Characterization of the Developmentally Regulated Osteoclast Support Potential of *In Vitro* Medullary Adipocytes

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Dedication

Dedicated to my parents Mrs. Betty Holt and Mr. Vance Holt-
  Appreciate the headstart you gave me.

  Gabrielle London Holt

Special Dedication:

Dr. Samuel Fan- Your memory will be with me forever. Thank you for
  your confidence in me; this I’ll never forget.
Table of Contents

- Dedication ............................................................... iv
- Acknowledgements.................................................... ix
- List of Abbreviations ................................................ xi
- Abstract ................................................................. xviii
- List of Tables ........................................................ xiii
- List of Figures ....................................................... xiv

Chapter 1 ................................................................................. 1
- Introduction ............................................................. 1
- Background .............................................................. 6

  Bone homeostasis and Bone Marrow cell physiology ..........6
  The Bone Marrow contains the Bone Modeling Unit and the
  Bone Remodeling Complex...............................................9
  Adipose tissue biology ..................................................14
  Aging and the adipocyte .................................................15
  Precursor pool changes with respect to age in adipose tissue
  ..................................................................................16
  Bone vs. Fat............................................................18
  Osteoblast and Osteoclast coupling: from migration to
  osteoclast and osteoblast maturity in the BMU.................19
  Elements of the Bone Modelling Unit can affect bone
  remodeling .................................................................23
    The marrow sinusoid ...................................................24
    The marrow stroma ...................................................26
    BRC lining cells .......................................................26
  Bone and Fat: Competing Hypotheses .........................27
Mesenchymal stem cell (MSC) and the MSC-derived Medullary Adipocyte Culture Model……………………31
Heterogeneous nature of in vitro adipocytes………………36

• Summary and Aims ………………………………………… 37

Chapter 2 ……………………………………………………………… 39

• The proposed role of the Medullary Adipocyte in Osteoclast development: A Thesis model ………… 39

• Materials and Methods ……………………………………… 45

\textit{Mesenchymal Stem Cell isolation and Culture} ..........45
\textit{MSC-derived medullary adipocytes} ..........................46
\textit{MSC-derived adipocyte culture enrichment and adipocyte ceiling culture} ................................. 46
\textit{Oil Red O staining} .................................................. 47
\textit{RNA isolation and quantitative PCR} .......................... 48
\textit{Immunofluorescence} .............................................. 49
\textit{PBMNC isolation and Osteoclast co-cultures} ............ 50
\textit{CyQuant Cell Counting} ........................................ 51
\textit{MSC-Dye-I labeling of HUVECs layered cultures} ...... 51
\textit{Confocal Imaging} ................................................... 52

Chapter 3 ……………………………………………………………… 53

• Results ………………………………………………………… 53

Medullary adipocyte differentiation from MSCs………….53
RANKL SDF-1, M-CSF, and OPG protein expression by MSC-derived medullary adipocytes………………..58
RANKL expression in MSC-derived adipocytes is based on lineage commitment: Model Validation ………… 64
RANKL, SDF-1, and M-CSF were expressed in distinct adipocyte populations…………………………….68
Preadipocyte cell proliferation……………………………………… 73
Adipogenic potential of RANKL expressing enriched cells….75
Early pro-adipogenic molecules in adipofibroblasts ............ 77
MSC marker expression among MSC-derived Adipocytes .... 82
RANKL expression among MSCs, Osteoblasts and Adipocytes ................................................................. 85
Adipofibroblast may support osteoclasts ......................... 92

Chapter 4 ................................................................................. 95
  ▪ Preliminary Data ............................................................... 95
  Exploring the role of the adipofibroblast in the BMU ........ 95
  Modeling the vascularized in vivo medullary adipocyte in vitro ..................................................................... 95
  Using Immunohistochemical analysis for giving insight into how the adipofibroblasts associate within the marrow .... 97
  Preliminary Conclusions: adipofibroblast-BMU association model ................................................................. 102

Chapter 5 .................................................................................... 104
  ▪ Discussion ........................................................................ 104
  Lineage markers and RANKL in the MSC derived adipocytes ........................................................................... 106
  A new cell: The RANKL-positive adipofibroblast .............. 108
  The adipofibroblast as an intermediate adipocyte ............ 110
  Pro-Adipogenic candidate genes are regulated differently in adipofibroblasts .................................................. 111
  The “Transitional” preadipocyte ........................................ 115
  Working model for MSC adipocyte Progression and RANKL/M-CSF/OPG expression .......................................... 117
  ▪ Future Directions ........................................................... 120
  Purifying the RANKL-positive adipofibroblast .................. 120
  Mimicking the fatty marrow in vitro: a 3D approach ......... 121
  Investigating the adipofibroblast in the aging adipose tissue .............................................................................. 122
• Conclusions .............................................................. 123
• Appendix .............................................................. 125
• Referenced Literature ............................................. 127
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List of Abbreviations

**MSC**- Mesenchymal Stem Cell

**AIM**- Adipogenic Induction Medium

**AMM**- Adipogenic Maintenance Medium

**GM**- Growth Medium

**BMU**- Bone Modeling/Multi-cellular Unit

**BRC**- Bone remodeling Compartment/Complex

**MA**- Medullary adipose/adipocyte

**RANKL**- Receptor Activator of NF-kb Ligand

**M-CSF**- Macrophage Colony Stimulating Factor

**OPG**- Osteoprotegerin

**SDF-1**- Stromal Derived Factor 1

**TNFα**- Tumor Necrosis Factor alpha

**IL-1β**- Interleukin 1 beta

**PPARγ**- Peroxisome Proliferating Activator Receptor gamma

**C/EBPα**- CCAAT Enhancer Binding Protein alpha
\textbf{C/EBP}\textsubscript{\textgreek{b}}- CCAAT Enhancer Binding Protein beta

\textbf{C/EBP}\textsubscript{\textgreek{d}}- CCAAT Enhancer Binding Protein delta

\textbf{FBS}- Fetal Bovine Serum
List of Tables

Chapter 1 .................................................................................................................. 1
  ▪ Table 1-1  Studies supporting the two hypothesized mechanisms of adipocyte regulation of bone remodeling 28

Chapter 2 ................................................................................................................. 39
  ▪ Table 2-1  Shows the various molecules involved in osteoclast regulation and formation leading to effective bone resorption, their specific role in the regulation of Osteoclast activity and the cells they are currently known to be expressed by................................. 44

Chapter 3 ............................................................................................................... 53
  ▪ Table 3-1  Depicts the average quantity of RANKL and PPARγ positive cells (n=3 donors) of several different fields; including the percentages of RANKL and PPARγ co-staining. ................................................................. 91
List of Figures

Chapter 1 ................................................................. 1

- Figure 1-1. Modeling and Remodeling schematic showing the mechanistic differences between bone modeling and bone remodeling .................................................. 8

- Figure 1-2. Schematic model of the important features and complexes of the Bone Multi-cellular Unit (or BMU) 11

- Figure 1-3. Histological section of bone marrow showing the layout of the BRC ........................................... 12

- Figure 1-4. Adipocyte spatial relationship to Bone remodeling Complex (BRC) ............................................. 13

- Figure 1-5. The hypothesized composition of adipose tissue and changes that occur to this population during the ageing process ...................................................... 17

- Figure 1-6. A classical representation of the Osteoblast and Osteoclast coupling dynamic ............................ 22

- Figure 1-7. Endothelial Cell (EC)-like adipocyte progenitor cell and pre-adipocyte capillary association within adipose vasculature ............................................. 25

- Figure 1-8. Figures representing each key result from in vitro studies showing a potential link between adipogenesis and osteoclast support ................................. 30

- Figure 1-9. The Mesengenic Process .......................... 32

- Figure 1-10. Lineage model of MSC differentiation into a mature lipid laden Adipocyte ................................. 34

- Figure 1-11. Gene expression profile of MSCs, MSC-derived adipocytes, and Medullary Adipocytes. ............ 35

Chapter 2 ................................................................. 39
• Figure 2-1. Osteoblast osteoclast regulating lineage model 42
• Figure 2-2. Adipocyte lineage thesis model ................. 43

Chapter 3 ........................................................................................................................................ 53
• Figure 3-1. Figure 12. MSC and MSC-derived adipocyte cultures at day 12 ................................. 55
• Figure 3-2. Culture timecourse ................................. 56
• Figure 3-3. Adipogenic lineage markers and osteoclast mediator expression profile in MSC-derived adipocytes 57
• Figure 3-4. RANKL protein expression varies as a function of MSC-derived adipocyte culture stage ................. 60
• Figure 3-5. M-CSF protein expression from MSC-derived adipocyte conditioned medium .......................... 61
• Figure 3-6. OPG protein expression from MSC-derived adipocyte conditioned medium .......................... 62
• Figure 3-7. SDF-1 protein expression: Donor and stage variability ................................................. 63
• Figure 3-8. RANKL expression in adipocyte cultures: model validation ............................................ 66
• Figure 3-9. RANKL expression in non-lipid-laden subpopulation throughout culture period ................. 69
• Figure 3-10. M-CSF protein expression in adipofibroblast sub-population conditioned medium ............... 70
• Figure 3-11. RANKL expression is based on adipocyte morphology .................................................. 72
• Figure 3-12. Growth Chart ...................................... 74
• Figure 3-13. Non-lipid-laden adipocyte subpopulation is capable of equivalent adipogenesis ............... 76
- Figure 3-14. RANKL and PPARγ mRNA expression are expressed in different adipocyte populations ............ 78
- Figure 3-15. Early adipogenic mRNA expression profile in both enriched MSC-derived adipocyte fractions ....... 81
- Figure 3-16. CD90 Expression in MSC-derived Adipocytes 83
- Figure 3-17. CD105 Expression in MSC-derived Adipocytes ................................................................. 84
- Figure 3-18. RANKL shows variant co-localization between MSC and adipocyte markers ................. 87
- Figure 3-19. Runx2 expression in RANKL positive MSC-derived adipocytes ............................................. 88
- Figure 3-20. Evidence of RANKL and PPARγ co-localization in MSC-derived adipocyte cultures .................. 90
- Figure 3-21. Osteoclast-like formation of adipofibroblast/PBMNC co-cultures ................................. 93

Chapter 4 ......................................................................................... 95
- Figure 4-1. HUVEC microtubule formation in layered co-cultures .............................................................. 98
- Figure 4-2. Dye-I labeled HUVECS from Marrow sections 99
- Figure 4-3. Dye-I labeled HUVECs and 12-day MSC-derived adipocyte layered co-cultures showing formation of microtubule formation in adipocytes after 3-days .100
- Figure 4-4. CD146 expression in enriched adipofibroblasts 101
- Figure 4-5. Schematic model of possible osteoclast regulatory cell niches during bone remodeling in the BMU ................................................................. 103

Chapter 5 ......................................................................................... 104
- Figure 5-1. Proposed model for MSC-derived medullary adipocytes .......................................................... 119
Characterization of the Developmentally Regulated Osteoclast Support Potential of *In Vitro* Medullary Adipocytes

Abstract by:

Vance Holt III

**Abstract**

In addition to its widely characterized role in lipid storage and energy metabolism, adipose tissue also has an important role as an endocrine organ. Obesity can alter normal systemic physiologic processes and has been linked to diseases such as cardiovascular disease, diabetes, and hypertension. Most studies have used extramedullary adipose tissue as the primary target for adipose characterization. A less-characterized adipose tissue source is the marrow derived medullary adipose tissue. The medullary adipose tissue depot can increase with age and this increase is associated with a decrease in total bone mass and bone mineral density in both mouse and human subjects. Using a well characterized mesenchymal stem cell (MSC)-derived *in vitro* medullary adipocyte model, the potential for medullary adipocytes to regulate osteoclast development was tested. Factors important in osteoclast development including RANKL, M-CSF and OPG were expressed by *in vitro* medullary adipocytes. Furthermore, the expression of these important osteoclast mediators was only observed
in a specific population of previously unidentified medullary adipocytes at a particular stage of differentiation. Thus, *in vitro* medullary adipocytes express osteoclast mediators at a restricted developmental stage within the adipocyte lineage that may play a role in bone remodeling.
Chapter 1

Introduction

Bone homeostasis involves the balanced processes of bone formation (generation) and bone resorption (breakdown). This process, also called bone remodeling, is controlled by a coupling behavior between osteoblasts and osteoclasts [1] and occurs in the bone marrow. The interaction between the osteoblast and osteoclast within remodeling marrow compartments is a developmentally regulated interaction in which each group of cells can induce or inhibit the developmental progression of the other cell through the direct and indirect actions of developmentally regulated factors [2–5].

A key factor in the coupling dynamic is Receptor Activator of NF-κB Signaling Ligand (RANKL), which is expressed by osteoblast progenitors [6–8]. RANKL signals through its receptor RANK that is expressed by osteoclast progenitors, and leads to the formation and maturation of osteoclasts [9]. In the bone marrow, RANKL is counteracted by its inhibitor, a soluble decoy receptor of RANKL called osteoprotegerin (OPG), which is expressed by mature osteoblasts [3].

Adipose tissue is an essential metabolic organ that has important regulatory functions in insulin sensitivity, lipid metabolism, and energy
homeostasis [10,11]. Recently, adipose tissue has been shown to have regulatory potential through the expression and secretion of cytokines and adipokines [11,12]. Indeed, adipose tissue has been associated with different physiological processes including appetite control, inflammatory responses, and angiogenesis [13]. Variations in normal adipose tissue homeostasis has been associated with physiological maladies such as hypertension, osteoporosis, diabetes, and various inflammatory diseases [14].

Medullary adipocytes, or those adipocytes located within the bone marrow, are present postnatal and steadily increase in number with age [15–17]. In late adulthood, the majority of bone marrow volume consists of medullary adipose tissue [17,18]. It is interesting that, increased bone marrow adiposity is correlated with age-associated decrease in bone mineral density [19–21], suggesting adipocytes may signal a decrease in bone density. This correlation between increased adiposity of the bone marrow and decreased bone density has been observed in vivo in both mice and humans [21–28] leading to the speculation that there is a possible link between medullary adipocytes and bone metabolism through osteoclast activity [23–25,29,30].

The medullary adipocyte and the osteoblast share a common progenitor, the Mesenchymal Stem Cell (MSC) and each have been
found to co-localize within similar regions inside marrow compartments [31–34]. Studies from in vitro co-cultures have shown that mouse stromal pre-adipocytes are capable of supporting osteoclast-like cell formation from osteoclast precursors [22,27].

In humans, a recent study showed that primary medullary adipocytes isolated from iliac crest marrow express the important osteoclast regulatory molecules OPG, M-CSF, and RANKL [28,35]. Further, marrow stromal RANKL expression is increased in corn oil-fed mice whose bone marrow has increased adiposity [36,37]. Collectively, these data suggest that medullary adipocytes may support osteoclast formation. However, identification of the developmental stage of the medullary adipocyte that can express these osteoclast mediators and, in turn affect osteoclast formation, is incomplete.

Characterization of bone marrow medullary adipose tissue and its role in bone metabolism is still in its early stages [26,38,39]. The role of adipose tissue within the marrow microenvironment remains unclear [26,39–42]. The process of differentiation of MSCs into adipogenic cells has been described, and it appears MSC-derived adipocytes and primary isolated medullary adipocytes share a very similar phenotype [31].
Primary MSC cultured cells can be induced to differentiate into adipocytes using a unique culture system developed by McKay et al. [43]. Using this culture method the adipocyte lineage progression of MSC-derived adipocytes both morphologically and by the expression pattern of various adipogenic markers along the culture time course has been completed [44]. This work thereby allows us to both investigate phenotypic and morphological changes of MSC-derived medullary adipocytes with respect to the adipocyte lineage.

