The role of Rac1 and Rac2 in determining bone quality in aged and osteoporotic female mouse models

by

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A thesis submitted in conformity with the requirements for the degree of Master of Science
Graduate Department of Dentistry
University of Toronto

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ABSTRACT

Thesis title: Determining the role of Rac1 and Rac2 in the bone quality of an aged and osteoporotic female mouse model
Joyce K. R. S. Magalhaes
Graduate department of Dentistry, University of Toronto, 2010
Degree: Master of Science

The osteoclasts, the bone cells responsible for bone degradation, have a crucial role in the age-related bone loss and post-menopause osteoporosis. Rac1 and Rac2, members of the Rho-family of small GTPases, are known for having a key role in osteoclast formation and activity, which could be translated to bone quality. In this study, we characterize the roles of Rac1 and Rac2 on bone quality using an aged and osteoporotic mouse model. Bones from wild type, Rac1KO and Rac2KO mice were harvested for mechanical tests, bone densitometry, micro-computed tomography and histomorphometric analyses to evaluate bone mineralization and architecture.

We observed that the deletion of Rac1 or Rac2 in pre-osteoclasts minimized bone loss in both age-related and post-menopause osteoporosis. These results highlight the importance of the two small GTPases in bone remodeling and identify Rac1 and Rac2 as potential targets for the development of new therapies for the treatment of osteoporosis.
ACKNOWLEDGEMENTS

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TABLE OF CONTENTS

ABSTRACT .................................................................................................................. ii

ACKNOWLEDGEMENTS......................................................................................... iii

TABLE OF CONTENTS............................................................................................ iv

LIST OF FIGURE AND TABLES............................................................................... vii

THEESIS FORMAT................................................................................................... ix

PUBLICATIONS FROM THESIS............................................................................... ix

AWARDS.................................................................................................................... x

ABREVIATIONS......................................................................................................... xi

CHAPTER 1 : INTRODUCTION ............................................................................... 1

1. Bone Biology ........................................................................................................ 2
   1.1 Bone Remodeling .............................................................................................. 3
   1.2 Bone Diseases .................................................................................................. 5
      1.2.1 Type I osteoporosis ...................................................................................... 6
      1.2.2 Type II osteoporosis ................................................................................... 7

2. Rac1 and Rac2 GTPases ....................................................................................... 7
   2.1 Rac GTPases and osteoclastogenesis ................................................................. 9

3. Bone Quality ......................................................................................................... 11
   3.1 Mechanical properties ...................................................................................... 12
   3.2 Material properties .......................................................................................... 13
      3.2.1 Bone Mineral Density ................................................................................. 13
   3.3 Structural properties ....................................................................................... 14
   3.4 Bone remodeling ............................................................................................. 14

4. Mouse model ......................................................................................................... 15
   4.1 Rac Knock out mouse model ........................................................................... 16

5 – Rac1 and Rac2: Possible targets to prevent the deterioration of bone quality in osteoporosis? ................................................................................................................ 17

CHAPTER 2: Determining the role of Rac1 and Rac2 in osteoclast mediated bone turnover, in vivo bone quality and age-related bone loss using a female mouse model

ABSTRACT ................................................................................................................ 19

INTRODUCTION ....................................................................................................... 21

MATERIALS AND METHODS .................................................................................. 23

   Animals.................................................................................................................... 23
   Bone mineral density (BMD) analysis................................................................. 24
   Mechanical testing............................................................................................... 24
CHAPTER 3: Deleting Rac1 and Rac2 improves vertebral bone quality in a murine osteoporosis model

ABSTRACT

INTRODUCTION

MATERIALS AND METHODS

Animals

Bone mineral density (BMD) analysis

Mechanical testing

Static and Dynamic Histomorphometric analysis

TRAP staining analysis

Back-scattered electron imaging

Micro-computed tomography

Statistical analysis

RESULTS

Rac1 and Rac2 deletion minimize the osteoporotic phenotype in vertebrae

CONCLUSION
Rac1KO osteoporotic vertebrae show more resistance to fracture compared to WT and Rac2KO

Rac2KO osteoporotic vertebrae have more trabecular bone compared to WT and Rac1KO

Osteoclasts in knock-out mice have less nuclei per cell and knock-out bones show lower bone formation rates

DISCUSSION

Osteoporotic Rac knock-out mice model

The deletion of Rac1 increases vertebral bone quality and attenuates the estrogen-deprivation-related bone loss

The deletion of Rac2 minimized the trabecular bone loss induced by osteoporosis

The deletion of Rac1 and Rac2 improve trabecular bone quality in an osteoporotic model in different way

CONCLUSION

CHAPTER 4: CONCLUSION

THESIS SUMMARY AND CONCLUSION

REFERENCES
LIST OF FIGURES AND TABLES

CHAPTER 1: Introduction

Figure 1.1: Bone remodeling cycle .................................................. 5

Figure 1.2: Rho activation and inactivation cycle ............................... 8

Figure 1.3: Relationship between bone quality and bone properties ........ 12

CHAPTER 2: Determining the Role of Rac1 and Rac2 in osteoclast mediated bone turnover, in vivo bone quality and age-related bone loss using a female mouse model

Figure 2.1: Bone Mineral Density (BMD) of whole body and isolated bones.. 29

Figure 2.2: Histograms of grey level generated at BSE .......................... 31

Figure 2.3: Bone formation parameters evaluated by static histomorphometry 34

Figure 2.4: Trabecular architecture analyzed at strut analysis ................. 36

Figure 2.5: Representative images of osteoclasts in TRAP stained slides...... 37

Table 2.1: Back-scattered electron microscopy (BSE) of 5th lumbar vertebrae ............................................................................................................. 30

Table 2.2: Bone mechanical properties from 3-point bending test .............. 32

Table 2.3: Bone mechanical properties from vertebral compression ........... 33

Table 2.4: Micro-computed tomography of vertebrae ............................. 35

Table 2.5: TRAP staining analysis .......................................................... 37

Table 2.6: Dynamic Histomorphometry ................................................ 38
CHAPTER 3: Deleting Rac1 and Rac2 improves vertebral bone quality in a murine osteoporosis model

Figure 3.1: Bone Mineral Density (BMD) of whole body and isolated bones... 57

Figure 3.2: Trabecular architecture analyzed at strut analysis....................... 60

Figure 3.3: Representative images of osteoclasts in TRAP stained slides ....... 63

Figure 3.4: Bone formation parameters evaluated by static histomorphometry... 63

Table 3.1: Bone mechanical properties from vertebral compression............. 59

Table 3.2: Bone mechanical properties from 3-point bending test ............... 60

Table 3.3: Micro-computed tomography of vertebrae .............................. 61

Table 3.4: Dynamic Histomorphometry................................................. 62

CHAPTER 4: Conclusion

Figure 4.1: Aged Model ........................................................................ 73

Figure 4.2: Osteoporotic Model .............................................................. 75
THESIS FORMAT

This Master of Science thesis is presented in the publishable style. Each of the chapters describing experimental procedures has been submitted for publication and they are presented in their original form. An introduction and thesis summary are included to contextualize the experimental results with our current knowledge. The contributions made by the collaborators are noted before each chapter. Those who provided technical support are listed in the acknowledgements section.

PUBLICATION FROM THE THESIS CHAPTERS


AWARDS

2009

Second place winner – Basic Science Category
Faculty of Dentistry Research Day
University of Toronto

2009

Investigator of the month – September 2009
Dental research institute
Faculty of Dentistry, University of Toronto

2008

University of Toronto Fellowship

2008

Graduate department of Dentistry
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>BMD</td>
<td>bone mineral density</td>
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<tr>
<td>BMM</td>
<td>bone marrow macrophage</td>
</tr>
<tr>
<td>BFR</td>
<td>bone formation rate</td>
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<tr>
<td>BSE</td>
<td>back-scattered electron</td>
</tr>
<tr>
<td>DEXA</td>
<td>Dual Energy X-ray Absorptiometry</td>
</tr>
<tr>
<td>FWHM</td>
<td>Full width at half the maximum height</td>
</tr>
<tr>
<td>KO</td>
<td>Knock-out</td>
</tr>
<tr>
<td>MS</td>
<td>mineralizing surface</td>
</tr>
<tr>
<td>%MS</td>
<td>percentage of mineralizing surface</td>
</tr>
<tr>
<td>MAR</td>
<td>mineral apposition rate</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.BS.</td>
<td>number of osteoclasts per bone surface</td>
</tr>
<tr>
<td>N.Oc.OcS</td>
<td>number of osteoclasts per osteoclasts surface</td>
</tr>
<tr>
<td>Oc.S.</td>
<td>osteoclast surface</td>
</tr>
<tr>
<td>% Oc.S.</td>
<td>percentage of osteoclast surface</td>
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<tr>
<td>OS</td>
<td>osteoid surface</td>
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<tr>
<td>OV</td>
<td>osteoid volume</td>
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<td>O.Th.</td>
<td>osteoid thickness</td>
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<tr>
<td>RANKL</td>
<td>receptor activator of NF-κB ligand</td>
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<td>Rac1KO</td>
<td>Rac1 knock-out</td>
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<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>Rac2KO</td>
<td>Rac2 knock-out</td>
</tr>
<tr>
<td>ROS</td>
<td>reactive oxygen species</td>
</tr>
<tr>
<td>TBV</td>
<td>trabecular bone volume</td>
</tr>
<tr>
<td>Tb.Th.</td>
<td>trabecular thickness</td>
</tr>
<tr>
<td>Tb.N.</td>
<td>trabecular number</td>
</tr>
<tr>
<td>Tb.Sp.</td>
<td>trabecular separation</td>
</tr>
<tr>
<td>TRAP</td>
<td>Tratrate-resistant acid phosphatase</td>
</tr>
<tr>
<td>TNF</td>
<td>tumoral necrosis factor</td>
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<tr>
<td>WT</td>
<td>wild-type</td>
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CHAPTER 1: Introduction
1 - Bone Biology

Bone is a complex composite material consisting of minerals embedded in an organic matrix. The mineral component of bone, which represents approximately 65% of bone material, is formed by small, impure, poorly crystalline and highly substituted hydroxyapatite crystals (Ca$_{10}$(PO$_4$)$_6$(OH)$_2$). The bone mineral phase largely contributes to the overall strength and stiffness of bone (Cowin, 2001). The organic matrix, which represents approximately 35% of bone material, includes proteins, cells and water (Cowin, 2001). Around 90% of bone organic matrix consists of type I collagen that provide elasticity and toughness to bone, and the remaining 10% consist of various non-collagenous proteins that are important to regulate mineral deposition and maturation (Cowin, 2001).

Bone is constantly being remodeled in response to mechanical forces and chemical mediators like hormones. This continuous remodeling activity guarantees the integrity and the bone quality important to preserve the skeleton through life. Bone remodeling is mainly mediated by the activities of osteoblasts and osteoclasts, leading to bone formation and/or resorption respectively. Any unbalance in these physiological mechanisms can lead to a variety of bone disorders (Mundy, 1995). The better understanding of the mechanisms involved in bone remodeling activity is essential to comprehend the pathophysiology of a variety of bone disorders, which is the first step toward the development of efficient and safe treatments. In the next topics, I will describe the mechanisms involved in bone remodeling and the diseases that are associated to defects in bone remodeling.
1.1 Bone Remodeling

The balance between mineral and organic phases of bone is maintained through a dynamic process known as bone remodeling or turnover. This phenomenon is continuously happening in the skeleton and it is responsible for a complete turnover of an adult human skeleton every 7-10 years (Rosen, 2003). Several studies have reported the role of multiple genes in bone remodeling process (Rosen et al., 2001; Turner et al., 2000).

The bone remodeling cycle (figure 1.1) starts with bone resorption by osteoclasts recruited through a signaling pathway. Osteoclasts are large, multi-nucleated cells that originate from hematopoietic stem cells and once activated they resorb bone through two mechanisms. The secretion of hydrogen (H+) ions dissolve the mineral phase, while the organic matrix is broken down by cathepsins (Cowin, 2001). Although the exact signaling pathways involved in the recruitment of osteoclasts is not completely clear, the understanding of osteoclast differentiation and activation has advanced considerably. Signaling by the receptor activator of NF-κB (nuclear factor-κB) ligand (RANKL) is known to be a key factor stimulating the differentiation and activation of osteoclasts and, therefore, is essential for bone remodeling (Suda et al., 1999). Moreover, the RANKL stimulation of bone marrow monocyte-macrophage lineage (BMM) cells transiently increases the intracellular level of reactive oxygen species (ROS) through a signaling cascade involving TNF (tumor necrosis factor) receptor-associated factor (TRAF) 6, Rac1 and NADPH (nicotin-amide adenine dinucleotide phosphate) oxidase (Nox)1 (Lee et al., 2005). The increased level of ROS acts as an intracellular signal mediator for...
osteoclast differentiation and activation (Lee et al., 2005). Once the resorption is complete, osteoblast are recruited to start bone formation. Osteoblasts originate from mesenchymal lineage and once activated they start bone formation by depositing unmineralized collagenous bone matrix, which is also known as osteoid (Cowin, 2001). Following this step, several non-collagenous proteins are involved in the initiation of late bone formation, known as mineralization stage. At this stage, mature osteoblasts are recruited and start to deposit minerals (Cowin, 2001). After mineralization, the bone reaches a quiescent stage (resting bone) and osteoblasts remain inactive. The inactive osteoblasts may remain on the quiescent bone surface, undergo apoptosis or may be embedded within the new bone matrix and become osteocytes. Osteocytes are the most abundant cell type in mature bone and they are believed to be responsible to send signals to initiate the remodeling cycle when microcracks or microdamages accumulate in bone (Cowin, 2001). Any alteration in the balance between the remodeling actions results in severe bone disorders (Mundy, 1995), and this explains the motivation to investigate and clarify the mechanisms involved in this process.
1.2 – Bone Diseases

Several bone disorders have been identified as a result of dysfunctions or imbalances in bone remodeling. Osteoporosis is a bone disorder characterized by compromised bone integrity, increased vulnerability to fractures that impairs person’s quality of life, and increased mortality (Center et al., 1999). This disorder represents a major public health issue of the modern society due to its impact in human physical abilities and consequently high costs to the healthcare system and national economy. It is estimated that 10 million people are affected by osteoporosis in the United States of America, being 80% women, and the disease is responsible for more than 1.5 million fractures per year, which means national care cost reaching $18 billion yearly (Owens et al., 2007). This condition is a result of a negative bone balance due to an increased osteoclast activity relative to the bone forming capacity of osteoblasts, leading to skeleton degradation. There are two forms of osteoporosis, the type I is known as postmenopausal...
osteoporosis, characterized by estrogen deprivation and excessive and trabecular bone loss mainly associated with vertebral fractures; and type II osteoporosis, known as age related osteoporosis, characterized by a gradual age-related bone loss that affects both men and women (Riggs et al., 1982).

