The *in vivo* effects of Rac1 and Rac2 on bone quality and aging

By

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A thesis submitted in conformity with the requirements for the degree of Masters of Science
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Abstract

Thesis title: the in vivo effects of Rac1 and Rac2 on bone architecture and mechanical properties

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Degree: Masters of Science (periodontics)

Introduction: The Rho family of small GTPases, including Rac1 and Rac2, are key regulators of osteoclast differentiation and function; however, little is known about their roles in bone quality and aging.

Methods: Male four and nine month old mice (n=10) with Rac1, Rac2 or both (DKO) isoforms deleted in osteoclast precursors were assessed using dual energy x-ray absorptiometry (DXA), scanning electron microscopy (SEM), micro computed tomography (microCT), compression, torsion and three-point bending testing, back scattered electron microscopy (BSE), Goldner’s trichrome and TRAP staining.

Results: All Rac null mice demonstrated decreased cortical structural properties and improved trabecular architecture. With age, Rac null mice demonstrated the ability to attenuate age-related bone loss.

Conclusions: Using an in vivo model with Rac1, Rac2 or both Rac isoforms deleted in osteoclasts, our findings demonstrate the deletion of Rac1 and Rac2 compromised cortical bone while improving trabecular bone properties and attenuated age-related bone loss.
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Table of Contents

Abstract ........................................................................................................... ii
Acknowledgements ........................................................................................ iii
Table of Contents ........................................................................................... iv
List of Figures ................................................................................................. vii
List of Tables ................................................................................................... viii
Awards ................................................................................................................ ix
Publications from this thesis .......................................................................... ix
List of Abbreviations ....................................................................................... x

1.0 INTRODUCTION .......................................................................................... 1

Background ...................................................................................................... 3
1.1 Bone .............................................................................................................. 3
1.2 Composition ................................................................................................ 3
1.3 Types of Bone .............................................................................................. 4
1.4 Bone Remodelling ...................................................................................... 7
1.5 Pathophysiology of bone remodelling ...................................................... 8
  1.6.1 Osteoporosis ........................................................................................... 9
  1.6.2 Hyperparathyroidism ........................................................................... 10
  1.6.3 Paget’s disease ..................................................................................... 11
  1.6.4 Osteopetrosis, osteosclerosis ............................................................ 11
1.7 Osteoclast formation and function ........................................................... 12
1.8 Actin cytoskeleton and the Rho family of Small GTPases ....................... 16
1.9 Rho, Cdc42 and Rac ................................................................................... 16
1.10 Rho GTPases and associated Human diseases ......................................... 17
1.11 Rac as a pharmacologic target ................................................................. 18
1.12 Rac1 and Rac2 .......................................................................................... 19
1.13 Rac and Osteoclasts ............................................................................... 20
1.14 Bone Quality ............................................................................................ 22
1.15 Bone Mineral Density ............................................................................. 24
1.16 Mechanical Properties ........................................................................... 24
1.17 Material Properties ............................................................................... 25
1.18 Structural Properties ............................................................................ 26
1.19 Bone Remodelling ............................................................................... 27
1.20 Mouse model .......................................................................................... 29
1.21 Hypothesis and Objectives ................................................................. 30

2.0 MATERIAL AND METHODS ................................................................. 33

2.1 Animal model ........................................................................................... 33
2.2 Experimental outline: techniques ............................................................ 35
2.3 Bone Densitometry ............................................................................... 36
2.4 Femur cortical geometry using Scanning electron microscopy ................. 38
2.5 Bone Mechanical testing ........................................................................ 38
2.5.1 Three point bending ........................................................................... 40
2.5.2 Torsion ................................................................................................ 40
2.5.3 Compression ....................................................................................... 41
2.5.4 Femoral neck fracture ....................................................................... 41
2.6 Trabecular architecture using Micro-computed tomography ........................................ 48
2.7 Static and Dynamic Histomorphometric analysis .................................................. 49
2.8 Bone Mineralization using Back-scattered electron imaging .................................. 51
2.9 Bone Connectivity using Strut Analysis .................................................................. 53
2.10 TRAP staining analysis ......................................................................................... 53
2.11 Statistical analysis ............................................................................................... 56

3.0 Results .................................................................................................................. 58
3.1 Bone mineral content is decreased in Rac2 null and Rac1 null femurs, with no differences in vertebral bone................................................................. 58
3.2 Rac deficient femurs demonstrate reduced cortical geometry compared to Wild-type mice .................................................................................................................. 62
3.3 Rac deficient femurs are smaller and demonstrate reduced structural properties, but prevents age-related loss of bone quality ....................................................... 65
3.3.2. Torsion .............................................................................................................. 69
Cortical testing summary ............................................................................................... 72
3.4 Rac deficient vertebrae demonstrate increased trabecular architecture and prevents age-related loss of bone quality .............................................................. 72
3.4.1 Micro Computed Tomography ........................................................................... 73
3.4.2 Strut analysis ...................................................................................................... 76
3.5 Rac deficient vertebrae demonstrate increased trabecular mechanical properties and attenuates age-related loss of bone quality ............................................ 79
3.5.1. Vertebral compression ...................................................................................... 79
3.5.2. Femoral neck fracture testing ........................................................................... 82
3.6 Rac deficient bone is hypermineralized, and more homogenous compared to Wild-types .................................................................................................................. 84
3.6.1 Back scattered electron microscopy................................................................. 84
3.7 Rac2 and Rac1 null mice demonstrate decreased bone turnover .............................. 87
3.7.1. Osteoclast quantification using TRAP staining .................................................. 87
3.7.2 Bone formation assessment using static histomorphometry ............................... 88
Bone turn over summary ............................................................................................... 91

4.0 Discussion .............................................................................................................. 92
4.1 The deletion of Rac1 resulted in smaller cortical bones and stronger trabecular bone......................................................................................................................... 94
4.2 The deletion of Rac1 in osteoclasts prevents age-related bone loss ......................... 97
4.3 The deletion of Rac2 compromises cortical bone structure .................................... 99
4.4 The deletion of Rac2 attenuates age-related bone loss .......................................... 101
4.5 Deletion of both Rac1 and Rac2 (Double knock-out) results in smaller cortical bones and stronger trabecular bones .............................................................. 102
4.6 The deletion of both Rac1 and Rac2 in double knock-out (DKO) mice attenuates age related bone loss .............................................................. 103
4.7 Conclusion ............................................................................................................ 103

5.0 Conclusions ......................................................................................................... 105
5.0 Thesis summary and conclusions .......................................................................... 106

6.0 Future work ......................................................................................................... 111
6.0 Future investigations ............................................................................................ 112

List of References ..................................................................................................... 113
Appendix

A) Immunoblot for Rac mouse genotyping

B) Identifying the Relative Contributions of Rac1 and Rac2 to Osteoclastogenesis. Yongqiang Wang, Dina Lebowitz, Chunxiang Sun, Herman Thang, Marc D Grynpas, and Michael Glogauer
List of Figures

Figure 1.1 Collagen fibrils.................................................................17
Figure 1.2 bone remodelling..........................................................19
Figure 1.3 Osteoclasts resorbing bone...........................................26
Figure 1.4 Bone fragility.................................................................35
Figure 1.5 Histomorphometry.......................................................40
Figure 2.1 Flow chart of experimental techniques..........................47
Figure 2.2 Dual energy x-ray absorptiometry.................................49
Figure 2.3 Representative graphs from mechanical testing............54
Figure 2.4a Three point bending....................................................55
Figure 2.4b Torsion testing.............................................................56
Figure 2.4c vertebral compression testing......................................57
Figure 2.5 Cortical bone properties: three point bending..............58
Figure 2.6 Cortical bone properties: torsion testing......................58
Figure 2.7 Trabecular bone properties: compression testing...........59
Figure 2.8 Trabecular and cortical mixed bone testing....................59
Figure 2.9 Micro computed tomography........................................60
Figure 2.10 Static histomorphometry............................................62
Figure 2.11 Back scatter electron microscopy.................................64
Figure 2.12 Strut Analysis...............................................................65
Figure 2.13 TRAP staining.............................................................67
Figure 3.1 Rac deficient mice demonstrate reduced femur bone content........73
Figure 3.2 Rac deficient femurs demonstrate reduced cortical geometry compared to Wild-type mice..............................76
Figure 3.3. Genetic deletion of Rac2 and Rac1 reduces structural properties but increased material properties.................................80
Figure 3.4. Genetic deletion of Rac2 and Rac1 reduces structural properties but increased material properties in cortical bone.............................................83
Figure 3.5 Deletion of Rac2 and Rac1 increases trabecular geometry. 87
Figure 3.6 Trabecular Connectivity................................................90
Figure 3.7. Rac deficient mice demonstrate improved structural and material properties.................................................................93
Figure 3.8. Rac deficient bone is hypermineralized compared to Wild-types........98
Figure 5.1.Rac1 and Rac2 deletion improves bone quality...............121
Figure 5.2 Attenuation of bone loss by Rac1 and Rac2.....................122
List of Tables

Table 1.1 experimental groupings and abbreviations...........................................46
Table 3.1 Genetic deletion of Rac2 and Rac1 results in reduced femur cortical Bone Mineral Content.................................................................72
Table 3.2. Rac deficient femurs demonstrate reduced cortical geometry compared to Wild-type mice.................................................................75
Table 3.3. Rac deficient femurs are smaller and demonstrate reduced structural properties but have increased material properties. ........................................82
Table 3.4 Genetic deletion of Rac2 and Rac1 reduces structural properties but increased material properties in cortical bone. ........................................79
Table 3.5 Trabecular architecture is increased in Rac2 null and Rac1 null groups compared to Wild-types. .................................................................86
Table 3.6. Trabecular connectivity.................................................................89
Table 3.7. Rac deficient mice demonstrate improved trabecular structural and material properties.................................................................92
Table 3.8. Rac1 and Rac2 are required for normal trabecular and cortical bone mechanical properties. .................................................................95
Table 3.9 All Rac deficient bones are more mineralized and uniform.....................97
Table 3.10 Rac2 and Rac1 null mice demonstrate increased quiescent bone surfaces...101
Table 3.11. Double null mice demonstrate increased osteoid parameters............102
Awards

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       University of Toronto, ON Canada

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### List of Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tbody>
<tr>
<td>BMC</td>
<td>bone mineral content</td>
</tr>
<tr>
<td>BMD</td>
<td>bone mineral density</td>
</tr>
<tr>
<td>BMP</td>
<td>bone morphogenetic protein</td>
</tr>
<tr>
<td>BMU</td>
<td>bone multicellular unit</td>
</tr>
<tr>
<td>BRONJ</td>
<td>Bisphosphonate related osteonecrosis of the jaws</td>
</tr>
<tr>
<td>BSE</td>
<td>back scattered electron</td>
</tr>
<tr>
<td>DKO</td>
<td>double knock-out</td>
</tr>
<tr>
<td>DXA</td>
<td>Dual energy x-ray absorptiometry</td>
</tr>
<tr>
<td>ES</td>
<td>eroded surface</td>
</tr>
<tr>
<td>FWHM</td>
<td>Full width at half maximum height</td>
</tr>
<tr>
<td>GEF</td>
<td>Guanine nucleotide exchange factor</td>
</tr>
<tr>
<td>IGF</td>
<td>Insulin-like growth factor</td>
</tr>
<tr>
<td>KO</td>
<td>Knockout</td>
</tr>
<tr>
<td>M-CSF</td>
<td>Macrophage colony-stimulating factor</td>
</tr>
<tr>
<td>NN</td>
<td>number of nodes</td>
</tr>
<tr>
<td>NNS</td>
<td>length of node-node struts</td>
</tr>
<tr>
<td>N.Oc</td>
<td>number of osteoclasts</td>
</tr>
<tr>
<td>O.Th.</td>
<td>osteoid thickness</td>
</tr>
<tr>
<td>OS</td>
<td>osteoid surface</td>
</tr>
<tr>
<td>OS/BS</td>
<td>osteoid surface (percentage of bone surface)</td>
</tr>
<tr>
<td>OV</td>
<td>osteoid volume</td>
</tr>
<tr>
<td>PTH</td>
<td>parathyroid hormone</td>
</tr>
<tr>
<td>RANKL</td>
<td>receptor activator for nuclear factor kappa beta- ligand</td>
</tr>
<tr>
<td>Rac1 null</td>
<td>Rac1 knock-out</td>
</tr>
<tr>
<td>Rac2 null</td>
<td>Rac2 knock-out</td>
</tr>
<tr>
<td>ROS</td>
<td>reactive oxygen species</td>
</tr>
<tr>
<td>SEM</td>
<td>scanning electron microscope</td>
</tr>
<tr>
<td>Tb.N.</td>
<td>trabecular number</td>
</tr>
<tr>
<td>Tb.Sp.</td>
<td>trabecular separation</td>
</tr>
<tr>
<td>Tb.Th.</td>
<td>trabecular thickness</td>
</tr>
<tr>
<td>TRAP</td>
<td>Tartrate-Resistant Acid Phosphatase</td>
</tr>
<tr>
<td>TBV</td>
<td>trabecular bone volume</td>
</tr>
<tr>
<td>TV</td>
<td>tissue volume</td>
</tr>
<tr>
<td>TNF</td>
<td>tumor necrosis factor</td>
</tr>
<tr>
<td>WT</td>
<td>wild-type</td>
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1.0 Introduction
1.0 INTRODUCTION

Bone is a specialized connective tissue essential for locomotion, support, and protection of internal organs. Formed by the concerted efforts of osteoblast and osteoclasts, these cells maintain bone homeostasis, a dynamic process involving the continual deposition and resorption of bone, respectively. The importance of this process is highlighted by diseases in which this balance is disturbed, resulting in the pathologic loss of bone structure as seen in osteoporosis and periodontal disease. The incidence of osteoporosis and periodontal disease increases with age, and is estimated to affect over 40% of the population over the age of 50, while costing in excess of $17 billion annually in North America alone (Canada 2004).

Currently, bisphosphonates are the most widely used antiresorptive drugs to target osteoclasts, the cells responsible for bone resorption. However, their use has been associated with side effects including osteonecrosis of the jaws after dental surgery, a condition for which there is no effective treatment (Nase and Suzuki 2006).

The targets of bisphosphonates have been identified as the Rho family of small GTPases, of which Rac is a member. Although Rac’s role has been established in the remodelling of the actin cytoskeleton in fibroblasts and the oxidative burst in neutrophils, its role in osteoclasts is largely unknown (Wang, Lebowitz et al. 2008).

There are two main Rac GTPase isoforms in osteoclasts, Rac1 and Rac2. Both isoforms share 92% amino acid homology, with the differences located in the C-terminal domain (Haeusler, Blumenstein et al. 2003; Sun, Downey et al. 2004; Fukuda, Hikita et al. 2005). 

*In vitro* experiments using dominant negative models of Rac1 have established its importance in the formation of actin rings, where its inhibition results in abnormal cell
morphology and defective bone resorption (Razzouk, Lieberherr et al. 1999; Fukuda, Hikita et al. 2005; Wang, Lebowitz et al. 2008). Further studies have determined Rac1’s crucial role in early osteoclastogenesis through the formation of reactive oxygen species (ROS) (Lee, Choi et al. 2005). However, the role of Rac2 has remained largely undefined, with some reports suggesting its redundancy (Fukuda, Hikita et al. 2005; Lee, Choi et al. 2005). Recently, the roles of Rac1 and Rac2 in vitro have been reported in early osteoclastogenesis by using a murine knock-out model. The authors report that Rac1 is the predominant isoform involved with early osteoclastogenesis, with Rac2 contributing little to the process (Wang, Lebowitz et al. 2008).