In this thesis, the MSC-derived adipocyte culture system developed and characterized by MacKay et al. [43], was employed to address the question of the potential role of medullary adipose tissue in the regulation of bone remodeling. Specifically, the objective of this study was to test how osteoblasts and adipocytes regulate osteoclast formation. More importantly, the aim of this project was to characterize the potential MSC-derived adipocytes have in supporting osteoclast formation by elucidating the expression patterns of RANKL, OPG, M-CSF, and SDF-1 along the MSC-derived adipogenic lineage.

The central hypothesis of this study is that cells of the medullary adipocyte lineage can regulate osteoclastogenesis in a developmentally regulated manner through the expression of osteoclastogenic mediators such as RANKL, OPG, M-CSF, and SDF-1. The findings from this study provide a novel insight into the adipocyte’s potential role in
age-related bone loss. Furthermore, by identifying molecules related to osteoclast regulation in adipocyte cultures, a powerful *in vitro* model has been identified to elucidate mechanisms by which adipocytes can affect bone resorption in adipocyte-rich marrow microenvironments.
Background

Bone homeostasis and Bone Marrow cell physiology

Bone remodeling is an active process that ensures bone integrity throughout adulthood and occurs within very distinct microenvironments. This process occurs primarily through the actions of three distinct cell populations; the bone forming osteoblast, the bone resorbing osteoclast, and the hematopoietic supporting stromal cell [45–49]. Osteoblasts are capable of depositing matrix protein elements which, in turn, promotes bone mineralization and formation [50]. Osteoclasts are cells that resorb bone matrix thereby facilitating bone degradation [51]. The third cell type, the stromal cell—which represents a mixed population of cell types, provides hematopoietic support and maintains bone marrow homeostasis [52,53].

The events of bone formation change gradually with age [54,55]. The earliest stage of bone metabolism, endochondral bone formation, involves an intricate balance between cartilage formation and matrix deposition, which forms bone both in a longitudinal and latitudinal space [56]. During adolescence and early adulthood bone metabolism is now primarily defined by the separate actions of the osteoblast and osteoclast. During this stage of bone metabolism, bone has a formation rate higher than that of the resorption rate [57] which, in
turn, gives bone the potential to reach its peak density. Around the
time bone reaches optimal density (which varies between individuals)
bone modeling undergoes a mechanistic change [50,54]. In late
adulthood, the bone modeling process is now accomplished via a
synchronous relationship between the osteoblast and osteoclast
(termed coupling) in which the rates of activity of the osteoblast and
osteoclast are similar and occur in the same microenvironmental
space—this is called bone remodeling [57–60](Figure 1-1).
Figure 1-1. **Modeling and Remodeling schematic showing the mechanistic differences between bone modeling and bone remodeling**

Bone modeling usually occurs between early childhood and early adulthood. Bone remodeling becomes the primary mechanism of bone metabolism by mid-adulthood and remains the primary mechanism of bone homeostasis up to old age.
The Bone Marrow contains the Bone Modeling Unit and the Bone Remodeling Complex

The bone marrow is comprised of a complex network of tissues, cellular niches and cell types [34,45,48,61]. These complexes function to direct cellular differentiation and physiological processes inside and outside of the marrow [48] while also to regulating bone metabolism [45]. Cells in the bone marrow can be localized in anatomic structures called bone multicellular units—or the BMU (Figure 1-2) that form the primary sites in which bone remodeling takes place [60]. BMUs contain a variety of cell types that includes the macrophages, T-cells, vascular endothelial cells, medullary adipocytes, stromal precursors and the primary bone remodeling cells; the osteoblast and osteoclast [62]. Active remodeling sites are contained in specific microenvironments within the BMU and are known as bone remodeling complexes (or the BRC) [60,63] (Figure 1-3). The BRC acts as a controlled structure that allows these actions to take place in a regulated, synchronistic and stepwise manner.

The BRC is comprised of osteoblasts, osteoclasts, bone lining stromal cells, the marrow sinusoid complex, and the endosteal surface of the bone itself [60,61,64] (Figures 1-2 & 1-3). The marrow sinusoid acts as a blood supply to the remodeling site, allowing easy trafficking
of progenitor cells to the active remodeling that facilitates the remodeling process [60,61].

Beyond its obvious role as substrate to osteoclasts and canvas to osteoblasts, the endosteal/bone surface also serves as a key regulatory point. The osteocyte network is contained within the endosteum and embedded deep within bone. As resorption takes place this osteocyte network has exclusive access to the BRC and cells contained within it. Signals from this now exposed network help regulate osteoclast and osteoblast formation and provide signaling molecules which might promote migration of nearby cells to active remodeling sites [45,50].

The cells that initially cover the endosteal surface eventually retract during the remodeling process and act as a barrier that separates the marrow compartment from the remodeling site [65,66]. The primary function of the bone lining cells, however, is not fully understood. Some hypothesize that, in addition to providing a barrier between the BRC and marrow, these cells may be a source of osteoblast precursors [45,61,66,67].

Another major cell type within the marrow compartment is the medullary adipocyte. With aging, this fat tissue source becomes the most abundant stromal-cell population within the marrow [55,68,69].
The medullary adipocyte is in very close proximity to the endosteum lining cells and is closely associated with the bone lining cells (Figure 1-4) [70]. Whether or not the adipocyte has some influence in bone remodeling remains unclear, and is, in part, one focus of the present study.

Figure 1-2. **Schematic model of the important features and complexes of the Bone Multi-cellular Unit (or BMU)**
Illustration includes the marrow capillary blood vessels (marrow sinusoid), the multi-cellular heterogeneous nature of the bone marrow stroma—which contains; the osteoblasts, osteoclast, stromal cells, hematopoietic progenitors, red and white blood cells, and the Bone Remodeling Complex (BRC).
Figure 1-3. **Histological section of bone marrow showing the layout of the BRC**
Black stain represents NCAM (adhesion molecule) positive bone lining cells while the brown staining represents CD34 positive (endothelial marker) capillaries that reside near the BRC. The box shows a TRAP positive Osteoclast. The lower panel shows the point of contact (black arrow) between a capillary of the marrow sinusoid and the BRC canopy. *(Andersen TL et al.)* [60].
Figure 1-4. **Adipocyte spatial relationship to Bone remodeling Complex (BRC)**

(A) Goldne’s trichrome stained histological section of a subject’s bone marrow with hyperparathyroidism with a corresponding adipocyte-rich stroma showing calcified bone (blue); Osteoid protein (red); cell nuclei (black).

(B) Close-up view that shows of the spatial relationship between multi-ocular and uniocular adipocytes (black arrows) to the BRC (red arrows). (Hauge et al.) [66]
Adipose tissue biology

Adipose, or fat tissue is composed of a variety of cell types including mature adipocytes, endothelial cells, adipocyte precursors, fibroblasts, MSCs, and macrophages [13,71–74]. There are two main types of adipocytes; brown adipocytes and white adipocytes [13]. The brown adipocyte is unique in that it stores fewer lipid than the white adipocyte and contains multiple mitochondria—which gives it its brown appearance. In addition, the brown adipocyte’s main physiological role is to serve as a metabolic source for heat and is usually present in larger amounts in hibernating capable mammals and newborn babies [13]. Although there have been reports of the existence of brown adipocytes within the adult bone marrow [75], its potential role within the bone marrow is not clear [38]. By contrast, the white adipocyte does not contain as many mitochondria as the brown adipocyte, can store much larger amounts of lipid [13] and can be utilized as an energy source [13] while maintaining secretory capabilities [11,13,76]. The white adipose tissue has three major repositories; visceral (surrounding organs) subcutaneous (under skin) and medullary (within the bone marrow) [11,13,77,78].

Notably, there are phenotypic differences in white adipose tissues based on their depot of origin [78–83] For instance, differences in cytokine expression patterns have been observed between omental
and subcutaneous fat depots [78–83]. What’s more, is that these phenotypic variations between adipocyte depots are present even after culture [78,82]. Despite some known inter-depot differences between adipocytes, little is known about the differences between medullary (marrow restricted) and extramedullary (non-marrow localized) adipocytes and characterization of the medullary adipocyte is still in its early stages.

**Aging and the adipocyte**

There have been several changes shown to be associated with the aging process in adipose tissue. First, there is a fat tissue depot redistribution from relatively abundant subcutaneous sites to visceral (ectopic), omental (mesenteric), and medullary sites such as the liver, stomach and the bone marrow respectively [15,84]. Second, with aging, there is an observed increase in migration of monocytes infiltrating adipose tissue [85]. Finally, and a possibly more intriguing notion, there is thought to be a change in the phenotype of the progenitor pool that makes up adipose tissue during the aging [86,87].
Precursor pool changes with respect to age in adipose tissue

As it turns out, the preadipocyte pool is one of the largest precursor pools within the body. Normally preadipocytes make up approximately 15-20% of total adipose tissue [88]. While aging is associated with a loss in adipose tissue depot size (at certain depots) the decrease in size is thought to be primarily due to a decrease in lipid vacuole size rather than a decrease in the precursor pool population. In fact during aging, in rat epididymal fat depots, the preadipocyte pool has been observed to be sustained or even increased (Figure 1-5) [89]. These data might indicate a loss in the ability of these cells to make mature lipid-laden adipocytes. Indeed, pre-adipocytes did show a loss in both proliferative capacity and plasticity when aged in vitro [87,90].
Figure 1-5. The hypothesized composition of adipose tissue and changes that occur to this population during the ageing process

The top panel schematic illustrates the asymmetrical replication in which one adipocyte precursor daughter cell is capable of maintaining its pre-adipocyte (plastic) phenotype while the other is committed to differentiation into a mature lipid-laden adipocyte. During the aging process, it has been proposed that there is a medullary depot specific increase in both lipid containing adipocytes and pre-adipocyte pool. [86,90]. The bottom panel implies the occurrence of precursor pools in mouse epididymal adipose tissue during aging. As mice get older the size of the fat depot is increased primarily by increase adipocyte and pre-adipocyte numbers; rather than adipocyte lipid size. [86]. (Figure adapted from Kirkland JL.) These figures might give insight into what takes place in certain adipose tissue depots in humans.
Bone vs. Fat.

The first evidence that bone density was reduced with age was based on early 20th century pathological studies[16,26,91]. It was not until an epidemiological study in 1971 that a strong correlation between marrow fat, bone density, and age was made [21]. In this study bone sections from numerous subjects grouped by age and sex were compared. This group found that not only were subjects with low bone density had corresponding high marrow fat content, but increased age was correlated with both the low bone density and high fat content [21]. These early observations have since been confirmed with contemporary approaches in both mice and humans [20,25,34,69,92]. These early observations sparked questions as to the role of the adipocyte in the bone [25]. However, since these observations the medullary fat in the aging bone marrow still unresolved and remains a source of controversy. Some argue that a direct relationship between adipocytes and bone metabolism exists while others argue that the correlation between fat and bone is primarily because of some indirect relationship [25,26]. Whether the correlation between the bone and fat is due to a direct or indirect relationship, however, is still not well understood [26,41,93,94]. The idea that adipocytes can have a direct relationship with bone
metabolism can partly be attributed to the role the closely related osteoblast has in bone.

**Osteoblast and Osteoclast coupling: from migration to osteoclast and osteoblast maturity in the BMU**

The relationship between the osteoblast and osteoclast is a unique one, in that the activity and formation of each cell is regulated by the activity and formation of the other. This reciprocal relationship between osteoblasts and osteoclasts is referred to as “coupling” ([Figure 1-6](#)) [60]. In the coupling model, stepwise molecular events are obligatory for the control of each cell’s activity [46]. The stepwise control of bone remodeling occurs in very distinct steps that is all contained within the BMU; migration of osteoclast progenitors into the BRC, bone resorption and formation, and termination [46].

Migration of osteoclast progenitors is believed to be controlled, in part, by stromal derived factor 1 (SDF-1), derived from both MSCs and stromal cells located in the bone marrow [45,95–97]. Endothelial cells may also play a role in the migration of precursors into the BRC through the expression of receptor activator of NfκB ligand (RANKL) [98]. After migration into the BRC, osteoclast precursors come into contact with additional differentiating factors including macrophage
colony stimulating factor (M-CSF) and RANKL-expressing stromal-like osteoblast precursors [99–102]. M-CSF, which signals through the c-fms receptor expressed by monocytes, promotes both the proliferation and (myeloid) commitment of cells into the osteoclast lineage [102].

The RANKL/RANK signaling axis is essential for the maturation and activity of developing osteoclasts [102]. RANKL is a member of the tumor necrosis factor cytokine family and can be expressed on the membrane of stromal like osteoblast progenitors or can be secreted by activated T-cells [103]. RANKL signals through the RANK receptor expressed on osteoclast progenitor cells [64,104,105] Sustained signaling by RANKL also helps with osteoclast survival. If RANKL expression is inhibited or down regulated, osteoclast survival decreases. Interestingly, as coupling between the osteoclast and osteoblast progresses, the coupling dynamics between each cell changes. I don’t understand this last sentence

As osteoblasts mature in the BRC, they no longer support osteoclast formation but, instead, inhibit osteoclast formation. This process is regulated through the down regulation of RANKL and the upregulation of osteoprotegerin (OPG)—a soluble receptor for RANKL which inhibits RANKL-mediated signaling [106]. Mature osteoclasts also have some influence on osteoblast activity through the expression of ephrinb2. Ephrinb2 is a ligand that is part of a bi-directional
signaling that includes its receptor EphB4—which is expressed on mature osteoblasts [6,107]. The EphB4/ephrinB2 bi-directional signaling axis promotes osteoblast mineralization in the forward (ligand-to-receptor) signaling direction while it inhibits further osteoclast resorption activity in the reverse signaling (receptor-to-ligand) direction [45,108].

These stepwise molecular and cellular events are essential in maintaining the sensitive balance between osteoblast and osteoclast activity. In fact, inhibiting bone resorption can reduce the rates of bone formation [109], while prematurely depleting osteoblast numbers negatively affects osteoclast progenitor numbers and resorption activity [110–112]. How adipocytes might affect this process through the alteration of this dynamic (either by direct or indirect means) is the focus of the present study.
Figure 1-6. **A classical representation of the Osteoblast and Osteoclast coupling dynamic**

Shows the relationship between the MSC/Stromal cell and osteoclast progenitor cells during their developmental progression toward the osteoblast and osteoclast respectively. This particular schematic also shows the importance in the expression balance between the osteoclast inductive molecule RANKL and osteoclast inhibiting molecule OPG during the lineage progression of the MSC to the mineralizing osteoblast. This process of coupling has been intensely studied since its first discovery in 1995 by Takashi et al.
Elements of the Bone Modelling Unit can affect bone remodeling

As discussed, the BMU defines the microenvironment whose various elements are each important during the remodeling process. The importance of understanding the role of each respective structure within the BMU lies in identifying potential targets (cellular or otherwise) that may regulate the remodeling process. Currently, it remains unclear which cells in the stroma signal to osteoclast precursors and promotes their differentiation and maturation. To that end, there is still some speculation as to whether the coupling between early osteoblasts and osteoclast progenitor cells takes place within the BRC, in the marrow cavity, or in the marrow sinusoid prior to entry into the BRC [61,113].

