Osteoclast-specific inactivation of the Integrin-Linked Kinase (ILK) inhibits bone resorption

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Abstract

Bone resorption requires the adhesion of osteoclasts to extracellular matrix (ECM) components, a process mediated by the αvβ3 integrin. Following engagement with the ECM, integrin receptors signal via multiple downstream effectors, including the Integrin-Linked Kinase (ILK). In order to characterize the physiological role of ILK in bone resorption, we generated mice with an osteoclast-specific ILK gene ablation. Mice with one inactivated ILK allele (ILK⁺/-) were mated with TRAP-Cre transgenic mice. Progeny from this cross (TRAP-Cre;ILK⁺/-) was bred to mice homozygous for a floxed ILK allele (ILK⁺/floxed) to yield mutant mice with ILK-deficient osteoclasts (TRAP-Cre;ILK⁺/floxed). The mutant animals thus had one ILK allele inactivated in all tissues, and both alleles disrupted in osteoclasts. Mutant mice appeared phenotypically normal, but histomorphometric analysis of the proximal tibia revealed an increase in bone volume and trabecular thickness. Osteoclastogenesis, assessed by TRAP staining of bone sections or in vitro cultures, was not affected. Indeed, osteoclast-specific ILK ablation was associated with an increase in osteoclast number both in vitro and in vivo. Primary cultures of osteoclasts were generated on synthetic calcium phosphate discs, as well as dentin, and the mutant cells displayed a decrease in resorption activity. We also measured decreased serum concentrations of the C-terminal telopeptide of collagen, a marker of osteoclastic activity, in mice with ILK-deficient osteoclasts. Our results show that ILK is important for the function, but not the differentiation, of osteoclasts. The characterization of the molecular mechanisms responsible for the observed phenotype will identify novel pathways regulating bone resorption.
Résumé

La résorption osseuse requiert l’adhésion des ostéoclastes aux composantes de la matrice extracellulaire (ECM), un procédé qui dépend de l’intégrine α₃β₃. Après liaison avec l’ECM, les récepteurs intégrines transmettent leur signal via des messagers secondaires, incluant Integgin-linked kinase (ILK). Afin de caractériser le rôle physiologique de ILK dans la résorption osseuse, nous avons généré un modèle de souris avec une ablation spécifique du gène ILK dans l’ostéoclaste. Les souris avec un allèle ILK inactivé (ILK⁺⁻) ont été croisée avec des souris transgéniques TRAP-Cre. La progéniture de ce croisement (TRAP-Cre;ILK⁺⁻) a été croisée avec des souris homozygotes pour l’allèle ILK modifié (ILKfloflo) pour produire des souris mutantes avec des ostéoclastes déficients en ILK (TRAP-Cre;ILK⁻⁻). Les souris mutantes avaient donc un allèle ILK inactivé dans tous les tissus et les deux allèles ILK inopérants dans les ostéoclastes. Le phénotype des souris mutantes apparaissait normal mais l’analyse histomorphométrique du tibia proximal a révélé une augmentation du volume osseux et de l’épaisseur trabéculaire. L’ostéoclastogénèse, évaluée par coloration TRAP de section d’os ou de cultures in vitro, n’était pas affectée. En effet, l’ablation de ILK spécifique aux ostéoclastes est associée avec une augmentation du nombre d’ostéoclastes autant in vitro que in vivo. Les cultures primaires d’ostéoclastes ont été générées sur des disques synthétiques de phosphate de calcium ainsi que sur dentine et les cellules mutantes ont démontré une diminution de l’activité de résorption. Nous avons également mesuré une diminution des concentrations sériques du télopeptide C-terminal du collagène, un marqueur de l’activité ostéoclastique, chez les souris avec ostéoclastes déicients en ILK. Nos résultats démontrent que ILK est important pour la fonction mais non la différenciation des ostéoclastes. La caractérisation des mécanismes moléculaires responsable du phénotype observé permettra l’identification de nouvelles voies de signalisation impliquées dans la résorption osseuse.
Acknowledgements

