plasticity. An adult stem cell can cross lineages and transform into another cell that is similar to the alternative tissue-specific stem cell (Sozer et al., 2008). Many adult tissues contain populations of stem cells that have the capability to self-renew after injury, disease or aging and these cells can be found within the tissues or in other tissues that serve as stem cell reservoirs.

The bone marrow is a source of adult hematopoietic stem cells (HSCs) and mesenchymal stem cells (MSCs)(Pittenger et al., 1999). MSCs give rise to those cells that form the mesenchymal tissues, including bone, cartilage, tendon, muscle, ligament and marrow stroma (Burder et al., 1994). They express a fibroblastic morphology and are obtained as a heterogeneous population from bone marrow after the removal of the non-adherent contaminating haematopoietic cells (Fox et al., 2007). MSCs are characterised as plastic-adherent when maintained in standard culture conditions. MSCs must also express CD105, CD29, CD73 and CD90 and lack expression of CD45, CD34, CD14 or CD11b, CD79 or CD19 on their surface. They must also be able to differentiate to osteoblasts, adipocytes and chondroblasts in vitro (Dominici et al., 2006). There is a belief that mesenchymal-like stem cells can be isolated from a number of vascular tissues and that these are pericytes which surround blood vessels and which express CD146, a characteristic stem cell marker (Shi and Gronthos, 2003). For characterising MSCs, a combination of few positive and negative markers are selected (Table 1.1).

Stem cells are widely being used to investigate the treatment of bone disorders and injuries. Stem cells from the bone marrow as well as adipose tissue have been used to heal defects using collagen scaffolds (Niemeyer et al., 2010). Interestingly most of these studies have utilised a cocktail of Platelet Rich Plasma (PRP) and stem cells to enhance bone regeneration as well as use genetically modified stem cells (Niemeyer

et al., 2010, Yamada et al., 2004, Gao et al., 2014), highlighting their potential clinical use in patients.

Table 1.1: Main group of CD markers used to characterise MSCs. A combination of positive and negative markers is used to specifically identify a stem cell population (Ode et al., 2011, Dominici et al., 2006, Chamberlain et al., 2007).

Positive MSC markers	**Biological role**
CD29	Involved in MSC migration in vivo
CD90	Wound repair, cell-cell and cell-matrix interactions.
CD105	Vascular homeostasis and modulates TGF-beta functions.
CD73	Catalyses production of extracellular adenosine from AMP.
Negative MSC markers	**Used to exclude**
CD34	Primitive hematopoietic cells and endothelial cells
CD45	Leukocytes
CD14 and CD11b	Monocytes and macrophages
CD79 and CD19	B cells

1.3.5 Bone formation

There are two separate methods for bone formation; Endochondral ossification and Intra-membranous ossification. In intramembranous ossification, bone is formed in a matrix of haphazardly arranged collagenous fibrils. Just before ossification occurs, the mesenchymal stem cells proliferate and start to differentiate into osteoblasts, and the intracellular matrix becomes denser and homogenous. The osteoblasts then produce more matrix, which is then calcified. Some of the cells then become trapped in the matrix and transform into osteocytes (Sumner-Smith, 2002).

Endochondral ossification occurs predominantly when bone elongates at the growth plate and it entails the replacement of cartilage with bone. Endochondral ossification can occur during fracture repair. During endochondral ossification, chondrocytes proliferate, undergo hypertrophy and die. The cartilage extracellular matrix is then

invaded by blood vessels, osteoclasts, bone marrow cells and osteoblasts. The osteoblasts deposit bone on the calcified cartilage matrix (Mackie et al., 2008, Sumner-Smith, 2002). The chondrocytes deposit an extracellular matrix (ECM) which is made up of Type II collagen and aggrecan and then the chondrocytes differentiate into hypertrophic chondrocytes that deposit an ECM that contains type X collagen. This matrix is then partially mineralized, resorbed by osteoclast like cells often termed chondroclasts and replaced with a matrix consisting of Type I collagen by osteoblasts (Behonick et al., 2007). Endochondral ossification in the growth plate is controlled by various transcription factors and locally secreted systemic factors such as IGFs, Wnts, BMPs and this in turn influences the signalling pathways that control the proliferation and hypertrophy of chondrocytes (Mackie et al., 2008). In the growth plate but not necessarily during endochondral ossification during fracture repair, other locally secreted factors that control proliferation and maturation of the chondrocytes in a time dependent manner include parathyroid related peptide, Indian hedgehog and a number of fibroblast growth factors. These factors act to control proliferation and maturation of the chondrocytes so that the orientation of the growth plate is maintained (Kozhemyakina et al., 2015).

The important role of chondrocytes in the initial stages on bone formation has been highlighted in many studies. Harada and co-workers implanted MSCs pre-differentiated to chondrocytes in vitro before implanting them in Poly(lactic-co-glycolic) acid (PLGA) scaffolds in a 5mm and 15 mm rat defect site. The strategy of the study was to mimic endochondral ossification. After 8 weeks, in the 15mm gap, they observed a 75% biomechanical strength in comparison to the normal bone and union occurring in a both gap sizes (Harada et al., 2014). Van der Stok et al, 2014 also looked at differences in non-union fracture healing when undifferentiated and chondrogenic differentiated MSCs were implanted into a 6mm defect site in mice. The chondrogenic differentiated implants had improved bone formation compared to the

groups that received undifferentiated MSCs (van der Stok et al., 2014). These studies show that cells differentiated to chondrocytes encourage bone formation as it initiates the stages of endochondral ossification.

It is well known from humans that osteoporosis affects fracture healing. Animal models, which do not recapitulate human osteoporosis but rather develop a more osteopenic bone structure have been used to investigate the effects of osteoporosis on fracture healing. Animal studies conducted on ovariectomised rodents as well as sheep have shown a delay in ossification in tibia or femur osteotomy, a decrease of 20-40% in callus area and a reduction in BMD by around 20%. They also observed a decrease in mechanical strength of the callus, decrease peak failure load and decreased bending stiffness. Most importantly the architecture of the callus in the osteotomy in the osteopenic animals was different to the control animals, with disrupted and thinner trabeculae and reduced connectivity (Lill et al., 2003, Cao et al., 2002, McCann et al., 2008a).

Studies have also investigated the release of growth factors that influence bone formation at a fracture site in an ovariectomised model. Xu and co-workers,observed a lower BMD at the fracture site in osteopenic Wistar rats. However, most importantly, 3-4 weeks after the fracture, they observed a reduction in TGFβ expression in the vicinity of the bone trabeculae in these rats, showing that osteoporosis affects release of bone growth factors, therefore affecting bone remodelling (Xu et al., 2004).

1.3.6 Signals regulating MSCs during bone regeneration

Bone regeneration is a complex process involving cells and factors from many different compartments of bone. Both local and distant progenitor cells contribute to repair and various soluble factors are important components of bone repair. In addition, physiologic stimuli such as hypoxia and mechanical loading influence the signalling for the migration, proliferation and differentiation of the MSCs to the site of

injury. These factors include; fibroblast growth factor, sclerostin, Bone Morphogeneic Protein (BMP), Wnt/β-catenin, and Notch signalling pathways (Knight and Hankenson, 2013).

Under the control of transcription factors RUNX2 and osterix, Vitamin D, BMPs and Wnt signalling, MSCs differentiate to osteoblasts. Mature osteoblasts then proceed to produce an extracellular matrix rich in type I collagen that is mineralised and serves as a scaffold. Osteocytes are embedded in the mineral matrix where they act as mechanosensors and regulate mineral homeostasis.

RANKL is a key osteoclast regulator and is expressed on MSCs, osteoblasts and osteocytes. Osteoclasts differentiate from haemopoietic stem and progenitor cells in the presence of macrophage colony-stimulating factor and receptor activator of NF-kB ligand (RANKL). In oestrogen deficiency and vitamin D insufficiency, RANKL is up-regulated and this therefore stimulates RANK on osteoclasts, encouraging their proliferation and activation, increasing the number of active osteoclasts. Both RANK and RANKL are bound to the cell surface but a soluble factor osteoprotegerin (OPG) that is secreted by stromal cells including osteoblasts, bind with RANKL preventing osteoclastogeneis (Hofbauer et al., 2014).

Oestrogen is important for epiphyseal closure during puberty in males and females, as well as regulation of bone turnover. Oestrogen acts through 2 receptors; oestrogen receptor α (ERα) and oestrogen receptor β (ERβ). ERα has been shown to be the primary mediator of oestrogen's actions on the skeleton. As well as acting on the BMU, its direct role on stem cells has also been identified. Oestrogen affects bone production by altering the T cell cytokine production and the production of RANKL or OPG from stromal or osteoblastic cells, by inhibiting the differentiation of osteoclasts

and also by stimulating bone formation by enhancing the response to mechanical forces of osteoblasts and osteocytes (

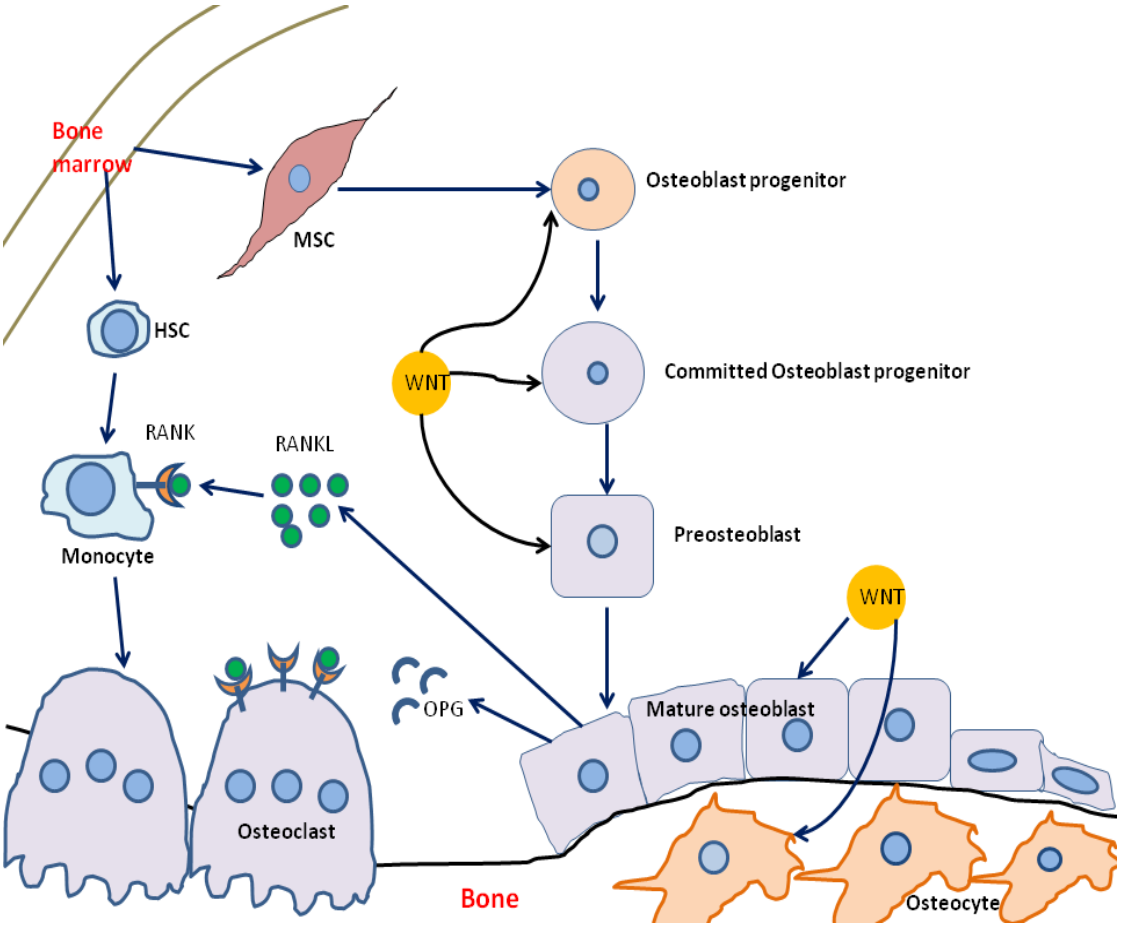

Figure 1.2) (Raisz, 2005).

Figure 1.2: The main pathways for bone formation that control the function of osteoblasts, osteoclast and osteocytes. The RANK/RANKL pathway is important for osteoclast proliferation and activation. The WNT signalling pathway encourages the formation of osteoblasts from MSCs.

1.3.7 Biomechanics of Bone

Resisting fractures while optimising the weight of the skeleton is an important function of the bone. Stiffness (resistance to deformation) and strength (maximum stress to failure) are necessary to accommodate large loads while toughness or ductility allows the bone to absorb the energy from impact loads. Higher tissue mineral content as well as a change in cross-linking structure of collagen would produce a stiffer but more brittle bone (Boskey and Coleman, 2010). Bone strength depends on age, gender, and species of animal and location of bone such as the humerus versus femur. Moreover, bone strength also depends on properties of bone such as inherent composition, microscopic morphology of bone components, bonds between fibres and matrix and bonds at points of contact of fibres. The structural properties of bone also determine bone strength such as geometry of whole bone, bone length, and bone curvature. Therefore, impaired bone strength might result from a reduction of bone mass, changes in bone micro-architecture or geometry (Fonseca et al., 2014, Smith, 1985). However, these bone properties may also have a U-shaped relationship with bone strength; for example, too low or too high mineralisation may both cause bone to become fragile.

Bisphosphonates cause osteoclast death, decrease bone turnover but this decreases fracture risk. Teriparatide increases bone turnover, but instead leads to an average decrease in bone tissue mineralisation, yet it has been shown to decrease fracture risk. The use of these drugs therefore proves that there are other determinants of bone strength apart from mineralisation (Arlot et al., 2005, Finkelstein et al., 2006, Maruotti et al., 2012).

The micro-architecture of bone also affects the biomechanical strength of bone. Plate-type trabecular bone has been shown to be much more mechanical efficient compared to rod-type trabecular bone. Thinning and loss of trabeculae is therefore associated with trabecular failure and a decrease in strength. The trabecular bone

properties are also affected by the degree of mineralisation (Hernandez and Keaveny, 2006). Reducing the number of trabeculae has a greater deteriorating effect on the strength of bone compared to reducing the thickness of the trabeculae, hence the importance of maintaining the trabecular number (Silva and Gibson, 1997).

1.4 Osteoporosis and Fractures

Fragility fractures are a major problem in osteoporosis and probably its most distinguishable factor. These fractures can be a socioeconomic and health burden to the patient as well as the health care system as they would most likely result into non- and delayed union. Oestrogen deficiency during growth and ageing is a cause of bone fragility fractures in women. The biological and mechanical environment is really important for fracture repair and influence the processes of cell and tissue proliferation and differentiation (Augat et al., 2005). However, fracture healing in an osteoporotic bone is more complex than in normal bone as the differentiation potential of the stem cells and structural capability of bone is compromised (Almeida and O'Brien, 2013, Féron and Mauprivez, 2016). This therefore presents as a problem in osteoporotic patients.

1.4.1 Fracture healing

The process of fracture healing involves the action and interaction of many cells, regulated by biochemical and mechanical signals (Geris et al., 2008). Fracture healing is divided into three main phases; inflammatory, repair and the remodelling stage. When injury occurs to the bone, bleeding occurs due to numerous blood vessels rupturing. A haematoma develops in the fracture site during the first few hours and days after injury. The fracture site becomes hypoxic and osteocytes and damaged tissues become deprived of their nutrients. This then triggers an inflammatory response, whereby inflammatory cells such as leukocytes and macrophages are brought to the site. These inflammatory cells secrete fibroblast growth factor (FGF), tumour necrosis factor-α (TNF-α), PDGF and TGF-β and also

release a variety of cytokines such as interleukin-1 (IL-1) and interleukin-6 (IL-6)(Komatsu and Warden, 2011, Geris et al., 2008).

In addition, granulation tissue is formed, vascular tissue develops and mesenchymal cells migrate into the fracture site from blood, periosteum, bone marrow and other tissue niches. The mesenchymal stem cells then proliferate and differentiate into osteoblasts and chondrocytes (Dimitrou et al., 2011). Osteoblasts from the cortex and osteoprogenitor cells from the periosteum proliferate and differentiate to form immature woven born. This type of bone is characterised by the haphazard organisation of the collagen fibres, which have a woven appearance and by large rounded osteoblasts which typically secrete extracellular matrix in a non-directed manner. Fibroblasts begin to lay down a stroma that helps support vascular ingrowth. As vascular ingrowth progresses, a collagen matrix is laid down while osteoid is secreted and subsequently mineralized, which leads to the formation of a soft callus around the repair site. The callus is then ossified by the osteoblast, forming a bridge of mineralised woven bone between the fracture fragments (Kalfas, 2001, Geris et al., 2008, Planell et al., 2009, Einhorn and Gerstenfeld, 2015).

In the remodelling phase, the original shape and mechanical strength of the healing bone is restored and the blood supply reverts to a normal state(Geris et al., 2008, Planell et al., 2009). Initially the trabecular bone is first resorbed by osteoclasts creating a resorption pit. Osteoblasts enter the resorption pit and manufacture new bone matrix of either the woven or lamellar type, starting from the outer surface and moving inwards, hence balancing the external removal with the addition on new bone to the internal surfaces(Kraus and Kirker- Head, 2006, Geris et al., 2008).

1.4.2 Influence of mechanical environment on fracture healing

There are many mechanical factors that influence fracture healing. The fracture geometry, which is portrayed by fracture type and gap size, is an important

mechanical factor that affects fracture healing. The mechanical environment influences the progress and outcome of the healing as it manipulates the differentiation and proliferation of cells in the defect region. The size of the fracture gap is another important factor. With increasing distance between the fracture surfaces, the ability for a fracture to repair decreases. Smaller gaps demonstrate a faster and more successful healing process than larger gaps (Claes et al., 1997, Harrison et al., 2003). Differentiation of tissue in the gap is also determined by the inter-fragmentary strain. According to Perren, strains below 2% result into direct healing, strains below 10% stimulate healing through endochondral ossification and strains between 10-100% lead to granulation tissue and non-union (Gitajn and Rodriguez, 2011, Perren, 1979).

There are two types of fracture healing: Primary or direct fracture healing and secondary or indirect fracture healing. Indirect (secondary) fracture healing is the most common form of fracture healing and consists of both endochondral ossification and intramembraneous bone healing and does not require rigidly stable conditions. Primary fracture healing, however, involves direct contact and requires stabilisation for healing (Féron and Mauprivez, 2016).

Most importantly, in this context, the mechanical quality of the entire bone affects the fracture geometry and the inter-fragmentary movement. Hence the poor strength of osteoporotic bone influences the initial stability and fracture stabilization is reduced. In ageing postmenopausal women, periosteal bone formation increases the cross sectional area of the bone, hence decreasing the load per unit area of bone. The bone size and volumetric BMD is reduced to below a critical level and the tolerance of bone's structural ability to sustain these loads decreases (Seeman, 2002, Augat et al., 2005). Lill et al, 2003 demonstrated no difference in bone healing at the beginning of the healing process between osteopenic and normal sheep. However, in normal sheep at 4 weeks, stiffness of the healing bone was higher compared to the

osteoporotic group, with callus formation affected in the early bone healing period and callus mineralisation in the later healing period. This illustrates the role of osteoblast function in bone formation and how osteoporosis prolongs the remodelling periods and affects fracture healing (Lill et al., 2003).

With increasing age and particularly with osteoporosis, bone mass diminishes. Osteopenia in rats has been shown not to significantly delay fracture healing compared to non-ovariectomised rats and an early healing process was found to be similar in ovariectomised and non-ovariectomised rats. However interestingly, there was reduced mechanical strength of bone after completion of healing in the osteopenic rats (Waters et al., 2000, Wheeler et al., 2000, Cao et al., 2002). In secondary osteoporosis, steroid treatment delays fracture repair with respect to callus mineralisation and biomechanical properties. Meyer and co-workers also measured the biomechanics of fractured femora over a time period. They observed no difference in rigidity and breaking load between OVX rats and their control counterparts. However, importantly they noticed a significant variability between different age groups of the rats. The femora from the younger rats healed much better and much faster in comparison to those from older rats (Meyer et al., 2001).

Therefore, there is no clear indication whether fracture healing in osteoporotic patients is slower due to their condition or their age. The lack of oestrogen as well as steroids in secondary osteoporosis could impair the function of osteoblasts hence affecting bone formation. Whether this affects the biomechanics during healing is still to be answered.

1.4.3 Fracture, ageing and osteoporosis

Ageing and osteoporosis are two inter-related yet separate degenerating conditions. Not all elderly patients are osteoporotic and an osteoporotic individual may not be elderly. Therefore, it is not clear whether poor healing capability of bone is due to

ageing or osteoporosis. Juvenile bone has a better healing response compared to adult bone due to a thicker and a more cellular periosteum as well as a better vascular supply. Since biological factors such as cells play an important role in fracture healing, the repair of bone is questionable in osteoporotic patients as their cells have a lower differentiation potential(Féron and Mauprivez, 2016, Almeida and O'Brien, 2013). However, it would be controversial to fully blame osteoporosis for the poor healing capacity of bone. This is because these patients suffer from other conditions such as age, rheumatism, thyroid and parathyroid disorders, and malignancy. The nutritional status of elderly patients is also compromised, which would further impair bone healing (Meyer et al., 2001, Paiva et al., 2016). A study by Meyer et al, 2000 showed that in older rats, neither sham operated or ovariectomised rats regained their mechanical strength in their fractured femora, 24 weeks after fracture. This was not the case with younger ovariectomised rats. This shows that age could also be a contributing factor in fracture healing (Meyer et al., 2001). Ovariectomy has been shown to delay fracture healing in rats. McCann and co-workers showed that OVX animals had a lower BMD, slower fracture repair, reduced stiffness in the fractured femur and strength after 8 weeks post-fracture (McCann et al., 2008a).

Age, rheumatism, thyroid and parathyroid disorders, and malignancy may influence the function of bone cells and impede healing through poor vascularisation. Tissue vascularisation is important for successful bone healing. Ageing affects the surface density of blood vessels but also signalling factors such as VEGF, HIF-1 and MMP-9 that regulate the process of angiogenesis. VEGF and HIF-1 also play important roles in endochondral ossification (Lu et al., 2008).

Summary: Mesenchymal stem cells play an important role in bone healing as they differentiate to osteoblasts and they can also form bone through the endochondral ossification. Even though the commitment of MSCs to osteoblasts is influenced by

various genes and pathways, the question remains whether their differentiation ability is affected by ageing and osteoporosis.

1.5 Osteoporosis and Ageing

1.5.1 Ageing of bone

In healthy individuals, the osteoblast progenitors, namely mesenchymal stem cells (MSCs), occur in low number (0.01 %–0.001 % of the nucleated portion) in the bone marrow (Wexler et al., 2003). In the aging population, the number of MSCs further decreases, thus leading to a significant reduction in bone formation (Garvin et al., 2007). Bone remodelling is the key method in bone structural reorganisation and changes in bone remodelling can disrupt the mechanical strength of bone. Osteons reflect different bone remodelling patterns. Young individuals have high osteon diameter, osteon area, and osteon wall thickness than osteoporotic and old individuals. Osteoporotic patients have thin walled osteons in their bone structure. Young individuals also display higher numbers of osteocyte lacunae per osteon than the aged individuals (Bernhard et al., 2013).

1.5.2 Stem Cells and Ageing

Impaired migration of MSCs has been shown in various studies and there is need to upregulate the expression of important homing molecules (Devine et al., 2001, Lapidot and Kollet, 2002, Petit et al., 2002b). However, what many of these studies have failed to answer is if this is due to the poor expression of homing molecules such as CXCR4 and whether this is associated with age or osteoporosis. Previous reports have suggested that with age, the proliferation, differentiation and the in vivo bone formation ability of stem cells reduce and their senescence increases. However, it is unclear how many passages, population doublings, or senescent-associated molecular changes are associated with ageing (Wagner et al., 2010, Sethe et al., 2006). Additionally, it is important to determine how age affects the proliferation rate

of MSCs, so as to enable MSCs from these older patients to be used in autologous transplantation, in age related diseases (Fossett et al., 2016). There are mixed results on the effect of ageing on the proliferation of MSCs. Stolzing et al, 2008 investigated the proliferation of MSCs from three different groups of patients; young (7-18 years old), adult (19-40 years) and aged (over 40 years). They observed a similar initial phase of growth up to 5 weeks in culture. However after 5 weeks the proliferation in the 'aged' MSCs began to decline while the MSCs from the 'adult' donors continued to proliferate throughout (Stolzing et al., 2008).

MSC deficiency in cellular function may be one reason that the repair and regeneration of musculoskeletal injury and disease is more impaired in older patients. It is not just the structure of the bone and the osteoprogenitor cells that are affected with age, but the bone marrow as well. Histological examination of the bone marrow in the proximal tibia of 26 month old mice showed significantly more fat cells compared to the marrow of 8 month old mice. The stem cells from the bone marrow of the old mice are more likely to form fat and chondrogenic matrix in vitro due to the increase in sensitivity of the PPAR-γ (Moerman et al., 2004b, Zheng et al., 2007). Shi and co-workers observed greater attachment and proliferation as well as adipogenic differentiation of juvenile adipogenic derived stem cells (ADSCs) isolated from 6 day old juvenile mice in comparison to adult ADSCs from 60 day old adult mice. However, both types of cells had similar osteogenic differentiation ability (Shi et al., 2005).

The link between osteoporosis, ageing and stem cells is debatable. There has been speculation that osteoporosis could partly be caused by a deficiency in BMSC osteogenesis. It has been reported that MSCs from osteoporotic postmenopausal women have poor osteogenic differentiation ability in comparison to MSCs from 'healthy' control individuals (Rodriguez et al., 1999). However, Stenderup and co-workers reported no difference in number, proliferation and osteogenic differentiation between osteoprogenitor cells from young, postmenopausal and osteoporotic

humans and concluded that there may be other mechanisms responsible for the defective osteoblast function in osteoporosis (Stenderup et al., 2001). The differences in the results from these studies could be because of differences in age and sex of patients. Stenderup's study used female as well as male subjects. The results may therefore be subjective as men have a different bone structure compared to women, at different time points in their life. This would therefore imply that the osteoprogenitor cells from male subjects may not be directly comparable to female subjects of the same age.

Another important factor to consider is whether age affects the pathway for bone formation. Meorman and co-workers showed increased sensitivity to PPAR-γ as well as a significant decrease in mRNA expression of the cytokines TGF-β2 and TGF-β3 with aging (Moerman et al., 2004b). Osteoporosis not only results in the decrease in the number and activity of stem cells but also a decrease in signalling molecules such as oestrogen, IGH, TGF-B, and calcitropic hormones. Notch signalling increase bone formation over bone resorption and therefore raising the question of whether its loss may contribute to age-related osteoporosis(Engin et al., 2008).

1.6 The role of Oestrogen in bone formation

Oestrogen has a significant influence on bone formation in vivo, and reduced levels result in considerable bone loss due to increased bone resorption. Oestrogen deficiency has been shown to cause the development of osteoporosis in elderly men and in postmenopausal women. This deficiency causes an increase in bone resorption associated with increased osteoclast number, activity and reduced osteoclast apoptosis (Riggs, 2000, Almeida et al., 2013). Conversely an increase in oestrogen also causes an increase in osteoblast number, therefore implying its critical role in maintaining a balance between bone formation and resorption. The full role of oestrogen in osteoporosis therefore remains unknown because it either exerts its influence on bone through anti-resorptive actions or additional bone-forming effects

(Almeida et al., 2013). Lack of oestrogen causes T-cells to produce tumour necrosis factor (TNF) which increases the effect of RANKL mediated osteoclastogenesis (Roggia et al., 2001).

The deletion of the oestrogen receptor-α (ERα) from cells of the osteoclast lineage has been shown to accelerate bone resorption and loss of cancellous bone. It has been shown the inactivation of the ER-α gene in female mice, results in a significant decrease in the trabecular and cortical bone volumes compared to control mice. However, in male mice it was shown that the osteoblastic ER-α is not required for the regulation of bone formation during skeletal growth but it is necessary for the maintenance of the trabecular bone volume (Maata, 2013 & Melville, 2014).

In vivo, bone goes through an adaptive change to loading conditions and this response is less effective in the absence of the α-form of the oestrogen receptor (ER-α). Osteoblast-like cells require ER-α to proliferate in response to mechanical strain in vitro. Oestrogen concentration determines the ER-α expression in osteoblasts and osteocytes. This therefore causes the ER-α activity in bone cells to reduce after menopause, limiting their anabolic response to mechanical loading. Lee et al, 2003 showed an increased proliferation of a monolayer culture of osteoblast-like cells when applied with a single period of dynamic strain and there was improved response when the osteoblasts were transfected with ER-α. This response was inhibited by ER-α blockers (Lee et al., 2003).

Oestrogen withdrawal causes an increased production of cytokines such as IL-7 and TNF, which limit the activity of mature osteoblasts. RANKL and M-CSF are important cytokines required for osteoclast formation and they are produced by stem cells, osteoblasts and activated T-cells. They induce the differentiation and proliferation of early osteoclasts. RANK-L binds to a receptor RANK, expressed on the surface of osteoclasts and osteoclast precursors. RANK-L also binds to OPG. During oestrogen

deficiency, additional cytokines produced or regulated by T-cells are responsible for the up regulation of osteoclast formation. TNF increases the osteoclastogenic activity of RANKL and together they up regulate RANK expression in osteoclast precursors (Figure 1.3) (Weitzmann and Pacifici, 2006).

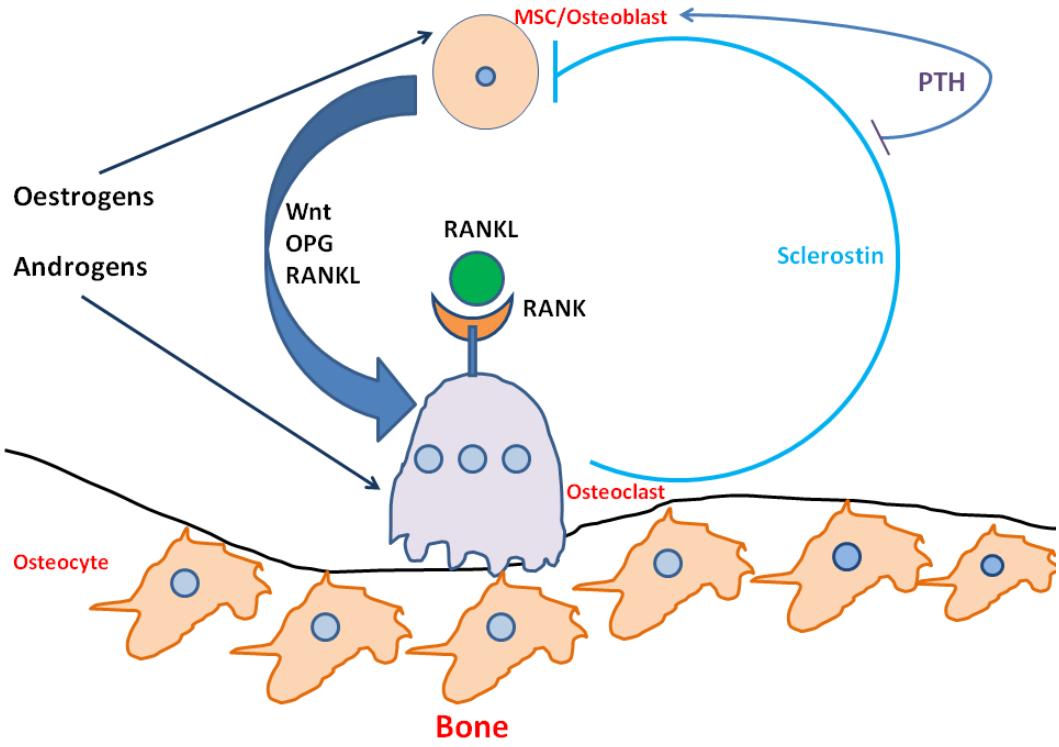

Figure 1.3: The role of oestrogen in bone formation. Proliferation of osteoclast is influenced by WNT signalling pathway and RANK/RANKL pathway. PTH which is applied in a continuous or an intermittent manner, stimulates modelling and remodelling of bone by acting on osteoblasts.

1.7 Current treatments of Osteoporosis

Bone mass can be changed in two ways; the anti-catabolic route prevents the loss of bone caused due to ageing and reduction in sex hormones. The second route is the anabolic route, which increases bone mass through net bone formation. The current treatment of osteoporosis is mainly anti-catabolic agents such as bisphosphonates, which increase bone mineral density by inhibiting osteoclast activity. The four most

commonly used bisphosphonates clinically include: alendronate, ibandronate, risedronate, and zoledronate (Favus, 2010). The action of bisphosphonates is explained by their binding to bone mineral. Osteoclasts then uptake bisphosphonates by endocytosis, resulting into osteoclast apoptosis (Russell et al., 2008). This consequently leads to a reduction in bone remodelling, which slows bone loss and also leads to alteration in the bone material properties reducing fracture risk. However, the prolonged use of this treatment can cause "bisphosphonate fractures", which is a cortical expansion in the subtrochanteric region leading to femoral neck fractures (Dosier et al., 2015). Importantly bisphosphonate treatment seeks to only reduce bone resorption and does not encourage bone formation. A number of agents act in an anabolic manner and increase bone formation such as anti-sclerostin antibody or cathespin K. Teriparatide and Abaloparatide have a dual anti-catabolic and anabolic effect (Bhargava et al., 2007, Zhang et al., 2014).

Parathyroid Hormone (PTH)

Parathyroid hormone and its N-terminal analogues (PTH1-34 and PTH 1-84) are used in the treatment of osteoporosis in postmenopausal women and both have shown to reduce the risk of new vertebral fractures (Nakajima et al., 2002). PTH is an anabolic agent that stimulates both remodelling and modelling that result in the formation of new bone, leading to an increase in bone mass (David B. Burr, 2013). PTH can be applied in a continuous or an intermittent (iPTH) manner and both types of administration lead to increased bone turnover at trabecular and cortical sites. However, unlike continuous dosing that results in increased osteoclast activation and lifespan, and thus enhanced endosteal resorption, iPTH results in increased trabecular bone volume, facilitated by the osteoblast. The primary receptor for PTH and PTHrP is the G-protein coupled receptor PTH1R, known to be expressed on the surface of osteoblasts, osteocytes, stromal cells, T-cells and macrophages. At low concentrations PTH binds preferentially to PTHR1 on cells of the osteoblastic lineage,

thus driving osteoblastic bone formation (Jilka et al., 2007, Bellido et al., 2003). Additionally, the rapid degradation of the hormone ensures that osteoclastic bone resorption is not activated via this mechanism. Intermittent PTH also induces the activation of RUNX2. This transcription factor not only drives the differentiation of stem cells down the osteoblastic lineage, it also maintains osteoblast maturity and thus has a role in increasing bone formation in both osteoporotic and increased bone turnover states (Powell et al., 2011, Mason et al.,2010).

Anti-Sclerostin therapy

Anti-Sclerostin therapy has been shown to be effective at increasing bone density and strength in animal models with osteopenia and patients with osteoporosis. Sclerostin is produced by osteocytes embedded in the bone matrix. Sclerostin inhibits BMP modulated bone formation by antagonising Wnt signalling. It acts by binding to intracellular LRP5 and/or LRP6, thereby impairing further signalling through B-catenin. This decreases osteoblastic activities, reducing the production of new bone and mineralization. It also acts by blocking the maturation of osteocytes and controls the expression of genes involved in bone matrix mineralization (Shah et al., 2015, Poole et al., 2005, van Bezooijen et al., 2007). A number of studies have investigated the effect of anti-sclerostin on fracture repair and healing. Systemic administration of sclerostin antibody has been shown to enhance bone repair in a critical-sized femoral defect in a rat model at 12 weeks (Virk et al., 2013). Liu and co-workers also investigated the effect on MSCs of rats injected with anti-sclerostin. They found MSCs isolated from rats injected with anti-sclerostin proliferated faster and expressed greater levels of ALP than cells isolated from non-treated rats. Additionally, the same authors demonstrated that anti-sclerostin increases bone mass, bone strength and bone formation in an osteopenic closed fracture model in Sprague Dawley rats, at a fracture site at 8 weeks compared to rats treated with saline (Liu et al., 2016).

Denosunab

Denosumab (Prolia*) is a human monoclonal RANKL antibody that causes the inactivation of osteoclasts, apoptosis, and reduction in osteoclasts' differentiation by preventing RANKL from binding to RANK. This drug mimics osteoprotegerin that binds to RANKL, preventing RANKL from binding to RANK on the osteoclast surface, therefore preventing osteoclast differentiation. The reduced RANK-RANKL binding, inhibits bone resorption and therefore bone mass is increased (Lipton and Goessl, 2011, Bone et al., 2011). However, the use of Denosumab has been associated with immunity and infection problems. There have been reports of serious infection and skin reaction with long term use of Denosumab which may be associated with disruption of the receptor sites of RANK on cells of the immune system (T cells). There have also been concerns of fractures when patients discontinue the use the denosumab due to the rapid reversibility of the anti-resorptive effect (Leder et al., 2017, Zaheer et al., 2015).

Hormone Replacement Therapy (HRT)

Another treatment option available for osteoporotic patients is Hormone Replacement Therapy (HRT). In this treatment therapy Oestrogen or Oestrogen in combination with progesterone is given to relieve symptoms of menopause. In HRT regimens, the oestrogen is taken daily and with the sequential or daily addition of progesterone. Indeed, it has been shown to improve bone density in post-menopausal women. However, it has been linked to breast cancer, stroke, thrombo-embolic events and cardiovascular disease (Nelson et al., 2002, Hickey et al., 2012, Prince et al., 1991).

Strontium Ranelate

This drug has been developed to reduce the risk of vertebral and non-vertebral fractures in postmenopausal women with osteoporosis (Meunier et al., 2004). It stimulates a dual action on bone formation by encouraging the differentiation of

osteoblasts and inhibits osteoclast differentiation, hence causing a negative effect on osteoclast resorption activity. (Reginster et al., 2005, Bonnelye et al., 2008). It therefore increases bone mineralisation and has also been shown to increase the trabeculae thickness and reduction in trabeculae separation in patients treated for 3 years (Jiang et al., 2006). Strontium ranelate acts on bone metabolism by interfering with the bone formation pathway by acting on RANKL/OPG ratio. It has been shown to reduce RANKL levels and increase OPG concentration, hence preventing the interaction of RANKL to RANK and reducing bone resorption by osteoclastic mechanisms (Marie et al., 2001, Brennan et al., 2009).

1.7.1 Stem cell therapy

Bone remodelling occurs through two mechanisms; one in the trabecular bone next to the red marrow, where the osteoprogenitor cells migrate from the marrow directly to the bone surfaces and a second mechanism within the cortical bone, where the osteoprogenitor cells arrive at the bone via an alternate route. As well as stem cells from the bone marrow, remodelling occurs in vascularised bone-remodelling compartments. Cells destined to become osteoblasts enter these compartments via the capillaries rather than the bone marrow. Circulating osteoblastic cells and haematopoietic stem cells enter the defect sites via these capillaries. Also it has been stated precursor cells within the capillary walls differentiate into osteoblastic progenitors. Some of these osteoprogenitor cells also originate from the perivascular compartments (pericytes) into the vascular system, and also contribute to bone formation (Fayaz et al., 2011, Mödder and Khosla, 2008).

There are various sources of stem cells that could contribute to bone formation and stem cells derived from these tissues have been used from bone regeneration.

Bone marrow Derived Mesenchymal stem cells (BMSCs)

Mesenchymal stem cells (MSCs) are multipotent adult stem cells that can be obtained from various sources such as bone marrow, adipose, muscle and umbilical cord blood tissue. They are pluripotent progenitor cells that divide many times and give rise to skeletal tissue: cartilage, bone, tendon, ligament, marrow stroma and connective tissue (Caplan, 2007). MSCs can be differentiated into several different cell types in vitro and their relative ease of expansion in culture, as well as immunologic characteristic makes them a good source for tissue repair and gene therapy. Recruitment of inflammatory cells, endothelial cells and MSCs from the tissues, circulation and the bone marrow is essential for bone healing. Stem cells can be used to restore tissue function either by integrating into the target tissue or acting as a medium to deliver complex signals to a target tissue without integrating into the tissue itself (Daley and Scadden, 2008). Unique to bone regeneration, MSCs differentiate to become either chondrocytes or osteoblasts and they are therefore a critical component for bone healing (Knight and Hankenson, 2013).

Perinatal derived stem cells

Perinatal derived stem cells can be derived from the umbilical cord, the placenta, as well as Wharton's jelly. Wharton's jelly is the mucoid connective tissue surrounding the umbilical cord vein and its main function is to protect the vasculature from pressure. Umbilical cord blood-derived stem cells (UCB) have also recently generated tremendous interest in the orthopaedic field. UCB can be harvested non-invasively without any risk to the donor, while possessing a low immunogenic potential, giving them a diverse application in cell therapy. An et al, demonstrated a 30% increase in bone mineral density (BMD) in mice when human UBC-MSCs were injected in OVX mice after 4 and 8 weeks, with increases in trabecular volume, number and thickness compared to mice treated with sham injections (An et al., 2013).

Placenta derived MSCs have been shown to tri-differentiate and express stem cell-like CD markers. They have also been capable of differentiating to osteoblasts and lay a mineralised matrix (Zhong et al., 2012). Interestingly when implanted with hydroxyapatite/tricalcium phosphate particles, the cells were capable of forming ectopic bone 8 weeks after transplantation in mice, with high expression of bone markers such as osteocalcin and osteopontin (Kusuma et al., 2015).