1.2.1 – Type I osteoporosis

Type I osteoporosis, also known as post-menopausal osteoporosis, occurs when the arrest of ovarian function leads to a natural depletion of estrogen production in women followed by an increased rate of bone loss, especially in trabecular bone (Marx, 1980; Purohit and Reed, 2002; Turner et al., 1994). The estrogen deprivation characteristic of postmenopausal phase in women has been reported not only to increase TNF production in the immune system, leading to osteoclastogenesis stimulation, but also to increase osteoclasts’ lifespan by inhibiting their apoptosis (Hughes et al., 1996; Weitzmann and Pacifici, 2006). Those combined processes lead to an overexpression of osteoclasts and an unbalance in bone remodeling. The loss of trabecular connectivity is a consequence of the accelerated bone loss, resulting in compromised structural integrity and increasing the risk of bone fracture (Manolagas et al., 2002; Riggs et al., 1982). While the exact mechanisms behind postmenopausal osteoporosis are not yet defined, the strong connection between ovarian hormones and the skeleton is well established. The estrogens play an integral role in reproduction, sexual dimorphism of the skeleton and maintenance of bone mass. It is also accepted that estrogen levels strongly influence internal and external bone architecture (Bouxsein et al., 2005; Laib et al., 2001).
Although the exact mechanisms of estrogen action on bone at the cellular level are still unclear, estrogen has been shown to decrease bone remodeling (Turner et al., 1994).

1.2.2 – Type II osteoporosis

Type II osteoporosis, also known as age-related osteoporosis, is characterized by an increased bone fragility and susceptibility to fracture in aged individuals. Several studies have demonstrated the effects of age in the differentiation and function of osteoblasts, describing a decrease in the osteoprogenitor pool of the bone marrow with advanced age (Bergman et al., 1996; Jilka et al., 1996). Another important and characteristic feature of the aging bone marrow is a shift from osteoblastogenesis to adipogenesis, leading to the accumulation of bone marrow fat and decline in osteoblast formation (Ducy et al., 1997; Moerman et al., 2004). Moreover, several authors believe that age-related bone loss in mice is associated with an increase in the osteoclast progenitor pool possibly related to increased mRNA expression of RANKL (Cao et al., 2005; Perkins et al., 1994). The combination of these factors leads to an uncompensated bone resorption and decreasing bone mass typically found in age-related osteoporosis, a bone disorder that affects both men and women (Bar-Shira-Maymon et al., 1989; Halloran et al., 2002).

2 – Rac1 and Rac2 GTPases

During the past 15 years, substantial data have led to the description of the roles of Rho small GTPase family members in cytoskeleton reorganization (Bokoch, 1995;
The Rho GTPase family has 3 major members, Cdc42, Rho and Rac, and at least 10 other proteins. These 20 – 30 kD GTPases were first discovered in the late 1980s by the analysis of small molecular weight proteins that bind guanosine-5’-triphosphate (GTP) (Spiegel, 1987). Afterwards, it was concluded that these molecules had a particular behavior, cycling between inactive GDP and active GTP-bound conformations, inferring that they functioned as ‘molecular switches’ in the transduction of signals originating from cell-surface receptors (figure 1.2) (Hall et al., 2002; Takai et al., 2001).

**Figure 1.2: Rho activation and inactivation cycle.** In an initial stage, Rho GDI remains attached to the C-terminus of a Rac molecule, inhibiting GDP exchange and maintaining its cellular localization. In a second step (2) towards signal activation, a GEF promotes a GDP-GTP exchange. During the GTP-bound state, Rac is activated and changes are observed in the Switch 1 (S1) and Switch 2 (S2) regions. Upon GAP stimulation (4), Rac hydrolyses GTP into GDP, returning to the initial GDP-bound inactive state. Adapted from Hall et al., 2002.
During the past few years, supported by newly available data on signaling pathways and the development of new techniques such as the use of transgenic mice (Glogauer et al., 2003; Li et al., 2002), scientists focused their efforts directly on the comprehension of the cytoskeletal dynamics and the signaling pathways related to efficient cell migration, chemotaxis and cell differentiation. The small GTPases have important functions in a wide range of biological processes, regulating essential cellular functions, cytoskeleton reorganization, cell growth, gene transcription, motility, adhesion, and production of reactive oxygen species (ROS) (Hall, 1992; Hall et al., 2002; Takai et al., 2001). The Rac subgroup of small GTPases is formed by three main isoforms: Rac1, Rac2 and Rac3. Rac1 is ubiquitously expressed while Rac2 expression is restricted to cells of the hemapoietic lineage. Rac1 and Rac2 share 92% amino acid identity with the major divergence occurring in the C terminus (Glogauer et al., 2003).

2.1 – Rac GTPases and osteoclastogenesis

Since 1970’s, when Walker first demonstrated that the bone resorptive cells in mice come from hematopoietic lineage (Walker, 1975a; Walker, 1975b), the osteoclasts have been extensively studied in order to better comprehend the signaling mechanisms involved in bone degradation. In 1990’s, it was established the monocyte-macophoge lineage origin of osteoclats (Suda et al., 1992; Udagawa et al., 1990). Further studies focused on identifying the osteoclastogenic cytokines and demonstrated the major importance of RANKL in promoting osteoclast differentiation. As a member of Tumoral Necrosis Factor (TNF) superfamily, the receptor activator of NF-κB ligand (RANKL) is a protein expressed on osteoblasts and stromal cells’ membrane and it activates the integral
membrane receptor RANK on bone marrow macrophages (BMM), leading to osteoclast differentiation (Dougall et al., 1999; Lacey et al., 1998; Yasuda et al., 1998). The signaling pathways activated upon RANKL activation are tightly regulated by members of the small GTPases (Coxon and Rogers, 2003; Lee et al., 2006; Lee et al., 2005). Lee et al have found a key role of Rac1 in stimulating a physiological production of reactive oxygen species (ROS) in macrophages and monocytes through the RANKL-TRAF6-Rac1-NADPH axis, a crucial step in osteoclastogenesis (Lee et al., 2005). The small GTPases have been shown to be indispensable in the formation of actin rings and resorption lacunae in mature osteoclasts (Razzouk et al., 1999). Additionally, further reports have demonstrated the importance of Rac1 in mediating survival signaling of osteoclasts and in regulating their bone resorptive activity (Fukuda et al., 2005). Recently, Wang et al, used a transgenic mouse model to confirm that Rac1 is the primary Rac isoform regulating ROS production and the cytoskeleton organization during the complex process of osteoclastogenesis (Wang et al., 2008). Although substantial progress is being made at the molecular level, a detailed analysis of the in vivo bone characteristics still needs to be performed to identify the role of Rac GTPases in bone remodeling cycle. The analysis of bone properties in Rac knock-out mice enables the investigation of how these small GTPases affect bone quality, especially in scenarios when the bone homeostasis is shifted toward bone resorption such as in age-related and post-menopausal osteoporosis.
3 – Bone Quality

Bone quality is defined by mechanical, structural and material properties regulated by the remodeling activity. Although the bone mineral density (BMD) measurement has been traditionally used for bone disorder diagnosis, several studies have shown that it is insufficient to assess bone quality only by evaluating bone density (Jepsen et al., 2003; Nielsen, 2000; Ott, 1993). Ideally, an overall bone evaluation is needed, combining density with underlying material and structural properties (Boskey et al., 2003). The flowchart presented in figure 1.3 summarizes the relationship between the bone properties that determine bone quality. As observed in figure 1.3, all of the underlying bone properties are directly or indirectly connected to bone quality. Following this idea, the bone remodeling affects both material and structural bone properties and both of them directly affect bone mechanical properties, which ultimately determine bone quality. The material properties refer to the bone composition and mineralization and the structural properties refer to trabecular connectivity and architecture. The complex interactions of underlying bone properties are more evident upon consideration of theories of bone adaptation. According to Wolf’s Law, bone adapts or arranges itself into a structure resulted from the most efficient distribution of load (Martin, 1993). The following sections will define the analyses used to characterize bone properties.
3.1 – Mechanical Properties

Measurement of mechanical properties through mechanical testing of whole bone specimen is useful but only feasible in animal models. Several mechanical tests are performed to determine the overall skeletal fragility, which leads to bone fractures. Typically three-point bending is performed to evaluate cortical bones and vertebral compression is useful to evaluate trabecular bones mechanical properties. From these mechanical tests, the failure load, deformation, energy to failure and stiffness of bone are measured to determine the structural mechanical properties. When the results are normalized to bone cross-sectional area, the material mechanical properties such as ultimate stress, failure strain, toughness and modulus are determined.
3.2 – Material Properties

The bone material properties are determined by composition and quality of both mineral and organic bone phases. The bone mineral composition contributes more to the mechanical properties than the bone organic content (Burr, 2002). In terms of bone mineralization distribution, it has been shown that the high mineralization is associated to increased strength (Cowin, 2001; Grynpas, 1993; Turner et al., 2000). The quality of the organic phase has been associated with toughness of bone (Wang et al., 2002). Reduced collagen cross-linking and disordered arrangement of collagen fibers have been correlated with reduced mechanical properties (Jepsen et al., 1997). Aged bones suffered drastic changes in material properties in both phases which determine an increased skeletal fragility of aging bones (Martin, 1993). The degree of bone mineralization has been shown to increase with age due to reduced new bone formation and an increase in secondary mineralization (Grynpas, 1993). This increase in mineralization increases the stiffness component, thereby helping to explain the increase in brittleness of bone with age.

3.2.1 – Bone Mineral Density

Bone mineral density (BMD) is a measure of the amount of bone mineral present in a given bone area. Measures of bone mineral density (BMD) have been demonstrated to explain a substantial portion of the risk of osteoporotic fractures (Faulkner et al., 1993; Melton et al., 1993). It is well established that BMD is strongly associated with bone resistance to fracture (Leichter et al., 1982; Marshall et al., 1996), however it is only one of the many factors that contribute to bone strength (Cheung and Detsky, 2008) and it is
limited for not providing information on bone geometry, architecture and microstructure. The assessment of BMD is a non-invasive, repeatable measurement that has traditionally been considered the gold-standard in clinics for diagnosis of bone disorder and predicting bone fracture.

3.3 – Structural Properties

Bone quality largely depends on how the bone is structurally organized. The structural properties of cortical bones depend on cortical thickness, cross-sectional area and moment of inertia. Typically bones with larger cross-sectional area require more load to be fractured (Turner et al., 2000). However it is important to consider how the bone mass is distributed in relation to the direction of the applied force (i.e. moment of inertia). In trabecular bones, the structural properties highly influence bone quality. Trabecular structure is determined by the overall microarchitecture of the trabecular bone, as well as the trabecular connectivity. As expected, an increased number of thicker, less separated and highly connected trabeculae are associated with more resistance to fracture (Thomsen et al., 2002).

3.4 – Bone Remodeling

The amount of remodeling activity is usually measured by static and dynamic histomorphometry on sections of bone tissue. New bone formation is related to increased bone toughness, due to the increased amount of unmineralized organic matrix (Viguet-Carrin et al., 2006). Altered rates of bone resorption are related to decreased failure strength and stiffness, expressed in metabolic disorders such as defective resorption of
osteopetrosis (de Vernejoul and Benichou, 2001) and excessive resorption of post-menopausal osteoporosis (Sommerfeldt and Rubin, 2001). With aging, metabolic processes tend to slow down and bone remodeling rates tend to decrease, which is observed in age-related osteoporosis (Halloran et al., 2002). After peak bone mass is reached, bone resorption exceeds bone formation, and this imbalance leads to an overall bone loss with age, which could result in compromised bone quality.

4 – Mouse Model

To investigate bone disease, an animal model is essential. The use of an animal model allows effective in vivo testing in a controlled environment and enables the study of the etiology and underlying mechanisms of diseases in genetically modified animals. In particular, the mouse model is extensively used because of its genetic, practical and experimental utility. Mice share 60-70% genetic similarity with humans, and therefore are an useful model to study genetic determinants of human disease (Rosen et al., 2001). By knocking out single genes it is possible to alter certain bone physical characterization and create a bone phenotype. Although very practical to be studied, there are some disadvantages of using a mouse model. A major disadvantage of the mouse model is the difference in skeletal system between mice and humans. Mice are devoid of Haversian system (Jee and Yao, 2001) and therefore lack substantial intracortical remodeling. Moreover, the significant small size of their skeleton could compromise the techniques used to assess bone quality, which could be a challenge to generate precise and repeatable results.
Mouse ovariectomy (OVX) has proven to successfully simulate menopause in female. Generally, mice experience a rapid loss of trabecular bone following OVX, and this loss can be prevented by estrogen therapy (Bain et al., 1993). Usually after ovariectomy, bone mechanical properties are affected and there is a decrease in stiffness and load to failure, more evident in trabecular bone (Fonseca and Ward, 2004). Moreover, similar to the metabolic effect observed in humans, the rate of bone remodeling in mice is affected after ovariectomy. Ovariectomized mice experience a significant increase in bone formation rate, although at a slower rate than the increase in bone resorption (Daci et al., 2000).

4.1 – Rac knock-out mouse model

The development of transgenic mice with neutrophils and monocytes lacking either Rac1 or Rac2 has allowed us to dissect the specific functions of these molecules in the regulation of different cellular processes (Glogauer et al., 2003). The Rac1 knockout (KO) mouse used in this thesis was generated by using a conditional Rac1c/-LysMcre in which the Cre-recombinase was expressed under the control of the murine lysozyme M gene regulatory region. This approach generated Rac1 deletion in neutrophils at birth (Glogauer et al., 2003). The Rac2 KO was described by Roberts et al. (Roberts et al., 1999). After crossbreeding with Rac1KO, the resulting offspring enabled the generation of neutrophils deficient in either Rac1 or Rac2 (Roberts et al., 1999). This transgenic mice model enables the investigation of in vivo bone quality in the absence of the small GTPases Rac1 and Rac2 in the hematopoietic cell lines, which will enhance the insight of the importance of these GTPases in bone physiology.
5 – Rac1 and Rac2: Possible targets to prevent the deterioration of bone quality in osteoporosis?

