Mechanisms underlying stem cell depletion in ageing.

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A thesis submitted in partial fulfillment of the requirements for the Master of Science degree in Pathology and Laboratory Medicine
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Abstract

As we age, our bodies lose the ability to repair damaged tissues. This impairment may be due to age-related deficits in our regenerative stem cells. Stem cells reside in specialized areas called stem cell niches. Ageing may cause alterations in the stem cells directly or may alter the stem cell niche to generate deficits. However, systematic studies mapping stem cell deficits and alterations to stem cell niche are lacking. Here, I investigated the bone marrows of young and aged mice to determine age-related changes in the microenvironment that may alter resident stem cells.

To achieve my research objective, I examined the bone tissues of male and female C57BL/6N mice at different ages, ranging from 8 to 71 weeks of age. This corresponds to 20-75 human years. I performed bone marrow morphometric analyses and show an increase in marrow adiposity with advanced ageing in both male and female mice. I then stained bone tissues and noted increased immunoreactivity to stem cell antigen (SCA1) only at the middle age in female mice. This increase was not seen in male mice. Screening for various genes in the marrow flush samples indicated a possible role of growth and sex steroid hormone receptors in mediating some of the changes. However, empirical evidence will be needed to determine the causative agent in increased bone marrow adiposity.

My studies may allow for a better understanding of how ageing affects the composition and function of marrow resident stem cell populations.

Keywords

Ageing, stem cells, bone marrow microenvironment, adiposity.
Summary for Lay Audience

Ageing is a complex process which occurs in all organisms. As we age, our bodies lose the ability to repair tissue damage, which contributes to the occurrence of age-related diseases in elderly populations. Since stem cells are responsible for replenishing the aged and damaged cells, the decline of regenerative stem cells may contribute to our inability to repair tissues. Recent studies have indicated that stem cell fate is indirectly influenced by the composition of their cellular environment, the niche. Since the bone marrow houses different regenerative stem cell types and has a rich microenvironment that is known to change with ageing, it is an excellent model to investigate the mechanisms which may contribute to stem cell deficits with ageing. Therefore, I hypothesize that age-related cellular changes in the bone marrow are associated with the marrow resident stem cell ageing phenotype.

To understand how ageing negatively impacts regenerative stem cells, I examined the bone tissues of male and female mice at different ages. I harvested the bones from the limbs of mice at 8, 24, 48, 58-61, and 67-71 weeks of age. These timepoints correspond to approximately 20 to 75 human years. My results showed that the bone marrows of mice become fatty as they age. The detection of cellular and molecular changes associated with ageing suggest that the bone marrow microenvironment might regulate the stem cell ageing process.

Overall, my studies will allow for a better understanding of how ageing effects the composition and function of marrow resident stem cell populations. I anticipate that these studies will lead to the identification of targets for the prevention of ageing-related ailments.
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<td>ANGPTL2</td>
<td>Angiopoietin-like protein 2</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>AR</td>
<td>Androgen receptor</td>
</tr>
<tr>
<td>BAT</td>
<td>Brown adipose tissue</td>
</tr>
<tr>
<td>BM</td>
<td>Bone marrow</td>
</tr>
<tr>
<td>BMAT</td>
<td>Bone marrow adipose tissue</td>
</tr>
<tr>
<td>BMSC</td>
<td>Bone marrow mesenchymal stem cell [also known as bone marrow stromal cells]</td>
</tr>
<tr>
<td>c-BMAT</td>
<td>Constitutive bone marrow adipose tissue</td>
</tr>
<tr>
<td>CT</td>
<td>Cycle threshold [also known as crossing point, Cp]</td>
</tr>
<tr>
<td>CTCF</td>
<td>Corrected total cell fluorescence</td>
</tr>
<tr>
<td>C57BL/6</td>
<td>C57 black 6 mice</td>
</tr>
<tr>
<td>DAPI</td>
<td>4’,6-diamidino-2-phenylindole</td>
</tr>
<tr>
<td>DEXA</td>
<td>Dual-energy X-ray absorptiometry</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>ECFC</td>
<td>Endothelial colony forming cell</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediminetraacetic acid</td>
</tr>
<tr>
<td>EPC</td>
<td>Endothelial progenitor/precursor cell</td>
</tr>
<tr>
<td>EPO</td>
<td>Erythropoietin</td>
</tr>
<tr>
<td>EPOR</td>
<td>Erythropoietin receptor</td>
</tr>
<tr>
<td>ESR</td>
<td>Estrogen receptor</td>
</tr>
<tr>
<td>FGF</td>
<td>Fibroblast growth factor</td>
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<tr>
<td>GAPDH</td>
<td>Glyceraldehyde 3-phosphate dehydrogenase</td>
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<tr>
<td>H&amp;E</td>
<td>Hematoxylin and eosin</td>
</tr>
<tr>
<td>HSC</td>
<td>Hematopoietic stem cell</td>
</tr>
<tr>
<td>HSPCs</td>
<td>Hematopoietic stem and progenitor cells</td>
</tr>
<tr>
<td>IGF</td>
<td>Insulin-like growth factor</td>
</tr>
<tr>
<td>IL17RA</td>
<td>Interleukin 17 receptor A</td>
</tr>
<tr>
<td>IMV</td>
<td>Interstitial/microvasculature area</td>
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<td>LT-HSC</td>
<td>Long-term hematopoietic stem cell</td>
</tr>
<tr>
<td>MSC</td>
<td>Mesenchymal stromal cell</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger RNA</td>
</tr>
<tr>
<td>PDGF</td>
<td>Platelet derived growth factor</td>
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<tr>
<td>PLIN1</td>
<td>Perilipin 1</td>
</tr>
<tr>
<td>PPARD</td>
<td>Peroxisome-proliferator-activated receptor D</td>
</tr>
<tr>
<td>Term</td>
<td>Description</td>
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<tr>
<td>PTPRC</td>
<td>Protein tyrosine phosphatase [also known as cluster of differentiation-45, CD45]</td>
</tr>
<tr>
<td>qPCR</td>
<td>Quantitative polymerase chain reaction</td>
</tr>
<tr>
<td>r-BMAT</td>
<td>Regulated bone marrow adipose tissue</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>RUNX2</td>
<td>Runt-related transcription factor 2</td>
</tr>
<tr>
<td>SCA1</td>
<td>Stem cell antigen 1</td>
</tr>
<tr>
<td>SD</td>
<td>Standard deviation</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of the mean</td>
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<tr>
<td>SOX2</td>
<td>SRY-box 2</td>
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<tr>
<td>ST-HSC</td>
<td>Short term hematopoietic stem cell</td>
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<tr>
<td>TGF-β</td>
<td>Transforming growth factor-beta</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
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<tr>
<td>WAT</td>
<td>White adipose tissue</td>
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Chapter 1

1 Introduction

1.1 Ageing

Ageing is a complex process that occurs in all organisms, and is marked by a gradual decline in cell, tissue, and organ function. The number of people aged 80 years or older is predicted to triple between 2020 and 2050\(^1\). In addition, ageing is a known risk factor for major human diseases such as cancer, cardiovascular, musculoskeletal, and neurodegenerative diseases\(^2\). With the global expansion of the elderly population, the incidence of age-related diseases is increasing\(^3\). Therefore, a major current research goal is to understand how age-related diseases arise and to extend a healthy life.

There are seven intertwined processes which are known to drive ageing in some capacity: macromolecular damage, alteration of metabolism, stem cells deficits and impaired regenerative processes, proteostasis, altered intercellular communication, inflammation, and epigenetic changes\(^3,4\). Macromolecular damage refers to the interference of molecular (e.g., lipids, proteins, DNA) function, which can further impair cellular processes\(^5\). As all organisms age, there is also the dysregulation of energy homeostasis\(^6\). For example, in elderly populations, there is often a greater proportion of fat mass compared to lean mass\(^7\). As a result, the prevalence of obesity increases with lifespan, which further heightens the risk for chronic disease\(^6\). Ageing is also associated with shifts in metabolic function, which contributes to the elevated production of reactive oxygen species (ROS) and oxidative stress\(^8\). Since stem cells are responsible for replenishing all damaged and aged cells, age-related stem cell deficits may underly the impairment of repair mechanisms. Stem cell and regeneration deficits will be discussed in greater detail in the following sections. Another hallmark of ageing is the dysfunction of protein homeostasis, known as proteostasis. During the ageing process, the accumulation of external and endogenous stresses leads to compromised proteome integrity, altered protein synthesis, and the accumulation of protein aggregates\(^9\). Ageing is also associated with changes in intercellular communication, through direct cell-cell contact or endocrine,
neurohormonal, or neuroendocrine function\textsuperscript{4}. For instance, neurohormonal insulin-like growth factor (IGF) signaling is often dysregulated in aged patients, which has consequences on cardiovascular, muscle, bone, and brain function and regulation\textsuperscript{10}. Dysregulation of inflammatory pathways is another mechanism which is known to drive the normal ageing process\textsuperscript{11}. Coined “inflamm-ageing”, in aged individuals, a low-grade sustained inflammation occurs despite the absence of infection or injury\textsuperscript{12}. The continuous generation of inflammatory factors contributes to pathologies by numerous conditions. For instance, inflamm-ageing leads to the exhaustion of adaptive immune responses, and further immunosenescence\textsuperscript{13}. The final hallmark of ageing is epigenetic changes, which include DNA methylation, histone modification, chromatin remodeling, non-coding RNA regulation, and RNA modification\textsuperscript{14}. There is commonly histone modification, DNA methylation changes, and global chromatin remodeling observed in ageing models\textsuperscript{15}. Complex bi-directional relationships exist between the seven age-associated processes. For example, age-related changes in epigenetic dynamics and immunometabolism has been linked to an altered cellular response to stimuli\textsuperscript{11}. Understanding these complex dynamics which occur between the hallmarks of ageing, the mechanisms behind the normal ageing process are poorly understood. However, investigating the causes which underly ageing will lead to interventions which may allow us to develop interventions against human pathologies along the lifespan. The remainder of this thesis focuses on regenerative stem cells and ageing and explores how it connects to the other ageing processes.

1.2 Dysfunction of stem cells

Regenerative stem cells are responsible for differentiating into cells from the tissues they reside in. This process maintains tissue homeostasis and repair following injury\textsuperscript{16}. With ageing, there is a decline in stem cell number and function, except for hematopoietic stem cells (HSCs), where there is an increase in proliferation, and change in function\textsuperscript{16}. The ageing stem cell phenotype varies depending on the type of stem cell but is often characterized by a decline in self-renewal ability and altered/skewed lineages. During self-renewal, a stem cell enters the cell cycle and at least one of the daughter cells remains in an undifferentiated state\textsuperscript{17,18}. Self-renewal maintains the pool of stem cells,
which promotes long-term tissue regeneration and homeostasis. There may also be disruptions in the balance of differentiated progenies during ageing, thus altering tissue homeostasis. Therefore, stem cell deficits contribute to age-related aberrations through impaired replenishment of damaged and aged cells.