Adipose derived Mesenchymal stem cells (ADMSCs)

The vast majority of pre-clinical studies have investigated the use of implanted bone marrow derived stem cells, though a growing body of work is exploring the use of adipose derived stem cells (Brennan et al., 2013, Hicok et al., 2004). Studies have suggested superior cell yield (6-fold), differentiation capacity and immune-modulation of adipose derived cells versus those from bone marrow, hence increasing interest in their in vivo osteogenic capacities (Fraser et al., 2006). Subsequently, canine and murine studies have demonstrated the efficacy of allogenic adipose derived cells in the healing of long bone and calvarial defects, though little work has demonstrated the use of autologous minimally manipulated adipose derived cells to improve bone formation in human studies (Cui et al., 2007, Levi et al., 2010, Mesimaki et al., 2009, Lendeckel et al., 2004, Thesleff et al., 2011).

Similarly, ADMSCs are also easy to isolate and they produce a large cell yield. Ye et al, transplanted autologous ADMSCs in alginate in the femoral condyles of rabbits, and found that it enhanced bone mass, increased cancellous bone formation and trabecular bone structure and decreased bone loss at 8 weeks in comparison to the groups with only alginate transplanted in the condyles. Additionally, at 12 weeks, the BMD reached 88% of the normal rabbit's value, much higher than the alginate-injected group. Additionally, they also demonstrated that ADMSCs in alginate composites positively induced a BMP signalling pathway that could favour

osteogenesis and inhibit adipogenesis of stem cells from osteoporotic rabbits (Ye et al., 2014). Jeong et al. injected ADMSCs into OVX rats through the lateral tail vein. An increase in the BMD and trabecular thickness was seen in the stem cell group when compared with the control; denoting the significance of stem cells in the pathophysiology and treatment of osteoporosis (Jeong et al., 2015).

Embryonic stem cells (ES cells)

Embryonic stem cells are derived from the inner cell mass of blastocyst, and are self-renewing and pluripotent. Their ability to differentiate to any tissue makes them ideal for regenerative purposes (Wu, 2015). The osteogenic differentiation of ES cells on scaffolds has been examined in vivo. Jukes and co-workers, 2008, differentiated ES cells into a cartilage matrix and transplanted these into critical-cranial defects in rats. They observed calcification of the cartilage and bone formation in the defect sites. They reported that pre-differentiation of ES cells to chondrocytes, to mimic endochondral ossification, encouraged bone formation in vivo. This was not observed with ES pre-differentiated to osteoblasts, even though these cells easily differentiated to bone in a tissue culture dish (Jukes et al., 2008)

ES cells have also been stimulated mechanically by cyclic loading and culturing them on BioFlex plates to encourage successful differentiation of ES cells to osteoblasts in vitro (Ehnes et al., 2015, Li et al., 2013). However, results from in vitro studies need to be considered with caution as they do not predict in vivo bone formation. Additionally, derivation of human ES cells have several ethical limitations as it involves the destruction of human embryos and it is not possible to generate patient-specific lines (Wu, 2015, Yamanaka, 2009).

Induced Pluripotent stem cells (iPS)

iPS cells are pluripotent stem cells derived from fibroblasts. In 2006, Shinya Yamanaka showed that mouse embryonic and adult fibroblasts acquire properties

similar to ES cells after retrovirally introducing genes encoding Oct3/4, Sox2, Klf4, and c-Myc (Takahashi and Yamanaka, 2006). This has increased the potential to derive pluripotent stem cells from easily accessible somatic cells. In vivo osteogenic differentiated iPS cells have been transplanted in a critical calvarial bone defect in SCID mice and were shown to form new bone in the defect site. However, if the iPS cells were not treated with irradiation before transplantation, they caused teratoma formation (Hayashi et al., 2012). In vitro their ability to differentiate to bone has been proven by many studies, however in vivo and clinically, their effectiveness to differentiate to bone still needs to be established (Grigoriadis et al., 2010, Li et al., 2010, Tashiro et al., 2009).

1.7.2 Gene therapy for bone regeneration

To improve the function by enhancing growth-factor delivery, recent work has involved genetically modifying stem cells. Gene therapy offers an alternative means to achieve controlled delivery of specific proteins for bone regeneration through the transfer of nucleic acids to somatic cells for a continuous therapeutic expression of osteoinductive factors. This regulates cell activity at a specific site by the release of appropriate concentrations of functional growth factors. Gene therapy has many possible applications in orthopaedic practice, including the enhancement of bone healing (Evans et al., 2005, Betz et al., 2005, Vo et al., 2012). The controlled delivery of growth factors and cells, at sites of bone injuries, can enhance and accelerate functional bone formation. Gene transfer can be performed using a viral (transfection) or a non-viral (transduction) vector, and by either an *in vivo* or *ex-vivo* gene transfer strategy (Dimitriou et al., 2005). In vivo transfer strategies to enhance bone formation include delivery of growth factors such as BMP-2, 4 and 7 directly to the site of repair either by injection or by incorporation on a scaffold. This approach has many disadvantages such as short half-life, instability which results in the repeated administration of the growth factors and safety issues. Gene therapy where cells are

transfected in vitro with a virus carrying a gene which has perceived beneficial effects, before being transplanted in vivo, could be advantageous for delivery of growth factors (Hao et al., 2009).

Since cells do not take up genes spontaneously, vectors have to be used for gene transfer into the cell. Viruses are effective vectors for delivering cDNA as they are designed to efficiently infect cells and transmit genetic material (Pensak and Lieberman, 2013). Commonly used viral vectors include; Oncoretrovirus and Lentivirus which are retrovirus, adeno-associated virus (AAV), adenovirus and Herpes simplex virus (Betz et al., 2005). Clinically adenoviruses have been used to treat cystic fibrosis in patients (Knowles et al., 1995, Zabner et al., 1996). Retroviruses integrate their genetic material into the chromosomal DNA of the cells they infect. This is advantageous when long-term transgene expression is required. However there is a low chance that the insertion site is random which may result in insertional mutagenesis, raising huge concerns about the safety of these vectors (Betz et al., 2005).

Bone healing does not require long-term transgene expression and therefore the application of non-integrating vectors such as adenovirus and adeno-associated virus (AAV), can be used. The advantages of using an adenoviral vector system for this type of gene therapy include; extremely high transduction efficiencies compared to liposome-mediated gene transfer methods and the ability to accommodate large (up to 7.5kb) cDNAs. The virus can also be grown to high titers, which can readily be prepared and used to efficiently infect a number of cell types. Unlike retrovirus transfection, adenovirus has a low pathogenicity in humans and does not integrate into the host genome which avoids potential problems associated with insertional mutagenesis. This is important because the viral genome will not be replicated into the host cells and would eventually be lost by dilution, due to cell division and apoptosis (Luo et al., 2007, Franceschi et al., 2000, Evans et al., 2007). Adenoviral

transfection has been used in bone regeneration studies such as adenoviral gene transfers of bone morphogenetic protein-2 (BMP-2) to enhance fracture healing. Egermann and co-workers, created osteotomies in osteoporotic sheep and transplanted osteoblasts and MSCs transduced with a recombinant adenovirus carrying BMP-2 cDNA (Ad.BMP-2). Ad-BMP-2 treatment caused increased mineralisation and stiffer callus formation (Egermann et al., 2006). Lieberman and co-workers transplanted adenoviral transfected murine MSCs with BMP2 and demineralised bone (DBM) into femoral defects and showed successful healing of the large bone defects in comparison to empty defects (Lieberman et al., 1998). Ho and co-workers, 2014 adenovirally transfected SDF-1 into MSCs and transplanted these modified cells into fracture sites and showed improved bone formation when genetically modified cells were used compared to an empty gap (Ho et al., 2014a). Tang et al, 2008 also transplanted hBMP-2 transfected bone marrow MSCs into scaffolds and implanted them into a defect site in the ramus of the mandible of osteoporotic rats and they showed improved bone formation (Tang et al., 2008).

Genetic engineering can also be achieved using non-viral methods (transduction). They have an advantage over the viral methods since they produce low immunogenic reactions; they have a nearly unlimited size of transgene and a well-established, straightforward transduction procedure. Such therapies include the use of photoporation, magnetoporation, hydroporation and chemical carriers. Lipids have also been developed for use in gene therapy. Lipid carriers developed to deliver genes directly into cells, have a positively charged hydrophilic head and a hydrophobic tail. The positively charged head binds with the negatively charged phosphate group in nucleic acids. However, the problem is that the positively charged liposomes interact non-specifically with negatively charged serum proteins and enzymes, resulting in low transfection rates. Additionally, the use of the other currently

available non-viral vectors does not fulfil the ideal vector properties and they have been proven to be very expensive (Ramamoorth and Narvekar, 2015).

Summary: In conclusion, the current treatment such as bisphosphonates, are catabolic and other treatments like HRT and Denosunab have been associated with problems. There has been research on using stem cells as well as gene therapy for bone regeneration. However, it still remains to be seen whether stem cell therapy is effective in osteoporosis. There is evidence showing that stem cells have the potential to regenerate in fractures but as osteoporosis affects bones throughout the body, and as such is not site specific, intravenous administration or endogenous mobilisation may be an effective way of treating it.

1.8 Osteoporotic animal model

To select an adequate animal model to study a disease, it is important to reproduce the disorder as closely as possible. In orthopaedic research, the choice of species is varied. After analysing 21,500 mammals, Martini et al showed that from 1970 – 2001 rats are most commonly used (36%), followed by mice (26%), rabbits (13%), dogs (9%), primates (3%), sheep, pigs and cats (2% each) (Martini et al., 2001). There are several factors to be considered when choosing an appropriate animal model for osteoporosis research:

- Cost and availability
- Ethical and societal implications
- Transferability of information
- Genetic uniformity of organisms where applicable
- Reproducibility

The main types of osteoporosis in humans are postmenopausal osteoporosis, disuse osteoporosis, and glucocorticoid-induced osteoporosis. Animal models for postmenopausal osteoporosis are achieved by ovariectomy to emulate the loss of

hormonal balance that interrupts the bone formation cycle. In the adult human skeleton, bone formation is coupled to bone resorption. Therefore, a 'perfect' animal model for an osteoporosis study would be a living animal in which spontaneous or induced bone loss is caused due to a deficiency of oestrogen, and in which the characteristics of the bone loss and its progression resemble those found in postmenopausal women in one or more respects (Kalu, 1991, Jee and Yao, 2001). The ovariectomised rat is considered an appropriate model for investigating human postmenopausal osteoporosis because of many similarities in their pathophysiological mechanisms. The rat skeleton demonstrates a gradual transition from modelling to remodelling that relates to age progression in both cancellous and cortical bone (Comelekoglu et al., 2007, Han et al., 1998, Zhang et al., 2007, Francisco et al., 2011, Lelovas et al., 2008).

Rats reach skeletal maturity at the age of 2.5 months but their skeleton is considered mature after the age of 10 months. However, this does work to the advantage of using the rat as an osteoporotic model, because skeletally immature rats have a low peak bone mass which is a high risk factor for human osteoporotic fractures. The skeletally mature rat, on the other hand, is considered an appropriate model for the research of postmenopausal and immobilization osteoporosis (Turner et al., 2001). However, the biggest drawback of the rat skeleton is that some bones maintain a lifelong growth and their epiphyses do not fuse. Trabecular bone and Haversian bone do not remodel in a rat skeleton and they also have a slow developing cortical bone loss compared to humans. In a rat's cortical bone, bone gain occurs in the periosteum and cortical bone is lost at the endosteum (Jee and Yao, 2001, Lelovas et al., 2008, Erben, 1996). The rat OVX model has been used in several studies to test treatment regimens for osteoporosis. Khajuria and co-workers tested the combination effect of Zoledronic acid and Propranolol on trabecular microarchitecture. The female Wistar rats were ovariectomised at 3 months and treatment regimens were applied 12 weeks post

ovariectomy. The bones from the rats were analysed 12 weeks after drug administration (Khajuria et al., 2014). Several other studies have also used the ovariectomised rat model to study the therapeutic effects of other drugs for osteoporosis such as anti-Sclerostin, teraparatide, and the bisphosphonate risedronate (Sugie-Oya et al., 2016, Li et al., 2014, Chen et al., 2015).

Mice, another commonly used animal model in orthopaedics are attractive due to their low cost, ease of handling, and the availability of genetic knockout varieties. However, they lack a Haversian canal system and there is concern about their size and how results from a small animal can be translated into humans (Viateau et al., 2008).

Large animals such as rabbits, dogs and primates have a similar Haversian remodelling to humans. However, these animal models are not used that much in studies because of high cost of maintenance, ethical dilemmas and also because they are inappropriate models for postmenopausal osteoporosis. Dogs have similar bone composition, remodelling and architecture as humans, but have lamellar and plexiform bone. Additionally their bones have different biomechanical properties as well as a variable remodelling rate from humans (Pearce et al., 2007, Kuhn et al., 1989).

A sheep OVX model has been used to model bone loss in osteoporosis (Newton et al., 2004). Ewes ovulate spontaneously and have a similar sex hormone profile to women. Sheep also present fewer ethical controls than domestic pets such as cats, dogs and non-human primates. Older sheep also display Haversian bone remodelling and they are genetically more identical to humans compared to rodents (Wilke et al., 1997, Newman et al., 1995).

Although the trabecular and cortical bone of older sheep (> 1 year) is composed of a well-developed Haversian system as well as bone remodelling by bone multicellular units (BMUs), the cortical bone of young sheep (<1 year) which is plexiform is

significantly different from the human cortical lamellar bone. Plexiform bone is characterised by a combination of woven and lamellar bone, which allows the animals to grow rapidly under optimal mechanical properties (Turner et al., 2001, Oheim et al., 2012). Additionally, the main problem with using sheep as an OVX model is that sheep attain bone loss in 3-8 years within their adult life while humans have a 10-fold longer period of development. Sheep also develop osteopenia at various sites and to different extents (Newton et al., 2004, Zarrinkalam et al., 2009). Additionally in comparison to a small animal such as a rat, which takes around 12 weeks to attain osteopenia, in sheep osteopenia takes around 12 months to develop (Zarrinkalam et al., 2009).

Therefore, for this study, I would be using a rat model for osteoporosis. The stem cells harvested from OVX rats would be genetically modified and injected intravenously into an OVX rat to investigate whether it would improve bone formation.

1.9 Migration of MSCs - The role of SDF1 and CXCR4 in cell homing and bone regeneration.

Cellular movement and re-localization are crucial for many important physiologic properties such as, embryonic development, neovascularisation and angiogenesis, immunologic responses, wound healing, and organ repair. Both local MSCs from the injured tissue and circulating MSCs collaborate in the healing of organs during organ regeneration. Cell movement is regulated by chemotaxis, which causes directional migration via signalling molecules called chemokines (Dar et al., 2006, Ito, 2011). During organ regeneration, it has been suggested that local MSCs derived from the injured tissue and circulating MSCs work together in healing of the damaged organs. Stem cells sense the tissue injury, migrate to the site of damage and undergo differentiation (Shyu et al., 2006, Ito, 2011) and this may explain the profound increment of stem cells found in damaged tissues compared to normal healthy tissues

such as impaired sites in the brain after Hypoglossal Nerve injury (Ji et al., 2004) and cerebral injury (Imitola et al., 2004). As a result of injury, the surviving cells may produce chemoattractants such as SDF-1 that may direct the migration of MSCs to the injury site through the interaction with CXCR4 (Imitola et al., 2004).

Cytokines and chemokines play important roles in maintaining the mobilization, trafficking and homing of stem cells. Stromal cell-derived factor SDF-1 and its receptor, CXCR4, are important in the mobilization of MSCs (Liu et al., 2009) (Figure 1.4). SDF-1 is produced in many organs, including the bone marrow and it therefore has a role in leukocyte trafficking and retention of hematopoietic stem and progenitor cells within the bone marrow (Petit et al., 2002a, Zou et al., 1998). CXCR4 has been shown to mediate the migration of MSCs to the bone marrow. Wynn and co-workers, (2004) showed that neutralizing anti-CXCR4 antibody inhibited MSC migration by approximately 46%. This showed that MSCs express functionally active CXCR4 receptor, which causes the migration of MSCs to the bone marrow. Additionally, the migration of MSCs in a transwell assay was also studied to investigate the response of CXCR4 receptor to the SDF1. It was found that dose dependent migration of MSCs occurred with maximum migration occurring at a SDF-1 concentration of 30ng/ml. However it was found that CXCR4 receptor is present at low levels on the cell surfaces of MSCs and to improve the engraftment of MSCs to bone marrow and bone, an increase in its functional expression may be required (Wynn et al., 2004b).

A study by Kitaori and co-workers (2009), investigated the role of SDF-1 and its receptor, CXCR4, in bone healing. It was found that SDF-1 promoted endochondral bone repair by recruiting mesenchymal stem cells in the periosteum of the injured bone and administration of anti-SDF-1 antibody resulted into cell migration being inhibited with decreased callus formation(Kitaori et al., 2009). In addition, Granero-Molto et al (2009) showed that implanted MSCs migrated to a fracture site and this was driven by CXCR4. The MSC transplant improved fracture healing by increasing

the material toughness of the callus and causing it to be less brittle. Interestingly they separated CXCR4(+) and CXCR4(-) from a stem cell population isolated from the bone marrow. They then injected these cells separately into rats with stabilized tibial fractures. They showed that CXCR4(-) MSCs were not capable of migrating to the fracture site, up to 14 days post-injection (Granero-Molto et al., 2009). This therefore proves that systematically transplanted MSC are able to home to fracture sites, but this migration is reliant on CXCR4.

The role of SDF-1/CXCR4 in recruiting stem cells has been studied for various injured tissues such as myocardial injury. It has been observed that SDF-1 expression is rapidly increased within minutes to an hour after myocardial infarction, providing a signal for stem cell recruitment and engraftment into the injured tissue. When treated with SDF-1 expressing cardiac fibroblasts, an increase in left ventricular mass and a significant increase in the anterior-wall thickness was observed (Askari et al., 2003). CXCR4 also serves as a guidance molecule for neural stem cells in injured sites in the central nervous system. The expression of CXCR4 on human neural stem cells and the exposure of these cells to the ligand SDF-1 led to the increased migration and homing of the neural stem cells, in vitro and in vivo in injured cerebral parts of the brain. Additionally hMSCs homed to areas of increased SDF-1 expression in the injured cortex (Imitola et al., 2004).

In a study by Yamaguchi and co-workers, 2003 the effect of SDF-1 on endothelial progenitor cells (EPCs) was investigated. Three days after the administration of local SDF-1, there was accumulation of EPCs and neovascularisation was significantly greater in the SDF-1 treatment group compared to the control group. Moreover it was observed that, EPCs widely expressed CXCR4 and local administration of SDF-1 improved vasculogenesis, neovascularisation and recruitment of transplanted EPCs in ischemic tissues in vivo (Yamaguchi et al., 2003).

Chemokine mediated MSC migration has also been demonstrated in Myocardial Infarction, whereby the levels of CXCL12 (SDF-1) have been shown to increase significantly in the left ventricle of mice, after MI (Abbott et al., 2004). Abbott et al (2004) induced MI of the left anterior descending coronary artery and then intravenously introduced BM-derived MSCs into the mice 48 hours later. They observed MSC migration within 72 hours to the infarcted tissue and the administration of AMD3100, a CXCR4 receptor antagonist, inhibited the MSC migration to the infarcted site. Additionally the introduction of CXCL12 into the myocardium led to a significant increase of MSCs in the heart (Abbott et al., 2004).

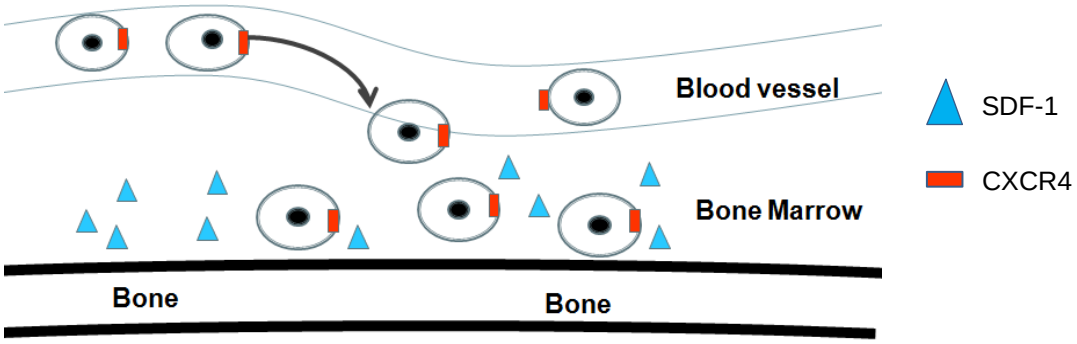

Figure 1.4: Diagram showing the localisation of SDF-1 in the bone marrow, and how this aids in attracting CXCR4 expressing MSCs. The SDF-1 is released by stromal cells and endothelial cells in the bone marrow.

The problem is only a small proportion of systemically administered MSCs actually reach and remain in the target tissue. This could be because the CXCR4 expression on the membrane of MSCs is very low, with some groups claiming there is no CXCR4 expression at all (Wynn et al., 2004a, Von Luttichau et al., 2005). Many groups have therefore designed transfection or transduction techniques, whereby CXCR4 plasmids are non-virally or virally introduced into the cells. CXCR4 over-expression improved the homing of adipose-derived MSCs to the bone marrow after intra-cardiac

injection in a NOD/SCID transplant mice. Not only was CXCR4 shown to improve migration to the bone marrow, but 90% of the transfected MSCs were retained in the bone marrow and maintained their ability to differentiate to osteocytes (Bobis-Wozowicz et al., 2011).

Lien and co-workers over-expressed MSCs with CXCR4 or Rank-Fc and injected these cells in glucocorticoid-treated mice to evaluate bone formation in an osteopenic mice model. The CXCR4-expressing MSCs were found to home and retain in the bone marrow (Lien et al., 2009b). A similar study by Cho et al, 2009 demonstrated enhanced cell trafficking to bone in ovariectomised mice, by over-expression of MSCs with CXCR4. Further bone production was enhanced by co-overexpressing the MSCs with RANK-Fc (Cho et al., 2009b). In both these studies, MSCs were intravenously introduced into the mice through the tail vein, and MSCs over-expressing CXCR4 promoted in vivo cell trafficking to bone in OVX mice. The interesting and ingenious aspect about these studies included; the therapeutic effect of CXCR4 protecting against bone loss, and bone formation was further boosted by the effect of Rank-Fc and Cbfa1 (Cho et al., 2009b, Lien et al., 2009b).

A problem with intravenous delivery of stem cells is that a large number become trapped in the spleen and in the lungs. In order to combat this effect, sodium nitroprusside, which dilates blood vessels, has been used. Cell size is an important factor and determines how many cells get trapped in organs such as lungs and therefore expanded MSCs tend to be larger than normal cells. This emphasises the importance of using sodium nitroprusside when expanded cells are administered intravenously (Gao et al., 2001, Fischer et al., 2009, Schrepfer et al., 2007).

1.9.1 The SDF-1/CXCR4 pathway.

It has been reported that stromal cells and endothelial cells residing in the bone marrow, produce the chemoattractant, SDF1, which acts as a chemoattractant for haemapoeitic stem cells to their niche.

SDF-1 is expressed in several organs including lung, liver, skin and Bone marrow. CXCR4, a receptor of SDF-1 is expressed in numerous tissues, including mature and immature hematopoietic and endothelial cells, EPCs, and smooth muscle cell (SMC) progenitors, which all have direct or indirect pro-angiogenic properties. Indeed, the SDF-1/CXCR4 pathway plays an important role in stimulating the trafficking and engraftment of hematopoietic stem cell and reconstitution of haematopoiesis. This implies that the expression of SDF-1 in a large number of tumors and injured tissues activates CXCR4 therefore promoting neo-angiogenesis (Ratajczak et al., 2006).

SDF-1 is released from the stromal cells into the intravascular compartment and it is transported from the plasma to the Bone marrow compartment. This process is known as transcytosis and it is accomplished through a complex vesicular transport system (Dar et al., 2005). When SDF-1 enters the BM microenvironment, it induces the activation of matrix metalloproteinase-9 (MMP-9) and the release of soluble kit-ligand (sKitL). Subsequently, sKitL induces the release of more SDF-1, enhancing mobilization of the CXCR4+ and c-Kit+ cells to the circulation (Heissig et al., 2002).

Additionally, the SDF-1 response is activated through the nitric oxide (NO) pathway. SDF-1 causes the endothelial cells to release NO which causes an upregulation of MMP-9. NO induces vasodilatation of BM endothelium which induces the release of cells (Aicher et al., 2003). In bone repair, SDF-1 is induced in the periosteum of injured bone and promotes endochondral bone repair by recruiting mesenchymal stem cells to the site of injury. Moreover, activation of osteoclasts and cathepsins also causes

the mobilization of the hematopoietic and endothelial progenitor cells (Urbich et al., 2005, Kollet et al., 2006).

1.9.2 Mobilization of stem cells

The importance of Mesenchymal stem cells, haematopoeitic stem cells as well as Haematopoeitic progenitor cells, in bone formation has been highlighted earlier in this chapter. SDF-1 secretion by stromal cells in the marrow enhances retention of these stem cells in the bone marrow. Haematopoetic stem cells reside in the bone marrow but can be forced into the blood using cytokines such as granulocyte colony stimulating factor (GCSF) and are then harvested in large number of cells for transplantation (Suarez-Alvarez et al., 2012). Mobilization of progenitor cells is a multistage process, which involves their release from the bone marrow niche and then the active migration across the bone marrow sinusoidal endothelium. The chemokine axis SDF1/CXCR4 has an important role in the retention of the hematopoietic stem cells within the bone marrow (Levesque et al., 2003, Pitchford et al., 2009). It is therefore important to emphasize how these cells can be mobilised from the blood. AMD3100 is an antagonist of CXCR4 that binds to its receptor, SDF1. A dose dependant increase in Haematopoeitic stem and progenitor cell (HSPCs) mobilization was also observed in healthy human volunteers, when AMD3100 was administered. There was an increase in CD34+ cells, identified using flow cytometry, as well as an increase in colony forming units (CFUs) (Liles et al., 2003). AMD3100 is currently being used in patients as Plerixafor (Mozobil, AMD3100). It has been approved to be used with G-CSF for stem cell mobilization in patients with myeloma and lymphoma (Hopman and DiPersio, 2014, Devine et al., 2004).

AMD3100 has been shown to induce rapid mobilization and engraftment of human and murine HPCs and this was further enhanced with G-CSF induced HPCs (Broxmeyer et al., 2005). GCSF disrupts the SDF1/CXCR4 axis by reducing the CXCR4 expression on HPCs and SDF1 levels in the bone marrow. It also encourages

the release of proteolytic enzymes, neutrophil elastase (NE) and cathespin G (CG), which cleave various molecules such as VCAM-1 that are responsible for HSC retention in the bone marrow (Levesque et al., 2003). Indeed Petit and co-workers, 2002 demonstrated that GCSF caused an up-regulation of CXCR4 expression and reduced the amount of SDF1 in the bone marrow (Petit et al., 2002b). Administering IGF1, PDGF, SCF or VEGF followed by AMD3100 all resulted in a larger number of colony forming MSCs, with the greatest response observed with IGF1. A follow on in vivo study demonstrated that AMD3100 with IGF1 treatment significantly improved bone formation in a mouse segmental defect model compared to each of the growth factors administered separately. Interestingly AMD3100 + IGF1 also increased proliferation, in vitro migration of MSC isolated from the blood and CXCR4 expression of MSCs isolated from tibial fracture demonstrating its critical role in improving the homing capability of MSCs to the required sites (Kumar and Ponnazhagan, 2012).

Apart from GCSF and IGF1, VEGF has also been used as a mobilization agent (Figure 1.5). Pitchford and co-workers, 2009 noticed a significant increase in circulating stromal progenitor cells when mice were given VEGF treatment in combination with AMD3100. Pitchford investigated three subsets of cells mobilised from the bone marrow; Haemapoetic progenitor cells (HPCs), Endothelial progenitor cells (EPCs) and stromal progenitor cells (SPCs). Interestingly his study found that a different regime was required for optimal mobilisation for each set of progenitor cells. GCSF treatment with AMD3100 stimulated a significant mobilization of HPCs and EPCs, while VEGF treatment with AMD3100 induced the release of EPCs and SPCs and not HPCs(Pitchford et al., 2009).

The SDF-1-CXCR4 pathway and mobilising agents seem to be influential in tissue regeneration. However, it would be interesting to investigate whether the SDF-1/CXCR4 pathway can be manipulated to mobilise stem cells and cause them to migrate to the bone marrow to improve bone formation in osteoporosis.

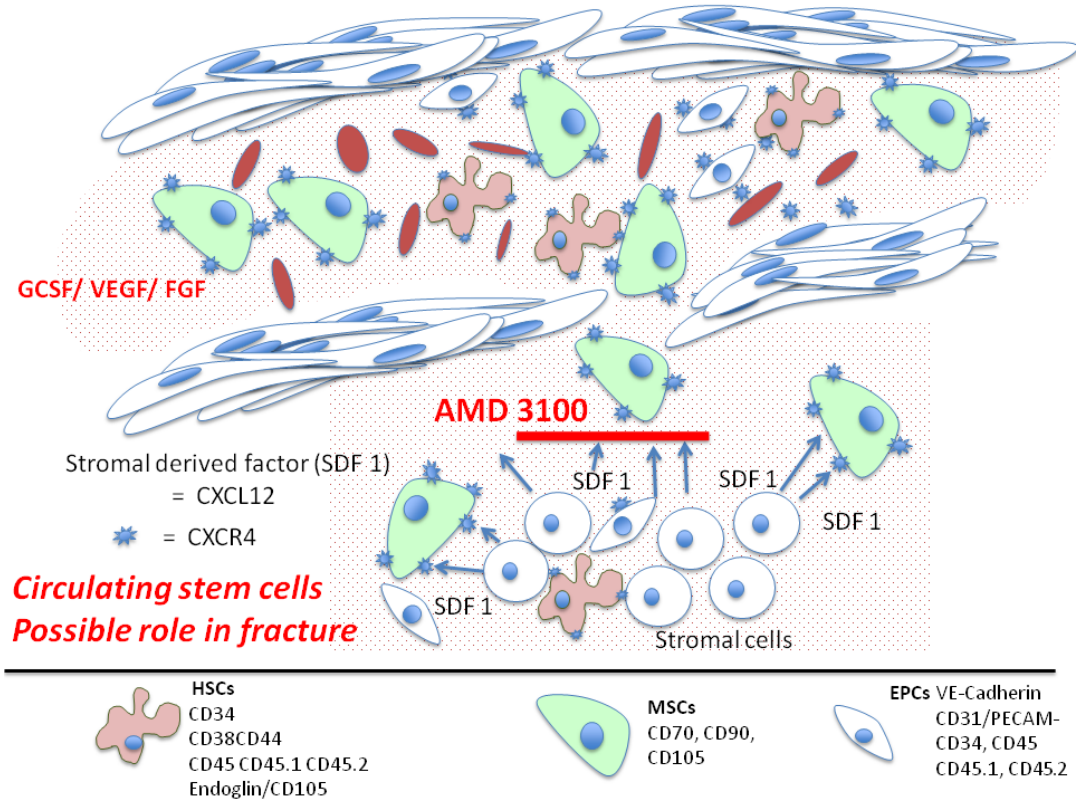

Figure 1.5: The mobilisation mechanism of cells from their niche. There are different modes of mobilisation of stem cells. circulating stem cells can be mobilised using GCSF/VEGF/FGF or AMD3100 (an antagonist of stem cells).

1.10 Research gap

The current treatment for osteoporosis such as bisphosphonates inhibits the catabolic activity of osteoclasts and subsequent bone resorption, but does not increase bone formation. There is therefore interest in using anabolic factors such as stem cells to augment fracture repair. Mesenchymal stem cells (MSCs) from postmenopausal women have a slower growth rate and osteogenic differentiation ability and this may possibly explain delayed unions in osteoporotic patients. Another factor associated with healing is the retention and migration of stem cells to the site of injury. Local mesenchymal stem cells (MSCs) from injured tissues and circulating MSCs aid in healing of injured tissues. Cytokines and chemokines such as SDF1 and its receptor CXCR4 play important roles in maintaining mobilization, trafficking and homing of

stem cells from bone marrow to the site of injury. Literature fails to clearly demonstrate the differences in proliferation, differentiation and migration as well as CXCR4 expression in MSCs from young, aged and OVX rats. Additionally, studies by Lien and Cho have shown a significant improvement in bone formation when OVX mice have been injected with CXCR4 transfected MSCs (Lien et al., 2009a, Cho et al., 2009b). However, these studies failed to show whether injecting MSCs from OVX rats is beneficial in bone formation and strength.

1.11 Aims and Hypothesis

The aim of this study was therefore:

- To establish an osteopenic rat model and to isolate stem cells from osteopenic rats, control rats and young rats and investigate the differences in proliferation, differentiation and migration of these cells in vitro.
- To transfect these MSCs with CXCR4 using an adenovirus and investigate whether viral transfection affects their differentiation, proliferation and CD marker expression.
- To investigate whether CXCR4 transfection improves in vitro migration of MSCs and whether differentiating MSCs to osteoblasts affects their migration towards SDF1 in a Transwell chamber.
- To inject transfected stem cells intravenously in OVX rats and investigate whether they improve bone formation and strength.

The hypothesis of this study is that the migration of MSCs from young, adult and ovariectomised (OVX) rats will have different migratory abilities but this will increase when they are genetically modified to over-express CXCR4.

Additionally, when CXCR4 transfected MSCs are injected intravenously into OVX rats, they would enhance bone formation and strength.

Chapter 2. **The Effect of Ovariectomy on Bone Mineral Density, Bone Strength and Morphological Structure.**

2.1 Introduction

A ‘perfect’ osteoporosis animal model would be a living animal in which spontaneous or induced bone loss is caused due to a deficiency of ovarian hormone, and in which the characteristics of bone loss and its progression resemble those found in postmenopausal women in one or more respects (Kalu, 1991, Jee and Yao, 2001). However, in nature animals do not suffer from osteoporosis in the same way as humans. The reason for this remains unclear. However, in an attempt to replicate the human condition so that interventions can be studied, a number of animal models have been used. The ovariectomised rat is considered the most appropriate model for investigating human postmenopausal osteoporosis because of many similarities in pathophysiological mechanisms. The rat skeleton demonstrates a gradual transition from modelling to remodelling that relates to age progression in both cancellous and cortical bone (Comelekoglu et al., 2007, Han et al., 1998, Zhang et al., 2007, Francisco et al., 2011, Lelovas et al., 2008).

Bone mineral density (BMD) is widely used for detecting osteoporosis and dual X-ray absorptiometry (DEXA) is a non-invasive method for measuring BMD. Bone mineral density measurements are used to assess osteoporosis, fracture risk and as a diagnosis for prescribing osteoporosis treatment. Together with bone strength, BMD is an important parameter for assessing bone quality. Bone strength can be measured using mechanical testing techniques where bone strength, stiffness, energy absorption capacity, and elastic modulus (young modulus) are quantified (Comelekoglu et al., 2007). Clinically BMD measurements are used to categorize the onset and extent of osteoporosis; however trabecular bone microarchitecture is also a key component of bone quality and is an important determinant of bone strength (Kanis, 1994, Dempster, 2000). Therefore, BMD as well as bone histomorphometry are important factors to evaluate the extent of osteoporosis.

The aim of this chapter was to describe the changes that occur in bone architecture and bone density in osteopenic Wistar rats 4 months post-ovariectomy.

The hypothesis was that ovariectomy induced osteopenia results in a reduction in BMD as well as a decrease in the mechanical strength of bone. This is important because one of the goals of my thesis is to investigate the effect of stem cell therapy on osteopenic bone. The results presented in this chapter allows me to investigate changes in bone density and architecture in animals before stem cell therapeutic intervention. In addition, this model would allow me to get stem cells (rat MSCs) from these rats to be used for further experiments in this thesis.

2.2 Methodology

2.2.1 Validation of Rat Ovariectomy

6 to 9 month old female Wistar rats were weighed, anaesthetised and shaved over the dorsal spine lumbar spine area (n=5). Using aseptic techniques, a longitudinal skin incision was made over the centre of the spine. The incision was reflected laterally on the right hand side to expose the peritoneal cavity, which was perforated by an incision and adipose tissue surrounding the ovary was exposed and retracted. The area around the distal uterine horn was tied using non-resorbable suture and the ovary removed. The procedure was then repeated and the left ovary removed through the same skin incision. Two experimental groups were investigated: (i) ovariectomised (OVX) rats were compared to (ii) non-OVX control rats of the same age (n=5). At 4 months post-ovariectomy and following euthanasia, femoral bone mineral density was measured using peripheral quantitative computed tomography pQCT (Stratec XCT1000), compression testing was carried out on L4 and L5 vertebrae and the medial gastrocnemius muscle was carefully excised from the left and right leg, weighed and results compared between groups. Bone specimens were

processed for decalcified histology and trabecular length, thickness, connectivity, distance between trabeculae and percentage soft tissue were quantified.

The skin and soft tissue around the right and left femur were removed and BMD measured within each femoral condyle in vitro using pQCT (XCT 2000, Stratec, Pforzheim, Germany). The samples were fixed in formalin before been scanned and were placed in a sealed tube during the scan. As a standard procedure a scout view was obtained to locate the desired scan position. A reference line was then positioned through the adjacent joint region at the femoral condyle. The total length of the bone was obtained prior to scanning and the femoral condyle of each sample was scanned symmetrically at slice intervals of 0.5mm. 3 slices were acquired at 10% and 1 slice was acquired at 20% (of the femoral length) from the proximal femur end. The samples were analysed using XCT software.

2.2.2 Compression Testing and Muscle Weight

L4 and L5 vertebrae were obtained from six adult and eight OVX rats. Tests were carried out on fresh samples on the day of retrieval and stored in saline before testing. Each vertebra was carefully separated from the rest of the vertebral column by halving the discs and dissecting away adjacent tissue and ligaments. The thickness of the vertebrae was then measured before being loaded onto the machine. The vertebrae were positioned between the parallel base plate and circular metal plate and a pre-load of 2N was applied (Figure 2.1). Each vertebrae was loaded in compression at a velocity of 5mm/min until failure (Zwick Roell 5T, UK). Load to failure and structural stiffness of the vertebrae was obtained from the load-deformation graph obtained from TestXpert testing software (Zwick, Roell, UK) (Figure 2.2).

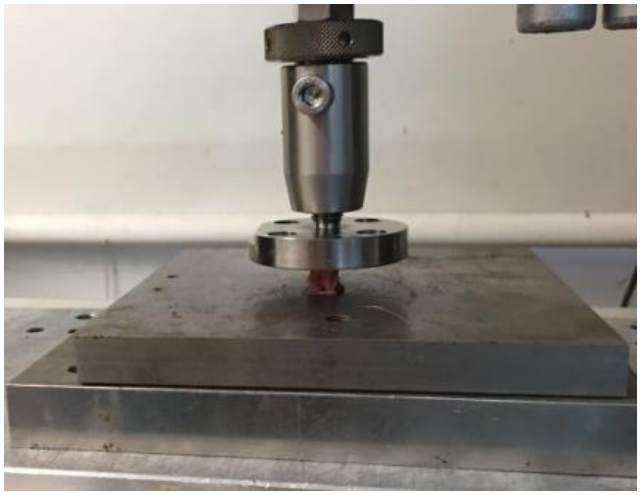

Figure 2.1: A photograph showing the compression test setup of a L4 vertebrae. L4 and L5 vertebrae was compressed until failure to obtain a force/deformation curve using a zwick machine.

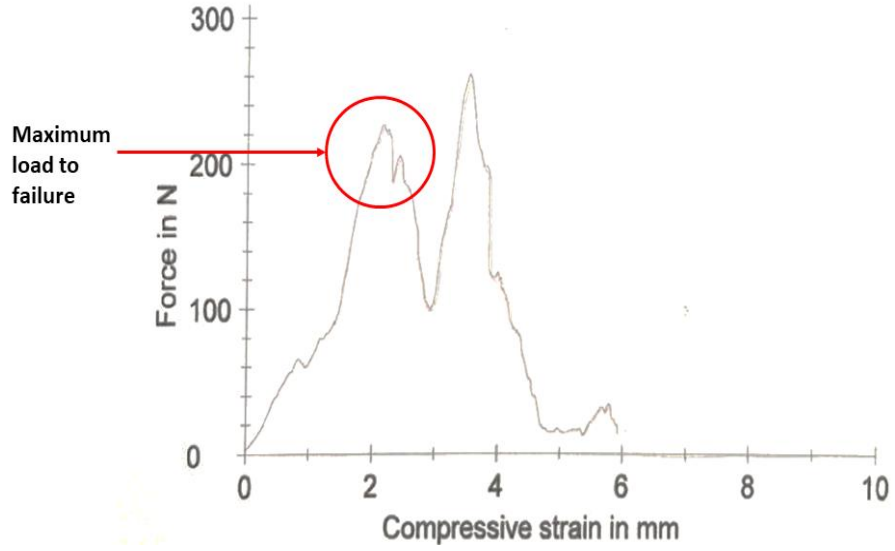

Figure 2.2: A graph showing how the maximum load to failure of a vertebrae sample was obtained from a compressive testThe first peak highlights the point where the first cortical bone of the vertebrae fails.

The medial gastrocnemius muscle was carefully excised bilaterally and each weighed. The weight of the muscle from the two groups of rats was compared. Muscle weight is important because the maintenance of adequate bone strength, density and

balance is associated with adequate muscle strength and mass (Szulc et al., 2005, Blain et al., 2010).