As previously noted, many reports have demonstrated the importance of Rac1 and Rac2 in osteoclast formation and activity (Fukuda et al., 2005; Razzouk et al., 1999; Wang et al., 2008). Following this concept, we hypothesize that the deletion of Rac1 or Rac2 will compromise the osteoclast activity and bone resorption in these animals, and will affect the bone quality in RacKO mice. In the next chapters, I will describe experiments that confirm that the compromised osteoclast activity in RacKO mice (Wang et al., 2008) attenuates the effects of age and hormone depletion in bone quality. Taken together, these results provide some important information toward the full understanding of the roles of small GTPases in bone remodeling and may lead to the development of new targets for anti-osteoporosis treatments.
CHAPTER 2: Determining the Role of Rac1 and Rac2 in osteoclast mediated bone turnover, in vivo bone quality and age-related bone loss using a female mouse model

Joyce K. R. S. Magalhaes, Marc D. Grynpas and Michael Glogauer
ABSTRACT

Rho small GTPases are important regulators of a variety of cell functions, including osteoclast differentiation. Recent evidence has suggested a key role for the Rac1 and Rac2 small GTPases in osteoclastogenesis. In order to elucidate the specific roles of the Rac1 and Rac2 isoforms in bone remodeling activity as well as age-related bone loss, we investigated bone quality in female young and aged mice in which Rac1 or Rac2 have been deleted in monocytes. Methods: 4-month old and 1-year old female mice from 3 groups – wild type (WT), Rac1 null (Rac1KO) and Rac2 null (Rac2KO) were studied. After sacrifice, the bones were harvested for mechanical tests, bone densitometry and histomorphometric analyses. In addition, back-scattered electron imaging (BSE) and micro-computed tomography were used to evaluate bone mineralization and architecture. The results were compared between groups using ANOVA and LSD post hoc test. Results: we observed that both young and aged Rac1KO cortical bones showed higher bone mineral density (BMD) and young Rac1KO femurs and aged Rac1KO vertebrae showed higher mechanical properties compared to control. Higher mineral density was also observed in Rac1KO trabecular bone in both young and aged group, and they showed increased trabecular connectivity and structural parameters such as trabecular bone number and volume compared to the controls. In contrast, young and aged Rac2KO showed lower BMD in both cortical and trabecular bones compared to Rac1KO. Rac2KO bones were less mineralized and displayed lower structural properties in the mechanical tests but no difference was found when material properties were considered. Consistent with our previous in vitro data from these mice, the Rac knock-out samples showed lower
numbers of multi-nucleated osteoclasts (osteoclasts with more than three nuclei) which suggest lower bone resorptive efficiency. Conclusion: our results demonstrate that the differences observed between young groups persisted after aging and confirm higher bone quality in knock-out bones compared to control at different stage of life. We conclude that when Rac1 or Rac2 are eliminated net osteoclast activity is reduced leading to a reduction in the normal bone resorption typically observed in aging bones. Our results suggest that Rac1 and Rac2 are key regulators of bone homeostasis and the pathways they regulate may be important targets for future therapeutics aimed at regulating bone resorption during aging.
INTRODUCTION

Bone is a dynamic tissue that is constantly being remodeled by osteoblasts, the bone cells responsible for bone formation, and by osteoclasts, the bone cells responsible for bone resorption (Mundy, 1995). Any unbalance in these two physiological mechanisms can lead to a variety of bone disorders. More specifically, the osteoclasts, due to its bone degrading activity (Roodman, 1996), have crucial role in the pathophysiology of numerous skeletal disorder including age-related bone loss, a physiological condition characterized by a decreased osteoblastogenesis that compromises the compensation of bone lost during bone resorption (Khosla and Riggs, 2005).

Over the past 30 years, osteoclasts have been extensively studied in order to better understand the signaling mechanisms involved in bone turnover (Walker, 1975a; Walker, 1975b). In the current literature, it is well established that the Rho-family of small GTPases, including Rho, Rac and Cdc42, have a critical role in regulating the actin cytoskeleton reorganization in a variety of cells (Fenteany and Glogauer, 2004; Hall, 1998; Ridley et al., 1992). The Rho GTPases Rac1 and Rac2 have been shown to be indispensable in the formation of actin rings and resorption lacunae in mature osteoclasts (Razzouk et al., 1999). Fukuda et al have reported the importance of Rac1 in mediating survival signaling of osteoclasts and in regulating their bone resorptive activity (Fukuda et al., 2005). Additionally, Wang et al used a transgenic mouse model to show that Rac1 is the principal Rac isoform involved in the regulation of reactive oxygen species production and the cytoskeleton organization during osteoclastogenesis (Wang et al., 2008). Despite the high molecular similarity between Rac1 and Rac2, previous
literature suggests that these two isoforms have different roles during actin remodeling (Sun et al., 2007), which triggers the question whether these two small GTPases play different roles in osteoclast differentiation and function.

Although substantial progress is being made at the molecular level regarding the role of Rac small GTPases in osteoclast differentiation and function, in vivo studies using animal models still need to be performed to analyze the specific roles of Rac isoforms in bone quality. In order to explore the role of the Rho GTPases in bone properties we used a transgenic mouse model deficient in Rac1 or Rac2 to analyze the specific role of each Rac isoform (Glogauer et al., 2003). As the bone’s characteristics result from the interplay between genetics and environmental factors (Judex et al., 2004; Slemenda et al., 1992), the genetically manipulated mouse model represents an important in vivo model for studying the genetic contribution to bone mass and turnover in a controlled environment.

Age-related bone loss is the primary underlying cause of fractures in elderly (Raisz, 2005) when there is reduced bone turnover, enhanced bone fragility and increased fracture risk due to the reduced bone formation and uncompensated bone resorption (Chan and Duque, 2002). The reported role of Rac GTPases in osteoclast formation led us to determine if these signaling intermediates are potential targets for preventing age-related bone loss. In order to investigate this hypothesis it is important to differentiate the roles of Rac1 and Rac2 in bone homeostasis.

In this study, we characterize the effects of the small GTPases Rac1 and Rac2 on bone quality using a young and aged female mouse model. To perform a complete analysis of bone quality, mechanical, structural and material bone properties were quantified and analyzed in association with bone densitometry and histomorphometry.
These *in vivo* studies show that the knock out of either of the Rac isoforms attenuated the aging-related bone loss. While the data shows that Rac1 plays a more significant role in osteoclast resorptive activity, Rac2 may play an important role in bone formation.

**MATERIAL AND METHODS**

*Animals*

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. Previously generated and characterized mice in which Rac1 was selectively deleted in neutrophils and monocytes (Sun et al., 2004), Rac1<sup>−/−</sup>LysM<sup>cre</sup>, were bred with Rac2<sup>−/−</sup> mice (Roberts et al., 1999) and the resulting offspring were bred over at least 6 generations to generate optimal breeding pairs (Rac1<sup>−/−</sup>LysM<sup>cre</sup>Rac2<sup>+/−</sup> x Rac1<sup>−/−</sup>LysM<sup>cre</sup>Rac2<sup>+/−</sup>), which enabled the generation of Rac1KO (Rac1<sup>−/−</sup>LysM<sup>cre</sup>Rac2<sup>+/−</sup>), Rac2KO (Rac1<sup>+/−</sup>LysM<sup>cre</sup>Rac2<sup>−/−</sup>), DKO (Rac1<sup>−/−</sup>LysM<sup>cre</sup>Rac2<sup>−/−</sup>), and wild-type mice (Rac1<sup>+/−</sup>LysM<sup>cre</sup>Rac2<sup>+/−</sup>) from the same litters, as previously described (Wang et al., 2008). This breeding strategy allows for the controlling of background variations. Genotyping for Rac1, Rac2, and LysM alleles was carried out as described previously (Glogauer et al., 2003; Roberts et al., 1999). All experiments were performed on 74 female mice divided into two groups: the young group (base-line data) with 42 4-month old mice (15 wild type, 12 Rac1 KO, 15 Rac2 KO) and the aged group with 32 1-year old mice (10 wild type, 11 Rac1 KO, 11 Rac2 KO). Although we started with 15 mice for each phenotype per group, some mice were lost prematurely due to infections. Any animal that presented any sign of infection was eliminated from the study. Three and ten
days before euthanasia the mice were injected intra-peritoneally with calcein-green (30mg/Kg), a bone marker important for bone dynamic histomorphometric analysis. Animals were sacrificed by CO₂ asphyxiation at four-month and 1 year of age.

**Bone Mineral Density (BMD) analysis**

Dual energy x-ray absorptiometry (DEXA) was performed using an animal PIXIImus densitometer (Lunar; GE Copr.) for the whole animal body right after euthanasia and for the isolated femur and lumbar vertebrae after dissection for determination of bone mineral density (BMD) and bone mineral content (BMC). To perform the analysis, a consistent region of interest was selected to maintain uniformity between the samples.

**Mechanical testing**

Mice’s right femurs were tested in three-point bending to evaluate the mechanical properties of cortical bones. The sixth lumbar vertebrae were tested in compression to evaluate the properties of trabecular bones.

Three-point bending and vertebral compression were performed using an Instron 4465 materials testing machine (Instron Canada Inc.). A pre-load of less than 1 N was applied to establish the samples’ contact with the upper device. Further load was applied by an 100N cell load at a speed of 1mm/min and load versus time data were collected every 0.1 seconds by LabView data acquisition software (National Instruments Corp.; Austin, TX) until the sample failure. Based on the speed and time, displacement was automatically calculated and a load-displacement graph was generated to evaluate bones’
structural mechanical properties such as ultimate load, failure displacement, energy to failure and stiffness. Afterwards, data was normalized to the bone cross-section area and a stress-strain graph was generated to evaluate bones’ material properties such as ultimate stress, failure strain, toughness and modulus.

*Static and Dynamic Histomorphometric analysis*

The fifth lumbar vertebrae were isolated from each animal and fixed in 70% ethanol. Samples were dehydrated in ascending concentrations of acetone followed by ascending ratios of unpolymerized spurr resin and acetone. Afterwards, bones were embedded in block of spurr resin and left to polymerize in a 60 C oven for 48 hours. Using a semiautomatic microtome (Leica RM 2265), three 5-micron thick coronal sections were cut from each samples and placed on gelatinized slides for Goldner’s trichrome staining (Holmes et al., 2007) and one 7-micron thick coronal section were cut, placed on gelatinized slide and kept unstained for dynamic histomorphometric analysis. Trabecular bone was analyzed using a 25x objective lens connected to a video camera (Retiga 1300). Serial fields using the Leitz Bioquant morphometry system (Bioquant Nova Prime version 6.50.10) were analyzed from each sample to determine the following static histomorphometric parameters: trabecular bone volume (TBV), trabecular thickness (Tb.Th.), trabecular number (Tb.N.) and trabecular separation (Tb.Sp.); and the formation parameters such as osteoid volume (OV), osteoid surface (OS) and osteoid thickness (O.Th.). Dynamic histomorphometry was done using fluorescence microscopy to measure the bone labels generated by the calcein-green injected before the animal euthanasia. The single and double calcein-green labels were measured on bone trabeculae.
to calculate mineralizing surface (MS), percentage mineralizing surface (%MS), mineral apposition rate (MAR) and bone formation rate (BFR) on unstained slides. All these parameters are in accordance with the histomorphometric nomenclature and definition of the American Society of Bone Mineral Research (ASBMR) (Parfitt et al., 1987).

**TRAP staining analysis**

The fourth lumbar vertebras were isolated from each animal and fixed in 4% paraformaldehyde (PFA). The samples were decalcified and embedded in wax. Three 5-micron thick coronal sections were done in each sample and placed in glass slides for Tartrate-Resistant Acid Phosphatase (TRAP) staining and hematoxilin counterstaining. The osteoclasts selectively express and stain positive for the TRAP enzyme. The Leitz Bioquant morphometry system was used to quantify the number of nuclei per osteoclast, osteoclasts surface (Oc.S.), percent osteoclasts surface (%Oc.S.), number of osteoclasts per bone surface (N.Oc.BS) and number of osteoclasts per osteoclasts surface (N.Oc.OcS).

**Back-scattered electron imaging**

The fifth vertebrae, embedded in spurr resin blocks, previously used for static histomorphometric analysis were then polished, carbon-coated and imaged using backscattered electron (BSE) imaging. To evaluate the mineralization distribution in each sample, the backscattering of the mineralized tissues was compared to a standard between each sample and histograms of the grey level distribution were created. From each histogram, the peak of mineralization value was determined to evaluate the overall degree
of mineralization from each sample. Higher the grey level of the histogram, more mineralized is the sample. The full width at half the maximum height (FWHM) of the histogram was also determined to evaluate the samples’ mineralization heterogeneity (Boyde and Jones, 1983; Lundon et al., 1994).

The images collected from the backscattered electron microscopy were also analyzed to determine the vertebral bone connectivity by performing strut analysis (Mellish et al., 1991). Parameters such as number of node, length of node-node strut, length of node-free strut, number of free end and length free-free strut were determined to assess trabecular bone connectivity.

**Micro-computed tomography**

The third lumbar vertebrae were isolated and trimmed to leave only the vertebral body. The vertebral bodies were mounted in microtubes and scanned using SkyScan 1172 micro-CT scanner. Scanned images were reconstructed and calibrated using a hydroxyapatite standard. Final three-dimensional images were analyzed using SkyScan CT-Analyser versio 1.6.1 software. For each sample, the density parameters considered was volumetric bone mineral density (BMD), and the structural parameters analyzed were percentage of bone volume (BV/TV), trabecular thickness (Tb.Th.), trabecular number (Tb.N.), trabecular separation (Tb.Sp.).

**Statistical analysis**

For all analyses SPSS (version 17.0) was used. Two-way Analysis of Variance (ANOVA, general linear model) and Post-hoc multiple comparisons LSD test was used
to compare the measured parameters between the three genotypes groups within the young group and the aged group. A p-value of <0.05 was required to consider a significant difference. All results are presented as mean ± standard deviation (SD).