In recent years, studies have sought to identify, individually, the role of the various structural elements within the BMU during the remodeling process [1,60]. Unfortunately, it is still not well understood the exact mechanism by which each element of the BMU regulates the other during the remodeling process [46,61,64]. What remains even more unclear is how the increasingly abundant adipocyte population in the aging marrow can affect each element’s influence on bone remodeling [26,38]. Each structural element of the BMU is outlined
below including the evidence that may suggest that adipocytes could play an influential role in their role in bone remodeling.

**The marrow sinusoid**

The marrow sinusoid is a complex that lies within the marrow cavity outside of the BRC complex [60,62,66,95]. This complex contains vascularized capillary-like elements that are integral in directing the circulating osteoclast and osteoblast progenitor cells into the active BRC [60]. Recently, RANKL expression in endothelial cells, a component of the marrow vascular network, was observed [98,100,101]. In this study, they identified a potential of endothelial cells to express RANKL upon induction of cytokines such as TNFα. This expression of RANKL in endothelial cells is believed to help direct and prime osteoclast progenitor cells to commit to the osteoclast lineage and migrate to the BRC.

An interesting new development in adipose tissue biology is the identification of endothelial cell-like adipocyte progenitors [114]. A study done by Tran et al. was able to show that endothelial-like cell progenitors and intermediate adipocyte-like cells are closely associated within the embedded capillary network of adipose tissue (Figure 1-7) [114]. In addition to this, this group was able to show that the endothelial-like cell was capable of giving rise to both endothelial cells and adipocytes. If adipocytes associate with the endothelium the
potential exists that an increasing population of these adipocytes may have a role in upregulating RANKL expression within the sinusoid which would increase, or at least maintaining, osteoclast trafficking into the marrow cavity even in the absence of osteoblast influence [29,115–117].

Figure 1-7. Endothelial Cell (EC)-like adipocyte progenitor cell and pre-adipocyte capillary association within adipose vasculature
Adipose tissue section highlighting pre-adipocyte (pa) containing small lipid droplets (L), poorly differentiated pericytes with glycogen granules (p), and endothelial-like elongated cells (e) contain tight junctions (tj). *Asterisks indicate capillary vesicles. An original EM vascular-adipocyte micrograph depictions published by Tran K. et al. [114]
The marrow stroma

Whether or not the marrow stroma has a stimulating role in the development and migration of osteoclast progenitors is not completely clear [60]. It has been suggested however, that osteoclast lineage maturation can occur within the stroma prior to entry into the active BRC site via RANKL expression by stromal cells near the BRC [45,64,118]. This provides an intriguing possibility; because the increase in medullary adipose tissue is seen primarily in the stroma of the marrow BMU. Therefore, if developing adipocytes can express RANKL, as is proposed here, these cells might exist in abundance within the increasingly adipocyte-rich aging stroma.

BRC lining cells

Beyond their function as barrier cells to the marrow stroma during active remodeling, the exact role of the endosteum lining cells during the remodeling process is not yet been fully characterized [45,60]. There is evidence to suggest, however, that these cells are early stromal osteoblasts and thus have the potential of being the primary source of RANKL for migrating osteoclast precursors[66]. In the marrow, adipocytes are closely related, spatially, to these lining cells both in the quiescent and active BMU [50]. Based on previous studies, the adipocyte has the potential to affect these lining cells in the marrow. It has been shown that adipocyte-rich environments can
have deleterious effects on osteoblast differentiation while retaining a propensity towards the adipocyte pathway [20,119–123]. In fact, age accelerated SAMP6 mice who have high marrow adiposity and low bone mineral density, show that undifferentiated stromal cells isolated from their marrow have higher basal PPARγ expression, lower basal RUNX2 expression levels and a higher tendency to differentiate into the adipocyte lineage when compared to controls [124]. Thus, there is a possibility that adipocytes might have some role in altering BRC lining cell phenotype in an increasingly adipocyte-rich environment seen in aging bone.

**Bone and Fat: Competing Hypotheses**

Several studies have been designed to test whether there is a link between medullary stromal adipocytes and bone metabolism [22,23,27,38,93,125–132]. Although there may be a multitude of factors which may contribute to the adipocyte-mediated decrease in bone density, the most accepted hypotheses largely deal with an indirect method in which the adipocyte could affect bone metabolism. From these studies, there have been several suggested mechanisms that attempt to explain the correlation between high marrow fat content and loss of bone density. In the first, high leptin signaling from adipocytes to the hypothalamaus is proposed to form an
endocrine/hormone signal that reduces bone density. In the second proposal, adipocyte-induced microenvironmental cues including TNFalpha act on adjacent cells to reduce bone density. (Table 1-1)

<table>
<thead>
<tr>
<th>Type of interaction</th>
<th>Primary mode of action</th>
<th>Suggested mechanism</th>
<th>Citation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sympathetic</td>
<td>Endocrine</td>
<td>Leptin signaling from adipocytes to hypothalamus</td>
<td>[133][134][135][135]</td>
</tr>
<tr>
<td>Microenvironment</td>
<td>Paracrine</td>
<td>Adipocyte induced microenvironment (e.g. TNFα expression)</td>
<td>[136][93][24]</td>
</tr>
</tbody>
</table>

Table 1-1  **Studies supporting the two hypothesized mechanisms of adipocyte regulation of bone remodeling**

Still others suggest a potential direct effect of adipocytes on the osteoclast, in an interaction similar to the direct relationship found between the osteoblast and osteoclast [22,23,27,132]. These mechanisms are explored in greater detail here.

Most of the data that support a direct relationship between stromal adipocytes and osteoclast formation have come from studies that have utilized mouse cell cultures [22,23,27,132]. In initial studies, clonally-expanded stromal-derived mouse adipocytes were generated and, at different stages of adipogenesis, were then placed into co-cultures with osteoclast progenitors. From these studies, it appears that early lineage “early preadipocytes”—as was defined by the study—were capable of supporting osteoclast-like cell formation while
late lineage— or “mature” adipocytes— gradually lost this capability. These studies did not show, however, the mechanism by which these adipocytes and osteoclasts might interact nor did they provide a molecular characterization of the cells that supporting osteoclast formation in these co-cultures.

In 2008, Takagi et al. showed co-localization of RANKL and PPARγ in a population of stromal-derived mouse primary medullary adipocytes [22]. The observations made by this group suggested that some stromal adipocytes cell might regulate bone formation directly. In humans, Hozumi et al. observed RANKL, M-CSF, and OPG expression in primary medullary adipose tissue [28]. Collectively, these studies support a hypothesis that medullary adipocytes may directly support osteoclast formation. Based on these observations, it remained important to learn if human MSC-derived adipocytes also expressed these factors; and to provide a more stringent characterization of the RANKL, OPG, and M-CSF expression pattern within the medullary adipogenic lineage (Figure 1-8).
Figure 1-8. **Figures representing each key result from in vitro studies showing a potential link between adipogenesis and osteoclast support**

Top left panel shows a figure from Kelly MA et al. [27] depicting the declining adipocyte-mediated support of osteoclast formation in co-cultures as a function of culture time. Bottom left panel shows a figure from Takagi K. and Kudo A. [22] displaying the presence of PPARγ- and RANKL-positive medullary adipocytes isolated from mouse marrow. Right panel shows table published by Sakaguchi K. et al. [23] which summarizes the phenotypic identity and osteoclast support capabilities of stromal cell clones derived from mouse bone marrow.
Mesenchymal stem cell (MSC) and the MSC-derived Medullary Adipocyte Culture Model

The highly plastic pluripotent embryonic stem cell (ESC) is capable of differentiating into most cell types and is plentiful only in embryos. Unlike the ESC, the Mesenchymal Stem Cell (MSC), an adult stem cell, is a multipotent stem cell whose differentiation capacity is limited to the skeletal and connective tissue phenotypes [137,138] (Figure 1-9). Like ESCs, the MSC, can retain the ability to self renew and thus maintain its plasticity throughout its life cycle. In the marrow, it is believed that MSCs also give rise to the medullary adipocytes [22,43,70,139–141].
Figure 1-9. The Mesengenic Process
An illustration that describes the differentiation capacity of the adult Mesenchymal stem cell as first described by Haynesworth and Caplan et al. [137,142] (adapted illustration with permission Caplan AI 1991).
Several in vitro studies have aimed to characterize the lineage progression of MSCs to the mature medullary adipocyte in detail [43,140,143–149]. Among the factors important for adipogenesis, the two major transcription factors, C/EBPα and PPARγ2 are thought to be the master regulators of adipogenesis [13,144,147,150]. Recently, other factors such as the Krüppel Like Factors (i.e. KLF4, KLF5, and KLF15) and the notch signaling pathway have been shown to be important for adipogenesis. Indeed, both KLFs and notch responding molecules, along with PPARγ and C/EBPα, are some of the early markers for adipogenesis believed to be expressed in preadipocytes [76,144,151–153] (Figure 1-10).

A unique cell culture model of medullary adipocyte development has been developed recently by MacKay D. et al. [43]. The approach used by Mackay et al. incorporated both morphological characteristics as well as adipogenic marker expression to assess the progression of MSCs along the adipogenic pathway. By comparing primary medullary adipocytes isolated from the bone marrow to MSC-derived adipocytes at various timepoints along the culture period, MacKay et al. observed a variety of adipogenic markers that have a similar expression profile to that of primary isolated medullary adipocytes (Figure 1-11).
Figure 1-10. **Lineage model of MSC differentiation into a mature lipid laden Adipocyte**

Through several studies a lineage map was developed showing the progression of the Mesenchymal Stem Cell to adipocyte *in vitro* following adipogenic induction via the expression of the Kruppel Like co-Factors and master regulators C/EBPα and PPARγ. Krox20, KLF4, and Srebp1c are the earliest known factors associated with the pre-adipocyte. C/EBPα and PPARγ are both necessary and sufficient master regulators of adipogenesis. Other factors, such as the co-repressors KLF3 KLF2, and the repressor Pref-1 are important negative feedback regulators of adipogenesis.(Schematic from Cook A. et al. 2009) [13].
Figure 1-11. **Gene expression profile of MSCs, MSC-derived adipocytes, and Medullary Adipocytes.**
This figure is depicted by MacKay et al. [43] in which a novel MSC-derived adipocyte culture method was used and gene expression was compared to that of primary isolated medullary adipocytes from human marrow aspirates. The gene profile of the MSC-derived adipocyte at various timepoints during the culture period is similar to that of the medullary adipocyte (MA).
Furthermore, MacKay et al. mapped the pattern of protein expression of the adipokines, leptin and adiponectin, as a function of adipogenic differentiation and found distinct, developmentally regulated expression patterns for each (data not published). Adiponectin was expressed during the developing phase of the adipogenic lineage and leptin was expressed only during the later/mature stages of the lineage (MacKay, unpublished data 2007; with permission). Similar results were obtained using similar culture methods *in vitro* [140,146]. This cell culture model provides an useful way to study the medullary adipocyte lineage *in vitro*.

**Heterogeneous nature of *in vitro* adipocytes**

Adipose tissue is a highly heterogeneous tissue containing many lipid-containing (lipid-laden) and non-lipid-containing (non-lipid-laden) cell types [13,73,74,154,155]. Included within this heterogeneous population of cells are MSC-like cells [73,156], committed early and late adipocytes [13,75,77,78,90,155,157], and uncommitted progenitor pools [74]. *In vitro*, adipocytes also exhibit this heterogeneity in cell population, at least morphologically. In culture, MSCs appear spindle shaped and fibroblastic while preadipocytes
appear polygonal with shifted nuclei. As preadipocytes develop, they start to acquire lipid vacuoles indicating adipocyte maturation. Currently, there have not been any substantial attempts to characterize the diverse population of non-lipid- and lipid-containing cells in adipocytes cultures, although some have postulated that some of these cells persist in culture may represent uncommitted precursors [144]. In addition, there are studies, both in vivo and in vitro, that suggest there are cells from adipose tissue that maintain an early osteogenic and adipogenic phenotype, even after culturing for an extended amount of time [74,125].

**Summary and Aims**

Adipose tissue is a complex organ that holds some significance in endocrine function and has, seemingly, important roles in the homeostasis and regulation of many physiological processes. In the marrow, the adipocyte function remains unclear. What is known, however, is that with aging the adipocyte population increases in the marrow. This increase in adipocyte numbers is correlated with a decrease in bone mineral density presumably through some change in bone metabolism. In adults, this process of bone metabolism is an
intricate and synchronized process that is controlled by the balanced activities of two specialized bone cells: the osteoblast and osteoclast.

With what is known about the influence of adipose tissue on physiological process, in states of dysregulation, and the importance of the balance of the osteoblast and osteoclast during modeling; this study set out to characterize a potential role that adipocyte may have on the osteoclast-mediated control of remodeling. It is proposed that the medullary adipocyte can affect osteoclast formation in a manner similar to that of the osteoblast in that they can regulate osteoclast activity in a developmentally regulated manner.

To test this hypothesis, a previously described in vitro model for the medullary adipocyte was used to investigate whether or not this cells has the potential to regulate the remodeling process. In doing so, two aims were tested. The first aim was to asses the expression pattern of the important osteoclast regulating molecules (e.g. RANKL, OPG, M-CSF and SDF-1) in the lineage progression MSC-derived medullary adipocyte model. The second aim was to show, in in vitro co-cultures with osteoclast progenitors, that adipocytes, at defined lineage stages, are capable of supporting osteoclast formation.
Chapter 2

**The proposed role of the Medullary Adipocyte in Osteoclast development: A Thesis model**

Osteoblast support of osteoclast formation is a lineage regulated process [6,7,107,158] where the expression of RANKL has been shown to vary among cells in the osteoblastic lineage, with high levels in early stage osteoblasts and low levels in late lineage stage cells [159]. With age, the ratio of osteoblast bone forming activity to osteoclast bone resorptive activity is decreased, as osteoblast activity is lost and osteoclast numbers and activity are either sustained or slightly decreased [20,115]. Also with the process of aging is the accumulation of a large medullary adipose tissue volume and decrease in bone mineral density in both the cortical and trabecular bone. A clear-cut link between the adipocyte and osteoclast activity, however, has not yet been established [25,26].

Interestingly, the idea that the support of osteoclast formation is not exclusive to the osteoblast lineage has been suggested [160]. In fact, it has been shown that other cell phenotypes of the MSC lineage can express RANKL (e.g. chondrocytes) [161] and thereby hold the potential to support osteoclast development. In our case, both the osteoblast and adipocyte share a common stem cell in the MSC, which
leads to the speculation that adipocytes may also share this osteoclast support capacity.

As previously discussed, adipocyte support of osteoclast formation has been suggested by others in both mice and humans [22,23,27,28,35]. What’s lacking from these previous studies is a stringent characterization of the specific cell phenotype that can express these osteoclast regulatory factors and allow support of osteoclast formation.

The studies in this thesis are designed to test the hypothesis that 1) the developmentally regulated support of osteoclasts by osteoblasts is shared by adipocytes and 2) the adipogenic lineage shows a differential support potential of osteoclasts based on its specific lineage stage. The objective of this thesis is to test the hypothesis that medullary adipocytes have the potential to support osteoclast formation through expression of important osteoclast developmental mediators. Another objective is to understand if there is a specific developmental stage at which the support potential of osteoclasts takes place.