2.2.3 Histological Analysis

Both left and right tibiae were retrieved and specimens fixed in 10% buffered formaldehyde before decalcification in ethylenediaminetetraacetic acid (EDTA) (Sigma Aldrich, UK). An EDTA solution was made by first dissolving 250g EDTA in 1,750ml distilled water followed by 25g of sodium hydroxide (Sigma Aldrich, UK). Decalcification was confirmed by radiography, after which the specimens were dehydrated, treated with chloroform to de-fat the tissue and then embedded in wax. Longitudinal sections measuring 5µm thick were made using a microtome (Life Technologies, UK).

Following sectioning, samples were de-waxed twice in xylene, placed in two changes of 100% alcohol and then hydrated in serial dilutions of alcohol. After hydration, samples were stained in haematoxylin (Solmedia labs, UK), a nuclear stain, for 5-10 minutes. Excess stain was removed by immersing slides in running water for 5 minutes. Samples were then differentiated in 0.5% HCL acid (made up in 70% alcohol) and washed using water. Samples were then counterstained with 1% eosin (Sigma Aldrich, UK) for 3-4 minutes, washed in water and dehydrated in serial dilutions of alcohol. Finally, samples were cleared with xylene and mounted under coverslips using Pertex Mounting Medium (CellPath plc, UK). Samples were observed under light microscopy (KS-300 Zeiss, UK). All samples were analysed blind of experimental group. The following parameters were quantified:

- Length of the trabeculae
- Percentage area of soft tissue compared to calcified tissue
- Connectivity of the trabeculae
- Distance between neighbouring trabeculae.

- Thickness of the trabeculae

To measure the connectivity of the trabeculae and percentage area of soft tissue, a line intercept method using a 1cm by 1cm grid on a x2.5 magnification image, whereby tissue under each interception point was counted and converted to a percentage. A region of interest (ROI) was selected close to the growth plate. Since the region selected was site specific, close to the growth plate and with x2.5 magnifications, it was possible to view the whole region to carry out the histomorphometric analysis. Only one region of interest was analysed per rat. Length, thickness and distance between trabeculae were measured using ImageJ software (ImageJ 1.47v, USA).

2.2.4 Statistical Analysis

The normality was checked using Kolmogorov- Smirnov and Shapiro Wilkinson test and where the data was normal, comparison was made using independent student T-test. Where the data was non-parametric comparison was made using Mann-Whitney U test with a Bonferroni correction. All data was analysed using SPSS version 24 (Chicago, USA).

2.3 Results

No surgical complications and no macroscopic signs of infection were observed in any of the rats investigated. At 4-months post-surgery, the OVX rats weighed 430.0 ± 33.8 g and adult control rats weighed 400.8 ± 55.3g.

2.3.1 Bone Mineral Density (BMD)

The mean femoral BMD measured in the OVX rat group was significantly lower (538.2 ± 23.3g/cm^3) when compared with control rats (666.9 ± 46 g/cm^3) (p=0.0002). BMD was seen to reduce by 19.3 ± 9% (Figure 2.3 & Figure 2.4).

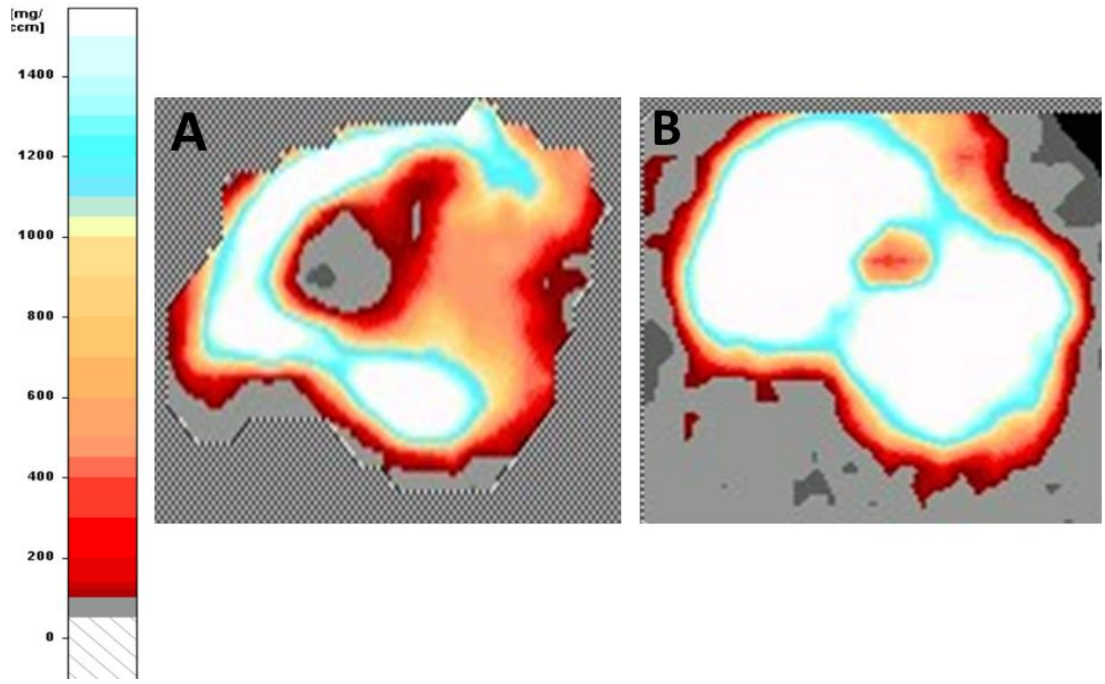

Figure 2.3: pQCT images showing the differences in BMD between OVX (A) (n=5) and adult control (B) (n=5) rats of the femurs. The XCT software was used to analyse the BMD in the femoral condyles of rats from each group of rats. BMD is scaled from a range of red to blue, with red representing the lowest BMD and blue representing the highest BMD.

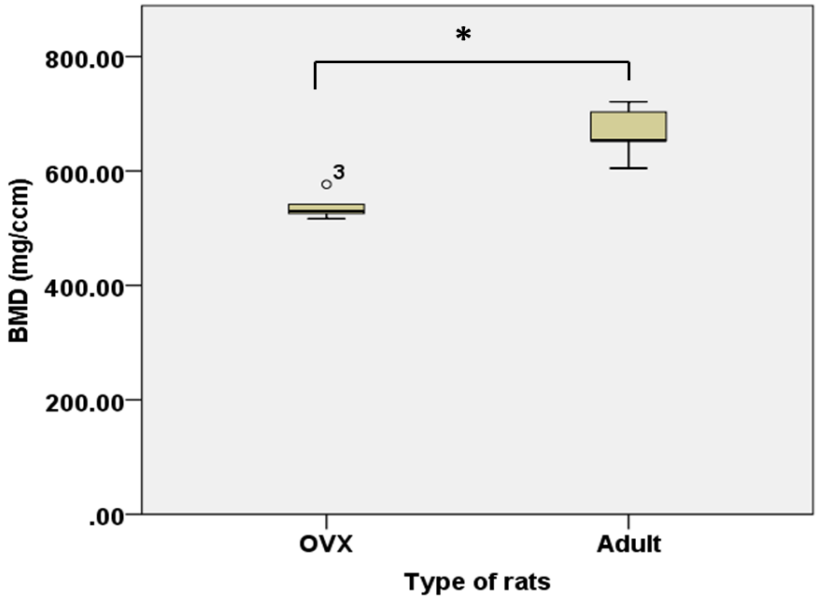

Figure 2.4: A graph showing mean BMD of the femurs measured using pQCT in the two experimental groups of rats (n=5) (* shows significance of p < 0.001). The OVX rats had significantly lower BMD compared to adult control rats.

2.3.2 Compression Testing and Muscle Weight.

The maximum load to failure for the L4 and L5 vertebrae was significantly increased in the adult non-OVX control rats compared to the OVX rats (p=0.0009 and p=0.001 respectively). The maximum load to failure for the L4 and L5 vertebrae in OVX rats was 126.7±34 N and 115±41.4N respectively, while in the control group, load to failure was 231.7±78.8N and 248.5±74.5N respectively. However, the stiffness was only significantly (p=0.02) higher for the L4 vertebrae in the non-OVX rats (130.7±30.9 N/mm) compared to the OVX rats (82.1±24.4 N/mm). Although the stiffness of the L5 vertebrae from the OVX rats (75.1±20.7 N/mm) was lower than those from non-OVX adult rats (101.6±21.8 N/mm), this was insignificant (p=0.08) (Figure 2.5 & Figure 2.6). There was no significant difference in muscle mass (Figure 2.7) between the two groups of rats.

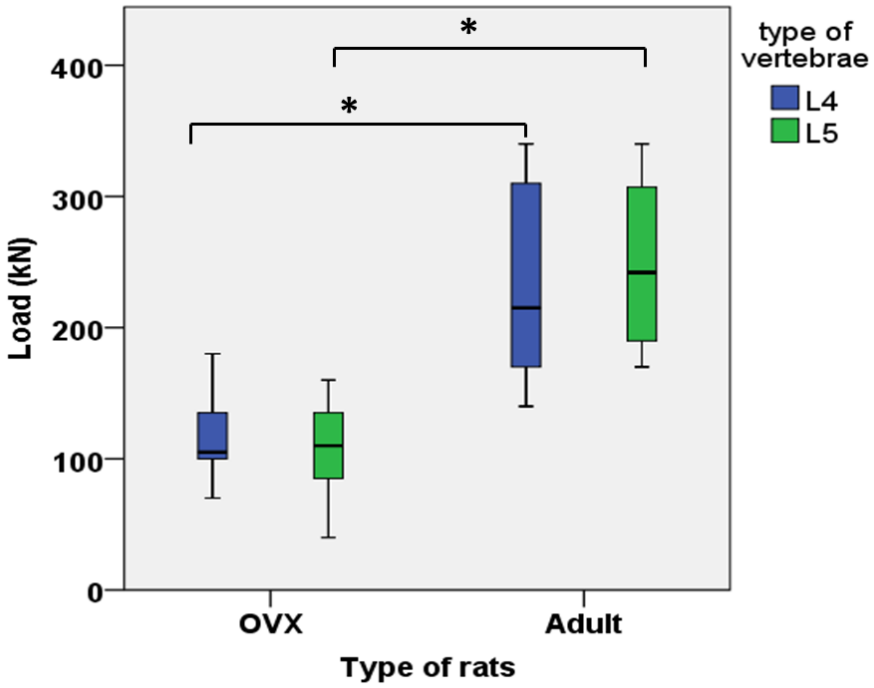

Figure 2.5: Maximum load to failure of L4 and L5 vertebrae from OVX and adult control rats. Vertebrae from the OVX rats had significantly lower load to failure compared to vertebrae from adult control rats. * shows significance of p < 0.001. (n=5).

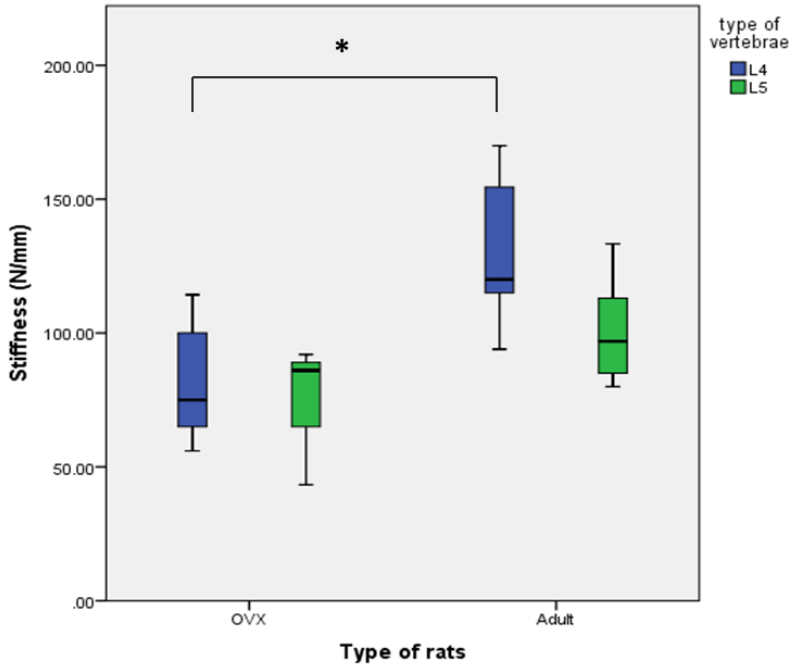

Figure 2.6: The stiffness of the L4 and L5 vertebrae from OVX and adult control rats. The L4 vertebrae from the OVX rats had significantly lower stiffness compared the adult control rats. However, this significant difference was not observed in the L5 stiffness. * shows significance of p < 0.05 (n=5).

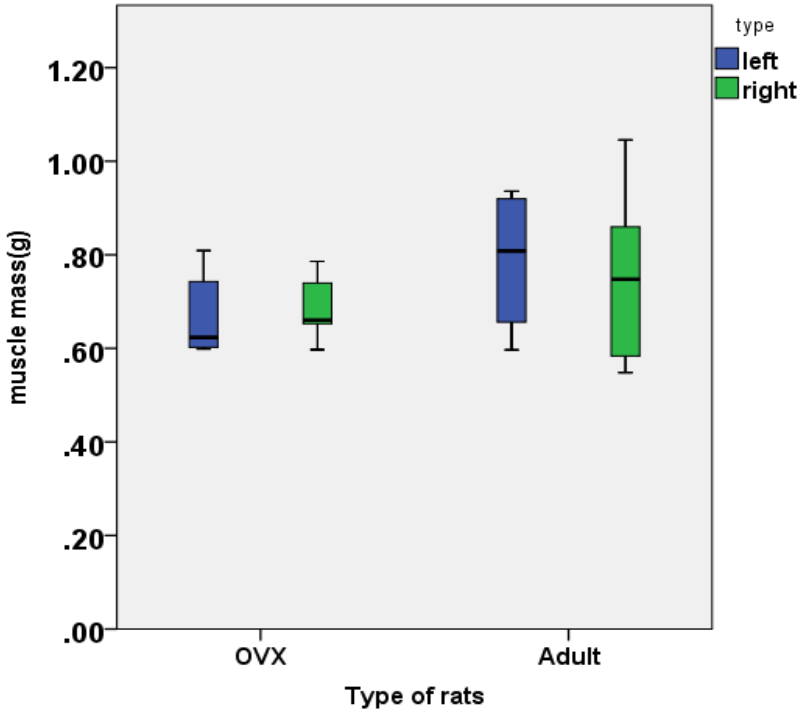

Figure 2.7: The mass of the left and right gastroscenemius muscle from the OVX and adult control rats. There was no significance between OVX and adult rats and also between the left and right muscles. (n = 5).

2.3.3 Histological Analysis.

Loss of trabeculae is a characteristic feature in osteoporosis. Results demonstrated significantly higher trabecular thickness (p=0.0008) in OVX bone when compared to the adult control rat group. Mean percentage soft tissue increased in the OVX group, however no significant difference was seen when the two groups were compared. Trabeculae length and distance between trabeculae was significantly less in OVX rats compared to the adult control rats (p=0.004 and p=0.0008 respectively). No significant difference in trabecular connectivity was observed when the two groups were compared (p=0.13) (Table 2.1, Figure 2.8).

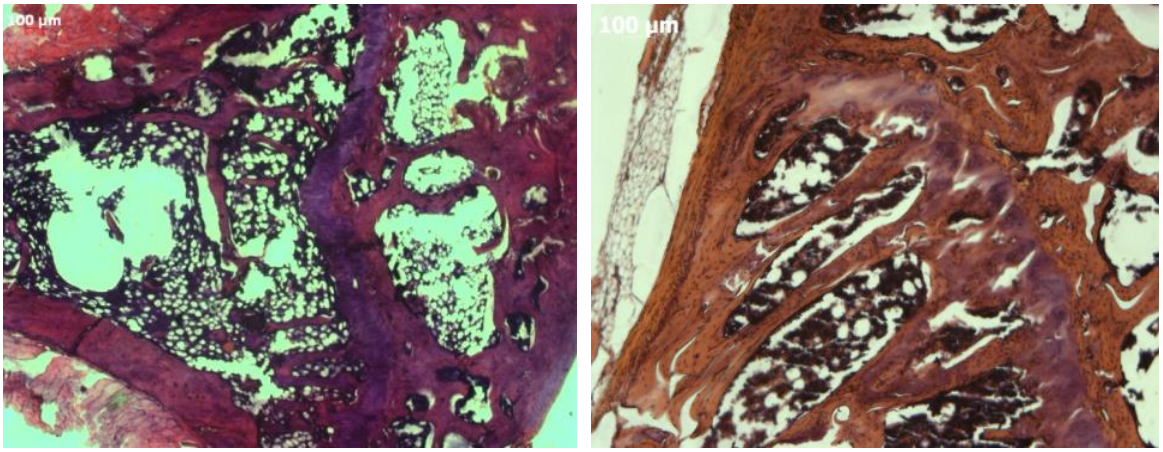

Figure 2.8: Histological sections of trabecular bone from the femoral condyles obtained from the OVX (left) and adult control rat (right). The trabeculae from the OVX bone was shorter with no connectivity. The trabeculae from the adult control bone was longer and displayed more connectivity The bone from the OVX rats also displayed a higher soft tissue area compared to that from the adult control rats.

Table 2.1: Histology results from femur of OVX and control rats (n=5).

Parameter	Adult Control (n=5)	OVX (n=5)	P Value
Trabeculae length (um)	1136.4 ± 463.4	408.0 ± 101.3	0.004
Percentage soft tissue area (%)	38.8 ± 10.2	43.5 ± 11.9	0.26
Trabecular connectivity (%)	7.2 ± 4.03	4.7 ± 2.2	0.13
Distance between trabeculae (um)	448.6 ± 122.2	185.1 ± 82.7	0.002
Trabeculae thickness (um)	54.02 ± 12.1	93.6 ± 14.3	0.0008

2.4 Discussion

In this chapter, results showed that BMD, vertebral compression, trabecular length, thickness and distance between trabeculae were all significantly reduced in the OVX rat group. Bone density and quality are terms used to describe a composite of properties that together evaluate the property of bone. BMD is often used clinically to assess bone quality and accounts for approximately 70% of bone strength. Additionally, histomorphometric analysis of trabecular bone reflects the quality of the bone (Liu et al., 2015).

In this study, osteopenia was confirmed in this model as a significant reduction (20%) in BMD was seen in OVX rats when compared with the control group. The load to failure of L4 and L5 vertebrae was also significantly lower in the OVX group when compared with control. This was possibly due to rapid loss in trabecular bone mass, as shown histologically. Studies have reported that the cortical shell determines the flexibility and energy absorption of bone while trabecular bone affects the load bearing capacity. Due to the rapid loss of trabecular bone mass seen during menopause, the cortex is thought to play an important mechanical role during weight-bearing activities.

Roux et al. showed that despite a significant age related decrease in lumbar vertebral cortical thickness, there is evidence showing that the contribution of the shell to vertebral strength increases with age (Roux et al., 2010). A study by Comelekoglu and co-workers also found a 14% decrease in rat femoral BMD and a thinner cortex compared to the control rats. They also observed a reduced elastic modulus and ultimate stress and strain in the femora of OVX rats, implying that the biomechanical properties of bone are related to its material and geometric properties (Comelekoglu et al., 2007). Yang and co-workers also found similar results in trabecular and soft tissue structure in the mandible and tibia of OVX and control rats when investigated using micro-CT analysis (Yang et al., 2014).

Vertebral bodies consist of two main structural components: trabecular and cortical bone and an age-related loss in trabecular bone structure have been shown in several studies (Comelekoglu et al., 2007, Liu et al., 2015). The histomorphological analysis of OVX femora in this chapter showed a reduced trabecular bone mass, trabecular connectivity, trabecular length and as a result the trabecular shape was more rod-like rather than plate-like. The connectivity of the trabecular system is important as it forms a supportive interconnected system that is able to resist loads. During the early stages of osteoporosis, contraction and loss of trabeculae results in the thickening of neighbouring trabeculae so as to fulfil mechanical strain placed on the bone. In the later stages of osteoporosis, the bone trabeculae are thinned and reduced to a thickness of only a few bone lamellae (Marcu et al., 2011). Euler's principle states that the resistance of a bar during a vertical load is inversely proportional with the square of its whole length. Additionally, the strength of the trabecular structure is proportional to its radius squared. This concept can be applied to the trabeculae if they are considered as bars. Bone resorption due to osteoporosis results in loss of horizontal trabeculae causing an increase in the formation of vertical trabeculae,

which decreases resistance to bending (Marcu et al., 2011, Mosekilde, 2000) and results in bone with poor mechanical strength.

Due to the functional interdependence of the bone marrow and the trabecular bone, a decrease in bone density would result in an increase in adipogenic tissue as essentially there is more space available. In addition the increase in adiposity is a result of reduced osteoblastic differentiation of the stromal cells and an increase in adipogenic differentiation (Marcu et al., 2011, Gödri and Neica, 2010). This was evident in my study as the bones from the OVX rats had a higher soft tissue area in comparison to the adult control group.

Age and osteoporosis are a major determinant of vertebral bone strength, mass and microarchitecture and therefore vertebral fractures are very common in osteoporotic patients. The vertebral body is the load bearing part of the vertebra. With age, there is alteration in the internal trabecular bone mass and changes in bone architecture causing a reduced resistance to loading. Additionally, due to resorption of the endosteal bone, there is also age-related thinning of the end plates and of the cortical shell, which would therefore compromise the strength of the vertebrae (Mosekilde, 2000). It would have been interesting to look at the vertebrae histologically in this chapter, but this was not investigated because the vertebrae were used for the compression test and therefore there was a limited number of samples.

Natural menopause drives a loss of muscle tissue as well as skeletal mass. Loss of activity with age also causes muscle atrophy. In this chapter I found no significant difference in muscle mass of the left and right gastrocnemius muscle in the OVX and control rats. Osteoporosis is a metabolic bone disease and theoretically it should not affect muscle strength. However, the reduction in muscle strength may be attributed to the pain and skeletal deformity related to osteoporosis. It has been shown that the reduction in muscle strength could further increase the postural abnormalities caused

by the disease and possibly bone density. Additionally, the loss of muscle mass may also cause a loss of skeletal mass as it decreases the mechanical stress on the skeleton and hence causes a decline in strength (Sinaki et al., 1993, Trombetti et al., 2016).

The results in this chapter have demonstrated that ovariectomy reduced BMD and changed trabecular structure in a rat model. Similar changes are seen in humans with osteoporosis and therefore this model is often used to study various therapeutic regimens investigated to treat osteoporosis. However, a disadvantage in using a rat osteopenic model is that rats grow continuously throughout their life and therefore their growth plates do not completely close which causes poor remodelling of their cortical bone. This therefore suggests that caution should be used when comparing results obtained in the rat model with that seen clinically and emphasizes the importance in using larger animal models (in addition to smaller animal models) to investigate new therapies to treat osteoporosis in patients (Hartke, 1999). It is also important to understand that although the rat model is an osteopenic model rather than an osteoporotic model exhibited in humans, it is the cheapest and most reproducible.

The aim of this chapter was to establish an osteopenic rat model so that MSCs could be harvested from these rats to investigate and compare the in vitro differences in MSCs with adult control and young rats. Osteopenic rats similar to the ones used in this chapter would also be used to determine whether injecting CXCR4 transfected MSCs would help with improving their bone formation. This chapter is therefore important as it measures the baseline osteopenia, as a result of OVX, before intervention with stem cells.

Chapter 3. **The Influence of Age and Osteoporosis on Rat Bone Marrow Stem Cells.**

3.1. Introduction

Osteoporosis causes over 8.9 million fractures annually worldwide, characterised by loss of trabecular architecture and bone mass, the disease has a 40% lifetime risk for sustaining a fragility fracture (hip, distal radius and vertebral (Raisz, 2005, Randell et al., 1995, Sambrook and Cooper, 2006)). Type I osteoporosis, also known as postmenopausal osteoporosis, is characterised by increased bone turnover and accelerated cancellous bone loss, resulting in an increased risk of vertebral fractures. Type II osteoporosis affects both men and women and leads to an increased incidence of fractures; mortality and morbidity are thus increased accounting for nearly 3 million disability adjusted life years. Regardless of type, the underlying pathology of osteoporosis is aberrant bone turnover, secondary to imbalanced bone resorption and formation (Bonyadi et al., 2003). Osteoporosis can impact on a number of conditions where bone formation is required. These conditions are often challenging and therefore new techniques involving gene therapy, cell therapy and tissue engineering are being explored to improve bone regeneration (Chen et al., 2012).

Bone loss in osteoporosis is a consequence of the imbalance between bone formation by the osteoblast and bone resorption carried out by the osteoclast, resulting in net bone loss (Sandhu and Hampson, 2011). In osteoporotic patients, regeneration of damage bone is impaired as a consequence of this imbalance. There may be a number of reasons for this associated with: a) The number of MSCs within bone marrow, which decrease with age and this may be associated with a reduction in bone formation (Lane, 2006, Lippuner, 2012, Chen et al., 2007, Katsara et al., 2011) b) The ability of MSCs in osteoporotic patients to differentiate into osteoprogenitor cells and mature osteoblasts (Sethe et al., 2006, Brack and Rando, 2007, Muschler et al., 2001, Wang et al., 2006). c) The ability of MSCs to mobilise from their niche, home across the tissue endothelium and mature into active cell types that modulate the

fracture environment. The stromal derived factor-1/CXCR4 (SDF-1/CXCR4) axis has been found to be an important regulator of stem cell migration. SDF-1 is produced by a multitude of tissue types including stromal cells in the endosteum. In its active form SDF-1 is bound to the CXCR4 receptor found on MSCs. Amongst others Granero-Molto et al demonstrated that stem cell migration to the fracture site in a stabilised tibial osteotomy model was CXCR4 dependent. The over-expression of CXCR4 on mesenchymal stem cells led to significant increases in bone mineral density in an osteopenic mouse model indicating the clinical significance of the SDF-1/CXCR4 axis in the treatment of osteoporosis. One of the reasons for this may be that MSCs derived from osteoporotic patients may have reduced levels of CXCR4, however the role of aging and osteoporosis on CXCR4 cell expression and thus migration to SDF-1 has not been investigated (Granero-Molto et al., 2009).

Osteoporosis and ageing may be interlinked, and differences between stem cells from young and old patients have been reported. It is therefore important to compare the biological differences in stem cells from young, adult as well as osteoporotic patients. As yet studies have not compared the functional differences between MSCs from these three groups.

The aim of this chapter was establish an OVX model and examine the influence of age and osteopenia on the morphology, proliferation, differentiation, CXCR4 expression and migration of bone marrow derived MSCs. Results from OVX and non-OVX rats were compared with MSCs obtained from young rats.

The hypothesis was that MSCs from OVX rats have a lower proliferative and osteogenic potential, a lower CXCR4 expression and migratory capacity and will be more likely to differentiate into adipocytes compared to MSCs obtained from young rats.

3.2. Materials and Methods

3.2.1 Culture of Bone Marrow stem cells (Young Rats)

Rat bone marrow mesenchymal stem cells (rBMCs) were harvested from 2-4 week old young Wistar rat femora (n=6). Bone marrow cells were harvested by flushing the femora with Dulbecco's modified Eagle medium (DMEM), 20% fetal calf serum, 1% Penicillin Streptomycin (P/S) in a 25 cm^2 flask. The aspirate obtained from the flushing was directly plated and cultured. Cells were cultured at 37°C at 5% CO_2. Media was changed after 4 days to remove non-adherent cells and then continuously refreshed twice a week thereafter. After 10-14 days of primary culture and when the cells were 70-80% confluent, they were passaged using Trypsin-EDTA (Sigma-Aldrich, MO). rBMCs were passaged every 7-8 days. MSCs were characterised by differentiating them into osteoblasts, adipocytes and chondrocytes and were stained using Alizarin Red (Sigma Aldrich, UK), Oil Red O (Sigma Aldrich, UK) and Alcian Blue (Sigma Aldrich, UK) respectively. They were also phenotypically identified by immunocytochemistry using Anti-CD106, Anti-CD105, Anti-CD45 and Anti-CD34 markers, purchased from Abcam (Cambridge, UK).

3.2.2 Flow Cytometry Analysis (CD Marker Expression)

10,000 rBMCs from young, adult control and OVX rats (n = 3) were analysed for their surface expression of CD29, CD90, CD45 and CD34. The cells were labelled with Anti-mouse/Rat CD29-FITC (eBioscience), anti-mouse/Rat CD90-APC (eBioscience), Anti-Rat CD45-APC (eBioscience) and CD34-PE (Abcam). The CD expression was compared to the isotype control. 10,000 cells were fixed in 4% formalin for 15 minutes at room temperature, washed with 0.5% Bovine serum albumin (BSA), stained with the conjugated primary antibody for 1 hour at room temperature in the dark. After 1 hour the cells were washed with 0.5% BSA and analysed on flow cytometer (Cytoflex, Beckman Coulter) (Cell Signalling Technology).

As explained in the introduction, from a vast selection of CD markers used to characterise MSCs, CD29, CD90, CD45 and CD34 were chosen because they are expressed on MSCs irrespective of the cell passage and are commonly used to identify rat MSCs (Harting et al., 2008, Dominici et al., 2006, Karaoz et al., 2009).

3.2.3 Cell Morphology

Passage 2 and 3 rBMCs were cultured and their morphology assessed by measuring their 'Aspect Ratio', whereby the ratio of the length of a cell to its width was calculated using Image J software (n=3). This was calculated for cells from young, adult and OVX rats.

3.2.4 Cell Proliferation

An Alamar Blue assay (AbD Serotec, UK) was used to measure rBMC proliferation from young, adult and OVX rats (n = 3). 10000 MSCs were seeded in a 6 well plate and metabolic activity was measured using an Alamar Blue assay (AbD Serotec, UK) at day 3, 7, 10 and 14. 10% Alamar blue was added to the culture medium for 4 hours and excitation at 560 nm and emission at 590 nm were measured using a Tecan plate reader (Tecan, Infinite Pro 200 series, Switzerland). The mean absorbance was determined from triplicate samples. The absorbance was then normalised to the DNA assays and a comparison was made between the cell groups (Czekanska, 2011).

3.2.5 Osteogenic Differentiation

30,000 MSCs (n = 3) from each of the three experimental groups were cultured in a 48 well plate in osteogenic media that consisted of DMEM supplemented with 100nM dexamethasone, 50ug/ml L-ascorbic acid 2-phosphate and 10mM beta-glycerol phosphate (Pittenger et al., 1999, Jaiswal et al., 1997). The cells were grown at 37°C at 5% CO_2 and their Alkaline Phosphatase (ALP) activity was measured at 3, 7, 14 and 21 days. ALP was measured by freezing and thawing the cells three times, centrifuging and incubating the cell lysate in equal volumes of the ALP substrate

solution (*p*-Nitrophenyl Phosphate Liquid Substrate System, Sigma Aldrich, UK*)* for 15 minutes at 37 degrees on a shaker. This was then normalised against the DNA Hoescht assay (Sigma Aldrich, UK). The amount of DNA in samples was quantified using a fluorimetric dye. 2ng/ml Hoechst 33258 (Sigma 14530) was added to the cell lysates and fluorescence was measured at a wavelength of 460 nm (Moe et al., 1994). Additionally, calcium phosphate deposition was measured by quantification of Alizarin Red staining using cetylpyridium chloride (CPC). The cells were washed in phosphate buffered saline (PBS), fixed in 10% formalin for 15 minutes and then stained with Alizarin Red solution (pH 4.2) for 10 minutes at room temperature. The cultures were then rinsed five times with PBS (Stanford et al., 1995). The stained samples were photographed and then quantitatively de-stained using 10% CPC made in 10mM sodium phosphate, pH 7.0 for 15 minutes at room temperature. The samples were de-stained while being shaken. The Alizarin Red concentration was then read on a plate reader at a wavelength of 570 nm (Tecan, Infinite Pro 200 Series, Switzerland). The concentration of Alizarin Red was determined using a standard curve obtained from 10-fold serial dilution of Alizarin Red. The Alizarin Red analysis was repeated for cells seeded in normal media with FCS and P/S, to validate the calcium phosphate production of cells seeded in osteogenic media (Stiehler et al., 2008, Cotter et al., 2011).

3.2.6 Adipogenic Differentiation

30,000 cells from young, adult and OVX rats were cultured in a 48 well plate in adipogenic media that consisted of DMEM supplemented with 0.1mM dexamethasone, 50mM Indomethacin, 0.45mM IBMX and 10mg/ml Insulin. The cells were grown at 37°C at 5% CO_2 and the presence of lipid droplets within the cells confirmed by staining with Oil Red O at 7, 14 and 21 days (Jaiswal et al., 1997, Pittenger et al., 1999). Additionally, Oil Red O staining was quantified using 100% isopropanol. The cells were washed in PBS, fixed in paraformaldehyde for 5 minutes,

washed with 60% isopropanol for 10 minutes and then stained with Oil Red O solution for 15 minutes at room temperature. The cultures were then rinsed five times with PBS. The stained samples were photographed and then quantitatively de-stained using 100% isopropanol for 15 minutes at room temperature. The Oil Red O concentration was read on a plate reader at a wavelength of 510 nm (Tecan, Infinite Pro 200 Series, Switzerland). The concentration of Oil Red O was determined by using a standard curve obtained from a 10-fold serial dilution of Oil Red O stain. In order to compare the adipogenic differentiation of these cells this was repeated for cells seeded in normal media with FCS and P/S (Cotter et al., 2011).

3.2.7 Chondrogenic Differentiation

To promote chondrogenic differentiation, mesenchymal cells were centrifuged to form a pelleted mass and the cells were cultured without 2% serum. Chondrogenic differentiation was induced in the expanded mesenchymal cell cultures by treatment with 1x Insulin-Transferrin-Selenium (ITS) (ThermoFisher Scientific, UK), 40ug/ml Proline (Sigma Aldrich, UK), 100nM Dexamethasone (Sigma Aldrich, UK), 100um Ascorbate-2-phosphate (Sigma Aldrich, UK) and 10ng/ml TGF-β1 (Peprotech, UK). Positive chondrogenic differentiation was established using Alcian Blue staining. The pellets were washed with PBS (Sigma Aldrich, UK), fixed for 1 hour in cold methanol (Sigma Aldrich, UK), and stained overnight in 1% Alcian Blue solution (Sigma Aldrich, UK). The pellets were then washed extensively with PBS and pictures of the stained pellets were obtained (Jaiswal et al., 1997, Pittenger et al., 1999).

3.2.8 Flow Cytometry Analysis (CXCR4 Expression)

Young rBMCs (n = 5) from the third passage were trypsinised and centrifuged at 2000 rpm for 10 minutes before being re-suspended at 100,000 cells in PBS. Cell aliquots were incubated with primary CXCR4 antibody (Abcam, UK) for 30 minutes at room temperature. The cells were washed in PBS and then incubated in secondary goat anti-rabbit antibody (Abcam, Cambridge UK) for 30 minutes at room temperature. The

negative control consisted of cells incubated in the secondary antibody only. 10,000 cells were then analysed using flow cytometry (Cytoflex, Beckman Coulter, UK). This was repeated with MSCs from OVX and adult rats (n = 5) (Cell Signalling Technology).

3.2.9 Cell Migration

A chemoinvasion assay was used to evaluate the ability of rBMCs from young, adult and OVX rats, to migrate towards SDF-1. rBMCs from the three groups were loaded separately in serum free medium in the upper compartment of the Boyden chamber, which had a membrane with a 5µm pore size that separated it from the lower compartment (Corning, UK). The lower compartment was filled with 100ng/ml SDF-1 (Peprotech, UK) in DMEM, FCS and P/S. The chambers were incubated at 37°C, 5% CO_2 for 16 hours to allow for cell migration through the chamber. After 16 hours, the cells that migrated to the opposite side of the membrane were fixed in 10% formaldehyde (Sigma Aldrich, UK) and stained with Crystal violet (Sigma Aldrich, UK). The migrated cells were counted by selecting six random fields at x20 magnification and calculating the percentage average number of cells. For each cell type (young, adult and OVX), the experiment was repeated three times. For the control, both the top and bottom of the chamber were filled with normal media with no SDF-1 and the cells were loaded in the upper chamber as before (Chen, 2005, Shaw, 2005).

3.2.10 Statistical Analysis

The normality was checked using Kolmogorov- Smirnov and Shapiro Wilkinson test and where the data was normal, comparison was made using independent student T-test. Where the data was non-parametric comparison was made using Mann-Whitney U test with a Bonferroni correction. All data was analysed using SPSS version 24 (Chicago, USA).

3.3 Results

3.3.1 Flow Cytometry Analysis (CD Marker Expression)

There was no significant difference in the percentage of cells expressing the different CD markers when young, adult control and OVX rBMCs were compared. Flow cytometry showed high expression of CD29, CD90 and low expression of CD34 and CD45 in all three groups. CD marker expression was similar in all three groups (Table 3.1, Figure 3.1).

Table 3.1: Percentage CD marker expression of cells from the 3 different type of rats. Data is represented as mean ± Standard deviation.

Type of cells				
	CD29	**CD90**	**CD34**	**CD45**
Young MSCs	98 ±1.9	96.8±5.0	1.4±1.2	7.03±4.8
Adult MSCs	95.6±3.6	98.6±0.7	3.2±4.1	21.9±20.8
OVX MSCs	91.7±8.9	94.4±4.3	3.3±4.4	3.9±5.8

3.3.2 Cell morphology

MSCs from young rats at passage 2 and 3 were smaller and had more spindle-like features (longer) compared to MSCs from adult control and OVX rats. Although the MSCs from the older rats still had spindle like features, they were more spread out and this became more evident with increasing passaging (**Figure 3.2**). The mean aspect ratio measured in the young MSC group was 18.66±13.45, which was larger

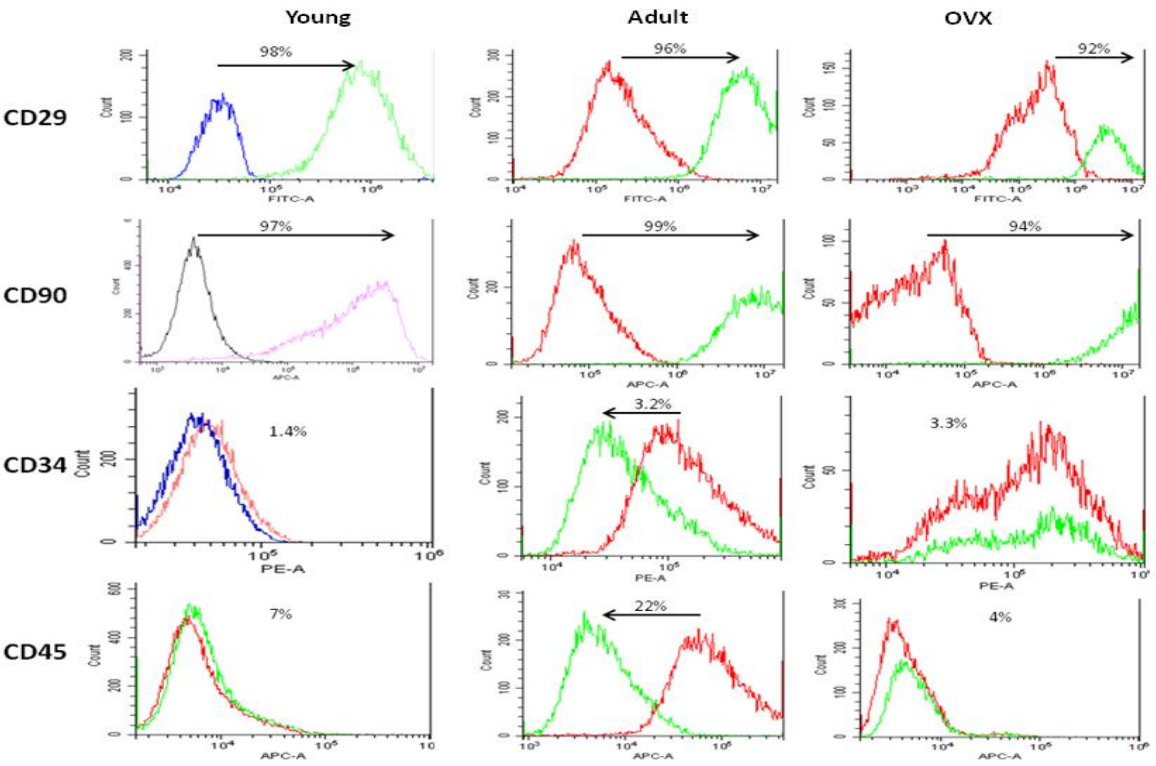

Figure 3.1: CD29, CD90, CD45 and CD34 expression of young (top), adult control (centre) and OVX (bottom) MSCs. MSCs from all three groups of rats had high expression of CD90 and CD29 and all low levels of CD45 and CD34 (n=3).

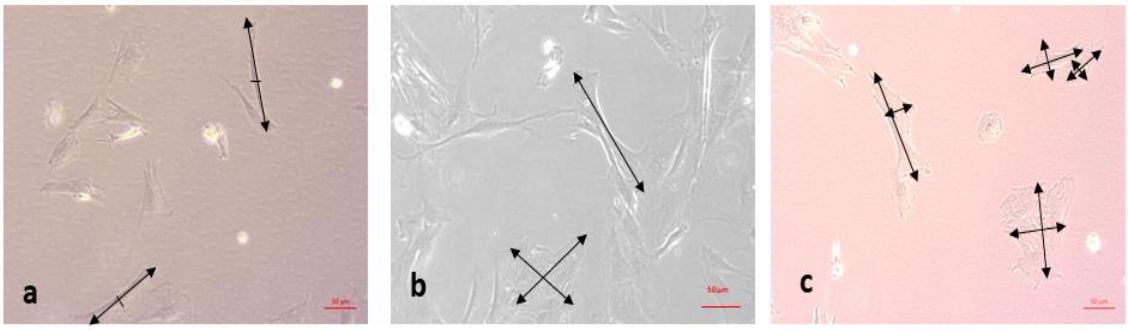

Figure 3.2: rBMCs from young (A), adult control (B) and OVX rats (C) x10 magnification. The young MSCs were longer and more spindle like shaped, the adult MSCs and OVX MSCs were wider (as demonstrated by the arrows). The young MSCs had the largest aspect ratio (length: width ratio) compared to cells from adult control and OVX rats.

when compared to cells obtained from adult (4.99±4.67, p=0.06) and this was significant when compared to MSC obtained from OVX rats (2.25±0.94, p=0.03).