RESULTS

Rac1 KO animals show a higher bone mineral density

WT, Rac1KO and Rac2KO animals were analyzed by dual energy x-ray absorptiometry (DEXA). Whole body analysis showed that both young and aged Rac1 knock-out mice had significantly higher bone mineral density (BMD) compared to Rac2 knock-out and controls (p<0.05) (fig. 2.1A). The same analysis of isolated femurs (cortical bone) showed that young Rac2KO had significantly lower BMD compared to all groups (p<0.05) but after aging Rac2KO bones showed BMD values similar to controls (fig 2.1B). Femur results were supported when isolated lumbar vertebrae (trabecular bone) were analyzed (fig. 2.1C). In order to confirm the BMD results, volumetric BMD was performed by the analysis of micro-computed tomography images, and the results clearly confirmed the higher BMD in Rac1KO vertebrae in the young group (p<0.05) and both Rac1KO and Rac2KO vertebrae showed higher BMD compared to the control after aging (p<0.05) (fig. 2.1D).
Figure 2.1 Bone Mineral Density (BMD) of whole body and isolated bones.
The Dual Energy X-ray Absorptiometry (DEXA) showed that Rac1KO animals have higher BMD of the whole body compared to Rac2KO and WT in both young (BL) and aged groups (1YO) (p<0.05) (Fig 2.1A). Similar results were observed when isolated vertebrae were analyzed (Fig 2.1C). The femurs were also isolated and scanned and the results showed that young Rac2KO bones have significantly lower BMD compared to the other two groups (p<0.002), but after aging Rac2KO femur showed no difference compared to control and both had lower values than Rac1KO femurs (p<0.05) (Fig 2.1B). In order to confirm the DEXA results, volumetric BMD were calculated using micro-CT images of vertebrae. Similarly to what was observed before, young Rac1KO showed higher volumetric BMD compared to the other groups (p<0.05) and aged Rac1KO and Rac2KO vertebrae had higher density compared to the control (Fig 2.1D).
Back-scattered electron microscopy (BSE) demonstrated that young Rac2KO vertebrae are around 10% less mineralized compared to Rac1KO and control (fig. 2.2). Additionally, both Rac1KO and Rac2KO histograms had narrower widths, indicating lower mineralization heterogeneity compared to controls (fig. 2.2). No difference was observed between groups after aging (table 2.1).

<table>
<thead>
<tr>
<th>4-month old</th>
<th>WT</th>
<th>Rac1KO</th>
<th>Rac2KO</th>
</tr>
</thead>
<tbody>
<tr>
<td>grey level at max. peak</td>
<td>165.25 (± 5.99)</td>
<td>165.78 (± 15.44)</td>
<td>148.69 (±6.83)*</td>
</tr>
<tr>
<td>FWHMH</td>
<td>28.25 (± 2.83)</td>
<td>25.67 (± 2.83)**</td>
<td>25.69 (± 1.70)**</td>
</tr>
<tr>
<td>1-year old</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>grey level at max. peak</td>
<td>164.3 (± 14.66)</td>
<td>165.6 (± 10.8)</td>
<td>165.27 (±15.04)</td>
</tr>
<tr>
<td>FWHMH</td>
<td>31.3 (±2.9)</td>
<td>31.3 (± 2.62)</td>
<td>29.45 (± 1.86)</td>
</tr>
</tbody>
</table>

FWHMH - full width at half of maximum height
values are means ± SD
* p<0.05 Rac2KO compared to WT and Rac1KO
**p<0.05 Rac1KO and Rac2KO compared to WT

Table 2.1. Back-scattered electron microscopy (BSE) of 5th lumbar vertebrae

*Rac1KO bones show more resistance to fracture compared to WT and Rac2KO bones.*

The mechanical tests evaluated both cortical and trabecular mechanical properties. Interestingly, both young and aged Rac2KO displayed weaker properties (lower values for ultimate load and stiffness) when cortical bones were tested in three-point bending but the differences did not persist when the results were normalized to bone cross-sectional area and the material properties were analyzed. Additionally, young Rac1KO cortical bones were stronger and more resistant to breakage compared to Rac2KO and control before and after normalization of the results but the same difference was not observed after aging (table 2.2). These outcomes led us to analyze the bone cross-section area and bone diameter between groups, which showed that Rac2KO cortical bones had significant
Figure 2.2 Histograms of grey level generated at BSE.

The representative histograms of grey level show the mineralization profile between the groups at younger stage of life using back-scattered electron microscopy images. The results show that while Rac1KO vertebrae mineralization profile is very similar to the control (WT), Rac2KO is around 10% less mineralized in the young group.
smaller geometric structure compared to all others, which explains the lower structural properties in this group (table 2.2).

When trabecular bones were tested, young Rac2KO vertebrae had lower failure displacement and failure strain compared to Rac1KO and control (table 2.3). After aging, Rac1KO vertebrae showed higher structural and material properties compared to Rac2KO and control groups (table 2.3). No differences were found in vertebral size between groups (data not shown).

<table>
<thead>
<tr>
<th>4-month old</th>
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<th>Rac1KO</th>
<th>Rac2KO</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Structural Properties</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ultimate load (N)</td>
<td>21.069 (± 3.787)</td>
<td>19.875 (± 2.968)</td>
<td>15.112 (± 1.846)*</td>
</tr>
<tr>
<td>failure displacement (mm)</td>
<td>0.368 (± 0.071)</td>
<td>0.497 (± 0.174)**</td>
<td>0.316 (± 0.098)</td>
</tr>
<tr>
<td>energy to failure (mJ)</td>
<td>5.345 (± 1.182)</td>
<td>6.766 (± 1.787)**</td>
<td>3.410 (± 1.224)</td>
</tr>
<tr>
<td>Stiffness (N/mm)</td>
<td>167.23 (± 44.35)</td>
<td>174.21 (± 19.99)</td>
<td>134.99 (± 24.70)*</td>
</tr>
<tr>
<td><strong>Material Properties</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ultimate stress (MPa)</td>
<td>192.31 (± 21.15)</td>
<td>198.54 (± 28.67)</td>
<td>207.61 (± 28.77)</td>
</tr>
<tr>
<td>failure strain (%)</td>
<td>7.355 (± 1.299)</td>
<td>9.775 (± 3.516)**</td>
<td>5.622 (± 1.825)</td>
</tr>
<tr>
<td>Toughness (MPa)</td>
<td>9.798 (± 2.032)</td>
<td>13.129 (± 2.912)**</td>
<td>8.190 (± 2.791)</td>
</tr>
<tr>
<td>Modulus (MPa)</td>
<td>7778.33 (± 2240)</td>
<td>8963.89 (± 1630)</td>
<td>10500 (± 2230)</td>
</tr>
<tr>
<td><strong>Femur Geometry</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>cross-sectional area</td>
<td>0.704 (± 0.086)</td>
<td>0.657 (± 0.063)</td>
<td>0.545 (± 0.056)*</td>
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<td>anterior-posterior diam.</td>
<td>1.206 (± 0.119)</td>
<td>1.177 (± 0.047)</td>
<td>1.064 (± 0.055)*</td>
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<table>
<thead>
<tr>
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<th>WT</th>
<th>Rac1KO</th>
<th>Rac2KO</th>
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<td><strong>Structural Properties</strong></td>
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<td></td>
</tr>
<tr>
<td>ultimate load (N)</td>
<td>26.982 (± 5.419)</td>
<td>28.819 (± 5.563)</td>
<td>22.889 (± 2.645)**</td>
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<td>failure displacement (mm)</td>
<td>0.215 (± 0.053)</td>
<td>0.241 (± 0.082)</td>
<td>0.243 (± 0.065)</td>
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<tr>
<td>energy to failure (mJ)</td>
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<td>4.75 (± 2.153)</td>
<td>3.757 (± 1.32)</td>
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<td>Stiffness (N/mm)</td>
<td>225.94 (± 38.51)</td>
<td>246.5 (± 31.76)</td>
<td>191.39 (± 17.4)*</td>
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<td><strong>Material Properties</strong></td>
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<tr>
<td>ultimate stress (MPa)</td>
<td>191.96 (± 19.67)</td>
<td>179.82 (± 13.85)</td>
<td>211.87 (± 35.85)**</td>
</tr>
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<td>failure strain (%)</td>
<td>4.429 (± 1.141)</td>
<td>5.528 (± 1.822)</td>
<td>4.97 (± 1.57)</td>
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<tr>
<td>Toughness (MPa)</td>
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<td>6.778 (± 3.128)</td>
<td>6.86 (± 1.945)</td>
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<tr>
<td>Modulus (MPa)</td>
<td>7877.98 (± 692)</td>
<td>6833.67 (± 1631)</td>
<td>8851.98 (± 1777)**</td>
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<td><strong>Femur Geometry</strong></td>
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<tr>
<td>cross-sectional area</td>
<td>0.856 (± 0.139)</td>
<td>0.976 (± 0.087)</td>
<td>0.804 (± 0.085)*</td>
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<tr>
<td>anterior-posterior diam.</td>
<td>1.232 (± 0.069)</td>
<td>1.381 (± 0.093)**</td>
<td>1.214 (± 0.092)</td>
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values are means ± SD
*p<0.05 Rac2KO compared to Rac1KO and WT
**p<0.05 Rac1KO compared to Rac2KO and WT
***p<0.05 Rac2KO compared to Rac1KO

Table 2.2 Bone mechanical properties from 3-point bending test
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<tr>
<td><strong>Structural Properties</strong></td>
<td></td>
<td></td>
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<tr>
<td>ultimate load (N)</td>
<td>25.8 (± 7.72)</td>
<td>32.27(± 9.51)**</td>
<td>26.94 (± 5.52)</td>
</tr>
<tr>
<td>failure displacement (mm)</td>
<td>0.777 (± 0.24)</td>
<td>0.771 (± 0.21)</td>
<td>0.531 (± 0.14)</td>
</tr>
<tr>
<td>energy to failure (mJ)</td>
<td>10.66 (± 4.14)</td>
<td>12.04 (± 5.00)</td>
<td>8.13 (± 3.50)</td>
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<tr>
<td>Stiffness (N/mm)</td>
<td>64.47 (± 18.99)</td>
<td>91.09 (± 30.28)**</td>
<td>98.56 (± 39.87)</td>
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<td><strong>Material Properties</strong></td>
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<tr>
<td>ultimate stress (MPa)</td>
<td>11.78 (± 3.39)</td>
<td>15.03 (± 4.79)**</td>
<td>12.73 (± 3.12)</td>
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<td>failure strain (%)</td>
<td>30.06 (± 8.69)</td>
<td>30.36 (± 8.91)</td>
<td>22.01 (± 6.04)</td>
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<tr>
<td>Toughness (MPa)</td>
<td>1.88 (± 0.66)</td>
<td>2.19 (± 0.94)</td>
<td>1.59 (± 0.73)</td>
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<tr>
<td>Modulus (MPa)</td>
<td>76.44 (± 23.43)</td>
<td>109.82 (± 41.78)**</td>
<td>114.68 (± 54.36)</td>
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</tbody>
</table>

Values are means ± SD
* p<0.05 Rac2KO compared to the other groups
**p<0.05 Rac1KO compared to Rac2KO and WT

Table 2.3. Bone mechanical properties from vertebral compression

Rac1KO trabecular bone is architecturally more connected and organized.

The vertebral architecture analysis done by micro-computed tomography (micro-CT) scanning is a very accurate test to measure the vertebral architecture because it is a three-dimension bone analysis. The micro-CT scans showed that young Rac1KO vertebras had higher percentage of bone volume (BV/TV) and trabecular number (Tb.N.) compared to Rac2KO and wild type (table 2.4). After aging, both Rac1KO and Rac2KO showed higher structural values compared to control (table 2.4).

The Goldener’s Trichrome stained sections of the samples showed results consistent with the micro-CT data. Both young and aged Rac1KO vertebras had a significantly higher percentage of trabecular bone (BV/TV) and trabecular number (Tb.N.) compared to Rac2KO and control (p<0.05) and lower trabecular separation.
(Tb.Sp.) compared to control (<0.05). No significant difference was found in the formation parameters (osteoid volume, osteoid surface and osteoid thickness) between young groups (fig 2.3A), but aged samples showed lower osteoid volume and osteoid surface in knock-outs groups compared to control (fig 2.3B).

Figure 2.3. Bone formation parameters evaluated by static histomorphometry.
The static histomorphometry showed no difference in osteoid volume (OV) and osteoid surface (OS) between groups at younger age (A). After aging, both knock-outs showed lower values for bone formation parameters (OV, OS) compared to the control (p<0.05) (B).
Once the trabecular connectivity was evaluated by performing strut analysis, young and aged Rac1KO showed more connected trabeculae compared to the other groups. Higher numbers of nodes between trabeculae was observed in Rac1KO compared to Rac2KO and control (fig. 2.4A). Additionally Rac1KO had significantly higher length of node-node strut compared to Rac2KO (fig. 2.4B).

Table 2.4. Micro-computed tomography of vertebrae

<table>
<thead>
<tr>
<th></th>
<th>4-month old</th>
<th>1-year old</th>
</tr>
</thead>
<tbody>
<tr>
<td>percentage of bone volume</td>
<td>17.033 (± 5.644)</td>
<td>9.751 (± 3.03)</td>
</tr>
<tr>
<td>trabecular thickness</td>
<td>0.067 (± 0.003)</td>
<td>0.0585 (± 0.003)</td>
</tr>
<tr>
<td>trabecular number</td>
<td>2.496 (± 0.737)</td>
<td>1.65 (± 0.454)</td>
</tr>
<tr>
<td>trabecular separation</td>
<td>0.288 (± 0.046)</td>
<td>0.363 (± 0.042)</td>
</tr>
<tr>
<td></td>
<td>WT</td>
<td>Rac1KO</td>
</tr>
<tr>
<td></td>
<td>25.74 (± 5.467)*</td>
<td>17.68 (± 2.93)***</td>
</tr>
<tr>
<td></td>
<td>0.07 (± 0.002)**</td>
<td>0.0609 (±0.002)**</td>
</tr>
<tr>
<td></td>
<td>3.672 (± 0.83)*</td>
<td>2.89 (± 0.42)***</td>
</tr>
<tr>
<td></td>
<td>0.262 (± 0.063)</td>
<td>0.287 (±0.027)***</td>
</tr>
</tbody>
</table>

Values are means ± SD
*p<0.05 Rac1KO compared to Rac2KO and WT
**p<0.05 Rac1KO compared to WT
***p<0.05 Rac1KO and Rac2KO compared to WT
Figure 2.4 Trabecular architecture analyzed at strut analysis. The strut analysis confirmed that both young (BL) and aged (1YO) Rac1KO trabecular bone are more connected and structurally organized. The results revealed that Rac1KO has higher number of nodes compared to WT and Rac2KO ($p<0.05$) (A), and higher length of node-node strut compared to Rac2KO ($p<0.05$) (B).