The approach chosen for this study is to first identify the essential factors that mediate the osteoblast regulation of osteoclast development, formation, and activity. Table 2-1 outlines these...
important factors that have been shown to contribute to the regulation of osteoclast activity from migration to resorption at various osteoblast development stages. The expression of these factors in medullary adipocytes may signify that these cells can regulate osteoclast formation and contribute to osteoclast development. These important osteoclast mediators include RANKL, OPG, SDF-1 and M-CSF. Using what is known about the osteoblast support of the osteoclast, a lineage map of the osteoblast and its corresponding osteoclast support potential is described (Figure 2-1). Clues from previous mouse reports [22,23,27] and what is known about adipogenic lineage progression in vitro, a similar map for the adipocyte lineage progression can be used to predict the potential roles each stage of the adipocyte lineage have on osteoclast activity (Figure 2-2).
<table>
<thead>
<tr>
<th>Factor</th>
<th>MSC (mix?)</th>
<th>Stromal cell Pre-osteoblast</th>
<th>Early Osteoblast</th>
<th>Mature Osteoblast</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rankl</td>
<td></td>
<td>++</td>
<td>+++</td>
<td>+</td>
</tr>
<tr>
<td>OPG</td>
<td></td>
<td>++</td>
<td>+++</td>
<td>?</td>
</tr>
<tr>
<td>SDF-1</td>
<td></td>
<td>++</td>
<td>+++</td>
<td>++++</td>
</tr>
<tr>
<td>M-CSF</td>
<td></td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Type I Collagen</td>
<td></td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Osteocalcin</td>
<td></td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Bone salprotein</td>
<td></td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Alkaline Phosphatase</td>
<td></td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

**Figure 2-1. Osteoblast osteoclast regulating lineage model**
Schematic representation of the MSC derived osteoblast lineage stage cells and their potentiality for Osteoclast regulation via RANKL and OPG expression in a developmental stage dependent scheme.
Figure 2-2. Adipocyte lineage thesis model
A schematic representation and hypothesis model of the primary objective of this thesis. Based on previous studies in mouse one might predict the functional relationship between cells within the adipogenic lineage starting with the MSC and its potential and/or ability to support osteoclast formation \textit{in vitro}. MSCs, pre-adipocytes, and early adipocytes have been shown to support osteoclast formation \textit{in vitro} co-cultures whereas late adipocytes have not yet been shown to support this formation. Interestingly, mature/aged adipose tissue, although possibly blocking direct osteoclast formation has been associated with the migration of macrophages and early osteoclast progenitors. In each of the cell lineage’s presumed role in osteoclast formation it is possible that there is a difference in the expression of specific osteoclast bioactive factors. In this model I aim to map expression of regulatory factors important for osteoclast development during the previously defined MSC-derived medullary adipocyte developmental lineage progression.
The model describes the expression of key regulatory factors among specific differentiation stages in the adipogenic lineage. This model will clarify the in vitro medullary adipocyte’s potential role in osteoclast development with the hope of leading to a better understanding of one of the possible roles of the adipocyte in the bone marrow.

<table>
<thead>
<tr>
<th>Factor</th>
<th>Role in Remodeling</th>
<th>Expressed by</th>
</tr>
</thead>
<tbody>
<tr>
<td>SDF-1</td>
<td>Osteoclast migration; early Osteoclast formation</td>
<td>•Stromal cell/Osteoblast</td>
</tr>
<tr>
<td>M-CSF</td>
<td>Pre-OC formation and proliferation</td>
<td>•Stromal cell/Osteoblast</td>
</tr>
<tr>
<td>RANKL</td>
<td>Osteoclast maturation and survival</td>
<td>•Stromal cell/Osteoblast</td>
</tr>
<tr>
<td></td>
<td></td>
<td>•T-Cell</td>
</tr>
<tr>
<td>OPG</td>
<td>Inhibition of Osteoclast formation</td>
<td>•Mature Osteoblast</td>
</tr>
<tr>
<td>IL-1β/TNFα</td>
<td>Induction of RANKL expression/Enhancement of Osteoclast formation and activation</td>
<td>•MSC/Stromal Cell</td>
</tr>
</tbody>
</table>

Table 2-1  Shows the various molecules involved in osteoclast regulation and formation leading to effective bone resorption, their specific role in the regulation of Osteoclast activity and the cells they are currently known to be expressed by. The molecules that have been bolded have been analyzed to some degree and will be analyzed and described in this document.
Materials and Methods

Mesenchymal Stem Cell isolation and Culture

Iliac crest bone marrow aspirate was obtained by routine aspiration from normal human donors under an IRB-approved protocol with informed consent. MSCs were isolated on the day of harvest from 10- or 20-mL marrow samples according to methods reported previously [137]. In brief, 10 mL of diluted bone marrow aspirate were centrifuged at 550 g, and the supernatant was discarded. The remaining cell suspension was loaded onto Percoll® diluted to a concentration of 1.073 g/mL with Hank’s balanced salt solution. After centrifugation at 900 g, mononuclear cells were removed from the interface and washed with Tyrode’s salt solution with a final centrifugation at 900 g. The pelleted cells were plated in Growth Medium (GM), consisting of DMEM-LG (5 mM glucose) supplemented with 10% Fetal Bovine Serum (FBS) of selected lots [162]. These primary cells were plated at a concentration of 1.3 x 10^5 – 2.0 x 10^5 cells per cm^2, and the medium was changed twice weekly. Just prior to their reaching confluence, hMSCs were detached from the vessel with trypsin/EDTA and passaged into new flasks at a ratio of 1:3. All cell types discussed in these methods were incubated at 37°C in a humidified atmosphere containing 5% CO₂. Medium changes and subculturing procedures, as described [43], were followed for first and
second passages. By second passage, any contaminating hematopoietic cells are removed due to non-adherence, and cells were harvested and plated for experimentation during third passage.

**MSC-derived medullary adipocytes**

MSCs at second or third passage were induced to form adipocytes using Adipogenic Induction Medium (AIM) containing 1 μM dexamethasone, 100 μM indomethacin, 0.5 mM 3-isobutyl-1-methylxanthine (IBMX), and 10 μg/ml Insulin supplemented with 10% FBS in DMEM-high glucose (HG) for up to 12 days. In order to induce adipocyte maturation [43,146] AIM was replaced with Adipocyte Maintenance Medium (AMM) containing DMEM-HG and 10 μg/ml insulin supplemented with 10% FBS to promote adipocyte maturity [43]. Cultures were analyzed prior to addition of AIM on day 0 and at the specific time-points throughout a 25-day time-course.

**MSC-derived adipocyte culture enrichment and adipocyte ceiling culture**

Lipid-laden and non-lipid laden (adipofibroblasts) cell populations were enriched as described [43,163,164]. MSC-derived adipocyte cultures were trypsinized on day 12 and resuspended in AMM. For gross separation of cell populations, cells were centrifuged at ~1000 rpm for 5 minutes after which the supernatant containing the lipid-laden fraction was removed and placed in a separate tube for high speed centrifugation (~2000 rpm). The pelleted cells containing the
adipofibroblasts were then resuspended in AMM and mixed vigorously, and a low speed centrifugation step was done at ~ 300 rpm for 5-10 minutes. The supernatant was removed, medium replenished and the low speed centrifugation steps were repeated. The resulting adipofibroblast cells were plated for subsequent analysis in AMM, while the lipid-laden cells were placed in flasks for ceiling cultures for later analysis. The adipofibroblasts were cultured for up to 2 days before analysis.

Ceiling cultures were used to plate the enriched lipid-laden cells from MSC-derived adipocyte cultures. Isolated cells were placed in 50-mL conical tubes and suspended in AMM. The isolated cells were then placed into small T-12.5 flasks, which were filled to the top with additional AMM. The flasks were then inverted and placed in 37 °C incubators overnight. After overnight culturing, the medium was removed from the flasks and replaced with 5 mL of AMM. The flasks were then placed right side up for additional culturing time and analysis.

**Oil Red O staining**

A stock solution of 3.5 mg/ml Oil Red O was prepared in 100% Isopropanol, filtered through a 0.4-µm filter, and stored at 4°C. A working solution of Oil Red O was prepared by mixing 30 mL of stock solution and 20 mL of Milli-Q H₂O. For staining with working solution of
Oil Red O, cells were first fixed with 3% Paraformaldehyde for 20 min. at 4°C. Cells were then rinsed and the background was removed by a short wash with 60% Isopropanol. Cultures were incubated in working solution for 15 minutes at room temperature to stain lipid droplets/vacuoles. Cells were rinsed and destained with 100% Isopropanol, total lipid accumulation was measured with a spectrophotometer at a wavelengths of either 500 or 530 nm. To quantify lipid accumulation on a per cell basis, cells were counterstained with a nuclear staining dye (either hematoxylin stain or Hoechst 3342 fluorescent dye) and manually counted from random fields and averaged by total area.

**RNA isolation and quantitative PCR**

RNA was isolated from MSC-derived adipocytes, enriched adipofibroblasts and lipid laden adipocyte cultures at specific time-points using RNeasy® Mini Kit according to the manufacturer’s instructions (Qiagen). Cells were lysed on plates in GITC-containing buffer (Buffer RLT). Reverse Transcription was performed immediately after RNA isolation using Transcriptor First Strand cDNA synthesis kit using oligo-dT primers, according to manufacturer’s instructions (Roche, Branchburg, NJ). Briefly, 2-5 ng of diluted synthesized cDNA was added to Roche Sybrgreen master mix. 500nM of each primer pair was added to the Sybrgreen master mix and cDNA solution.
Expression levels of the housekeeping beta-actin gene were used to normalize mRNA expression for Real-Time PCR. Reactions were performed in a 20µl volume using the Applied Biosystems StepOnePlus thermocycler using the following conditions: 95°C for 10 min. and cycles of 95°C for 15 sec. 55-60°C for 30 sec and 72°C for 30 seconds for 37 cycles. The primers used for each gene is listed in Appendix

**Immunofluorescence**

At time points of interest, cells were fixed in 3% paraformaldehyde in Tyrode’s salt solution at 4°C for 20 minutes and rinsed with wash buffer (0.1% BSA in Tyrode’s salt solution). Cells were then permeabilized with 0.01% digitonin in Tyrode’s salt solution for 20 minutes at room temperature and rinsed in wash buffer. Blocking of non-specific antibody interaction was carried out using a 10% serum solution based on secondary antibody species. Blocking was carried out for 30-50 minutes. Anti-RANKL rabbit primary antibody (ab65024 abcam) in 10% goat serum was then added to each well for 1-2 hours at room temperature. For co-staining, RANKL primary antibody was added in a blocking solution with mouse derived antibodies for either Runx2 (ab76956), PPARγ, SH2 antibody [165,166], anti-CD90 antibody (gift from Michael Sorrell), or goat anti-C/EBPa (sc-9314). After three 5-minute rinses in wash buffer, cells were incubated for 2 hours at room temperature on a rocker with
fluorophore-conjugated secondary antibodies. Cell layers were then rinsed in wash buffer 3 times for 5-minutes each time prior to imaging. During the final wash a concentration of 3 µg/mL Hoechst 33342 (Sigma) diluted in wash buffer was added to cell layers. Antibody dilutions and incubations times can be seen in Appendix.

**PBMNC isolation and Osteoclast co-cultures**

Peripheral Blood Mononuclear Cells (PBMCs) were provided by the Cancer Center at Case Western Reserve University and isolated using their criteria. In short, PBMCs were collected from Ficoll enrichment layers. Peripheral blood was diluted 1:1 with PBS and layered over a Ficoll-Paque solution at room temperature. The Ficoll-gradient solution was then centrifuged at 1500 rpm for 30 minutes without brake. The mononuclear cells were collected from the Ficoll-Paque/plasma interface washed with PBS and used immediately for assay and analysis. To generate osteoclasts PBMNCs were plated at either 0.5 or 0.8 X 10⁶ cells/cm² on 48-well or 24-well plates in RPMI medium supplemented with 10% FBS. M-CSF and RANKL were added to these cultures at 25ng/mL and 30 ng./mL respectively. Medium was changed in osteoclast cultures every 3 days and for up to 14 days. At specified timepoints (day 0, 3, 9, & 14) cells were fixed and stained using TRAP staining kit as described by the manufacturer (Sigma-Aldrich) and counterstained with a hematoxylin solution. For co-culture
experiments, PBMNCs were placed on confluent layers of either adipofibroblasts or dermal fibroblasts and cultured for up to 21 days in RPMI: DMEM (1/1) medium supplemented with 10%FBS only.

**CyQuant Cell Counting**

Mesenchymal Stem Cells and MSC-derived adipocytes growth rates were assessed using CyQuant® NF according to the manufacturer’s protocol (Invitrogen). Briefly, using MSCs as standards, MSC-derived adipocytes were analyzed at days 0, 3, 6, 12, 21 & 24 on multi-well plates. The absorbance of multiple cyquant nuclei-labeled cultured wells for each condition (n=4) was read on the GENiosPro®/Tecan® multi-plate reader (Tecan, Durham, NC) using an excitation wavelength of ~485 nm and an emission wavelength of ~535 nm with gains normalized to the MSC standard cultures.

**MSC-Dye-I labeling of HUVECs layered cultures**

HUVECs were labeled with vital Dye Cm (or Dye-I; Di-I) using methods as described by the manufacturer (Invitrogen) with modifications as previously described [167,168]. Vascular support layered cultures were done as previously described [169]. For the MSC-derived adipocyte-HUVEC layers, MSCs were plated for adipogenic induction and allowed to differentiate for 12 days. After day 12 a 1:0 (HUVECs only) or 1:1 (HUVEC:MSCs) mixture of Dye-I labeled HUVECs and MSCs, respectively, were layered on the MSC-
derived adipocyte cultures at a density of 10,000 cells/cm² with Bishop’s Medium as described [170] and gently mixed for even distribution. Layered cultures were allowed to form microtubules and fed once. After 5-7 days images of cultures were taken.

**Confocal Imaging**

To provide a more sensitive imaging of immunofluorescent antibody stained cultures confocal images of fluorescently stained MSC-derived adipocyte were acquired using a Leica TCS SP2 AOBS filter free UV/spectral confocal laser scanning inverted microscope. Images were taken either on day 9 or day 12. All images were generated with a 20x-60x N.A. 0.5 water immersion Achroplan® objective.
Chapter 3

Results

Medullary adipocyte differentiation from MSCs

MSCs progressively differentiate into mature medullary adipocytes in culture during a 24-day time-course (Figure 3-1). MSCs placed in adipogenic medium showed morphological changes early on in culture. By day 3, cells began to appear rounded (polyganol) and a subset began to acquire small lipid droplets. By day 12 the adipocyte cultures contained a morphologically diverse population of cells including: spindle, fibroblastic; rounded cells; and lipid containing cells. To characterize adipocyte differentiation, mRNA was isolated from cells and conditioned medium was collected at specified timepoints from day 1-24? (Figure 3-2). Adipogenic markers were quantified using qPCR.. PPARγ, C/EBPα, adiponectin, and leptin mRNA were each expressed along the 24-day time course (Figure 3-3A). PPARγ mRNA expression was first observed at day 3 and peaked at day 12. Adiponectin expression was initially relatively low but also--much like PPARγ-- peaked at day 12 as expected [43]. In culture, adipocyte maturity is commonly linked to increased leptin expression and decreased adiponectin expression [43,146]. Indeed, PPARγ, C/EBPα, and adiponectin expression decreased during the later culture
stages (days 21-24) while leptin mRNA expression was increased and peaked during the latest stage of culture (d24) (Figure 3-3Aiv).