3.3.3 Tri-differentiation

The isolated young rat MSCs positively differentiated to osteoblasts, adipocytes and chondrocytes. Osteogenic differentiation was evident by calcium deposits which stained red with Alizarin Red. Under adipogenic conditions for 3 weeks, the formation of intracellular micro-droplets stained positive for Oil Red O. After 21 days in chondrogenic media, the cells were positively differentiated to chondrocytes, which was confirmed by Alcian Blue staining for cartilage (Figure 3.3).

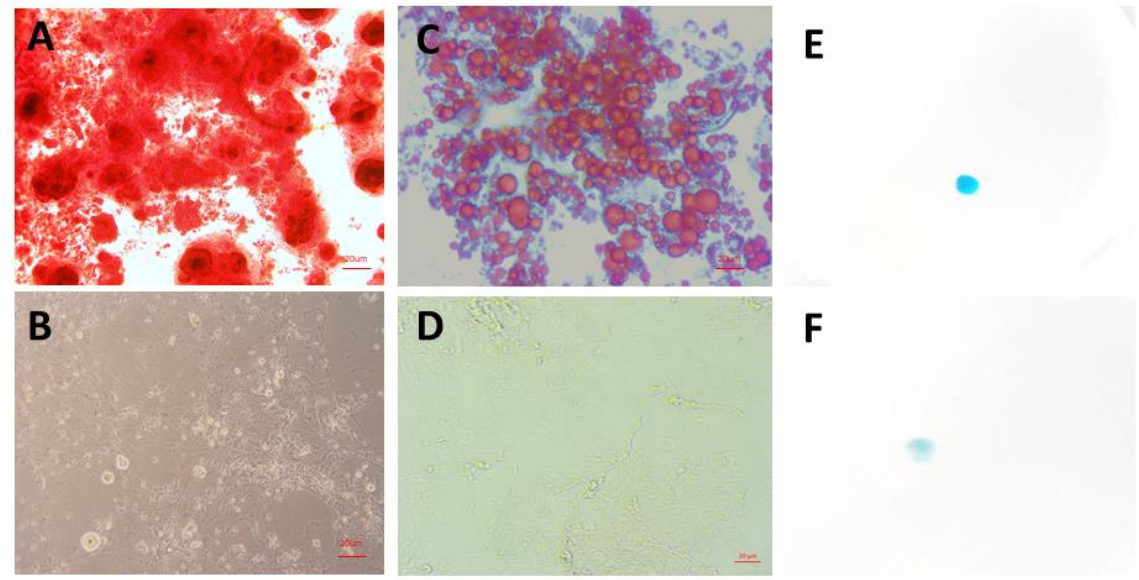

Figure 3.3: Characterisation of bone marrow MSCs from young rats using tri-differentiation after 21 days. Red positive stain for Alizarin red for calcium phosphate deposition after osteogenic differentiation (A), Red Oil Red O stain for fat droplets after adipogenic differentiation (B) and bright blue Alcian Blue stain to show chondrogenic differentiation of the pellet (C). Their control counterparts are shown by negative staining (B, D and F respectively).

3.3.4 Cell Proliferation

Histograms of mean absorbance were plotted to examine cell proliferation. After 3 days in culture, the rBMCs showed time dependent growth in all samples until day 7. Overall there was no significant difference in proliferation between rBMCs from

young, adult control and OVX rats. An increase in cell proliferation was seen in all groups between days 3 and 7 (Figure 3.4).

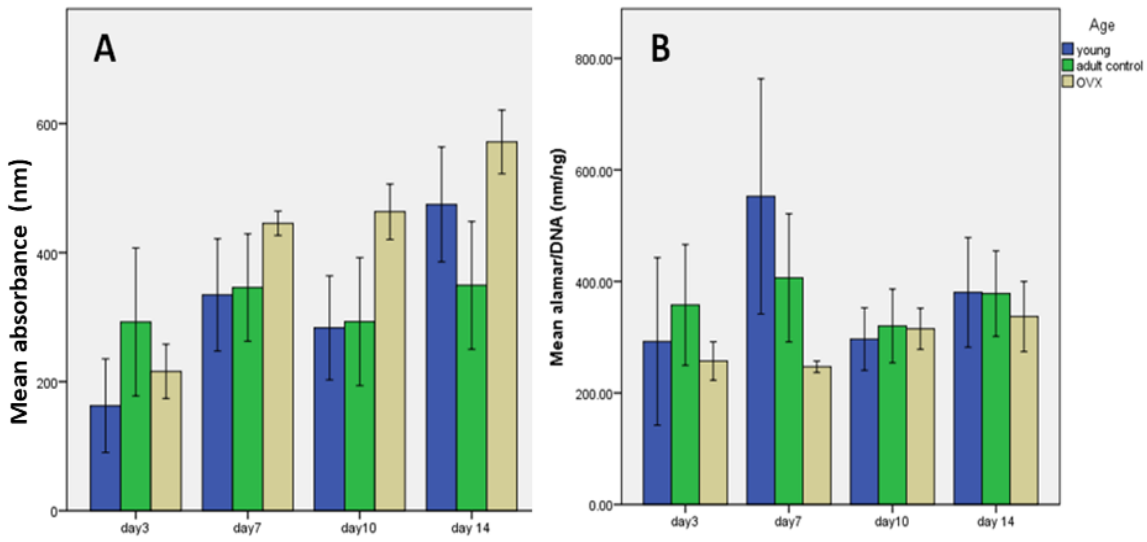

Figure 3.4: Proliferation of MSCs from young, adult control and OVX rats at Day 3, 7, 10 and 14 (n = 3) as measured by the reduction of Alamar Blue and corresponding absorbance (A) and mean absorbance readings normalised against DNA to reflect their metabolic activity (B).

3.3.5 Osteogenic Differentiation

The amount of Alizarin Red staining (measured in mM) indicated the production of calcium phosphate mineral. Mineralisation of the extracellular matrix in young, adult and OVX rBMCs increased significantly from day 7 to day 21 in all the three experimental groups. These results were compared to mineralisation in the control group, which received normal media and where no calcium phosphate was seen (Figure 3.5, Table 3.2). At day 7, the amount of calcium phosphate produced was relatively low and was similar between the three groups of cells. At day 14 and day 21 there was a significant increase in the amount of calcium phosphate produced by all groups. At day 14 MSCs from young rats produced nearly three times the amount of mineral compared to cells isolated from ovariectomised animals (p=0.004). This significant difference in mineralisation was also apparent at 21 days (p = 0.0018) but the difference was less.

Table 3.2: Quantification of Alizarin Red staining measured in nM indicating osteogenic differentiation of young, adult and OVX MSCs at day 7, 14 and 21 for cells differentiated to osteoblasts as well as control cells grown in normal media.

	Young MSCs			Adult MSCs			OVX MSCs		
	Day7	Day14	Day21	Day7	Day14	Day21	Day7	Day14	Day21
Osteo (mM)	2.7±1.7	4.6±1.4	5.4±0.9	1.2±0.6	4.0±0.4	4.9±0.6	1.1±0.8	1.7±1.5	3.6±0.4
Control (mM)	0.2±0.2	0.1±0.1	0.1±0.1	0±0	0.1±0.2	0±0.3	0±0	0±0	0.03±0.1

Results showed that when MSCs from young, adult and OVX rats were differentiated to osteoblasts, a large variability in ALP expression was seen between days 3 and 21 in all 3 groups of cells. In the young group, the percentage ALP change decreased from day 3 to day 7 and increased thereafter up to day 14, after which it dropped at day 21. However, this pattern was not observed in the adult control and OVX groups. A peak increase in ALP expression was seen in the OVX MSC groups on day 7, which later decreased at day 14. There was a significant difference in ALP change between young and OVX MSCs at day 14 ($p = 0.003$). However due to a large variability seen in data obtained from the adult MSC group, this significance was not observed at day 14 when the young group was compared with the adult MSC group and also when the adult group was compared with the OVX MSC group.

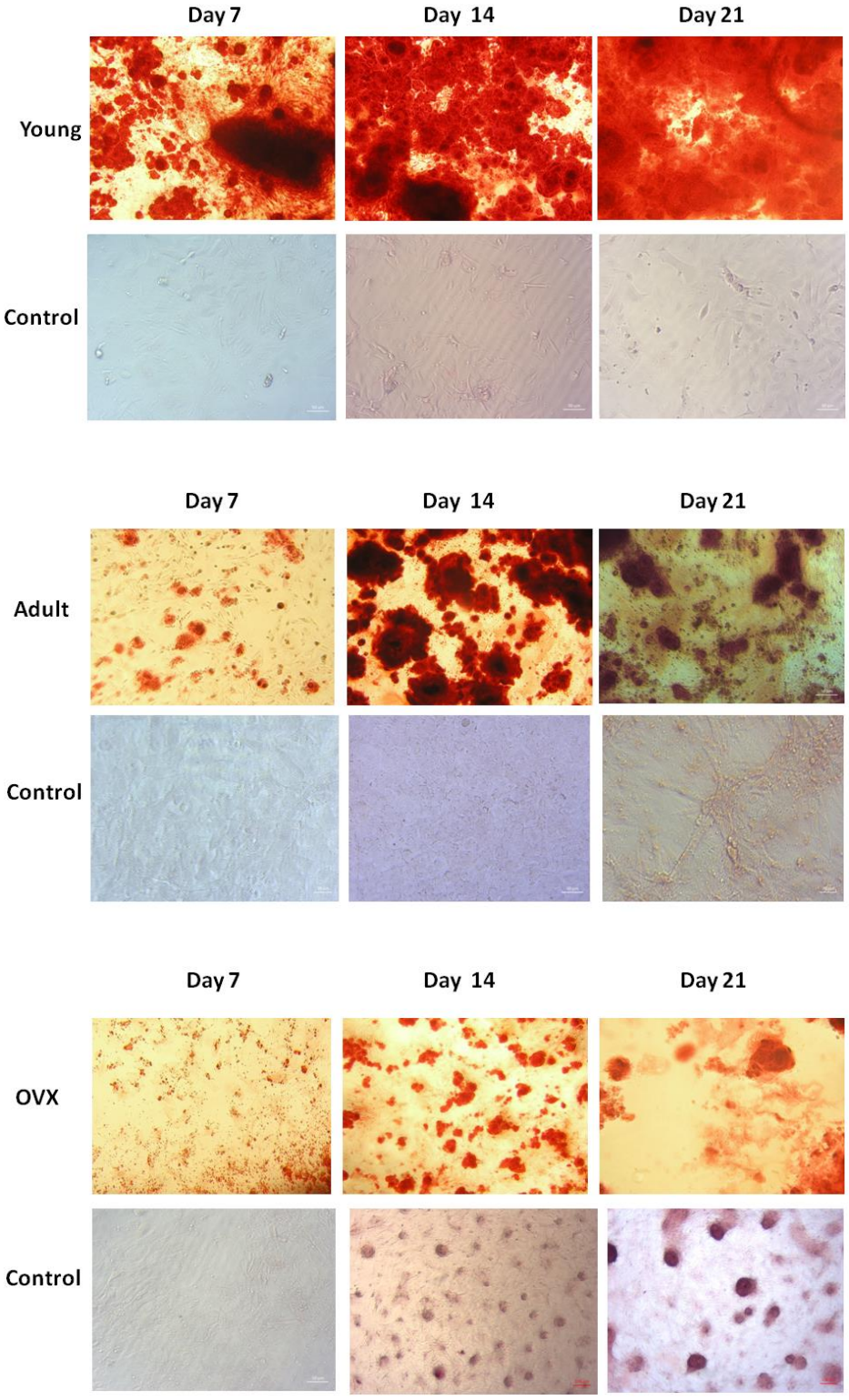

Figure 3.5: Alizarin staining of calcium phosphate deposition following osteogenic differentiation of Young, adult control and OVX MSCs at day 7, 14 and 21. The young MSCs had significantly more calcium phosphate deposition at day 7, 14 and 21 compared to MSCs from adult and OVX MSCs.

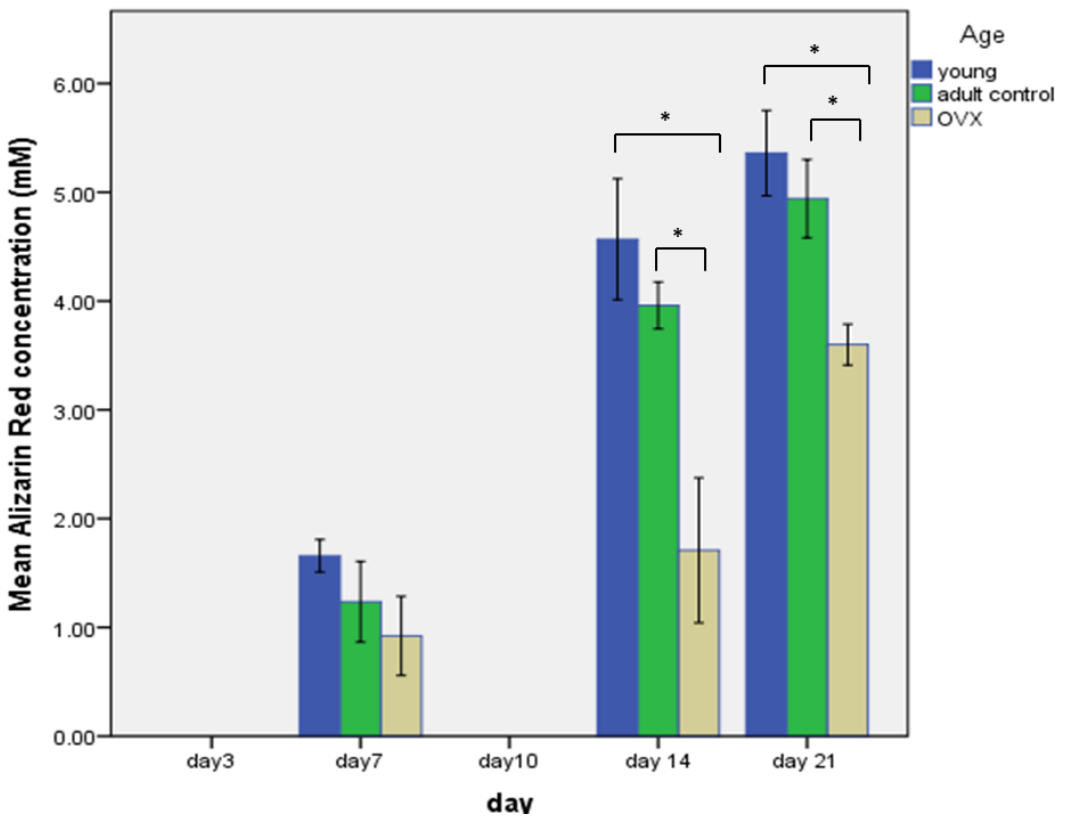

Figure 3.6: Graph showing the average Alizarin Red production when MSCs from young, adult control and OVX rats (n=3) differentiated to osteoblasts, at day 7, 14 and 21. (* and ** shows significant differences p<0.05).

It is worth noting that two of the adult MSC cultures at day 14 showed very low expression whereas in one adult culture the ALP expression was very high. Interestingly, ALP expression in the young stem cell cultures was less variable over all the time periods investigated. There was a percentage increase in ALP expression in OVX MSCs at day 7 (37.5 ± 43.5%), and this was significantly more than young MSCs ($p = 0.02$). However, this surge in ALP was still not as high as that expressed by young MSCs at day 14 (123 ± 21.9%).

The average ALP values for the young MSCs (9.52±6.97 to 3.20±1.74 U/ng) and adult MSCs (16.83±14.73 U/ng to 13.13±14.65 U/ng) decreased from day 3 to day 7 and increased at day 14 (7.34±4.3 U/ng and 18.15±20.91 U/ng) respectively. However, in the OVX MSC group, the average ALP readings increased at day 7 (1.64 ± 1.96 U/ng)

and then decreased at day 14 (1.50 ± 1.49 U/ng). At day 14, the young MSCs had significantly higher ALP readings compared to OVX MSCs (p = 0.04). However, although the young MSCs had a higher average ALP reading at day 14 compared to the adult MSCs, this difference was insignificant due to large variability in the readings (Figure 3.7).

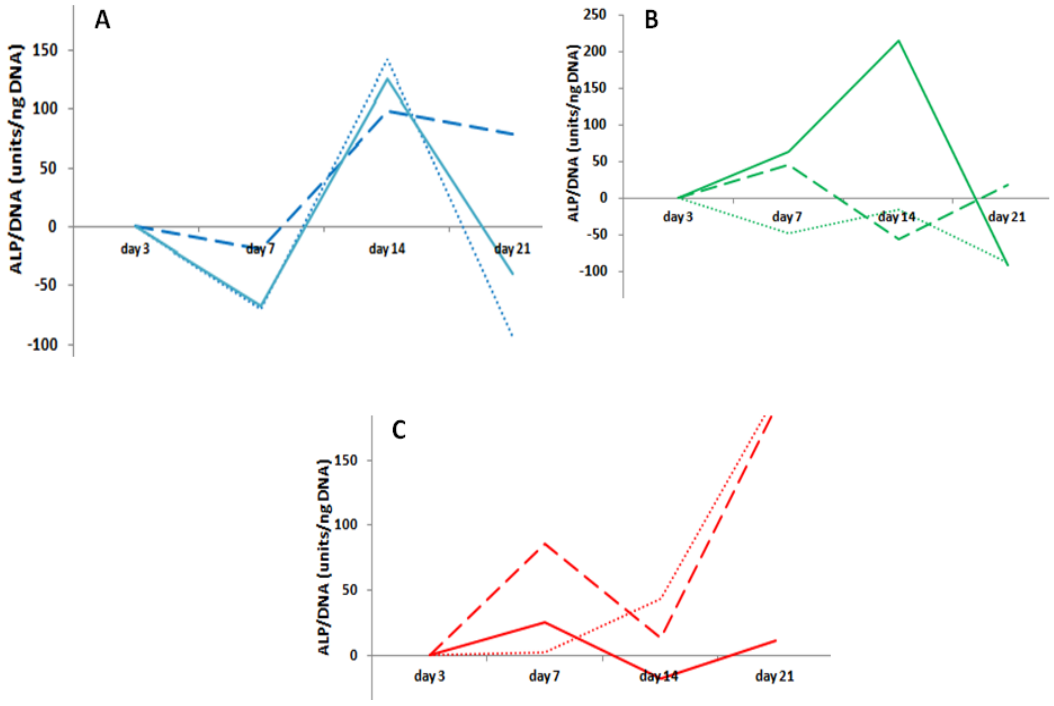

Figure 3.7: Percentage ALP/DNA change for young (a), adult (b) and OVX (c) MSCs differentiated to osteoblasts at day 3, 7, 14 and 21. Each line represents the results from a single culture.

3.3.6 Adipogenic Differentiation

At days 14 and 21, adipogenic differentiation was significantly greater in MSCs isolated from young animals compared to cells isolated from adult control and OVX animals (Figure 3.8). MSCs from young rats formed lipid droplets significantly faster from day 7 compared to the other two groups of cells.

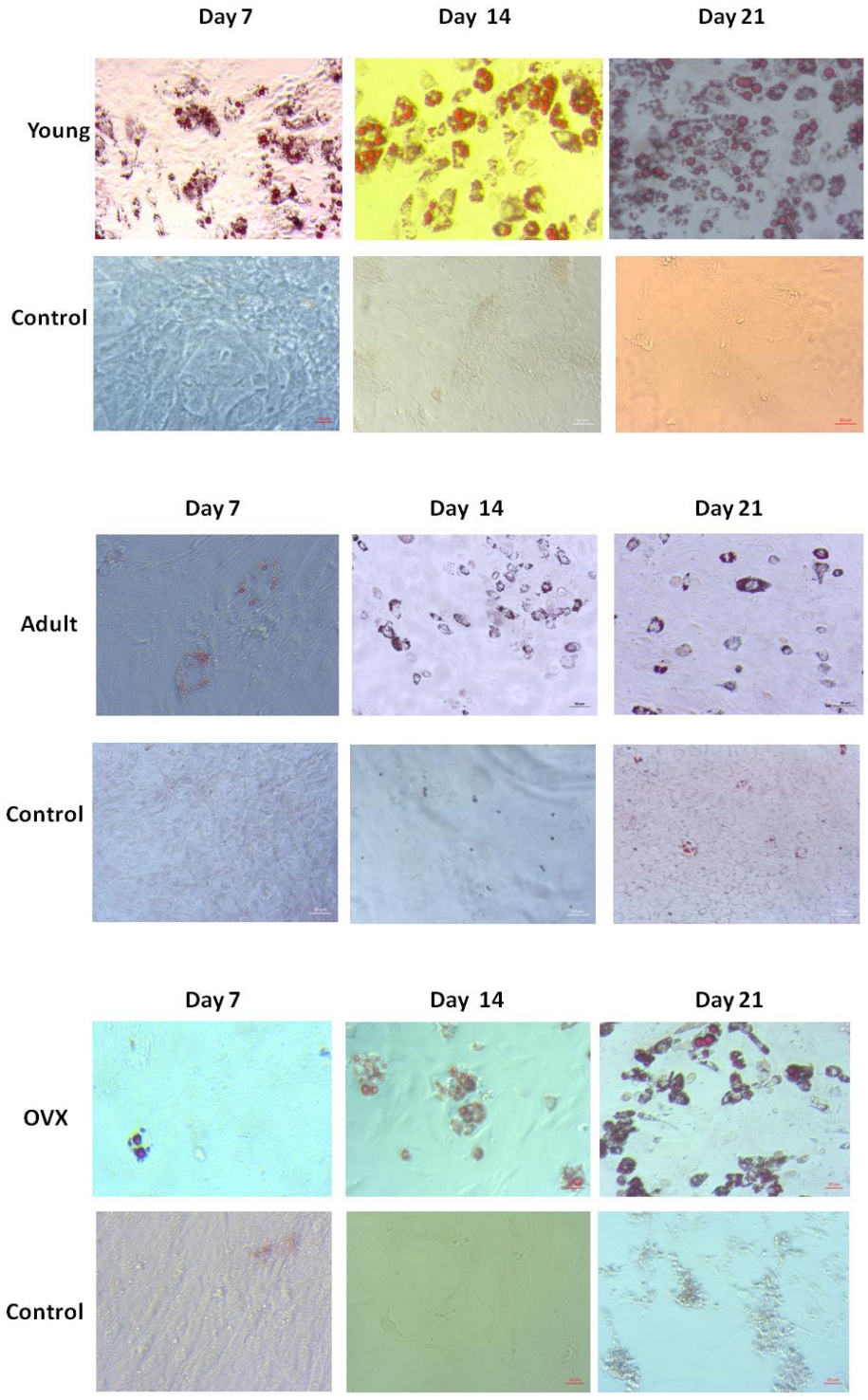

Figure 3.8: Oil Red O staining following osteogenic differentiation of Young, adult control and OVX MSCs at day 7, 14 and 21. These images demonstrated that by day 7, the young MSCs had differentiated to adipocytes significantly more compared to MSCs from OVX and adult control rats.

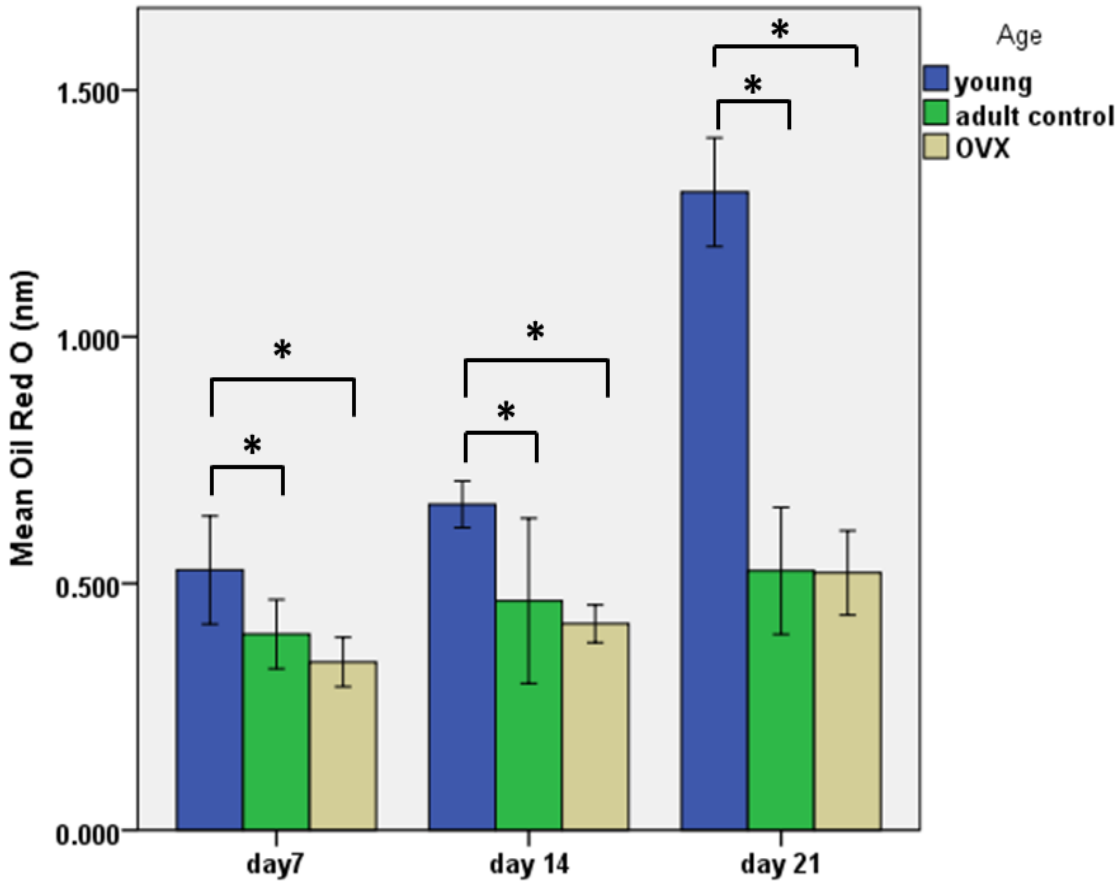

Figure 3.9: Adipogenic differentiation of young, adult and OVX MSCs at day 7, 14 and 21 measured using Oil Red O stain of fat droplets. MSCs from young rats had significantly higher lipid droplet accumulation compared to MSCs from adult control and OVX rats at days 7, 14 and 21. No differences in adipogenic differentiation between MSCs from OVX and adult control rats (* and ** shows significant differences p<0.05).

Additionally, lipid droplet accumulation significantly accelerated from day 14 to day 21 when young MSCs were differentiated while MSCs from adult and OVX rats showed no increase in lipid accumulation at day 7, 14 and 21. Fat droplet production was also assessed in the control group, which were seeded in normal media. On average all three groups of MSCs had minute amounts of fat droplet production in the control media (Figure 3.9, Table 3.3).

Table 3.3: Values of adipogenic differentiation (nm) of young, adult and OVX MSCs at day 7, 14 and 21 for cells differentiated to adipocytes as well as control cells with normal media.

	Young MSCs			Adult MSCs			OVX MSCs		
	Day 7	Day14	Day21	Day7	Day14	Day21	Day7	Day14	Day21
Adipo (nm)	2.7±1.7	4.6±1.4	5.4±0.9	1.2±0.6	4.0±0.4	4.9±0.6	1.1±0.8	1.7±1.5	3.6±0.4
Control (nm)	0.3±0.1	0.4±0.1	0.3±0.1	0.4±0.1	0.4±0.1	0.4±0.02	0.3±0.1	0.5±0.03	0.4±0.04

3.3.7 Flow Cytometry Analysis (CXCR4 Expression)

Flow cytometry results demonstrated that rBMCs from adult ($p < 0.001$) and OVX rats ($p<0.001$) expressed significantly lower levels of CXCR4 compared to rBMCs from young rats (Figure 3.10). rBMCs isolated from adult rats expressed a significantly greater amount of CXCR4 in comparison to cells obtained from OVX rats ($p= 0.04$). On average, 32.1±6.2% and 19.4±9.8% of the rBMCS from adult and OVX rats respectively expressed CXCR4, while 87.5±5.1% of the young rBMCs expressed CXCR4.

3.3.8 Cell Migration

Results demonstrated that significantly lower numbers of OVX MSCs migrated towards SDF-1 compared to the adult and young rat groups. The migration to SDF-1 was 2-fold greater in young cells compared to adult rat cells ($p = 0.023$) and was four times higher when compared to cells isolated from OVX rats ($p = 0.013$) (Figure 3.11).

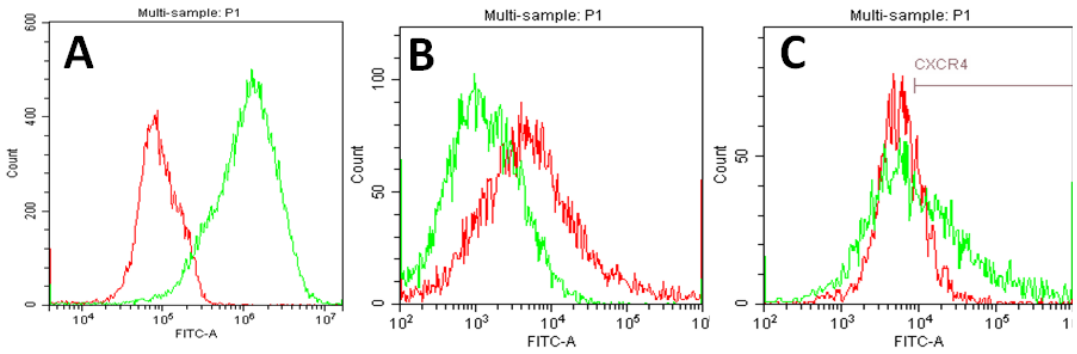

Figure 3.10: Flow cytometry showing CXCR4 surface expression in MSCs from young (A), adult control (B) and OVX rats (C). Young MSCs have the highest expression of CXCR4 (87%), followed by MSCs from adult control (32%) and MSCs from OVX rats (19%) had the lowest expression of CXCR4 (n = 3).

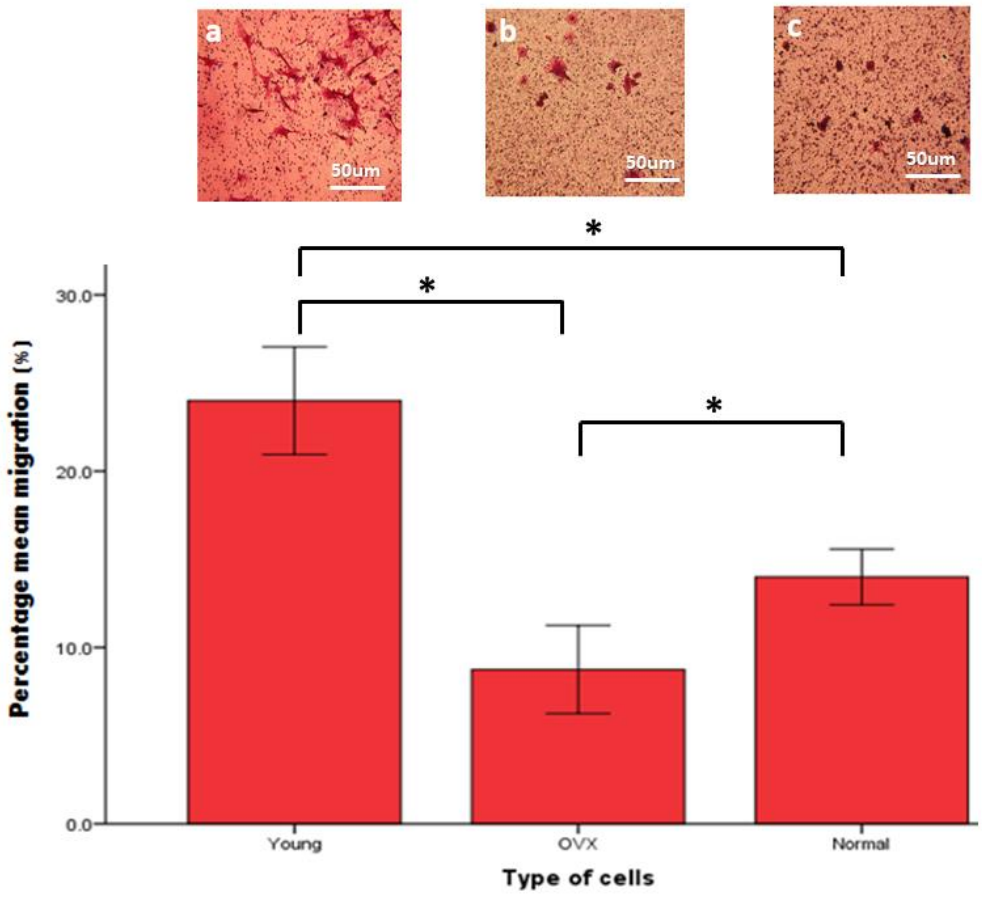

Figure 3.11: Mean percentage migration of uninfected MSCs from OVX, adult control and young rats in a Transwell chamber towards SDF1. * and ** represent significance p<0.05. The images of young (A), adult (B) and OVX (c) MSCs migrated towards SDF-1 in a Boyden chamber and stained with Crystal Violet stain. Scale:

3.4 Discussion

Impaired MSC recruitment from the stem cell niche, reduced differentiation to bone or decreased proliferative activity of mature stem cells are all possible causes of reduced bone formation (Yellowley, 2013a). The results obtained in this chapter have highlighted that during ageing and osteoporosis, the proliferative potential of the stem cells is maintained but their differentiation ability is compromised. Many studies have investigated differences in stem cell populations obtained from old and young animals however, the novelty in this study includes comparing stem cell function between young, normal adult and OVX rats. Results from this chapter have demonstrated that there are differences between MSCs isolated from young, adult and OVX rats. Although all cells positively expressed CD90 and CD29 and negatively expressed CD45 and CD34, they showed significant differences in their osteogenic and adipogenic differentiation potential. Further I showed that expression of CXCR4 and the migration of cells towards SDF-1 was much greater in the young stem cell group when compared with older adult cells. Cells isolated from OVX rats showed a reduction in migration compared with adult cells. It is interesting to note that CXCR4 expression of MSCs from OVX and adult rats was significantly lower than those obtained from young rats hence impairing their migration. CXCR4 expression was measured using flow cytometry and although it is quantitative, it measures the number of cells expressing CXCR4 rather than the amount expressed by each individual cell. Individual CXCR4 cell expression can be measured using ELISA or immunostaining assays and when combined with migration over a known period of time, the rate of migration of each of the cell types can be quantified and compared.

The capability of cells to differentiate to bone and their use in cell based therapy is vital in many orthopaedic treatments such as osteoporosis and osteoarthritis, especially in the ageing population. There is contradictory data on the differentiation potential of MSCs from young and aged rats. In this chapter I hypothesized that MSCs

obtained from OVX rats would have a lower osteogenic potential and would more likely differentiate to adipocytes when compared to MSCs isolated from young rats however, this was not the case. MSCs from OVX rats had a lower osteogenic differentiation potential but they also had a lower adipogenic differentiation ability compared to MSCs obtained from young rats. Asumda and Chase did not look at the differentiation potential of MSCs from osteopenic rats, but they showed similar differences to this study in terms of osteogenic and adipogenic differentiation between MSCs from young and old rats. They demonstrated a reduced differentiation ability in cells from old rats compared to MSCs from young rats (Asumda and Chase, 2011). However Singh et al. (2016) found no observable difference in osteogenic and adipogenic differentiation between cells from young and old mice (Singh et al., 2016). Likewise, Beane et al (2014) showed no differences in Alkaline Phosphatase expression as well as Alizarin Red staining between bone marrow derived MSCs from young and old rabbits but they found that age affected the adipogenic differentiation of the same cells (Beane et al., 2014). Moerman reported changes in the expression of phenotype specific gene markers in bone marrow mesenchymal stem cells from older mice that encouraged differentiation to fat rather than bone in the bone marrow niche. Bone marrow from old mice had increased levels of PPAR-γ, which stimulated cells to turn to fat rather than bone and inhibited osteoblast function. Bone marrow obtained from young mice on the other hand, had increased levels of BMP-2 and TGF-β, which induced MSCs towards the osteoblastic lineage (Moerman et al., 2004a). My results demonstrated that differentiation is associated with the origin of the cells and that this is independent of the cytokine profile in their environment.

In this study, no significant difference in proliferation was seen in the different types of MSCs investigated. This is in contrast to several studies that have shown that MSCs from older rats have a significantly lower proliferation rate compared to MSCs isolated from young rats (Asumda and Chase, 2011, Kretlow et al., 2008). Beane and

co-workers (2014) investigated MSCs from young and old rabbits from the bone marrow, muscle (MDSC) and fat (ADMSCs) and demonstrated a relative reduction in proliferation with age of MSCs from the bone marrow but not from the other two sources (Beane et al., 2014). Georgen (2014) found that MSCs from OVX rats had a lower proliferation rate than their control counterparts and therefore concluded that the low proliferation rate would correlate with reduced self-renewal capacity which might cause a gradual depletion of MSC sources in the bone marrow of OVX animals (Goergen et al., 2013). In addition, variability between same species of animals is another important factor to consider. Stem cells from every individual would be different and this variability could possibly have affected the significance of ALP expression between MSCs from young, adult control and OVX rats. To overcome this a large number of rats would have to be used within each group.

This study showed that MSCs from OVX rats have a lower *in vitro* migration and CXCR4 expression compared to MSCs from young rats and the adult rats. SDF-1 is a chemokine receptor for CXCR4 and the SDF-1/CXCR4 biological axis plays an important role in the migration of stem cells and the wound repair of tissues and organs (Molyneaux et al., 2003, Toupadakis et al., 2012, Shao et al., 2011, Lien et al., 2009b, Wynn et al., 2004a). The impaired migration capacity of MSCs from 4-month post-ovariectomy rats could be due to their low expression of CXCR4 and may explain the impaired bone formation seen in osteoporotic patients as cells from these patients have a reduced capacity to migrate to the site of bone loss. SDF1 is produced in the periosteum of injured bone and encourages endochondral bone repair by recruiting mesenchymal stem cells to the site of injury. Therefore mobilization of osteoblastic progenitors to the bone surface is an important step in osteoblast maturation and the formation of mineralised tissue (Kitaori et al., 2009).

This study supports the notion that although stem cells remain active with age, their differentiation ability is affected, therefore impairing their regenerative and

differentiation capacity. The contradictory results reported in various studies could be due to patient variability, osteoporotic models used and source of stem cells. Further *in vivo* studies are necessary to clarify whether the *in vivo* bioactivity of MSCs obtained from young patients are efficacious in the regeneration of bone in osteoporotic patients. The rat OVX model is an osteopenic model and does not fully represent human osteoporosis. This study could therefore be validated by investigating MSCs isolated from human osteoporotic, old non-osteoporotic and young patients.

Using cells from younger hosts could be an option for cellular and genetic therapies for bone degenerative diseases. Another stem cell therapy factor to consider is whether use of an allogenic cell source from young patients would be compatible in osteoporotic patients? Although we have shown that stem cells from OVX rats are similar in their proliferation and expression of CD markers to their younger counterparts, their inability to migrate to the site of bone loss as well as their reduced capacity to differentiate reduces their ability to form bone.

Chapter 4. Genetic Modification of rBMCs to Over-express CXCR4.

4.1 Introduction

The controlled delivery of growth factors and cells at sites of bone injury can enhance and accelerate functional bone formation. Current bone regeneration therapies include delivery of growth factors such as BMP-2, 4 and 7 to the site of repair and have many disadvantages such as short half-life and instability which results in the repeated administration of the growth factors. The focus is therefore moving towards gene therapy where cells are transfected *in vitro* with a virus carrying a gene which has perceived beneficial effects, before being transplanted *in vivo* (Hao et al., 2009).

Gene therapy offers an alternative means to achieve controlled delivery of specific proteins for bone regeneration through the transfer of nucleic acids to somatic cells for a continuous therapeutic expression of osteoinductive factors. This regulates cell activity at a specific site by the release of appropriate concentrations of functional growth factors. There are two main gene therapy strategies for bone repair; stem-cell based gene therapy and direct gene therapy. Stem-cell based gene therapy involves the delivery of genetically engineered cell populations that act as the carrier for therapeutic genes. On the other hand, direct gene therapy involves the direct delivery *in vivo* via viral or non-viral vectors. The efficiency of direct gene therapy for bone repair has been demonstrated with different vectors in various animal models (Vo et al., 2012). Viruses are effective vectors for delivering cDNA as they are designed to efficiently infect cells and transmit genetic material (Pensak and Lieberman, 2013).

The advantages of using an adenoviral vector system for this type of gene therapy include; extremely high transduction efficiencies compared to liposome- mediated gene transfer methods and the ability to accommodate large (up to 7.5kb) cDNAs. The virus can also be grown to high titers, which can readily be prepared and used to efficiently infect a number of cell types. Unlike retrovirus transfection, adenovirus has a low pathogenicity in humans and does not integrate into the host genome, which avoids potential problems associated with insertional mutagenesis. This is important

because the viral genome will not be replicated into the host cells and would eventually be lost by dilution, cell division and apoptosis (Luo et al., 2007, Franceschi et al., 2000).