Firstly, I would like to thank my supervisor, Dr. René St-Arnaud, for giving me the opportunity to be a part of his research team. He has provided me with invaluable guidance, knowledge and the skills required to approach my future tasks with the utmost confidence. I am grateful to him for all that I have learned during my time here. I would like to thank my committee members, Dr. Francis Glorieux and Dr. Svetlana Komarova for their guidance and direction throughout my project. I would also like to thank Alice Arabian for the creation of the ILK KO and ILK fl/fl strains as well as her insight and technical advice. Much appreciation goes to Dr. Eunice Lee for performing the electron microscopy studies. I would like to extend my gratitude to Dr. Denis Roodman for providing us with the TRAPCre transgenic strain and Dr. Shoukat Dedhar for providing us with the ILK-inhibitor molecule. Thanks to the illustration staff, especially Guylaine Bédard for preparing all of the thesis figures. Many thanks to the animal health care technicians: Mia Esser, Louise Marineau, Judith Cowen and Mireille Malouin. A special thanks goes to all of my colleagues and friends at the Shriners Hospital for the stimulating conversations and unforgettable laughs. I would most importantly like to thank my parents for their unconditional love and support throughout all of my academic endeavors, as none of this would have been possible without them. And lastly, my brother Irfan, who has taught me that perseverance is the key to success.
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<tr>
<td>a-NAC</td>
<td>Nascent polypeptide-associated complex</td>
</tr>
<tr>
<td>ABD</td>
<td>Actin-binding domain</td>
</tr>
<tr>
<td>ALP</td>
<td>Alkaline phosphatase</td>
</tr>
<tr>
<td>AP-1</td>
<td>Activating protein-1</td>
</tr>
<tr>
<td>ATF4</td>
<td>Activating transcription factor 4</td>
</tr>
<tr>
<td>bHLH</td>
<td>Basic helix-loop-helix</td>
</tr>
<tr>
<td>bHLH-Zip</td>
<td>Basic helix-loop-helix zipper</td>
</tr>
<tr>
<td>Ca2+</td>
<td>Calcium ion</td>
</tr>
<tr>
<td>CAII</td>
<td>Carbonic anhydrase II</td>
</tr>
<tr>
<td>CaSR</td>
<td>Calcium-sensing receptor</td>
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<td>CATK</td>
<td>Cathepsin K</td>
</tr>
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<td>Cbl</td>
<td>c-Cbl</td>
</tr>
<tr>
<td>CH</td>
<td>Calponin homology</td>
</tr>
<tr>
<td>CH-ILKBP</td>
<td>Calponin homology domain-containing ILK-binding protein</td>
</tr>
<tr>
<td>CSF</td>
<td>Colony stimulating factor</td>
</tr>
<tr>
<td>Dpd</td>
<td>Deoxypyridinoline</td>
</tr>
<tr>
<td>dRTA</td>
<td>renal tubular acidosis</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
</tr>
<tr>
<td>EGMA</td>
<td>Ethylene glycol monoethyl ether acetate</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbant assay</td>
</tr>
<tr>
<td>ERK</td>
<td>Extracellular signal-regulated kinase</td>
</tr>
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<td>FA</td>
<td>Focal adhesion</td>
</tr>
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<td>Focal adhesion kinase</td>
</tr>
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<td>GM-CSF</td>
<td>Granulocyte macrophage colony stimulating factor</td>
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<td>GSK-3</td>
<td>Glycogen synthase kinase 3</td>
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<td>Interleukin-1</td>
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<td>ILK</td>
<td>Integrin-linked kinase</td>
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<td>JNK</td>
<td>c-Jun N-terminal kinase</td>
</tr>
<tr>
<td>LD motifs</td>
<td>leucine-rich repeating sequences</td>
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<td>LEF-1</td>
<td>Lymphoid enhancer binding factor 1</td>
</tr>
<tr>
<td>LIM</td>
<td>Lin-11 Isl-1 and Mec-3</td>
</tr>
<tr>
<td>loxP</td>
<td>floxed stopper fragment</td>
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<tr>
<td>MAPK</td>
<td>Mitogen-activated protein kinase</td>
</tr>
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<td>MBP</td>
<td>Myelin basic protein</td>
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<td>Macrophage colony stimulating factor</td>
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<td>MEK</td>