The osteoclast mediators M-CSF, RANKL, and OPG were also expressed in a developmentally regulated manner. M-CSF mRNA was initially increased with adipogenic differentiation (compared to MSC controls) but decreased during the mature adipogenic stages (days 21 and 25) (Figure 3-3Bi). SDF-1 mRNA expression did not increase in MSC-derived adipocytes when compared to day 0 MSCs, however downregulation was observed during the later (days 21 and 25) timepoints (Figure 3-3Bii). RANKL mRNA expression was induced in the adipocyte cultures compared to MSC controls (day 0) at day 3 (Figure 3-3Biii). During the adipogenic induction timecourse, RANKL expression increased up to day 12, and then dramatically declined during the late culture timepoints (days 21 and 25). Conversely, OPG mRNA expression was low during the earlier timepoints and dramatically increased after day 12 (Figure 3-3Biv). These results indicate that important osteoclastogenic mediators have a distinct temporal expression pattern in adipocytes that correlates with the MSC-derived adipocyte lineage stage.
Figure 3-1. **Figure 12. MSC and MSC-derived adipocyte cultures at day 12**

human Mesenchymal stem cell cultures after day 12 in either (A). Growth medium (GM) w/ 10% FBS or (B). Adipogenic Medium (AIM) w/10% FBS; 1µM Dexamethasone; 100µM Indomethacin; 0.5mM IBMX; 10µg/mL Insulin. MSC-derived adipocyte cultures show a heterogeneous mixture of cell morphology including lipid-laden cells (black arrow) and non-lipid laden cells (white arrow)
Figure 3-2. **Culture timecourse**

Schematic outlining the data collection timepoints along the MSC-derived adipocyte culture timecourse.
Figure 3-3. Adipogenic lineage markers and osteoclast mediator expression profile in MSC-derived adipocytes

mRNA from MSC-derived medullary adipocyte was isolated at specific timepoints during the 25-day culture timecourse and analyzed for the (A) known adipogenic lineage markers (Ai) PPARγ2; (Aii) C/EBPα; (Aiii) adiponectin; and (Aiv) leptin. (B) Osteoclast development regulator expression patterns were mapped using the same culture timepoints and analyzed.
for (Bi) M-CSF; (Bii) SDF-1; (Biii) RANKL; and (Biv) OPG. All measurements of mRNA expression levels were done by quantitative PCR and compared to MSCs at day 0 at all timepoints. Dashed lines indicates switch from adipogenic medium (AIM) to maintenance medium (AMM).

**RANKL SDF-1, M-CSF, and OPG protein expression by MSC-derived medullary adipocytes**

To test whether the mRNA changes were reflected in protein increases with adipogenic differentiation, MSC-derived adipocytes were assayed by immunocytochemistry at the same culture-defined adipocyte lineage stages. RANKL was expressed at day 3, day 9, and day 12 compared to MSCs on day 0 and dermal fibroblast controls (Figure 3-4A). Immunofluorescent staining of RANKL showed RANKL protein located at the cell membranes and within the cell cytoplasm. The percentage of RANKL-positive cells (Figure 3-4B) increased day 9 but then declined with further culture, similar to its pattern of mRNA expression profile (n=3 donors). The presence of secreted, M-CSF, OPG and SDF-1 proteins in conditioned medium were analyzed using ELISA at timepoints indicated (n=3 donors) (Figure 3-2). M-CSF, and OPG protein were each detected in conditioned medium at varying levels based on adipogenic stages and generated profiles similar to the mRNA expression patterns of each factor (Figures 3-5 & 3-6). M-CSF
protein expression was increased in the MSC-derived adipocytes at all stages when compared to MSCs. M-CSF protein levels decreased in mature adipocyte conditioned medium (Figure 3-5). By contrast, OPG protein levels were low in early MSC-derived adipocyte cultures (days 3-12) but increased in the mature cultures (day 24). Interestingly, OPG consistently showed a higher level of protein expression in the MSC controls compared to the MSC-derived adipocytes (Figure 3-6). Notably, SDF-1 expression by medullary adipocytes never exceeded the expression levels of MSC cells at any point during the culture period (Figure 3-7A-C). In none of the three different donors tested did SDF-1 protein levels show an adipocyte developmental stage dependent pattern of expression, indicating that SDF-1 expression is not developmentally regulated in adipocytes (Figure 3-7A-C).
Figure 3-4. **RANKL protein expression varies as a function of MSC-derived adipocyte culture stage**

MSC cultures were induced to form MSC-derived adipocytes, and RANKL protein expression was analyzed using Immunofluorescence; cells were counterstained with Hoechst33342 to quantify RANKL expression (average positive expression per total cells counted over several fields) RANKL positive versus total cell population was counted manually. Analysis was done on (Ai) day 0, (Aii) day 3, (Aiii) day 9, (Aiv) day 12 and (Av) day 18. There is an increase in both the number and intensity (white arrows) of RANKL positive staining between day 3-day 12 to quantitative RANKL expression. RANKL positive staining was not detected in (Ai) MSCs at day 0 and remained negative in the (Av) dermal fibroblasts throughout the culture timecourse. (B) A representative graph depicting percentage of RANKL-positive cells. Error bars represents the standard deviation of 3-5 fields of one donor population.
Figure 3-5. **M-CSF protein expression from MSC-derived adipocyte conditioned medium**

Representative data showing protein levels of M-CSF from 3-day of both MSC-derived adipocyte and MSC (in GM) conditioned medium as measured by ELISA at indicated timepoints n=3 donors.
Figure 3-6. **OPG protein expression from MSC-derived adipocyte conditioned medium**
Representative data showing protein levels of OPG from 3-day conditioned medium of both MSC-derived adipocytes (*black bars*) and MSC (in GM) (*gray bars*) as measured by ELISA at indicated timepoints. Error bars depict standard deviation of triplicate wells.
Figure 3-7. **SDF-1 protein expression: Donor and stage variability**

To take a closer look at the variable SDF-1 protein expression (A,B,C) all donors tested are shown. There is both high donor and stage dependent protein level variability as measured by ELISA of 3-day conditioned medium of MSC-derived adipocytes at the indicated culture day. (D) Shows variant SDF-1 protein levels of non-lipid-laden adipocytes (Fibroblast fraction) and lipid-laden adipocytes (Adipocyte fraction) 3 days after enrichment.
**RANKL expression in MSC-derived adipocytes is based on lineage commitment: Model Validation**

Hormones such as Vitamin D3, PTH, PGE2, and glucocorticoids are known inducers of osteoclast formation through the regulation of RANKL and/or OPG expression. Dexamethasone (dex) is a synthetic steroid hormone that is an essential component of adipogenic induction medium and used as a potent glucocorticoid substitute in most adipogenic induction protocols [43,140,146]. Moreover, *in vitro* Dexamethasone treatments in both human and mice marrow-derived stromal cell clones as well as mouse primary isolated calvarial osteoblasts have been shown to strongly induce RANKL expression while also inhibiting OPG expression [171–174]. Thus, it remained important to learn if RANKL expression reflected adipocyte differentiation.

To verify that RANKL and OPG expression in the MSC-derived adipocytes was primarily due to entry of MSCs into the adipogenic lineage and not solely due to the presence of dexamethasone in the adipogenic induction medium, MSCs were treated with either Growth Medium (DMEM w/ 10%FBS), Adipogenic Medium (DMEM-HG w/10% FBS, IBMX, $10^{-6}$ M dexamethasone, and 10µg/ml Insulin), or Growth Medium + Dexamethasone (Growth Medium + $10^{-6}$ M Dexamethasone) for up to 3 days to simulate normal induction kinetics. RANKL
expression increased slightly (up to 4 fold over baseline) by 24 hrs, similar to what has been seen previously in mice and humans [173,175]. In the AIM cultures, RANKL expression was significantly higher than both baseline and cultures with comparable concentrations of Dexamethasone (Figure 3-8A) for all time-points.

To confirm that MSCs entered the adipogenic lineage during this early window of induction, a marker for early adipogenesis, pre-adipocyte factor 1 (Pref 1), was measured by PCR. Pref-1 mRNA was detected in cells cultured in adipogenic induction medium, but was absent in both the GM controls and Dexamethasone treated cells (Figure 3-8B). These results indicate that RANKL expression during the adipogenic induction period is primarily a function of adipogenic stage rather than Dexamethasone influence.

To confirm these results and determine if the pattern of protein expression in response to AIM and dexamethasone differed from the mRNA results, RANKL immunocytochemistry was performed during the timepoints of optimal RANKL expression (days 3-12) (Figure 3-8C). Quantification of RANKL-positive cells per total population showed that the number of RANKL positive cells was higher in adipocyte cultures versus cultures given just dexamethasone in all three timepoints tested (Figure 3-8C).
Figure 3-8. **RANKL expression in adipocyte cultures: model validation**

RANKL expression was assessed using (A,B) PCR and quantitative PCR during early induction timepoints. (A) Shows RANKL mRNA expression in Adipocytes ( ), Medium with dexamethasone ( ), and the baseline control MSCs ( ). (B) Adipogenic lineage entry assessed by pre-adipocyte factor 1 (Pref-1) expression using PCR. Shows that at both 12- and 24-hrs. pref-1 is primarily expressed in the adipocyte cultures. (C) RANKL protein expression was assessed and quantified at the indicated timepoints using immunofluorescent staining. Graph represents percentage of RANKL positive cells per total cells per field (n=2 donors) for each indicated culture condition. (AIM) Adipogenic induction medium, (Dex) DMEM-HG complete medium supplemented only with dexamethasone, or (GM) complete Growth medium control. Error bars represent standard deviation of 3-5 fields per each donor analyzed.
RANKL, SDF-1, and M-CSF were expressed in distinct adipocyte populations

Although there is an overall progression of medullary adipocyte differentiation and maturity along the 24-day time course as measured by morphology, cell lipid content and expression of lineage-stage adipogenic markers, the population of adipogenic cells remains heterogeneous in all of the metrics at each time point (Figure 3-9A-C). For example, at day 12, when many lipid laden adipocytes are observed, there remains a subpopulation of cells that are devoid of lipid vacuoles. To determine the contribution of expression of osteoclastogenesis mediators among subpopulations of adipogenic lineage cells, day 12 MSC-derived adipocytes were fractionated and enriched based on their lipid vacuole content, and conditioned medium from these enriched populations was collected and analyzed for both SDF-1 and M-CSF protein expression. Both SDF-1 and M-CSF proteins were primarily expressed by the non-lipid-laden adipocyte fibroblast-like cell cultures, as measured by ELISA (Figures 3-7D & 3-10).
Figure 3-9. **RANKL expression in non-lipid-laden subpopulation throughout culture period**

Immuno fluorescent staining of RANKL (*green*) counterstained with Hoechst 3342. *(A,B,C)* both the lipid-laden (*grey arrows*) and non-lipid (*white arrows*) diverse population was present throughout the MSC-derived adipocyte cultures (from day 3- day 12). RANKL staining was highest (on average) at day 9 and was present in the non-lipid-laden adipocyte-like cells throughout the culture time *(A) day 3 (B) day 9 (C) day 12*. RANKL positive cells per total field declined after day 9 but RANKL staining intensity either remained or was increased by day 12 as compared to *(D)* SaOs-2 positive control.
Figure 3-10. **M-CSF protein expression in adipofibroblast sub-population conditioned medium**

**(A)** Representative ELISA results of 3-day conditioned medium from 12-day cultures of MSC-derived adipocytes (in AIM) and MSC controls (in GM). Both the lipid-laden and non-lipid-laden (adipofibroblasts) adipocyte populations were enriched. **(B)** After enrichment an ELISA was performed from 3-day conditioned medium of both fractions was collected at the indicated culture day after re-plating was done showing differences in protein levels from each enriched population. Error bars represent standard deviation of triplicate wells. (n=2 donors).
Similarly, at day 12 RANKL expression was present primarily within the non-lipid-laden cells (Figure 3-11A), and this expression was maintained primarily in the non-lipid-laden populations throughout the culture period where RANKL expression was optimal (days 9-12) (Figures 3-9 & 3-11C). Further, mRNA from both the enriched lipid-laden and non-lipid-laden cells (Figure 3-11B) was isolated and analyzed by quantitative PCR for RANKL. RANKL expression was present largely within the non-lipid-laden adipocyte fraction (Figure 3-11D). Thus osteoclastogenic mediators are differentially expressed between the two morphologically distinct populations present in MSC-derived adipocyte cultures during mid-stage adipogenesis. In contrast, OPG protein expression showed no population bias as measured by ELISA (data not shown).
Figure 3-11. **RANKL expression is based on adipocyte morphology**

(A) RANKL immunostaining (green) of day 12 MSC-derived adipocyte cultures. Cells containing lipid vacuoles (white arrows) that are negative for RANKL. Cells that do not contain lipid vacuoles show evidence of RANKL positivity (gray arrows) (B) Enrichment populations of MSC-derived adipocytes showing both the (Bi) non-lipid-laden and (Bii) lipid-laden MSC-derived adipocyte populations. (C) Graph depicting percentages of lipid-containing cells that were positive for RANKL immunostaining (gray bars) vs. total RANKL positive cells (black bars) during MSC-derived adipocyte culture timecourse confirming that lipid containing adipocytes consistently represent a very low percentage of the total RANKL-positive cell populations. (D) mRNA quantitative PCR analysis of both the non-lipid-laden (gray bar) and lipid-laden (white bar) enriched adipocyte populations show an approximately 10-fold difference in RANKL expression levels.
Preadipocyte cell proliferation

Cell growth rate was measured using CyQuant®. MSCs and MSC-derived adipocytes were quantified over the adipogenic lineage timecourse. Both MSC and adipocyte populations show a maximum of 1 population doubling throughout the 24 day time-course (Figure 3-12). Furthermore, adipocytes show a slight growth arrest early in the cultures when compared to that of hMSCs, which might reflect the period of adipogenic lineage commitment where proliferation is thought to be inhibited [140,144,146]. These data show that growth patterns of MSCs and MSC-derived adipocytes are different and that there was no major growth arrest in the MSC-derived adipocyte cultures.
Figure 3-12. **Growth Chart**
Chart shows both the MSC-derived adipocytes (in adipogenic medium) and MSCs (in growth medium) growth properties over a 24-day timecourse in culture.
**Adipogenic potential of RANKL expressing enriched cells**

To assess whether the non-lipid-laden cells that express RANKL ([Figure 3-11](#)) and M-CSF ([Figure 3-10](#)) retained adipogenic potential. MSC-derived adipocyte day 12 cultures were enriched and the non-lipid-laden cell populations were re-induced to undergo adipogenesis (secondary induction) by culturing cells in AIM for another 12 days. The secondary induced cells were compared to MSC-derived adipocytes at primary induction. RANKL expression in the non-lipid-laden “adipofibroblasts” was higher on a per cell basis when compared to culture matched MSCs ([Figure 3-13A&B](#)) from each donor (approx. 50% ± 20% compared to 17% ± 5% in MSC’s; n=3 donors).