The hypothesis of this study was that using an adenovirus vector system to transfect rBMSC with cDNA encoding for CXCR4 would result in a larger number of cells expressing CXCR4 and that transfected cells would express increased levels of CXCR4 compared to non-transfected cells. In addition, the nature of these cells in terms of CD marker expression, their proliferation rate and the ability of cells to differentiate down an osteoblastic lineage would not be affected by the incorporation of cDNA encoding for CXCR4.

The overall aim of this chapter was to over-express CXCR4 in MSCs from young, adult and OVX rats by transfecting using an adenovirus carrying CXCR4 gene and to determine whether this affected phenotype, migration, proliferation and differentiation of these cells.

Therefore, I investigated the following:

- The establishment of the CXCR4 gene in MSCs using an adenoviral vector system.
- The expression of CXCR4 expression in infected and uninfected MSCs from young, adult control and OVX rats.
- The effect of CXCR4 expression on cell proliferation, differentiation, CD marker expression in MSCs from young rats.

In subsequent chapters, the effect of cell migration on osteopenic bone generated following ovariectomy will be investigated.

4.2 Methods

4.2.1 Cloning the CXCR4 gene into the pShuttle vector

The human CXCR4 cDNA (pCMV-XL5-CXCR4 Origene) was digested by restriction endonucleases NotI and XhoI (ThermoFisher Scientific, UK) and then inserted into pShuttle-CMV (AdEasy XL Adenoviral vector system; Stratagene, United States) to form pShuttle-CMV-CXCR4. The human CXCR4 gene and the pShuttle-CMV vector were both cut by restriction endonuclease NotI and XhoI (ThermoFisher Scientific, UK) and were then repaired to form blunt ends. The cuts were confirmed by gel electrophoresis. Human CXCR4 cDNA was then cloned into the pShuttle-CMV vector using DNA ligase (ThermoFisher, Scientific, UK). The presence of the insert was confirmed by restriction digest as well as Sanger sequencing. The incorporated shuttle vector was then linearised with PmeI restriction endonuclease and transformed into BJ5183-AD-1 competent cells using electroporation (Figure 4.1). An empty plasmid was created using similar steps but the cDNA sequence was not inserted into the pShuttle-CMV vector.

Following selection of the smallest colonies, the recombinant plasmid was collected using a miniprep kit (ThermoFisher Scientific, Loughborough, UK) and cut using the PacI restriction enzyme (ThermoFisher Scientific, UK) (Figure 4.2). The correctly identified copies were then expanded in bulk using the recombinant-deficient XL10-Gold strain.

4.2.2 Production of the viral particles

Purified recombinant plasmid DNA, cut with PacI was used to transfect AD-293 cells. The viral particles were generated by alternate freezing and thawing of the infected AD293 cells and collecting the supernatant. An anti-hexon antibody stain (Stratagene, USA) was used to determine the plaque forming units/ml (pfu/ml) of the infected cells by counting the number of dark infected cells.

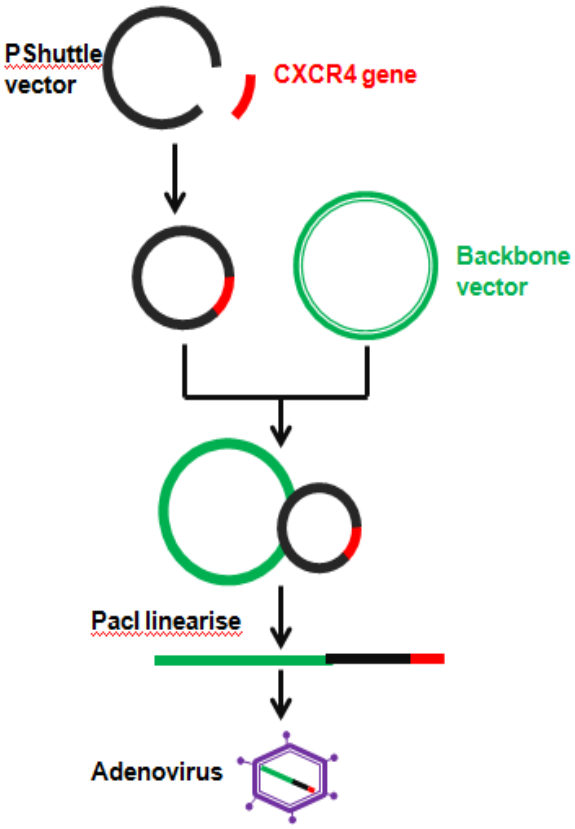

Figure 4.1: A schematic diagram showing the production stages of the recombinant adenovirus using the AdEasy XL adenoviral vector system.

4.2.3 Sequencing method

A polymerase chain reaction (PCR) was carried out on 3.5µl (500ng) of the purified plasmid DNA (pShuttle-CMV- CXCR4) using the forward and reverse primer, sequencing buffer (ABI 4336699) and BigDye v3.1 (ABI 4337455). PCR products were then purified using ethanol precipitation and centrifuged at 3000rpm for 30 minutes. The products were dried and washed with 70% ethanol, spun down and resuspended in HiDi Formamide (ABI 4440753) and analysed in a Sanger sequencer. A negative control with no DNA was also sequenced to validate the results.

4.2.4 Analysis of CXCR4 Expression

4.2.4.1 Analysis using Flow Cytometry

In order to validate whether transfection had increased CXCR4 expression in MSCs from young, adult control and OVX rats, CXCR4 expression was analysed in uninfected and infected MSCs (n = 3).

Young rBMCs from the third passage were infected with the CXCR4 adenovirus at a Multiple of Infection (MOI) of 800, trypsinised and centrifuged at 2000 rpm for 10 minutes before being resuspended at 100,000 cells in phosphate buffered saline (PBS). Cell aliquots were incubated with primary CXCR4 antibody (Abcam, UK) for 30 minutes at room temperature. The cells were washed in PBS and then incubated in secondary goat anti-rabbit antibody (Abcam, UK) for 30 minutes at room temperature. The negative control consisted of cells incubated in the secondary antibody only. 10,000 cells were then analysed using a flow cytometry machine (Cytoflex, Beckman Coulter, UK).

In order to measure the natural expression of CXCR4 on young, adult control and OVX cells, rBMCs from the third passage were trypsinised and centrifuged at 2000 rpm for 5 minutes before being resuspended at 100,000 cells in phosphate buffered saline (PBS). The cells were fixed in 4% formaldehyde for 10 minutes at room temperature. After 10 minutes, the cells were washed in PBS to remove the formaldehyde. Cell aliquots were permeabilised in ice cold 90% methanol (Sigma Aldrich, UK) for 10 minutes, washed with blocking buffer (0.5% Bovine Serum Albumin in PBS) and incubated with primary CXCR4 antibody (Abcam, UK) for 1hour at room temperature. The cells were then washed with blocking buffer and then incubated in secondary goat anti-rabbit antibody (Abcam, UK) for 30 minutes at room temperature.

For the negative control, cells were treated in the same manner but only incubated in the secondary antibody for an hour. All cells were then resuspended in PBS and analysed using flow cytometry (Cytoflex, Beckman Coulter). The above procedure was repeated for young, adult control and OVX rBMCs from the third passage that were infected with the CXCR4 adenovirus at a MOI of 800.

4.2.4.2 Immunocytochemistry

CXCR4 expression on these cells was also analysed using immunocytochemistry. Briefly the CXCR4 infected cells were fixed with 4% formalin (Sigma Aldrich, UK), incubated in a blocking buffer (1% Bovine serum albumin (Sigma Aldrich, UK)) for an hour and then incubated with primary CXCR4 antibody (Abcam, UK) for 2 hours at room temperature. The cells were washed with PBS and then incubated in secondary goat anti-rabbit antibody (Abcam, UK) for 30 minutes at room temperature. DAPI was used to counter stain cell nuclei. The cells were imaged using a fluorescent microscope (Zeiss – Axiovision 4.5) (Cell Signalling Technology) .

4.2.5 CD Marker expression of CXCR4 infected MSCs

10,000 young MSCs from passage 3 infected with CXCR4 adenovirus at MOI of 800 were analysed for CD29, CD90, CD45 and CD34, to investigate whether infection with an adenovirus affected their stem cell features (n = 3). As described in chapter 2, the cells were labelled with Anti-mouse/Rat CD29-FITC (eBioscience, UK), anti-mouse/Rat CD90-APC (eBioscience, UK), Anti-Rat CD45-APC (eBioscience, UK) and CD34-PE (Abcam, UK). CD expression was compared to the isotype control. The cells were fixed in 4% formalin for 15 minutes at room temperature, washed with 0.5% BSA-PBS, stained with the conjugated primary antibody for 1 hour at room temperature in the dark. After 1 hour the cells were washed with 0.5% BSA and analysed using a flow cytometer (Cytoflex, Beckman Coulter, UK) (Cell Signalling Technology).

4.2.6 Differences in proliferation between infected and uninfected cells.

10000 CXCR4 infected and uninfected young MSCs (n=3) were seeded in a 6 well plate and metabolic activity was measured using an Alamar Blue assay (AbD Serotec, UK) for 4 hours. Excitation at 560 nm and an emission at 590 nm were measured using a Tecan plate reader. The mean absorbance was determined from triplicate samples. The absorbance was then normalised to the DNA assays and a comparison was made between the infected and uninfected cells (methodology described in detail in chapter 3) (Czekanska, 2011).

4.2.7 Differences in ALP production between infected and uninfected cells.

Transfected and non-transfected young MSCs were harvested and differentiated to osteoblasts (n = 3). Their ALP activity was measured at 0, 7, 14 and 21 days. Briefly *p*-Nitrophenyl Phosphate (Sigma Aldrich, UK) was added to the cell lysate and the absorbance was measured at 405 nm. This was then normalised against DNA readings of each sample (Oreffo et al., 1998) (described in detail in chapter 3).

4.2.8 Statistical analysis.

Data was analysed using a student T-test using SPSS version 21 and results considered significant at the 0.05 level. The normality of the data was checked using a parametric test (Shapiro Wilkinson test).

4.3 Results

4.3.1 Production of the CXCR4 adenoviral vector system

The CXCR4 gene was successfully cloned into the pShuttle-CMV vector and the presence of the CXCR4 insert was confirmed by Sanger sequencing (Figure 4.4) as well as HindIII and Sal1 restriction enzymes, which generated a band of approximately 1.8 kb, the size of the CXCR4 insert using gel electrophoresis. The pShuttle-CMV-CXCR4 plasmid was linearised with PmeI and recombined into the Ad-plasmid in BJ5183 cells (e.coli strain). This insert was confirmed by a PacI restriction

cut which generated a band of 3 kb and 23 kb (Figure 4.3). The plasmid was then linearised and inserted into the AD293 cells (packaging cell line) and the sign of viral production was observed by a colour change in the medium from red to orangish yellow, compared to the negative control. Additionally, a further sign of viral infection was shown by some of the cells rounding up, detaching from the plate and floating in the media. Anti-hexon antibody staining generated dark brown cells which were counted to calculate the pfu/ml of the CXCR4 and empty plasmid virus (n=2) (Figure 4.5, Figure 4.6). Immunocytochemistry further confirmed the presence of CXCR4 in the AD293 cells (n=3) (Figure 4.8).

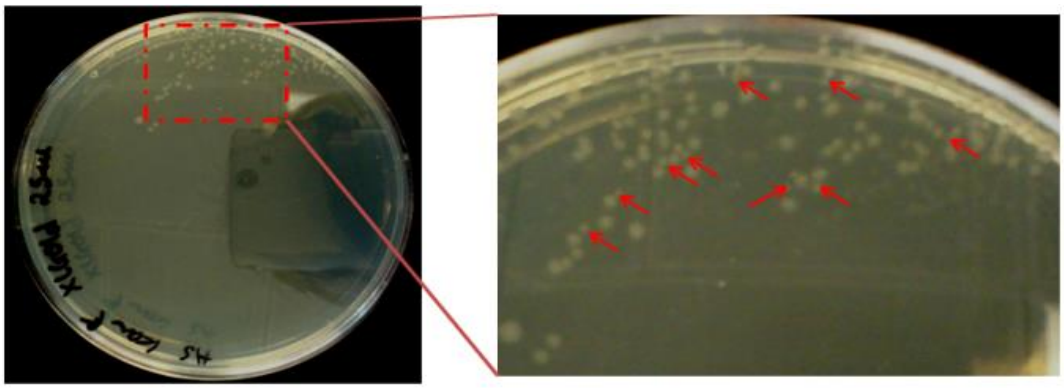

Figure 4.2: Screening of the potential adenovirus recombinants after recombination in AdEasier cells. The small colonies most likely contain the recombinant plasmid. The larger colonies are background clones.

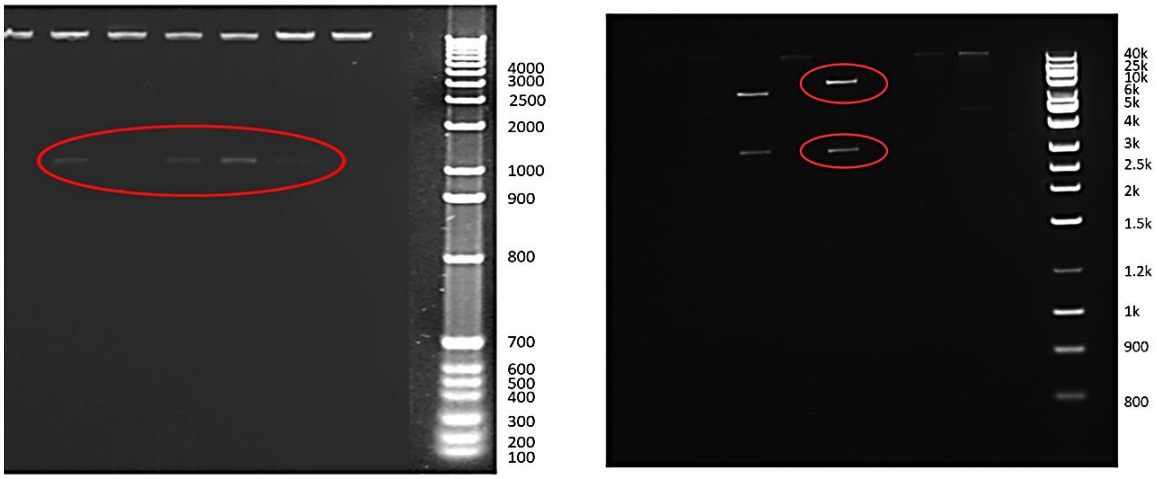

Figure 4.3: Gel electrophoresis confirming the CXCR4 insert into the Pshuttle-CMV vector, using SalI and HindIII restriction enzyme resulting into a band size of 1.8 kb (left). The recombination of the pShuttle-CMV-CXCR4 with the Ad plasmid was also confirmed with the 3 kb and 23 kb bands.

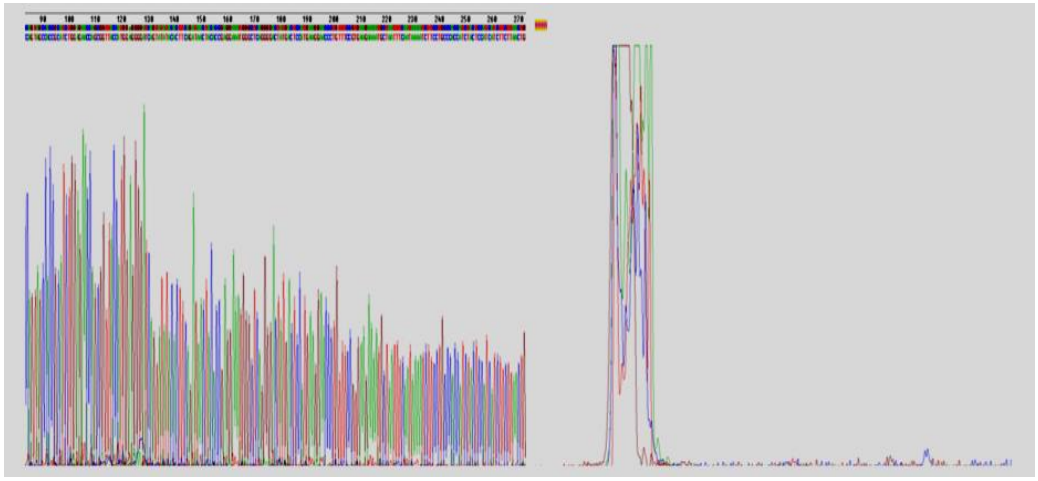

Figure 4.4: The insert of the gene was further confirmed by an electropherogram from Sanger sequencing (left) and was also validated using the negative control, which was the empty insert (right).

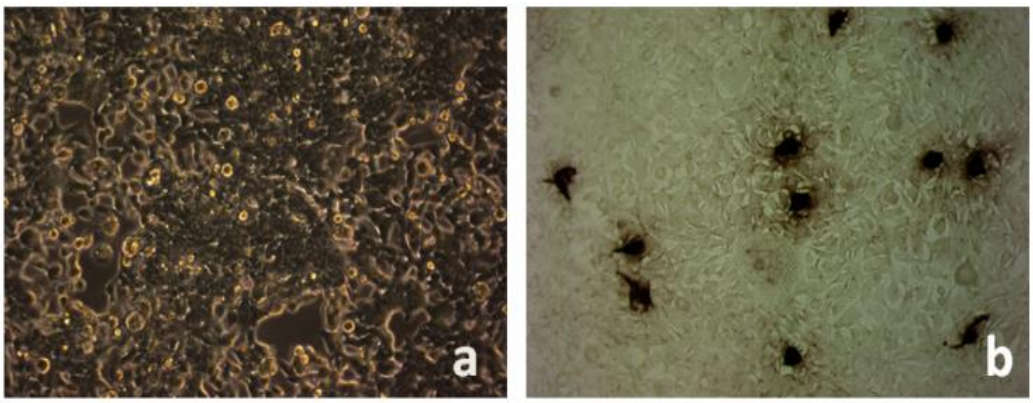

Figure 4.5: Ad-293 cells, 10 days after transfection with linearised pShuttle-CMV-CXCR4 plasmid (a), anti-hexon staining of the infected Ad293 cells (b) x20magnification, immunocytochemistry staining for CXCR4 expression of uninfected AD293 cells (c) and infected AD293 cells (d) (x100 magnification).

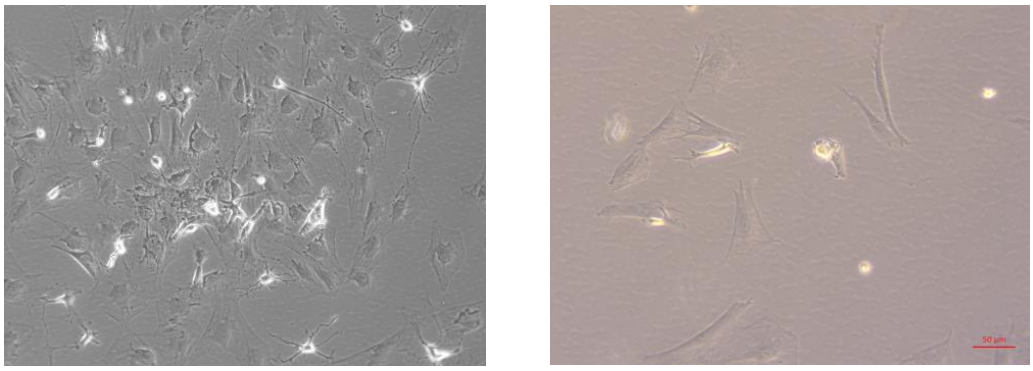

Figure 4.6: CXCR4 infected MSCs has a much rounder and larger nucleated morphology (left), compared to their uninfected counterparts that have retained their spindle like morphology (x10 magnification).

4.3.2 CXCR4 expression of infected and uninfected cells

Surface expression of CXCR4 on these cells was analysed by flow cytometry and immunocytochemistry using an anti-CXCR4 antibody (n=3). The CXCR4 infected cells over-expressed CXCR4 evident by immunocytochemistry and flow cytometry in comparison to uninfected cells and empty infected cells. This was also evident when AD293 cells were infected with a CXCR4 adenovirus. The AD293 and MSCs infected

with the empty virus showed no expression of CXCR4 when compared to their infected counterparts (Figure 4.8).

After transfection with the CXCR4 virus at MOI 800, 92±2.6%, 44.2±8.2% and 40±16.1% of young, adult control and OVX MSCs respectively over-expressed CXCR4. CXCR4 expression in OVX MSCs was doubled while in young MSCs it only increased by 5% after CXCR4 transfection (Table 4.1, Figure 4.7). This could be because the young MSCs already expressed high levels of CXCR4 and over-expression did not affect further CXCR4 expression.

Table 4.1: The percentage change in CXCR4 expression before and after CXCR4 transfection in young, adult control and OVX MSCs. * & ** & * represents significance p<0.05 (n=3).**

Type of cells	**CXCR4 expression before transfection (%)**	**CXCR4 expression after transfection (%)**	**Percentage increase (%)**
Young MSCs	87.5±5.1*, **	92±2.6***	5.1
Adult MSCs	28.1±1.5*	44.2±8.2***	57.2
OVX MSCs	20.2±9.8**	40±16.1***	97.9

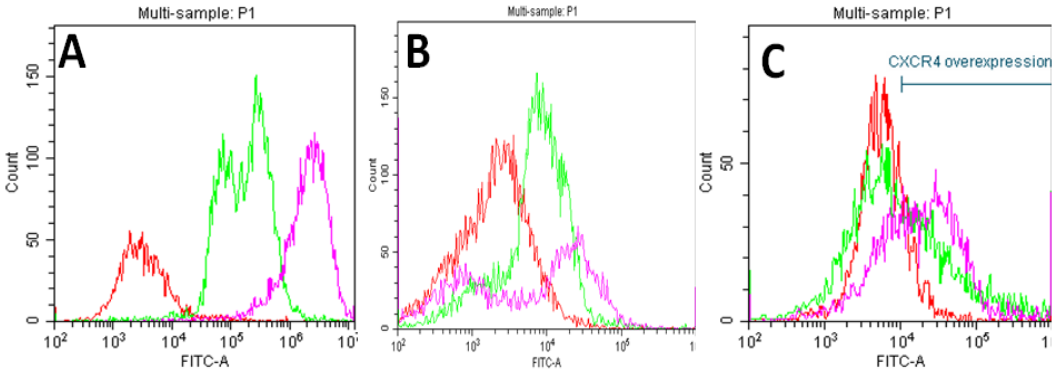

Figure 4.7: FACS analysis of CXCR4 infected cells and uninfected cells of young MSCs (a); adult control (b) and OVX MSCs (c). The secondary isotype is represented by red, CXCR4 expression in MSCs is represented by green and pink represents the CXCR4 over-expression in the infected MSCs.

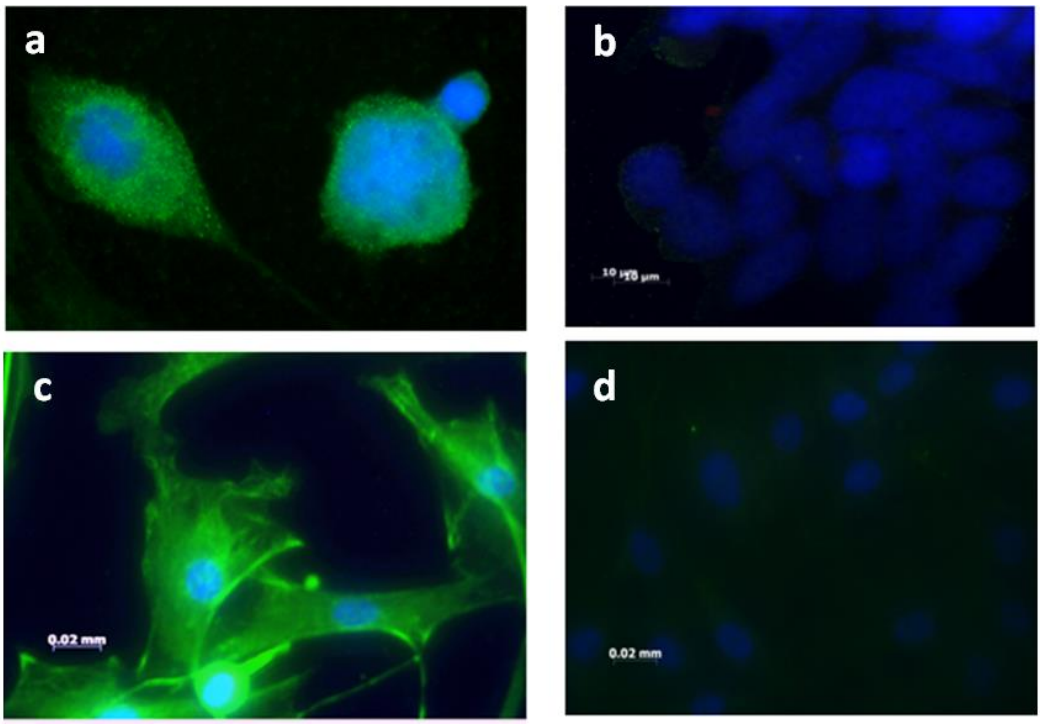

Figure 4.8: Immunocytochemistry of CXCR4 expression in CXCR4 infected (a), empty infected AD293 cells (b) (x100 magnification), infected young rBMCs (c), and 'empty plasmid' infected rBMCs (d) (x40 magnification).

4.3.3 CD Marker expression of CXCR4 infected MSCs

To investigate whether adenovirus infection of MSCs effected 'stem' phenotype of MSCs, the expression of CD29, CD90, CD34 and CD45 from the infected groups was compared to the uninfected young cells (n = 3). Overall the CD marker expression of the infected cells was not significantly different from uninfected cells. On average 81.97±6.97% of the infected and uninfected cells expressed CD29, 94.67±7.6% of the cells expressed CD90, 2.47±3.24% of the cells expressed CD34 and 26.9±18.24% of the cells expressed CD45 (

Figure 4.9).

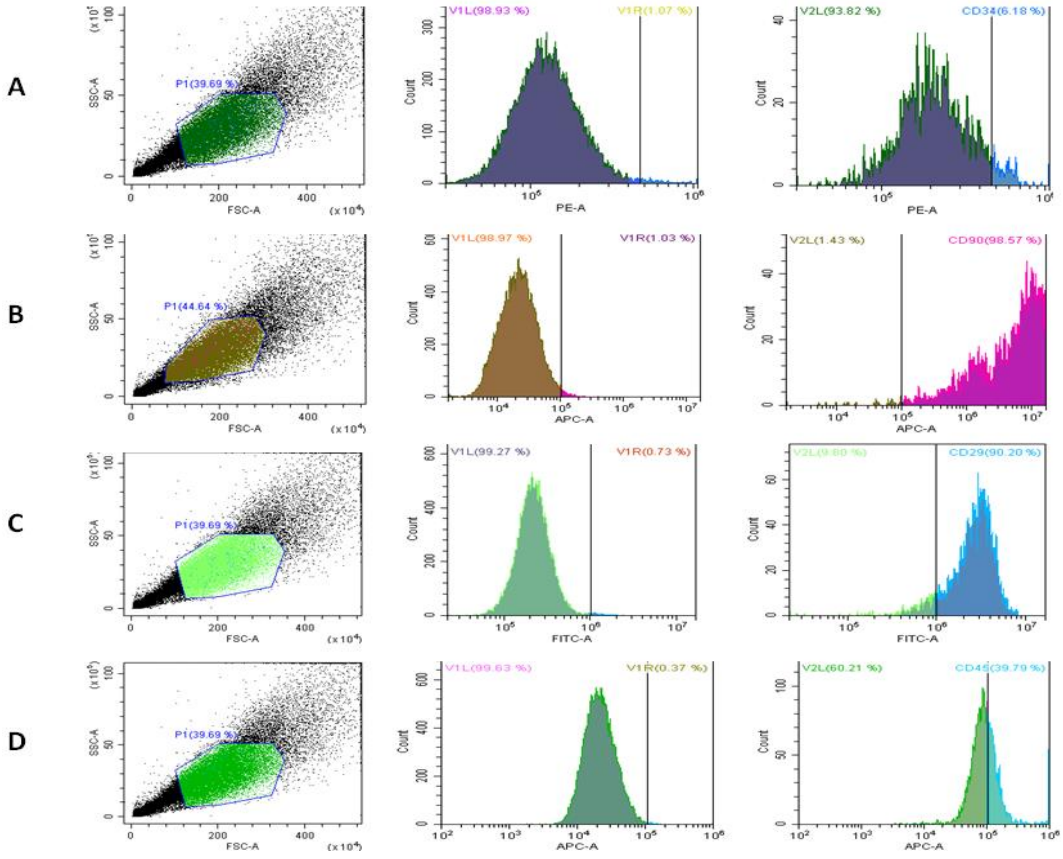

Figure 4.9: CD Marker expression of CXCR4 infected young MSCs from passage 3. CD34 (A), CD90 (B), CD29 (C) and CD45 (D) expression shown using FACS analysis, with histograms in the middle showing the secondary isotype control and histograms on the far right showing the expression of the CD markers (n=3).

4.3.4 Differences in proliferation between infected and uninfected cells

To measure cell proliferation, the Alamar blue assay normalised against the DNA content was measured for the CXCR4 infected cells and the uninfected cells (n=3). It was observed that the infected cells had higher cell proliferation in comparison to the uninfected cells at day 5 with a decrease at day 7 and thereafter increased again at day 14. In contrast, the uninfected cells had lower proliferation at day 5, which peaked at day 7 and then reduced at day 14 (Figure 4.10a). The differences in proliferation between the infected and uninfected cells were not significant at day 5 ($p = 0.063$), day 7 ($p = 0.08$) and day 14 ($p = 0.25$), showing that infecting rBMCs with CXCR4 adenovirus did not affect their proliferation.

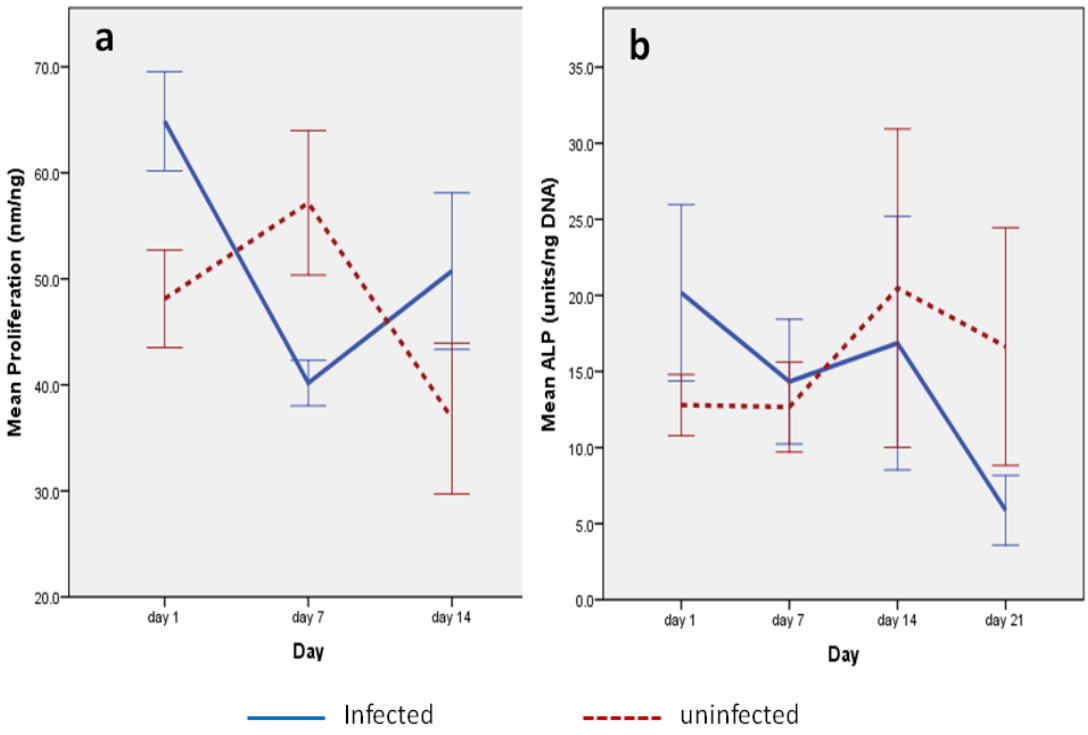

Figure 4.10: Average Alamar blue readings normalised against DNA content for CXCR4 infected and uninfected cells (a) and ALP normalised against DNA content (b) for CXCR4 infected and uninfected cells when they were differentiated to osteoblasts and control cells which were grown in normal media (n=3).

4.3.5 Differences in ALP production between infected and uninfected cells

To ensure that infecting rBMCs with CXCR4 did not affect osteogenic differentiation, the CXCR4 infected cells and the uninfected cells were differentiated into osteoblasts and their ALP expression was measured from day 0 to day 21 (n=3). The ALP readings were normalised against the DNA content. The uninfected cells had a peak increase in ALP at day 14. However, the infected cells showed higher ALP at day 1, which was observed to decrease by day 7 and rise by day 14. For both the infected and uninfected cells the ALP readings were observed to decrease at day 21(Figure 4.10b). No significant difference in ALP expression of the infected and uninfected cells at day 0 ($p = 0.21$), day 7 ($p = 0.55$), day 14 (0.79) and day 21 ($p = 0.25$) was found, implying that CXCR4 infection did not affect ALP expression of rBMCs.

4.4 Discussion

The aim of this chapter was to establish a reliable system for modifying MSCs in order to make these cells over-express CXCR4 so as to increase their migration ability to sites of injury or to osteopenic bone. An adenovirus is an appealing transfection system for gene therapy because of its high transfection efficiency for a wide range of cell types and most importantly the virus does not integrate with the DNA of the cell (Luo et al., 2007). The cytopathic effect of AD293 cells during the adenovirus production procedure demonstrated the assembly of the virus particles in the cells. This was observed in stem cells during viral transduction. MSCs were seen to have an altered morphology; with a rounder and larger nucleus. The uninfected MSCs maintained a spindle like morphology (Figure 4.6).

Adenovirus infects cells by the attachment of Cocksackie adenovirus receptor (CAR) on the cell surface. Following receptor-mediated endocytosis, adenovirus escapes from the endosomes to the cytoplasm and translocates into the nucleus where viral transcription and replication begin. Completion of the virus life cycle is marked by cell death and the release of progeny viruses, which was clearly demonstrated when the

AD293 cells became rounder and detached from the surface of the flask (Luo et al., 2007). The AD293 cells were used to amplify the adenovirus, by collecting viruses from small numbers of AD293 cells and infecting a larger number of AD293 in a bigger flask and thereby using virus isolated after passage 2. After confirming the adenovirus' infection ability on AD293 cells using anti-hexon staining, we transfected MSCs and tested the expression of CXCR4 in both the infected (MOI 800) and uninfected MSCs using flow cytometry and in 'empty plasmid' infected MSCs using immunocytochemistry. MSCs from OVX and adult control rats demonstrated an average 20% increase in CXCR4 expression after CXCR4 infection in comparison to the uninfected cells. After transfection, CXCR4 expression was 40% greater in the OVX and adult control MSC groups. It was interesting to note that an increase in CXCR4 expression after transfection in young MSCs was not as prolific as that observed in OVX and adult control cells. This may be because young MSCs already have a high expression of CXCR4 and infecting them would only increase their CXCR4 expression per cell, which was captured by immunocytochemistry. Immunocytochemistry demonstrated high amounts of CXCR4 in the infected MSCs and AD293 cells in comparison to the cells infected with an empty plasmid.

Although the infected MSCs had a rounder morphology compared to the uninfected cells, their CD marker expression was unchanged. The transfected cells retained their high expression of CD90 and CD29 and low expression of CD34 and CD45. There was also no significant difference in proliferation and ALP expression when infected and uninfected cells were compared. This proved that the viral vector system and CXCR4 over-expression did not affect cell physiology and function, especially the ability of the cells to differentiate to osteoblasts and the MSCs retained their stem cell characteristic post-transfection. This was an important characteristic to investigate because the CXCR4 transfected MSCs would be used to augment bone formation in OVX rats.

In this chapter, a reliable adenoviral gene modifying system has been established to transfect rat MSCs with CXCR4, with expression of CXCR4 effectively quantified using flow cytometry. The effect of CXCR4 on the migration of young, adult control and OVX MSCs will be tested in vitro in Transwell chambers. The goal of this work is to use these transfected cells in vivo in osteopenic rats to test the effect of over-expressing CXCR4 in stem cells potentially making their migration and homing ability more efficient. This would retain transfected cells at sites producing SDF-1 that are likely to be associated with injury. Potentially this could lead to better bone repair and regeneration.

Chapter 5. Migration of Stem Cells is Enhanced by CXCR4.

5.1 Introduction

It has been suggested that MSCs from postmenopausal women have a slower growth rate and osteogenic differentiation ability compared to MSCs from premenopausal women, which causes lower bone density and reduced fracture healing capacity in osteoporotic humans (Rodriguez et al., 1999, Rodriguez et al., 2000). Wang et al. injected MSCs embedded within a calcium alginate gel, into the femora of OVX rabbits. After 2 months, an increase in bone apposition, trabecular thickness and bone stiffness was seen in femora from rabbits treated with MSCs, compared to the controls (Wang et al., 2006). Similar results were shown by Ocarino Nde et al. when GFP labelled MSCs were injected into the femoral bone marrow cavity in OVX rats. Most interestingly, the GFP labelled cells were shown to line the surface of the newly formed femoral bone (Ocarino Nde et al., 2010). This highlights the importance of stem cell movement for bone healing.

Cellular movement and re-localization are crucial for many important physiologic properties such as embryonic development, neovascularisation and angiogenesis, immunologic responses, wound healing, and organ repair. Both local MSCs from the injured tissue and circulating MSCs can contribute to tissue and organ regeneration. Cytokines and chemokines play important roles in maintaining the mobilization, trafficking and homing of stem cells from the bone marrow to the site of injury (Dar et al., 2006, Ito, 2011, Liu et al., 2009). During tissue regeneration, it has been suggested that local MSCs derived from the injured tissue and circulating MSCs work together to heal damaged organs. Stem cells detect the tissue injury, migrate to the site of damage and undergo differentiation (Shyu et al., 2006, Ito, 2011) and this may explain the increase of stem cells found in damaged tissues such as impaired sites in the brain after hypoglossal nerve injury (Ji et al., 2004) and cerebral injury (Imitola et al., 2004) when compared to normal healthy tissues. As a result of injury, the surviving

cells may produce chemoattractants such as SDF-1. With its receptor CXCR4, SDF-1 directs the migration of MSCs to the injury site (Imitola et al., 2004).

Wynn et al.(Wynn et al., 2004a) showed that when a neutralizing anti-CXCR4 antibody was used to block CXCR4 expression, it inhibited MSC migration to the bone marrow by approximately 46%. This study also demonstrated a dose dependent migration of MSCs towards SDF-1 in a transwell assay, when MSCs were seeded in the upper chamber of a Boyden chamber and SDF-1 was used as a chemoattractant. Blocking CXCR4 expression significantly reduced cell migration towards SDF-1 (Wynn et al., 2004a). Lien and co-workers transfected murine MSCs with CXCR4 and Cbfa-1 and injected these modified cells into osteopenic mice. A complete recovery of bone stiffness and strength in these animals was seen after 4 weeks (Lien et al., 2009b). A similar study by Cho et al. also showed that over-expression of MSCs with CXCR4 and RANK-Fc improved bone mineral density in ovariectomized mice, with CXCR4 acting as an important migratory factor and enhancing the therapeutic effects of RANK-Fc for bone loss (Cho et al., 2009b). However, information on differences in the ability of MSCs to migrate in osteopenic, adult and young rats are limited.

The purpose of this part of my thesis was to investigate the scope of CXCR4 in improving the migration of MSCs from these three different groups of rats. In patients with osteoporosis, there is often an increased incidence of delayed union associated with fragility fractures. This may be associated with recruitment of stem cells to the fracture site and their differentiation to bone. Considering the significance of the SDF-1/CXCR4 axis in homing and engraftment of bone marrow cells and the potential of MSCs in bone regeneration therapy, I investigated the novel concept of using over-expression of CXCR4 in stem cells to affect and enhance the migratory capacities of MSCs from three rat populations: young, adult and osteopenic.

The first hypothesis of this chapter was that young MSCs transfected with CXCR4 would migrate more towards SDF-1 compared to CXCR4 infected MSCs from OVX and adult rats.

The second hypothesis was that uninfected MSCs differentiated to osteoblasts would not migrate towards SDF-1 in a Boyden Chamber, however CXCR4 infected MSCs differentiated to osteoblasts would migrate towards SDF-1 in a Boyden Chamber.

5.2 Methods

5.2.1 Migration of Infected and Uninfected Cells from Young, Adult and OVX Rats

For this part of the study, the *in vitro* migration of MSCs was investigated in the following experimental groups:

- Groups A – Uninfected Young MSCs
- Group B – CXCR4 infected young MSCs
- Group C – Uninfected adult MSCs
- Group D – CXCR4 infected adult MSCs
- Group E – Uninfected OVX MSCs
- Group F – CXCR4 infected OVX MSCs
- Group G – 'Empty plasmid' infected Young MSCs.