Although these studies have demonstrated the roles of Rac1 and Rac2 in early osteoclastogenesis and function, there are no current studies to examine their in vivo effects on bone quality.

Using a transgenic mouse model of Rac1 (Rac1 null), Rac2 (rac2 null), or both (Double knock out) genes deleted in osteoclast cells, our project focuses on determining the in vivo contributions of Rac1 and Rac2 to bone quality.
Background

1.1 Bone

The skeletal system serves many physiological purposes including acting as a reservoir for calcium, physical protection, and providing a framework for locomotion. Bone is a biomechanically complex material with its composition, remodelling and structural characteristics determining its structure and function.

1.2 Composition

Bone is a composite material consisting of a mineral phase embedded within an organic matrix. Bone consists of approximately 65% mineral and 35% organic matrix (Jee, 2001). The mineral component of bone is formed by hydroxyapatite crystals ($\text{Ca}_{10}$$\text{(PO}_4\text{)}_{6}$$\text{(OH)}_2$), and the organic component made up of proteins, cells and water (Kaplan et al., 1994).

The organic matrix of bone consists mainly (~ 90%) of Type I collagen, a triple helix formed from two identical $\alpha 1$ chains and one unique $\alpha 2$ chain. A ubiquitous protein, collagen consists of three polypeptide chains of approximately 1000 amino acids and is the major structural component of the bone, blood vessels and connective tissues. Each collagen molecule in bone is aligned in a quarter-staggered array to produce a collagen fibril, producing a 40nm gap, termed “hole zones” exist. The formation of mineralized matrix is speculated to occur between the ends of the molecules in these spaces. (Kaplan et al., 1994) (Figure 1-1).
1.3 Types of Bone

At a microscopic level, bone is made up of two forms: woven and lamellar. Woven bone is considered immature, newly formed bone. Woven bone is commonly found during embryology in newborns, newly formed fracture callus, and in the metaphyseal region of growing bone (Kaplan et al., 1994). Woven bone is coarse-fibered and contains no uniform orientation of collagen fibres. It exhibits more cells per unit volume than lamellar bone and its mineral content varies with its cells randomly arranged. The relatively disoriented collagen fibres of woven bone give it uniform, or
isotropic, mechanical characteristics. When a load is applied mechanically, the behaviour of woven bone is similar regardless of the orientation of the applied forces (Turner and Burr, 2001; Jee, 2001).

In contrast, lamellar bone is a more mature form of bone, which begins to form one month after birth. Lamellar bone is formed by actively replacing woven bone during the process of bone turnover (Kaplan et al., 1994). As seen in humans, the majority of skeletal bone is lamellar (Kaplan et al., 1994). Lamellar bone is thus more mature bone that results from remodelling of woven or previously existing bone. Further, collagen fibres in lamellar bone are highly organized, able to resist mechanical stresses due to the organized orientation of collagen bundles. Due to this organization, lamellar bone maintains anisotropic properties, where the mechanical behaviour can differ depending on the orientation of the applied force. That is, the greatest strength of lamellar bone is greatest parallel to the longitudinal axis of collagen fibres (Turner and Burr, 2001).

Further, lamellar bone is structurally organized into trabecular (spongy or cancellous) bone and cortical (dense or compact) bone. Cortical bone is found as the surface ‘covering’ around bones such as vertebrae, and is the predominant bone type in the diaphysis of long bones including the femur (Kaplan et al., 1994). Cortical bone is subject to bending and torsional forces as well as to compressive forces during normal function. In humans, cortical bone is organized into Haversian systems, where bone forming and resorbing cells are arranged as cylindrical units surrounding a central vascular canal (Haversian canal). These haversian canals lie parallel to the length of the bone and form concentric layers of bone during remodelling. A single cylindrical unit is known as an osteon, and numerous osteons are then organized to form cortical bone (Jee,
It is important to note that bone structure and remodelling can differ between various animals. For instance, many mammals including rodents such as mice do not possess nor remodel via Haversian systems in their cortical bones (Kalu, 1991). Thus, studies using animal models may not directly translate into identical findings in humans.

Trabecular bone is found principally at the proximal and distal ends of long bones, as well as in cuboid bones including vertebral bodies (Jee, 2001). Surrounded by cortical bone, the internal structure of trabecular bone is organized into a three-dimensional network of struts which branch and connect with one another. The organization of these struts has been found to form along the lines of stress, demonstrating the adaptive nature of bone to applied loads. Thus, bone remodelling via bone turnover can improve bone properties by increasing the organization of trabeculae (Jee and Yao 2001; Turner 2002).

Bone remodelling is a surface event, where by bone is both resorbed and deposited from the superficial surfaces inwards. Bone resorbing cells or Osteoclasts degrade bone through attachment to bone surfaces, while bone depositing cells or Osteoblasts produce immature bone in its place. Being a surface event, bone remodelling is 8 fold greater in trabecular bone due to its increased surface area as compared to cortical bone (Jee and Yao 2001). Conversely, due to a decreased rate of bone metabolism, cortical bone demonstrates four times greater bone mass as compared to trabecular bone (Jee, 2001). Due to the increased rate of bone turnover, the effects of diseases involving bone remodelling are more readily observed in trabecular bone (Turner and Burr 1993).
1.4 Bone Remodelling

Bone is constantly in a dynamic state of remodelling. This process is vital in the maintenance of structural integrity as well as to mobilize the reserves of calcium and phosphorous stored in bone. Skeletal remodelling can be initiated by changes in mechanical forces, in order to repair micro fractures, or by hormonal regulation in response to changes in systemic homeostasis. This process is a highly regulated and coordinated event involving bone forming osteoblasts cells and bone resorbing osteoclasts cells. These two cell types form the bone multicellular unit (BMU) and follow a sequence of events involving cell activation, resorption, reversal, formation, and mineralization (Figure 1-2). Abnormalities in this process can result in a variety of skeletal disorders and are the basis of diseases including osteoporosis and periodontal diseases.

Figure 1.2: Bone remodelling (Newine, 2005)

Bone remodelling commences with resorption led by osteoclast cells, characterized by their large size (20 to 100 µm in diameter) and their multiple nuclei (Kaplan et al., 1994). These cells are derived from pluripotential cells of hematopoietic origin. Activation involves a highly regulated process involving macrophage colony
stimulating factor (M-CSF) and receptor activator for nuclear factor kappa B-ligand (RANK-L) found on osteoblast surfaces. Activated osteoclasts resorb via mineral dissolution through the secretion of hydrogen ions (H+) and proteolytic digestion via intracellular enzymes (Kaplan et al., 1994).

Bone resorption causes the activation of bone forming cells, osteoblasts, initiating the **reversal phase** of bone remodelling. Osteoblasts originate from a mesenchymal lineage (Kaplan et al., 1994) and form lining cells along the resorption pit. During the bone **formation** stage, active osteoblasts deposit unmineralized bone matrix, known as osteoid (Jee, 2001). **Mineralization** stage begins as mature osteoblasts deposit mineral onto osteoid tissue which further mineralizes and matures (Jee, 2001). Osteoblasts may become trapped in mineralized bone and become regulating cells termed osteocytes, or remain as lining cells on bone surfaces.

A resting phase or **quiescence** is reached when neither formation nor resorption is occurring. The majority of bone surfaces are at this quiescent or resting stage in the bone remodelling cycle (Jee, 2001); (Kaplan et al., 1994). During the quiescent phase, additional mineralization can occur. This additional stage is known as secondary mineralization, and is responsible for the mineralization of the remaining 30% of the osteoid (Jee, 2001), which can occur over 3-6 months.

### 1.5 Pathophysiology of bone remodelling

The skeleton is a metabolically active organ which undergoes remodelling throughout life (Raisz 1999; Hadjidakis and Androulakis 2006; Sipos, Pietschmann et al. 2009). The cycles of bone resorption and deposition are tightly coupled in order to maintain structural integrity of bone, as well as to serve as a storehouse for essential minerals.
This process is highly regulated by local and systemic cytokines and hormones including estrogen, parathyroid hormone, calcitonin, vitamin D and glucocorticoids (Raisz 1999). Any abnormalities in bone homeostasis and regulation can produce a variety of skeletal and metabolic disorders.

1.6.1 Osteoporosis

Osteoporosis is a multifactorial, age-related metabolic bone disease characterized by low bone mineral density, the deterioration of the micro architecture of trabecular bone, and changes in the material properties of bone. These decreases in bone properties can leading to increased bone fragility and risk of fracture (Grynpas et al., 2000). There are two forms of osteoporosis with different etiologies: type I osteoporosis, also called postmenopausal osteoporosis, and type II osteoporosis also termed age-related osteoporosis (Marx 1980; Hadjidakis and Androulakis 2006). The loss of bone mass and strength observed in osteoporosis can be attributed to either a failure to reach peak bone mass in young adults, excessive bone resorption, or impaired bone remodelling response (Sipos, Pietschmann et al. 2009).

Type I post menopausal osteoporosis is by far the most common metabolic disorder of the skeleton, and has been linked to a deficiency of estrogen after cessation of ovarian function. The bone loss is characterized by an acceleration in bone turnover, without an increase in bone formation resulting in an over all loss of bone. Due to its increased surface area and thus bone turnover, the effects of osteoporotic bone loss is more readily observed in trabecular bone (Marx, 1980). This may lead to increased susceptibility to vertebral fractures observed in postmenopausal women.
Type II age-related osteoporosis, affects older women as well as older men. Bone loss can result in an increased risk of pathologic hip fracture, which can have a greater mortality and morbidity than type I osteoporosis (Grynpas et al., 2000). Osteoporotic bone loss continues for about 20 years, during which women lose 0.5 to 1.5 % of their peak bone mass every year (Marx, 1980). The rate of loss may eventually decrease; however, the skeleton may be significantly weakened already.

As observed in type I osteoporosis, estrogen deficiency causes an increase in bone remodelling activity characterized by increased osteoblastic and osteoclastic activity (Eastell et al., 1988). Increased osteoclastic activity leads to deeper resorption bays and can cause erosion and perforations in the trabecular struts, leading to a less connected and weaker trabecular network. Reduction in connectivity, decreases in trabecular number and thickness coupled with decreased trabecular bone volume causes a decrease in the structural integrity of the bone and leads to an increase in fracture risk (Parfitt, 1987).

1.6.2 Hyperparathyroidism

Systemic regulation of bone metabolism is controlled by various hormones affecting the resorption and excretion of calcium, including parathyroid hormone (PTH). PTH regulates serum calcium concentration and is a potent stimulator of bone resorption. However, both PTH and thyroid hormones have been observed to stimulate bone formation as well as bone resorption. Further, studies examining the administration of PTH intermittently have demonstrated bone formation, indicating possible anabolic properties of PTH (Marx 1980; Jee and Yao 2001).
1.6.3 Paget’s disease

The deregulation of bone homeostasis can be observed in Paget’s disease of bone, where osteoclastic resorption and osteoblastic deposition of bone has been abnormally activated (Sipos, Pietschmann et al. 2009). The result is the formation of irregular new bone which results in an increase in bone density. However, due to its irregular architecture, bone strength is decreased and pathologic fractures may occur (Turner, Hsieh et al. 2000). Further, a genetic component to Paget’s disease may also increase these patients’ risk for osteosarcoma.

1.6.4 Osteopetrosis, osteosclerosis

In contrast to a loss of bone density due to a decrease in bone turnover, reduced bone remodelling can also lead to osteopetrosis and osteosclerosis, conditions in which an increase in bone density is observed due defective bone resorption (Sipos, Pietschmann et al. 2009). Despite increased bone density, these bones become excessively hard but brittle, resulting in an increased susceptibility to fracture (McLean and Olsen 2001).

The importance of normal bone turnover and remodelling is emphasized by these systemic diseases of bone metabolism. Osteoclasts are essential for the normal bone formation and function, where any defect in osteoclast function can result in increased bone fragility and fracture.
1.7 Osteoclast formation and function

Osteoclasts are bone resorptive cells derived from hematopoietic monocyte/macrophage precursors found in blood serum (Boyle, Simonet et al. 2003). Cell formation termed Osteoclastogenesis involves fusion and differentiation of these precursor cells under the regulation of two main cytokines: macrophage colony stimulating factor (M-CSF) and receptor activator for nuclear factor kappa B-ligand (RANK-L) (Boyle, Simonet et al. 2003). Both cytokines are required and together, are sufficient to induce osteoclastogenesis in vitro (Boyle, Simonet et al. 2003; Tanaka, Nakamura et al. 2003). M-CSF, a polypeptide growth factor and RANK-L, a Tumor necrosis factor (TNF) related cytokine, are produced by bone marrow mesenchymal cells including osteoblasts. Their interaction with RANK receptor expressed on hematopoietic precursor cell surfaces initiates the activation of osteoclast cell differentiation, leading to the development of mature osteoclasts (Boyle, Simonet et al. 2003). This intimate relationship has been well documented (Wang, Lebowitz et al. 2008; Wang, Belsham et al. 2009) and can be blocked by the addition of soluble Osteoprotegrin (OPG) which acts as a decoy receptor blocking RANKL binding to RANK (Boyle, Simonet et al. 2003). Animal studies targeting the disruption of the RANK/RANKL/OPG interaction have resulted in severe osteopetrosis (Tanaka, Nakamura et al. 2003), highlighting its importance in normal osteoclast differentiation. To further emphasize this relationship, it has been demonstrated that fully differentiated osteoclasts will undergo rapid apoptosis in the absence of supporting cells such as osteoblasts or bone marrow cells (Tanaka,
Nakamura et al. 2003). Osteoblasts expressing RANKL are essential for the
differentiation and survival of osteoclasts.

Osteoclasts are characterized by their relatively large size (20 to 100 µm), the presence
of multiple nuclei, and are located in resorptive bays on bone surfaces termed Howships
lacunae (Figure 1.3) (Kaplan et al., 1994).
Figure 1.3: Osteoclasts resorbing bone. Multinucleated giant osteoclast cells (arrows) are observed resorbing bone (tartrate resistant acid phosphatase (TRAP) stain, decalcified specimen 100x mag)
Following fusion and differentiation, osteoclasts migrate and polarize onto bone surfaces, a process which requires intimate physical contact with bone matrix (Boyle, Simonet et al. 2003). Cell attachment to bone surfaces are mediated by integrins via membrane protrusions called podosomes (Faccio, Novack et al. 2003). The formation of these cell extensions aids in the formation of a characteristic region called the Sealing zone, which involves the remodelling of F-actin in the cytoskeleton to form a ring structure (Chambers 2000). Within the sealing zone, the formation of the ruffled border enables tight bone contact, as well as to allow the extracellular transport of intracellular vesicles across the cell membrane. After osteoclast fusion and attachment onto bone surfaces, activated osteoclasts resorb bone via acidification and proteolysis of the region bound by the ruffled border (Chambers 2000; Boyle, Simonet et al. 2003). This zone allows the isolation of a region of bone through which a gradient of hydrogen ions (H+) and proteolytic enzymes are excreted to degrade hydroxyapatite crystals and bone mineral matrix. Further resorption of collagen fibers within bone matrix is mediated by the release of lyzosomal cathepsins and collagenases across the cell membrane into the ruffled border (Kaplan et al., 1994).