Stem cell ageing and exhaustion may be caused by cell-intrinsic and/or -extrinsic changes\textsuperscript{19}. Since stem cells are long-lasting in the body, they are at an increased risk of acquiring injuries such as DNA damage, and metabolic and epigenetic changes\textsuperscript{19,20}. DNA damage in stem cells is commonly caused by complications associated with DNA replication due to the high occurrence of self-renewal\textsuperscript{20,21}. This is because stem cells are repetitively being activated from a quiescent state, and thus are more susceptible to acquiring DNA damage\textsuperscript{20,21}. In addition, the use of glycolysis for metabolic function in stem cells are associated with a greater production of reactive oxygen species (ROS), which places stress on the mitochondrial genome\textsuperscript{22}. Epigenetic drift has also been noticed in the stem cell genome during the ageing process\textsuperscript{19}. For instance, whole-genome DNA methylation analysis of HSCs showed there was an increase in global DNA methylation in old mice compared to young\textsuperscript{23}. Mutations of epigenetic regulators and can lead to the expansion of mutated stem cells, an increased risk of tissue dysfunction, and cancer\textsuperscript{19,24}. Increasing evidence suggest that the accumulation of intracellular damage occurring in stem cells with advanced age is driven by intercellular communication through extrinsic factors such as systemic factors and cellular changes which arise within the surrounding microenvironment (stem cell niche) overtime\textsuperscript{25}.

### 1.3 Stem cell-niche interactions

Adult stem cells reside in specialized microenvironments, also called niches. Components of the stem cell niches communicate with and support stem cells to protect the integrity of the cells, and to regulate self-renewal and differentiation\textsuperscript{26-28}. The specific composition of stem cell niches depends on the specific tissue. Stem cell niches have been described in trabecular bone marrow, skin, the central nervous system, the gut, and muscle\textsuperscript{29-33}. The general niche model involves the tissue-resident stem cells, stromal support cells, extracellular matrix (ECM) proteins, blood vessels, and neural cues\textsuperscript{34}. Stromal support cells communicate with stem cells through cell-cell adhesion molecules and secreted
soluble factors. ECM proteins act as anchors and scaffolds to provide structural support and to transmit signals to stem cells. Blood vessels function as a mode of transport for nutrients and systemic signals between the niche and extramedullary spaces. The vasculature also facilitates the mobilization of stem cells to and from the niche. Neural cues relays messages from other organs to the niche, and inputs signals for the trafficking of stem cells out of their niches to target locations.

Stem cell-niche interactions occur by physical cell-cell contact or by diffusible factors emitted from the niche. Cell-cell interactions are facilitated through transmembrane proteins called cadherins. Stem cell-niche interactions have been shown to cause changes in stem behaviour. For instance, adhesion between neural stem cells and endothelial cells is associated with cycle arrest in the stem cells. In drosophila, interactions between stem cell cadherins and niche cells were shown to alter the mitotic spindle orientation during cell division, which has an effect on the choice of symmetric or asymmetric cell division. Symmetric cell division refers to the generation of identical daughter cells (ie all undifferentiated, or all differentiated cells). In asymmetric cell division, one daughter cell is usually differentiated, while the other remains undifferentiated.

The stem cell-matrix interactions also cause changes in stem cell function. The niche provides structural support, facilitates adhesion, and secretes molecules which sends external cues to regulate stem cell behaviour. A primary way in which the ECM influences stem cell behaviour is through the acquisition of locally and systemically produced diffusible factors such as growth factors, chemokines, and other regulatory molecules. These factors act through molecular pathways including Wnt/beta-catenin, bone morphogenetic protein, notch, angiopoietin-1, and growth factors such as fibroblast growth factor (FGF), insulin-like growth factor (IGF), vascular endothelial growth factor (VEGF), transforming growth factor-beta (TGFβ) and platelet derived growth factor (PDGF). The signals provided from the stem cell niche are received by the stem cells to regulate actions such as self-renewal, quiescence, migration, cell death, or differentiation. For example, the Wnt signaling pathway has been shown to effect HSC activity. Wnt ligands bind to a receptor complex on the stem cells, which leads to the stabilization of the protein β-catenin, which accumulates and translocates to the nucleus.
where it activates targeted transcript level\(^{45}\). In the inactivated state, beta-catenin is degraded by the beta-catenin destruction complex\(^{45}\). In stem cells, the Wnt/beta-catenin pathway is known to regulate the stability of the beta-catenin and beta-catenin-mediated gene transcription, which supports self-renewal. This demonstrates the effect that signaling molecules within the niche can have on stem cell function.

The niche needs to be highly specialized to regulate the balance of stem cell function, however there are many gaps in our knowledge pertaining to the mechanisms active in the niche. In addition, the dynamics involving the regulation of molecular signaling pathways within the niche in abnormal physiological and pathological conditions are poorly understood. Therefore, a goal for current day research is to investigate the relationship between stem cells and their surrounding microenvironment. To study stem cell-niche interactions, the bone marrow is an excellent model since it is an enclosed reservoir that includes heterogenous population of stem cell types, defined stromal cells, blood vessels, and neural elements. Furthermore, there is evidence of age-related changes in the bones and the bone marrow. Our current knowledge in this system will be presented here.

1.4 The bone marrow niche

Enclosed within the cavities of bones, the marrow acts as a reservoir for a complex combination of cells which includes hematopoietic and non-hematopoietic stem cells (Figure 1.4.1)\(^{46}\). The marrow develops prenatally during the process of bone formation (osteogenesis) and continues to evolve throughout life. There are two different modes of osteogenesis, intramembranous ossification, and endochondral ossification\(^{47}\).

Intramembranous ossification occurs to form flat bones such as the skull and ribs\(^{48}\). In this process mesenchymal cells differentiate into osteoblasts, which secrete a collagen-proteoglycan matrix called osteoid needed to bind and sequester calcium\(^{48}\).\(^{49}\). Osteoblasts get entrapped within the calcified osteoid matrix, to yield osteocytes\(^{48}\). A membrane called the periosteum forms around the developing bone, and hard cortical bone forms parallel to the spongy trabecular bone\(^{48}\). Blood vessels are interspersed within the trabecular bone, MSCs, and osteoid, forming the red bone marrow\(^{48}\). Due to the structure
of flat bones, bones which develop via intramembranous ossification do not have a bone marrow cavity\textsuperscript{48}. Endochondral ossification involves the formation of cartilage tissue from mesenchymal cells before the development of bone\textsuperscript{50}. Mesenchymal cells differentiate into chondrocytes, which then divide and secrete a cartilage-specific ECM\textsuperscript{50}. Blood vessels invade the cartilage, leading to chondrocyte apoptosis, and creation of the marrow cavity. Osteoblasts then begin setting the bone matrix on the degraded cartilage\textsuperscript{51,52}. Endochondral ossification forms the vertebral column, the pelvis, and limbs\textsuperscript{50,51}. The primary function of the bone marrow niche is to house and support the self-renewal and differentiation of resident stem cells. The remainder of this section will discuss the different cell types found within the bone marrow niche and their functional role.

![Schematic diagram illustrating the marrow resident stem cells.](image)

**Figure 1.4.1:** Schematic diagram illustrating the marrow resident stem cells.

The bone marrow is a rich reservoir for hematopoietic and non-hematopoietic stem cells. Hematopoietic stem cells give rise to all other blood cells through a process called hematopoiesis. The non-hematopoietic stem cells are believed to include endothelial colony forming cells (ECFCs; endothelial precursors) and mesenchymal stromal cells (MSCs; precursors of adipocytes, osteoblasts, and chondrocytes). The image was created in Biorender.
The best known (and most studied) stem cell population in the bone marrow are the HSCs. HSCs are responsible for replenishing the body’s pool of blood cells throughout life. The first pool of hematopoietic stem cells and hematopoietic progenitor cells (HSPCs) is formed during embryonic development through a process called primitive hematopoiesis. Erythroid and myeloid progenitors are generated, followed by HSCs in the aorta-gonad-mesonephros (AGM) region of the embryonic mesoderm. Additional sources of hematopoietic stem cells (HSCs) during embryogenesis include the placenta and yolk sac. HSPCs then circulate to the fetal liver before migrating and colonizing the bone marrow. Throughout adult life, HSCs self-renew to maintain the existing pool of undifferentiated cells and differentiate into myeloid and lymphoid progenitor cells to meet the demands of extramedullary sites (places outside of the bone marrow). HSC generate two different subtypes of cells, long-term (LT) and short term (ST) HSCs. LT-HSCs undergo asymmetric cell division to self-renew to sustain the stem cell pool into old age, or differentiate into ST-HSCs. ST-HSCs have limited self-renewal ability and can give rise to progenitors which can differentiate into all mature blood cell types. Lymphoid progenitors differentiate into lymphocytes, and include T cells, B cells, and natural killer (NK) cells. B and NK cells are continually produced in the bone marrow throughout life, then achieve maturity in peripheral lymphoid organs. Lymphoid progenitors migrate to the thymus to form T-cells. Myeloid progenitors develop in the bone marrow and generate megakaryocytes, erythrocytes, granulocytes, and macrophages. HSPCs exist in the bone marrow until called to extramedullary spaces by various signaling mechanisms. For instance, vascular endothelial growth factor (VEGF, typically VEGF-A form) stimulates mobilization of HSPCs.

The marrow-resident non-hematopoietic stem cells are rather elusive and ill-defined. There is substantial evidence showing that there are progenitor cells generated in the bone marrow which were not derived from HSCs. This demonstrates that non-hematopoietic stem cells also exist in the marrow space. For example, we know that the bone marrow contains mesenchymal stromal cells and endothelial colony forming cells (ECFCs). Mesenchymal stromal cells (MSCs; sometimes referred to as mesenchymal stem cells) are precursors which do not express hematopoietic markers or factors but differentiate into adipocytes, osteoblasts, and chondrocytes. These cells are
appropriately characterized as stromal cells rather than stem cells since self-renewal capacity is difficult to demonstrate. In vitro, MSCs are identified by the abundance of select markers which include the cluster of differentiation (CD)105, CD73 and CD90, and lack the gene abundance of HSC markers (CD45 and CD34) and endothelial markers (CD31). MSCs can maintain tissue homeostasis and regenerate damaged tissue. Within the bone marrow, MSCs are located around blood vessels to provide physical support to HSPCs. In addition, through the differentiation into adipocytes, osteoblasts, and chondrocytes, MSCs are involved with the functional remodeling of the bone marrow niche. MSCs also regulate hematopoiesis through direct cell-cell contact and indirectly by secreting soluble factors. In addition, MSCs modulate activities of the immune system. For instance, it has been reported that MSCs induce the expression of immunosuppressive molecules such as interleukin (IL)-10 and programmed cell death 1 ligand 1. MSCs have also shown to suppress proinflammatory molecules such as tumor necrosis factor-alpha, interferon gamma, and interleukin-1beta. In addition to regulating its own environment, MSCs aid in systemic tissue repair by migrating and homing to target tissues through the vasculature.

Previous work has also suggested the presence or derivation of endothelial precursor cell known as endothelial colony forming cells (ECFCs). ECFCs have a high proliferative capacity and circulating ECFCs form vascular tubules both in vitro and in vivo. Isolated bone marrow ECFCs have been shown to display surface markers consistent with endothelial cells, have a high potential for proliferation. In addition, when cultured with fetal bovine serum, BM-ECFCs demonstrated patterns of spontaneous capillary-like structure formation. One study recruited 5 male recipients and engrafted allogenic bone marrow from female donors and used the detection of X (donor) and Y (recipient) chromosomes to mark the origin of vessel wall endothelial cells. The findings suggested that circulating EPCs are the origin of vessel wall cells. However, another study recreated the sex-mismatched transplantations and found that the outgrowth of endothelial cells from blood was primarily derived from transplanted marrow-derived cells. Although there is evidence that ECFCs originate from the bone marrow and are capable of blood vessel formation, the cellular source of ECFCs is not fully defined. Despite the gaps in
knowledge surrounding bone marrow ECFCs, the regenerative and angiogenic potential makes ECFCs an attractive area for research.