A chemoinvasion assay was used to evaluate the ability of CXCR4 infected MSCs to migrate towards SDF-1 in comparison to uninfected MSCs. 10,000 cells (i) transfected with CXCR4, (ii) with an empty plasmid at MOI 800, and (iii) uninfected cells were introduced into the chamber. The lower compartment of the Boyden chamber was filled with 100ng/ml SDF-1 in DMEM, FCS and penicillin/streptomycin. The chambers were incubated at 37°C, 5% CO_2 for 16 hours. After 16 hours, cells that migrated to the opposite side of the membrane were fixed in 10% formaldehyde

and stained with crystal violet. The migrated cells were counted by selecting six random fields at x20 magnification and calculating the percentage average number of cells. The normalised number of cells was calculated by dividing the infected cells by the uninfected cells of the same group. Each experiment was repeated three times with cells from three different rats. In the control group, both the top and bottom of the chamber were filled with normal media with no SDF-1 and the cells were loaded in the upper chamber as before. This was to create an environment with no SDF-1. The migrated cells were stained and counted after 16 hours. Data was analysed for both studies using SPSS and results considered significant at the 0.05 level.

5.2.2 CXCR4 expression of Osteogenic differentiated rBMCs and CXCR4 infected rBMCs differentiated to osteoblasts

CXCR4 infected and uninfected young rBMCs from the third passage were treated with osteogenic media for 21 days. The cells were thereafter trypsinised and centrifuged at 2000 rpm for 5 minutes before being re-suspended at 100,000 cells in phosphate buffered saline (PBS). The cells were fixed in 4% formaldehyde for 10 minutes at room temperature. The cells were then washed in PBS to remove the formaldehyde. Cell aliquots were permeabilised in ice cold 90% methanol for 10 minutes, washed with blocking buffer (0.5% Bovine Serum Albumin in PBS) and incubated with primary CXCR4 antibody (Abcam) for 1hour at room temperature. The cells were then washed with blocking buffer and incubated in secondary goat anti-rabbit antibody (Abcam) for 30 minutes at room temperature.

For the negative control, cells were treated in the same manner but only incubated in the secondary antibody for an hour. All the cells were then resuspended in PBS and analysed using a flow cytometry machine (Cytoflex, Beckman Coulter). The above procedure was repeated for young rBMCs seeded in normal media for 21 days.

CXCR4 expression was therefore investigated in three groups of cells in this part of the study:

- Group A – Young MSCs grown in normal media (Control).
- Group B – Uninfected Young MSCs differentiated to osteoblasts.
- Group C – CXCR4 infected young MSCs differentiated to osteoblasts.

Young MSCs were only used for this part of the study because they had very high initial levels of CXCR4 and any observable differences in CXCR4 expression after osteogenic differentiation could easily be compared to controls.

5.2.3 Migration of rBMCs and CXCR4 Infected rBMCs grown in Osteogenic Media

A chemoinvasion assay was used to evaluate the ability of undifferentiated and osteogenic differentiated MSCs to migrate towards SDF-1. To confirm osteogenic differentiation of rBMCs, cells were stained with Alizarin red and their osteocalcin expression analysed after 21 days. For the chemoinvasion assay 25000 osteogenic differentiated rBMCs and undifferentiated rBMCs were loaded in serum free medium in separate upper compartments of the Boyden chamber. The lower compartment of the Boyden chamber was filled with 100ng/ml SDF-1 in DMEM, FCS and penicillin/streptomycin. The chambers were incubated at 37°C, 5% CO_2 for 16 hours. After 16 hours, the cells that migrated to the opposite side of the membrane were fixed in 10% formaldehyde and stained with crystal violet. The migrated cells were counted by selecting six random fields at x20 magnification and calculating the percentage average number of cells. Each experiment was repeated three times with cells from three different rats. For the control, both the top and bottom of the chamber were filled with normal media with no SDF-1 and the cells were loaded in the upper chamber as before. This was to create an environment with no SDF-1 (Figure 5.1).

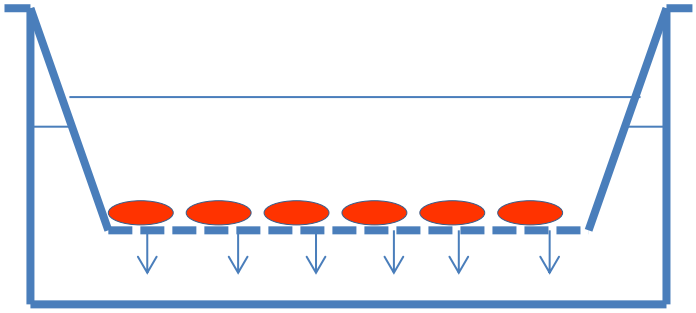

Figure 5.1: A schematic diagram demonstrating a Boyden Chamber. The cells loaded in the upper chamber, would migrate to the bottom depending on whether the bottom chamber is filled with SDF-1 or normal media. The number of cells that migrated to the opposite side of the membrane would be counted using crystal violet stain.

5.2.3 Statistical Analysis

The normality was checked using Kolmogorov- Smirnov and Shapiro Wilkinson test and where the data was normal, comparison was made using independent student T-test. Where the data was non-parametric comparison was made using Mann-Whitney U test with a Bonferroni correction. All data was analysed using SPSS version 24 (Chicago, USA).

5.3 Results

5.3.1 Migration of Infected and Uninfected Cells from Young, Adult and OVX rats

The effect of SDF1 on the migration of CXCR4 infected cells, empty plasmid-infected cells and uninfected cells was investigated using the transwell migration assay. The data was normalised to the uninfected cells. Over-expression of CXCR4 significantly increased the migration of young rBMCs towards SDF-1 by 87±27% in comparison to uninfected cells ($p = 0.006$). Similarly, the effect of infecting the cells with an empty plasmid on their migration towards SDF-1 was measured. Although, significantly

fewer 'empty plasmid' infected cells migrated towards SDF-1 in comparison to the uninfected cells (p = 0.03), there was a significant decrease in the migration of 'empty plasmid' infected cells towards SDF-1 when compared with infected cells (p = 0.003), demonstrating that over-expression of CXCR4 increased cell migration towards this cytokine. Most importantly the effect of CXCR4 was significantly more profound in rBMCs from OVX rats. Five times more rBMCs from OVX rats migrated towards SDF-1 after being over-expressed with CXCR4 compared to uninfected cells (p = 0.025) (Figure 5.3 & Figure 5.4).

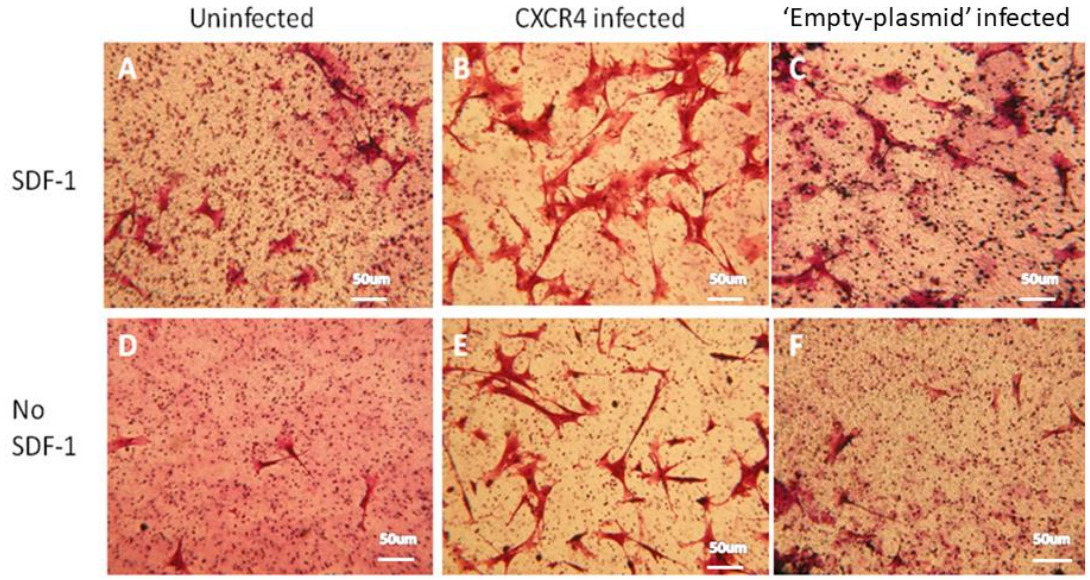

Figure 5.2: Crystal violet staining of migrated Young MSCs on the opposite side of the Boyden Chamber; uninfected (A), CXCR4 infected (B) and 'empty plasmid' infected MSCs (C) towards SDF-1 and control uninfected (D), CXCR4 infected (E) and 'empty-plasmid' infected (F) towards plain media. CXCR4 transfection significantly improved migration of young MSCs compared to 'empty plasmid' infected and uninfected MSCs (x20 magnification).

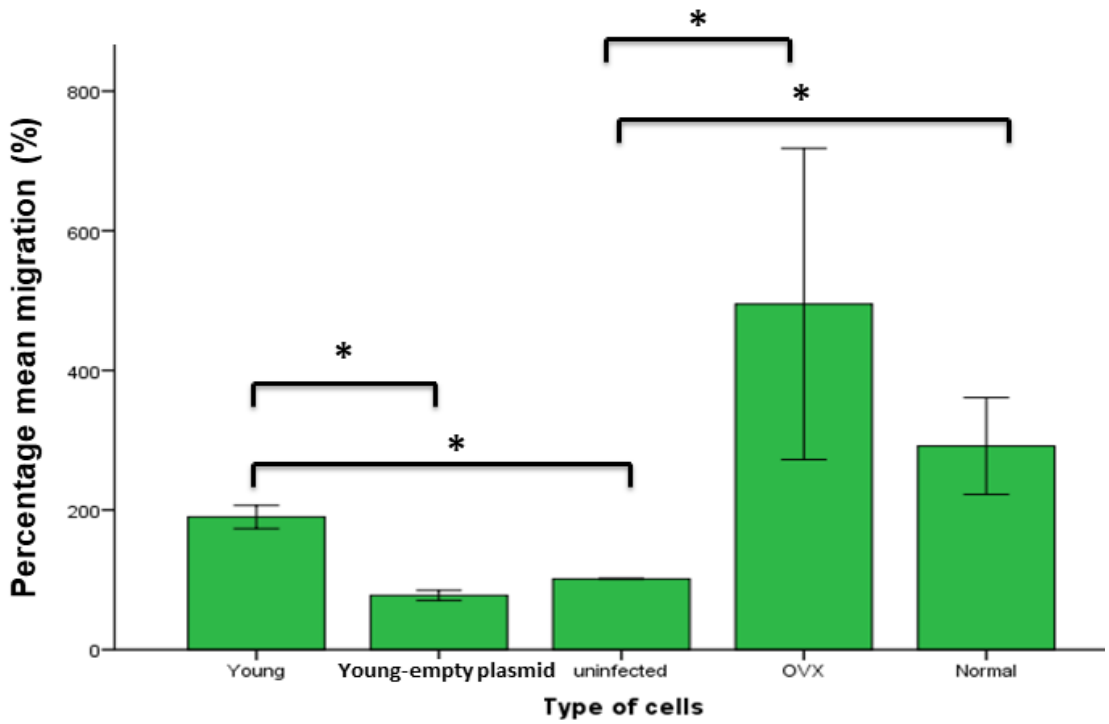

Figure 5.3: The mean percentage migration of infected cells normalised against uninfected cells. Cells were normalised by dividing infected with their uninfected counterparts. *, ** and * represent significance p<0.05.**

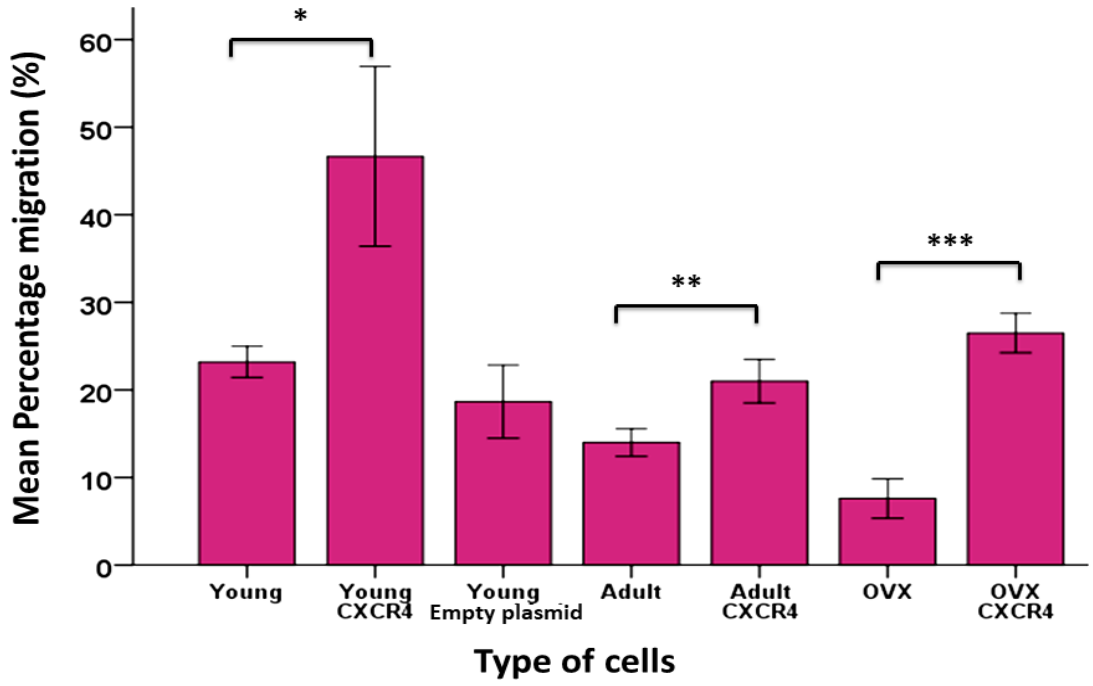

Figure 5.4: The mean percentage migration of CXCR4 infected cells compared to uninfected cells. * and ** represents significance p<0.05 and * represents significance p<0.001.**

5.3.2 CXCR4 Expression of Infected and Uninfected rBMCS Differentiated to Osteoblasts

To investigate whether osteogenic differentiation affects CXCR4 expression of differentiated rBMCs, the CXCR4 levels were measured using flow cytometry. Differentiation of young rBMCs to osteoblasts was confirmed by osteocalcin and alizarin red staining at day 21 (Figure 5.6). A control group of young rBMCs grown in normal media for 21 days was used to investigate whether culturing cells for 21 days affected CXCR4 expression. Overall, flow cytometry analysis demonstrated that the CXCR4 expression of young MSCs dropped significantly when differentiated to osteoblasts irrespective of the fact that these MSCs were transfected with the CXCR4 virus (Table 5.1, Figure 5.5). Culturing young MSCs for 21 days in normal non-osteogenic media (68.7±15.4%) did cause their CXCR4 expression to drop compared to culturing them for 7 days until confluency (84.4±8.2%), but this was not a significant drop.

Table 5.1: Flow cytometry results demonstrating the percentage CXCR4 expression of CXCR4 infected and uninfected MSCs differentiated to osteoblasts for 21 days. * and ** represent significance p<0.05.

Type of cells	**Undifferentiated MSCs**	**Uninfected osteoblasts**	**CXCR4 infected osteoblasts**
Percentage CXCR4 expression.	68.7 ± 15.4 * **	6.3 ± 0.6 *	8.0 ± 5.2**

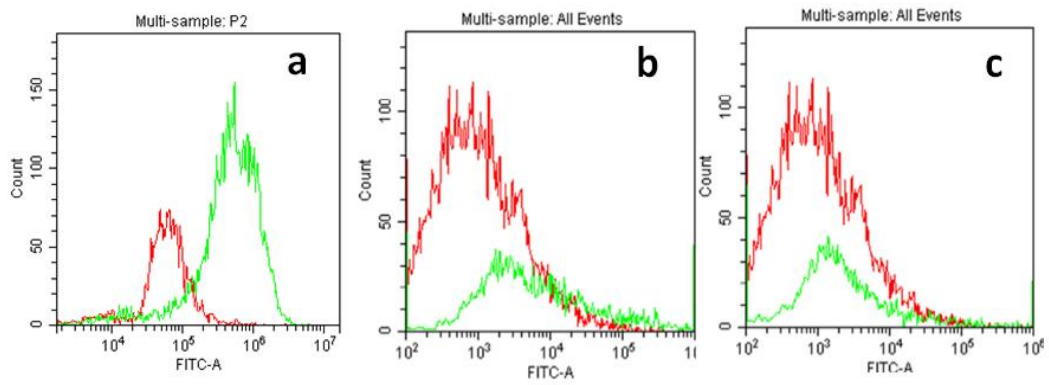

Figure 5.5: Spectra obtained following flow cytometry and analysis of CXCR4 expression in young rBMCs cultured for 21 days in non-osteogenic media (a), uninfected MSCs differentiated to osteoblasts (b) and CXCR4 infected MSCs differentiated to osteoblasts (c). The red peak represents the secondary control while the green peak represents the cells labelled with the primary anti-CXCR4 antibody.

5.3.3 Migration of Osteogenic Differentiated rBMCs and CXCR4 Infected rBMCs Differentiated to Osteoblasts

In order to determine whether osteogenic differentiation of rBMCs affected migration towards SDF-1 and whether transfection with CXCR4 increased their migratory capacity, I repeated the migration assay on stem cells grown in osteogenic medium. CXCR4 infected cells were also differentiated to osteoblasts and their migration measured using a Boyden chamber. It was observed that differentiating rBMCs to osteoblasts significantly reduced their migration towards SDF-1 (6.7 ± 2.3%) compared to undifferentiated rBMCs (23.2 ± 4%) ($p = 0.0006$). Results also showed that when CXCR4 infected rBMCs were differentiated to osteoblasts, their migration capability towards SDF-1 did not significantly improve (11.25 ± 8.6%), whereas transfection significantly improved the migration of undifferentiated rBMCs (47 ± 17%) (Figure 5.6).

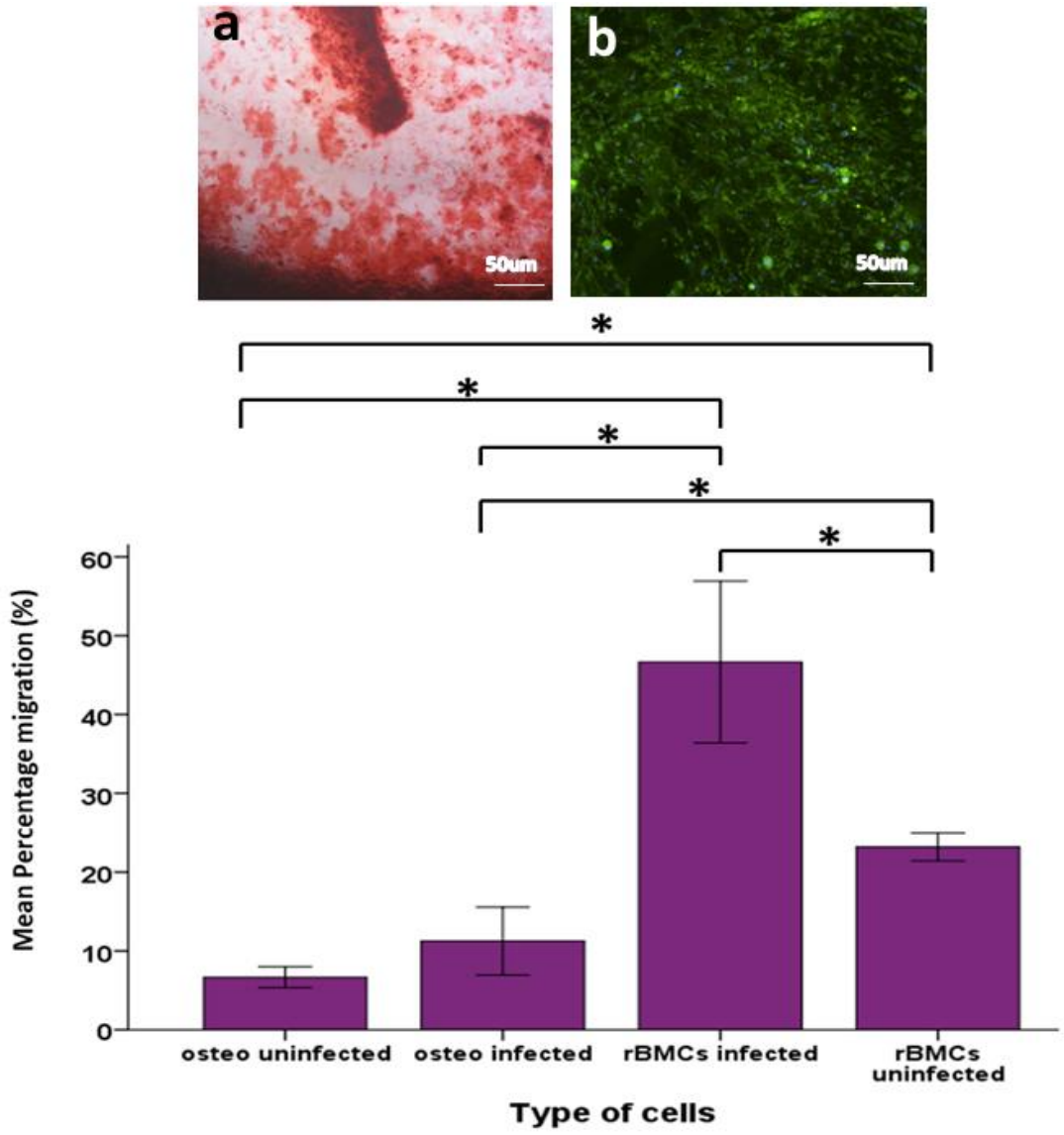

Figure 5.6: The mean percentage migration of infected and uninfected rBMCs, osteogenic differentiated rBMCs and CXCR4 infected rBMCs differentiated to osteoblasts towards SDF1 in a Boyden chamber. * represent significance $p<0.05$. Positive Alizarin Red (a) and Osteocalcin staining (b) expressed at day 21 of osteogenic differentiated rBMCs.

5.4 Discussion

This *in vitro* study has highlighted the pivotal role of the SDF-1/CXCR4 axis in the homing of stem cells and the potential significance this may have in improving bone formation in osteoporosis. Increased migration was seen in cells transfected with

CXCR4 when compared to uninfected cells. CXCR4 therefore plays a crucial role in stem cell migration as identified in these transwell *in vitro* assays. During aging, the composition, structure and function of bone deteriorates, resulting in osteoporosis (Raisz, 2005). It has also been demonstrated that MSCs from osteoporotic humans have impaired migration towards chemoattractants such as fetal calf serum (FCS), BMP-2 and BMP-7 in Boyden chambers as well as in micro-slide chemotaxis chambers (Haasters et al., 2014). SDF-1 has previously been shown to recruit more host stem cells to a fracture defect site and encourage osteogenic differentiation and production of bone (Ho et al., 2014a).

When considering the effect of age on cells, the work in this chapter has also demonstrated that stem cells from OVX and adult rats express lower levels of CXCR4 on their surface compared to stem cells from young rats, which might explain their impaired migration towards SDF-1. CXCR4, a receptor of SDF-1 is expressed in numerous tissues, including mature and immature hematopoietic and endothelial cells, EPCs, and smooth muscle cell (SMC) progenitors, which all have direct or indirect pro-angiogenic properties. SDF-1/CXCR4 pathway plays an important role in stimulating the trafficking and engraftment of hematopoietic stem cell and reconstitution of haematopoiesis (Ratajczak et al., 2006).

SDF-1 is released from the stromal cells into the intravascular compartment and it is transported from the plasma to the bone marrow compartment (Dar et al., 2005), where it induces the activation of matrix metalloproteinase-9 (MMP-9) and the release of soluble kit-ligand (sKitL). Subsequently, sKitL induces the release of more SDF-1, enhancing mobilization of the CXCR4+ and c-Kit+ cells to the circulation (Heissig et al., 2002). SDF-1 also activates the nitric oxide (NO) pathway, causing the endothelial cells to release NO which causes an upregulation of MMP-9. NO induces vasodilatation of BM endothelium which induces the release of cells (Aicher et al., 2003). In bone repair, SDF-1 is induced in the periosteum of injured bone and

promotes endochondral bone repair by recruiting mesenchymal stem cells to the site of injury (Urbich et al., 2005, Kollet et al., 2006).

Liang and co-workers intravenously injected lethally irradiated young or old Ly-5.1 mice with old or young Ly-5.2 bone marrow cells. 24 hours later, they analysed the marrow from the recipients for Ly-5.2+ stem cells that had homed there. It was found that the older cells migrated 2 to 3 times less to the bone marrow niche compared to the young cells (Liang et al., 2005). This was most likely to be due to defective CXCR4 expression developing during aging, reducing the number of CXCR4-positive cells in the bone marrow and the circulation which would eventually cumulate towards reduced angiogenesis and vessel repair (Shao et al., 2011). In this study I improved the mobility of stem cells obtained from OVX rats towards SDF-1 by increasing their CXCR4 expression. It is believed that SDF-1 causes cell migration by binding with CXCR4. Therefore, increased secretion of SDF-1 at the site of injury creates an environment that mediates the homing of circulating CXCR4-positive stem cells. The cells will subsequently differentiate into the surrounding tissue type and effect repair at the injury site (Stich et al., 2009). Poor homing ability of the stem cells to bone could result in a significant reduction in bone formation which ultimately contributes to osteoporosis (Antebi et al., 2014). This poor migration may also lead to reduced fracture healing in these patients.

Oestrogen deficiency is the main factor that causes postmenopausal osteoporosis. Oestrogen improves the osteoblastic differentiation of hBMSCs through ER-α (Hong et al., 2006) or through activating Wnt/β-catenin signalling (Bhukhai et al., 2012). A deficiency in oestrogen also increases the circulating levels of cytokines such as IL-1, TNF-α and granulocyte macrophage colony stimulating factor (GM-CSF). Increased levels of these cytokines, due to a reduction in oestrogen, enhance bone resorption (Kular et al., 2012, Manolagas and Jilka, 1995). The Notch signalling pathway has been shown to induce osteogenic differentiation and inhibit adipogenic

differentiation of BMSCs (Ugarte et al., 2009). A study performed by Fan (Fan et al., 2014) has shown that oestrogen up-regulated Notch signalling enhanced the proliferation and differentiation of hBMCs. This could further explain the difference in migration between the MSCs from the OVX and those from adult and younger rats, where the cells from OVX rats showed significantly reduced migration. However, the current study did not measure the effect of oestrogen on the migration of stem cells from young, OVX or from senile rats.

The differentiation of MSCs to osteoblasts is a complex process that involves the interaction of numerous hormones, autocrine and paracrine processes and systemic growth factors (Mannello et al., 2006). We found that stem cell migration towards SDF-1 is reduced after osteogenic induction even though cells were over-expressed with CXCR4 prior to differentiation. The diminished migration of the differentiated MSCs may be a result of increased extra-cellular matrix deposition, and subsequently greater surface adherence. Matrix metalloproteinases (MMP) play a significant role in several differentiation events. MMPs are also involved in regulating osteoblast proliferation and apoptosis (Karsdal et al., 2002, Batouli et al., 2003). However, it has been shown that MMPs inactivate SDF-1 and this may have an implication on the migration of osteoblasts (Abbott et al., 2004). This could further imply that SDF-1/CXCR4 axis is no longer required once the osteogenic differentiation pathway has been set into motion, (Liu et al., 2013). Additionally, the role of an osteoblast is to secrete osteoid in an orientated manner and the migration of cells may impair this localised production. Liu and co-workers found that when the cells were pre-treated with anti-SDF1 and anti-CXCR4 antibody for 6 hours, BMP-9-induced Runx2 and OSX expression, which are markers of osteogenesis, was reduced significantly, highlighting the importance of SDF1/CXCR4 in initiating osteogenesis (Liu et al., 2013). However, although the CXCR4/SDF1 axis may be involved in the induction of

early stages of osteogenic differentiation, their levels were seen to diminish during osteogenesis (Kortesidis et al., 2005, Liu et al., 2013).

The importance of CXCR4 in improving bone loss in osteopenic animals has been highlighted in previous studies. Lien's study looked at the migration of MSCs transfected with CXCR4 and Cbfa-1 in osteopenic mice and Cho's study similarly looked at the migration of stem cells transfected with CXCR4 and RANK-Fc in osteopenic mice (Lien et al., 2009b, Cho et al., 2009b). However, this study emphasizes the differences in CXCR4 expression between MSCs from ovariectomised rats, adult and young rats, as well as their differences in migration *in vitro*, which was improved after CXCR4 transfection. MSCs from OVX rats have poor migration ability and CXCR4 was observed to be the most effective in improving their migration compared to MSCs from young rats. Moreover, the migration of osteoblasts showed that CXCR4 over-expressing differentiated rat MSCs does not improve their migration.

The *in vitro* work in this chapter showed that MSCs from osteopenic individuals have a poor migration capacity. This may be associated with the inability of osteoporotic patients to heal fractures. The reduced migration to SDF-1 producing cells may possibly indicate that there may be a reduced stem cell number at the fracture site compared to non-osteoporotic individuals. Nevertheless, the maintained migration response upon up-regulation of CXCR4 illustrates the therapeutic potential of CXCR4-expressing MSCs in the treatment of osteoporotic fractures.

Chapter 6. **CXCR4 enhances bone formation in osteopenic rats.**

6.1 Introduction

The long term cure for osteoporosis would entail encouraging a positive bone balance with each remodeling cycle, resulting in a sustained increase in total bone mass and a skeleton that no longer fractures with minimal forces (Lelovas et al., 2008). The major complication of osteoporosis, fracture, is due to lower bone strength and therefore any treatment of osteoporosis must encourage improvement in bone strength. Bone strength is determined by bone geometry, cortical thickness and porosity, trabecular bone morphology, and intrinsic properties of bony tissue (Ammann and Rizzoli, 2003).

Homing of bone marrow stromal cells (MSCs) to bone and bone marrow after transplantation is important for the correction of bone and cartilage disorders. For a MSC transplantation to generate a clinical benefit, MSCs must engraft at the site of bone injury or the bone marrow. Chemokines play significant roles in controlling cellular migration. SDF-1 and its ligand CXCR4 has been shown to be important in causing the migration of cells to the bone marrow. It has been shown that CXCR4 is important for MSC migration to the bone marrow (Wynn et al., 2004b, Yellowley, 2013b). Transplantation of MSCs over-expressing RANK Fc(an antagonist of RANK L) or CXCR4 (Lien et al., 2009a) as well as, CXCR4 and Cbfa1 (Cho et al., 2009a) have been shown to restore bone mass and strength in osteopenic mice. In both these studies, MSCs were intravenously introduced into the mice through the tail vein, and MSCs over-expressing CXCR4 promoted in vivo cell trafficking to bone in OVX mice. This therapeutic effect of CXCR4 transfected MSCs therefore protects against bone loss and increases bone formation.

In this chapter I compare bone formation using MSCs from young rats, MSCs from OVX rats transfected CXCR4 and un-transfected OVX MSCs in an osteopenic rat model. The comparison between the cell types is an important aspect of my thesis because it investigates the beneficial effects of autologous cells from osteoporotic

patients and whether CXCR4 modified cells would provide an advantage benefit in the treatment of osteoporosis.

The aim of this chapter is to investigate the effect of stem cells transfected with CXCR4 on the migration and bone formation in osteoporosis.

The hypothesis was that stem cells transfected with CXCR4 have increased migration to the bone marrow and they would enhance bone formation in osteoporosis.

6.2 Methodology

30 rats with bilateral ovariectomy were left for 16 weeks to become osteopenic. The rats were injected with 0.5ml Sodium Nitroprusside (25mg/ml), followed by cells suspended in 2ml sterile PBS or saline and finally 0.2ml of saline to clear any cells left in the syringe (Figure 6.2).

The rats in this study were divided into two parts (Figure 6.1):

a) Cell tracker group
b) Experimental treatment group

a) **Cell tracker group:** 6 rats were injected with DiI labelled cells. This group of rats was used to track the cells 7 days after injection. This was the 'cell tracker group' (Day 0 control rats). The rats were euthanised 7 days post-injection and the samples retrieved from these rats were also used to obtain baseline data. The rats were divided into 2 groups:
    - DiI labelled Young MSCs + Empty Plasmid (n=3)
    - DiI labelled Young MSCs + CXCR4 (n=3)

b) **Experimental treatment group:** The rest of the 24 OVX rats were divided into 4 groups; Groups A, B and C were injected with cells and Group D

received saline injection. The rats in this group were euthanized after 11 weeks.

The treatment groups were divided as follows:

- Group A – Young MSCs + CXCR4 (n=6)
- Group B – OVX MSCs (n=6)
- Group C – OVX MSCs + CXCR4 (n=6)
- Group D – Saline (n=6)

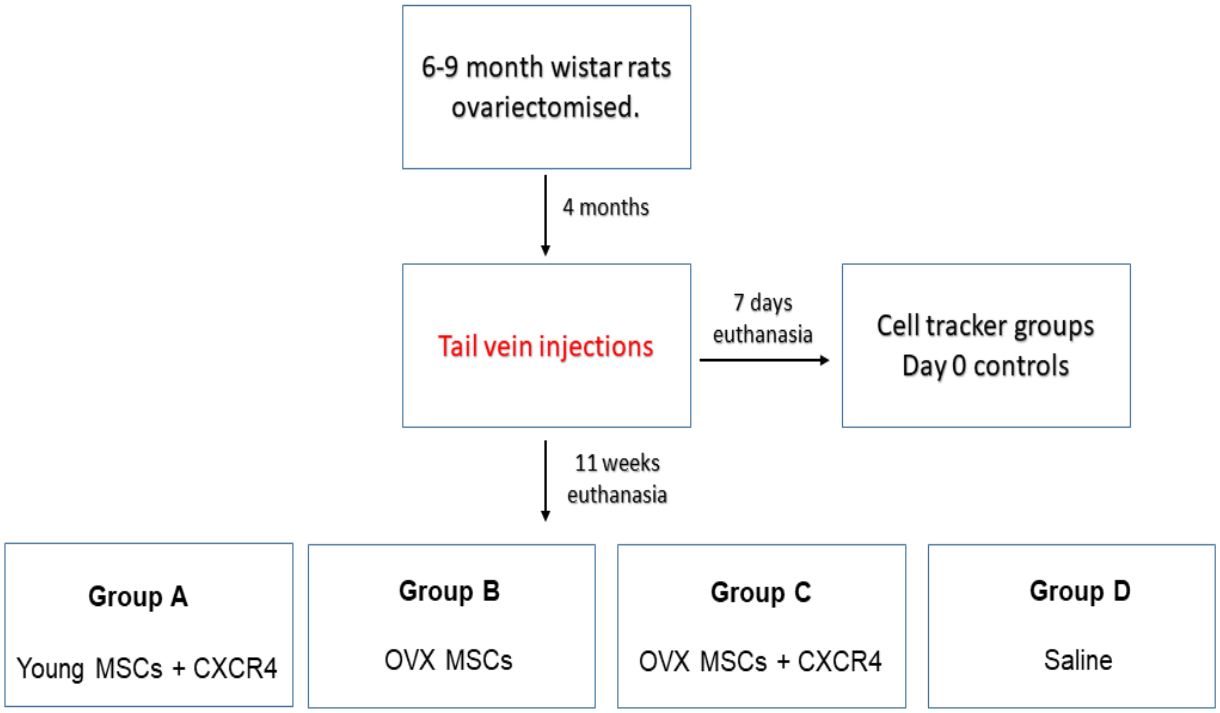

Figure 6.1: A schematic illustration showing the different groups of rats in the in vivo study. Cell tracker group (day 0 controls), were euthanised after 7 days, while the rest of the rats were euthanised after 11 weeks.

From all the rats in both groups, the following samples were retrieved; the left and right femur and tibia, the left humerus and L4 and L5 vertebrae.

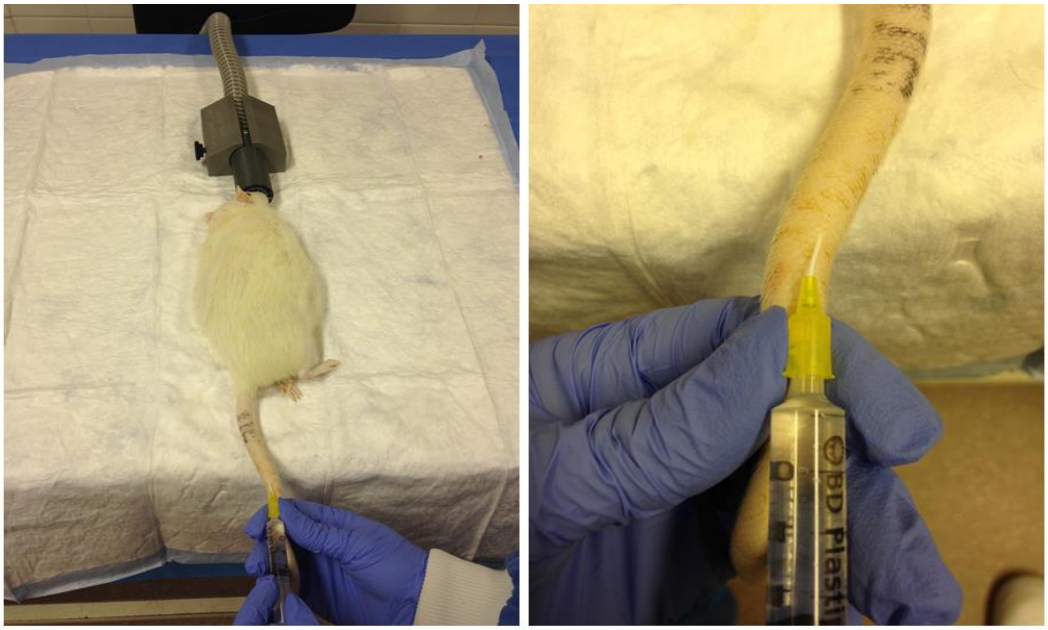

Figure 6.2: Tail vein injection of the rats. The rats were anaesthetised during the injection and were injected with Sodium Nitroprusside, followed by cells suspended in 2ml of saline, and finally a saline flush.

6.2.1 Vertebral compression

Each vertebra was carefully separated from the rest of the vertebral column by halving the discs and cutting the ligaments. The facet joints and spinous processes were preserved to maintain physiological motion of the vertebrae during compression. The vertebrae were compressed at a speed of 0.75mm/min until failure and the ultimate compressive strength was measured (Figure 6.3). The stiffness of the vertebrae was obtained by calculating the gradient of the force deformation graph (Figure 6.4). The mean compressive strength of each vertebrae was obtained from the graphs as explained in chapter 2.

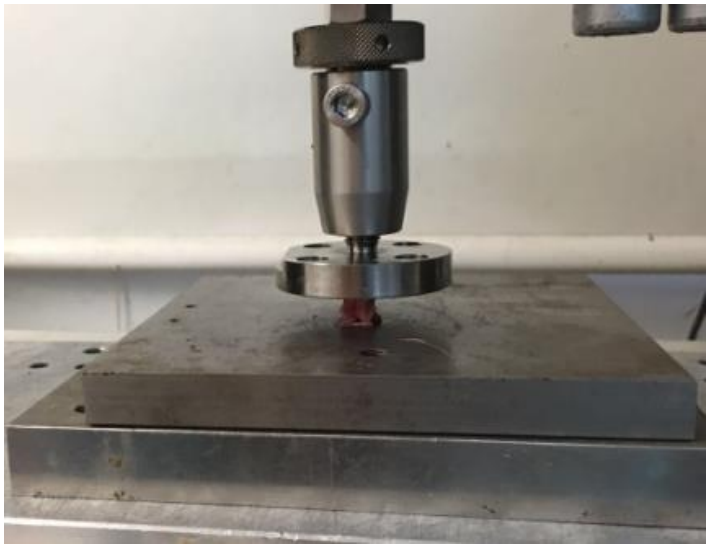

Figure 6.3: A setup to measure the compression of the L4 and L5 vertebrae. L4 and L5 vertebrae was compressed until failure to obtain a force/deformation curve using a Zwick machine.

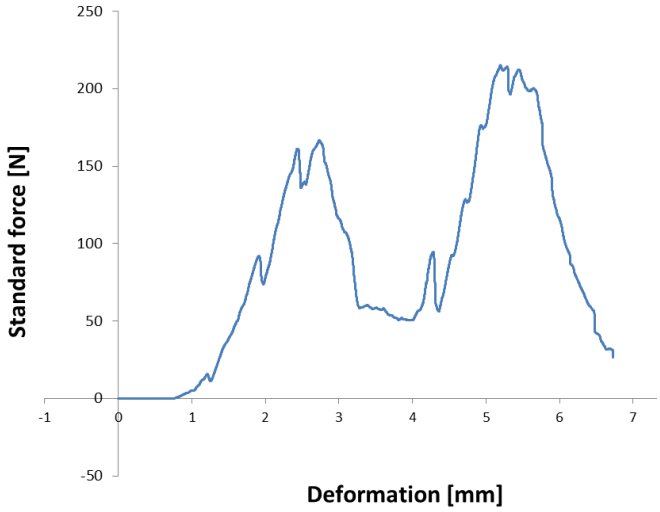

Figure 6.4: A typical Force against Deformation curve for L4 vertebrae.

6.2.2 Three-point bending test of humerus, tibia and femur

The humerus, femur and tibia were retrieved, and all the tissues and muscles around the bone were cleared. The length of the bones was measured, the loading pin was set up at the bone's mid shaft and the bones were loaded at a speed of 0.5mm/min, until failure point when the bone fractured (Figure 6.5). The span between the two

lower supports was set 10 mm apart. A force against displacement graph was generated and it was used to calculate the stiffness and maximum loading force of the bones (Figure 6.5). The stiffness was calculated by working out the gradient of the force displacement curve.