Representative data are shown in [Figure 3-13E](#). Lipogenesis was quantified on both a per cell basis and by total lipid vacuole accumulation ([Figure 3-13F&G](#)). Adipogenesis in the ‘adipofibroblast’ cultures was comparable to that of primary adipogenic induction in both total lipid accumulation and percent lipogenesis indicating that adipogenic potential is maintained even in the non-lipid-laden enriched fractions of MSC-derived adipocyte cultures.
Figure 3-13. **Non-lipid-laden adipocyte subpopulation is capable of equivalent adipogenesis**

MSC-derived adipocyte cultures were allowed to undergo adipogenesis for 12 days under. The potential for adipogenesis in the enriched non-lipid-laden (B,D) RANKL-positive subpopulation of MSC-derived adipocyte day 12 cultures was compared to that of (A,C) culture matched MSCs after both populations were re-induced for adipogenesis 1 day after enrichment. RANKL expression was observed to be higher in the enriched sub-populations by both (A,B) Immunofluorescence and (E) quantitative PCR (MSCs-dark bar; adipofibroblasts- white bar). Lipid accumulation was quantified by extracting Oil Red O adipocyte stain and averaging total cell counts from several fields or to quantify percentage of lipogenesis cells containing lipid vs. total cell count in several fields was counted manually. Both lipid-laden and non-lipid-laden populations showed an ability to form lipid laden adipocytes as observed by (C,D) Oil Red O staining in both. (F) Total lipid accumulation was measured from Oil Red O-stained adipocyte cultures and was shown to be comparable in culture matched MSCs and parent MSC-derived adipocyte cultures.
Early pro-adipogenic molecules in adipofibroblasts

Previous studies using mouse marrow adipose tissue-derived stromal cell cultures have identified cells that were capable of both RANKL and PPARγ expression [22]. To determine if the RANKL-expressing non-lipid-laden MSC-derived adipocyte cultures were also capable of PPARγ expression, mRNA was isolated from both the lipid-laden and non-lipid-laden fractions of MSC-derived adipocyte cultures and tested for both RANKL and PPARγ (Figure 3-14). As expected, both RANKL and PPARγ mRNA are upregulated in AIM when compared to GM MSC controls by day 12. Furthermore, RANKL expression was present only in the non-lipid-laden fractions. It is interesting to note that PPARγ mRNA expression was exclusively present in the lipid-laden fraction and absent from the RANKL-expressing non-lipid-laden fractions.
Figure 3-14. **RANKL and PPARγ mRNA expression are expressed in different adipocyte populations**
mRNA was isolated from both the lipid-laden (Adip fract) and non-lipid-laden (Pellet) and analyzed for PPARγ2 and RANKL expression using PCR. While PPARγ2 was present in the lipid-laden RANKL-negative fractions when, PPARγ2 was absent in the RANKL-positive non-lipid-laden adipocytes. All experimental samples were compared to donor matched MSC-derived adipocyte (Adip d12) and MSC in growth medium 12-day cultures (MSC d12) controls. HUVECs served as positive control for RANKL.
This study demonstrates that RANKL expression is primarily limited to the non-lipid-laden adipocytes (Figure 3-11). Because PPARγ was not expressed in the RANKL-positive non-lipid-laden cells (Figure 3-14), that still retain an adipogenic potential similar to MSCs, it remains possible that these cells are held in an intermediate state of adipogenesis, in part, due to some inhibitory process that is associated with adipocytes in culture [76,144,176].

To test the hypothesis that the non-lipid-laden cells are held in some early pro-adipogenic state prior to PPARγ expression, a gene expression profile testing expression of adipogenic factors and cofactors was conducted, along with markers that are have been candidates of adipocyte lineage progression inhibition. The adipogenic marker expression for MSCs (as controls) MSC-derived adipocytes (at day 12), the lipid-laden enriched adipocyte populations, and the non-lipid-laden enriched adipocyte populations was analyzed for comparison (Figure 3-15). Pro-adipogenic factors including KLF4, KLF9, and KLF5 mRNA were observed in the non-lipid-laden adipofibroblast enriched population (Figure 3-15). Although we showed earlier that, PPARγ expression was absent in the non-lipid-laden adipocyte fractions, the PPAR target gene adipsin was present (Figure 3-15). The presence of adipsin mRNA expression may be due to low-lipid-containing cells that have contaminated this population due to
their similar density to those cells that are completely devoid of lipid. It is also possible that there is some small degree of PPARγ protein activity within a subset of these cells. The Hes-1 target gene of pref-1 [143,177,178] was present at low levels in adipofibroblasts, and Hes1 expression in the lipid-laden adipocytes was noticeably greater.
Figure 3-15. **Early adipogenic mRNA expression profile in both enriched MSC-derived adipocyte fractions**

The expression of early pro-adipogenic and intermediate-associated adipocyte marker expression in day 12 MSC (column 1) MSC-derived adipocytes (column 2) and enriched adipocyte subpopulations: 'adipofibroblasts' (column 3) and lipid-laden adipocytes (column 4) using PCR from RNA isolations of each group was analyzed. The enriched non-lipid-laden and lipid-laden adipocyte adipocyte populations were cultured for 24 hours before RNA was isolated and PCR was performed. Check marks indicate the pro-adipogenic factors expressed by the non-lipid-laden enriched adipocyte populations as highlighted by the blue box. *Srebp1 expression can be induced by insulin which was used for the non-lipid-laden enriched adipocyte population cultures.
**MSC marker expression among MSC-derived Adipocytes**

MSC-like cells are present in adipose tissue [73,144,179]. In culture, adipocytes are morphologically heterogeneous and the presence of MSC-like cells in cultures of adipocytes has not been reported [144]. Non-lipid-laden populations of MSC-derived adipocyte exhibit a morphology similar to MSCs. To test for the presence of MSCs in these cultures, immunocytochemistry for CD90 and CD105, two markers associated with the MSC phenotype [180] was performed on cultures after 12 days in differentiation medium. MSC-derived adipocytes were stained for both CD90 ([Figure 3-16](#)) and CD105 ([Figure 3-17](#)). While MSC-derived adipocyte cultures included MSC-like cells, there were markedly fewer than those in the MSC controls in Growth Medium for 12 days (~30% ± 5% vs. ~90%± 10% in MSCs; n=3 donors) These results are shown in representation in Figures 3-16C & 3-17C. CD90 and CD105 positively stained cells within the MSC-derived adipocyte cultures were mainly contained within the non-lipid-laden adipocyte subpopulations ([Figure 3-16b](#) & [3-17b]). These results indicate that there are non-lipid-laden MSC-like cells maintained within these MSC-derived adipocyte cultures. There is a possibility, therefore, that these CD90-/CD105-positive cells account for both the RANKL-positive cells and, at least in part, the maintained adipogenic capabilities shown to be present within this non-lipid-laden population.
Figure 3-16. **CD90 Expression in MSC-derived Adipocytes**

MSC-derived adipocyte 12 day cultures were stained with an immunofluorescent tagged antibody for CD90 (red) compared to MSC controls. (A) CD90 staining in MSC confluent cultures. (B) CD90 expression detection in MSC-derived adipocyte cultures. (C) Representative graph depicting the quantification of CD90 staining positivity of MSC-derived adipocytes (gray bars) and MSCs (black bars) vs. total population in each condition respectively. CD90 staining was done using mouse anti-CD90 and an Alexa Fluor- 594 tagged secondary goat anti-mouse antibody. Nuclei were counterstained using Hoechst 33342.
Figure 3-17. **CD105 Expression in MSC-derived Adipocytes**

MSC-derived adipocyte 12 day cultures were stained with an immunofluorescent tagged antibody for CD105 (red) compared to MSC controls. (A) CD105 staining in MSC confluent cultures. (B) CD90 expression detection in MSC-derived adipocyte cultures. (C) Representative graph depicting the quantification of CD90 staining positivity of MSC-derived adipocytes (gray bars) and MSCs (black bars) vs. total population in each condition respectively. CD105 staining was done using mouse anti-CD105 and an Alexa Fluor-594 tagged secondary goat anti-mouse antibody. Nuclei were counterstained using Hoechst 33342.
RANKL expression among MSCs, Osteoblasts and Adipocytes

To assess whether the non-lipid-laden RANKL-positive cells found in MSC-derived adipocyte cultures were undifferentiated MSCs, double label immunocytochemical staining of RANKL and the common MSC markers CD90 and CD105 was performed (Figure 3-18A&B). The majority of RANKL-immunoreactive cells (80% ± 3%; n=3 donors) did not express either CD90 or CD105 The few cells that did show MSC marker co-localization with RANKL had a lower staining intensity for both CD90 (Figure 3-18Aii), CD105 (Figure 3-18Bi) and vice versa (Figure 3-18Ai).

Because osteogenic lineage cells might be present in these cultures and may account for the RANKL staining [125], the presence of RUNX2 in RANKL-positive cells was assessed. Although RUNX2 was present within the adipocyte cultures, the majority of RANKL positive cells were completely devoid of both RUNX2 staining (Figure 3-19) and Osterix (OSX) staining (Figure 3-19Bi&ii).

To test whether the RANKL positive cells were committed to the adipocyte lineage and determine if there was any evidence of transient PPARγ protein activity, cells were assayed for RANKL and PPARγ and C/EBPα protein expression, the two transcription factors important for adipocyte commitment (Figure 3-18C&D). Interestingly, there was little co-localization between RANKL and PPARγ (Figure 3-18C). In
contrast, RANKL co-localization with C/EBPα was observed (Figure 3-18D).
**Figure 3-18. RANKL shows variant co-localization between MSC and adipocyte markers**

RANKL Immunofluorescent co-staining was done to identify potential RANKL (green) co-localization with common MSC markers (A) CD90 (red) and (B) CD105 (red) and the adipocyte transcription factors (C) PPARγ (red) and (D) C/EBPα (red). The majority of RANKL-positive cells did not co-localize with (A) CD90-positive cells (B) CD105, or (C) PPARγ but showed some co-localization with (D). C/EBPα (gray arrows). (Ai-Ci) Insets shows representative instances in which there was some co-localization.
Figure 3-19. **Runx2 expression in RANKL positive MSC-derived adipocytes**

Co-staining of RANKL (green) and RUNX2 (red) was done in order to show potential co-localization between the two molecules. **(A)** In the Osteosarcoma (SaOs-2) positive controls, co-localization (black arrow) was expected in the Osteosarcoma. **(B)** In MSC-derived adipocyte cultures at day 12 highly RANKL-positive cells (white arrows) showed no evidence of RUNX2 expression. In cells that were positive for RUNX and RANKL showed RUNX2 expression mainly in the cytoplasm of cells (black arrows). **(Bi,ii)** Shows immunofluorescent co-staining of Osterix (red) and RANKL (green). **(Bi)** Although there was some evidence of Osterix expression in MSC-derived adipocytes **(Bii)** RANKL-expressing adipocytes were did not co-localize with Osterix.
RANKL co-localization with PPARγ has been previously shown in mouse medullary adipocytes [22], and indeed there was some RANKL co-localization with PPARγ in the present cultures (Figure 3-18Ci). To more rigorously test for co-localization, the more sensitive visualization technique of confocal imaging was used to study the RANKL/PPARγ immunoreactive cultures. With this approach, co-localization RANKL and PPARγ in MSC-derived adipocyte cultures was readily observed (Figure 3-20). However RANKL and PPARγ in MSC-derived adipocytes was observed only in a minority of cells, Table 3-1.
Figure 3-20. **Evidence of RANKL and PPARγ co-localization in MSC-derived adipocyte cultures**

Fluorescent co-staining of RANKL (green) and PPARγ (red) reveals co-localization was observed between both after leica laser imaging (*left panel*) and confocal imaging (*right panel*).
Table 3-1 Depicts the average quantity of RANKL and PPARγ positive cells (n=3 donors) of several different fields; including the percentages of RANKL and PPARγ co-staining.

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Adipofibroblast may support osteoclasts

Co-cultures of adipofibroblasts and peripheral blood mononuclear cells (PBMNCs) were used to test if ‘adipofibroblasts’ support formation of TRAP-positive multi-nucleated osteoclast-like cells. When compared with positive controls of PBMNC supplemented with recombinant RANKL and M-CSF, co-cultures with adipofibroblasts exhibited comparable levels of TRAP-positive staining and multinucleated osteoclast-like cell formation (Figure 3-21 A&C). Dermal fibroblasts served as a negative control and did not show comparable levels of multi-nucleation and TRAP positivity in co-cultures with PBMNCs (Figure 3-21 B&D).
Figure 3-21. **Osteoclast-like formation of adipofibroblast/PBMNC co-cultures**

Osteoclast formation potential of adipofibroblast MSC-derived adipocyte subpopulation was assessed by TRAP staining after 15 days of co-culture of enriched adipofibroblasts and peripheral blood mononuclear cells and compared to positive control cultures were given 50 ng/ml of M-CSF and 60 ng/ml RANKL in DMEM:RPMI (1/1) medium. (C) Multinucleated osteoclast-like cells formed after treatment with M-CSF and RANKL for 15 days shows a distinct osteoclast-like cell phenotype including multi-nucleation, TRAP positivity and large diameter. Positive osteoclasts-like cells were numerous in positive controls (black arrows) while scarce in (D) co-cultures with dermal fibroblasts (A). Multi-nucleated TRAP positive osteoclast-like cells formed in ‘adipofibroblast’ PBMNC co-cultures after 15 days in culture. Osteoclast-like cell formation was comparable to that of the (B) Positive controls while no significant formation of multi-nucleated TRAP positive osteoclast-like cell formation was seen in (B) dermal fibroblast and PBMNC co-cultures. The inserts in each panel show a close-up view of a cell representative of an osteoclast that was common in each respective culture conditions.
**New Adipocyte lineage thesis model.** Updated thesis model described in Chapter 2 (Figure 2-2) illustrating new findings based on data presented within this document. Osteoclast mediators RANKL, OPG, M-CSF, and SDF-1 are expressed throughout the MSC-derived adipocyte culture. Furthermore, RANKL, OPG, and M-CSF show a developmental dependent expression pattern in the *in vitro* Adipocyte cultures. Unlike M-CSF and SDF-1, RANKL and OPG pattern of expression in the adipocyte lineage was highly distinct. RANKL expression was upregulated early in culture and is lost during maturation. OPG expression showed that expression levels were absent early on in the adipocyte lineage and increases during maturation of the culture. SDF-1 was present throughout culture but did not appear to have any developmentally regulated pattern of expression. M-CSF expression with respect to the adipocyte culture maturation is less distinct but does show some upregulated expression during adipogenesis and some slight decrease during culture maturation.
Chapter 4

Preliminary Data

Exploring the role of the adipofibroblast in the BMU

In the marrow, adipocytes are closely related spatially to several elements of the remodeling BMU; including the marrow sinusoid (vascular network), the marrow stroma, and the BRC lining cells [50]. Due to this relationship, it remains important to learn what role the RANKL-positive non-lipid-laden adipocyte-like cells, identified here, have in the remodeling environment, in vivo. The current challenge is to set-up a model that has representations of each of these BMU elements in in vitro cultures containing adipofibroblasts. One approach may be to use a more complex vascularized co-culture system containing adipocytes, vascular elements and the non-lipid-containing adipofibroblasts.

Modeling the vascularized in vivo medullary adipocyte in vitro

In a previous study, a co-culture system using human umbilical vein endothelial cells (HUVECS), MSCs, and dermal fibroblasts was shown to mimic angiogenesis and vascularized tissue in vitro, in hopes of mimicking the in vivo dermal vascularized layers [181]. In our hands this culture-mimicked vascular network can be prepared in
similar co-cultures using MSCs, adipocytes and HUVEC cells only (Figure 4-1). In contrast to the dermal fibroblast-HUVEC-MSC vascular-mimicking layered cultures, the formation of capillary-like tubes in the adipocyte-HUVEC layered cultures are less uniform and tubules form in a more sporadic assortment (Figures 4-1 & 4-2), similar to what is seen in isolated marrow sections with Dye-I labeled endothelial cells (Figure 4-3) [182].