Cellular processes including the differentiation, fusion of precursor cells, and bone resorption, forms a series of complex events necessary for the formation and function of osteoclasts. These processes include cell fusion, attachment, movement, ruffled border and sealing zone formation, all of which require the remodelling of the cytoskeleton. It is readily apparent that any alteration to normal cytoskeleton remodelling can affect the formation and function of the osteoclast, and thus affect normal bone remodelling.
1.8 Actin cytoskeleton and the Rho family of Small GTPases

Cellular structure and shape are determined by the formation and remodelling of the cytoskeleton. Composed of an array of actin filaments (F-actin), the cytoskeleton mediates a number of crucial cell functions including providing a structural framework, motility and cell division (Hall 1998). These functions are possible due to the dynamic nature of F-actin, capable of elongating and severing during a process called tread milling (Hall 1998). This process begins with the uncapping of an actin barbed-end and the addition of actin monomers, leading to rapid actin polymerization required during lamellipodium formation, cell movement and fusion (Wherlock and Mellor 2002). This process is mediated by a family of molecular switch proteins called the Rho family of small GTPases. Within the family of Rho GTPases are three main members: Rho, Cdc42 and Rac. Not surprisingly, significant cross-talk between the members of the Rho GTPase super family has been reported (Hall 1998).

1.9 Rho, Cdc42 and Rac

Part of the Ras super family of GTPases, Rho GTPases function by switching between active GTP-bound states and inactive GDP-bound states (Wherlock and Mellor 2002), mediated by guanine nucleotide exchange factors (GEFs) (Haeusler, Blumenenstein et al. 2003) and GTPase activating proteins (GAPs) (Rivero and Somesh 2002). In unstimulated cells, inactive or GDP-bound GTPases, remain in the cytoplasm bound to Guanine nucleotide dissociation inhibitors (GDIs) (Takai, Sasaki et al. 2001). Upon stimulation, Rho GTPases dissociate from GDIs and translocate to cytoplasmic membranes where GEFs mediate the exchange of GDP with GTP, rendering them active.
Activation results in a conformational change that allows the GTPase to activate other downstream signal transduction molecules (Hall 1998; Yamauchi, Marchal et al. 2005).

Rho, the first to be studied was shown to activate contractile actin-myosin filaments in fibroblasts, while Cdc42 was found to induce actin-rich surface protrusions called filopodia. Rac has been extensively studied in fibroblasts where its importance has been demonstrated in the assembly of F-actin at the cell periphery, producing lamellipodia and membrane ruffling (Hall 1998; Wherlock and Mellor 2002; Yamauchi, Marchal et al. 2005). Further, studies have shown that the inhibition of Rac GTPase results in the blockage of actin surface extensions, demonstrating the importance of Rac in lamellipodia formation (Hall 1998). It is apparent that Rac is a crucial member of the Rho GTPase family, required for normal remodelling of the actin cytoskeleton.

1.10 Rho GTPases and associated Human diseases

The Rho family of small GTPases has been studied in the context of disease development due to its crucial role in cell polarization, cell to cell adhesion and cytoskeletal regulation, all of which are involved with the migration, invasion and metastasis of human diseases and cancers (Boettner and Van Aelst 2002). Further, over expression of Rac1 isoform has been described in colorectal tumors, indicating a possible oncogenic link (Jordan, Brazao et al. 1999). Other molecular studies have implicated Rac isoforms with the spread of metastatic breast cancer (Mira, Benard et al. 2000), proliferation of lymphoma/leukemia (Habets, Scholtes et al. 1994) and neuroblastoma (Yan, Chen et al. 2008).
The ubiquitous expression of Rac and its implication in a variety of diseases highlights the need to further investigate the role of Rac isoforms in the hopes of understanding the pathogenesis and spread of human diseases.

1.11 Rac as a pharmacologic target

The deregulation of bone homeostasis is the basis of diseases including osteoporosis and periodontal disease. These diseases are characterized by imbalanced osteoblastic bone deposition and osteoclastic bone resorption. The treatment of osteoporosis, in addition to various other metastatic bone cancers, focuses on the use of bisphosphonates to target osteoclast survival.

Bisphosphonates are pyrophosphate analogues which are potent inhibitors of osteoclastic bone resorption. These pyrophosphate analogues have the ability to bind divalent metal ions including calcium in bone (Roelofs, Thompson et al. 2006). During osteoclastic bone resorption, bisphosphonates enter osteoclasts through endocytosis where osteoclastic activity is suppressed and apoptosis is induced. These drugs, and the more potent nitrogen containing bisphosphonates, target the Mevalonate pathway inhibiting protein modification, specifically post-translational prenylation. This process involves the attachment of hydrophobic isoprenoid lipid groups (geranlygeranyl and farnesyl) to the C-terminal domain of molecules. These prenylated molecules are essential for the association of small GTPases to the cell membrane, where the exchange of GTP is thought to occur (Coxon and Rogers 2003; Roelofs, Thompson et al. 2006).

Recent studies have identified the targets of these drugs to include the Rho family of small of GTPases, of which Rac is a member (Reszka and Rodan 2004; Dunford, Rogers
et al. 2006). Bisphosphonate therapy has been reported to reduced bone turnover by 80-
90%, and cause a gain in bone mineralization (Turner 2002; Roelofs, Thompson et al. 2006), resulting in increased bone properties including bone rigidity. Although the targets of the drugs are known, very little is actually know about these molecules. Further, patients taking high doses of bisphosphonates for metastatic cancers are at high risk for Bisphosphonate related osteonecrosis of the jaws (BRONJ) after routine dental surgery (Ruggiero and Drew 2007). Currently, there is no known treatment for this condition.

Through a better understanding of the molecules involved with osteoclast function and survival, further targeted pharmacologic interventions can be elucidated in order to minimize side effects and co-morbidities associated with current drugs (Fukuda, Hikita et al. 2005).

1.12 Rac1 and Rac2

There are three main isoforms of Rac described in mammals: Rac1, Rac2 and Rac3, of which Rac1 is ubiquitously expressed, Rac2 is expressed mainly in haematopoietic cells (Haeusler, Blumenstein et al. 2003), and Rac3 is expressed mainly in brain tissues (Haataja, Groffen et al. 1997). As osteoclasts are derived from haematopoietic cells, our discussion will be limited to Rac1 and Rac2, which share 92% amino acid homology (Glogauer, Marchal et al. 2003; Koh, Sun et al. 2005). Rac 1, the predominant isoform, has been reported to be primarily responsible for the rearrangement of the actin cytoskeleton (Hall 1998; Wherlock and Mellor 2002), where as Rac 2 has been found to be responsible for the regulation of the oxidative burst in neutrophils, as
well as contributing to cell chemotaxis (Glogauer, Marchal et al. 2003; Sun, Downey et al. 2004; Sun, Magalhaes et al. 2007).

Rac1 and Rac2 contain identical GEF binding effector domains at amino acids 26-45 (Yamauchi, Marchal et al. 2005); however, association-dissociation affinities have been reported to be six-fold greater in Rac2 (Haeusler, Blumenstein et al. 2003). This difference has been attributed to the C-terminal domains of Rac1 and Rac2, where the greatest divergence in amino acid sequence is observed (Glogauer, Marchal et al. 2003; Yamauchi, Marchal et al. 2005; Sun, Magalhaes et al. 2007).

Due to the high degree of homology between Rac1 and Rac2, it remains unclear how each Rac isoform independently functions, and whether both isoforms are in fact required for normal cell function (Fukuda, Hikita et al. 2005). Further, with few studies investigating the roles of Rac1 and Rac2 in osteoclasts, their function during osteoclastogenesis and their impact on bone formation are still relatively unknown.

1.13 Rac and Osteoclasts

Actin cytoskeletal remodelling is imperative in the formation and function of multinucleated osteoclast. Using in vitro models studying osteoclast function, it has been established that dominant negative inhibition of Rac1 results in reduced F-actin reorganization in podosome formation (Ory, Munari-Silem et al. 2000), resulting in rounded cells with disrupted actin rings (Razzouk, Lieberherr et al. 1999). In addition to abnormal cell morphology, other studies have found Rac1 deficient osteoclasts to be functionally defective when subjected to resorption assays on dentine slices (Razzouk, Lieberherr et al. 1999; Wang, Lebowitz et al. 2008). Further, Rac1 has been identified to
regulate early osteoclastogenesis (Razzouk, Lieberherr et al. 1999; Wang, Lebowitz et al. 2008), as well as cell apoptosis through M-CSF signalling (Fukuda, Hikita et al. 2005). Rac1’s role in intracellular vesicle transport, an essential process in osteoclastic bone resorption has also been reported (Sun, Buki et al. 2005).

The role of Rac2 has been described in neutrophils where it has been shown to be vital in the production of reactive oxygen species (ROS) through the assembly of the NADPH oxidase complex (Lee, Choi et al. 2005; Yamauchi, Marchal et al. 2005). Further, studies by Lee et al have demonstrated that the production of ROS by osteoclast precursors is linked to early osteoclastogenesis (Lee, Choi et al. 2005; Yamauchi, Marchal et al. 2005). However in osteoclastogenesis, this ROS production has been attributed to the Rac1 isoform rather than Rac2, as in monocytes such as neutrophils. This finding that Rac1 and not Rac2 as the predominant isoform in the activation of the NADPH oxidase complex is also echoed by Zhao et al (Zhao, Carnevale et al. 2003). Although the importance of Rac1 in dynamic actin remodelling and early osteoclastogenesis has been established, the role of Rac2 in these processes is still relatively unknown.

Studies attempting to decipher their individual contributions have focused on the use of dominant negative models of both Rac1 and Rac2. These studies have concluded that due to their high amino acid homology, Rac2 is redundant and not necessary for normal osteoclast function (Razzouk, Lieberherr et al. 1999; Fukuda, Hikita et al. 2005; Lee, Choi et al. 2005; Sun, Buki et al. 2005). Conversely, it has also been suggested that due to the 92% homology, the use of standard dominant negative mutants in the study of Rac1 and Rac2 may in fact mask both isoforms, rendering it impossible to determine their individual contributions (Sun, Downey et al. 2004).
Further, the use of RAW cell lines in the study of osteoclastogenesis may pose additional difficulties in the study of Rac1 and Rac2. One study reported a wide degree of heterogeneity within groups of RAW cell clones, with only select cells able to form morphologically normal osteoclasts (Cuetara, Crotti et al. 2006). Finally, all previous studies reported *in vitro* findings, with a dearth of studies reporting the *in vivo* effects of osteoclasts deficient in Rac1 and Rac2 (Wang, Lebowitz et al. 2008).

Recently, the roles of Rac1 and Rac2 have been reported in early osteoclastogenesis using a murine knock-out model. Using a conditional knock-out of Rac1, Rac2 and both isoforms in haematopoietic cell lines, *in vitro* analysis of M-CSF and RANKL induced osteoclastogenesis was examined. The authors report that Rac1 is the predominant isoform involved with early osteoclastogenesis, with Rac2 contributing little to the process (Wang, Lebowitz et al. 2008).

Although these studies have demonstrated the roles of Rac1 and Rac2 in osteoclastogenesis and osteoclast function, there are no studies to examine their in vivo effects on bone quality.

### 1.14 Bone Quality

Bone quality is determined by its mechanical, material and structural properties, all regulated by the bone remodelling process. Previous methods to evaluate bone mass in humans relied on bone mineral density measurements (BMD) using dual energy x-ray absorptiometry (DXA). Although a relatively quick and non-invasive method of determining bone mass, recent studies have implied that DXA is an insufficient means to predict the risk of bone fracture (Heaney 1993; Jepsen, Akkus et al. 2003). It has been
suggested that combining bone mineral density with underlying material and structural properties be used in determining the quality of bone (Figure 1-4)

Figure 1.4: Relationship between bone fragility and various bone properties (adapted from Cowin SC. Bone mechanics handbook, 2001)
As indicated in Figure 1-4, bone remodelling is the underlying process that determines the structural and material properties of bone, which affects the overall fragility of bone.

1.15 Bone Mineral Density

Bone mineral density (BMD) as measured by Dual Energy X-Ray Absorptiometry (DXA) is a measure of the amount of bone mineral density projected in a given area. This represents a relatively non-invasive, quantitative measurement of bone mass as an alternative to more invasive techniques, including bone biopsies. It is of note that BMD is a two dimensional parameter, and may not be representative of the density differences in all dimensions of a particular bone.

DXA is the most common method of assessing BMD, as studies have demonstrated that a decrease in BMD is correlated to the risk of fracture in osteoporotic women (Council of the National Osteoporosis Foundation, 1996). In the research environment, small animal DXA machines are commercially available to examine entire rat or mouse specimens, as well as excised bones (Nagy, Prince et al. 2001)

1.16 Mechanical Properties

Bone fragility or mechanical properties, are important parameters in assessing bone quality as it is directly related to fracture risk. However, mechanical properties can not be determined without the irreversible, non-repeatable fracture testing of the bone. The mechanical testing of bone is invasive and therefore can only be performed using animal models.
Mature bone demonstrates organization and can remodel in response to stress (Turner and Burr 1993). This directional organization of bone is called anisotropy, and gives bone the ability to withstand loading differently depending on the direction of force application. In order to assess mechanical properties of a given bone, anisotropy must be taken into account by loading the specimen in directions relevant to its normal physiologic loading. As such, a variety of mechanical tests are performed on various bone sites to generate a broad overview of skeletal fragility.

Three-point bending tests are performed with the load applied perpendicular to the long bones in both tension and compression. Compression testing is typically performed on trabecular bone found in vertebral bodies of the spine, due to their relatively large size and uniform geometry. Torsion tests can be performed to test the behaviour of cortical bone subjected to a shearing type force. From these mechanical tests, the failure load, deformation, energy to failure and stiffness of bone are determined. Normalizing these data using the geometry of the bone provides information about the material properties including stress, strain, elastic modulus and toughness.

1.17 Material Properties

The composition of bone determines the material properties of bone, and can be described in terms of its mineral and organic phases. As bone is composed of 65% mineral and 35% organic (Jee and Yao 2001), the contribution of the mineral phase to mechanical properties has traditionally held more importance than the organic phase in the study of bone material characteristics (Burr, 2002). The quality of the mineral phase can be assessed by determining the degree of mineralization and its distribution.
Back scattered electron imaging (BSE) is a method to obtain a mineralization profile. The bone surfaces are scanned using an electron microscope where the incident beam of electrons is deflected by the constituent atoms in the bone. The degree of deflection depends on the atomic weight of the atom and the intensity of detected electrons is related to the atomic number of the constituents. Since calcium has the heaviest nuclei, the intensity of detected electrons is related to calcium concentration and therefore, the degree of mineralization (Boyde and Stewart 1963).