Osteoclasts in Rac knock-out mice have less nuclei per cell and knock-out bones show lower bone formation rates

The osteoclast evaluation by performing analysis of TRAP stained slides showed that all young knock-out groups had lower percentage of osteoclast surface (osteoclast surface/bone surface ratio) compared to the control (table 2.5). The TRAP stained slides were also analyzed at high magnification (X100) to evaluate osteoclast nuclei number per osteoclast cell. It was clearly observed that knock-out samples have fewer osteoclasts with more than three nuclei compared to the controls in both young and aged groups (fig. 2.5), which is consistent with the increased bone volume observed in knock-outs groups.
<table>
<thead>
<tr>
<th></th>
<th>WT</th>
<th>Rac1KO</th>
<th>Rac2KO</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>4-month old</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Osteoclast surface</td>
<td>1.62 (± 0.76)</td>
<td>1.53 (± 0.59)</td>
<td>1.58 (± 0.32)</td>
</tr>
<tr>
<td>Percentage of osteoclast surface</td>
<td>22.073 (± 9.8)</td>
<td>16.015 (± 5.14)*</td>
<td>16.59 (± 2.61)*</td>
</tr>
<tr>
<td># Osteoclasts per bone surface</td>
<td>7.48 (± 3.09)</td>
<td>6.43 (± 1.48)</td>
<td>6.1 (± 1.29)</td>
</tr>
<tr>
<td># Osteoclasts per osteoclast surface</td>
<td>35.63 (± 6.96)</td>
<td>41.87 (± 8.03)</td>
<td>36.75 (± 5.07)</td>
</tr>
<tr>
<td><strong>1-year old</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Osteoclast surface</td>
<td>0.899 (± 0.34)</td>
<td>1.13 (± 0.57)</td>
<td>0.868 (± 0.44)</td>
</tr>
<tr>
<td>Percentage of osteoclast surface</td>
<td>7.45 (± 2.76)</td>
<td>6.44 (± 2.84)</td>
<td>5.79 (± 2.61)</td>
</tr>
<tr>
<td># Osteoclasts per bone surface</td>
<td>2.79 (± 0.64)</td>
<td>2.37 (± 0.94)</td>
<td>2.22 (± 0.85)</td>
</tr>
<tr>
<td># Osteoclasts per osteoclast surface</td>
<td>39.31 (± 7.06)</td>
<td>37.25 (± 4.50)</td>
<td>39.97 (± 7.80)</td>
</tr>
</tbody>
</table>

Values are means ± SD

* p<0.05 Rac1KO and Rac2KO compared to WT

Table 2.5. TRAP staining analysis

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**Figure 2.5. Representative images of osteoclasts in TRAP stained slides**

Osteoclasts (black arrow) from Rac1KO and Rac2KO TRAP stained slides show fewer nuclei per cell compared to control either at 4 month or 1 year of age, which suggest the lower osteoclast resorptive activity in these groups compared to the control in different ages.
The calcein-green labels were analyzed in unstained slides for dynamic histomorphometric measurements. As shown in table 2.6, both young and aged Rac1KO and Rac2KO mice had significantly lower values for mineral appositional rates (MAR) and bone formation rates (BFR) compared to the control. Although no statistically difference was found in BFR between knock-outs groups, a lower mean value was observed in Rac2KO (table 2.6).

<table>
<thead>
<tr>
<th>4-month old</th>
<th>WT</th>
<th>Rac1KO</th>
<th>Rac2KO</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mineralizing surface</td>
<td>2.130 (± 0.66)</td>
<td>2.272 (± 0.506)</td>
<td>2.050 (± 0.429)</td>
</tr>
<tr>
<td>% Mineralizing surface</td>
<td>24.156 (± 5.54)</td>
<td>21.240 (± 5.06)</td>
<td>19.384 (± 3.78)</td>
</tr>
<tr>
<td>MAR</td>
<td>1.151 (± 0.126)</td>
<td>0.929 (± 0.16)*</td>
<td>0.970 (± 0.085)*</td>
</tr>
<tr>
<td>BFR</td>
<td>0.278 (± 0.073)</td>
<td>0.202 (± 0.069)*</td>
<td>0.189 (± 0.047)*</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>1-year old</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Mineralizing surface</td>
<td>1.425 (± 0.49)</td>
<td>1.655 (± 0.39)</td>
<td>1.396 (± 0.32)</td>
</tr>
<tr>
<td>% Mineralizing surface</td>
<td>14.22 (± 3.19)</td>
<td>15.25 (± 4.25)</td>
<td>11.64 (± 2.55)</td>
</tr>
<tr>
<td>MAR</td>
<td>0.91 (± 0.158)</td>
<td>0.759 (± 0.76)*</td>
<td>0.711 (± 0.146)*</td>
</tr>
<tr>
<td>BFR</td>
<td>0.129 (± 0.038)</td>
<td>0.115 (± 0.032)</td>
<td>0.084 (±0.032)**</td>
</tr>
</tbody>
</table>

MAR - mineral appositional rate; BFR - bone formation rate
values are means ± SD
* p<0.05 Rac1KO and Rac2KO compared to WT
** p<0.05 Rac2KO compared to WT

Table 2.6. Dynamic Histomorphometry
DISCUSSION

The Rho family of small GTPases is critical to intra-cellular signaling in diverse cell populations from cells of mesenchymal origin to hematopoietic origin. Rac1 and Rac2 have been demonstrated to regulate actin cytoskeleton dynamics, cell movement, phagocytosis, cell growth, cell adhesion, gene transcription and reactive oxygen species (ROS) production (Guo et al., 2008; Sun et al., 2007; Wang et al., 2008; Wheeler et al., 2006), crucial steps in a variety of cell differentiation, including osteoclasts formation. Aging bone has reduced osteoblast formation, increased bone marrow adiposity, a shorter life span for mature osteoblasts, and a higher frequency of osteoblast apoptosis (Chan and Duque, 2002), which explains the imbalance in bone homeostasis that trends toward bone resorption. By better understanding the mechanisms regulating osteoclast activity in vivo we may be able to identify novel therapeutic targets that will enable us to overcome the negative aging effects on the skeleton and attenuate the age-related bone loss. The work described here is focused on determining if Rac1 and or Rac2 are potential therapeutic targets for blocking the age related skeletal effects that mediate increased fracture risk.

For this study we utilized a previously described knock out mouse model in which Rac1 or Rac2 are deleted in monocytes. Using this model we recently reported that Rac1 is the primary Rac isofrom required for in vitro osteoclastogenesis (Wang et al., 2008). Using an aged murine model we evaluate here the role of the two Rac small GTPase in bone regulation associated with the physiological lower bone turnover typical in aging adults. In this study we further demonstrate that although these two proteins have high homology they may play differing roles in osteoclastogenesis and osteoclast function leading to the different bone quality observed between Rac1KO and Rac2KO groups.
The present investigation furthers our understanding of how these two key signaling elements regulate *in vivo* bone physiology through osteoclast regulation at different stages of life and we believe that it could help future investigation to enhance our knowledge in prevention and treatment of age-related bone loss.

The deletion of Rac1 increases bone quality at different ages and attenuates the age-related bone loss

Our findings indicate that the deletion of Rac1 in monocyte-osteoclast precursors results in stronger cortical bones with increased trabecular connectivity in trabecular bone compared to WT control bones. This phenotype was observed in young and aged animals and may be explained by the role of Rac1 in osteoclast differentiation (Wang et al., 2008). This is supported by our observation of reduced numbers of multinucleated osteoclasts in femur sections from the Rac1KO mice compared to the WT controls. It has also been shown that Rac small GTPases regulate osteoclast functions. Fukuda et. al. demonstrated the importance of Rac1 in mediating resorptive activity and survival signaling in osteoclasts (Fukuda et al., 2005). The attenuated resorptive activity of osteoclasts in Rac1KO may also explain the increased bone mineral density and resistance in the mechanical tests in this group. Moreover, the compromised bone resorption is consistent with the increased bone mass in trabecular bone. The static histomorphometry, strut analysis and micro-computed tomography showed that the absence of Rac1 leads to higher number of trabeculae, which are also more connected and structurally organized. The results are consistent with our prior work in which we demonstrated that Rac1 deletion manifests at the tissue level with increased trabecular bone volume and
trabeculae number (Wang et al., 2008). More importantly, the fact that we observed the same differences during aging suggest that the absence of Rac1 attenuated the age-related bone loss, leading to higher bone quality in Rac1KO aged group. When we compare the outcomes from the young group to the aged group, it is clear that all genotypes suffered the natural consequences of the process of aging. Aged bones are significantly weaker in all tests and have a poorer structure compared to young bones. Nonetheless, the deletion of Rac1 diminished the physiological aging effect and results in stronger bones compared to the control of the same age.

Similarly to the conclusions in previous studies (Lee et al., 2006; Wang et al., 2008), we observed here that the Rac knock-out osteoclasts have fewer nuclei per cell, which means that they are significantly less effective in resorbing bone. Dynamic histomorphometric analysis demonstrated that Rac1KO had lower bone formation rates compared to the control, which is likely a consequence of the decreased activity of osteoclasts on those animals and a natural tissue response to maintain the appropriate balance between bone resorption and bone formation (Chambers, 2000; Teitelbaum, 2007). In summary, the analysis of Rac1KO group suggests that Rac1 deletion decreases bone remodeling, but the effect on bone resorption is dominant, resulting in increased bone mass and bone quality, which is especially important in aging bone.

The deletion of Rac2 compromises bone structure and negatively affects bone quality in young mice.

We observed that in the absence of Rac2, cortical bones were smaller in size compared to WT and Rac1KO samples in young and aged bones. The effect of Rac2 on
bone dimension has not been described previously in the literature. We hypothesize that the absence of Rac2 may negatively affect the release of anabolic factors normally produced by activated osteoclasts. Garimella et al have reported the importance of bone morphogenic proteins (BMP) expressed in activated osteoclasts involved in the initiation of the anabolic phase of bone remodeling (Garimella et al., 2008). The exact role of Rac2 in regulation of osteoclast functions remains unclear, however our result suggests that this small GTPase might play a role not only in osteoclast resorptive activity but also in osteoclast mediated promotion of bone formation. Kawano et. al. recently observed an increased anabolic response to para-thyroid hormone (PTH) in Rac2KO mice (Kawano et al., 2008). Kawano et. al. credited the increased anabolic response to PTH in Rac2KO samples to the diminished osteoclast resorptive activity in this group. Additionally, they found an increased number of osteoblasts per total area in Rac2KO samples treated with PTH, compared to wild type animals, which led to the hypothesis that altered Rac2 expression could be responsible for an enhancement of bone tropic release factors from osteoclasts. Based on our results, we believe that PTH could neutralize the absence of Rac2, and therefore bone anabolism should overcome bone resorption in samples treated with PTH. Although Kawano et. al. reported different results from ours, they did not investigate differences in bone size between Rac2KO and the control, which would be important to confirm the significant anabolic difference between the groups. Comparing Kawano et. al. results to this study, we believe that the increased anabolism in Rac2KO reported by Kawano et. al. may be due to effects of PTH in bone physiology, knocking out the effects of Rac2 absence. Further investigation of PTH effects in bone cells of Rac2KO is needed to demonstrate this effect. We hypothesize that the effects of Rac2 in
modulating osteoclast activity by regulating the release of anabolic factors could be more significant than the inhibition of bone resorption that Rac2 knock-out could promote. This effect could explain the significantly reduced size of Rac2KO femurs not observed in the Rac1KO group.

Similar to what was observed in Rac1KO group, both young and aged Rac2KO bones also showed osteoclasts with less nuclei per cell, consistent with lower resorptive activity. Rac2KO bones also displayed decreased bone formation rates in dynamic histomorphometric analysis similarly to Rac1KO samples. Our results showed that although Rac2KO bones apparently have lower quality compared to Rac1KO in earlier stage of life, their phenotypes become closer to Rac1KO as the mice age. This suggests that Rac2 might indirectly affects bone formation and the effects on bone quality are more evident at younger age. This difference supports the hypothesis that Rac2 deletion protects bone from the natural consequences of aging but less effectively than Rac1 deletion did.

*In vivo bone quality in Rac knock-outs*

In order to evaluate the *in vivo* role of Rac1 and Rac2 in bone quality, a previously described Rac knock-out mouse model was used (Glogauer et al., 2003). The crucial importance of these two proteins in immune system development made the knock-out mice breeding a difficult process due to their susceptibility to infections. This explains the loss of some mice in the study, especially in the aged group. As suggested in our previous study (Wang et al., 2008), the Rac knock-out mouse model is a valid model
to dissect the specific roles of Rac1 and Rac2 in osteoclast differentiation and function which will certainly help in the identification of drug targets for osteoclasts.

Using this model, we show that the increased bone quality found in Rac1KO group in both young and aged groups suggests a key role of Rac1 in regulating bone turnover. Conversely, Rac2KO showed lower bone quality in younger stage of life but their bone characteristics improved with aging compared to the others at the same age. The decreased bone quality observed in the Rac2KO group highlighted the negative impact on bone structure in the absence of Rac2. The present results are consistent with our previous finding of an *in vitro* model showing that Rac1 and Rac2 play different and nonoverlapping roles in osteoclast activity. These new results do not support the reduction in osteoclast formation between Rac knock-outs groups but they show important reduction in the number of osteoclast nuclei in Rac deficient animals which confirm the poor osteoclast function in this group also reported in previous *in vitro* studies. We believe that Rac GTPases play a more significant role in osteoclast activity rather than osteoclast formation *in vivo*.

*Bone quality and aging bone*

Bone quality is maintained through the balance between bone formation and bone resorption. During growth the skeleton undergoes several changes to adjust to the body’s need in each phase of life. At early ages bone formation exceeds bone resorption, and in adulthood to elderly the balance shifts and more bone resorption is observed. This physiological change leads to significant cortical and trabecular bone loss resulting in a structurally weaker bone (Bar-Shira-Maymon et al., 1989; Halloran et al., 2002), which is
more significant and evident in females (Glatt et al., 2007). Comparing the results observed in the young group (base-line data) to the result of the aged group in the different analysis performed in this study, we clearly observe the effects of aging on the skeleton. The aged bones generally show higher bone mineral density similarly to previous studies that revealed a peak in the BMD in mice model at 12 months of age (Glatt et al., 2007). Moreover, the mechanical tests show that aged bones are materially weaker, which could be a result of osteopenia typically found at later ages (Weiss et al., 1991). The analyses of the trabecular architecture performed by histomorphometry and micro-computed tomography also revealed that aged bones have important trabecular bone loss, as shown in the literature (Bar-Shira-Maymon et al., 1989; Glatt et al., 2007).