There have been studies that suggests both early and mature adipocytes can associate with the vasculature [114,169,176,183]. To that end, the model of vascularization, described here, could provide some key answers as to how these RANKL-positive adipofibroblasts—if it exists in the adipocyte population in vivo—might associate in the marrow. If RANKL-positive adipofibroblasts associate closely with the tubes formed in co-cultures, this might indicate that this particular cell type might associate with the marrow sinusoid—similar to a perivascular cell.
Using Immunohistochemical analysis for insight into how the adipofibroblasts associate within the marrow

By contrast, detailed immunohistochemistry may reveal that adipocytes may not associate with the marrow sinusoid in vivo. Markers like CD146, expressed on cells that associate with endothelial cells in vivo (i.e. pericytes), including the Mesenchymal Stem Cell [184], have been identified in the stromal vascular fraction of adipose tissue [73,156]. RANKL co-staining with CD146 shows that the vast majority of RANKL-positive adipofibroblasts show no significant co-localization with CD146 (Figure 4-4). This might indicate that these adipofibroblasts do not associate with the marrow sinusoidal complex in vivo, however more data is needed in order to confirm this. In contrast to CD146, another MSC associated marker, CD271, describes MSCs localized away from the marrow sinusoid and within the marrow stroma [184]. Like CD146, there are studies that have identified CD271 expression in the in vivo adipose tissue [156,176]. It would be interesting to determine whether CD271 is expressed in in vitro MSC-derived adipocytes and whether it can co-localize with the RANKL-positive adipofibroblasts cells. If so, this suggests that high marrow fat content might correlate with an increase in RANKL positive cells in the marrow stroma.
Figure 4-1. **HUVEC microtubule formation in layered co-cultures**
HUVEC formation of microtubules in cultures of either MSCs with Dermal Fibroblasts (Left panel) or with MSC-derived adipocytes (Right panel). HUVEC were labeled with Dye-I as described (Sorrel JM et al. 2009) and layered on 12-day cultures of either MSCs and Dermal fibroblasts in GM or MSCs induced for adipogenesis in AIM. After 3 days images were taken to show microtubule formation of HUVEC cells.
Figure 4-2. **Dye-I labeled HUVECS from Marrow sections**

A section of bone marrow depicting Dye I-Ac-LDL endocytosed by sinusoids. The sinusoidal endothelial cells can now be identified individually (white arrows). Due to intramedullary hemorrhaging other cell types have taken up the dye such as macrophages (yellow arrows). This image gives an example of the marrow sinusoidal network in an ex vivo setting (*Li Xm et al. 2009*).
Figure 4-3. Dye-I labeled HUVECs and 12-day MSC-derived adipocyte layered co-cultures showing formation of microtubule formation in adipocytes after 3-days Microtubule formation, though sparse, is seen (gray arrows) within cultures. Both lipid-laden and non-lipid laden adipocytes can be seen to be associated with the HUVEC cells (red).
Figure 4-4. **CD146 expression in enriched adipofibroblasts**
MSC-derived adipocyte enriched adipofibroblasts were co-stained for CD146 (red) and RANKL (green). The majority of CD146 positive cells were negative for RANKL staining.
Preliminary Conclusions: adipofibroblast-BMU association model

Using these preliminary data, results from this study and what is known about how adipocytes associate within the BMU, a schematic of the non-lipid-containing-adipocyte-BMU relationship in the remodeling bone is proposed (Figure 4-5). In this model, the potential of the RANKL positive non-lipid-laden “adipofibroblast” to affect bone remodeling is characterized by where it has the potential to associate with each remodeling element within the BMU microenvironment (highlighted by the numbered arrows in Figure 4-5).

The preliminary data described here, suggest that the adipofibroblasts do not associate with the marrow sinisoid. In view of the fact that a phenotypic heterogeneity even lays within the adipofibroblast population itself, it is possible that adipofibroblasts might be capable of associating within multiple niche elements of the BMU; there is no mutual exclusivity to their localization. More data is needed to define their relative space within the marrow and, more importantly, their role, if any, in the remodeling process.
Figure 4-5. Schematic model of possible osteoclast regulatory cell niches during bone remodeling in the BMU

Osteoclast development and formation during the bone remodeling process in increase adiposity can be regulated at several different niches within the Bone modeling Unit. Represented here is the various sites that RANKL mediated osteoclast formation could likely take place. 1). Marrow sinusoid: containing RANKL expressing MSC (as pericytes), endothelial-associated pre-adipocytes and endothelial cells (as adipocyte progenitors/vasculature). 2). Marrow stroma: containing a population of hematopoietic and RANKL-expressing supportive cells. 3). Endosteal lining cells: containing osteogenic and MSC derivative cells that act as barrier cells for the Bone Remodeling Complex. Each BMU complex has been show to have some osteoclast support capabilities and adipogenic connection in the literature.
Chapter 5

Discussion

The medullary adipocyte is believed to play an important, yet uncharacterized, role in the marrow. In particular, adipose tissue dysregulation is associated with various maladies such as hypertension, diabetes, and inflammation and cardiovascular diseases [14]. With aging, there is an increase in medullary adipocytes such that they become the most abundant stromal cell source within the marrow [21,185]. This increase in adipose tissue is associated with a decrease in bone mineral density in trabecular bone and to a smaller degree, cortical bone [121,186].

It has been proposed that an increase in medullary adipocytes in the marrow can directly affect bone density by affecting bone metabolism through activity of the osteoclast. Indeed, the notion that there is a relationship between bone and fat is supported by both in vivo and in vitro mouse studies [20,23,27,130,185]. A strong correlation between marrow adiposity and osteoclast numbers has been shown in vivo [24]. Other studies looking at cultured adipocytes from both mice and humans showed a potential for medullary adipocytes to support osteoclast formation via expression of factors
that are important for osteoclast development and osteoclast-like cell formation [28,35]. What remained missing was a stringent characterization of an adipocyte cell that can affect osteoclasts, and an understanding of whether adipocyte regulation is developmentally regulated, similar to what has been seen in osteoblasts.

The studies in this thesis were designed to characterize the relationship between adipocytes and osteoclasts. Studies tested the hypothesis that cells of the medullary adipocyte lineage influence osteoclastogenesis through the expression of positive and/or negative osteoclast mediators based on their developmental stage, similar to the expression of osteoclastogenic mediators by cells along the osteogenic lineage [6,7]. Using a well-characterized model of medullary adipocyte differentiation from marrow-derived MSCs, mRNA and protein expression of several key osteoclastic mediators were measured in adipogenic cells at various lineage stages along their differentiation from MSCs [43]. Indeed, developmentally regulated patterns for RANKL, OPG, and M-CSF were identified in adipocyte lineage cells. Thus, this study shows that adipocytes have the capability to regulate osteoclast development; and, like osteoblasts, can support osteoclasts in a developmentally regulated manner. Furthermore, this provides a new insight as to how adipocytes can interact with bone.
Lineage markers and RANKL in the MSC derived adipocytes

RANKL is one of the essential molecules needed for osteoclast formation and maturity [187]. In osteoblasts, RANKL expression has been shown to be induced in cells that are very early in the lineage; and once these cells progress down the osteogenic lineage, RANKL expression is decreased [6,7,107]. In MSC-derived adipocyte cultures, RANKL expression showed an initial increase when cells entered the adipogenic lineage. This increased expression in RANKL mRNA and protein remained consistent throughout the developing adipocyte period. Once the adipocytes matured, RANKL expression gradually decreased. This pattern of RANKL expression confirmed that osteoclast mediator expression varied based on adipocyte differentiation stage in these cultures.

Unlike the osteogenic program [137,138,188], adipogenic lineage progression in culture is poorly characterized [13,144]. The classic model of adipocyte maturation starts with the expression pattern of PPARγ and, to a lesser extent, C/EBPα. PPARγ expression and activity in adipocytes has been shown to be optimal during early development stages of adipocytes [13,43,144,189]. Once adipocytes mature, PPARγ expression is decreased. The present data show that RANKL expression was highest during higher PPARγ expression and
low during lower PPARγ expression in culture; although there was no direct correlation between RANKL and PPARγ expression.

OPG, like RANKL, has a very distinct pattern of expression in osteoblasts, but unlike RANKL, OPG expression is upregulated only during the mature stages of osteoblast differentiation [8]. In cultured MSCs, OPG expression was clearly suppressed once MSC-derived adipocyte cultures entered into the adipogenic program and was enhanced only after cells were allowed to mature in adipocyte maintenance medium. Unexpectedly, OPG expression was markedly higher in MSC controls. This result may suggest that MSC-like (or stromal cells) have a role in downregulating osteoclast activity. However, there are currently no studies that have investigated whether or not MSCs can express RANKL or if they are capable of regulating osteoclastogenesis through OPG expression either in vivo or in vitro. This raises the question as to whether the results of OPG expression in adipocytes is physiologically relevant and if the level of OPG expression seen in MSC-derived adipocytes is sufficient to inhibit osteoclast formation in co-cultures.

M-CSF promotes the lineage commitment and proliferation of osteoclast progenitors [102]. Unlike RANKL and OPG, M-CSF expression in MSCs was less clear in the osteogenic program. In MSC-derived adipocytes, M-CSF expression was increased once cells
entered the adipogenic program. As seen with RANKL expression, M-CSF expression was consistently increased throughout the early- to mid-stages of adipogenesis and decreased (modestly in this case) once cells were allowed to mature in adipocyte maintenance medium.

The expression pattern of M-CSF was similar to that of RANKL in these cultures, as expression was largely dependent on developmental stage. However, unlike RANKL in MSC-derived adipocytes, changes in M-CSF expression as a function of culture time were less dramatic. There are no data to suggest that M-CSF protein levels increase with high marrow adiposity. There is evidence on increase osteoclast numbers in adipocyte-rich marrow [24,190]. Whether or not an increase osteoclast numbers in adipocyte-rich marrow is, in part, mediated by an increase in M-CSF protein secretion would be an avenue worth further investigation.

Collectively, these results show that, like cells in the osteogenic lineage, adipocytes have the potential to support osteoclast formation in early stages of the lineage.

**A new cell: The RANKL-positive adipofibroblast**

RANKL expression was primarily observed on a previously uncharacterized cell among medullary adipocytes. These RANKL positive cells appear to be early-stage, pre-lipid-laden adipocytic
cells—termed here as adipofibroblasts. In fact, adipose tissue is a highly heterogeneous tissue and contains a diverse population of many different cell phenotypes [13,73,74,154,155]. Included within this heterogeneous population of cells are MSC-like cells [73,156], committed pre-adipocytes [13,75,77,78,90,155,157], and other non-lipid-containing cell types [74]. To determine which of these non-lipid-laden cell types, common in adipose tissue, contributes to the observed RANKL expression, a series of co-localization studies using antibodies to RANKL and phenotypic markers that identify MSCs, osteoblast lineage cells, and cells of the adipogenic lineage was performed.

Two markers associated with the MSC phenotype, CD90 and CD105, although present in culture, did not co-localize with the RANKL expressing cells. There was also no evidence of RANKL expressing cells having an early osteoblastic lineage phenotype as characterized by Osterix (data not shown) and/or Runx2 expression. The majority of RANKL-positive cells expressed C/EBPα but did not show significant co-localization with PPARγ. Collectively, these data show that the majority of RANKL expressing cells observed in our MSC-derived medullary adipocyte cultures are from non-lipid-laden fibroblast-like cells that appear to be very early stage medullary adipocyte lineage cells based on their positive expression of C/EBPα and lack of PPARγ expression.
Studies looking at a potential direct relationship between marrow stromal derived adipocytes and osteoclasts have been conducted in both mice and humans [22,27,35]. However, primary isolated medullary adipocytes contain a heterogeneous mixture of cell types before and after culture [144], thereby preventing reliable tissue characterization due to multiple stromal cell phenotypes. To that end, there are no studies that were able to identify RANKL expression in adipose tissue in the non-lipid-containing populations. However, recently, a group looking at corn oil fed mice did observe an increase in RANKL expression in a population of non-lipid-containing cells closely associated with adipocytes in marrow with increased adiposity [24]. Their interpretation of these data was an increase in RANKL mediated by an adipocytic microenvironment. These RANKL expressing cells may be the ‘adipofibroblasts’ identified in the present MSC-derived adipocyte cultures. In future studies, it may be possible to isolate a pure population of RANKL-positive adipofibroblasts and characterize their gene profiles in adipocytic-rich marrow.

The adipofibroblast as an intermediate adipocyte

An interesting feature of the adipofibroblast in these MSC-derived adipocyte cultures was that these non-lipid-laden adipocyte-like cells were present throughout the timecourse. When non-lipid-laden cells were enriched and induced to form adipocytes, the majority
of these adipofibroblasts differentiated into mature, lipid-laden adipocytes; while a subpopulation of the adipofibroblasts remained at similar percentages as the original adipocyte cultures. These novel findings suggest the presence of a mechanism within the adipocyte microenvironment that prevents the maturation of the adipocyte-like cells to form fully lipid-containing mature adipocytes. These data suggest that they may reflect an early stage or intermediate stage adipocyte phenotype.

The existence of an early stage intermediate adipocyte present in adipose tissue is a relatively new concept. Other studies mention this cell type as a common feature among adipocytes in vitro. In the present study, a preliminary characterization was undertaken of the adipofibroblasts by enriching the non-lipid-laden portion of the MSC-derived adipocyte cultures and testing the gene expression profile of early pro-adipogenic candidate genes.

**Pro-Adipogenic candidate genes are regulated differently in adipofibroblasts**

Krüppel-Like Factors (KLFs) are a large family of zinc-finger proteins that regulate apoptosis, proliferation and differentiation [191,192]. In adipocytes, KLFs act as co-factors that can either promote or repress adipocyte development [13,144,193]. A range of KLFs can act as promoters of early adipogenesis. These factors include
KLF4 [194,195], KLF5 [153], and KLF9 [192]; while KLF15 acts on both differentiation and glucose metabolism [13,144,152]. KLF activity appears important only during the very early stages of adipogenesis, thus, KLF expression in cells may represent early stage adipocytes and the absence of KLF expression may represent undifferentiated cells and late stage adipogenesis. The present data demonstrate that KLF 4, 5, & 9 is expressed in the gross population of non-lipid laden fractions of adipocytes and, as predicted, absent in the ‘mature’ lipid-laden adipocyte fractions.

The Krox20 (also known as Egr2) transcription factor first identified in serum-stimulated NIH3T3 fibroblasts [196] is highly enriched in adipose tissue [149]. Krox20 activity in mouse 3t3-l1 pre-adipocytes is present only during the initial adipocyte differentiation phase, and during maturation its expression is rapidly down regulated [149]. While Krox20 is expressed in the MSC-adipocyte-like controls, in the adipofibroblast, Krox20 expression is reduced. These data suggest that the adipofibroblast population may be in an intermediate stage in the adipocyte lineage that is beyond the earliest adipogenic lineage stage where Krox20 expression is beginning to reduce.