1.18 Structural Properties

Not only is the amount and quality of bone mass important in defining mechanical integrity, but fragility also is dependent on how the bone is geometrically organized and arranged. Structural properties of cortical bone, specifically in shafts of long bones, include cortical thickness, cross-sectional area and moment of inertia. Typically, the thicker and larger the cross-sectional area of a bone, the more load is required to fracture (Turner and Burr, 1993).

With regards to trabecular bone, the micro architecture contributes to the structural properties of bone. The number, thickness and separation of individual trabeculae, as well as their relative connectivity contribute to their fracture resistance. For example, an increased number of thicker, less separated, and highly connected trabeculae are associated with higher failure loads (McCalden, McGeough et al. 1997). Disconnectivity or thinning and perforation of trabeculae cause the weakening of the trabecular network, a process observed in osteoporosis.

Strut analysis is used to measure trabecular connectivity, which is determined from a two dimensional skeletonized image of trabecular bone. Strut analysis determines
the number of nodes, or connections between separate trabeculae, and the number of free ends or disconnections (Garrahan, Mellish et al. 1986). The loss of connectivity can be seen by a decrease in the number of nodes and an increase in the number of free ends (Compston, Garrahan et al. 1993)

### 1.19 Bone Remodelling

Bone turnover in the process of remodelling can be determined using Histomorphometry, a method of quantifying bone formation parameters as well as structural properties. Histomorphometric analysis using undecalcified samples stained using Goldner’s trichrome allows the differentiation between mineralized bone and newly formed bone (osteoid) (Figure 1.5). Formation is determined by the measurement of osteoid volume, surface and thickness (Parfitt et al., 1987). Analyzing formation parameters allows an overall assessment of bone remodelling.
Figure 1.5: Histomorphometry. Goldner’s trichrome staining of undecalcified sections allows the identification of calcified (green) and newly formed osteoid (red, arrows). 25x magnification
1.20 Mouse model

The use of transgenic animal models has allowed the study of a variety of diseases through selective gene mutations. Advances in genetic technology have allowed the generation of mouse knock-out models to manipulate individual, or multiple genes in order to facilitate their study (McLean and Olsen 2001). Further, mice share 60-70% genetic homology with humans, and are thus a useful model in the study of genetic diseases affecting humans (Rosen, Beamer et al. 2001). With respect to Rac GTPases, the amino acid sequence of murine Rac1 and human Rac1 are reported to be identical. In addition, murine Rac2 differs from human Rac2 by only two amino acids, an aspartate/glutamate substitution at position 148 and proline/alanine at position 188 (Yamauchi, Marchal et al. 2005).

However, despite these striking similarities, obvious and subtle differences exists, which may limit the extent to which animal studies can translate into human application. For instance, mice do not remodel via Haversian systems in cortical bones (Jee and Yao 2001) Further, the significant overall size differences between both models may pose technical difficulties as assays and techniques used to assess bone quality in mice and humans differ significantly. Despite these limitations, the use of knock-out mice models allows the advancement of studies beyond in vitro cell cultures to determine the overall effect in vivo.

The development of a knock-out mouse model lacking in either Rac1 (Rac1 null) Rac2 (Rac 2 null) or both Rac1 and Rac2 (double knock-out, DKO) has allowed us to study the individual and specific roles of these molecules in osteoclasts, and their role on in vivo
bone properties. BL6/129 knock-out mice were generated as previously studied and described (Roberts, Kim et al. 1999; Sun, Downey et al. 2004). Briefly, Rac isoforms were selectively deleted in myeloid cells, including osteoclast precursors, by mating Rac2 null mice (Rac2\(^{-/-}\)) (Roberts, Kim et al. 1999) with conditional Rac1 null mice expressing Cre-recombinase downstream of a myeloid promoter, lysozyme M (Rac1\(^{c/-}\)LysM\(^{cre}\)) (Glogauer, Marchal et al. 2003; Sun, Downey et al. 2004). The resulting offspring were subsequently bred over at least 6 generations to create optimal breeding pairs (Rac1\(^{c/-}\)LysM\(^{cre}\)Rac2\(^{+/+}\) x Rac1\(^{c/-}\)LysM\(^{cre}\)Rac2\(^{+/+}\)), which enabled the generation of Rac1null (Rac1\(^{c/-}\)LysM\(^{cre}\)Rac2\(^{+/+}\)), Rac2null (Rac1\(^{+/+}\)LysM\(^{cre}\)Rac2\(^{+/+}\)), DKO (Rac1\(^{c/-}\)LysM\(^{cre}\)Rac2\(^{-/-}\)), and wild-type mice (Rac1\(^{+/+}\)LysM\(^{cre}\)Rac2\(^{+/+}\)) from the same litters. This breeding strategy allows for the controlling of background variations both between and within subject groups. Using this mouse model of Rac1 (Rac1 null), Rac2 (rac2 null), or both (Rac1/2 null) genes deleted in osteoclast cells, our project focuses on determining the in vivo contributions of Rac1 and Rac2 to bone architecture and fragility. Further, the use of young and aged cohorts allows us to determine the effects of aging on bone quality.

1.2.1 Hypothesis and Objectives

Using a transgenic mouse model with Rac1 (Rac1 null), Rac2 (rac2 null), or both (DKO) genes deleted in osteoclast cells, our project focuses on determining the in vivo contributions of Rac1 and Rac2 to bone quality.

Null hypothesis: The are no differences in bone quality between Wild-type controls and Rac deficient mice.
The specific objectives of the study are:

i. To determine the effects of the deletion of Rac 1 and Rac2 in osteoclasts on bone mass, geometry and structure.

ii. To determine the mechanical properties of Rac1 and Rac2 deficient bones.

iii. To determine osteoclast and bone formational parameters in Rac1 and Rac2 deficient bone.

iv. To determine the effects of age in Rac1 and Rac2 deficient mice in vivo.
2.0 MATERIALS AND METHODS
2.0 MATERIAL AND METHODS

2.1 Animal model

All procedures were carried out in accordance with the ‘Guide for the Humane Use and Care of Laboratory Animals’ and were approved by the University of Toronto Animal Care Committee. B6/129 knock-out mice were generated as previously studied and described to selectively delete Rac1 (Rac1null), Rac2 (Rac2null), or both Rac1 and Rac2 (DKO) (Roberts, Kim et al. 1999; Sun, Downey et al. 2004). Rac isoforms were selectively deleted in myeloid cells, including osteoclast precursors, by mating Rac2 null mice (Rac2^{-/-}) (Roberts, Kim et al. 1999) with conditional Rac1 null mice expressing Cre-recombinase downstream of a myeloid promoter, lysozyme M (Rac1^{cre/LysM^{cre}}) (Glogauer, Marchal et al. 2003; Sun, Downey et al. 2004). The resulting offspring were subsequently bred over at least 6 generations to create optimal breeding pairs (Rac1^{cre/LysM^{cre}Rac2^{-/-}} x Rac1^{cre/LysM^{cre}Rac2^{+/+}}), which enabled the generation of Rac1null (Rac1^{cre/LysM^{cre}Rac2^{+/+}}), Rac2null (Rac1^{cre/LysM^{cre}Rac2^{+/+}}), DKO (Rac1^{cre/LysM^{cre}Rac2^{-/-}}), and wild-type mice (Rac1^{+/+}LysM^{cre} Rac2^{+/+}) from the same litters, as previously described (Glogauer, Marchal et al. 2003; Sun, Downey et al. 2004; Wang, Lebowitz et al. 2008). This breeding strategy allows for the controlling of background variations both between and within subject groups.

Genotyping for Rac1, Rac2, and LysM alleles were confirmed using RT-PCR and immunoblotting as previously described (Roberts, Kim et al. 1999; Glogauer, Marchal et al. 2003; Sun, Downey et al. 2004).

All Experiments were performed on male mice in order to minimize sex and cyclic hormonal fluctuations that may affect bone turnover. A total of 46 healthy male four
month old male mice (11 wild type, 12 Rac1 KO, 11 Rac2 KO and 12 DKO) were studied to establish a baseline cohort. A second cohort of 46 healthy, adult 9 month old male mice (11 wild type, 13 Rac1 KO, 11 Rac2 KO and 10 DKO) were studied to determine the effects of aging on Rac deficient mice (Table 1-1).

Table 1.1: experimental grouping and abbreviations

<table>
<thead>
<tr>
<th>Age</th>
<th>Group</th>
<th>Abbreviation</th>
<th>Number of mice</th>
</tr>
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<tbody>
<tr>
<td>4 month</td>
<td>Wild type</td>
<td>4WT</td>
<td>11</td>
</tr>
<tr>
<td></td>
<td>Rac2 null</td>
<td>4Rac2</td>
<td>12</td>
</tr>
<tr>
<td></td>
<td>Rac1 null</td>
<td>4Rac1</td>
<td>11</td>
</tr>
<tr>
<td></td>
<td>Double knock-out</td>
<td>4DKO</td>
<td>12</td>
</tr>
<tr>
<td>9 month</td>
<td>Wild Type</td>
<td>9WT</td>
<td>11</td>
</tr>
<tr>
<td></td>
<td>Rac2 null</td>
<td>9Rac2</td>
<td>13</td>
</tr>
<tr>
<td></td>
<td>Rac1 null</td>
<td>9Rac1</td>
<td>11</td>
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<tr>
<td></td>
<td>Double knock-out</td>
<td>9DKO</td>
<td>10</td>
</tr>
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</table>

All animals where phenotypically healthy, with no overt signs of pathology. Any unhealthy or diseased mice were eliminated from the study. All animals were housed and maintained under identical conditions, with unlimited access to food and water. Sterile conditions were maintained and daily monitoring was performed at the Division of Comparative Medicine, University of Toronto.

Ten and two days prior to euthanasia, intra-peritoneal injections with Oxy-tetracycline (30mg/Kg) were performed as a dynamic bone marker for subsequent histomorphometric analysis (Tam and Anderson 1980; Hori, Takahashi et al. 1985). All mice were
humanely sacrificed using CO₂ asphyxiation and cervical dislocation at exactly the specified times of four and nine months of age. All animals were maintained on ice and immediately analyzed.

2.2 Experimental outline: techniques

Experiments were performed using the techniques as outlined in Figure 2.1. All samples were handled and stored in the same manner, and analyses were performed using a semi-blinded coding system.

Figure 2.1. Flow chart of experimental techniques utilized to assess bone quality
2.3 Bone Densitometry

Dual energy x-ray absorptiometry (DXA) was performed using a small animal PIXImus densitometer (Lunar; GE Corporation, London ON, Canada) for entire animal cadavers immediately after sacrifice and for isolated femur and lumbar vertebras after dissection. Bone mineral density (BMD), and bone mineral content (BMC) were analyzed for whole body scans and isolated bones (figure 2.2).

The DXA densitometer employs a cone beam x-ray source that generates two energies: 35 (low) and 80 (high) KeV. Incident photons transversing tissue are either absorbed or scattered, depending on their attenuation properties. To distinguish bone mineral, a threshold value is calibrated and set by the PIXImus to distinguish between areas that are considered bone and those that are soft tissue. Calibration using an aluminum/lucite phantom (Lunar, GE Corporation, London ON, Canada) was completed and a quality control scan was passed prior to each scanning session. The amount of bone mineral content (g) is then divided by the projected bone area (cm$^2$) to give an areal bone mineral density (g/cm$^2$) measurement (Griffin, Kimble et al. 1993; Ladizesky, Zeni et al. 1994). Dissected right femurs and lumbar vertebrae 4-6 were placed individually on a polystyrene plate to simulate soft tissue thickness, and scanned. Data were analyzed with uniform regions of interest selected to maintain consistency between samples.

The PIXImus densitometer was not available at the time of sacrifice for the 9 month group, thus comparisons between age cohorts were made using dissected femurs and vertebrae to determine the effects of age on bone mineral properties.
Figure 2.2: DXA scan of entire body (a) and isolated bones (b). Uniform regions of interest have been selected to isolate only the femur or vertebrae.
2.4 Femur cortical geometry using Scanning electron microscopy

Femur geometry was determined using isolated right femurs. Cross sectional cuts were made at femur mid-shafts using an Isomet precision saw (Buehler, Lake Bluff, IL USA) and embedded in epoxy resin (Buehler, Lake Bluff, IL USA). Femurs were carbon coated for scanning electron microscopy (FEI XL300E SEM, FEI Company, Hillsboro, OR, USA) and scanned at 120x magnification, spot size 7, 20kV with a 15mm working distance. Images were analyzed using ImageJ software (National Institutes of Health, Bethesda MD, USA) to determine femur cortical bone parameters including cross sectional thickness (cTh), length (medial-lateral diameter), width (anterior-posterior diameter) and moment of inertia (I).

2.5 Bone Mechanical testing

Fracture testing under mechanical loading was performed on mouse femurs and vertebrae to determine cortical and trabecular properties respectively. Cortical properties were determined using dissected femurs and trabecular bone properties were determined using the 6th lumbar vertebrae, the largest vertebral body. Analysis of combined cortical-trabecular bone was completed using the proximal ends of femurs which contain the femoral head and neck to simulate a clinically relevant ‘hip fracture’ under controlled conditions. Mechanical testing was performed using a materials testing machine, Instron 4465 (Instron Canada Inc.) fitted with a 100N load cell. Right femurs were pre-loaded with
less than 1 N to establish primary contact with the load cell and subsequently fractured at a speed of 1 mm/min.

Torsion assays were performed using a torsion apparatus fitted with a 100N load cell, with left femurs tested to failure at a speed of one degree per second (Turner and Burr 1993; Turner 2002).

Load (N) versus time (sec) data were collected every 0.1 seconds via LabView data acquisition software (National Instruments Corp.; Austin, TX) until sample fracture. A load-displacement curve was generated for every sample tested (see Figure 2.3a). The slope of the linear region of this curve is termed the stiffness or rigidity ($S; \text{N/m}$), and represents the elastic behaviour of the sample: under loading, the specimen will return original shape when removed. Under further load, the sample undergoes irreversible transformation and enters the plastic region, where the sample does not return to its original shape when the load is removed. This transition point is termed the yield point.

The Ultimate load ($F_u; \text{N}$) represents the maximum force applied to the specimen, while failure load ($F_f; \text{N}$) represents the force at which the specimen undergoes failure or fracture. The summation of the entire area under the load-displacement curve, up to the point of failure, is termed the energy to failure ($U_f; \text{mJ}$), and represents the amount of energy required to cause failure (Figure 2.3b). All of the above parameters determined from the load-displacement curve provide a comprehensive view of the mechanical integrity of bone (Turner and Burr 1993; Turner 2002).

To further evaluate the mechanical properties of bone, geometric factors were mathematically removed through data normalization to determine the material properties of each sample. The Load-displacement curve can be transformed or normalized into a
stress-strain graph using standard engineering formulae (Figure 2.4). Normalization produces analogous mechanical parameters, where the ultimate and failure force are represented by the ultimate ($\sigma_u$; MPa) and failure stress ($\sigma_f$; MPa), respectively and the slope or stiffness is termed the elastic or Young’s modulus ($E$; MPa). Finally, the area under the stress-strain curve is termed toughness ($U_f$; J/mm$^3$), which represents the amount of energy required to cause failure per unit volume of bone.

Due to the complex shape and organization of trabecular and cortical bone, several different mechanical tests were performed. Cortical properties were determined using both femurs, where right femurs were used in three-point bending assays, and left femurs for torsion analysis. Trabecular properties were determined using vertebral body compression assay and femoral neck fracture testing.