Some previous studies explained the age-related bone loss as a result of changes in osteoblast and osteoclast intra-cellular signaling regulation. It has been reported that in the aging process there is an increased expression of RANKL (Cao et al., 2003), a member of TNF family, important in the stimulation of osteoclast differenciation and activation (Dougall et al., 1999), which could be one of the factors responsible for the shift toward bone resorption in bone homeostasis. Considering the changes in the cellular regulation after aging, the increased osteoclastogenesis could be a result of the enhanced activation of RANK and its downstream elements such as Rac1 and Rac2 (Brazier et al., 2006; Lee et al., 2006), well known for their crucial role in osteoclast formation. Comparing our results with the previous reports we observed that the deletion of the two Rac GTPases might affect the cellular changes typically found in aging, which attenuated the age-related bone loss. These results help to improve our understanding of the mechanisms behind age-related bone loss and show the importance of RacGTPases in
future investigation toward the development of alternatives to minimize the effects of age on bone quality.

CONCLUSION

The in vivo analysis of bone properties suggests that Rac1 deletion works as a bone protector, increasing bone quality, which seems to be an effective alternative to attenuate the changes in the skeleton due to age-related bone loss. Further studies are needed to clarify the exact role of this small GTPase on osteoclast function, as Rac1 or more likely its downstream signaling elements could be important targets for designing drugs to prevent bone loss. Conversely Rac2 deletion acted as bone protector only after aging, which shows that it also attenuated the age-related bone loss, although not so significantly as Rac1 deletion did. Rac2 seems to be more related to bone formation process, which show that it is also an important element for future bone related research.
CHAPTER 3: Deleting Rac1 and Rac2 improves vertebral bone quality in a murine osteoporosis model

Joyce K. R. S. Magalhaes, Marc D. Grynpas and Michael Glogauer
ABSTRACT

Rho small GTPases, including Rac1 and Rac2, are key regulators of a variety of cell functions, including osteoclast differentiation and function. In order to investigate the roles of the Rac1 and Rac2 isoforms in a high bone turnover state such as postmenopausal osteoporosis, we investigated bone quality in female ovariectomized mice in which Rac1 or Rac2 have been deleted in monocytes. Methods: 1-year old female mice from 3 groups – wild type (WT), Rac1 null (Rac1KO) and Rac2 null (Rac2KO) – were studied in control and an osteoporotic group (mice ovariectomized at 4 month of age). After sacrifice at 12 months of age, the bones were harvested for mechanical tests, bone densitometry, micro-computed tomography and histomorphometric analyses to evaluate bone mineralization and architecture. The results were compared between groups using ANOVA and LSD post hoc tests. Results: We observed that Rac1KO mice showed higher bone mineral density (BMD) in the vertebrae compared to Rac2KO and WT in the control group and after the induction of osteoporosis. Consistent with this finding Rac1KO vertebrae showed higher mechanical properties and increased trabecular connectivity compared to WT in both groups. Rac2KO mice have structurally weaker cortical bones, but they are materially stronger as observed in mechanical tests in both control and osteoporotic groups. Moreover the micro-CT analysis revealed that Rac2KO osteoporotic vertebrae have more trabecular bone. Consistent with previous in vitro analysis, the Rac knock-out samples showed lower numbers of multi-nucleated osteoclasts (osteoclasts with more than three nuclei) in both control and osteoporotic groups, which suggest lower bone resorptive efficiency. Conclusion: Our results demonstrate that the deletion of Rac1 and Rac2 increases vertebral bone quality.

compared to WT bones in an osteoporosis model. The results suggest that both Rac1 and Rac2 or their downstream signaling partners may be important targets for anti-osteoporotic drug design.
INTRODUCTION

Osteoporosis is a metabolic disorder of the adult skeleton resulting from the imbalance between the resorptive and formative phases during bone remodeling (Parfitt, 1982). Estrogen deficiency has been reported as a major risk for the development of postmenopausal osteoporosis in women due to the steroidal hormone’s effect on bone homeostasis (Manolagas, 2000; Riggs et al., 2002). The estrogen deprivation characteristic of postmenopausal phase in women not only increases TNF production leading to stimulation of osteoclastogenesis, but also increases osteoclasts’ lifespan by inhibiting apoptosis (Hughes et al., 1996; Weitzmann and Pacifici, 2006). Those combined processes lead to an increase in osteoclasts and a misbalance in bone remodeling, resulting in rapid bone loss during early menopause and the slow bone loss associated with aging (Khosla and Riggs, 2005; Riggs et al., 2002).

Osteoclasts have been extensively studied in order to better understand the signaling mechanisms involved in bone turnover (Walker, 1975a; Walker, 1975b). Rac1 and Rac2, members of the Rho-family of small GTPases, are key regulators of the actin cytoskeleton in a variety of cells (Fenteany and Glogauer, 2004; Hall, 1998; Ridley et al., 1992), including osteoclasts (Razzouk et al., 1999). Fukuda et al. demonstrated that Rac1 regulates osteoclast bone resorptive activity (Fukuda et al., 2005), which was confirmed by Wang et al. using a transgenic mouse model (Wang et al., 2008). Although both Rac1 and Rac2 have been reported to regulate bone remodeling, a previous study suggests that these two highly homologous isoforms have non-overlapping functions (Sun et al., 2007). This leads to the question of the roles played by these two small GTPases in determining bone quality.
In vivo bone analysis of Rac1 null and Rac2 null mice will allow us to gain important insights into how Rac1 and Rac2 affect bone quality during the development of osteoporosis, which could be important for the identification of new drug targets for this bone disorder.

MATERIAL AND METHODS

Animals

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. Previously generated and characterized mice in which Rac1 was selectively deleted in neutrophils and monocytes (Sun et al., 2004), Rac1\textsuperscript{c/-}\textsuperscript{LysM\textsuperscript{cre}}, were bred with Rac2\textsuperscript{c/-} mice (Roberts et al., 1999) and the resulting offspring were bred over at least 6 generations to generate optimal breeding pairs (Rac1\textsuperscript{c/-}\textsuperscript{LysM\textsuperscript{cre}}Rac2\textsuperscript{+/-} x Rac1\textsuperscript{c/-}\textsuperscript{LysM\textsuperscript{cre}}Rac2\textsuperscript{+/-}), which enabled the generation of Rac1KO (Rac1\textsuperscript{c/-}\textsuperscript{LysM\textsuperscript{cre}}Rac2\textsuperscript{+/-}), Rac2KO (Rac1\textsuperscript{+/-}\textsuperscript{LysM\textsuperscript{cre}}Rac2\textsuperscript{+/-}), DKO (Rac1\textsuperscript{c/-}\textsuperscript{LysM\textsuperscript{cre}}Rac2\textsuperscript{+/-}), and wild-type mice (Rac1\textsuperscript{+/-}\textsuperscript{LysM\textsuperscript{cre}}Rac2\textsuperscript{+/-}) from the same litters, as previously described (Wang et al., 2008). This breeding strategy allows for the controlling of background variations. Genotyping for Rac1, Rac2, and LysM alleles was carried out as described previously (Glogauer et al., 2003; Roberts et al., 1999). All experiments were performed on 61 female mice divided into two groups: the control group with 32 1-year old mice (10 wild type, 11 Rac1 KO, 11 Rac2 KO); and the osteoporotic group with 29 1-year old mice (10 wild type, 8 Rac1 KO, 11 Rac2 KO) ovariectomized at 4 month of age. Although we
started with 15 mice for each phenotype per group, some mice were lost prematurely due to infections. Any animal that presented any sign of infection was eliminated from the study. The animals from osteoporotic group were ovariectomized at 4 month of age to induce premature menopause and osteoporosis. The surgical procedure was performed under aseptic conditions following the University of Toronto Animal Care protocol. Three and ten days before euthanasia the mice were injected intra-peritoneally with calcein-green (30mg/Kg), a bone marker important for bone dynamic histomorphometric analysis. Animals were sacrificed by C02 asphyxiation at 1 year of age.

**Bone Mineral Density (BMD) analysis**

Dual energy x-ray absorptiometry (DEXA) was performed using an animal PIXIImus densitometer (Lunar; GE Copr.) for the whole animal body right after euthanasia and for the isolated femur and lumbar vertebrae after dissection for determination of bone mineral density (BMD) and bone mineral content (BMC). To perform the analysis, a consistent region of interest was selected to maintain uniformity between the samples.

**Mechanical testing**

Mice’s right femurs were tested in three-point bending to evaluate the mechanical properties of cortical bones. The sixth lumbar vertebrae were tested in compression to evaluate the properties of trabecular bones. Three-point bending and vertebral compression were performed using an Instron 4465 materials testing machine (Instron Canada Inc.). A pre-load of less than 1 N was applied to establish the samples’ contact
with the upper device. Further load was applied by a 100N cell load at a speed of 1mm/min and load versus time data were collected every 0.1 seconds by LabView data acquisition software (National Instruments Corp.; Austin, TX) until the sample failure. Based on the speed and time, displacement was automatically calculated and a load-displacement graph was generated to evaluate bones’ structural mechanical properties such as ultimate load, failure displacement, energy to failure and stiffness. Afterwards, data was normalized to the bone cross-section area and a stress-strain graph was generated to evaluate bones’ material properties such as ultimate stress, failure strain, toughness and modulus.

*Static and Dynamic Histomorphometric analysis*

The fifth lumbar vertebras were isolated from each animal and fixed in 70% ethanol. Samples were dehydrated in ascending concentrations of acetone followed by ascending ratios of unpolymerized spurr resin and acetone. Afterwards, bones were embedded in block of spur resin and left to polymerize in a 60 C oven for 48 hours. Using a semiautomatic microtome (Leica RM 2265), three 5-micron thick coronal sections were cut from each samples and placed on gelatinized slides for Goldner’s trichrome staining (Holmes et al., 2007) and 7-micron thick coronal sections were cut, placed on gelatinized slide and kept unstained for dynamic histomorphometric analysis. Trabecular bone was analyzed using a 25x objective lens connected to a video camera (Retiga 1300). Serial fields using the Leitz Bioquant morphometry system (Bioquant Nova Prime version 6.50.10) were analyzed from each sample to determine the following static histomorphometric parameters: trabecular bone volume (TBV), trabecular thickness
(Tb.Th.), trabecular number (Tb.N.) and trabecular separation (Tb.Sp.); and the formation parameters such as osteoid volume (OV), osteoid surface (OS) and osteoid thickness (O.Th.). Dynamic histomorphometry was done using fluorescence microscopy to measure the bone labels generated by the calcein-green injected before the animal euthanasia. The single and double calcein-green labels were measured on bone trabeculae to calculate mineralizing surface (MS), percentage mineralizing surface (%MS), mineral apposition rate (MAR) and bone formation rate (BFR) on unstained slides. All these parameters are in accordance with the histomorphometric nomenclature and definition of the American Society of Bone Mineral Research (ASBMR) (Parfitt et al., 1987).

**TRAP staining analysis**

The fourth lumbar vertebrae were isolated from each animal and fixed in 4% paraformaldehyde (PFA). The samples were decalcified and embedded in wax. Three 5-micron thick coronal sections were cut from each sample and placed in glass slides for Tartrate-Resistant Acid Phosphatase (TRAP) staining and hematoxilin counterstaining. The osteoclasts selectively express and stain positive for the TRAP enzyme. The Leitz Bioquant morphometry system was used to quantify the number of nuclei per osteoclast, osteoclasts surface (Oc.S.), percent osteoclasts surface (%Oc.S.), number of osteoclasts per bone surface (N.Oc.BS) and number of osteoclasts per osteoclasts surface (N.Oc.OcS).
Back-scattered electron imaging

The fifth vertebrae, embedded in spurr resin blocks, previously used for static histomorphometric analysis were then polished, carbon-coated and imaged using backscattered electron (BSE) imaging. To evaluate the mineralization distribution in each sample, the backscattering of the mineralized tissues was compared to a standard between each sample and histograms of the grey level distribution were created. From each histogram, the peak of mineralization value was determined to evaluate the overall degree of mineralization from each sample. The higher the grey level of the histogram, the more mineralized the sample is. The full width at half the maximum height (FWHM) of the histogram was also determined to evaluate the samples’ mineralization heterogeneity (Boyde and Jones, 1983; Lundon et al., 1994).

The images collected from the backscattered electron microscopy were also analyzed to determine the vertebral bone connectivity by performing strut analysis (Mellish et al., 1991). Parameters such as number of node, length of node-node strut, length of node-free strut, number of free end and length free-free strut were determined to assess trabecular bone connectivity.

Micro-computed tomography

The third lumbar vertebrae were isolated and trimmed to leave only the vertebral body. The vertebral bodies were mounted in microtubes and scanned using SkyScan 1172 micro-CT scanner. Scanned images were reconstructed and calibrated using a hydroxyapatite standard. Final three-dimensional images were analyzed using SkyScan CT-Analyser versio 1.6.1 software. For each sample, the density parameters considered
was volumetric bone mineral density (BMD), and the structural parameters analyzed were percentage of bone volume (BV/TV), trabecular thickness (Tb.Th.), trabecular number (Tb.N.), trabecular separation (Tb.Sp.).

Statistical analysis

For all analyses SPSS (version 17.0) was used. Two-way Analysis of Variance (ANOVA, general linear model) and Post-hoc multiple comparisons LSD test was used to compare the measured parameters between the three groups. A p-value of <0.05 was required to consider a significant difference. All results are presented as mean ± standard deviation (SD).

RESULTS

Rac1 and Rac2 deletion minimize the osteoporotic phenotype in vertebrae

WT, Rac1KO and Rac2KO animals were analyzed by dual energy x-ray absorptiometry (DEXA) to evaluate bone mineral density (BMD). Considering the control group, we observed higher BMD in the whole body, isolated femurs and vertebrae of Rac1KO mice compared to Rac2KO and WT (fig 3.1). After osteoporosis was induced by ovariectomy the lumbar vertebrae from Rac knockout mice have higher BMD compared to WT (fig. 3.1). In order to confirm the BMD results, volumetric BMD was measured using micro-computed tomography images. This analysis revealed higher BMD in both Rac1KO and Rac2KO vertebrae compared to WT in the control group, and higher BMD in Rac2KO vertebrae in the osteoporotic state (fig. 3.1).
Figure 3.1. Bone Mineral Density (BMD) of whole body and isolated bones.