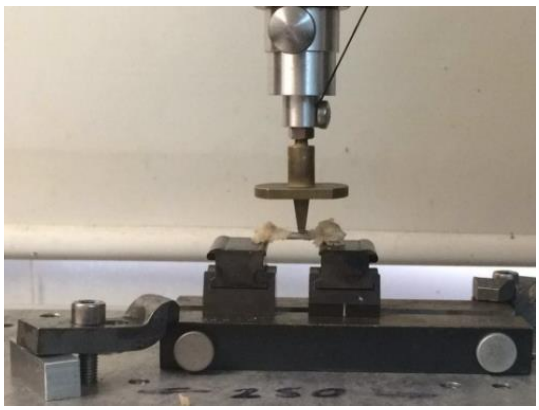

Figure 6.5: A set up to show the 3-point bending test being carried out on a rat femur. Femur, humerus and tibia were loaded until the bones fractured to obtain a force/deformation curve using a Zwick machine.

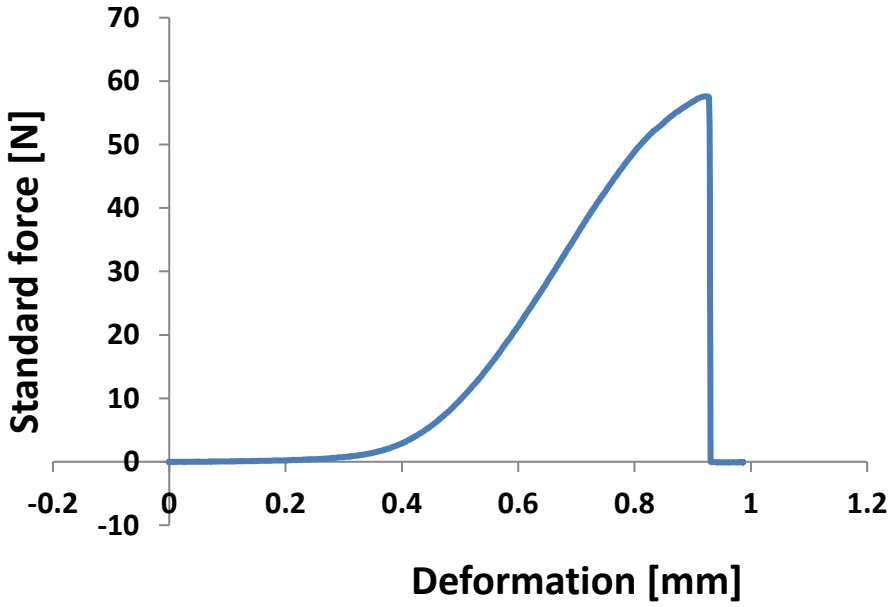

Figure 6.6: A typical Force against Deformation curve for a 3-point bending test of a humerus.

6.2.3 Peripheral Quantitative Computed Tomography (pQCT) of the tibia and femur

The skin and soft tissues around the right and left tibia were removed and the bones were examined in vitro using XCT 2000 pQCT (Stratec, Pforzheim, Germany). As a standard procedure the scout view was obtained to locate the desired scan position. A reference line was then placed through the adjacent joint region (articular face of the distal tibia or femur). The total length of the bone was obtained prior to the measurements and the CT was positioned at the femoral head. The femoral head of each sample was scanned symmetrically and the interval between each slice was 0.2mm (More detail provided in chapter 2).

6.2.4 MicroCT of the metaphysis and epiphysis of the femur

The femurs of rats were fixed in formalin. During the scanning process, the samples were wrapped in cling film, placed in a tube and placed on a sample holder. The x-ray source was set at 70kV with a current of 120uA. To reduce beam hardening, a 0.5mm aluminium filter was used during the scan. During the scan a rotation degree of 0.6°, a frame averaging of 2 and a voxel size of 6um was applied (Figure 6.7).

The NRecon software (Bruker, Belgium) was used to reconstruct the scanned images with the following parameters: smoothing = 2, beam hardening factor correction = 12% and ring artefact reduction = 41%.

The trabecular bone was analysed using a CtAn software (Bruker, Belgium) at two regions of the femur; the proximal (metaphysis) and distal (epiphysis). The growth plate was selected as a reference point (Figure 6.8). To maximise the trabeculae and to ensure that the growth plate was not included in the analysis the following parameters were selected:

- The metaphysis - a volume of 150 slices were selected, 200 slices above the growth plate

- The epiphysis - a volumetric region of 200 slices were analysed which was located 100 slices below the growth plate.

The following was measured:

- Bone Volume (BV)
- Percentage Bone Volume (BV/TV)
- Bone surface (BS)
- Trabecular thickness (Tb.Th)
- Trabecular number (Tb.N)
- Trabecular separation (Tb.Sp)
- Total Porosity

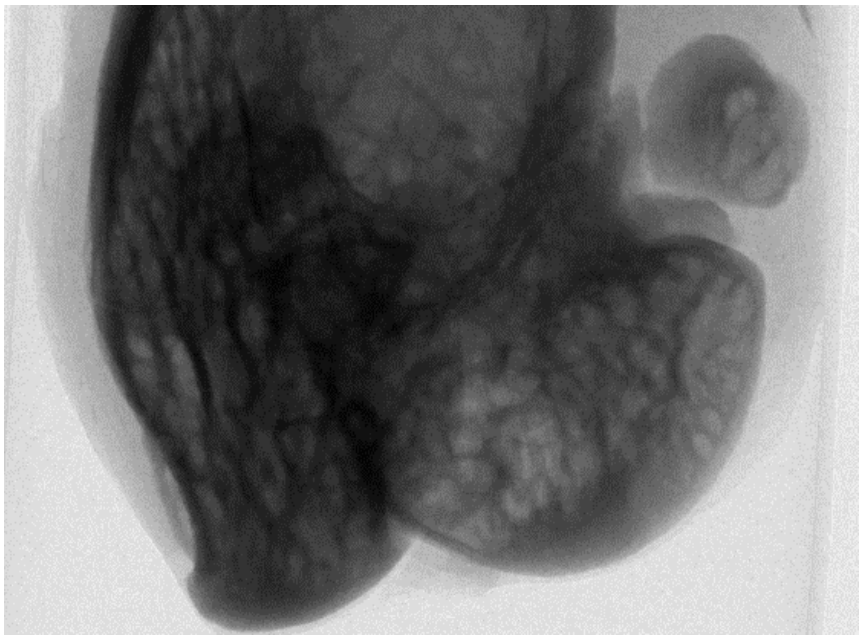

Figure 6.7: A scan illustrating a scout view of the femoral condyle from the OVX rats using the SkySCan 1172 (Bruker). The femoral condyles were scanned at 6µm pixel size.

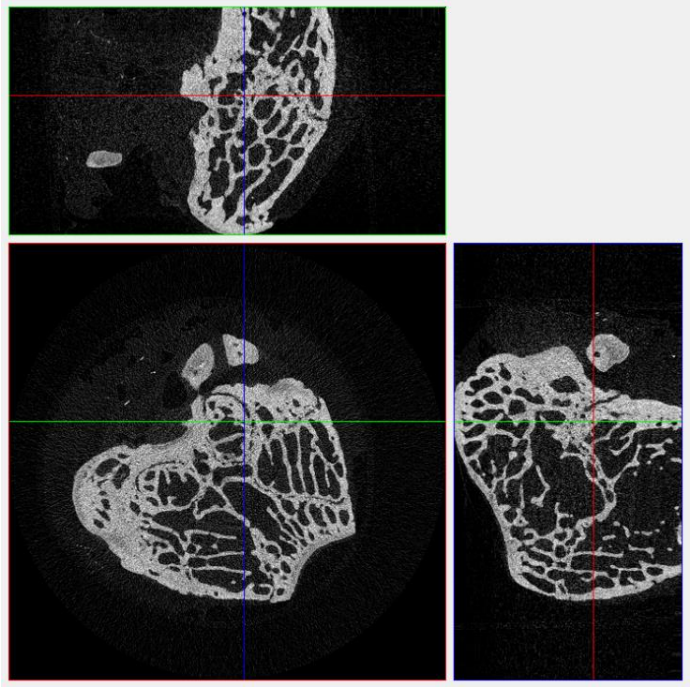

Figure 6.8: The sagittal, coronal and transaxial display of a reconstructed image of the growth plate, whereby a reference point was selected for analysis of bone morphometry.

6.2.5 Fate of systematically injected MSCs

To investigate the homing ability of MSCs, cells were labelled with Vybrant DiI cell-labelling solution (ThermoFisher Scientific, UK). $1x10^6$ cells were plated in a T225 flask. 100ul of the DiI solution suspended in 5ml media and the solution was then added to the cells and left for 30 minutes. The DiI labelled cells were then injected systemically through the tail vein as described above.

The rats were euthanised 7 days (Day 0 controls) post-injection and their femurs and tibias retrieved.

The specimens were fixed in 10% buffered Formaldehyde, and decalcified in ethylenediaminetetraacetic acid (EDTA). Decalcification was confirmed by radiography, after which the specimens were dehydrated in increasing alcohol concentrations, treated with chloroform for 2 days to de-fat the tissue and then

embedded in wax. Sections measuring 5µm thick were made using a sledge microtome (ThermoFisher Scientific, UK).

Sections were de-waxed twice in xylene, placed in two changes of 100% alcohol and then hydrated in serial dilutions of alcohol. After hydration, samples were stained in haematoxylin, a nuclear stain, for 5-10 minutes. The excess stain was washed off by immersing the slides in water for 5 minutes. Samples were then differentiated in 0.5% HCL acid (made up in 70% alcohol) and washed using water. After removing the acid-alcohol, samples were counterstained in 1% eosin for 3-4 minutes, washed in water and dehydrated by serial dilutions of alcohol. Finally, samples were cleaned by xylene and mounted under coverslips using Pertex Mounting Medium (CellPath plc, UK). Samples were observed under a light microscope (KS-300 Zeiss, UK). This was used to observe the DiI labelled that had migrated to the bone (See section 6.2.6 below). The H&E images observed under a light microscope allowed the location of the fluorescent cells to be identified in the bone.

To observe the fluorescent cells, slices were imaged under a fluorescent microscope (Zeiss Apatome 2). The slides were stained with Hoechst 3342 (Thermofisher Scientific, UK) and mounted with Fluoromount^{TM} Aqueous Mounting Medium (Sigma Aldrich, UK) and viewed under an emission wavelength of 617nm.

6.2.7 Statistical analysis

The normality was checked using Kolmogorov- Smirnov and Shapiro Wilkinson test and where the data was normal, comparison was made using independent student T-test. Where the data was non-parametric comparison was made using Mann-Whitney U test with a Bonferroni correction. All data was analysed using SPSS version 24 (Chicago, USA).

6.3 Results

6.3.1 Vertebral compression

The mean compressive strength of both the L4 and L5 vertebrae was higher for the group of rats injected with Young CXCR4 MSCs (179.9±41.5 N and 179.9±75.78 N), followed by the rats injected with OVX-CXCR4 MSCs (164.98±51.47 N and 158.4±48.2 N) respectively. Overall the rats injected with OVX MSCs (141.93 ± 43.1 N and 126.78±29.7 N) and saline (149.4 ± 40.95 N and 136.6 ± 43.63 N) had the weakest L4 and L5 vertebrae respectively (Figure 6.9). Although there was a visible trend whereby the rats injected with young MSCs transfected with CXCR4 had the strongest vertebrae and the groups injected with saline and OVX MSCs were the weakest, these differences were not significant.

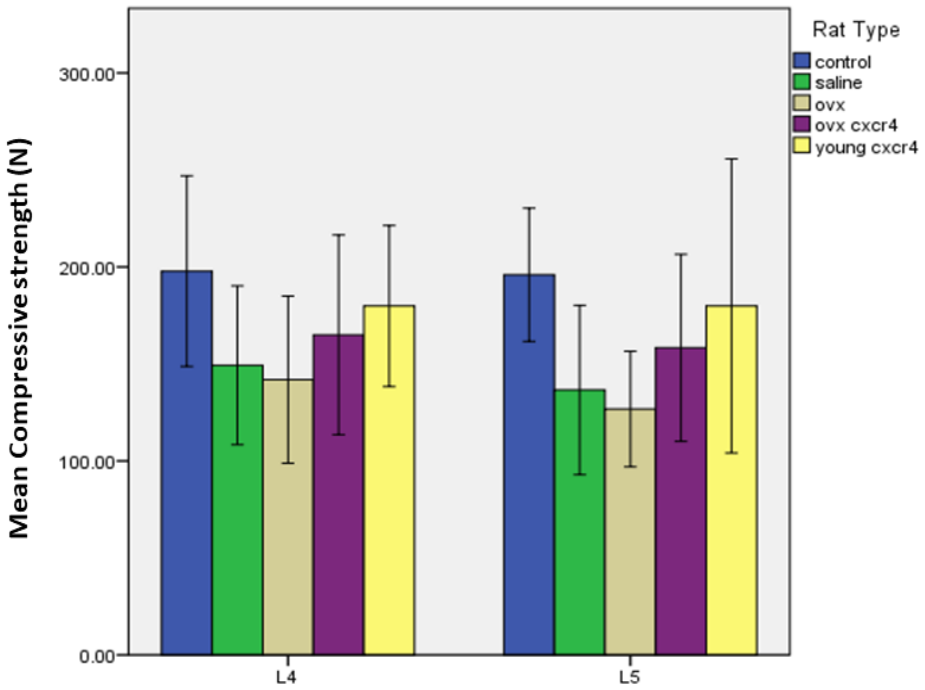

Figure 6.9: The mean compressive strength of L4 and L5 vertebrae. There was no significant difference between the treatment groups. Error bars represent ±1 Standard deviation.

This similar trend was observed for the stiffness of the vertebrae. The stiffness of the L4 vertebrae was significantly higher ($p = 0.008$) for the group that received the Young-CXCR4 MSCs (251.5 ± 61.7 N/mm) in comparison to the rats that received a sham saline injection (99.2 ± 16.2 N/mm) (Figure 6.10).

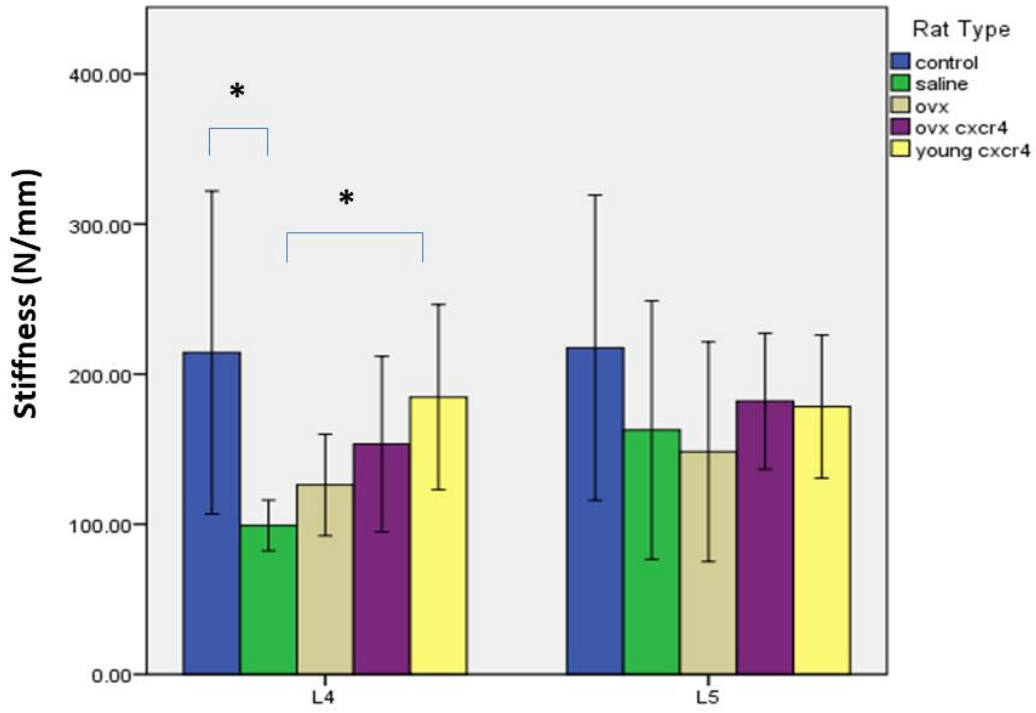

Figure 6.10: The stiffness of the L4 and L5 vertebrae calculated from the gradient of the compression graph (n=6). Error bars represent ±1 Standard deviation. * shows significance $p < 0.05$.

6.3.2 Three-point bending test of the humerus, tibia and femur

The stiffness of the humerus, tibia and femur were obtained by calculating the gradient of the force displacement graph. No significance difference was seen between the different groups for strength and stiffness of the bones. However, there was a trend, whereby the rats receiving the saline injections had the lowest stiffness compared to those that received OVX-CXCR4 MSCs and young-CXCR4 MSCs for the tibia and femur (Figure 6.11, Figure 6.12).

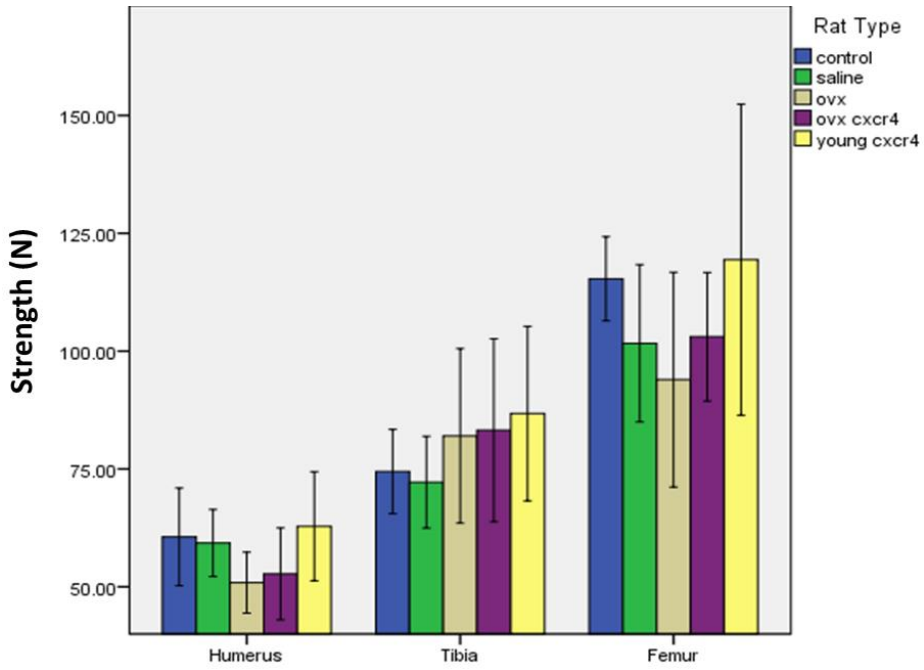

Figure 6.11: The mean strength for humerus, tibia and femur for rats that had either received saline, OVX MSCs, OVX-CXCR4 MSCs or young-CXCR4 MSCs (n=6). Error bars represent ±1 Standard deviation.

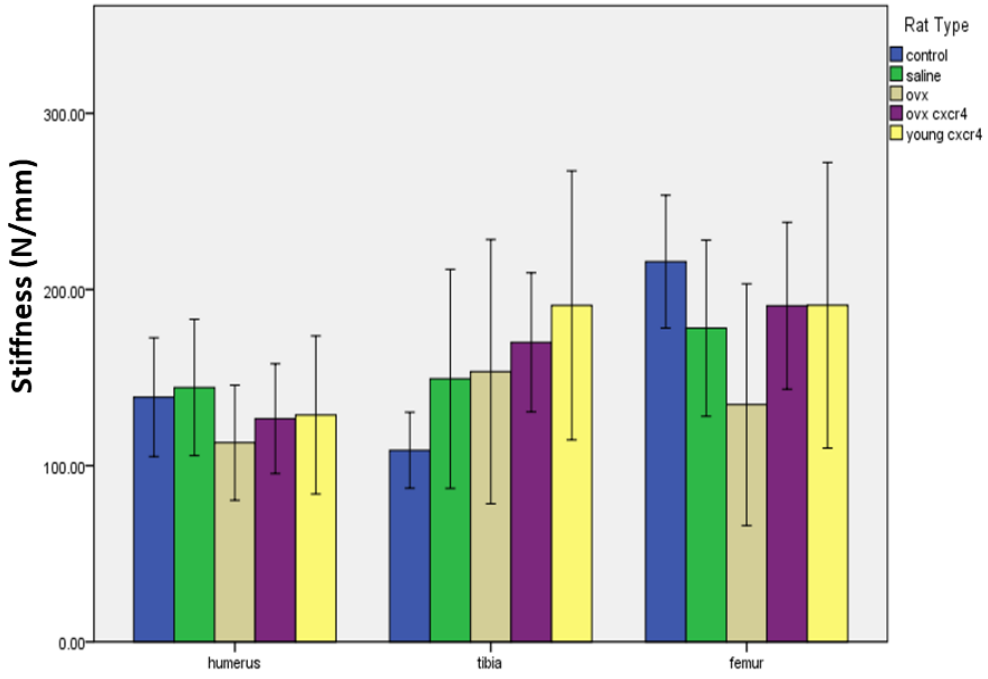

Figure 6.12: The mean stiffness calculated from a force displacement graph for humerus, tibia and femur. The rats had either received saline, OVX MSCs, OVX CXCR4 MSCs or young CXCR4 MSCs (n=6). Error bars represent ±1 Standard deviation.

6.3.3 PQCT of the tibia and femur

pQCT data demonstrated that injecting young MSCs transfected with CXCR4 significantly improved the BMD of the bone in the femoral condyles of the OVX rats, in comparison to injecting them with saline. This significant difference in BMD was seen for the trabecular bone (p=0.005), cortical/subcortical bone (p=0.02) and total BMD (p=0.02). OVX rats injected with young-CXCR4 MSCs transfected had a trabecular BMD of 716.4±105.7 mg/ccm, total BMD of 694±80.1mg/ccm and Cort/sub cort BMD of 715.2±45.4 mg/ccm, while OVX rats injected with saline had a BMD of 535.5±61.6mg/ccm, 563.4±82.9 mg/ccm and 576.2±11 mg/ccm respectively (Figure 6.12 & Figure 6.13).

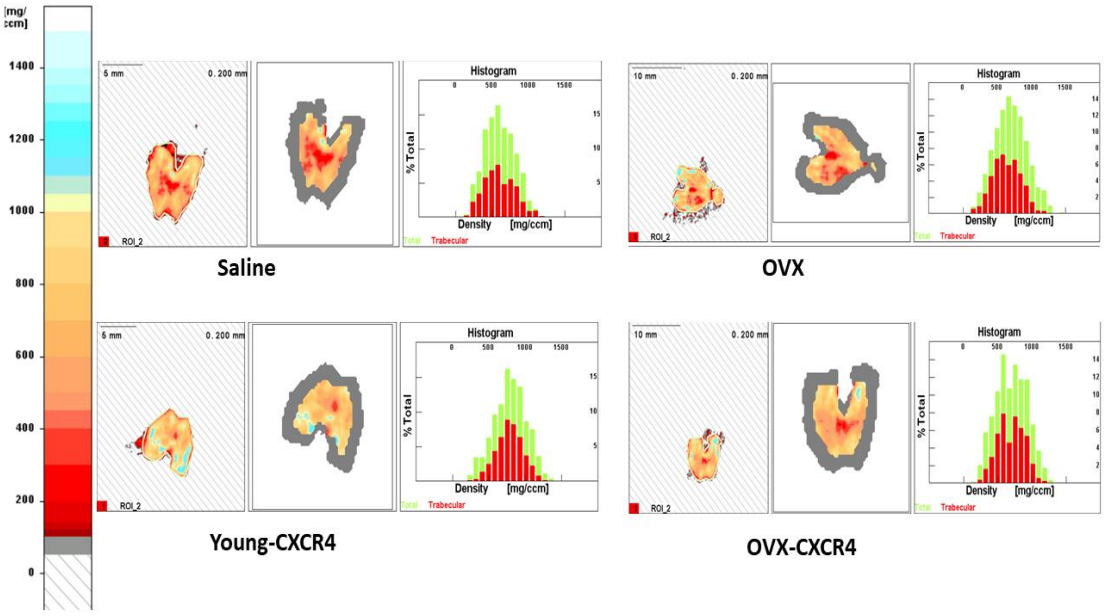

Figure 6.13: A typical scan result generated from PQCT of the femurs from rats injected with saline, OVX MSCs, OVX-CXCR4 and Young-CXCR4 MSCs. The blue regions represent the highest BMD and red represents the lowest BMD.

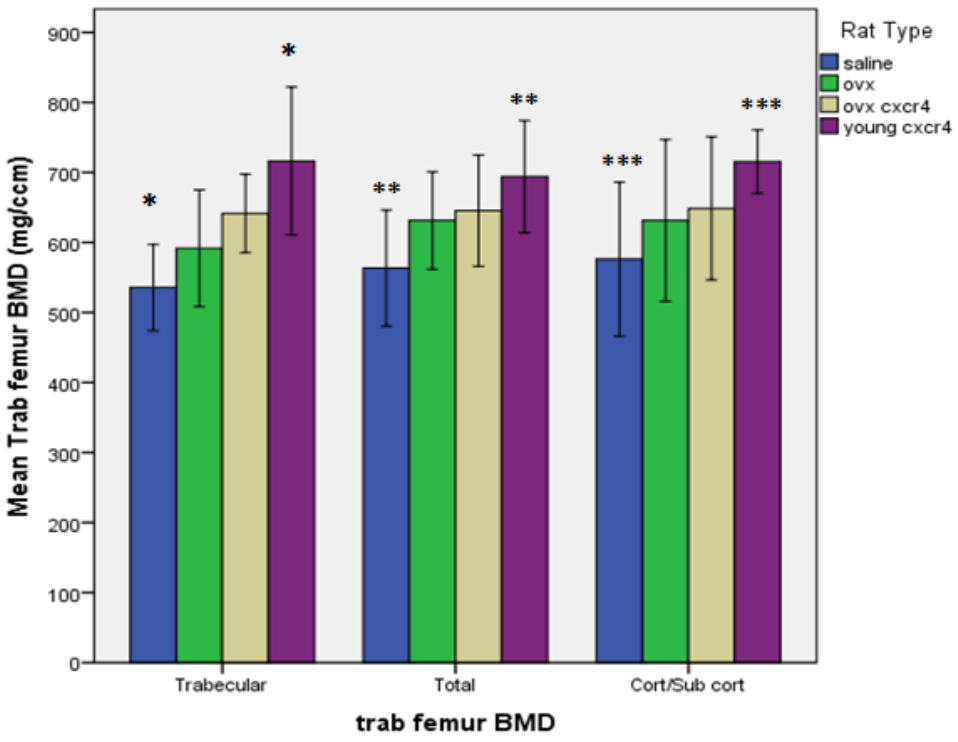

Figure 6.14: The mean BMD of the femur measured using PQCT. The BMD was measured for the femur and tibia and trabecular, cortical and total bone density was analysed for both bones. Significant increase in BMD was observed between rats injected with saline and young-CXCR4 MSCs. Error bars represent ±1 Standard deviation. *, **, ** show significance of p<0.05.

Although there was no significant difference between the groups that received young MSCs, OVX MSCs and OVX-CXCR4 MSCs, there was a general pattern, where the rats that received the OVX MSCs had the lowest femoral BMD, followed by the OVX-CXCR4 in comparison to young MSCs group. This trend was also observed for the tibial BMD. However, there was no significant difference between any of the groups (Figure 6.15).

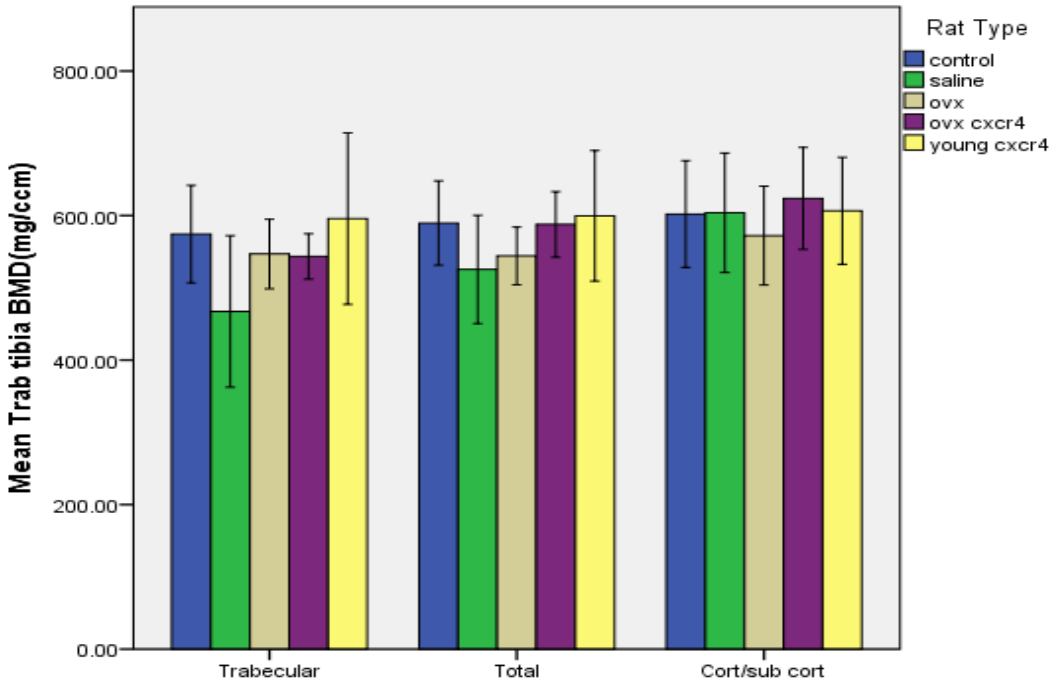

Figure 6.15: The mean BMD of the tibia measured using PQCT. The BMD was measured for the femur and tibia and trabecular, cortical and total bone density was analysed for both bones. Error bars represent ±1 Standard deviation. There was no significance between any of the data.

6.2.4 MicroCT analysis of the femoral condyles

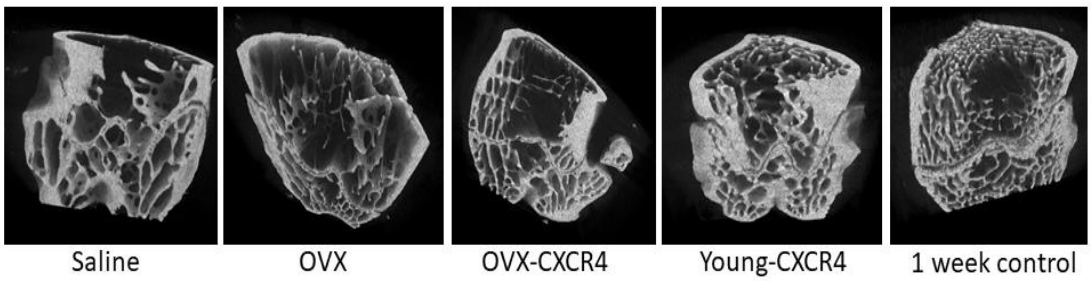

Figure 6.16: Three dimensional images of the femoral heads to illustrate the trabeculae structure of the metaphysis and epiphysis. The rats injected with Young-CXCR4 cells had a denser meshwork of trabeculae compared to those injected with Saline, OVX cells and OVX-CXCR4 cells.

MicroCT analysis of the femoral condyles was carried out on the metaphysis and epiphysis region. Overall the effect of stem cells injection on the bone volume was

subtle compared to saline injection but the groups injected with saline had the worst bone histomorphometric structure compared to the other groups (Figure 6.16).

6.2.4.1 Histomorphometric Analysis of the metaphysis

MicroCT examination of the metaphysis of the femur showed that injecting rats with saline caused a decrease in cancellous bone volume, percentage bone volume, trabecular thickness and increase in total porosity. Injection of Young-CXCR4 MSCs caused an increase in surface area and volume of the cancellous bone in the metaphysis, but this difference was insignificant compared to rats injected with saline and OVX MSCs. Even though there was no difference in trabecular number between groups receiving different types of injections, it was observed that the trabecular thickness was highest for the rats that received the cell injection while the rats injected with saline had the least developed trabecular thickness. Even though this difference was not significant, it implies that cell injections enhanced trabecular thickness in the metaphysis region of the femoral condyles.

There was a general trend in the mean bone surface (BS) area. Although these differences were not significant, the rats treated with young-CXCR4 MSCs had the highest BS area ($9.5x10^7 \pm 5.3x10^7$ μm^2) compared to rats treated with OVX-CXCR4 MSCs ($7.6x10^7 \pm 5.0x10^7$ $\mu m^{2)}$, OVX MSCs ($7.1x10^7 \pm 1.7x10^7$ μm^2) and saline ($7.3x10^7 \pm 5.5x10^7$ μm^2). As expected the Time 0 controls had the highest bone surface area because they were euthanised 7-days post-injection while the other groups of rats were euthanised 5-weeks post-injection (Table 6.1).

Table 6.1: Bone histomorphometry of the metaphysis of the femurs obtained using microCT. The values presented are mean ± standard deviation.

Metaphysis	Saline	OVX	OVX-CXCR4	Young-CXCR4	Day 0 controls
BV (um^3)	$1.1x10^9 \pm 8.5x10^8$	$1.4x10^9 \pm 2.8x10^8$	$1.7x10^9 \pm 1.4x10^9$	$1.5x10^9 \pm 7.0 x10^8$	$2.5x10^9 \pm 8.1 x10^8$
BV/TV (%)	7.2±3.4	8.6±2.0	8.7±4.8	8.5±4.1	13.9±3.1
BS (um^2)	$7.3x10^7 \pm 5.5 x10^7$	$7.1 x10^7 \pm 1.7 x10^7$	$7.6 x10^7 \pm 5 x10^7$	$9.5 x10^7 \pm 5.3 x10^7$	$1.7x10^8 \pm 6.7 x10^7$
Tb.Th (um)	44.8±21.1	56.9±11.2	59.3±23.3	50.7±10.9	44.8±5.6
Tb.N (1/um)	0.002±0.001	0.0015±0.0004	0.0014±0.0008	0.002±0.001	0.003±0.00087
Tb.Sp (um)	359.9±191.7	429.7±17.9	472.2±132.9	399.5±84.4	343.1±36.7
Total Porosity (%)	92.8 ± 3.4	91.9 ± 1.7	91.3 ± 4.8	91.5 ± 4.1	86.1±3.1

The rats injected with saline (p=0.03), OVX MSCs (p = 0.004) and OVX-CXCR4 (p = 0.02) MSCs had a significantly lower bone surface area compared to the rats injected with Young-CXCR4 MSCs (p = 0.07) (Figure 6.17). This demonstrates that with time, a further 11 weeks, the bone surface area of the cancellous bone in the femur reduced. The group injected with Young-CXCR4 MSCs had lower bone surface area compared to Time 0 controls but there was an increase in bone formation or retention of bone compared to the other injected groups.

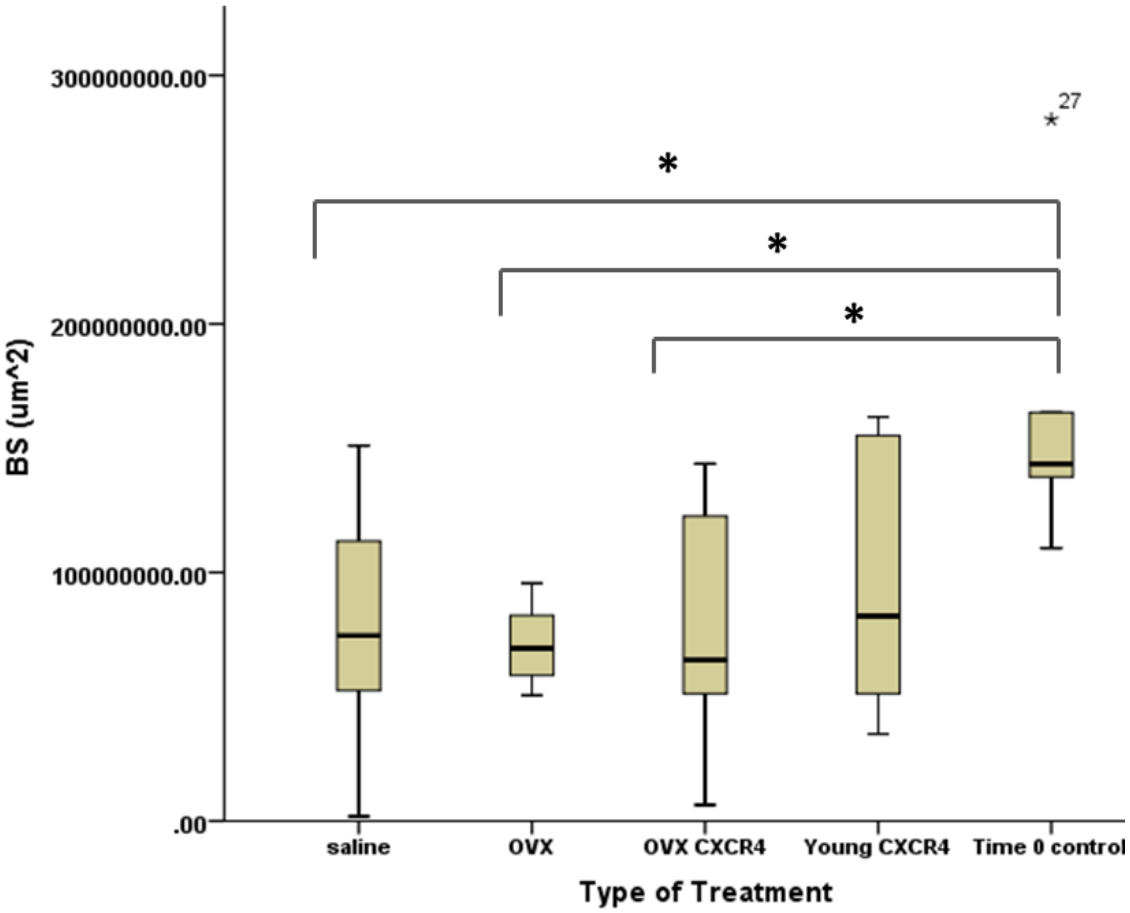

Figure 6.17: Data showing the mean Bone Surface (μm^2) of the femur obtained from rats treated with saline, OVX MSCs, OVX-CXCR4 MSCs and young CXCR4 MSCs. Compared to time 0 controls, all groups of rats, except the ones that received young-CXCR4 MSCs, had significantly lower Bone Surface Area. The error bars represent standard error of the mean * shows significance $p < 0.05$.

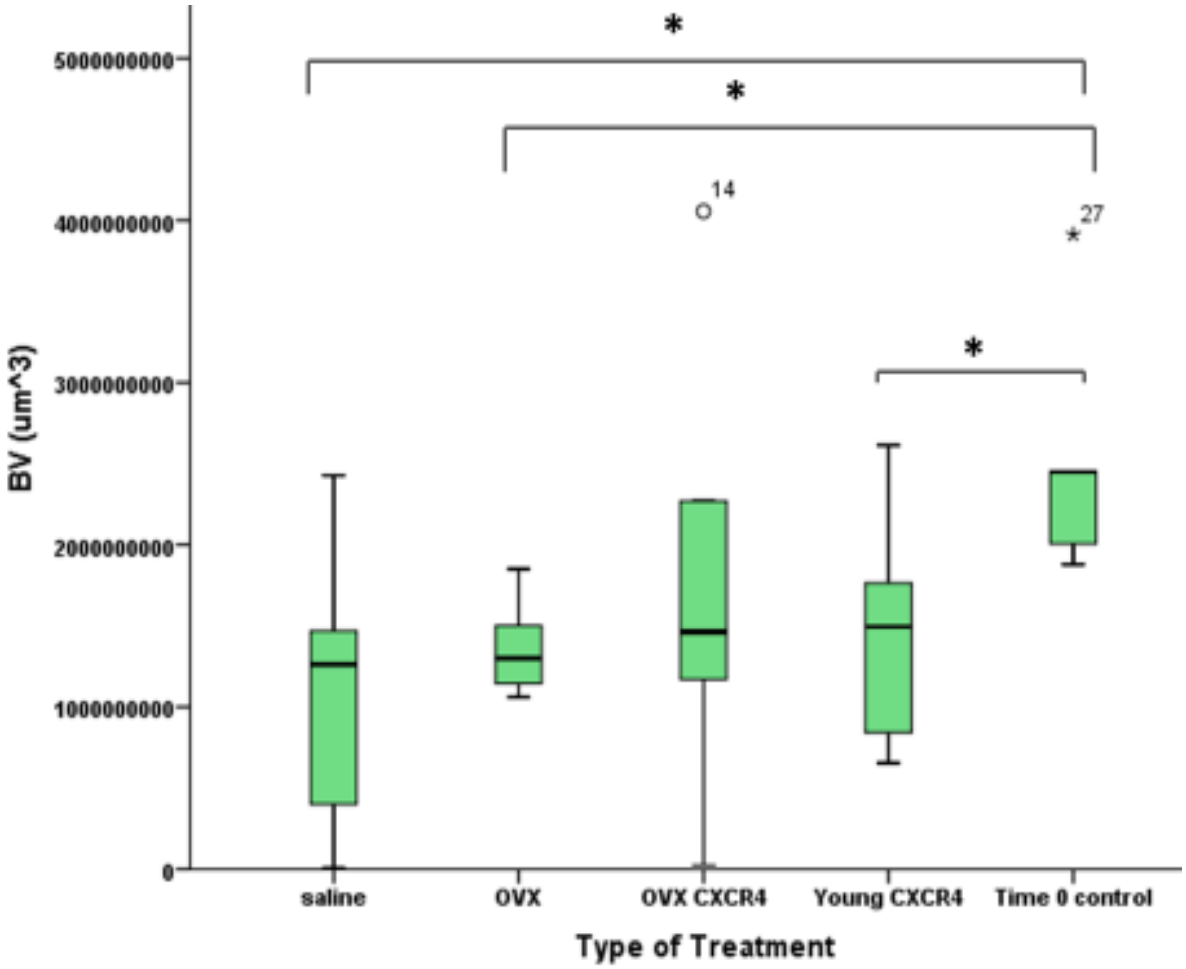

Figure 6.18: Data showing the mean Bone Volume (μm^3) of the femur obtained from rats treated with saline, OVX MSCs, OVX-CXCR4 MSCs and young CXCR4 MSCs. The error bars represent standard error of the mean. * shows significance p < 0.05.