Amongst the stem cells, there are other cellular elements such as adipocytes, osteoblasts, chondrocytes, endothelial cells, and cell products including cytokines, growth factors, and matrix proteins, which all play a supporting role in hematopoiesis. Bone marrow adipose tissue (BMAT) functions to take up space in the bone marrow cavity, and to maintain bone metabolism. Marrow adipocytes believed to originate from MSCs, and act as an energy reservoir and support for hematopoiesis and osteogenesis. The expression of transcriptional factors peroxisome proliferator-activated receptor-gamma (PPARG) and CCAAT-enhancer-binding protein-alpha (CEBPA) in MSCs controls adipogenesis. BM adipocytes secrete proteins such as adiponectin, RANK ligand, and stem cell factor, which effects local and systemic metabolism. In addition, the metabolism of fatty acids acts as a source of energy in the bone. BMAT has been shown to be distinct from the other adipose tissue types, white (WAT) and brown adipose tissue (BAT). WAT and BAT are different in appearance and function. White adipocytes have a spherical shape and contains a singular large lipid droplet, while brown adipocytes contain multiple lipid droplets. In addition, WAT functions to store and release energy, and has endocrine functions, while BAT is involved with thermoregulation. BMAT has been shown to be functionally distinct from WAT and BAT since it resists cold- and insulin-stimulated glucose uptake. There are two subtypes of BMAT; constitutive BMAT (cBMAT) and ‘regulated’ BMAT (rBMAT). cBMAT is characterized as adipose tissue which is formed early in life and located at the distal skeletal regions including the hands, feet, distal tibia, and tail in rodents. Histologically, cBMAT accumulates in a dense formation and contains more unsaturated lipids and adipocytes which are larger in size. rBMAT develops after cBMAT and forms interspersed within the hematopoietic bone marrow. The morphology of rBMAT is also more susceptible to change in response to external stimuli such as temperature. Adipocytes typically make up 70% of bone marrow volume, however the accumulation of BMAT fluctuates under different physiological and clinical conditions. Adipogenesis in bone marrow spaces is associated with ageing and pathologies such as diabetes, anorexia nervosa, and growth hormone deficiencies.
Within the bone cavity there are also osteoblasts. In adult bone marrow, osteoblasts are formed from MSCs by the action of transcription factors runt-related transcription factor 2 (RUNX2), osterix, alkaline phosphatase, and collagen type I\(^95,96\). Osteoblasts are responsible for bone formation and resorption by signaling to produce osteocytes and osteoclasts, respectively\(^97\). Osteocytes form endosteal bone and provide systemic structural support. Interestingly, osteocytes have been shown to have an effect on bone marrow niche dynamics and overall health of mice\(^98\). The conditional deletion of osteocytes resulted in the emergence of severe sarcopenia, osteoporosis, and degenerative kyphosis. In addition, osteocyte declines lead to the reduced commitment of MSCs to osteoblasts, and an increase in HSC differentiation to myeloid progenitors\(^98\). This demonstrates the significant role that osteoblasts and the downstream production of osteocytes have on the homeostasis of the bone marrow niche. Intercellular communication between osteoblasts and HSPCs has also been observed\(^96\). Labeled HSPCs were shown to be positioned close to endosteal osteoblasts\(^99\). In addition, the ablation of osteoblasts in mice lead to a loss of hematopoietic stem cells, and lymphoid, erythroid, and myeloid progenitors in the bone marrow\(^100\). Therefore, in addition to the modulation of cortical and trabecular bone, osteoblasts may play a regulatory role in hematopoiesis within the bone marrow niche.

The bone marrow ECM primarily consists of proteoglycans, fibrous proteins (for example, collagens, fibronectins, elastins, and laminins), glycosaminoglycans (for example, hyaluronic acid, chondroitin sulfate, heparan sulfate, keratan sulfate, dermanatan sulfate, and heparin), and matricellular proteins (for example, osteopontin and periostin)\(^101\). Proteoglycans are defined as macromolecules which consists of a core protein with at least one glycosaminoglycan chain attached\(^102\). These macromolecules function by facilitating signaling pathways by binding to growth factors and cytokines which regulates matrix assembly and bone marrow niche dynamics, such as bone formation\(^103\). Fibrous proteins fibronectin, collagens, laminin, and elastin are the most abundant proteins in the ECM, and they function to provide structural support within the ECM\(^101\). Glycosaminoglycans are long, linear polysaccharides which have a variety of functions\(^104\). Therefore, the specific role of glycosaminoglycans within the bone marrow is not fully known. However, it is suggested that matrix glycosaminoglycans are involved
with bone remodeling\textsuperscript{104}. Matricellular proteins refers to proteins secreted into the ECM which have no effect on the matrix structure\textsuperscript{105}. Instead, these proteins function to moderate cell activities by interacting with structures in the ECM such as cell-surface receptors, proteases, hormones, and structural matrix proteins\textsuperscript{105}. External soluble molecules such as growth factors are also secreted in the matrix to facilitate activities within the niche such as bone remodeling and hematopoiesis. Growth factors are small glycoproteins synthesized and released in the bone marrow matrix by many different cell types such as stem and progenitor cells, osteoblasts, fibroblasts, and endothelial cells. Common growth factors in the bone marrow microenvironment include insulin like growth factor 1 (IGF1), epidermal growth factor (EGF), and transforming growth factor beta (TGF\textbeta). In the bone marrow, IGF1 is produced from osteoblasts and chondrocytes, however it is unknown whether other bone marrow resident cells also act as a source of IGF1. IGF1 is associated with influencing bone formation and preventing fat accumulation within the marrow\textsuperscript{106}. TGF\textbeta signaling plays a key role in bone remodeling\textsuperscript{107}. Growth factors have an effect on the function of the bone marrow niche by promoting cellular proliferation and differentiation\textsuperscript{108}. For instance, one study found that the binding of TGF\textbeta to proteoglycans, biglycan and decorin leads to the sequestration of TGF\textbeta\textsuperscript{103}. Bi et al. removed biglycan and decorin in mice\textsuperscript{103}. This led to excess TGF\textbeta within the marrow, resulting in an increase in TGF\textbeta signaling transduction pathway\textsuperscript{103}. Excess TGF\textbeta signaling lead to the apoptosis of MSCs, which caused decreased osteoblast and bone formation\textsuperscript{103}. BM-derived cytokines are proteins that trigger the inflammatory response to injury\textsuperscript{108}. 
The bone marrow houses a variety of components which aim to support stem cell function. These include nerve fibers which provide neural input, blood vessels for the transport of substances into and out of the niche, extracellular matrix proteins, and supporting cells (e.g., mesenchymal stromal cells, adipocytes, and osteoblasts). Made in biorender

Figure 1.4.2: Schematic diagram illustrating the bone marrow niche.

1.5 The ageing phenotype of marrow-resident stem cells

Over a lifetime, HSCs function to meet the changing demands for blood cells. During postnatal development, HSCs rapidly self-renew to establish the growing population of blood cells in the vasculature and bone marrow\textsuperscript{109}. Into and during adulthood, most HSCs are dormant, with cells cycling to maintain the blood homeostasis as required\textsuperscript{109}. In adult ageing, the HSC populations have been shown to increase or remain stable, but studies have reported dysfunction in abilities to repopulate specific progenitors\textsuperscript{109,110}. In addition, transcriptomics revealed that there is an upregulation in genes involved with stress,
inflammation, and protein aggregation and down-regulated profiles of genes associated with the preservation of genomic integrity and chromatin remodeling in older HSCs compared of young. In addition, it has been theorized that increased HSC proliferation with ageing is prompted to compensate for the decline in HSC function. Aged HSCs are also known to have impaired homing abilities when transplanted to irradiated bone marrow. Another common characteristic of HSC ageing is lineage skewing, aged HSCs are known to exhibit a myeloid-biased differentiation at the expense of the lymphoid lineage. These changes in lineage potential adds to systemic ageing deficits. The ageing of HSCs contributes to susceptibility to age-related immune diseases such as myeloid and lymphoid leukemias, anemia, autoimmunity, and infectious disease.

The ageing phenotype of non-hematopoietic bone marrow resident stem cells are not fully understood, but research suggests there are stem cell deficits associated with the ageing of MSCs. Many studies found there is a decrease in MSC proliferation with ageing. When comparing MSCs from adult bone marrow and neonatal tissues (placenta and umbilical cord), it was found that umbilical cord MSCs have a higher proliferation capacity than bone marrow-MSCs. In addition, flow cytometric analysis has shown that murine platelet-derived growth factor receptor (PDGFR)-A+SCA1+ cells (a subtype of mesenchymal stromal cell) are most prevalent in developing bone, then are seen at lower frequencies in adult bone marrow. A study also reported a decline in proliferation, adipogenic potential, and cell surface abundance of SSEA4, CD146 and CD274 in human bone marrow MSCs with increasing donor age. MSCs exhibit a loss of proliferative abilities compared to embryonic stem cells (ESCs) in vitro.

Transcriptomic profiling of the bone marrow stromal cell microenvironment of early postnatal, adult, and aged mice revealed that inflammatory transcriptional programs are increased in stromal cells with advanced age. It was also suggested that adult and fetal stromal progenitors have different developmental potentials for niche formation. Cell culture work has shown that aged MSCs are associated with telomere shortening, increased number of senescent cells, and a reduced resistance to oxidative stress. In addition, there is a shift towards adipogenic potential at the expense of osteogenesis in MSCs with ageing. As a result, the ageing of MSCs have been suggested to contribute
to age-related bone abnormalities such as osteoporosis and osteoarthritis\textsuperscript{122,125}. Adding on, it was found that bone marrow MSCs from patients with osteoporosis have less sensitivity to IGF and a loss of osteogenic potential\textsuperscript{126}. Therefore, it is possible that cellular changes in the bone marrow cavity drive MSC ageing, but the mechanisms which drive MSC age-associated traits are poorly understood.

Sex-related differences have also been reported in the ageing process of HSPCs\textsuperscript{127}. Flow cytometry counts showed that bone marrow-resident HSC expansion occurred at middle age in female mice, but old age in male mice\textsuperscript{127}. The same study also found there was a decline in HSC associated transcript level which occurred earlier in life in the bone marrow of male mice\textsuperscript{127}. Interestingly, sex-mismatched BM transplantations showed that the bone marrow microenvironment was responsible for the sustained increased abundance of HSC genes in female mice\textsuperscript{127}. This demonstrates that sex-related differences of HSC frequency are attributed to cell-extrinsic factors. There were also sex differences involved with the ageing of non-hematopoietic stem cells. In vitro, human BM-MSCs demonstrated a reduced colony forming unit-fibroblast (CFU-F) number in female compared to male donors\textsuperscript{119}. Hormonal changes which occur in the bone marrow microenvironment causes the sex-related differences observed in the ageing of marrow-resident stem cells\textsuperscript{127,128}. For instance, the decline in estradiol levels during perimenopause, menopause, and post menopause has been shown to account for sex-related differences in ageing phenotype of marrow-resident stem cells\textsuperscript{128}. In vitro, estrogen promoted the differentiation of human MSCs into osteoblasts in comparison to adipocytes\textsuperscript{129}. In addition, when cultured with estrogen, bone marrow MSCs from postmenopausal patients with osteoporosis displayed an increased proliferation and osteoblastic differentiation\textsuperscript{130}. Therefore, to understand how the ageing stem cell niche influences the stem cell ageing phenotype it is important to investigate the sex-related differences in the ageing of stem cells and their surrounding microenvironment.