Notch signaling is widely believed to play a role in cell fate and tissue development, and in adipocytes, Notch signaling appears to inhibit adipogenesis in precursor cells [144]. Hairy and enhancer of
split-1 (Hes-1) is one of several proteins known to be directly induced by the Notch receptor. In 3T3-L1 pre-adipocytes, Hes-1 has been shown to be inhibitive during lineage progression [143,178]. If cells were in fact held in an intermediate or pre-adipocytic state, Hes-1 may represent a practical read out molecule and would be expected to be present in adipofibroblast. However, Hes-1 was decreased in both the adipofibroblast and MSC-derived adipocyte populations when compared to MSCs in GM under similar culture conditions. An alternative mechanism may include Notch receptor endocytic trafficking in which internalization of the Notch receptor can down regulate notch signaling [197–199].

In developing adipocytes, Hes-1 downregulation is upstream of PPARγ and C/EBPα activation and is necessary for adipocyte differentiation [143,145,178]. Since Hes-1 downregulation was observed in both the MSC-derived adipocyte cultures and adipofibroblast enriched populations, but not the MSC and mature lipid-laden adipocyte cultures, it is possible that these adipofibroblasts have entered an early adipocyte lineage stage; possibly through the downregulation of Notch signaling. Future studies will be directed to clarify the notch pathway during this differentiation process in adipocytes. It would be important to understand whether the pattern of
Hes-1 expression is important in the RANKL-expressing adipofibroblast phenotype.

These gene expression results along with the differentiation marker expression results suggest that the adipofibroblast has properties consistent with early adipocytes. There are several mechanisms that have been associated with the maintenance of cells in an intermediate pre-adipogenic state (54; 65–68). For instance, pre-adipocyte factor 1 (pref-1), expressed by early adipocytes, has been shown to inhibit progression down the adipogenic lineage in a paracrine/juxtacrine manner by, among other mechanisms, inhibiting PPARγ expression and activity (69–71). It remains important to consider that 1) the ‘adipofibroblast’ present within our cultures maintain an adipogenic potential that is comparable to primary induced adipocyte cultures and 2) these cells are largely PPARγ negative while capable of expressing other related early pro-adipogenic factors including C/EBPα. Thus, there may be an inhibitory mechanism in place, perhaps mediated by pref-1 and/or other factors, that controls adipogenic commitment and PPARγ expression that thereby inhibits adipogenesis progression.

In addition to pref-1, both canonical and non-canonical Wnt signaling has been identified as a candidate regulator of adipogenesis (66; 72). Wnt10b and its receptor Lrp5 have been implicated in the
maintenance of adipose tissue by inhibiting adipogenesis and maintaining a precursor pool; this is known as pro-adipogenic Wnt signaling (73). Additional experiments are needed to identify the mechanism by which these cells are maintained in this proposed intermediate pre-adipocyte-like state and whether this state intensifies the RANKL-positive phenotype.

**The “Transitional” preadipocyte**

Based on the present studies, MSC-derived adipocytes are a diverse population of cells, perhaps representing multiple stages in a differentiating pathway. MSC-derived adipocyte cultures contain non-lipid-laden adipocytes, moderately-lipid-laden adipocytes, and heavily-lipid-laden adipocytes. Phenotypically, the majority of the non-lipid-laden adipofibroblasts were RANKL positive, PPARγ negative, CD90 negative, CD105 negative, and RUNX2 negative. Some non-lipid-laden cells however, expressed low levels of PPARγ. It was striking that the majority of these low-level-expressing PPARγ cells contained a very small amount of lipid even after 12 days of culture. This suggests that a population of cells were still transitioning from the non-lipid-laden pre-adipocyte to a more mature lipid-containing adipocyte even in the later stages of the culture.

Using these observations, a model is proposed in which a population of “transitional adipocytes”, present throughout culture,
represent cells that are initially held in an early stage of adipogenesis then slowly progress into the mature adipocyte lineage phase at a rate slower than neighboring mature heavily-lipid-laden adipocytes. This may represent a mechanism present in adipocytes that corresponds to adipocyte turnover; where there is believed to be a constant replenishment of mature adipocytes by preadipocytes in the \textit{in vivo} adipose tissues \cite{77,200}.

Additional support for the existence of a transitional pre-adipocyte exists in the MSC marker expression data. In mice and humans, CD90, CD105 and CD146 were found to be expressed in a range of early non-lipid-containing pre-adipocytes \cite{156,201–203}. In our cultures, some cells did show co-localization between RANKL and CD90/CD146. The co-localization between RANKL and the CD-markers was relatively infrequent however, and when co-localized RANKL or the surface markers had low overall expression. In addition, these cells did not contain any lipid vacuoles but did show a polygonal shape common in pre-adipocytes \cite{204}. Thus, because all CD-marker-positive non-lipid-laden cells were contained within the MSC-derived adipocyte cultures, maintained polygonal cell morphology and were either RANKL-negative or had very low levels of RANKL, these cells may be another type of transitioning pre-adipocyte. We postulate that this particular transitional pre-adipocyte can express CD90, CD146, and/or
CD105 (as pre-adipocytes) and quickly transitions through a RANKL-positive lineage stage.

**Working model for MSC Adipocyte Progression and RANKL/M-CSF/OPG expression**

These studies demonstrate the potential of adipocytes to support osteoclast-like cell formation in a developmentally regulated manner. Osteoclast regulating molecules are expressed in adipocytes at specific stages of adipogenesis [43]. The compilation of the expression data in the present study leads to a new model of the adipogenic lineage as it relates to expression of osteoclast mediator RANKL (Figure 5-1). In this model, the MSC-adipocyte progression is characterized by the presence or absence of CD-markers, adipocyte master regulating molecules, lipid content, and RANKL. For example, RANKL expression was restricted to the non-lipid-laden, and in some instances, low-lipid-laden adipocytes in virtually all instances. Thus it appears that RANKL expression is present only in the very early stages of adipogenesis, where lipid content is low, and PPARγ expression is absent. The model allows the description of two types of early adipocytes within these MSC-derived adipocyte cultures:

- **The intermediate adipocyte:** RANKL expression is high and present throughout the culture. This early adipocyte does not express PPARγ, is non-lipid-laden, shows low C/EBPα expression,
and none of the CD-markers tested. This cell type may be held at this stage through some inhibitory mechanism present in developing adipocytes.

- **The transitional adipocyte**: RANKL expression is low or absent throughout culture. This early adipocyte expresses PPAR\(_\gamma\), has some lipid content (low to intermediate), and can express C/EBP\(_\alpha\), CD-105 and CD-90 marker expression. This particular cell type may be actively transitioning into a mature adipocyte; whether it is from a RANKL expressing intermediate stage, or directly from an MSC.
Figure 5-1. Proposed model for MSC-derived medullary adipocytes.
MSC-derived adipocytes can express RANKL very early-on in the adipogenic lineage in non-lipid-vacuole-containing adipocyte cultures. There was a divergent population in these non-lipid-laden adipocytes where RANKL expression was either high or absent/Lo, representing 2 types of early adipocytes. The first is an intermediate early adipocyte population that can express high levels of RANKL and C/EBPα; the second is a low RANKL expressing pre-adipocyte cell that can express both PPARγ, C/EBPα, and the stem cell markers CD105 and CD90. In this model, MSCs may (1) transition directly to the RANKL-low/absent “transitional” pre-adipocyte and/or (2) rest at the RANKL-hi intermediate pre-(early) adipocyte stage before slowly progressing into a transitional pre-adipocyte before maturation.
**Future Directions**

**Purifying the RANKL-positive adipofibroblast**

The study presented here provides a partial characterization of a RANKL-positive non-lipid-laden cell that is present in a morphologically diverse population of cultured MSC-derived adipocytes. Given that, *in vivo*, adipose tissue is also represented by a morphologically diverse population of cells, the existing goal would be to establish an *in vivo* correlation to the results observed in this *in vitro* study. Indeed, there is data to suggest that these newly characterized RANKL-positive non-lipid-laden adipocyte cells identified in this study may also be present *in vivo* [24]. For example, in high marrow adiposity, there is a higher concentration of RANKL-positive non-lipid-laden cells when compared to lower marrow adiposity [24]. The question, therefore, is; whether the cells identified here are those observed in fatty marrow. To fully characterize this non-lipid-laden RANKL-positive cell and to assess whether these cells are similar to marrow adipocyte-like cells, it would be necessary to purify these cells based on their RANKL-positive phenotype. Such a cell might offer an attractive cell or molecular target for investigating the presumed adipocyte-mediated increase in bone resorption.
Mimicking the fatty marrow *in vitro*: a 3D approach

A relatively new advancement in *in vitro* culture modeling was recently described by Hammoudi T. et al [205]. This group described a 3-D in vitro culture system designed to mimic interactions between adipocytes, osteoblasts and MSCs in the marrow microenvironment. They were able to show changes in the MSC gene expression pattern depending on the cellular makeup of their 3-D culture system. One particularly interesting result was a change in MSC phenotype when the MSCs were layered with adipocytes only (as opposed to layers with both osteoblasts and adipocytes). When layered with adipocytes, the PPARγ basal expression of MSCs was significantly increased. This technique could be useful in trying to mimic what takes place in aged marrow where there is an increase in adipocyte populations. If one could successfully mimic the adipocyte-rich aged marrow in this 3-D *in vitro* model system, then one could assess changes in the MSC phenotype primarily focusing on the expression pattern changes of bone remodeling regulating molecules such as OPG, RANKL, M-CSF and SDF-1.

In addition, because adipose tissue is so closely related to bone lining cells [50], this type of study could give further insight as to how, if at all, BRC lining cells would change in response to an increased adipocytic microenvironment. This would either support or refute the
notion that closely related adipocytes could affect the lining cells by influencing their resting lineage state and promoting these cells away from an osteogenic stage and into an early adipogenic RANKL-expressing stage. By this assertion these cells would retain their function as barrier cells but also have an enhanced potential as osteoclast recruiting/supporting cells through their expression of RANKL and M-CSF. In order to support this logic, more studies are needed to identify the phenotype of these barrier cells, isolate these cells, determine their basal expression profile and assess whether or not their basal phenotype changes when placed in an adipocyte-layered 3D culture.

Investigating the adipofibroblast in the aging adipose tissue

The current model of adipogenesis during the aging process includes an asymmetric-like differentiation shift from cells that are capable of forming mature lipid-laden adipocytes to a large pool of senescent intermediate non-lipid-laden adipocyte-like cells (Figure 1-5) [90]. We identified a non-lipid laden RANKL-positive population of cells that are contained within MSC-derived adipocyte cultures. The question, therefore is not only if these RANKL-positive cells we identified exist in adipose tissue, but do these adipofibroblasts share characteristics with the intermediate preadipocyte thought to exist in the aging adipose tissue [86,90]. These data, along with the evidence
that suggests that there is redistribution of adipose tissue to sites that includes the bone marrow occurs with aging, hints towards the idea that this proposed ‘adipofibroblast’ might exist in abundance in the aging bone marrow. A study investigating whether these cells become prominent in the aging adipose tissue needs to be done. One way of doing this is identifying a candidate lineage marker for these RANKL-positive adipocyte-like cells and lineage tracing these cells in older and younger mice.

**Conclusions**

The classical view of bone remodeling involves a coupling relationship between the osteoblast and osteoclast, in which early osteoblasts express RANKL in order to support osteoclast formation, and late osteoblasts express OPG while reducing expression of RANKL to inhibit osteoclast formation. It has been reported here and by others [160,161] that there is evidence that RANKL expression may not be exclusive to the osteoblast lineage and may be shared by other early stage cell phenotypes of the MSC lineage.

In summary, RANKL is expressed in a distinct subpopulation of MSC-derived medullary adipocyte lineage cells, and these, previously uncharacterized cells, are capable of supporting osteoclast-like cell
formation in co-cultures. These data confirm earlier studies that suggest a role for medullary adipose tissue in the regulation of osteoclastogenesis and identifies a possible cellular target to further the characterization of the osteoclast support potential of medullary adipocytes \textit{in vivo}.
### Appendix

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward Sequence</th>
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<th>Tm</th>
</tr>
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<tr>
<td>Pref-1</td>
<td>CTGGACGGGTGGCCTATGAATG</td>
<td>ATCATCCAGCGAGGTGCCTC</td>
<td>55°C or 60.8°C</td>
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<tr>
<td>PPARγ2</td>
<td>ATTGACCCAGAAAGCGATTC</td>
<td>CAAAGGAGTGGGAGTGTCT</td>
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<td>C/EBPa</td>
<td>GCAAACTCACCCTGGCTATG</td>
<td>TTAGGTCAACGGCAGAGTC</td>
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<td>C/EBPδ</td>
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<td>CCAAGGTGCTCAGAGGTGCT</td>
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<tr>
<td>C/EBPβ</td>
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<td>TGCTGCGTCCTCAGAGGTGCT</td>
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<tr>
<td>Adiponectin</td>
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<td>CAGGACTCCAGGGCTTGAAGT</td>
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<td>Leptin</td>
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<td>RANKL</td>
<td>CAGCACATCAGACAGAGAAAGC</td>
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<tr>
<td>OPG</td>
<td>GAAGGGGCCTACCTTGAGAT</td>
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<tr>
<td>M-CSF</td>
<td>CAGGCCCCCTGCCCCCTTTTA</td>
<td>ACGGAGAGACGTGTGTGCTGCT</td>
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<tr>
<td>SDF-1</td>
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<td>TGCCAACTCAGGGCCGAT</td>
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<td>Hes-1</td>
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<td>GCCCGGGGTAGGTCTATGACA</td>
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<td>Krox20</td>
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<td>KLF15</td>
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<tr>
<td>COL I</td>
<td>AGCGCTGTTTCTGACTTCATCC</td>
<td>CATCCGAGGGCTGGACCTTC</td>
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<tr>
<td>RUNX2</td>
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<td>Osteocalcin</td>
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<td>β-actin</td>
<td>TGTGCCCATCTACAGGGGTATGC</td>
<td>GGTACATGCTGCGCCGAGCA</td>
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<tr>
<td>Cyclophilin A</td>
<td>CTCGTGGCCGGTTTGCAGACGCC</td>
<td>TCCTGACACCCCGGGCCAT</td>
<td>55-60°C</td>
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**Abbreviations:** C/EBPa/β/δ, CCAAT/enhancer-binding protein alpha/beta/delta; PPARγ1/2, peroxisome proliferator-activated receptor gamma 1/2; APM1, adipose most abundant gene transcript 1, also called adiponectin; RANKL, Receptor activator of Nfκb ligand; OPG, Osteoprotegerin; M-CSF, Macrophage colony stimulating factor; SDF-1, Stromal derived factor-1; Hes-1, hairy and enhance of split-1; KLFs, Krüppel-like factor 4/5/9/15; COL I, Collagen Type 1; RUNX2, Runt-related transcription factor 2

<table>
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<th>Antibody Target</th>
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<th>2° Ab dilution/Incubation time</th>
</tr>
</thead>
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<tr>
<td>RANKL</td>
<td>1:100/1-2hr</td>
<td>Alexa Fluor 488 (X-crossed)</td>
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<tr>
<td></td>
<td></td>
<td>1:750</td>
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<tr>
<td>PPARγ1/2</td>
<td>1:100/2-3hr</td>
<td>Alexa Fluor 594 (X-crossed)</td>
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<td>1:500</td>
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<tr>
<td>CD90</td>
<td>N/A /1hr</td>
<td>Alexa Fluor 594 (X-crossed)</td>
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<td>1:500</td>
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<td>CD146</td>
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<tr>
<td>CD105/SH2</td>
<td>N/A /1hr</td>
<td>Alexa Fluor 594 (X-crossed)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1:500</td>
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</table>
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