2.5.1. Three point bending
Three-point bending using femurs applies both tension and compression at the point of application, in this case, the midpoint (Figure 2.5). As the mid shaft of the femur is composed of entirely cortical bone, a gauge length of 6mm (or middle 40% of the femur) was used to isolate cortical bone and remove any influence of the mixed cortical-trabecular composition of the proximal and distal ends. Although providing valuable information regarding the cortical properties of the femur, three point bending only tests the isolated length of the femur at the mid shaft, and is not representative of the mechanical properties of the entire femur length (Turner and Burr 1993; Turner 2002).

2.5.2. Torsion
Torsion testing represents a mechanical test that evaluates the shear behaviour of cortical bone applied along the entire length of the femur (Figure 2.6). This assay allows
the failure to occur at the weakest point along the entire cortical bone shaft and may provide clinically relevant information regarding the true nature of the mechanical properties of the femur (Turner and Burr 1993; Turner 2002).

2.5.3. Compression

Lumbar vertebrae were isolated and subjected to compression testing due to their high content of trabecular bone, relative to its cortical component (Figure 2.7). While both cortical and trabecular bone are present in individual vertebra, vertebral compression is generally accepted as a test for trabecular bone mechanical properties (Turner and Burr 1993). As compressive fractures are often seen in osteoporotic patients, this test can be considered to be a clinically relevant assay to determine the trabecular mechanical properties of bone (Marx 1980).

2.5.4 Femoral neck fracture

Finally, femoral heads from the proximal end of the femur were selected due to their mixed composition of trabecular and cortical bone and subjected to a cantilevered fracture testing (figure 2.8). The femoral neck represents a site that is especially sensitive to skeletal fragility, where its effects are observed in patients with traumatic hip fractures (Jamsa, Koivukangas et al. 1999). Due to the complex geometry of this region, normalization can not be performed on these samples, but testing does provide information regarding the structural properties of combined cortical and trabecular bone. All four mechanical tests were performed on the excised bones of all sample groups.
Figure 2.3 Representative graphs from mechanical testing. Force-displacement (A) curves were generated for each sample undergoing three-point bending, torsion, compression and femoral neck fracture testing to determine the structural properties of bone. After geometry analysis and normalization, stress-strain (B) curves were created to determine the material properties of each sample.
\[ \sigma_f = \frac{F_f l_o \phi_{AP}}{8I_{xx}} \]

\( \sigma_f \): Failure stress (MPa)
\( F_f \): Measured failure load (N)
\( l_o \): Gauge length (mm)
\( \phi_{AP} \): External diameter in anterior – posterior direction (mm)
\( I_{xx} \): Elliptical moment of inertia (mm\(^4\))

\[ \epsilon_f = \frac{6\phi_{AP} \delta_f}{l_o^2} \cdot 100 \]

\( \epsilon_f \): Failure strain (%)
\( \phi_{AP} \): External diameter in anterior – posterior direction (mm)
\( \delta_f \): Failure displacement (mm)
\( l_o \): Gauge length (mm)

**Figure 2.4a** Three point bending
\[
\sigma_f = \frac{\phi_{ML}}{2} \frac{T_f}{J}
\]

\(\sigma_f\) : Failure shear stress (MPa)
\(T_f\) : Failure torque (N \cdot mm)
\(\phi_{ML}\) : External diameter in medial – lateral (major) direction (mm)
\(J\) : Polar moment of inertia (mm^4)

\[\varepsilon_f = \frac{\phi_{ML} \cdot \alpha_f}{2} \frac{l_o}{100}\]

\(\gamma_f\) : Failure shear strain (%)
\(\phi_{ML}\) : External diameter in medial – lateral (major) direction (mm)
\(\alpha_f\) : Failure rotation (mm)
\(l_o\) : Gauge length (mm)

Figure 2.4b Torsion testing
\[ \sigma_f = \frac{F_f}{A_{xs}} \]

\( \sigma_f \): Failure stress (MPa)
\( F_f \): Bending moment (N \cdot mm)
\( A_{xs} \): Cross-sectional area (mm\(^2\))

\[ \varepsilon_f = \frac{\delta_f}{h_v} \cdot 100 \]

\( \varepsilon_f \): Failure strain (%)
\( \delta_f \): Displacement at failure (mm)
\( h_v \): Height of vertebra (mm)

Figure 2.4c Vertebral compression testing
Figure 2.5 Determination of cortical bone mechanical properties: three point bending testing. Right femurs from Wild-type, Rac2 null, Rac1 null and DKO mice were subjected to forced fracture analysis along the femoral mid shaft using an Instron mechanical testing apparatus. Structural and material properties were determined using load-displacement graphs and normalized to generate stress-strain graphs respectively.

Figure 2.6 Determination of cortical bone mechanical properties: Torsion testing. Left femurs from Wild-type, Rac2 null, Rac1 null and DKO mice were subjected to shear fracture testing using an Instron mechanical testing apparatus. Structural and material properties were determined using load-displacement graphs and normalized to generate stress-strain graphs respectively.
Figure 2.7 Determination of trabecular bone mechanical properties: compression testing. Lumbar vertebral bodies from Wild-type, Rac2 null, Rac1 null and DKO mice were subjected to compression testing using an Instron mechanical testing apparatus. Structural and material properties were determined using load-displacement graphs and normalized to generate stress-strain graphs respectively.

Figure 2.8 Determination of mixed trabecular and cortical bone mechanical properties: femoral neck fracture testing. Right femoral heads from Wild-type, Rac2 null, Rac1 null and DKO mice were subjected to shear fracture testing of the femoral neck using an Instron Instron mechanical testing apparatus. Structural properties were determined using load-displacement graphs; however, normalization was not performed due to the complex geometry of the femoral neck.
2.6 Trabecular architecture using Micro-computed tomography

Three dimensional architecture was determined using dissected third lumbar vertebral bodies (figure 2.9). Vertebrae were mounted in 1mL microtubes and immobilized for scanning in epoxy resin (Buehler, Lake Bluff, IL USA). High resolution scans were performed using a GE eXplore Locus micro-CT scanner (GE Healthcare, London ON, Canada). Scanned images were calibrated using a hydroxyapatite, water and air standard and reconstructed using three-dimensional image analysis software (Microview Version 2.0, GE Healthcare, London ON Canada). In order to remove the contribution of cortical bone, a three dimensional region of interest was selected to isolate only trabecular bone. Volumetric analyses were performed to determine parameters analyzed including volumetric bone mineral density (vBMD) and content (vBMC), as well as trabecular architecture including trabecular thickness (Tb.Th.), number (Tb.N.), separation (Tb.Sp.) and bone volume fraction (BV/TV).

Figure 2.9 Micro computed tomography of Lumbar vertebrae. Vertebral bodies were scanned using a micro CT scanner and digitally reconstructed for three-dimensional analysis.
2.7 Static and Dynamic Histomorphometric analysis

Dissected vertebral bodies were isolated from fifth lumbar vertebrae and immediately fixed in 70% ethanol. Bones were subsequently embedded in Spurr resin over 6 weeks in ascending ratios of unpolymerized spurr resin and descending concentrations of acetone. To ensure uniform penetration of embedding medium in these undecalcified samples, all processing was performed under vacuum. Final processing was completed using 100% spur resin and polymerization performed at 60°C for 48 hours. Undecalcified slide preparation was performed using a semi-automated microtome (Leica RM 2265), creating consecutive 5-micron and 7-micron thick coronal sections. Samples were placed onto gelatinized slides, with the 5-micron slides stained using Goldner’s trichrome staining (figure 2.10) for static histomorphometric analysis (Holmes, Khan et al. 2007), and 7-micron slides left unstained for dynamic histomorphometric analysis. Analysis was performed using a semi-automated histomorphometric software (Bioquant Nova Prime version 6.50.10) using a 25x objective lens connected to a digital video camera (Retiga 1300). Adjacent serial fields were analyzed using the Bioquant morphometry system to determine bone static parameters including trabecular bone volume (TBV), trabecular thickness (Tb.Th.), number (Tb.N.) and separation (Tb.Sp.); and dynamic formation parameters such as osteoid volume (OV), surface (OS) and thickness (O.Th.) (Parfitt, Drezner et al. 1987).
Dynamic histomorphometry was performed via fluorescence microscopy using a 420μm D-filter to detect Oxy-tetracycline labeled bone. Dual labeled bone measurements were used to calculate trabecular growth parameters including mineralizing surface (MS), percentage mineralizing surface (%MS), mineral apposition rate (MAR) and bone formation rate (BFR) (Parfitt, Drezner et al. 1987). However, due to inadequate labeling of Oxy-tetracycline in trabecular bone, dynamic histomorphometry could not be performed on both 4 and 9 month cohorts.

Figure 2.10 Static histomorphometry. Static grown parameters were measured using undecalcified sections stained with Goldner’s Trichrome. Immature bone or osteoid (red, arrows) can be readily identified from mature bone (green).
2.8 Bone Mineralization using Back-scattered electron imaging

Mineralization of trabecular and cortical bone were determined using dissected fifth lumbar vertebral bodies. Bones were embedded in spurr resin as previously described above (figure 2.11a) and diamond paste polished (Buehler, Lake Bluff, IL USA) to a 2um finish and subsequently carbon-coated. Imaging was performed using an electron microscope fitted with a backscattered electron (BSE) detector (FEI XL300E SEM, FEI Company, Hillsboro, OR, USA) with a beam intensity of 20kV and 7.0 spot size at 150x magnification.

Bone mineralization and distribution was calibrated between each sample using a silicone dioxide standard. Histograms were generated from grey level distributions (figure 2.11b) to determine parameters including degree of bone mineralization (maximum peak) and heterogeneity of mineralization (full width at half the maximum height, FWHM) (Boyde and Jones 1983). Cortical, trabecular, and total bone mineralization were analyzed for each sample.
Figure 2.11 Back Scatter electron microscopy. Lumbar vertebrae were analyzed using back scatter electron microscopy to determine relative hyper/hypo mineralization of each group. Further, the Full width at half maximum (FWHM) height provides information regarding the relative homogeneity of each sample.
2.9 Bone Connectivity using Strut Analysis

Bone connectivity was analyzed from scanning electron microscopic images of trabecular bone using Strut analysis (Mellish, Ferguson-Pell et al. 1991). Fifth lumbar vertebral bodies embedded in resin were processed in epoxy resin as described above, and coronal scans were performed at 150x magnification using an electron microscope. Trabecular connectivity parameters were measured including number and length (mm) of trabecular nodes, free ends and struts Figure 2.12.

Figure 2.12 Strut analysis. Cross-sections of dissected vertebrae were imaged at 150x magnification using a scanning electron microscope (SEM). Connectivity of trabecular struts were analyzed and determined by the comparison of free-free struts, free-node struts and node-node struts.
2.10 TRAP staining analysis

Histologic analysis was performed using dissected fourth lumbar vertebral bodies. Isolated bones were immediately fixed in 4% paraformaldehyde, and decalcified in 0.5M Ethylene-diamine-tetra acetic acid (EDTA) over 2 weeks (Sigma-Aldrich, St.Louis MO, USA). Decalcification was verified using Faxitron bone scan (Faxitron X-Ray Series Hewlett-Packard, Lincolnshire, IL USA), with further histologic processing performed after wax infusion and embedding in paraffin wax. Consecutive 5-micron coronal sections were cut using a manual microtome (Reichert-Jung, Buffalo NY USA) and fixed onto electrostatically charged glass slides (Superfrost Plus, Fisher Scientific, Ottawa ON, Canada). Osteoclasts were stained using a commercially available kit for Tartrate-Resistant Acid Phosphatase (TRAP) and counterstained with hematoxalin (Sigma-Aldrich, Oakville ON Canada). Histologic analyses were performed using the Leitz Bioquant morphometry system as described above at 25x magnification. Consecutive adjacent fields of view were analyzed and resorption parameters were measured, including number of osteoclasts (#Oc), osteoclasts surface (Oc.S.), percent osteoclasts surface (%Oc.S.), and number of osteoclasts per bone surface (N.Oc.BS). Further, the number of osteoclasts with greater than 3 nuclei were identified and enumerated at 100x magnification using the above microscope system (Figure 2.13).
Figure 2.13 Tartrate resistant acid phosphatase (TRAP) staining of osteoclasts. Decalcified lumbar vertebral sections were stained using TRAP to identify osteoclasts. Further, osteoclasts with greater than 3 nuclei were identified at 100x magnification (arrows)
2.11 Statistical analysis

Data comparisons between Wild type, Rac1null, Rac2null and DKO genotypes were analyzed using One-way Analysis of Variance (ANOVA, general linear model) with Post-hoc testing performed using multiple comparisons LSD (SPSS version 17.0). Data analysis to determine the effect of age between baseline four month and 9 month groups were performed using an unpaired Student’s T-test. A ‘p’-value of <0.05 was considered significant. Results are presented as mean ± standard deviation (sd).
3.0 Results
3.0 Results

The in vivo effects of Rac1 and Rac2 on bone quality were assessed using transgenic mice with targeted deletion of Rac1 and Rac2 in osteoclast precursor cells. Genotypes were confirmed using PCR and Western blot analysis (Appendix A). Wild-type (WT) mice were used as a control and were compared to Rac1 null (Rac1), Rac2 null (Rac2) and double null (DKO) mice (n=11 per group). The effects were studied over a four and 9 month time period to determine the effects of age on bone quality in Rac deficient mice.

All data are expressed as mean ± standard deviation (SD), with a ‘p’ value <0.05 considered statistically significant.

3.1 Bone mineral content is decreased in Rac2 null and Rac1 null femurs, with no differences in vertebral bone.

In order to assess bone mineral properties, wild-type (WT), Rac1 null (Rac1), Rac2 null (Rac2) and double knock-out (DKO) mice were scanned using dual energy x-ray absorptiometry (DXA). Results from whole body scans were confirmed with dissected femur and vertebral scans to assess Bone mineral density (BMD) and bone mineral content (BMC) in cortical and trabecular bone, respectively. Femurs were selected for their cortical content, while vertebrae were used for their high trabecular bone contribution.

Compared to Wild-type groups, all Rac null femurs demonstrate reduced BMC at both four and 9 months time points (p<0.05). With age, all groups demonstrated increased
BMD and BMC (P<0.05). Femoral BMD was decreased at 4 months time, but this
difference was not observed at 9 months. No differences noted in vertebral BMC or
BMD at both time points (Table 3.1).

DXA results indicate a reduction in cortical bone mineral content in femurs with no
differences in trabecular bone mineral density or content. The deletion of Rac1 and Rac2
in osteoclasts appears to affect vertebral trabecular bone and femur cortical bone
differently, with their deletion resulting in decreased femoral bone mineral properties at 4
and 9 months, with no effect on vertebral bone mineral density or content. With age, all
mice demonstrated improved BMC and BMD (Figure 3.1).