1.6 Bone marrow stem cell-niche interactions

Research has demonstrated that the manipulation of extrinsic factors within the bone marrow microenvironment effects HSC function. A study published in 2021 aimed to determine how the ageing bone marrow causes the HSC ageing phenotype\textsuperscript{114}. The
authors found that by middle age, there is a lineage skewing of HSCs towards myeloid progenitors and a decline in insulin like growth factor (IGF1) levels in the bone marrow of crushed tibiae, femurs, and iliac crests. Subsequently, through the reciprocal transplantation of HSCs in young and middle-aged mice, it was concluded that the middle-aged bone marrow microenvironment was responsible for the HSC lineage skewing. Lastly, they found that the direct stimulation of middle-aged HSCs with IGF1 was able to restore HSC functionality. This shows that the aged bone marrow microenvironment triggers the stem cell ageing phenotype. Another study found that altering a component of the bone marrow microenvironment effected HSC behaviour. The expression of angiopoietin-like protein 2 (Angptl2) from endothelial cells in the bone marrow niche hindered the repopulating ability of HSCs. Mechanistically, it was suggested that ANGPTL2 increases the expression of peroxisome-proliferator-activated receptor D (PPARD) which further prevents HSC from entering the cell cycle.

HSPC behaviour is also influenced by changes in the cellular composition of its surrounding niche. It was found that HSPCs have a reduced frequency when located in adipocyte rich areas in comparison to adipocyte-free spaces. It was also found that age increased myeloid progenitors, the majority of which were found in adipocyte dense regions. Previous work in our laboratory showed that adipogenesis in the tibia of mice with diabetes precedes stem cell depletion. This increased adipogenesis was associated with the suppression of TGFB signaling. This suggests a mechanism, which may justify how adipogenesis interacts with stem cells in the bone marrow microenvironment. Previous studies have also suggested that bone marrow non-hematopoietic stromal cells are regulators of HSPCs. For instance, CXCL12 abundant reticular cells (CARs), a subset of stromal cells has been demonstrated to support the activities of HSPCs.

Altogether, the bone marrow microenvironment may regulate stem cell health and function through cellular changes and downstream molecular mechanisms. However, it is poorly understood how the marrow niche effects non-hematopoietic stem cell behaviours.
1.7 Known cellular changes within the aged bone marrow niche.

Enhanced adipogenesis is a key cellular change which occurs with increasing age in most animals\textsuperscript{137}. The analysis of osmium-stained tibiae revealed that adipocyte accumulation arises in specific regions of the bone depending on age in mice\textsuperscript{93}. In addition, there were differences in the types of adipose tissue that would form in these spaces overtime\textsuperscript{93}. It was observed that cBMAT develops first during early life at the distal end of the bone, then rBMAT develops in the middle and proximal tibia from middle to old age in mice\textsuperscript{93}. In addition, the adult rBMAT is interspersed in the hematopoietic marrow, while the cBMAT exists in tight clusters\textsuperscript{93}. A similar pattern of adipogenesis occurs with the ageing of rats, rabbits, and humans\textsuperscript{93}. However, the rate of BMAT formation decreases as the lifespan/size of the animal increases\textsuperscript{93}. The amount of BMAT that forms and extends into the skeleton also increases as the size of the animal increases\textsuperscript{93}. By the approximate age of 25 years in humans, BMAT is present in 50 to 70\% of total bone marrow volume\textsuperscript{137}. However, in mice, BMAT forms by middle age, and takes up a smaller portion of the marrow volume. Throughout human development, the rate of BMAT also depends on the type of bone. cBMAT develops earlier in long bones than in the axial skeleton (sternum, ribs, pelvis, and vertebral bones)\textsuperscript{138}. Age associated adipogenesis can have negative effects on overall health. For instance, there is a positive correlation between osteoporosis and BMAT formation\textsuperscript{137}. Perhaps the most exciting study linking adipocytes to stem cell function in the bone marrow is the characterization of A-ZIP/F1 fatless mice\textsuperscript{132}. Compared to A-ZIP/F1 fatless mice, wild-type mice with high bone marrow adiposity have fewer hematopoietic progenitors, reduced HSC functionality, lower peripheral leukocyte counts, and slower peripheral hemoglobin recovery following lethal irradiation of the bone marrow, which suggests that an increase in adipocytes suppresses hematopoiesis\textsuperscript{132}.

With advanced age, there is also a loss in bone mass, and a reduction in mineralized components\textsuperscript{139}. This is consistent with the presence of age-related diseases such as osteoporosis, osteoarthritis, etc. In aged mice, there was a decrease in osteoblast progenitors\textsuperscript{140}. While there is an overall loss of bone with ageing in both males and
females, there are sex-related differences associated with the ageing bone. In murine models, females had a lower bone volume fraction and increased marrow fat area in the tibia compared to age-matched males\textsuperscript{141}. Due to the loss of estrogen which begins at perimenopause, menopausal females are more susceptible to osteoporosis\textsuperscript{142}. Estrogen deficiency has shown to increase bone resorption\textsuperscript{143}. Therefore, sex is a factor which impacts the age-related changes within the bone marrow microenvironment.

In summary, since increased adipogenesis is a key change which occurs in the bone marrow microenvironment with age which has been previously shown to alter stem cell behaviour. Therefore, it is a promising candidate mechanism for investigating the ageing of stem cells.

1.8 Purpose of Thesis

While it is known that stem cell health and function decline with increasing age, the mechanisms which drive stem cell ageing are currently poorly understood. Recent studies found that changes in the stem cell microenvironment were responsible for the hematopoietic stem cell ageing phenotype\textsuperscript{114}. For instance, mismatched transplantations of LT-HSCs into young and middle-aged bone marrow demonstrated that the middle-aged bone marrow microenvironment was responsible for the HSC ageing phenotype of increased myeloid progenitors\textsuperscript{114}. However, the causes behind non-hematopoietic stem cell ageing remains unknown. Furthermore, it is not clear whether changes to the microenvironment lead to or simply associate with changes to stem cells. However, it is known that the marrow space becomes populated with adipocytes with advanced age. The purpose of my study is to investigate the correlation between the marrow resident stem cell ageing phenotype and changes within the bone marrow microenvironment (Figure 1.8). I hypothesize age-related increase in adipogenesis, and stem cell deficits occur in the bone marrow microenvironment.
Figure 1.8.1. Schematic diagram illustrating the study hypothesis.

*Ageing is associated with reduced regenerative capacity, likely through a reduction in stem cell frequency/number. I hypothesize that ageing-associated changes to bone marrow microenvironment may indirectly lead to stem cell depletion/exhaustion.*

1.9 Objectives of Thesis

Overall, I sought to investigate the mechanisms underlying stem cell deficits/ageing. My studies aimed to characterize changes within the bone marrow microenvironment and whether marrow resident hematopoietic or non-hematopoietic stem cell ageing phenotype is correlated. My specific objectives were:

1. Map the cellular changes within the bone marrow microenvironment.

2. Investigate the hematopoietic and nonhematopoietic stem cell changes.

3. Explore candidate molecular mechanisms which may connect the stem cell ageing phenotype within the bone marrow microenvironment.
2 Materials and Methods

2.1 Mouse models and dissection

All studies were conducted following approval by the Research Ethics Board at Western University, London, Ontario, Canada [Animal Use Protocol: 2021-081]. A total of fifty-eight C57BL/6N mice were obtained from Charles River Canada. These mice were at 7-, 23-, 47-, 57-60-, 66-70-weeks of age. Mice were acclimated for at least 1 week and euthanized at 8- (n = 7 females and 7 males), 24- (n = 7 females and 7 males), 48- (n = 7 females and 7 males), 58-61- (n = 4 females and 4 males), and 67-71- (n = 4 females and 4 males) weeks of age. This range corresponds to 20-75 human years. Of these, 8- and 24-week-old mice (corresponds to 20 and 26 human years, respectively) was selected to represent young mice, since sexual maturity occurs at around 8-weeks in C57BL/6[14].

Forty-eight-weeks was selected to represent middle-age because typical signs of ageing begin at this point[145]. For example, decreased bone mass and intraarticular resorption occur at ages 42 and 52 weeks, respectively. C57BL/6 generally show age-related diseases at around 86-weeks age[146]. Therefore, 58-61- and 67-71-weeks age was selected to represent mice of old age, to minimize potential confounders. In addition, male and female mice were included because bone composition and ageing are sexually dimorphic due to reproductive senescence, which occurs at around 50- and 60-weeks in male and female C57BL/6 respectively[147–149]. The mice were divided into groups by age and sex (n=4 or 7). After arrival, the mice were placed in a room with a light dark (12h/12h) cycle at 21°C. The mice were monitored daily for body condition scores and overall health. All mice remained at a body condition score of 3 (well-conditioned). The mice were euthanized by CO₂ inhalation with a secondary form of euthanasia being removal of the heart. The hind legs were dissected from the body, with one leg processed for the bone marrow flush, while the other fixed in formalin for paraffin embedding and morphometric analyses (Figure 2.1).
Male and female C57BL/6 [C57BL/6N, Charles River] of 7-70 weeks of age were acquired and allowed to acclimate for at least one week prior to euthanasia. Corresponding human years are indicated. The tibias and femurs were harvested. Tissues from one side were fixed in formalin for histology and the other side for bone marrow flush preparation for transcript level analysis. This image was created in Biorender.
2.2 Bone marrow flush and transcript level profiling

The soft tissue was removed from the tibia and femur using surgical scissors. The tibia was detached from the femur and the calcaneum at the joints. A cut was made into the posterior side of the femur and tibia at the epiphyseal plates, allowing for extraction of the marrow. The bones were then placed into 0.5 mL centrifuge tubes nested in a 1.5 mL centrifuge tubes. A small hole was created at the bottom of the 0.5 mL tubes with an 18G needle, prior to adding the tissues. Tubes containing bone tissues were then centrifuged at 1000 xg for 10 s to flush the bone marrow. To determine whether this procedure fully removes the marrow cells, processed/emptied bones were fixed in formalin, embedded in paraffin, sectioned, and stained with hematoxylin and eosin (Materials and Methods 2.3 and Figure 5.1-APP). To the extracted marrow cells, a total of 500 µL of a cell freezing media (90% fetal bovine serum and 10% dimethyl sulfoxide, DMSO; Millipore; Cat# S-002-5F) was added. The flush samples were then stored at 80°C. For transcript level analyses, marrow flush samples were thawed and used immediately.