The Dual Energy X-ray Absorptiometry (DEXA) showed that Rac1KO whole body has higher BMD compared to the others in the control group (C), but no difference was observed between osteoporotic groups (OVX) (Fig 1A). Similar results were observed when isolated femurs were analyzed (Fig 1B). The lumbar vertebrae were also isolated and scanned and the results showed that both Rac1KO and Rac2KO vertebrae have significantly higher BMD compared to WT in both control and osteoporotic groups (p<0.05) (Fig 1C). In order to confirm the DEXA results, volumetric BMD were calculated using micro-CT images of vertebrae and it showed the higher BMD in both knock-out compared to WT in the control group, but in osteoporotic group only Rac2KO vertebrae had higher BMD compared to the others (p<0.05) (Fig 1D). Moreover, the results show higher values in the control group (C) compared to the osteoporotic group (OVX).
Back-scattered electron microscopy (BSE) demonstrated no difference in the mineralization profile of the samples in the control group. In the osteoporotic group, the histogram of the grey level generated by Rac2KO vertebrae analysis had narrower width, indicating higher mineralization homogeneity in this group compared to Rac1KO and WT (data not shown).

*Rac1KO osteoporotic vertebrae show more resistance to fracture compared to WT and Rac2KO*

The mechanical tests evaluated both cortical and trabecular mechanical properties. When trabecular bones were tested under compression, Rac1KO vertebrae showed higher mechanical properties compared to Rac2KO and WT in the control group and the difference persisted after the induction of osteoporosis (table 3.1). This result suggests that Rac1KO vertebrae are structurally and materially stronger when loaded to fracture even in an osteoporotic model. These outcomes are explained by strut analysis of trabecular connectivity where Rac1KO vertebrae show more connected trabeculae compared to WT in both control and osteoporotic group (higher numbers of nodes between trabeculae and higher length of node-node struts) (fig. 3.2).

When cortical bones were tested to fracture we observed that the ovariectomy did not change the results between groups and similar outcomes were observed in both control and osteoporotic groups. The only significant difference observed was that Rac2KO femurs from both control and osteoporotic groups displayed weaker structural properties (lower values for ultimate load and stiffness) in three-point bending but higher material properties when the results were normalized to bone cross-sectional area.
(Rac2KO showed higher values for ultimate stress and modulus). This result suggests that Rac2KO cortical bones are structurally weaker but materially stronger compared to WT (table 3.2). These outcomes were explained when we analyzed the bone cross-section area and bone diameter between groups, which showed that Rac2KO cortical bones have significant smaller geometric structure compared to the other groups (table 3.2) and this difference remains in the osteoporosis-induced condition.

<table>
<thead>
<tr>
<th>Control</th>
<th>WT</th>
<th>Rac1KO</th>
<th>Rac2KO</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Structural Properties</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ultimate load (N)</td>
<td>25.8 (± 7.72)</td>
<td>32.27 (± 9.51)*</td>
<td>26.94 (± 5.52)</td>
</tr>
<tr>
<td>failure displacement (mm)</td>
<td>0.777 (± 0.24)</td>
<td>0.771 (± 0.21)</td>
<td>0.531 (± 0.14)</td>
</tr>
<tr>
<td>energy to failure (mJ)</td>
<td>10.66 (± 4.14)</td>
<td>12.04 (± 5.00)</td>
<td>8.13 (± 3.50)</td>
</tr>
<tr>
<td>Stiffness (N/mm)</td>
<td>64.47 (± 18.99)</td>
<td>91.09 (± 30.28)*</td>
<td>98.56 (± 39.87)</td>
</tr>
<tr>
<td><strong>Material Properties</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ultimate stress (MPa)</td>
<td>11.78 (± 3.39)</td>
<td>15.03 (± 4.79)*</td>
<td>12.73 (± 3.12)</td>
</tr>
<tr>
<td>failure strain (%)</td>
<td>30.06 (± 8.69)</td>
<td>30.36 (± 8.91)</td>
<td>22.01 (± 6.04)</td>
</tr>
<tr>
<td>Toughness (MPa)</td>
<td>1.88 (± 0.66)</td>
<td>2.19 (± 0.94)</td>
<td>1.59 (± 0.73)</td>
</tr>
<tr>
<td>Modulus (MPa)</td>
<td>76.44 (± 23.43)</td>
<td>109.82 (± 41.78)*</td>
<td>114.68 (± 54.36)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>OVX</th>
<th></th>
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</thead>
<tbody>
<tr>
<td><strong>Structural Properties</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>failure displacement (mm)</td>
<td>0.613 (± 0.085)</td>
<td>0.829 (± 0.085)</td>
<td>0.513 (± 0.142)</td>
</tr>
<tr>
<td>energy to failure (mJ)</td>
<td>5.01 (± 2.081)</td>
<td>8.092 (± 2.882)*</td>
<td>5.083 (± 2.252)</td>
</tr>
<tr>
<td>Stiffness (N/mm)</td>
<td>44.817 (± 16.05)</td>
<td>75.857 (± 30.58)**</td>
<td>72.293 (± 26.18)**</td>
</tr>
<tr>
<td><strong>Material Properties</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ultimate stress (MPa)</td>
<td>8.007 (± 2.617)</td>
<td>11.545 (± 2.991)*</td>
<td>8.19 (± 2.667)</td>
</tr>
<tr>
<td>failure strain (%)</td>
<td>19.933 (± 2.304)</td>
<td>20.81 (± 2.927)</td>
<td>17.013 (± 4.44)</td>
</tr>
<tr>
<td>Toughness (MPa)</td>
<td>0.802 (± 0.273)</td>
<td>1.285 (± 0.45)*</td>
<td>0.746 (± 0.314)</td>
</tr>
<tr>
<td>Modulus (MPa)</td>
<td>68.615 (± 19)</td>
<td>109.15 (± 42.36)**</td>
<td>99.36 (± 42.3)</td>
</tr>
</tbody>
</table>

Values are means ± SD
* p<0.05 Rac1KO compared to Rac2KO and WT
** p<0.05 Rac1KO compared to WT
*** p<0.05 Rac1KO and Rac2KO compared to WT

Table 3.1. Bone mechanical properties from vertebral compression
<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>WT</th>
<th>Rac1KO</th>
<th>Rac2KO</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Structural Properties</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ultimate load (N)</td>
<td>26.982 (± 5.419)</td>
<td>28.819 (± 5.563)</td>
<td>22.889 (± 2.645)***</td>
<td>191.39 (± 17.4)*</td>
</tr>
<tr>
<td>Stiffness (N/mm)</td>
<td>225.94 (± 38.51)</td>
<td>246.5 (± 31.76)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Material Properties</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ultimate stress (MPa)</td>
<td>191.96 (± 19.67)</td>
<td>179.82 (± 31.81)</td>
<td>211.87 (± 35.85)***</td>
<td>8851.98 (± 1777)***</td>
</tr>
<tr>
<td>Modulus (MPa)</td>
<td>7877.98 (± 692)</td>
<td>6833.67 (± 1631)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Femur Geometry</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>cross-sectional area</td>
<td>0.956 (± 0.139)</td>
<td>0.976 (± 0.087)</td>
<td>0.804 (± 0.085)*</td>
<td></td>
</tr>
<tr>
<td>anterior-posterior diam.</td>
<td>1.232 (± 0.069)</td>
<td>1.381 (±0.093)**</td>
<td>1.214 (± 0.092)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>OVX</td>
<td></td>
<td>Rac1KO</td>
<td>Rac2KO</td>
</tr>
<tr>
<td><strong>Structural Properties</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ultimate load (N)</td>
<td>21.657 (± 3.327)</td>
<td>20.346 (± 2.486)</td>
<td>18.499 (± 2.976)*</td>
<td>172.38 (± 29.10)*</td>
</tr>
<tr>
<td>Stiffness (N/mm)</td>
<td>197.01 (± 16.95)</td>
<td>187.80 (± 23.18)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Material Properties</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ultimate stress (MPa)</td>
<td>159.93 (± 17.50)</td>
<td>153.00 (± 19.85)</td>
<td>188.96 (± 27.58)*</td>
<td></td>
</tr>
<tr>
<td>Modulus (MPa)</td>
<td>7165.35 (± 774)</td>
<td>6454.90 (± 996)</td>
<td>9300.98 (± 1365)*</td>
<td></td>
</tr>
<tr>
<td><strong>Femur Geometry</strong></td>
<td></td>
<td></td>
<td>Rac1KO</td>
<td>Rac2KO</td>
</tr>
<tr>
<td>cross-sectional area</td>
<td>0.885 (± 0.071)</td>
<td>0.798 (± 0.074)</td>
<td>0.712 (± 0.055)*</td>
<td></td>
</tr>
<tr>
<td>anterior-posterior diam.</td>
<td>1.228 (± 0.050)</td>
<td>1.318 (± 0.045)</td>
<td>1.135 (± 0.046)*</td>
<td></td>
</tr>
</tbody>
</table>

Values are means ± SD
* p<0.05 Rac2KO compared to Rac1KO and WT
**p<0.05 Rac1KO compared to Rac2KO and WT
***p<0.05 Rac2KO compared to Rac1KO
° Rac2KO compared to WT

Table 3.2. Bone mechanical properties from 3-point bending test

![Strut Analysis](image)

**Figure 3.2. Trabecular architecture analyzed at strut analysis.**
The strut analysis confirmed that Rac1KO trabecular bone is more connected in both control (C) and osteoporotic (OVX) groups. The results revealed that Rac1KO vertebrae have higher number of nodes (A) and higher length of node-node strut (B) compared to WT (p<0.05).
**Rac2KO osteoporotic vertebrae have more trabecular bone compared to WT and Rac1KO**

The micro-computed tomography (micro-CT) scanning is a very accurate test to measure the vertebral architecture because it quantifies the trabecular structure in three-dimensions. The micro-CT scans showed that while in the control group both Rac1KO and Rac2KO showed higher structural values compared to WT, after the ovariectomy only Rac2KO vertebrae have a higher percentage of bone volume (BV/TV) and trabecular number (Tb.N.) and lower trabecular separation (table 3.3). Although all three phenotypes lost trabecular bone after ovariectomy compared to control, Rac2KO osteoporotic vertebrae showed significantly better trabecular structure compared to the Rac1KO and WT.

<table>
<thead>
<tr>
<th>Control</th>
<th>WT</th>
<th>Rac1KO</th>
<th>Rac2KO</th>
</tr>
</thead>
<tbody>
<tr>
<td>percentage of bone volume</td>
<td>9.751 (± 3.03)</td>
<td>17.68 (± 2.93)*</td>
<td>18.37 (± 2.79)*</td>
</tr>
<tr>
<td>trabecular thickness</td>
<td>0.0585 (± 0.003)</td>
<td>0.0609 (± 0.002)</td>
<td>0.0598 (± 0.002)</td>
</tr>
<tr>
<td>trabecular number</td>
<td>1.65 (± 0.454)</td>
<td>2.89 (± 0.42)*</td>
<td>3.07 (± 0.474)*</td>
</tr>
<tr>
<td>trabecular separation</td>
<td>0.363 (± 0.042)</td>
<td>0.287 (± 0.027)*</td>
<td>0.264 (± 0.045)*</td>
</tr>
</tbody>
</table>

**OVX**

| percentage of bone volume | 7.42 (± 2.18) | 9.97 (± 3.12) | 14.29 (± 2.98)** |
| trabecular thickness | 0.0557 (± 0.002) | 0.0574 (± 0.001) | 0.0573 (± 0.002) |
| trabecular number | 1.33 (± 0.37) | 1.74 (± 0.54) | 2.49 (± 0.53)** |
| trabecular separation | 0.393 (± 0.057) | 0.353 (± 0.058) | 0.293 (± 0.051)** |

Values are means ± SD

* p<0.05 Rac1KO and Rac2KO compared to WT
**p<0.05 Rac2KO compared to Rac1KO and WT

Table 3.3. Micro-computed tomography of vertebrae
Osteoclasts in knock-out mice have less nuclei per cell and knock-out bones show lower bone formation rates

The TRAP stained slides were analyzed at high magnification (X100) to evaluate osteoclast nuclei number per osteoclast. It was clearly observed that knock-out samples have fewer osteoclasts with more than three nuclei compared to WT in both control and ovariectomized groups (fig. 3.3), which is consistent with the increased bone volume observed in knock-outs groups.

The calcein-green labels were analyzed in unstained slides for dynamic histomorphometric measurements. As shown in table 3.4, both Rac1KO and Rac2KO mice had significantly lower values for mineral appositional rates (MAR) and bone formation rates (BFR) compared to WT in both control and osteoporotic groups. In accordance to these results, both knock-out groups showed lower values for formation parameters (osteoid volume and osteoid surface) measured in Goldener’s Trichrome stained sections, compared to WT in both control and osteoporotic groups (fig 3.4).

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>WT</th>
<th>Rac1KO</th>
<th>Rac2KO</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mineralizing surface</td>
<td>Mineralizing surface</td>
<td>1.425 (± 0.49)</td>
<td>1.655 (± 0.39)</td>
<td>1.396 (± 0.32)</td>
</tr>
<tr>
<td>% Mineralizing surface</td>
<td>14.22 (± 3.19)</td>
<td>15.25 (± 4.25)</td>
<td>11.64 (± 2.55)</td>
<td></td>
</tr>
<tr>
<td>MAR</td>
<td>0.91 (± 0.158)</td>
<td>0.759 (± 0.76)*</td>
<td>0.711 (± 0.146)*</td>
<td></td>
</tr>
<tr>
<td>BFR</td>
<td>0.129 (± 0.038)</td>
<td>0.115 (± 0.032)</td>
<td>0.084 (± 0.032)**</td>
<td></td>
</tr>
</tbody>
</table>

|          | O VX     | Mineralizing surface | 1.478 (± 0.43) | 1.098 (± 0.41) | 1.049 (± 0.36) |
| % Mineralizing surface | 15.95 (± 4.99) | 11.62 (± 3.97)* | 10.78 (± 3.29)* |
| MAR      | 0.84 (± 0.108) | 0.64 (± 0.158)* | 0.67 (± 0.142)* |
| BFR      | 0.137 (± 0.053) | 0.075 (± 0.033)* | 0.074 (± 0.027)* |

MAR - mineral appositional rate; BFR - bone formation rate values are means ± SD
* p<0.05 Rac1KO and Rac2KO compared to WT
** p<0.05 Rac2KO compared to WT

Table 3.4. Dynamic Histomorphometry
Figure 3.3. Representative images of osteoclasts in TRAP stained slides
Osteoclasts (black arrow) from Rac1KO and Rac2KO TRAP stained slides show fewer nuclei per cell compared WT in both control and ovariectomized groups, which suggest the lower osteoclast resorptive activity in Rac knock-out groups compared to WT.