Although the use of DXA is a relatively non-invasive technique to provide preliminary
information regarding the quality of bone, it merely describes a two-dimensional
description of bone quality. Further evaluation using advanced imaging techniques are
required to help determine the effects of Rac1 and Rac2 on bone quality.
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<td>Femur BMC</td>
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<td>0.026±0.002**</td>
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Table 3.1 Deletion of Rac2 and Rac1 in osteoclasts results in reduced femur cortical bone mineral density and bone mineral content

Bone mineral density (BMD) and bone mineral content (BMC) were assessed in Wild-type, Rac2 null, Rac1 null, and double knock-out (DKO) mice using Dual Energy x-ray absorptiometry (DXA). BMD was decreased in Rac2 null and DKO groups, while all Rac deficient femurs demonstrated reduced BMC compared to WT groups at both four and 9 months (p<0.05). No differences were observed in vertebrae.

[mean±sd, *(p<0.05), **(p<0.001) versus wild-type, (n=11) per group]
Figure 3.1 All mice demonstrated increased bone mineral content with age
Bone mineral density (BMD) and bone mineral content (BMC) were assessed in Wild-type, Rac2 null, Rac1 null, and double knock-out (DKO) mice using Dual Energy x-ray absorptiometry (DXA). All femurs demonstrated increased bone mineral content with age (BMC), with no differences observed in vertebral BMD or BMC. DXA results show that BMC was reduced in all Rac deficient cortical bones, but demonstrated improved femoral and vertebral BMC and BMD with age. [*p<0.05], versus wild-type, (n=11) per group]
3.2 Rac deficient femurs demonstrate reduced cortical geometry compared to Wild-type mice

From our DXA analysis, it is clear that cortical bone mineral content in femurs from all Rac null groups are decreased compared to Wild-type controls. Further geometric analysis of cortical bone was performed using scanning electron microscopy (SEM). Femurs were cut in cross section at the mid-shaft and embedded for SEM imaging at 125x magnification. Cortical bone geometry including anterior-posterior diameter (minor), medial-lateral diameter (major), and cortical thickness were measured and analysed (Figure 3.2).

Femur geometry of Rac2 null, Rac1 null and DKO mice all demonstrate reduced size compared to Wild-types (P<0.05) (Table 3.2). This finding was consistent at both four and 9 month time points. With age, all groups demonstrated an increase in bone geometry. Interestingly, the cortical geometry of Rac2 femurs were found to be even further decreased to that of Rac1 and DKO groups at four months (P<0.05), indicating that the deletion of Rac2 in osteoclasts affected bone formation, decreasing cortical bone geometry. Further, the deletion of both Rac1 and Rac2 in DKO groups did not demonstrate decreases beyond those seen in single null groups, indicating that the deletion of both isoforms is not additive in vivo.

Rac deficient mice demonstrated decreased cortical bone geometric properties compared to WT groups. To determine if these reductions translated into decreased mechanical properties, fracture testing was performed to determine structural and material properties of Rac deficient cortical bone.
63

Table 3.2 All Rac deficient femurs demonstrate decreased cortical geometry.
Cortical geometry was analyzed using femurs from Wild-Type, Rac2 null and Rac1 null mice. Femurs were cut mid-shaft with cross sections analyzed using scanning electron microscopy (SEM) for major and minor diameters, and cortical thickness. All Rac deficient femurs were smaller compared to wild-types (P<0.05) at both four and 9 month time points (p<0.05). Further, cortical geometry of Rac2 null bone was less than that of both Rac1 null and DKO groups (P<0.05), suggesting that the deletion of Rac2 in osteoclasts decreased bone formation. All mice demonstrated improved cortical geometry with age. [mean±sd *(p<0.05), **(p<0.001) versus wild-type, (n=11) per group]

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Table 3.2 continued.
Figure 3.2 All Rac deficient femurs demonstrated reduced cortical geometry compared to Wild-type mice. Cortical geometry was analyzed using femurs from Wild-Type, Rac2 null and Rac1 null mice. Femurs were cut mid-shaft with cross sections analyzed using scanning electron microscopy for major and minor diameters, and cortical thickness. Representative images at 150x magnification shown (A). All Rac null mice demonstrated reduced size compared to wild-types (P<0.05) at both four and 9 month time points and with age, all mice demonstrated improved cortical geometry (B). [(*P<0.05) (n=11) per group]
3.3 Rac deficient femurs are smaller and demonstrate reduced structural properties, but prevents age-related loss of bone quality.

DXA scanning and SEM analysis have demonstrated that Rac1, Rac2 and DKO femurs have reduced cortical bone mineral content as well as decreased cortical geometry at four and 9 month time points. We next determined whether these reduced geometric properties affected their mechanical properties. Fracture testing using three-point bending and torsion testing were performed using femur cortical bone.

3.3.1. Three point bending analysis

Right mouse femurs from Wild-Type, Rac2 null, Rac1 null and DKO mice were dissected and subjected to three-point bend testing using an Instron mechanical testing machine until fracture. Load displacement data were generated and analyzed to determine structural properties including Ultimate load, failure load, stiffness, and energy to failure (Table 3.3).

Rac1 null, Rac2 null and DKO groups all demonstrated reduced cortical structural properties compared to WT mice (P<0.05) (Figure 3.3). Interestingly, Rac2 null femurs were even structurally weaker than Rac1 null and DKO mice (p<0.05), indicating that the deletion of Rac2 in osteoclasts had a greater negative effect on cortical bone than observed in Rac1 null and DKO groups.

With age, WT mice demonstrated decreased structural properties, indicating a loss of bone quality with age. This age-related decrease in structural properties was not observed in any Rac deficient mouse group; however, Rac deficient cortical bones were still structurally weaker than age matched WT controls at 9 months.
Material properties were determined by normalizing load-displacement data using cross-sectional geometry to generate Stress-Strain data to account for geometric differences between groups.

Rac1 null, Rac2 null and DKO cortical bone demonstrated no differences in material properties at four mouths when compared to wild-types (p>0.05). However with age, WT groups demonstrated decreased material properties compared to baseline, indicating a decrease in bone quality with aging (P<0.05) (Table 3.3). This decrease in age-related material properties of cortical bone was not observed in any Rac deficient group, suggesting that the deletion of Rac in osteoclasts attenuated the bone loss seen during aging. These age-related changes resulted in all Rac null groups demonstrating improved material properties (p<0.05) compared to wild-type controls at 9 months.
Table 3.4. All Rac deficient femurs were structurally weaker but demonstrated improved material properties with age.

Mechanical properties of cortical bone were analyzed using dissected femurs in a three-point bend test for Wild-type, Rac2 null, Rac1 null and DKO mice. Structural properties of Rac2null and Rac1 null femurs were decreased compared to WT mice (P<0.05) at both 4 and 9 months (p<0.05). Material properties were determined from normalized data to account for bone geometry. No differences were noted at 4 months; however, all Rac deficient bones demonstrated increased material properties (P<0.05) compared to wild-type mice at 9 months.

[mean±sd, *(p<0.05), **(p<0.001) versus wild-type, (n=11) per group]
Figure 3.3. Rac null femurs are structurally weaker but prevented age-related bone loss. To assess the mechanical properties of cortical bone, femurs were dissected from Wild-Type, Rac2 null, Rac1 null and DKO mice and subjected to three-point bend testing using an Instron mechanical testing device. With age, WT groups demonstrated a decrease in material properties (p<0.05), with no changes observed in any Rac deficient groups. Age-related decreases were not observed in Rac null mice, demonstrating that the deletion of Rac isoforms in osteoclasts attenuates age-related decreases in material properties. [(n=11) per group, *(p<0.05)]
3.3.2. Torsion

Where three point bending induces bone fracture at a specified section, torsional testing allows the fracture of cortical bone along its weakest point.

Left femurs from Wild-Type, Rac2 null and Rac1 null mice were dissected and subjected to shear forces in a torsion testing apparatus using an Instron mechanical testing machine until fracture. Load displacement data were generated and analyzed to determine structural properties including Ultimate load, failure load, stiffness, and energy to failure (Table 3.4).

Rac2 null and DKO groups demonstrated decreased torsional stiffness (P<0.05) with a non-significant trend towards decreased failure torque. No differences were observed in material properties at four months (p>0.05) (Figure 3.4).

With age, no differences were observed in structural properties; however, Rac2 null groups remained structurally weaker compared to age matched WT controls (P<0.05).
Table 3.3. Deletion of Rac2 in osteoclasts reduces structural properties in cortical bone. To assess mechanical properties of cortical bone, femurs were dissected from Wild-Type, Rac2 null, Rac1 null and DKO mice and subjected to torsional fracture testing. Rac2 null femurs demonstrate reduced structural properties (P<0.05) at 4 and 9 months, with no differences in Rac1 null compared to Wild-type groups. DKO groups demonstrated decreased torsional stiffness at four months, but this difference was not observed at 9 months.

Material properties were assessed after data normalization to account for geometric differences. No differences were observed in any Rac null group compared to age matched wild-type controls.

[mean±sd *(p<0.05), **(p<0.001), (n=11) per group]
Figure 3.4. Rac2 deficient mice demonstrate reduced structural properties.
To assess the mechanical properties of cortical bone, femurs were dissected from Wild-Type, Rac2 null, Rac1 null and DKO mice and subjected to torsional fracture testing. Rac2 null femurs demonstrate reduced structural properties (P<0.05) at 4 and 9 months, with no differences in Rac1 null compared to Wild-type groups. Material properties were assessed after data normalization to account for geometric differences. No differences were observed in any Rac null group compared to wild-types at both four and 9 months. Rac2 null groups were structurally weaker than WT controls.
[(n=11) per group]
Cortical testing summary

Rac1 null, Rac2 null and DKO mice demonstrated reduced cortical bone content as assessed by DXA, and further analysis using SEM revealed decreased geometric properties compared to WT groups. These decreases in both bone mineral content and size produced inferior structural properties when subjected to fracture testing at both four and 9 month time points using three-point bending and torsion testing. Although structurally weaker than WT groups, all Rac deficient mice demonstrated the ability to attenuate age-related loss of bone quality, resulting in increased bone material properties compared with WT controls.

3.4 Rac deficient vertebrae demonstrate increased trabecular architecture and prevents age-related loss of bone quality.

As bone remodelling is a surface event, bone turnover occurs 8 times faster in trabecular bone than in cortical bone (Jee and Yao 2001). In order to assess the effect of the deletion of Rac isoforms in osteoclasts on trabecular bone quality, vertebral bodies from Rac deficient mice were used.

Trabecular architecture was assessed using dissected lumbar vertebrae from Wild-type (WT), Rac1null (Rac1), Rac2null (Rac2) and double knock-out (DKO) mice. Trabecular properties were analyzed using three-dimensional micro computed tomography (uCT) to determine trabecular volume, thickness, number, and separation, while the connectivity of trabecular bone was assessed using strut analysis.
3.4.1 Micro Computed Tomography

Third lumbar vertebrae were dissected and scanned using a micro-CT (uCT) scanner. Vertebral bodies were embedded in 1mL microtubules and high resolution scans were performed to assess three-dimensional trabecular properties (figure 3.5).

Compared to WT controls, Rac1 null and DKO groups demonstrated increased trabecular volume and numbers, with a decrease in trabecular separation. These findings indicate improved trabecular architecture in Rac1 null and DKO groups compared to WT groups, which persisted with age (p<0.05) (Table 3.5). No differences were observed in Rac2 groups at four months; however with aging, trabecular numbers increased with decreased in trabecular separation, indicating an increase in trabecular properties over time. All Rac deficient groups demonstrated improved trabecular architecture with age.
Table 3.5 Trabecular architecture is improved in all Rac deficient mice.

Three-dimensional trabecular properties were assessed in vertebral bodies of Wild-type, Rac2 null, Rac1 null and DKO mice using micro computed tomography. 3rd lumbar vertebrae were dissected and scanned to determine trabecular properties. Rac1 and DKO groups demonstrated increased trabecular number with a decrease in separation, indicating improved trabecular architecture at four and 9 months compared to WT controls. Rac2 null mice demonstrated no differences at four months, but showed increased architecture at 9 months.

[mean±sd, *(p<0.05), **(p<0.001), (n=11) per group]
Figure 3.5 Genetic Deletion of Rac2 and Rac1 increases trabecular geometry.
Three dimensional trabecular properties were assessed in vertebral bodies of Wild-type, Rac2 null and Rac1 null mice using micro computed tomography. Vertebrae were dissected and scanned for trabecular analysis. Representative figures shown (A). Rac1 and DKO vertebrae demonstrated increased trabecular architecture at both four and 9 month time points (p<0.05). Rac2 groups demonstrated no differences at four months, but showed increased architecture at 9 months. [(n=11) per group]
3.4.2 Strut analysis

Dissected 4th lumbar vertebrae were embedded and coronal sections used in Strut analysis. Scanning electron microscope images were made at 150x magnification and the trabecular pattern was analyzed. Trabecular bone connectivity was assessed by determining the number of free-end struts, and multiple-points, indicating a relative decrease and increase in connectivity, respectively. The length and ratio of the free and multiple points were also assessed to determine the degree of connectivity (Figure 3.6). Rac1 null groups demonstrated increased trabecular connectivity compared to WT controls (P<0.05), with no differences observed in Rac2 null and DKO mice (Table 3.6). With age, WT controls demonstrated decreased trabecular connectivity, indicating age-related loss of bone quality. This age related decrease was not observed in any Rac deficient group, which demonstrated increased trabecular connectivity with age.
Table 3.6. Rac deficient mice demonstrate improved trabecular connectivity.
Trabecular bone connectivity was analyzed using strut analysis. Lumbar vertebral coronal sections were prepared and imaged using scanning electron microscopy. Rac1 null mice demonstrated increased trabecular connectivity at four and 9 months (p<0.05). Rac2 groups demonstrated no differences at four months, but showed increased connectivity at 9 months. [mean±sd, *(p<0.05), **(p<0.001), (n=11) per group]

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Figure 3.6. Rac deficient mice demonstrate improved trabecular connectivity.
Trabecular connectivity was analyzed using strut analysis. Lumbar vertebral coronal sections were prepared and imaged using scanning electron microscopy. WT controls demonstrated decreased trabecular connectivity with age. This age-related loss of connectivity was not observed in any Rac deficient group, indicating that the deletion of Rac isoforms in osteoclasts prevented age-related loss of trabecular connectivity. [(n=11) per group]
3.5 Rac deficient vertebrae demonstrate increased trabecular mechanical properties and attenuates age-related loss of bone quality.

Using uCT and Strut analysis, we have established that Rac null vertebral bone demonstrated improved trabecular architecture and connectivity. In order to determine whether these improvements infer increased fracture resistance, mechanical fracture testing was performed using a vertebral compression assay, as well as a femoral neck fracture test.

3.5.1. Vertebral compression

Dissected 5th lumbar vertebrae were subjected to a compression assay using an Instron materials testing apparatus. Loading was performed parallel to the long axis of the vertebrae to simulate physiologic loading of trabecular bone. Load-displacement data were generated to determine structural properties, while material properties were determined by normalizing load-displacement data to account for bone geometric differences (figure 3.7).

Compression testing revealed no differences in structural or material properties between Wild-type, Rac1null, Rac2null and DKO groups at four months (p>0.05) (table 3.7). With age, WT controls demonstrated decreased structural and material properties (P<0.05), indicating an age-related loss of bone mechanical properties. These decreases were not observed in any Rac null groups, which demonstrated improved structural and material properties compared to age matched controls. These findings indicate that the
Deletion of Rac isoforms in osteoclasts improves trabecular bone quality, and further, attenuates age-related loss of mechanical properties as seen in WT controls.