Total RNA from marrow flush samples was isolated using RNeasy Mini Plus Kit (Qiagen; Cat# 74134). Total RNA was measured using Qubit RNA Broad Range Assay (Thermo Fisher; Cat# Q10210) in a Qubit Fluorometer (Thermo Fisher; Cat# Q32857). cDNA was then synthesized using iScript cDNA Synthesis Kit (Bio-Rad Laboratories; Cat# 1708841). TaqMan-based chemistry was used to measure mRNA of target genes. The reactions consisted of 5 µL TaqMan Fast Advanced Master Mix (Thermo Fisher; Cat# 4444963), 0.5 µL of the gene probe (Table 2.2), 1 µL cDNA, and 3.5 µL nuclease-free H2O. All reactions were run on CFX Connect Real-Time PCR Detection System (Bio-Rad Laboratories). TaqMan-based reactions were performed for 40 cycles using the following temperature profiles: 95 °C for 3 s (initial denaturation), and 60 °C for 30 s (annealing and extension). Target gene mRNA data were normalized to Gapdh housekeeping gene and data was analyzed by CFX Manager software (Bio-Rad; Cat# 1845000) using the normalized (ΔCT) method.
Table 2.2: List of primers used for qPCR

<table>
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<th>Gene</th>
<th>Description</th>
<th>Source (catalogue #)</th>
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<td>Ar</td>
<td>Androgen receptor</td>
<td>Thermo Fisher (Mm00442688_m1)</td>
</tr>
<tr>
<td>Epor</td>
<td>Erythropoietin receptor</td>
<td>Thermo Fisher (Mm00833882_m1)</td>
</tr>
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<td>Esr1</td>
<td>Estrogen receptor 1</td>
<td>Thermo Fisher (Mm00433149_m1)</td>
</tr>
<tr>
<td>Gapdh</td>
<td>Glyceraldehyde 3-phosphate dehydrogenase [housekeeping gene]</td>
<td>Thermo Fisher (Mm99999915_g1)</td>
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<td>Perilipin 1</td>
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<td>Thermo Fisher (Mm03053810_s1)</td>
</tr>
<tr>
<td>Tgfb1</td>
<td>Transforming growth factor-beta1</td>
<td>Thermo Fisher (Mm01178820_m1)</td>
</tr>
</tbody>
</table>
2.3 Tissue processing for histology

For histological analyses, bone tissues were fixed in 10% neutral buffered formalin (Sigma-Aldrich; Cat# HT501128) overnight at room temperature. Then, tissues were decalcified in 0.5 mol/L ethylenediaminetetraacetic acid (EDTA; Fisher Scientific; Cat# S311) for 7 days at 4 °C. Tissues were then rehydrated in 30% sucrose solution (Sigma-Aldrich; Cat# 84100) overnight at 4 °C. Samples were moved to 70% ethanol and processed and embedded in paraffin. Tissues were sectioned at a 4-μm thickness and affixed to glass slides (Fisher Scientific; Cat# 1255017).

2.4 Immunostaining of bone tissues

The bone tissue sections were deparaffinized in xylenes and then rehydrated through an ethanol gradient. Bone tissues were placed in containers with citrate buffer (10 mmol/L sodium citrate, 1mmol/L EDTA, 0.05% Tween-20, pH 6.0) for heat-induced antigen retrieval using 2100 Retriever (Electron Microscopy Science, Hatfield, PA). Tissues were then blocked in 1% bovine serum albumin (Sigma-Aldrich; Cat# A7906) in PBS containing 0.1% Tween-20 (Sigma-Aldrich; Cat#P9416) for 1 hour. After blocking, the tissues were incubated with rabbit anti-SCA1 antibody (1:150, Abcam; Cat# Ab109211) at room temperature for 1 hour. Next, the tissues were incubated in FITC-conjugated anti-rabbit (1:200, Thermo Scientific; Cat# F-2765) secondary antibody for 1 hour at room temperature. Then, all sections were exposed to Vector TrueVIEW autofluorescence Quenching reagent (Vector Laboratories; Cat# SP-8400-15). The same procedure was completed for the negative control sections except for the primary antibody step. Fluorescence images were taken using an Olympus BX-51 microscope equipped with Infinity 3-1 Color CCD camera (Teledyne Lumenera, Ottawa, Canada) and Infinity Analyze software (Teledyne Lumenera). The same exposure time was used for all sections. ImageJ was used to measure fluorescence staining intensity per area of the sections.

2.5 Hematoxylin and eosin staining of tissues

The bone tissues were deparaffinized in xylene and rehydrated through an ethanol gradient. The tissues were stained using hematoxylin (Leica Biosystems; Cat#3801561)
and eosin (Leica Biosystems; Cat#3801601) for morphometric analysis. The stained slides were digitalized using Aperio slide scanning system (Aperio Technologies, Inc., Vista, CA) with a 20X and 40X objective and saved in Tagged Image File Format (TIFF).

### 2.6 Histomorphometric analysis

Histomorphometric analysis were performed on H&E-stained mouse bone marrow tibia and femur tissues (Figure 2.6). Selected scanned images were first uploaded onto the program QuPath. To account for differences in adiposity due to spatial variability, only sections where the epiphysis was visible was selected for histomorphometric analysis. Then, the tissue boundaries were annotated onto the images. The tissue boundaries for the tibia were defined as the first third of the bone. Due to the curvature of the tibia, a single cross section of the entire bone was not possible to capture for all models. Therefore, to maximize sample size, a third of the length of the bone was selected. The third of the length of bone was calculated using the measured length the whole bone (the number of pixels in an annotated line from the epiphysis to the tip of the distal tibia). The length of the entire bone was then multiplied by a third. The resulting length was then used for the tissue boundaries, starting from under the epiphysis. Since the femur is more robust, the entire bone was included in the annotated tissue boundaries. After the tissue boundaries were defined, all artifacts were annotated. Since MarrowQuant calculates the adipocytic space as all white spaces, the white spaces which were not adipocytes were annotated as artifacts (blood vessels, gaps in the tissue, etc.). Then, an orange rectangle labeled as background was placed in one of the white artifact annotations. MarrowQuant (a semi-automated image analysis plug-in for Qupath) was ran, and the percent adiposity (adipocyte area/marrow area) and percent interstitial/microvasculature area (%IMV) was analyzed. Finally, user/pathologist feedback was integrated to ensure that the adipocyte selections were correct.
## Image Preparation

<table>
<thead>
<tr>
<th>A</th>
<th>Acquire &amp; scan H&amp;E-stained bone sections.</th>
</tr>
</thead>
<tbody>
<tr>
<td>B</td>
<td>Select &amp; Annotate.</td>
</tr>
<tr>
<td>C</td>
<td>Run MarrowQuant.</td>
</tr>
<tr>
<td>D</td>
<td>Save Results output.</td>
</tr>
<tr>
<td>E</td>
<td>Integrate User/Pathologist feedback.</td>
</tr>
</tbody>
</table>

Slides scanned using Aperio slide scanning system.

| Slides scanned using Aperio slide scanning system. | Tissue Boundaries, Background, Artifacts | Background colour balance. Detection of cells, e.g., white space not marked as artifact for adiposity. | % Adiposity (Adipocytic Area/(Marrow Area)) | %IMV (Interstitium & Microvasculature/ (Marrow Area)) | Re-annotate and run if necessary |

**Figure 2.6: Workflow diagram of MarrowQuant.**

Morphometric analysis was performed using QuPath MarrowQuant. (A) The H&E-stained sections were uploaded to QuPath. (B) The ‘Tissue Boundaries’, ‘Background’, and ‘Artifacts’ were annotated on the images. To calculate the percent adiposity (adipocyte area/marrow area), all white spaces that were not identified as fat were marked as artifacts and therefore omitted from the quantification of adipocyte area. (C) Marrowquant was run with the user-adjusted parameters. (D) The measurements of interest (% Adipocytic Area/ (Marrow Area) and %IMV (Interstitium & Microvasculature/ (Marrow Area)) were saved. (E) User and/or pathologists’ feedback was integrated by correcting for false-positive adipocyte selection.
2.7 Statistical analysis

The data were expressed as means ± SEM. All tissues were analyzed from mice, without exclusion. When appropriate, student’s unpaired t-tests, analysis of variance (ANOVA) was performed using Graphpad Prism 9. Following ANOVA, Tukey post-hoc analyses were conducted. P values < 0.05 were considered statistically significant.
Chapter 3

3 Results

3.1 Increased adipogenesis in tibias of mice with ageing

My first objective was to observe the cellular changes which occur in the bone marrow with ageing, with the specific goal of observing emergence of adipocytes. Past studies have found that adipocytes accumulate in the tibia marrow of middle-aged mice. In addition, previous work in our laboratory found that increased adiposity in the bone marrow of diabetic mice may lead to the depletion of vascular regenerative stem cells. Therefore, I aimed to determine if age-related adipocyte accumulations could be correlated to the stem cell ageing phenotype. I performed morphometric analysis on H&E-stained tibia and femur sections to confirm and characterize when adipogenesis occurs in the bone marrow of ageing mice (Figure 3.1.1). Consistent with literature, BMAT, and specifically cBMAT, was observed in the distal end of the tibia tissues in mice of all ages (Figure 3.1.1). I also noted adipocyte accumulations occurring at the proximal ends, and there were little to no adipocytes in the middle section of the tibiae (Figure 3.1.1). Therefore, I used MarrowQuant plugin of QuPath to determine the percent adiposity within the first third of each tibia section, proximal to the epiphysis (Figure 3.1.2). There was a significant increase in adiposity by 67-71-weeks of age in both female and male tibia marrow sections (Figure 3.1.2B). In the female tibiae, there was a significant increase between all ages and the 67-71-week-old images, while there was only a significant increase in adiposity between the 8-week and 67-71-week old male tibiae.

To investigate if adiposity increases with age on a molecular level, I measured the mRNA levels of adipocyte marker perilipin 1 (Plin1) in the tibia flush samples using qPCR. There was no detected cycle threshold (CT) reading for many of the samples from mice under 48-weeks (Table 3.1.1). In contrast, there was a detected reading for both the older male and female samples (58-71weeks) (Table 3.1.1, Figure 5.2A-APP). There were also higher CT values (indicative of lower initial mRNA levels) for the samples which had positive Plin1 readings in the mice under 48-weeks of age, however not statistically
significant (Table 3.1.2, Figure 5.2-APP). This suggests that there is an increased in adiposity in tibia marrow of older mice (58-67-week-old).

The morphometric and transcript level results were compared to see if the cellular and molecular changes are correlated (Figure 3.1.3). The samples which showed a positive Plin1 CT value were associated with the tibiae which had an increased percent adiposity (adipocyte area/marrow area). A CT value was considered positive if there was detectable amplification of Plin1 in 40 cycles or less.

I also aimed to determine if there would be any age-related changes within the marrow of the femur (Figure 3.1.4). Surprisingly, I observed there were no differences in the presence of adipocytes within the H&E-stained marrow sections of the femurs with ageing. I selected the 24- and 67-71-week-old femur sections to perform morphometric analysis. There were no significant differences in percent adiposity (adipocyte area/marrow area) within the C57BL/6N femurs between the ages 8-, 24-, 48-, 58-61-weeks (Figure 3.1.5). Therefore, for the remaining studies, I focused on the tibia.
Figure 3.1.1: Representative whole images of H&E-stained mice tibias in different age groups.

The tibia of all C57BL/6N mice at 8- to 67-71- weeks of age were harvested, fixed in formalin, sectioned, and stained with hematoxylin and eosin (H&E). Whole slide images were acquired by Aperio slide scanning system. Representative images shown.
Figure 3.1.2: Increased adiposity in tibia marrow of ageing male and female mice.

The tibia of C57BL/6N mice at 8 to 67-71 weeks were harvested, fixed in formalin, sectioned, and stained with hematoxylin (H&E). (A) Representative images of the tibia epiphysis and proximal tibia. (B) The percent adiposity [calculated as adipocyte area/marrow area] of the H&E-stained tibia sections were quantified using QuPath [Mean ± SEM; n=4-7; a two-way ANOVA was performed; *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001].
Table 3.1.1: Percentage of tibia flush samples positive for *Plin1* and *Gapdh* mRNA detection by qPCR.