In all groups injected with stem cells bone volume (BV) was increased in comparison to saline injection. Although these differences were not significant, the rats treated with OVX-CXCR4 MSCs had the highest BV ($1.7x\ 10^9 \pm 1.4 \times 10^9\ \mu m^2$) compared to rats treated with Young-CXCR4 MSCs ($1.5 \times 10^9 \pm 7.0 \times 10^8\ \mu m^{2)}$, OVX MSCs ($1.4 \times 10^9 \pm 2.8 \times 10^8\ \mu m^2$) and saline ($1.1 \times 10^9 \pm 8.5 \times 10^8\ \mu m^2$).

6.2.4.2 Histomorphometric Analysis of the Epiphysis

MicroCT examination of the epiphysis of the femoral head revealed a mild but statistically insignificant effect of injection of transfected and un-transfected MSCs. The increase in percentage bone volume, bone surface and trabecular number was evident in groups injected with Young-CXCR4 MSCs but this was insignificant compared to the other groups especially the saline group. The variability within the groups produced data which affected the significance (Table 6.2).

Table 6.2: Bone histomorphometry of the epiphysis of the femurs obtained using MicroCT. The values presented are mean ± standard deviation.

Epiphysis	Saline	OVX	OVX-CXCR4	Young-CXCR4	Day 0 controls
BV (um^3)	$3.1x10^9 \pm 1.3x10^9$	$3.5x10^9 \pm 5.7x10^8$	$3.3x10^9 \pm 1.6x10^9$	$3.5x10^9 \pm 9x10^8$	$3.6\ x10^9 \pm 2.4\ x10^8$
BV/TV (%)	13.4 ± 3.3	15.9±3.0	15.2±7.6	15.1 ± 3.0	18.1±1.03
BS (um^2)	$2.1x10^8 \pm 1.8x10^8$	$1.7x10^8 \pm 3.8x10^7$	$1.6x10^8 \pm 7.5x10^7$	$2.4x10^8 \pm 1.9x10^8$	$2.2x10^8 \pm 3.68x10^7$
Tb.Th (um)	51.4 ±17.1	51.4±5.1	47.0±15.4	45.0±12.2	42.5±4.4
Tb.N (1/um)	0.003 ± 0.001	0.003±0.001	0.003±0.001	0.004±0.001	0.004±0.00053
Tb.Sp (um)	414.8 ± 133.5	398.9±19.5	401.4±46.4	424.5±116.9	372.3±25.8
Total porosity (%)	86.6 ± 3.4	84.1 ± 2.9	84.5 ± 7.0	84.9 ± 3.0	81.9±1.05

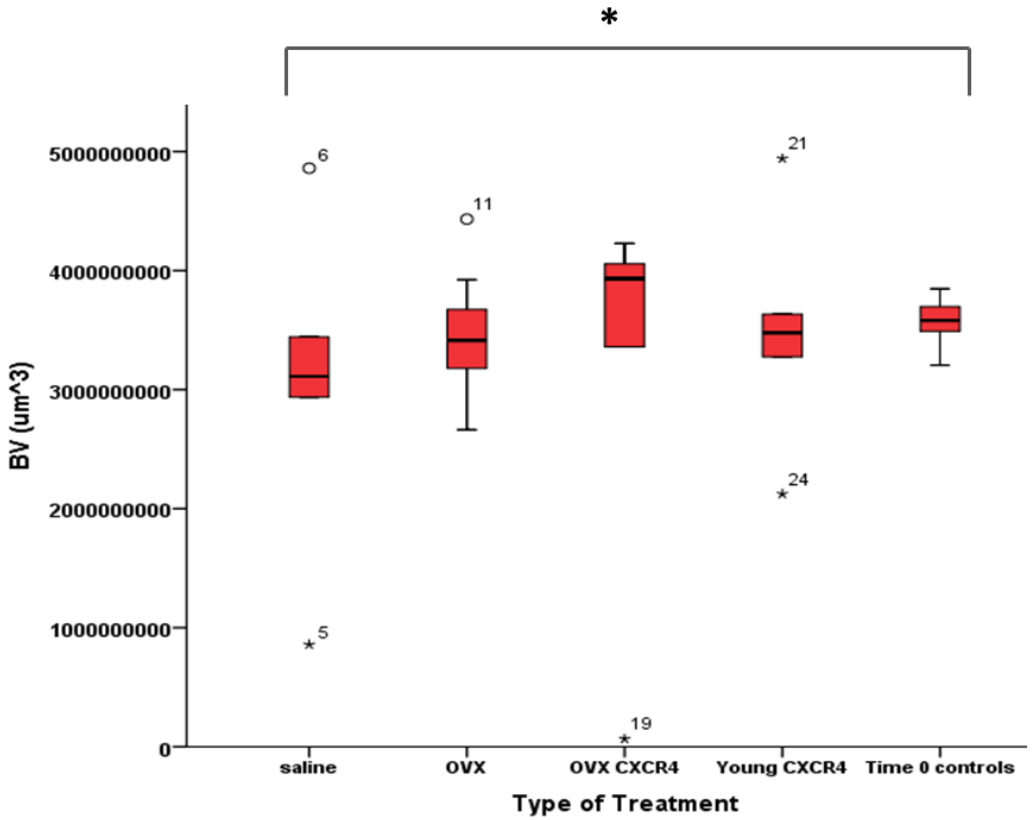

Figure 6.19: Bone volume of the epiphysis of the femur measured using MicroCT, for the different groups of rats treated with saline, OVX MSCs, OVX-CXCR4 MSCs and Young-CXCR4 MSCs. Compared to the Time 0 control rats, only the rats injected with saline had significant lower bone volume (BV), showing that cell injections that a positive effect on bone volume. The error bars represent standard error of the mean. * represents p<0.05.

Although there was variability in the mean bone volume within each group of rats, the mean bone volume of the epiphysis was improved with various cell treatments. The rats injected with the saline had the lowest bone volume ($3.1x10^9 \pm 1.3\ x10^9\ \mu m^3$) compared to rats injected with OVX MSCs ($3.46\ x10^9 \pm 5.7x\ x10^8\ \mu m^3$), OVX-CXCR4 MSCs ($3.26x10^9 \pm 1.6x10^9\ \mu m^3$) and young-CXCR4 MSCs ($3.49\ x10^9 \pm 9x10^8\ \mu m^3$). The rats from Time 0 group had significantly higher bone volume compared to those injected with saline injections ($p = 0.01$). There were no significant differences between the Time 0 rat group and rats injected with any cell type, irrespective whether they were transfected or un-transfected or from young or OVX rats (Figure 6.19).

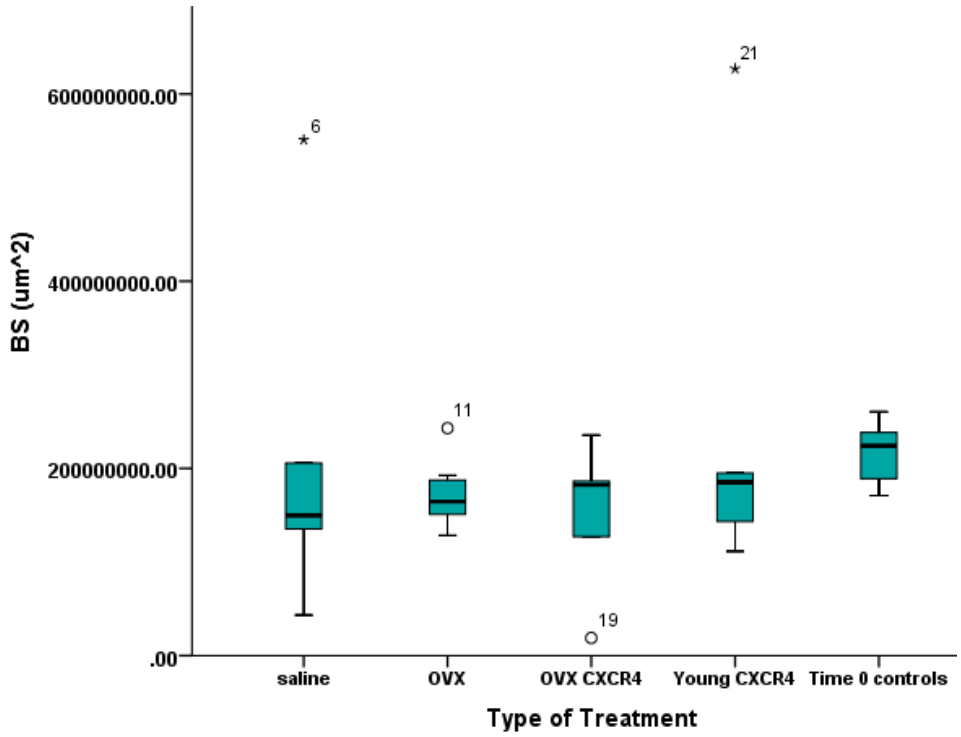

Figure 6.20: Bone surface area of the epiphysis of the femur measured using MicroCT, for the different groups of rats treated with saline, OVX MSCs, OVX-CXCR4 MSCs and Young-CXCR4 MSCs. The error bars represent standard error of the mean. * represents p<0.05.

The average epiphyseal bone surface was highest for the rats injected with Young-CXCR4 MSCs ($2.4x10^8 \pm 1.9 x10^8 \mu m^2$) in comparison to all the other groups including Time 0 controls. However this difference was not significant and this could be due to large variability in the data (Figure 6.20).

6.2.6 Fate of systemically injected MSCs

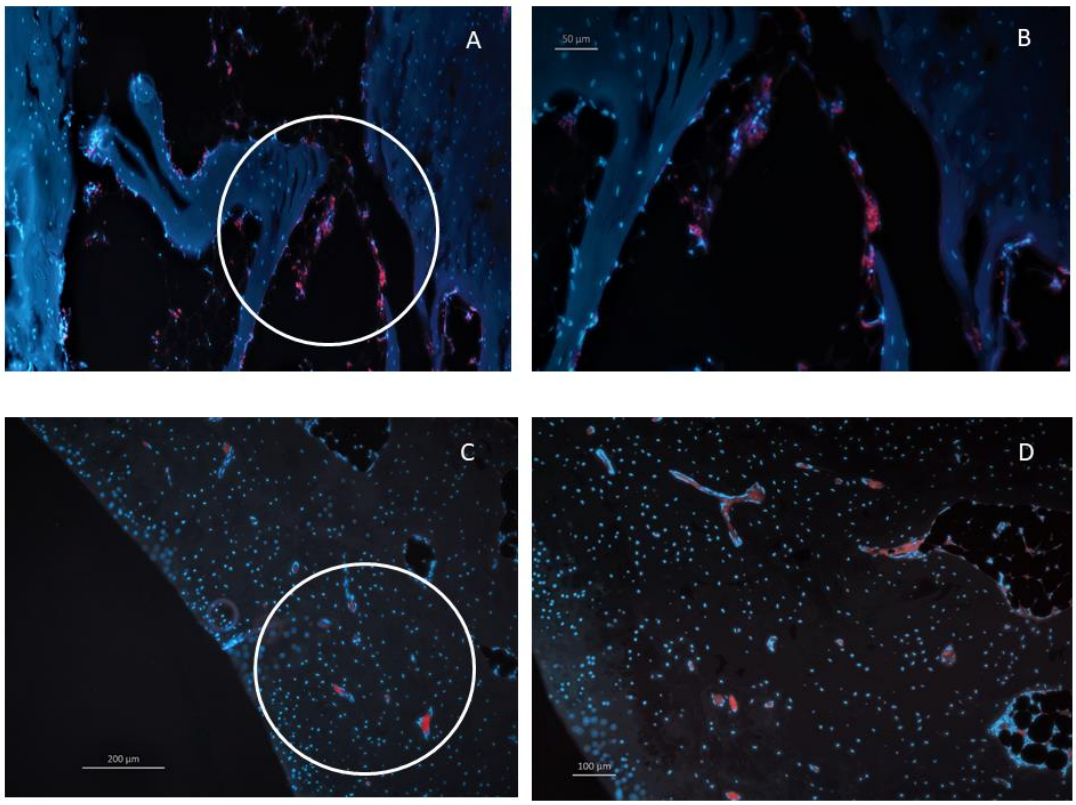

Figure 6.21: DiI labelled cells (Red) located in the femur of rats systemically injected with Empty plasmid-Young MSCs (A & B) and CXCR4-Young MSCs (C & D). These images were taken from 5um wax histology slides with the nucleus stained for Hoechst (Blue). 7 days post-injection, the DiI labelled cells were located in the blood vessels in the bone. Images A and C are taken at x10 magnification and Images B and D are taken at x20 magnification.

Fluorescent imaging proved that the DiI labelled MSCs homed to the bone after 7 days. In addition to blood cells, numerous DiI labelled nucleated cells were observed in the blood vessels in the cortical and trabecular bone of the femoral condyles. However, it would have been interesting to observe whether these cells engrafted and played a role in enhancing bone formation by keeping these labelled cells for a longer time in the rats. There did not seem to be any evident difference between Young MSCs transfected with an empty plasmid (Group D) or those transfected with CXCR4 (Group E) (Figure 6.21 & Figure 6.22). This could possibly be because

Young-MSCs already express high levels of CXCR4 as shown in chapter 3 and therefore have a high migration capacity. Additionally, no fluorescent cells were visible in slides from rats that were injected with unlabelled cells (Figure 6.23).

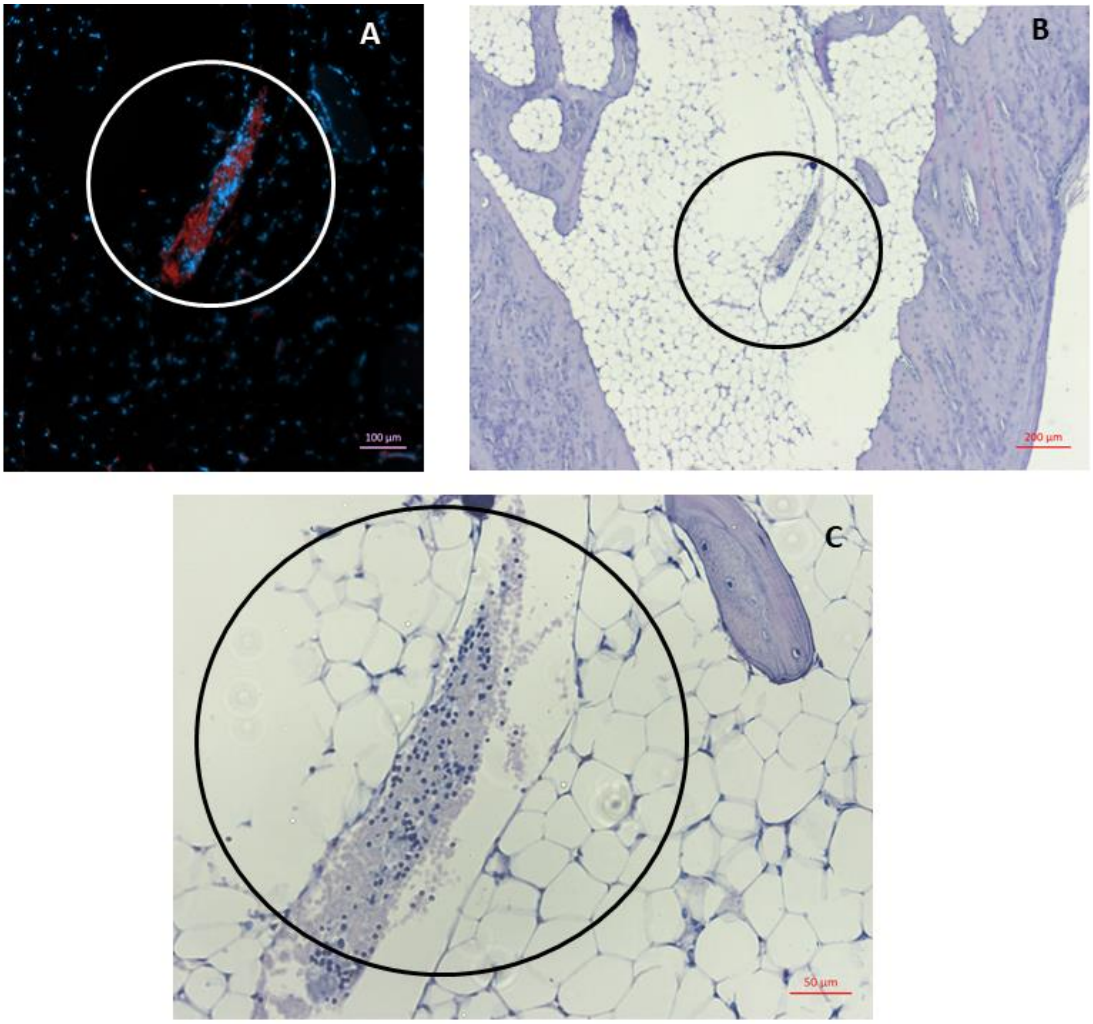

Figure 6.22: DiI labelled cells located in the blood vessels of the femur (A).H & E staining of these cells of wax histology slides of the femur. The dark stained cells are nucleated cells which correspond to the DiI labelled cells (B & C).

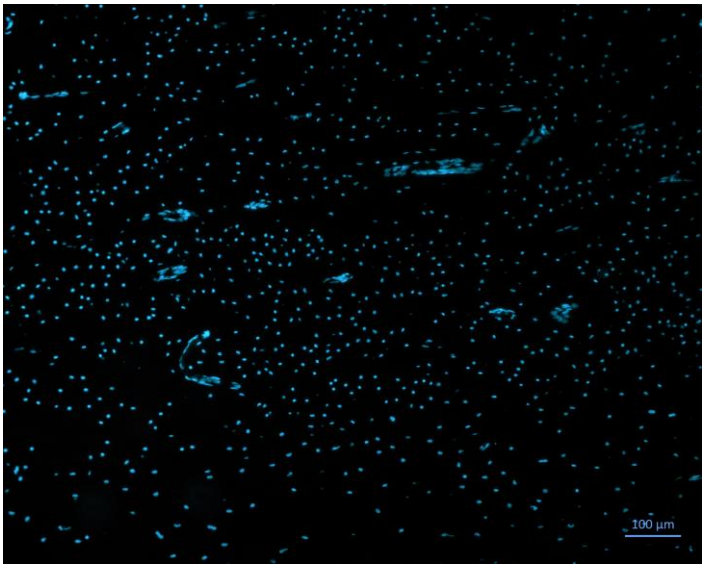

Figure 6.23: Fluorescent images of histological slides of femur from rats injected with unlabelled cells. There were no visible DiI labelled cells in the blood vessels of these rats.

6.4 Discussion

Osteoporosis causes thinning of the trabeculae and an increase in cortical porosity, bone fragility and fracture risk (Chen et al., 2013). A fracture occurs when an externally applied load to the bone exceeds the strength of the bone. The strength of bone therefore relies not only on bone mass and cortical strength but also on the trabecular structure which determines the microarchitecture of bone. In osteoporosis the bone remodelling process is compromised due to the imbalance between osteoclasts and osteoblasts (De Souza et al., 2005, Chen et al., 2013, Laib et al., 2001). The hypothesis of this study was that stem cells injected intravenously are able to contribute to bone formation by migration to the bone and differentiation into osteoblasts. I also hypothesis that bone formation would be further enhanced by transfecting stem cells with CXCR4 which would enhance their migration to bone.

Fragility fractures commonly occur in the metaphysis. The trabeculae strengthen the thin cortex and transfer loads from the joint to the thicker cortical bone in the diaphysis

(Bala et al., 2015). It is therefore important for any osteoporotic treatments to have an effect on the metaphysis of the bones such as proximal femur, distal tibia, proximal humerus and distal radius. The deteriorating changes of bone in the OVX rats injected with saline was reflected by the thinning of the trabeculae, reduced bone surface area and bone volume in the proximal femur. These micro-structural changes are common problems in osteoporotic patients, causing fragility fractures (Chen et al., 2013, Kiernan et al., 2016). However, the results from this study reflect the enhancement of trabecular thickness in the metaphyseal region of the femoral condyles in OVX rats injected with cells. OVX rats that received saline injections had no improvement in trabecular thickness. Although these results were insignificant, they highlight the positive effect of cell injections on the trabeculae.

The predominant issue with MSCs from OVX animals or osteoporotic patients is their poor capability to migrate, differentiate as well as be retained at the bone marrow site. The previous chapters in this study highlighted the poor CXCR4 expression as well as migration of MSCs from OVX rats. In this chapter, CXCR4 transfected MSCs were therefore intravenously injected into OVX rats, to see if their migration would be enhanced to the marrow, hence improving bone formation in these animals. The important aspect of osteoporotic treatments is not only to increase BMD but also to ensure this bone formation is retained and the bone microarchitecture is improved. In this chapter, I showed that injecting young MSCs transfected with CXCR4 significantly enhanced BMD ($p=0.02$) and L4 vertebral strength ($p=0.008$) compared to the group of rats that received a saline injection. It was also highlighted that injection of OVX and OVX-CXCR4 MSCs helped improve the BMD and trabeculae structure in the femoral condyles of the OVX rats compared to the groups that received saline injection.

The deteriorating bone structure of the OVX rat model was clearly observed as the Time 0 control rats had the most bone volume and density compared to the rest of the OVX rats which were treated for a further 10 weeks. However, the rats injected with cells had a higher bone surface area and volume compared to the rats injected with saline. There was an increased BMD in the animals injected with cells using pQCT which seemed to contradict the modest increase in bone volume observed by microCT. This is similar to results by Huang and co-workers, 2016 who injected allogeneic MSCs from 8 week old rats into OVX rats and looked at their bone formation. They concluded that although the systemic transfusion of allogeneic MSC is a safe procedure, administering cells only may not be the most effective procedure for preventing osteoporosis. They also observed an increase in BMD using PQCT but no difference in trabeculae structure using microCT (Huang et al., 2016). These differences could be because PQCT measures global bone formation while microCT is more specific as it measures slices specifically for analysing trabeculae structure.

Regenerating the trabeculae may not be as simple as it seems. Cunningham et al, 2005 investigated the recovery of the trabeculae in the right os-calcis in an ovine model. After removal of the trabeculae in the os-calcis animals were returned to their normal daily activities however the trabeculae did not regenerate and the os-calcis remained hollow but with increased density in the cortical bone. This could be because the load was distributed to the upper and lower beams of the bone, hence the increase in density and thickness of the cortical bone (Li et al., 2003, Kim et al., 2008, Cunningham et al., 2005). Once interconnectivity between trabeculae has been compromised it is difficult to reform cross-links because of the mechanisms of load transfer which is altered due to reduced number of bridging elements.

Injecting MSCs, irrespective of the fact that they were from OVX or young rats or uninfected or infected protected against bone loss associated with ovariectomy in rats compared to rats injected with saline. These results are similar to those reported by

Lien and co-workers and Cho and co-workers, 2009 who injected CXCR4-infected cells in OVX rats and observed increased BMD in rats injected with cells compared to sham injections. However, both these studies used an additional recombinant protein that improves bone formation, to not only improve migration but to also enhance the ability of the MSCs to differentiate to bone (Lien et al., 2009, Cho et al., 2009a).

Lien injected CXCR4 and Cbfa-1 transfected MSCs and Cho injected CXCR4 and RANK-fc. Both studies showed even more improved bone formation when they were injected with cells co-transfected with the recombinant proteins. The CXCR4 transfected MSCs were shown to migrate to the bone marrow, 7 days after injection and improved bone formation in OVX rats compared to the sham control rats. However, cells transfected with CXCR4 and the additional recombinant proteins, had the most improved bone formation, showing the therapeutic role of Cbfa-1 and RANK-Fc. They showed that, in addition to improving migration of MSCs, an additional factor is necessary to boost the differentiation potential of MSCs (Cho et al., 2009b, Lien et al., 2009).

Indeed, it is interesting to see whether these homed MSCs contribute to bone formation. Lien and co-workers labelled their cells with Qdot nanoparticles for short term tracking and fluorescently labelled them with Green Fluorescent Protein (GFP) for long term tracking of injected MSCs in mice. They observed homing and engraftment of labelled MSCs to the bone marrow which was enhanced by CXCR4 (Lien et al., 2009). However, it is unknown whether these MSCs have a paracrine function or whether they actually contribute to the bone mass.

Sheyn and co-workers, 2016 injected a combinational therapy of MSCs and PTH and showed that PTH aided with the migration of MSCs and also played an anabolic role in bone formation in vertebral defects in pigs (Sheyn et al., 2016). This highlights the

importance of improving migration of cells and also stimulating their ability to form bone. It is important for cells to home and engraft into the marrow cavity (Kim et al., 2006) as well as help with the bone loss (Cho et al., 2009a, Kim et al., 2006). Studies by Lien and Cho showed significantly improved BMD after injection of MSCs, however these results were based on pQCT data and unlike this work did not look at changes in the trabeculae using MicroCT. Additionally, they induced osteopenia in younger mice (around 3-4 months old) compared to this study. In this study the rats were ovariectomised when they were around 6-9 months old and were injected when they were 10-13 months. The work in this chapter has utilised much older rats, therefore the rats were injected when their bone structure would have been more deteriorated due to age as well as osteopenia.

Although significant differences were seen in this study, there were a number of trends noted that were not significant. The number of significant differences seen in this study may have been increased by increasing the number of animals in each group. A power calculation done in the main discussion (Chapter 7) demonstrates that n=10 would have helped the study to achieve a power of 80%.

However, the novelty and significance of the work in this chapter is that it investigates the prospect of injecting autologous stem cells in osteoporotic patients. Injecting OVX-MSCs may not be as beneficial as injecting Young-MSCs or Young-CXCR4 MSCs, but it still enhances bone formation although to a lesser extent.

Chapter 7. **Main Discussion**

At the moment treatments for osteoporosis focus on improving the catabolic bone remodeling that is, preventing bone resorption and most treatments do not encourage bone formation. Mesenchymal stem cells have a regenerative capacity and may be able to enhance anabolic bone formation. Information in the literature and presented in my thesis suggests that mesenchymal stem cells in patients with osteoporosis are down regulated and do not form bone as well as cells from normal individuals (Rodríguez et al., 1999). Cell recruitment, migration and homing are very important factors for neovascularisation, chondrogenesis, osteogenesis and therefore bone remodelling. MSCs are required to find their way from the periosteum, local bone marrow, the circulation and also the distant bone marrow to sites of injury, which is in part a function of the SDF1/CXCR4 pathway (Yellowley, 2013a).

The aim of this thesis was to investigate whether mesenchymal stem cells (MSCs) over-expressing CXCR4 would improve bone formation in osteoporosis. The results of the experiments in previous chapters support the hypotheses that:

1) Bone marrow MSCs from OVX rats have reduced migration and osteogenic differentiation capability and CXCR4 expression compared to MSCs from young and senile rats.

2) Genetically modifying MSCs to over-express CXCR4 would improve the migration of MSCs towards SDF-1.

3) MSCs genetically modified to over-express CXCR4, when injected intravenously in ovariectomised (OVX) rats would improve bone formation compared to those injected with saline.

This study has established the migratory and differentiation abilities of MSCs from young, adult and OVX rats. I hypothesized that MSCs from OVX rats would have reduced proliferation and osteogenic differentiation ability, but increased adipogenic

differentiation. However, the MSCs from the three groups of rats had no significant differences in proliferation and CD marker expression, showing that age and osteopenia do not affect the phenotypic properties of MSCs. However, MSCs from young rats were capable of greater adipogenic and osteogenic differentiation when compared to cells isolated from osteopenic rats. This is in agreement as well as in contrast to studies carried out by other groups (Goergen et al., 2013, Beane et al., 2014, Asumda and Chase, 2011, Singh et al., 2016). However, if findings from this study are correct then using autologous MSCs in patients that are old and osteoporotic may have an implication on subsequent bone formation as these cells would not be effective.

MSC migration from blood, periosteum and marrow to the site of fracture is particularly important in fragility fractures. These fractures are at greater risk of delayed healing due to the poor fixation of screws used to stabilise the fractures and the reduced ability of patients to form bone and unite the fracture (Giannoudis et al., 2007). The CXCR4-SDF1 pathway plays a crucial role in the retention and engraftment of MSCs during fracture repair. At any site of injury there is a release of SDF1, which attracts CXCR4 expressing MSCs (Wynn et al., 2004a). In this thesis results from flow cytometry demonstrated significantly lower expression of CXCR4 on MSCs from OVX rats in comparison to young and senile MSCs impairing their ability to migrate in vitro in a Boyden Chamber. This new information indicates that the poor bone formation associated with osteoporosis may be associated with an impaired CXCR4 expression in these cells. This migration was seen to be improved when the cells were transfected with CXCR4.

In the in vivo experiments the homing destination of the injected MSCs was investigated by tracking DiI labelled MSCs in a group of rats. Although the cells were detected in the blood vessels in the bone, this was a short-term study and the animals were euthanized after 7 days and it was not possible to determine if these cells

differentiated into osteoblasts and contributed directly to bone formation. This is a limitation and a longer-term study investigating the engraftment of CXCR4 transfected stem cells is warranted. It is possible that these cells either had a direct role in bone formation or that they may have been acting in a paracrine fashion. Indeed, it has been shown that osteoblasts or MSCs differentiated to osteoblasts have high α_2-integrin expression which is important for cell adhesion as it helps to facilitate mechanotransduction and serves as a mechanosensor from the cells to the matrix (Shih et al., 2011).

In vitro CXCR4 transfection was seen to have a more prominent effect in MSCs from OVX rats. MSCs from young rats have a high CXCR4 expression and therefore transfection with CXCR4 did not further increase migration. However, MSCs from older rats transfected with CXCR4 had higher migration than the non -transfected MSCs. Clinically this could be a significant finding as the poor migratory ability as well as differentiation ability of stem cells from osteoporotic patients could affect their bone formation. Transfecting MSCs with CXCR4 would not only improve the migration but also aid in the retention of MSCs at those sites. Lien and co-workers injected MSCs transfected with CXCR4 and Cbfa1 into young and adult osteopenic mice and showed significant improvement in bone formation in comparison to groups injected with just CXCR4 transfected MSCs or saline. In their study osteopenia was induced by the use of corticosteroids. They measured BMD using pQCT and bone mechanical strength using a four-point bending test. However unlike this study, they failed to measure the bone trabecular structure using histology or micro-CT (Lien et al., 2009b). Another study by Cho et al, 2009 injected MSCs transfected with CXCR4 as well as RANK-Fc into ovariectomised mice. They observed that CXCR4 improved in vivo trafficking and retention of MSCs, which then enhanced the therapeutic effect of RANK-Fc (Cho et al., 2009b). Although RANK-FC and Cbfa1 play an important therapeutic role in

improving bone, CXCR4 enhances the migration of cells, hence aiding the therapeutic role of these recombinant proteins.

Aging causes an increase in circulating inflammatory factors. SDF1, a chemoattractant for CXCR4 may have an important role in the homing and migration of stem cells to the bone marrow niche. Higher SDF1 levels in the plasma may perturb circulating stem cells and reduced rehoming to the stem cell niche over time. Low BMD in both men and women has been correlated to high SDF1 levels in blood, therefore implying that SDF1 is linked to bone homeostasis. As levels of SDF1 in the plasma increase, greater numbers of bone marrow stem cells are mobilized into the blood (Carbone et al., 2017). This means that SDF1 may mobilise stem cells away from the bone marrow niche into the blood stream in patients with osteoporosis thereby reducing bone formation. Using MSCs transfected with SDF1 the beneficial effect of SDF1 in fracture healing has been demonstrated (Ho et al., 2014b). My study has shown the relationship between ageing, osteoporosis and levels of CXCR4 on stem cells. However, the relationship between SDF1 and osteoporosis yet remains to be established.

To test the therapeutic treatments for osteoporosis, many animal models are utilised such as sheep, rats and mice. The rat OVX model is a commonly used model for osteoporosis (Lelovas et al., 2008, Kalu, 1991). The rat is an appropriate animal model for the research of postmenopausal osteoporosis. After OVX, bone resorption exceeds bone formation initially, causing bone loss. Soon thereafter, bone remodelling reaches a steady state, where resorption and formation are balanced (Lelovas et al., 2008). It must be noted that in osteoporotic women, the first and most severe bone changes occur in the spongy bone of the vertebral body, whereas in aged and mature rats they predominantly involve the trabecular bone of the metaphyseal region of the long bones and, more specifically, of the distal femur and proximal tibia. Changes in the thickness and porosity of cortical bone after OVX in

rats is also different to that seen in humans with osteoporosis. Nevertheless, the rat OVX model has been extensively used. In my study I used aged OVX rats but more precise replication of human osteoporosis is seen in senile OVX rats but this was not possible due to time constraints, cost and variability of the animals. A large number of studies on osteoporosis utilise mature rats which are 3 months old. The advantage of using an aged model is that it replicates the skeletal characteristics of women who are susceptible to postmenopausal osteoporosis. The skeletal properties in aged rats has stabilised and is therefore no longer subject to changes due to age (Bonucci and Ballanti, 2014).

The effect of CXCR4 on the migration of MSCs was tested in vivo in OVX rats by the injection of MSCs intravenously. The femoral bone mineral density and bone structure of these rats was analysed 11 weeks after cell administration and compared to the control group injected with saline. Three groups of cells were administered into the rats; Young-CXCR4 MSCs, OVX MSCs, and OVX-CXCR4 MSCs. Rats injected with young-CXCR4 MSCs had significantly improved BMD measured by pQCT and L4 vertebral strength compared to rats injected with saline. Rats that received young-CXCR4 MSCs, had higher BMD compared those that received OVX-CXCR4 MSCs and this could be attributed to the better migration and differentiation capability of young MSCs. Similar to studies by Lien and Cho (Lien et al., 2009b, Cho et al., 2009b), further work could also implement gene therapy to improve bone formation, in addition to CXCR4. In this way the migratory as well as bone formation capability of the MSCs would be improved.

The data from this work demonstrate that although use of isogenic MSCs in osteoporosis protects against bone loss, MSCs from the bone marrow have a declining function with age, proving to be challenging for autologous cell-based therapy. This problem could be tackled by using an allogeneic source of stem cells from young patients. However, using allogeneic stem cells may cause immunological

problems in the patient. Additionally, from a regulatory point of view using genetically modified CXCR4-MSCs would require ethical approval and stringent safety checks before being applied clinically. Although adenovirus vectors are currently being used for gene therapy to treat cancer, and as vaccines to express foreign antigens (Westphal et al., 2013, SM Wold and Toth, 2013), their role in bone formation still needs to be established in humans.

It was observed that there was an increase in BMD with injection of young CXCR4-MSCs, however there was no significant difference in the trabecular architecture using micro CT. The increase in BMD could be due to increase in thickness of cortical bone. Huang and co-workers, demonstrated an increase in BMD but no changes in trabecular structure when OVX rats were injected with young allogeneic MSCs (Huang et al., 2016). Of course the increase in trabecular structure, that is, trabecular thickness, number, length and connectivity, is important and these changes will enhance the microstructural properties if the bone as well as BMD. However, regenerating the trabeculae where they are no longer interconnected is difficult as shown by Cunningham et al, 2005, who measured the response of the bone after removal of the trabeculae from the oscalis of sheep. To compensate for the loss of trabeculae bone, the load was distributed via a thicker and denser cortex and a hollow structure was retained without the formation of trabecular cross struts. This is probably because there is no load distribution across non-connected trabeculae. Additionally, in the OVX model there is significant loss of the trabecular bone and the interconnectivity between the trabeculae is reduced, altering the loading of bone. Hence it is very difficult to form new trabeculae bridges (Cunningham et al., 2005, Kim et al., 2008). This may be associated with the trend towards an increase in thickness of the trabeculae in the MSCs-CXCR4 groups rather than an increase in number of trabeculae or an increase in connectivity.

In this in vivo study was that there was no significant difference in BMD between rats injected with young-CXCR4 MSCs and OVX-CXCR4 MSCs as well as those injected with OVX MSCs and OVX-CXCR4 MSCs. A higher BMD was observed for the CXCR4 group as well the young MSCs group. A number of measurement showed a trend but remained insignificant. A consideration for any future studies would be, the use of younger ovariectomised rats (not ex-breeders), would be important. In my study 6-9-month old ex-breeder rats were ovariectomised, left for 4 months and injected with the cell treatment and then left for a further 11 weeks until the end of the study. The older the rats, the more difficult it is to recover the trabeculae (McCann et al., 2008b, Tanaka et al., 2003). Although the age of the rats used in this study is reflective of a clinical scenario, the variability of age and type of rats used makes it difficult to obtain any significant data. Additionally, due to large variabilities between the wistar ex-breeder rats, it would have been useful to use inbred strains. A higher number of rats in each group for the in vivo study would also have helped to improve the significance in the data. An equation below shows an example of the power calculation for the percentage bone volume with data from my study and highlights that a higher sample size (number of rats) is needed to achieve a power of 80% (equation 1). In this case the number of individuals would have to be 10 in order to show a significant difference and in my study an N of 6 was routinely used.

Fragility fracture is a common problem in osteoporotic patients in both women and men, with 6 million hip fractures projected globally by 2020, leading to high costs, mortality as well as morbidity (SELECKI and EISMAN, Giangregorio et al., 2006, Pisani et al., 2016). As stem cells can be used for increasing BMD in osteoporotic patients but also for their ability to regenerate bone in fractures then this study is important as MSCs over expressing CXCR4 may be expected to be better retained at the site of injury.

$$n = \frac{2\sigma^2(Z_\beta + Z_{\alpha/2})^2}{\text{difference}^2}$$

$$\boldsymbol{n} = \frac{2x4^2(0.84 + 1.96)^2}{5^2}$$

Sample size = n =10

σ = standard deviation

N = sample size

Z_{β} = The desired power (Typically 0.84 for 80% power).

$Z_{\alpha/2}$ = Level of statistical significance (Typically 1.96).

Difference = Effect size (Difference in

Equation 1: An example of a power calculation equation showing the sample size of the rat number for percentage bone volume to achieve a power of 80% and significance of 95%.

An interesting future study would therefore be to investigate the effect of systemic administration of MSCs, in osteoporotic rats with femoral fractures. The fracture, stabilized with an external fixator would reflect the non-union problems associated in fragility fractures. This could then be compared to the administration of cytokines such as GCSF, VEGF and IGF1 that have been shown to cause mobilization of stem cells into the blood steam.

In conclusion, the work in this thesis has highlighted that injecting cells, be it from an older donor source or from a young donor, protects against bone loss caused due to osteoporosis. MSCs from OVX and aged rats have poor differentiation and migration ability, which makes it difficult for these cells to differentiate to bone. However, transfecting MSCs with CXCR4 and injecting them into OVX animals improves migration of cells and improves BMD and vertebral strength in OVX rats. Further work still needs to be carried out to help improve the trabeculae organization and regeneration leading to greater structural competence in osteoporotic bone.

Appendix

Publications

1. Osteoporosis and ageing affects the migration of stem cells and this is ameliorated by transfection with CXCR4. **Sanghani-Kerai A**, Coathup M, Samazideh S, Kalia P, Silvio LD, Idowu B, Blunn G. Bone Joint Res. 2017 Jun;6(6):358-365.
2. The Influence of Age and Osteoporosis on Bone Marrow Stem Cells from Rats. **Sanghani-Kerai A**, Blunn G, Osagie L, Coathup M. Submitted for publication to Plos One.
3. CXCR4 infected MSCs enhance bone formation in Osteoporosis. **Sanghani-Kerai A**, Coathup M, Samazideh S, Kalia P, Silvio LD, Idowu B, Blunn G. (Manuscript in preparation).

Conferences

1. Bone Formation in Osteoporosis can be improved by Stem Cells Transfected with CXCR4. **A Sanghani-Kerai**, M J Coathup, S Samizadeh, G Blunn. British Orthopaedic Research Society 2017.

2. Osteoporosis and Ageing Reduces the Migration of Stem Cells but this is Ameliorated by CXCR4. **Anita Sanghani**, Melanie J Coathup, Sorousheh Samizadeh , Priya Kalia, Lucy Di Silvio, Bernadine Idowu, Gordon Blunn . Orthopaedic Research Society 2016.

3. Osteoporosis and ageing affects stem cell function and migration. **Anita Sanghani Kerai**, Melanie J Coathup, Liza Osagie, Sorousheh Samizadeh, Gordon Blunn. European Orthopaedic Research Society 2016.

4. Osteoporosis affects stem cell migration that is ameliorated by CXCR4. **A Sanghani**, M Coathup, S Samazideh, P Kalia, L Di Silvio, G Blunn. British Orthopaedic Research Society 2015. Young Investigator's Award.

5. Osteoporosis and ageing affects stem cell differentiation and migration. **A Sanghani Kerai**, M J Coathup, L Osagie, S Samizadeh, G Blunn. British Orthopaedic Research Society 2016.

6. Osteoporosis and ageing reduces the migration of stem cells but this is ameliorated by CXCR4. **Anita Sanghani**, Melanie J Coathup, Sorousheh Samizadeh, Priya Kalia, Lucy Di Silvio, Bernadine Idowu, Gordon Blunn. World Biomaterials Congress 2016.

7. Osteoporosis affects stem cell migration that is ameliorated by CXCR4. **Sanghani A**; Coathup M; Samazideh S; Kalia P; Di Silvio L; Blunn G. 9Th Mesenchymal Stem Cell Meeting, 2015.

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