Table 3.7. Rac deficient mice demonstrated improved mechanical properties

Mechanical properties were determined using dissected lumbar vertebrae from Wild type, Rac2 null, Rac1 null, and DKO mice and subjected to compression testing. No differences were observed at 4 months; however, all Rac null groups demonstrated improved structural and material properties at 9 months compared with age matched WT controls.

[mean±sd, *(p<0.05), **(p<0.001), (n=11) per group]

<table>
<thead>
<tr>
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<th>4 month</th>
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<td>4DKO</td>
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Figure 3.7. The Deletion of Rac in osteoclasts attenuates age-related loss of trabecular bone mechanical properties.

Mechanical properties were determined using dissected lumbar vertebrae from Wild type, Rac2 null, Rac1 null, and DKO mice and subjected to fracture testing in a compression test. With age, structural and material properties were decreased in WT groups (p<0.05); however, these age-related decreases were not observed in Rac null mice. These findings indicate that the deletion of Rac isoforms in osteoclasts prevented age-related decreases in bone mechanical properties.

[(n=11) per group]
3.5.2. Femoral neck fracture testing

In order to assess the mechanical properties of combined cortical and trabecular bone, the proximal ends of femurs containing the femoral neck were fracture tested. The femoral neck contains a combination of cortical and trabecular bone, where its fracture serves as a test to simulate hip fractures sustained by some patients. This test represents a clinically relevant assessment of bone structural properties; however, due to its complex geometry the material properties of cortical and trabecular bone can not be determined through data normalization. Therefore, only structural properties are presented here.

Proximal ends of femurs from Wild-type, Rac2, Rac1 and DKO mice were dissected and subjected to fracture testing (Table 3.8). Compared to WT groups, Rac2 null and Rac1 null mice demonstrated inferior cortical and trabecular structural properties at both four and 9 months (p<0.05). These findings were in agreement with the decreased structural properties we demonstrated earlier in cortical bone from femurs, demonstrating that Rac null femoral bone are structurally weaker compared to WT mice.
Table 3.8. Rac1 null and Rac2 null mice are structurally weaker.
Structural properties of mixed cortical/trabecular bone were determined using proximal ends of femurs containing the femoral neck. Compared to WT groups, Rac2 and Rac1 mice demonstrated decreased structural properties at both four and 9 months. Due to the complex geometry of the femoral neck, normalization was not performed. [mean±sd, *(p<0.05), **(p<0.001),  (n=11) per group]

<table>
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<tr>
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<th>4Wt</th>
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<th>4Rac1</th>
<th>4DKO</th>
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<td>fail disp (mm)</td>
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Trabecular summary

In order to assess the effect of the deletion of Rac1 and Rac2 in osteoclasts on bone quality, trabecular bone was assessed using mouse vertebral bodies and proximal femoral heads containing the femoral neck. Data from micro computed tomography and strut analysis, have demonstrated increased trabecular connectivity and volume. Through mechanical testing, we have further demonstrated that Rac deficient trabecular bone have improved mechanical properties compared to WT controls. With age, Rac null mice did not demonstrate a loss of trabecular architecture and decreased mechanical properties as seen in WT mice, indicating that the deletion of Rac resulted in the attenuation of age-related loss of trabecular bone properties.

All Rac null groups demonstrated improved trabecular material properties with age and attenuated age-related decreases in bone mechanical properties observed in WT groups.

3.6 Rac deficient bone is hypermineralized, and more homogenous compared to Wild-types.

Bone is composed of approximately 65% mineral and 35% organic matrix (Jee and Yao 2001). In order to assess the mineral component in bone, we assessed the degree of bone mineralization and its relative distribution in Wild-type, Rac2 null, Rac1 null and DKO mice.

3.6.1 Back scattered electron microscopy

Dissected vertebrae were embedded in epoxy resin and coronal sections prepared for analysis using back scattered electron microscopy (BSE). Briefly, samples were imaged at 150x magnification using an SEM equipped with a BSE detector to obtain a
mineralization profile. As calcium nuclei are the heaviest, the deflected electrons from
the incident beam are measured using a BSE detector, and are correlated to the calcium
content, and hence the mineralization of bone.

Compared to WT groups, all Rac null mice demonstrated increased mineralization,
indicating relative hypermineralization at four months (p<0.05) (figure 3.9). Further,
Rac null bones demonstrated a decrease in the distribution of bone mineral compared to
WT groups, indicating an increase in bone mineral uniformity, or homogeneity, at 9
months (p<0.05) (Figure 3.8). Rac null bones demonstrated increased mineralization and
homogeneity as compared to WT groups at four and 9 months, respectively.

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<tr>
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<td>28.27±1.90**</td>
<td>28.17±2.16**</td>
<td>30.13±2.35**</td>
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</tbody>
</table>

Table 3.9. All Rac deficient bones are more mineralized and uniform.
Mineralization was compared in Wild-type, Rac2 null, Rac1 null and DKO mice using Back scattered electron
microscopy (BSE). Mineralization profiles were constructed and compared to WT controls. All Rac deficient
groups demonstrated increased mineralization at four months and increased homogeneity with age (p<0.05).
[mean±sd, *(p<0.05), ***(p<0.001), (n=11) per group]
Figure 3.8. All Rac deficient bones are hypermineralized compared to Wild-types. Mineralization was compared in Wild-type, Rac2 null, Rac1 null and DKO mice using Back scattered electron microscopy (BSE). Dissected vertebrae were embedded and cut in cross section for analysis (A). All Rac deficient groups demonstrated relative hypermineralization compared with WT groups demonstrating an increase in mineralization at four months (B). [(n=11) per group]
3.7 Rac2 and Rac1 null mice demonstrate decreased bone turnover

Using a transgenic mouse model with either Rac1, Rac2 or both isoforms deleted in osteoclasts, we have demonstrated the in vivo effects of their deletion on bone architecture and mechanical properties. However, their effect on osteoclast function is relatively unknown. In order to indirectly assess osteoclast function during bone turnover, osteoclast quantification and bone formation parameters were analyzed using histomorphometry.

3.7.1. Osteoclast quantification using TRAP staining

In order to assess osteoclast quantity, Wild-type, Rac1 null, Rac2 null and DKO vertebrae were decalcified and stained for tartrate resistant acid phosphatase (TRAP). Briefly, 4th lumbar vertebrae were dissected and decalcified using EDTA. After embedding in paraffin wax, 5 micron vertebral coronal sections were prepared and stained for TRAP with hematoxylin as a counter-stain. The total number of osteoclasts was determined, as well as the number of osteoclasts with greater than 3 nuclei. As osteoclasts are formed from the fusion of precursor cells, these results represented an indirect measure of osteoclastogenesis. Further, osteoclast functional parameters including eroded/quiescent bone surfaces were determined.

Our results demonstrated no differences in the total number of osteoclasts at both four and 9 month time points. However, DKO groups demonstrated fewer osteoclasts with greater than 3 nuclei (p<0.05) at four months indicating decreased osteoclastogenesis (Table 3.10). Further, bone formational parameters from histomorphometric analysis
demonstrated increased quiescent surfaces with decreased osteoclastic eroded surfaces in
Rac1 null and Rac2 null groups (p<0.05), indicating reduced osteoclast activity in Rac
deficient mice.

3.7.2 Bone formation assessment using static histomorphometry
Bone formation parameters were assessed in Wild-type, Rac1 null, Rac2 null and DKO
mice using Static histomorphometry. Briefly, undecalcified 5th lumbar vertebrae were
embedded in resin and coronal sections prepared for analysis. Sections were stained
using Goldner’s trichrome to identify mineralized bone, bone marrow and immature bone
(osteoid). Bone formation properties were assessed using osteoid volume surfaces and
thickness.
No differences were observed in Rac2 null and Rac1 null groups at both time points,
demonstrating no differences in bone formation compared to WT controls (Table 3.11).
However, an increase in osteoid volume and surface was observed in DKO mice as
compared with WT groups at 9 months, indicating increased new bone formation in DKO
groups (p<0.05).
Table 3.10 DKO mice had fewer multinucleated osteoclasts while Rac2 and Rac1 null mice demonstrate increased quiescent bone surface.

In order to assess osteoclast quantity, Wild-type, Rac1 null, Rac2 null and DKO vertebrae were decalcified and stained for TRAP. No differences were observed in the total number of osteoclasts; however, DKO groups contained fewer osteoclasts with greater than 3 nuclei (p<0.05) at four months. Further, Rac2 and Rac1 groups demonstrated increased quiescent surfaces with age, indicating a decrease in osteoclast function.

[mean±sd, *(p<0.05), **(p<0.001), (n=11) per group]
Table 3.11. DKO mice demonstrate increased bone formation.

Bone formation parameters were assessed in Wild-type, Rac1 null, Rac2 null and Double knock-out mice using Static histomorphometry. Mice vertebral coronal sections were stained with Goldner’s trichrome to identify newly formed osteoid and mature bone. No differences were observed in Rac2 and Rac1 null groups. An increase in osteoid volume and surface was observed in DKO mice indicating increased new bone formation with age.

[mean±sd, *(p<0.05), **(p<0.001), (n=11) per group]
**Bone turn over summary**

Using a transgenic mouse model with Rac1, Rac2 or both (DKO) isoforms deleted in osteoclast cells, we have reported that although all Rac null groups are able to form multinucleated osteoclasts, Rac1 null and Rac2 null mice demonstrated reduced osteoclastic bone resorption with no differences in bone formation. Thus, Rac deficient mice demonstrated an osteopetrotic-like phenotype at 9 months. DKO groups demonstrated decreased early osteoclastogenesis, with an increase in bone formation at 9 months.
4.0 Discussion
4.0 Discussion

Rac1 and Rac2 are members of the Rho family of small GTPases, which regulate cytoskeletal organization necessary for lamellapodia formation and membrane ruffling, essential processes required for the formation and function of osteoclasts (Razzouk, Lieberherr et al. 1999; Wherlock and Mellor 2002; Sun, Downey et al. 2004; Wang, Belsham et al. 2009). Previous studies have demonstrated that the deletion of Rac1 in osteoclasts results in reduced osteoclastogenesis and bone resorption (Wang, Lebowitz et al. 2008); however, little is known about these effects on bone quality in vivo. As aging results in bone loss leading to increased susceptibility to bone fractures (Marx 1980), a better understanding of the molecules involved in the regulation of osteoclasts in vivo will allow the identification of novel therapeutic targets to prevent bone fragility seen with aging.

Using a genetic mouse model previously described (Glogauer, Marchal et al. 2003) with Rac1, Rac2, or both (Double knock-out, DKO) isoforms deleted in osteoclast precursors, we have determined the effects of Rac deletion on bone quality in vivo. Further, with the use of baseline and aged groups, we were able to observe the effects of aging on bone quality in Rac deficient mice. We report that the deletion of Rac1 resulted in smaller, weaker bones compared to Wild-type (WT) mice. Rac2 deficient mice showed a similar bone phenotype to Rac1 deficient mice; however, improvements in bone quality were delayed.

In aged groups, WT groups demonstrated decreased bone quality with age; however, these decreases were not observed in Rac1 and Rac2 deficient mice. Our findings suggest that both Rac isoforms demonstrate the ability to attenuate age-related bone loss
and may be appropriate targets for the development of drugs to prevent the loss of bone as seen with aging.

4.1 The deletion of Rac1 resulted in smaller cortical bones and stronger trabecular bone

Our findings indicate that the deletion of Rac1 in osteoclast precursors resulted in smaller cortical bones and increased trabecular bone architecture compared to WT controls. The differences between cortical and vertebral bone growth may be attributed to the regulation of bone growth in these bone sub-types. As bone resorption is a surface event, trabecular bone has been reported to have an 8 fold increased rate of remodelling due to its increased surface area compared to cortical bone (Jee and Yao 2001). Further, the regulation of cortical and trabecular bone growth has been attributed to different genes (Turner, Hsieh et al. 2000). Using C3H and B6 mice known for their thick cortical bone and fine trabecular architecture, respectively, Turner demonstrated that cross breeding produced a variety of mice with combinations of thick and thin cortical and trabecular bone architecture, suggesting that different cortical and trabecular bone geometry are regulated by different genes (Turner, Hsieh et al. 2000). We report here that Rac1 deletion in osteoclasts affected cortical and trabecular bone growth differently, where cortical geometry was reduced and trabecular volume improved compared to WT controls.
Interestingly, the effect of Rac1 deletion on cortical bone size and growth *in vivo* has not been described previously in the literature. Our results suggest that the deletion of Rac1 in osteoclasts may not only affect bone resorption, but it may also reduce the formation of cortical bone by osteoblasts. Osteoblast stimulation of bone resorption has been established in the literature via the RANKL/RANK system (Raisz 1999; Jee and Yao 2001; Boyle, Simonet et al. 2003; Hadjidakis and Androulakis 2006; Kim, Zhao et al. 2006). The converse has also been reported with osteoclast stimulating bone formation via the release of growth factors including insulin-like growth factor (IGF-I), transforming growth factor- beta (TGF-beta) and bone morphogenic proteins (BMP) (Koh, Demiralp et al. 2005; Kawano, Troiano et al. 2008). Further, the importance of osteoclasts in bone formation was demonstrated by Koh et al. using osteoprotegrin (OPG), a competitive analogue of RANKL to block osteoclastogenesis. Koh et al. demonstrated that by blocking osteoclast formation, a reduction in bone formation was observed, even in the presence of parathyroid hormone (PTH), a potent bone anabolic protein. These findings demonstrate the importance of normal osteoclastogenesis to the formation of bone, where the inhibition of osteoclast formation resulted in reduced bone deposition (Koh, Demiralp et al. 2005). These studies support the hypothesis that impaired osteoclastogenesis reduced the release of growth factors by osteoclasts, resulting in smaller cortical bones in Rac1 deficient mice. We report that not only were Rac1 deficient cortical bones smaller, but they were structurally weaker compared to WT controls.
Although we have previously reported the effects of Rac1 deletion on trabecular architecture (Wang, Lebowitz et al. 2008), our current project demonstrated that these effects persisted with age and improved the quality of trabecular bone. We hypothesize that the improved trabecular architecture observed in Rac1 deficient mice were the result of decreased osteoclast formation and function. Previous studies have identified Rac1 as the key regulator of osteoclastogenesis (Wang, Lebowitz et al. 2008). Using osteoclast precursor cells from Rac1 null mice, we have previously reported that Rac1 deficient osteoclasts demonstrated decreased ability to produce multinucleated osteoclasts when stimulated with M-CSF and RANKL (Wang, Lebowitz et al. 2008). Further, these osteoclasts demonstrated reduced function as observed in the formation of fewer resorptive pits on dentin compared with wild-type controls (Wang, Lebowitz et al. 2008).