<table>
<thead>
<tr>
<th>Age [weeks]</th>
<th>Sex [M/F]</th>
<th>Sample size [n]</th>
<th>% samples positive for <em>Plin1</em></th>
<th>% samples positive for <em>Gapdh</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>8</td>
<td>F</td>
<td>7</td>
<td>57.1</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>M</td>
<td>7</td>
<td>28.6</td>
<td>100</td>
</tr>
<tr>
<td>24</td>
<td>F</td>
<td>7</td>
<td>57.1</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>M</td>
<td>7</td>
<td>14.3</td>
<td>100</td>
</tr>
<tr>
<td>48</td>
<td>F</td>
<td>7</td>
<td>14.3</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>M</td>
<td>7</td>
<td>28.6</td>
<td>100</td>
</tr>
<tr>
<td>58-61</td>
<td>F</td>
<td>4</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>M</td>
<td>4</td>
<td>75</td>
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<tr>
<td>67-71</td>
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<td>100</td>
</tr>
<tr>
<td></td>
<td>M</td>
<td>4</td>
<td>100</td>
<td>100</td>
</tr>
</tbody>
</table>

Table 3.1.2: Average amplification threshold cycles for *Plin1* and *Gapdh* mRNA in tibia flush samples.

<table>
<thead>
<tr>
<th>Age [weeks]</th>
<th>Sex [M/F]</th>
<th>Sample size [n]</th>
<th>Average <em>Gapdh</em> qPCR threshold cycle [Cq]</th>
<th>Average <em>Plin1</em> qPCR threshold cycle [Cq]</th>
</tr>
</thead>
<tbody>
<tr>
<td>8</td>
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<td>7</td>
<td>23.70</td>
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</tr>
<tr>
<td></td>
<td>M</td>
<td>7</td>
<td>22.61</td>
<td>36.38</td>
</tr>
<tr>
<td>24</td>
<td>F</td>
<td>7</td>
<td>24.73</td>
<td>37.16</td>
</tr>
<tr>
<td></td>
<td>M</td>
<td>7</td>
<td>25.24</td>
<td>37.31</td>
</tr>
<tr>
<td>48</td>
<td>F</td>
<td>7</td>
<td>25.56</td>
<td>36.30</td>
</tr>
<tr>
<td></td>
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<td>7</td>
<td>24.98</td>
<td>37.42</td>
</tr>
<tr>
<td>58-61</td>
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<td>22.47</td>
<td>34.93</td>
</tr>
<tr>
<td></td>
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<td></td>
<td>M</td>
<td>4</td>
<td>23.10</td>
<td>34.74</td>
</tr>
</tbody>
</table>
qPCR was performed on tibia marrow flush samples to detect the gene abundance of Plin1. The x-axis shows whether samples were positive (yes) or negative (no). Positivity was defined as threshold cycle < 40. Negative was defined as no detectable amplification in 40 cycles of qPCR. The % Adiposity [adipocyte area/marrow area] was quantified by QuPath MarrowQuant. Each point represents a mouse [Mean ± SD; a two-tailed student’s t-test was performed; **p<0.01].
Figure 3.1.4: Representative whole images of H&E-stained mice femurs in different age groups.

The femur of C57BL/6N mice at 8- to 67-71-weeks of age were harvested, fixed in formalin, section, and stained with hematoxylin and eosin (H&E). Whole slide images were acquired by Aperio slide scanning system. Representative images shown.
Figure 3.1.5: No significant age-related changes in femur adiposity in male and female mice.

The percent adiposity [adipocyte area/marrow area] of the H&E-stained femur tissues from 24- and 67-71-week-old mice was quantified using QuPath MarrowQuant [Mean ± SEM; n=4-7; a two-way ANOVA was performed]. No significant differences were noted.
3.2 Tibia osteogenesis and microvasculature

It is generally known that reductions in bone density and mineralization is associated with advanced age. Therefore, I measured the gene abundance of osteoblast marker, Runx transcription factor 2 (Runx2). In both female and male tibia marrow, there was a significant increase in Runx2 abundance at old age (58-61 weeks-age) compared to young (24 weeks-age) and middle (48 weeks-age) age (Figure 3.2.1A).

Since the function of stem cells is greatly impacted by vasculature, I measured the percent interstitial/microvasculature area (%IMV) in tibia sections of male and female 8-, 24-, 48-, 58-61-, and 67-71-week-old C57BL/6N (Figure 3.2.1B). In the female marrow, there was a significant decrease in %IMV by old age (58-61-weeks age). In the male marrow, there was a phasic change in %IMV with the peak %IMV values being at 24- and 58-61-weeks age.
Figure 3.2.1: Cellular changes appear in the bone marrow of mice tibiae at old age.

(A) mRNA levels of osteoblast-associated Runx2 were measured by qPCR in the bone marrow flush of C57BL/6N mice at 8-, 24-, 48-, 58-61-, and 67-71-weeks age. (B) The percentage of interstitial/microvasculature area (%IMV) of the H&E-stained tibia tissues was quantified using QuPath MarrowQuant [Mean ± SEM; n=4-7; a two-way ANOVA was performed; *p<0.05, **p<0.01].
3.3 The HSPC ageing phenotype

My second objective was to observe the hematopoietic and non-hematopoietic stem cell ageing phenotype in the bone marrow of male and female C57BL/6N mice. I first screened for the gene abundance of HSPC markers to determine if the hematopoietic stem cell ageing phenotype is evident in the tibia marrow of mice 8- to- 71-weeks age (Figure 3.3.1). I observed that there were no age-related changes in the gene abundance of myeloid progenitor marker erythropoietin receptor$^{150,151}$ (Epor) and lymphoid progenitor marker interleukin 17 receptor A$^{151,152}$ (Il17ra) in the tibia marrow flush samples (Figure 3.3.1a,b). In addition, there was no change in hematopoietic stem cell marker protein tyrosine phosphatase (Ptprc) in the tibia flush samples between the ages of 8 to 67-71 weeks (Figure 3.3.1c).
Figure 3.3.1: mRNA levels of HSPC genes in tibia of mice.

mRNA levels of hematopoietic stem cell-associated genes were measured by qPCR. Figure showing (A) Epor, (B) Il17ra, and (C) Ptprc in the tibia marrow flush of C57BL/6N mice at 24-, 48-, and 67-71-weeks age. [Mean ± SEM; n=4-7; Two-way ANOVA and Tukey Post hoc analysis were performed].
3.4 The stem cell ageing phenotype

I examined the gene abundance of stem cell markers in the tibia marrow of male and female mice. First, I performed immunofluorescence staining of male and female tibia tissues for stem cell antigen-1 (SCA1). SCA1 is perhaps the most reliable precursor cell marker in mice\(^{153–155}\). Previous studies have shown protein level of SCA1 in marrow stem cells but not adipocytes\(^{154–156}\). The corrected total cell fluorescence (CTCF) represents the fluorescent intensity of each cell per area. The tibia tissues showed an increased immunoreactivity to SCA1 by middle age in compared to the young and old aged female mice (Figure 3.4.1, Figure 5.4-APP). In addition, there was a significant decrease in SCA1 intensity between the 24- and 67-71-week-old male tibia sections (Figure 3.4.2B, Figure 5.4-APP). However, the mRNA levels of Sca1 increased from young to old age in the marrow flush of both males and females (Figure 3.4.2A). There was a decline in Sox2, another stem cell gene, between young to old age and no change in females and males, respectively (Figure 3.4.2B).
Figure 3.4.1: SCA1 immunoreactivity in tibias of mice in different age groups.

(A) Immunofluorescence staining for SCA1 (green) in tibias of female and male 8-, 24-, 48-, 58-61-, and 67–71-week-old C57BL/6N mice. (B) Quantification of the SCA1 fluorescence intensity [per area] as determined by ImageJ [Mean ± SEM; 2 images per mouse tissue were measured; a two-way ANOVA with Tukey Post hoc tests were performed; *p<0.05, **p<0.01, ***p<0.001].
Figure 3.4.2: mRNA levels of stem cell genes in the tibia of mice.

mRNA levels of stem cell genes (not restricted to hematopoietic stem cells) were measured by qPCR. Figure showing (A) Sca1 and (B) Sox2 in the tibia marrow flush of C57BL/6N mice. [Mean ± SEM; n=4-7; a two-way ANOVA with Tukey Post hoc tests were performed; *p<0.05, **p<0.01].
3.5 Levels of Igfl and Tgfb1 in tibia of mice

As outlined earlier, studies have suggested that cell-external factors, such as paracrine growth factors, may alter stem cell function in the marrow. Therefore, to investigate the indirect mechanisms which may facilitate the stem cell ageing phenotype, I measured the mRNA levels of insulin like growth factor 1 (Igf1) and transforming growth factor beta 1 (Tgfb). Recently, a study showed a decline in IGF1 protein levels in crushed bone tissues of middle-aged mice. It is not known whether this decline is due to reduced circulating IGF1 or reduced abundance in the bone marrow. Our laboratory has also shown increased adipogenesis in the tibia of mice with diabetes to be associated with suppressed levels of TGFB. In my examination, there was a significant increase in gene abundance of Igf1 at 58-61-weeks age compared to young (24 weeks) and middle aged (48 weeks) male mice (Figure 3.5.1A). Interestingly, there was an increase in Tgfb from young to middle age in the marrow of both female and male mice (Figure 3.5.1B).

There were sex-related differences observed in the ageing of stem cells (Results 3.4), it is likely that changes in sex steroid hormone signaling influences the activities within the bone marrow environment during the ageing process. Therefore, I explored the mRNA levels of estrogen receptor 1 (Esr1) and androgen receptor (Ar) in male and female marrow from 8- to 71-weeks of age (Figure 3.5.2). There was a significant increase from young to old age in transcript level of Esr1 and Ar in both male and female tibiae (Figure 3.5.2).
Figure 3.5.1: mRNA levels of *Igf1* and *Tgfb1* in the tibia of C57BL/6N mice.

*Genes were measured by qPCR. Figure showing (A) *Igf1* and (B) *Tgfb1* in the tibia marrow flush of C57BL/6N mice at 8-, 24-, 58-61-, and 67-71-weeks age. [Mean ± SEM; n=4-7; a two-way ANOVA with Tukey Post hoc tests were performed; *p<0.05, **p<0.01].*
Figure 4.5.2: mRNA levels of sex steroid receptors *Esr1* and *Ar* in the tibia of C57BL/6N mice.

mRNA levels were measured in marrow flush samples by qPCR. Figure showing mRNA levels of (A) estrogen receptor-1 (*Esr1*) and (B) androgen receptor (*Ar*) in 8-, 24-, 58-61-, and 67-71-week-old mice. Male and female mice are shown separately [Mean ± SEM; n=7 for 24-47; a two-way ANOVA and Tukey Post Hoc tests were performed; *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001].
4 Discussion

4.1 Cellular changes in the bone marrow microenvironment

My first objective was to elucidate the cellular changes which occur in the bone marrow microenvironment during normal ageing. Morphometric and gene abundance analyses of murine male and female tibia showed a positive correlation between marrow adiposity with age (Figure 3.1.2 and Table 4.1.1). However, there are differences in adipogenesis between the data of this study and previous findings. While it was previously shown that there is an increased adiposity in the proximal to middle tibia of C57BL/6 by middle age\(^93\), adipogenesis increased in female mice by old age (67-71 weeks) only in the proximal tibia in my study (Figure 3.1.2B). However, the percent adiposity was only calculated in the first third of each tibia below the epiphysis (Figure 3.1.2A). This was because the curvature of the tibia made it difficult to capture the entire bone in a singular cross section. Therefore, the lack of adipocyte clusters observed in the middle section of the tibiae, may have been attributed to the exclusion of that area upon initial screening for adiposity. Setting the tissue boundaries as the first third of each tibia allowed for a greater sample size to be included in the analysis.