Figure 3.4. Bone formation parameters evaluated by static histomorphometry.
The static histomorphometry showed that both Rac1KO and Rac2KO showed lower values for bone formation parameters in both control (C) and osteoporotic (OVX) groups. The osteoid volume (A) and the osteoid surface (B) of knock-out samples are significantly smaller compared to WT (p<0.05).
DISCUSSION

The Rho family of small GTPases is critical to intra-cellular signaling in diverse cell populations. The literature has reported the crucial role of Rac1 and Rac2 in actin cytoskeleton dynamics, cell movement, phagocytosis, cell growth, cell adhesion, gene transcription and reactive oxygen species (ROS) production (Guo et al., 2008; Sun et al., 2007; Wang et al., 2008; Wheeler et al., 2006), crucial steps in cell differentiation. During menopause, a physiological phase in female life cycle, the body faces a sudden and dramatic decrease in estrogen production due to the arrest of ovarian function. The loss of sex steroids leads to increase bone remodeling due to upregulation of osteoblastogenesis (Jilka et al., 1998) and osteoclastogenesis (Jilka et al., 1992). This increased post-menopausal bone turnover shifts bone homeostasis toward bone resorption, resulting in rapid trabecular bone loss (Manolagas et al., 2002; Raisz, 2005).

The work described here is focused on determining if Rac1 and or Rac2 are involved in the postmenopausal related skeletal effects that mediate increased bone fragility and fracture risk. This study will help determine if these two small GTPases and their signaling pathways are potential therapeutic targets for preventing osteoporosis.

Osteoporotic Rac knock-out mice model

In order to evaluate the in vivo role of Rac1 and Rac2 in bone quality, a previously described knock-out mouse model in which Rac1 or Rac2 are deleted in monocytes was used (Glogauer et al., 2003). Using this model we recently reported that Rac1 is the primary Rac isoform required for in vitro osteoclastogenesis (Wang et al.,
2008), and we demonstrated that the deletion of either Rac isoform leads to attenuation of age-related bone loss (Magalhaes et al., 2009).

Ovariectomy of the mouse has been proven to successfully simulate menopause in younger female mice. Generally, mice experience a rapid trabecular bone loss following ovariectomy, and estrogen therapy has been reported to prevent this bone loss (Bain et al., 1993). Using an ovariectomized murine model we evaluate here the role of each Rac small GTPase in bone regulation associated with the physiological higher bone turnover typical in the postmenopausal phase.

The study of osteoporosis has furthered our understanding of the mechanisms that cause bone loss and microarchitecture deterioration of skeletal structures. Riggs et al have brought the concept of two forms of osteoporosis, type I known as postmenopausal osteoporosis, characterized by estrogen deprivation and excessive and rapid trabecular bone loss mainly associated with vertebral fractures; and type II osteoporosis, known as age related osteoporosis, characterized by a gradual age-related bone loss that affects both men and women (Riggs et al., 1982). Comparing the results of the ovariectomy group to the results from control group that aged naturally, we observed that bones from the osteoporotic group had poorer mechanical and structural properties compared to the control group. As expected the effects of ovariectomy were more significantly observed in trabecular bones, the site more affected by type I osteoporosis. Although the bone quality generally decreased after ovariectomy, the effects of the estrogen deficiency in vertebrae were minimized in Rac knock-out groups compared to wild type, which suggest that the Rac deletion attenuates the effects of post-menopause induced osteoporosis.
The deletion of Rac1 increases vertebral bone quality and attenuates the estrogen-deprivation-related bone loss

Our results suggest that the deletion of Rac1 in monocyte-osteoclast precursors generates mechanically stronger trabecular bones with increased trabecular connectivity compared to WT bones in the osteoporotic mouse model. The crucial role of Rac1 in osteoclast formation observed in our previous study (Wang et al., 2008) may explain the Rac1KO phenotype observed in the present study. Rigg et al recently suggested that the up regulation of RANKL on bone marrow cells is a key determinant for the enhanced bone resorption induced by estrogen deficiency (Eghbali-Fatourechi et al., 2003). This is consistent with the idea that the deletion of Rac1 dampens the RANK signaling pathway, leading to attenuation of osteoclastogenesis and osteoclast resorptive activity. This would result in minimizing the effects of estrogen deprivation generated by the ovariectomy in Rac1KO animals compared to wild type controls.

Although the absence of Rac1 in an osteoporotic model did not make a significant difference in the bone quality of long bones, our results clearly show its impact on vertebral bone quality, which are the bones more clinically affected by postmenopausal osteoporosis (Manolagas et al., 2002; Raisz, 2005; Riggs et al., 1982). The attenuated resorptive activity of osteoclasts in the osteoporotic Rac1KO may explain the increased vertebral connectivity and increased bone strength in the mechanical tests in this group.

As in previous reports (Lee et al., 2006; Wang et al., 2008), we observed that the Rac knock-out osteoclasts have fewer nuclei per cell, which is consistent with the significantly less effective resorptive activity in these animals. Dynamic histomorphometric analysis clearly demonstrated that osteoporotic Rac1KO samples had
lower bone formation rates compared to WT, which is the expected consequence of the decreased activity of osteoclasts in those animals (Chambers, 2000; Teitelbaum, 2007). We believe that the reduced osteoclast activity in the Rac1 knock-out group slows down the high bone turnover present in the postmenopause state and consequently attenuates the vertebral bone loss caused by estrogen deprivation.

*The deletion of Rac2 minimized the trabecular bone loss induced by osteoporosis*

Our results revealed that Rac2KO vertebrae have very good structural parameters compared to Rac1KO and WT in the osteoporotic group, which was clearly observed by micro-CT analysis. Although these results were not translated to higher mechanical properties observed in vertebral compression, the higher BMD and higher trabecular mass observed in this group compared to wild type group led us to conclude that the deletion of Rac2 improves bone quality in a postmenopause state. While the deletion of Rac1 seems to directly affect the trabecular mechanical properties in osteoporotic bones, the deletion of Rac2 seems to reduce the typical trabecular bone loss caused by osteoporosis.

We also observed that in the absence of Rac2, cortical bones were smaller in size compared to WT and Rac1KO samples. The effect of Rac2 on bone dimension has been observed by us previously at early time points as reported in our previous analysis of bone quality in Rac2KO bone (Magalhaes et al., 2009). We hypothesize that the absence of Rac2 may negatively affect bone formation through osteoclast regulation of bone remodeling. Garimella et al reported the importance of bone morphogenic proteins (BMP) expressed in activated osteoclasts involved in the initiation of the anabolic phase.
of bone remodeling (Garimella et al., 2008). The exact role of Rac2 in regulation of osteoclast functions remains unclear, however our result suggests that this small GTPase might play a role not only in osteoclast resorptive activity but also in osteoclast mediated promotion of bone formation. We hypothesize that the effects of Rac2 in modulating osteoclast activity by regulating the release of anabolic factors could be more significant than the inhibition of bone resorption that Rac2 knock-out could promote. This effect could explain the significantly reduced size of Rac2KO femurs not observed in the Rac1KO group. Moreover, the reduce bone formation possibly present in Rac2KO group, associated to reduced bone resorption should increase the downregulation of bone turnover in this group. These factors may explain the preservation of the trabecular bone volume compared to WT after the development of osteoporosis.

Similar to what was observed in Rac1KO group, and in our previous investigation (Magalhaes et al., 2009), Rac2KO bones also showed osteoclasts with less nuclei per cell, consistent with lower resorptive activity. Moreover, Rac2KO bones also displayed decreased bone formation rates in dynamic histomorphometric analysis similarly to Rac1KO samples. Our results showed that although Rac2KO bones did not show the same phenotype compared to Rac1KO in the postmenopausal phase, the deletion of both Rac GTPases down-regulated the bone turnover and protected trabecular bone, the site more affected by post-menopausal osteoporosis, against the effects of estrogen deprivation.
The deletion of Rac1 and Rac2 improve trabecular bone quality in an osteoporotic model in different way

We clearly observed the positive effects in trabecular bone quality of an osteoporotic model when either Rac1 or Rac2 are deleted. Although the deletion of Rac2 attenuated the effects of bone loss typically present in osteoporosis, the higher bone mass and bone mineral density observed in Rac2KO vertebrae were not translated to higher performance in the mechanical tests. Several studies have shown that the ability of a bone to resist fracture at a given loading configuration depends not only on its mass but also on the spatial distribution of bone tissue and the intrinsic properties of bone material (Crawford et al., 2003; Lochmuller et al., 2008; Lochmuller et al., 2002). Moreover, a recent review concluded that the bone mass organization in vertebrae is likely to be more associated with vertebral strength, suggesting that one of the most important factors associated with an increased risk of bone fracture might be the individual’s ability and propensity to remodel the trabecular bone to local mechanical changes (Kreider and Goldstein, 2009). This could explain the different phenotypes observed between Rac1KO and Rac2KO vertebrae. We believe that while the absence of Rac2 minimized the trabecular bone loss in the osteoporotic model, the absence of Rac1 generated vertebrae with trabeculae that are optimally organized in a way that maximizes the vertebral strength even with lower bone mass. The combination of both effects generated by the deletion of Rac1 and Rac2 could compensate for the vertebral deterioration caused by osteoporosis.
CONCLUSION

The *in vivo* analysis of bone properties in an osteoporotic mouse model suggests that both Rac1 and Rac2 deletion preserve trabecular bone by increasing bone quality which effectively attenuate the effects of estrogen deprivation. While Rac1 deletion effectively improved the vertebral mechanical properties, Rac2 absence reduced the trabecular bone loss in these OVX mice. These results suggest that these two small GTPases or their signaling pathways are potential targets for the treatment of postmenopausal osteoporosis.
CHAPTER 4: CONCLUSION
4.1 – Thesis summary and conclusion

In this work we evaluated the bone quality of Rac1 and Rac2 deficient mice. The animals were challenged with aging and sexual hormone deprivation induced by ovariectomy to create the aged and the post-menopause model. By evaluating the bone quality of Rac knock-out mice with osteoporosis we were able to measure the bone properties in affected bones and assess the impact of Rac1 and Rac2 in regulating the mechanisms responsible for bone deterioration.

_in vitro_ studies have demonstrated the key role of Rac GTPases in osteoclast differentiation and activity, which lead to the hypothesis that Rac may play important role in bone remodeling and should be key determinant of bone quality in conditions characterized by enhanced activity of osteoclasts. Confirming this hypothesis, our analyses of bone quality in Rac1 and Rac2 knock-out animals revealed that the deletion of Rac generated higher bone quality in RacKO osteoporotic mice compared to WT. These results demonstrated that the _in vitro_ reports supporting the Rac regulation of osteoclasts can be translated to _in vivo_ bone analyses and the association of both should be essential to increase our understanding in the cellular mechanisms that controlled bone turnover and their expression in the bone properties.

We observed that all animals experienced the effects of aging in the skeleton, such as higher BMD, weaker mechanical properties and important trabecular bone loss compared to the younger matched animals. However, both Rac1KO and Rac2KO were less affected by the age-related bone loss compared to WT. When the aged model was analyzed, the uncompensated bone resorption typically observed in aging bones was
minimized in Rac knock-out mice due to the reduction in the osteoclast activity (fig. 4.1). Cao et.al. have reported the increased expression of RANKL with aging as one factor to justify the increased osteoclast activity in aged individuals (Cao et al., 2003). Therefore we believe that the deletion of Rac1 and Rac2 small GTPases, well known to be important elements in the RANKL signaling pathway (Brazier et al., 2006; Lee et al., 2006), should explain the reduction in the osteoclast hyperactivity during the aging process in RacKO mice.

Figure 4.1. Aged Model
The deletion of Rac attenuated the aging effects in trabecular bones compared to WT. After aging, both Rac knock-out vertebrae showed higher BMD and trabecular bone volume compared to WT. Moreover, aged Rac1KO vertebrae have more connected trabeculae.
In order to evaluate the effect of estrogen deprivation in bone quality, ovariectomized mice were compared to aged-matched control. The results confirmed the higher bone quality in RacKO bones compared to WT even in an osteoporotic state. Srivastava et. al. reported that estrogen down-regulates osteoclastogenesis by decreasing the responsiveness of osteoclast precursors to RANKL (Srivastava et al., 2001). Therefore we believe that the deletion of Rac minimizes the effects caused by the sudden drop in estrogen production and consequently high stimulation of osteoclast activity. While the deletion of Rac1 in the osteoporotic model generated more connected and resistant vertebrae, the absence of Rac2 generated vertebrae with higher BMD and more trabecular bone (fig 4.2). Although Rac1KO and Rac2KO generated different phenotypes, both attenuated the post-menopausal bone loss. These different phenotypes should be explained by the different roles that Rac1 and Rac2 might have in bone remodeling activity. While the deletion of Rac1 might preserve more the bone material, the deletion of Rac2 might prevent the high trabecular bone loss during osteoporosis. We believe that the association of both effects should be an optimal alternative to overcome the deleterious effect of post-menopausal osteoporosis.
Figure 4.2. Osteoporotic Model
The deletion of Rac attenuated the effects generated by ovariectomy in trabecular bones compared to WT. After post-menopause osteoporosis is induced, Rac1KO vertebrae show more connected trabeculae leading to higher mechanical resistance, and Rac2KO vertebrae show more trabecular bone volume with higher BMD compared to WT.

Summary

Based on the reports previously described, we observed the crucial importance of Rac GTPases in the control of bone homeostasis. The results presented in the previous chapters showed that the deletion of Rac1 or Rac2 in pre-osteoclasts minimized bone loss in both age-related and post-menopause osteoporosis. These results highlight the importance of the two small GTPases in bone remodeling and identify Rac1 and Rac2 as potential targets for the development of new therapies for the treatment of osteoporosis.
REFERENCES