Reduced osteoclast function in Rac1 deficient cells has also been reported in the literature, where it has been attributed to decreased podosome formation, ROS production and intracellular vesicular transport (Razzouk, Lieberherr et al. 1999; Lee, Choi et al. 2005; Sun, Buki et al. 2005). Impaired osteoclast formation and function in Rac1 deficient osteoclasts, coupled with normal bone formation as shown in our histomorphometric analysis may explain our observed increase in trabecular architecture as demonstrated in data from micro computed tomography and strut analysis. Consistent with this hypothesis, Rac1 deficient trabecular bones demonstrated improved connectivity and material properties compared to age matched WT controls. Improved trabecular architecture and material properties were observed at both baseline and aged groups, demonstrating that the deletion of Rac1 in osteoclasts affected bone remodelling

*in vivo.*
In contrast to previous studies examining the role of Rac1 in early osteoclastogenesis (Wang, Lebowitz et al. 2008; Wang, Belsham et al. 2009), our findings demonstrate Rac1 deficient mice were, in fact able to form multinucleated osteoclasts in an in vivo model. This difference may be attributed to the length of our current study examining the long-term effects of Rac1 deletion, compared to short-term in vitro studies examining early osteoclastogenesis over 5 days (Fukuda, Hikita et al. 2005; Lee, Choi et al. 2005; Wang, Lebowitz et al. 2008). Although able to form multinucleated TRAP positive cells, our findings agree with previous studies demonstrating decreased osteoclast bone resorption, as shown in our histomorphometric analysis revealing decreased osteoclast erosion surfaces (Wang, Lebowitz et al. 2008).

In summary, our findings suggest that the deletion of Rac1 in osteoclasts resulted in reduced cortical growth, while improving trabecular remodelling. With age, Rac1 deficient osteoclasts demonstrate decreased function, resulting in improved bone material properties, identifying Rac1 as a potential target for the development of drugs aimed at improving bone quality with aging.

4.2 The deletion of Rac1 in osteoclasts prevents age-related bone loss

Using baseline and aged groups, we report that Rac1 deficient mice demonstrated the ability to maintain bone quality with age, suggesting that the absence of Rac1 in osteoclasts attenuated age-related bone loss resulting in improved bone quality. These findings suggest that the deletion of Rac1 in osteoclasts prevented the physiologic effects
of aging, resulting in stronger bones compared age matched WT controls. We hypothesize that due to Rac1’s location downstream of RANK, the deletion of Rac1 attenuated RANKL stimulated bone resorption as seen with aging.

The effects of aging on bone has been previously characterized by decreased bone formation coupled with increased bone resorption, resulting in a net loss of bone structure and increased susceptibility to fracture (Marx 1980; Turner 2002). Previous studies have attributed this age-related imbalance in bone remodelling to increased expression of RANKL, a member of the tumour necrosis factor (TNF) super family involved with the stimulation of osteoclast differentiation (Lee, Choi et al. 2005; Brazier, Stephens et al. 2006; Tat, Pelletier et al. 2009). Using several cohorts of young and aged mice, Cao et al measured RANKL expression over time and demonstrated that with age, RANKL expression increased favouring increased osteoclast activation. The authors further correlated RANKL expression with bone volume and concluded that with age, an increase in RANKL expression favoured osteoclastic bone resorption resulting in reduced bone volume (Cao, Venton et al. 2003). As Rac1 is situated downstream to RANK (Brazier, Stephens et al. 2006), its deletion in osteoclasts may impair the RANK/RANKL system, preventing the activation of osteoclasts and bone resorption seen with age. We hypothesize that by altering intracellular signalling downstream to RANK receptor, the deletion of Rac1 prevented the bone loss observed in aged WT mice, identifying Rac1 as a possible target for pharmacologic therapy in the treatment of osteoporosis.
4.3 The deletion of Rac2 compromises cortical bone structure

The deletion of Rac2 in osteoclasts resulted in smaller cortical bones exhibiting weaker bone quality compared to both Rac1 null and WT groups. We further report that with age, bone quality in Rac2 deficient bones improved to Rac1 levels, suggesting that the deletion of Rac2 in osteoclasts negatively affected early bone formation, but demonstrated improved material properties with age. Interestingly, the effects of Rac2 deletion on bone quality have also not been reported in the literature. Further, the exact role of Rac2 in the regulation of osteoclast functions remain unclear (Lee, Choi et al. 2005; Wang, Lebowitz et al. 2008). Our results suggest that Rac2 is not only involved with bone resorption, but also with osteoclast mediated promotion of bone formation.

We hypothesize that the deletion of Rac2 in osteoclasts may negatively affect the release of bone anabolic growth factors required for bone formation.

Previous studies have established the importance of osteoclast released growth factors on bone remodelling, including BMP, IGF and TGF, where the inhibition of osteoclastogenesis caused a decrease in bone formation (Koh, Demiralp et al. 2005; Garimella, Tague et al. 2008; Kawano, Troiano et al. 2008). Conversely, Kawano et al. reported increased bone formation in Rac2 deficient mice stimulated with PTH (Kawano, Troiano et al. 2008). Using Rac2 deficient mice, Kawano et al reported increased cortical bone formation in response to PTH. The authors credited this increase to decreased osteoclast function in Rac2 deficient mice coupled with uninhibited PTH stimulated bone formation. Based on our observations, we believe that their observed increase in bone formation was due more so to the anabolic affect of PTH, rather than decreased osteoclast
function. We hypothesize that the effect of PTH overcame the absence of Rac2 in osteoclasts, resulting in increased cortical bone formation. Further investigation of PTH on bone quality in Rac deficient mice are required to demonstrate this effect.

We observed that although Rac2 null mice were able to form multinucleated TRAP positive cells, these Rac2 deficient osteoclasts demonstrated reduced osteoclast resorption as shown in decreased erosion surfaces in histomorphometric analysis. These findings are in agreement with previous studies demonstrating decreased osteoclast resorption in Rac2 deficient cells; however, controversy exists regarding the quantity of multinucleated osteoclasts formed. (Lee, Choi et al. 2005; Kawano, Troiano et al. 2008; Wang, Lebowitz et al. 2008). Wang et al. reported reduced multinucleated osteoclast formation in Rac2 deficient tibiae sections, where as Kawano reported increased or similar osteoclast numbers to WT controls. These differences were reported to be due to staining and histomorphometric techniques; however, our findings using vertebral cross sections demonstrated that Rac2 deficient mice formed similar numbers of TRAP positive cells and multinucleated giant osteoclasts compared to age matched WT controls. Further investigations using standardized histologic techniques are required to clarify this issue.

In summary, the deletion of Rac2 compromised cortical bone structure resulting in smaller and weaker bones than Rac1 null mice and WT controls. With age; however, bone properties in Rac2 deficient mice demonstrated improved material properties similar to Rac1 null mice. Improvements on bone quality in Rac2 deficient mice were only observed after aging, while Rac1 demonstrated improvement throughout life. These
findings suggest that the deletion of Rac2 in osteoclasts improved bone quality, but at a lower magnitude than Rac1 deletion.

4.4 The deletion of Rac2 attenuates age-related bone loss

Using baseline and aged groups, we report that the deletion of Rac2 in osteoclasts prevented the age-related bone loss observed in WT groups. These findings suggest that the deletion of Rac2 in osteoclasts attenuated the negative effects of aging on bone quality. Similar to our discussion of Rac1, we hypothesize that the deletion of Rac2 in osteoclasts inhibits RANKL stimulated osteoclast activation due to its location downstream to RANK receptor (Brazier, Stephens et al. 2006). As previously demonstrated, Cao et al attributed decreased bone volume observed with aging to increased RANKL expression resulting in increased osteoclast activation (Cao, Venton et al. 2003). In the absence of Rac2 downstream to RANK receptor, we hypothesize that osteoclast activation is decreased, preventing increased bone resorption as seen with aging. We hypothesize that by altering downstream signalling to the RANKL/RANK pathway though the deletion of Rac2 in osteoclast cells, the loss of bone quality observed with aging is prevented. Our findings suggest that the deletion of Rac2 attenuates age-related bone loss, and may be a valuable target for the development of drugs aimed at preventing bone resorptive diseases.
4.5 Deletion of both Rac1 and Rac2 (Double knock-out) results in smaller cortical bones and stronger trabecular bones

Similar to our findings in single Rac1 deficient groups, double knock-out (DKO) mice had smaller cortical bones with weaker structural properties and demonstrated improved trabecular architecture. With age, we also report that both cortical and trabecular material properties of bone improve. Interestingly, we observed that Rac DKO mice demonstrated bone phenotypes similar to Rac1 single knock-outs, suggesting that Rac1 is the dominant isoform in the regulation of \textit{in vivo} bone quality.

Previous studies have also identified Rac1 as the primary isoform in the regulation of osteoclastogenesis, ROS production and osteoclast cell survival (Hall 1998; Boyle, Simonet et al. 2003; Fukuda, Hikita et al. 2005; Lee, Choi et al. 2005; Wang, Lebowitz et al. 2008). Wang et al. reported that the targeted deletion of Rac1 in osteoclasts in both single and double knock-out models resulted in decreased osteoclastogenesis, whereas the deletion of Rac2 demonstrated little reduction (Wang, Lebowitz et al. 2008). However, these studies suggest an additive decrease, where the deletion of both Rac1 and Rac2 in DKO groups resulted in severe cellular defects, beyond those observed in single knock-outs. Contrary to these findings, we observed that Rac DKO groups did not demonstrate an additive defect beyond those observed in Rac1 single knock-out. Our data indicated reduced multinucleated osteoclast formation in DKO groups beyond single Rac1 and Rac2 deficient groups; however, these decreases did not persist with age. These differences may be attributed to the short duration of \textit{in vitro} osteoclastogenesis studies, compared to long term \textit{in vivo} reports. We report that DKO groups demonstrated
similar increases in bone quality to Rac1 deficient groups and that the deletion of both Rac1 and Rac2 did not result in an additive defect.

4.6 The deletion of both Rac1 and Rac2 in double knock-out (DKO) mice attenuates age related bone loss

Similar to single Rac1 and Rac2 deficient groups, we report that DKO groups also demonstrated the attenuation of age-related bone loss. Although we observed bone loss with aging in the WT groups, this decrease in bone quality was not seen in DKO groups suggesting that the deletion of both Rac1 and Rac2 attenuated the effects of aging on bone. In fact, bone material properties in DKO mice demonstrated improved trabecular architecture observed in both micro CT and strut analysis, as well as increased material properties as seen in bone mineralization. These improvements resulted in improved fracture resistance, indicating that the deletion of both Rac1 and Rac2 in DKO mice attenuated age-related bone loss and improved bone quality with age.

4.7 Conclusion

Using a previously described transgenic mouse model, we have described the effects of the deletion of Rac1 and Rac2 in osteoclast cells in vivo. In addition, by using baseline and aged groups, we have also determined their effects on bone quality with age. Our in vivo analysis of bone architecture and quality in Rac1 and Rac2 deficient mice demonstrate that the deletion of Rac1 protects bone quality, resulting in increased bone properties and preventing the bone loss observed with aging. The deletion of Rac2 compromised early bone quality, but demonstrated improved bone properties with age.
The deletion of Rac2 also attenuated age-related bone loss, but was less effective than the deletion of Rac1 as its improvements to bone quality were of a reduced magnitude. Further, we observed that the deletion of Rac2 in osteoclasts resulted in severely reduced bone formation, identifying Rac2 as a possible regulator of bone formation in osteoclasts. These findings help to improve our understanding of the mechanisms behind age-related bone loss and demonstrate the importance of Rac GTPases in the maintenance of bone homeostasis. Further investigation of these molecules may enable the development of targeted therapeutics to reduce the effects of aging on bone quality.
5.0 Conclusions
5.0 Thesis summary and conclusions

In this present study, we evaluated the effects of the deletion of Rac1 and Rac2 in osteoclasts on bone architecture and quality in male knock-out mice. By examining the effects on young and aged mice, we were able to observe the effects of age on bone quality, and assess the roles of Rac1 and Rac2 in this process.

Previous studies, and supported by in vitro studies from our lab, have established the importance of Rac GTPases in osteoclastogenesis and osteoclast function; however, the individual roles of Rac1 and Rac2 in vivo remain largely undefined. By altering osteoclast formation and activity, our findings suggest that the deletion of Rac may affect bone remodelling and turnover, thus affecting bone quality.

Objective 1: To determine the effects of the deletion of Rac1 and Rac2 in osteoclasts on bone mass, geometry and structure:

Our results confirm that in vivo, Rac GTPase deficient mice demonstrate decreased cortical bone geometry and improved trabecular bone architecture compared to WT controls (Figure 5.1). Our in vivo results support the in vitro conclusions that the deletion of Rac GTPases affects bone remodelling through the regulation of osteoclastogenesis and osteoclast activity.

Objective 2: To determine the mechanical properties of Rac1 and Rac2 deficient bones:
We have demonstrated that cortical bone were structurally weaker due to their smaller geometry, where as trabecular bone were structurally stronger due to improved trabecular architecture and connectivity. Further, Rac deficient mice demonstrated improved material properties compared to age matched WT controls.

**Objective 3: To determine osteoclast and bone formational parameters in Rac1 and Rac2 deficient bone:**

Rac deficient bones demonstrate increased bone quiescent surfaces indicating reduced osteoclast function compared to wild-type controls.

**Objective 4: To determine the effects of age in Rac1 and Rac2 deficient mice in vivo:**

With age, we observed that WT animals experienced deterioration in bone quality resulting in reduced bone architecture and weaker bones. However, Rac1 and Rac2 deficient mice were not affected by these decreases, suggesting that the deletion of Rac GTPases attenuates the bone loss associated with aging (figure 5.2). Bone loss associated with aging has been attributed by Cao et al to increased RANKL expression, resulting in increased osteoclast activation in aged individuals (Cao, Venton et al. 2003). As Rac1 and Rac2 are downstream members in the RANKL signalling pathway (Wherlock and Mellor 2002; Lee, Choi et al. 2005; Brazier, Stephens et al. 2006), we believe that the deletion of either Rac GTPase prevents the osteoclastic hyperactivity as seen in aged individuals, resulting in the attenuation of bone loss associated with aging.
These findings help to improve our understanding of the mechanisms behind age-related bone loss and demonstrate the importance of Rac GTPases in the maintenance of bone homeostasis. Further investigation of these molecules may enable the development of targeted therapeutics to reduce the effects of aging on bone quality.
Figure 5.1 Rac1 and Rac2 deletion improves bone quality.
The deletion of Rac1 and Rac2 in osteoclasts improved trabecular bone connectivity and fracture resistance compared with Wild-type controls.
Figure 5.2. Rac1 and Rac2 attenuates age-related bone loss.
With age, bone deterioration was observed in WT mice, resulting in decreased fracture resistance. These decreases in bone quality were not observed in Rac GTPase deficient mice, demonstrating that the deletion of Rac attenuated the aging effects of bone compared to WT controls.
6.0 Future work
6.0 Future investigations

Current work in our lab focuses on developing techniques to assess osteoclast activity in vitro. Although we have demonstrated reduced number of osteoclast resorption pits on dentine slices, the depth and extent of resorption by osteoclast has yet to be determined. Further data regarding the bone resorptive capacity of Rac deficient osteoclasts will provide further insight into the role of Rac GTPases on osteoclast function.

Further investigations will include the elucidation of other up-stream and down-stream intracellular signalling molecules to Rac that may be appropriate pharmacologic targets in the treatment of bone resorptive diseases.

Further investigation of these molecules may enable the development of targeted therapeutics to reduce the effects of aging on bone quality.
List of References


Appendix A
Appendix B