There was a wide range in sample variance in the calculation of percent adiposity in the 67-71-female and male biological replicates. For instance, while there was a significant increase in adiposity by old age in female mice, I observed that one of the mice from this group did not have many adipocytes in the tibia marrow (Figure 3.1.2 and Figure 5.3-APP). The sample variance may be due to confounders in the morphometric analysis. A confounder which could have skewed the morphometric analysis is the presence of gaps and holes in some of the tibia samples (Figure 5.3-APP). To calculate the percent adiposity, QuPath detected the area of white spaces in the tibia marrow. Therefore, any holes were marked as artifacts to be excluded in the calculation. However, that may have led to a misrepresentation of the tibia marrow landscape.

I also examined the effect of ageing on adipogenesis from a molecular perspective. Transcript abundance studies suggest that adipocyte marker Plin1 has a higher transcript level in older mice compared to young (Table 4.1.1). qPCR was not able to detect Plpn1
in all the marrow flush samples of mice 8- to 48-weeks of age, therefore it can be assumed that those samples have very low gene abundance of the adipocyte marker. In addition, the gene abundance of housekeeping gene Gapdh was detected in all samples. This shows that the lack of detection of Plpn1 was not due to damage of the samples.

As a quality control check, I compared the marrow flush samples which had a positive Plin1 reading (indicating presence of mRNA) to the percent adiposity quantified from the histological sections. The samples which did have a Plin1 reading were significantly correlated with a higher percent adiposity (Figure 3.1.3). This provides validity to the results. However, the 67-71-week-old female and male mice which had a low percent adiposity also had a positive Plpn value (Table 5.1-APP). This suggests that there could have been an increase in adiposity in those samples as well. Therefore, future studies with a larger sample size of older mice would be required to strengthen the claim that increased adiposity within the tibia occurs by older age in mice. It should be noted that I also examined transcript levels of other adipogenesis-related genes, including Pparg, Cebpa, Lep, and Fabp4 (data not included because it did not add any information). Like Plin1, I found these genes to be expressed at a low to undetectable level.

As expected, there was a stronger increase in adiposity by old age in the female mice than their male counterparts (Figure 3.1.2). This is consistent with current knowledge surrounding the metabolic changes which occur in postmenopausal women. Skeletal adiposity is associated with ageing in women due to the drop in estradiol which occurs after menopause\textsuperscript{157}. Estrogen supplementation in postmenopausal women was shown to decrease marrow adiposity by 5\%\textsuperscript{158}. This suggests that the changes involve sex steroid hormones may extend to the bone marrow microenvironment.

Interestingly, there was no significant differences in percent adiposity within the bone marrow of femur sections (Figure 3.1.5). Only a modest increase in adiposity was observed in the older femurs of both the male and female mice. However, chemical shift encoding-based water-fat MRI in ageing human participants showed that femur fat fraction increased in females, and not males\textsuperscript{159}. Contradictory to my observations, this study found that with ageing, BMAT increases in order from the axial skeleton to the
proximal then distal femur, and little adiposity exists in the tibia\textsuperscript{159}. The lack of significance noticed in morphometric analysis of the ageing murine femurs could be due to differences in species. Mice are known to display a decreased adiposity in comparison to humans\textsuperscript{160}. Therefore, it is justified that there would be less fat noticed in the femurs. In addition, QuPath calculated the percent adiposity as the adipocyte area/marrow area. Since the femurs have an overall greater area, the impact of adiposity is lower. In addition, I calculated the percent adiposity only within the proximal tibia (where the adipocyte accumulations were observed in the older mice). Since the femurs are more robust and anatomically straight bones, I was able to capture the entire section (Figure 3.1.4). Therefore, I set the tissue boundaries as the entire bone when quantifying the percent adiposity within the femurs. Overall, I observed there was a greater age-related increase in adiposity within the tibias in comparison to the femurs. Therefore, a future goal for research could be to dissect the differences in bone marrow niche dynamics between various bones.

Since there were no age-related changes observed in the femurs, I decided to focus on the tibiae for the remainder of my studies. I decided to investigate how ageing effects the presence of osteoblasts in the tibia marrow because reductions in osteoblasts is a key characteristic of cellular remodeling within the bone marrow during ageing\textsuperscript{161}. In addition, losses in estradiol in postmenopausal women is associated with a reduction in osteoblast activity\textsuperscript{162}. Therefore, I aimed to determine if changes in the detection of osteoblast markers in the bone marrow correlates to stem cell ageing. There was an increase in gene abundance of osteoblast marker Runx2 between the 24- and 48-week-old mice with the 58-61-week-old mice (Figure 3.2.1). A potential explanation for the increase in transcript level of Runx2 is that the fate of Runx2-expressing preosteobasts may be altered during ageing\textsuperscript{161}. It was previously shown that Runx2+ cells have the capacity to form lipid droplets under pathological and ageing conditions\textsuperscript{161}. Studies have shown that Runx2+ MSC-derived osteoblast cells expressed lipid droplets under the conditional deletion of the epigenetic regulator histone deacetylase 3 (Hdac3)\textsuperscript{163}. In addition, the depletion of Hdac3 levels in bone is associated with advanced age\textsuperscript{164}. Therefore, it is possible that the increase in Runx2+ cells observed in the older mice contributes to age-related adipogenesis. However, since bone density is known to
decrease with ageing and the osteoblasts play an important role in the replenishment of osteocytes, future studies may also measure bone density using technologies such as a dual-energy X-ray absorptiometry (DEXA) scan. A decrease in bone density and increase in osteoblast gene abundance in elderly mice would support the speculation of an altered osteoblast function occurs with advanced age.

The bone marrow microvasculature plays a major role in facilitating the systemic responsibilities of the marrow-resident stem cells. Morphometric analysis revealed a significant decrease and no change in the percent interstitial/microvasculature area (IMV) in the tibia marrow of old female and male mice respectively (Figure 3.3.2). This is consistent with the common ageing phenotype of a decline in function of the microvasculature and endothelial dysfunction. A reduction in blood vessels was found in the bone marrow old mice (66-120 weeks of age) compared to young mice (8-30 weeks of age). However, the sex of the mice was not disclosed. To date, there are no studies which investigate the differences between female and male microvasculature with ageing. In addition, a phasic trend of %IMV with ageing has not been reported before. The alteration of %IMV with old age may have been a result of endothelial progenitor or ECFC deficits. Therefore, further studies can investigate ageing of endothelial precursor cells. A decreased microvasculature with age would result in a hindered mobilization of stem and progenitor cells to the vasculature and contribute to the characteristic ageing phenotype. Therefore, this topic creates an avenue for future work.

4.2 The stem cell ageing phenotype

Interestingly, the transcript level analysis did not detect any age-related changes in the mRNA levels of HSPC markers (Figure 3.3.1). However, the known HSPC ageing phenotype is characterized as an increased proliferation of HSCs, but some other studies suggest that HSC frequency remains stable throughout adulthood. Therefore, the similar mRNA levels detected in the Piprc expression in the mice from young to old age reflects current knowledge. This suggests that changes in HSC activity during ageing is likely characterized as in an alteration in function rather than frequency in the bone marrow.
To investigate any age-related HSC lineage skewing, I measured the mRNA levels of myeloid and lymphoid progenitor markers *Epor* and *Il17ra*, respectively. *Epor* is present on the surface of immature erythroid cells. The binding of erythropoietin (EPO) to EPOR facilitates the survival, proliferation, and differentiation of premyeloid progenitor cells. The known HSPC ageing phenotype is characterized as an increase in myeloid progenitor frequency. In addition, previous work has found that *Epor* expression increased from infancy to adulthood in the neurons of rats. Therefore, it was expected that *Epor* expression would increase with ageing. The transcript level analysis of lymphoid progenitor marker interleukin 17 receptor A (*Il17ra*) also did not align with the known HSPC ageing phenotype. It was expected that lymphoid progenitors decrease with ageing. An explanation for differences between the data and literature found in transcript level analysis in this study could be due to limitations posed by the small sample size of the eldest group of mice (67-71-weeks of age). In addition, it is possible there is a disconnect between gene and protein levels with ageing. A previous study has found that in female mice, there was an expansion of bone marrow derived HSPCs using flow cytometry. They did not notice a change in transcript level of HSPC markers in the bone marrow of female mice. This demonstrates that there can be dimorphisms between protein and gene abundance of the same target.

Previous studies suggest that there is a reduction in MSC proliferation with increased ageing. To investigate the stem cell ageing phenotype, I measured the gene and protein levels of stem cell markers within the tibia marrow. I performed immunostaining to detect SCA1 in tibia sections, and qPCR to quantify the gene abundance of *Sca1* and *Sox2*. SCA1 is a cell surface protein which is known to be expressed on many stem and progenitor cell types. Particularly in the bone marrow, SCA1 expression is associated with HSPCs and MSCs. *SOX2* is a transcription factor which plays a role in maintaining the self-renewing abilities in stem cells. *SOX2* was originally found in embryonic stem cells, but its expression has also been seen in adult stem cells such as bone marrow MSCs. *SCA1* and *SOX2* are markers of cells which have the stemness characteristic. However, since transcript level studies analyzing HSC markers showed no significant changes with ageing (Figure 3.3.1), quantified mRNA levels of
Sox2 and Sca1 in the tibia marrow flush samples may detect expression patterns non-hematopoietic stem cell ageing.

The measurement of protein and gene abundance of these stem cell markers showed varying trends associated with ageing in males and females. There was a significant increase in SCA1 immunofluorescence at middle age in female stained tibia sections compared to the young and old counterparts (Figure 3.4.1). To date, there are no other findings which show an increase in stem cell expression at middle age. However, HSCs are known to increase in frequency with ageing\textsuperscript{176}. Therefore, a potential explanation could be that the increase noticed at middle age is attributed to HSC expansion. In addition, there was a significant decrease in SCA1 fluorescence intensity between the tibia samples of young and old male mice (Figure 3.4.1B). This suggests that stem cell depletion occurs in male mice. However, this data is limited since there were no positive controls used for the staining of these images. A future goal would be to dissect the differences between the hematopoietic and nonhematopoietic changes which occur in protein expression in the bone marrow with ageing. The transcript level analysis showed that mRNA levels of Sca1 increased from young to old mice in both male and female tibia flush samples (Figure 3.4.2A). In addition, there was a significant decrease in Sox2 mRNA levels between young and old mice in female tibia samples and no age-related change in the male flush (Figure 3.4.2B). The existence of sex-related differences in the gene and protein expression of stem cell genes with ageing suggest that hormonal changes may impact the stem cell ageing phenotype\textsuperscript{177}. In addition, the variability noticed in ageing trends between gene abundance of HSCs (Figure 3.3.1) and general stem cell markers (Figure 3.4.2) suggest that stem cell ageing occurs differently depending on the type of stem cell.

### 4.3 Igf1 and Tgfb1 in the marrow

It is proposed that cells within the bone marrow environment can indirectly communicate with stem cells through extrinsic factors such as growth factors. Therefore, to investigate potential mechanisms which may dictate stem cell ageing, I measured the gene abundance of Igf1 and Tgfb in the tibia marrow of C57BL/6N mice (Figure 3.5.1). Since most cellular changes occurred in the 24-week-old male and female tibia sections, I
decided to focus on this age to represent young mice. I selected Igf1 as a candidate factor because it was previously found that the age-related decline in IGF1 was responsible for the HSC ageing phenotype\textsuperscript{114}. IGF1 is a regulator of tissue growth and development\textsuperscript{178}. In the bone marrow, IGF1 is derived from osteoblasts and chondrocytes\textsuperscript{179}. In addition, it was found that the deletion of Igf1 from bone marrow MSCs resulted in decreased bone formation, impaired bone regeneration, and increased adipogenesis, which are common characteristics associated with bones of advanced age\textsuperscript{179}. A previous study found that the deletion of IGF1 in MSCs results in decreased bone mass\textsuperscript{180}. In addition, reductions in IGF1 have been associated with increased adipogenesis in the bone marrow\textsuperscript{181}. There was a significant increase in mRNA levels of Igf1 in tibia flush samples of male C57BL/6N (Figure 3.5.1A). There was no increase in Igf1 expression in female mice. This unexpected result may be due to a disconnect between gene and protein expression\textsuperscript{114}. Therefore, to elucidate how ageing impacts IGF1, future work can simultaneously compare how ageing impacts the expression of Igf1 and IGF1 protein levels. Since histomorphometry showed increased adipogenesis with ageing, it makes sense that IGF1 would decline with ageing. However, the increase in Igf1 with ageing in male mice suggests there could be alterations in translation of Igf1 to IGF1 during the ageing process.

Interestingly, in the male tibia samples, the significant age-related increase of Igf1 observed preceded the decrease in SCA1 intensity. There was a significant increase in Igf1 mRNA levels between the 48-week and 58-61-week-old male tibias (Figure 3.5.1A). The decrease in fluorescence intensity occurred in males between 24-weeks and 67-71 week old males. This suggest that activities involving Igf1 within the bone marrow may contribute to age-related stem cell declines in males.

Tgf-b1 was selected as a candidate mechanism since it is altered with ageing, and since it a key player in the bone marrow microenvironment which has been shown to alter stem cell behaviour. It was reported that TGF-B signaling increased in human subjects of advanced age compared to young subjects\textsuperscript{182,183}. In addition, previous work in the lab showed that the suppression of TGF-B signaling was associated with enhanced adipogenesis and a reduction in number of marrow-resident vascular stem cells\textsuperscript{134}. 

Interestingly, there was an increase in Tgf-b1 expression by old age in both the male and female tibia flush samples (Figure 3.5.1B). This is consistent with what is currently known about how ageing impacts Tgf-b. Since there was also an increased adipogenesis at similar age ranges, the increase in Tgf-b suggests is possibly correlated with age related adipogenesis.

Since there were sex-related differences observed in the ageing of stem cells and adipocytes, I investigated the expression of estrogen receptor 1 (Esr1) and androgen receptor (Ar) to elucidate the hormonal changes which occur in the bone marrow during the ageing process. There was a significant increase in Esr1 expression in the mice of old age in comparison to young and middle-aged mice (Figure 3.5.2.A). There was also an increase in Ar mRNA levels associated with old mice (Figure 3.3.5B). To date, there have been no reports of estrogen and androgen receptor frequency in the bone marrow with ageing. However, sex steroid hormones have been previously shown to impact the behaviour of stem and progenitor cells in the bone marrow such as MSCs. For instance, evidence has suggested that estradiol promotes the osteogenic differentiation of bone marrow MSCs through ER1184. In addition, the estrogen favours HSC cell self-renewal in males and females185,186. Since the decline in estrogens is associated with advanced age187, it was expected that Esr1 and Ar decline by old age in the tibia marrow of mice. An explanation for the increase in Esr1 could be that sex steroid hormones act independently of their receptors. Therefore, to further investigate the role of sex steroid hormones on bone marrow ageing dynamics, future studies can involve manipulation of the presence of circulating estrogens and androgens. However, it is interesting that there was a significant change by old age. This suggests that ageing did alter Esr1 and Ar transcript levels. Therefore, increases in Esr1 and Ar might be involved in the ageing of the bone marrow niche.

### 4.4 Limitations

A limitation to this study is the increased risk of confounders in the data pertaining to 58-61- and 67-71-week-old mice models. This is due to the potential of disease, sample size, and differences in treatment. At older ages, animals are more susceptible to developing age-related diseases. In terms of C57BL/6N mice, age-related diseases may include
cancer, dementia, Alzheimer’s, hearing loss, and even bone density changes, obesity and diabetes. Therefore, when studying aged models, there is the potential for pathology confounding the analysis of normal ageing. This may have led to the high variability noticed in some of the results. For instance, in the calculation of percent adiposity in the tibias of female and male 67-71-week-old mice (Figure 3.1.2). Another limitation of this study was sample size. Firstly, the variability in sample sizes across age groups may have confounded the data. The sample sizes of the two eldest groups were 4 mice, while the rest was 7 mice. It is more difficult to obtain and grow mice to older ages. Therefore, given the timeline of this project, the sample size of the two eldest groups was smaller than the rest. Another concern with sample size is that a sample size of 4 for the older mice reduces reliability. Differences in housing of the mice during the one-week acclimation period may have also created discrepancies in the results. Due to excessive fighting, the 58-61 and 67-71 male mice were all housed in their own cage, while the remaining mice were housed in groups of 4. To optimize the wellbeing of the mice, if possible, the mice were not housed alone. Stress or loneliness may have caused physiological changes in the mice that were housed alone. A previous study has found that compared to mice who were housed alone, group-housed mice showed greater variance in percentage of body fat, which was likely a result of changes in behaviour\textsuperscript{188}. This demonstrates that differences in social dynamics can alter physiological changes, specifically adipogenesis. Therefore, the single-housing of the male 58-61- and 67-71-week-old male mice may have impacted the analysis comparing how the male mice age, and the sex-related differences observed between the ageing of male and female mice.

4.5 Concluding remarks

The overall objective of this study was to observe and correlate the age-related changes which occur in the bone marrow microenvironment to the stem cell ageing phenotype. I showed that the adipogenesis is increased by old age in the tibia of male and female C57BL/6N. In addition, stem cell expression studies showed that stem cell expression decreased by old age in males and increased by middle age in females. My results also suggested that age related increases in \(\text{Igf1}\) and \(\text{Tgf-b}\) might be involved in stem cell ageing. Lastly, sex-related differences in the ageing of the bone marrow suggest that sex
steroid hormones play a major role in the ageing of the bone marrow microenvironment and stem cell deficits. Identifying the mechanisms which drive stem cell deficits during the ageing process would contribute to major advancements in extending health span for the growing elderly population. The prospective goal of this study would be to produce clinical implications which prevent stem cell deficits and further impaired repair mechanisms. This research shows that approaches for preventing age-related stem cell deficits mechanisms for ageing may differ depending on sex. Understanding how age-related stem cell deficits occur would allow for prevention plans.

4.6 Future directions

While the results from his study correlated age-related cellular and molecular changes in the bone marrow microenvironment to the stem cell ageing phenotype, it is still unknown whether these alterations cause stem cell deficits. Determining how the bone marrow microenvironment alters stem cell ageing be a major achievement for stem cell and ageing research. The results suggest changes in adipocytes, *Igf1*, and *Tgf-b* may influence stem cell ageing. An extension of this study would be to explore pathways relating to *IGF1*, *TGF-B* to determine how they relate to adipogenesis and the ageing phenotype of different types of stem cells.

There remains to be knowledge gaps pertaining to the ageing of the bone marrow microenvironment. A major goal for future research would be to dissect the sex-related differences in the relationship between ageing stem cells and the surrounding stem cell niche. This kind of research would involve an investigation into how sex steroid hormones and their receptors impact the stem cell microenvironment during the ageing process.

Overall, understanding the mechanisms underlying stem cell deficits could lead to the development of therapeutic targets for preserving stem cells, which could have an impact on endogenous repair.
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Figure 5.1-APP. Quality control for bone marrow flush preparation.

The femur (A) and tibia (B) of 8-week female C57BL/6N mice was harvested. Epiphyses were removed and bone tissues were placed in nested centrifuge tubes. Tubes were centrifuged at 10,000 xg for 15 s to flush the bone marrow. Bone tissues were then fixed in formalin. Image showing hematoxylin and eosin (H&E)-stained bone tissues after flushing the marrow.
Figure 5.2-APP: Quantification of increased adiposity in tibia marrow of ageing male and female mice.

qPCR was performed on tibia marrow flush samples to detect the expression of Plin1. (A) Samples positive and negative for Plin1 were recorded as “1.0”, and “0.0” respectively. [Mean ± SEM; n=4-7; a one-way ANOVA was performed; p<0.05, **p<0.01] (B) Average Plin1 qPCR threshold cycle (Cq) was recorded [Mean ± SEM; n=4-7; a two-way ANOVA was performed].
Figure 5.3-APP. An example of low adiposity in the tibia of 67-71-week-old female C57BL/6N mouse.

The tibia of a 67-71-week-old female mouse was harvested, fixed in formalin, and stained with hematoxylin and eosin (H&E).
Table 5.1-APP. Adiposity and *Plin1* mRNA expression in tibia of C57BL/6N mice in different age groups.

Adiposity (%) was measured by MarrowQuant. Plin1 mRNA was detected by qPCR for maximum of 40 cycles.

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58-61-week-old mice

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67-71-week-old mice

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Figure 5.4-APP: Nuclear counterstain in tibias of mice in different age groups.

*DAPI (blue) counterstaining of tibia sections from female and male C57BL/6N mice at 8-, 24-, 48-, 58-61-, 67-71-weeks of age.*
Curriculum Vitae

Name: Mackenzie Hsu

Post-secondary Education and Degrees:

Queen’s University
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Thesis: Mechanisms underlying stem cell deficits in ageing
Supervisors: Christopher Howlett, MD and Zia A. Khan, PhD

Honours and Awards

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The University of Western Ontario
2023

Western Graduate Research Scholarship
The University of Western Ontario
2021-2023

Related Work Experience

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The Department of Physiology and Pharmacology, The University of Western Ontario
London, Ontario, Canada
Course code: PHYSPHAR3000E
09/2021-04/2022
09/2022-04/2023

Abstracts and Conference Presentations

Pathology and Laboratory Medicine Research Day, London ON
Abstract: Molecular mechanisms of ageing-related stem cell deficits in the bone marrow
Authors: Hsu M., Howlett C.J., Khan Z.A.
03/2023

Pathology and Laboratory Medicine Research Day, London ON
Abstract: Molecular mechanisms of ageing-related stem cell deficits in the bone marrow
Authors: Hsu M., Howlett C.J., Khan Z.A.
03/2022
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<th>Departmental and Community Activities</th>
<th>Science Rendezvous, Western University</th>
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<td>Booth leader</td>
<td>Designed, organized, and ran the Pathology and Laboratory Medicine booth.</td>
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**Undergraduate Research in Natural and Clinical Science and Technology (URNCST) Journal**

**Mentor**

Met with (weekly) and provided guidance to undergraduate students with writing a manuscript for the URNCST journal.

01/2023- Present

**Western Pathology Association**

**VP Multimedia**

Worked with the WPA and Department of Pathology and Laboratory Medicine to support events and advertisements.

11/2023- Present