<td>MAP kinase/ERK-activating kinase</td>
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<tr>
<td>MITF</td>
<td>Microphthalmia transcription factor</td>
</tr>
<tr>
<td>MLC</td>
<td>Myosin light chain</td>
</tr>
<tr>
<td>MLCK</td>
<td>Myosin light chain kinase</td>
</tr>
<tr>
<td>MLCP</td>
<td>Myosin light chain phosphatase</td>
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<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<td>--------------</td>
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<tr>
<td>MMP-9</td>
<td>Matrix metalloproteinase-9</td>
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<tr>
<td>mRNA</td>
<td>Mitochondrial ribonucleic acid</td>
</tr>
<tr>
<td>NF-κB</td>
<td>Receptor-activator of NF-kappa B</td>
</tr>
<tr>
<td>NFAT2</td>
<td>Nuclear activator of activated T cells (NFATc1)</td>
</tr>
<tr>
<td>ODF</td>
<td>Osteoclast differentiation factor</td>
</tr>
<tr>
<td>OPG</td>
<td>Osteoprotegerin</td>
</tr>
<tr>
<td>OPGL</td>
<td>Osteoprotegerin ligand</td>
</tr>
<tr>
<td>OSTM1</td>
<td>Osteopetrosis associated transmembrane protein</td>
</tr>
<tr>
<td>OSX</td>
<td>Osterix</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate-buffered saline</td>
</tr>
<tr>
<td>PCNA</td>
<td>Proliferating cell nuclear antigen</td>
</tr>
<tr>
<td>PFA</td>
<td>Paraformaldehyde</td>
</tr>
<tr>
<td>PH</td>
<td>Pleckstrin homology</td>
</tr>
<tr>
<td>Pi</td>
<td>Phosphate</td>
</tr>
<tr>
<td>PIP</td>
<td>PINCH- ILK-α-parvin</td>
</tr>
<tr>
<td>PI(3)K</td>
<td>phosphatidylinositol 3-0H kinase</td>
</tr>
<tr>
<td>PKB</td>
<td>Protein kinase B</td>
</tr>
<tr>
<td>PTEN</td>
<td>Phosphatase and tensin homolog (detected on chromosome 10)</td>
</tr>
<tr>
<td>PTH</td>
<td>Parathyroid hormone</td>
</tr>
<tr>
<td>p130Cas</td>
<td>Cas, Crk-associated substrate</td>
</tr>
<tr>
<td>Pyk-2</td>
<td>PYK2/CAKβ/RAFTK</td>
</tr>
<tr>
<td>PINCH</td>
<td>Particularly interesting new cytoine-histidine-rich protein</td>
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<tr>
<td>RANK</td>
<td>Receptor-activator of NFκB</td>
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<td>Receptor-activator of NFκB ligand</td>
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<td>arg-gly-asp</td>
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<td>RT-PCR</td>
<td>Reverse transcriptase-polymerase chain reaction</td>
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<tr>
<td>Runx2/Cbfa1</td>
<td>Runt domain factor 2/core binding factor α1</td>
</tr>
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<td>SDS</td>
<td>Sodium dodecyl sulfate</td>
</tr>
<tr>
<td>SH2</td>
<td>Src-homology 2</td>
</tr>
<tr>
<td>SH3</td>
<td>Src-homology 3</td>
</tr>
<tr>
<td>Src</td>
<td>pp60^src, proto-oncogene of the Rous Sarcoma Virus</td>
</tr>
<tr>
<td>TGF-β</td>
<td>Transforming growth factor -β</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumor necrosis factor</td>
</tr>
<tr>
<td>TRAF</td>
<td>Tumor necrosis factor receptor associated factor</td>
</tr>
<tr>
<td>TRANCE</td>
<td>Tumor necrosis factor TNF-related activation-induced cytokine</td>
</tr>
<tr>
<td>TRAP</td>
<td>Tartrate-resistant acid phosphatase</td>
</tr>
<tr>
<td>v-ATPase</td>
<td>Vacuolar proton-ATPase</td>
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The TRAPCre transgenic strain was created within and obtained graciously from the Roodman Laboratory. The ILK KO and ILK fl/fl strains were both created within our laboratory, at the Shriners Hospital for Children, by Alice Arabian prior to my arrival.

Electron microscopy studies were also performed on-site, with the assistance of Dr. Eunice Lee and laboratory.

**Introduction**

**Bone Function**

Bone is a specialized and complex tissue with both a protective and supportive role. It has many essential functions, such as protecting vital organs, providing a microenvironment for hematopoiesis, as well as a storage for calcium and other minerals. The organic matrix of bone is made up of type I collagen in combination with proteoglycan and non-collagenous proteins. The skeleton has been designed in such a manner that it can provide maximal support with minimal mass, in order to cater to the physiological needs of any organism [1].

Two processes vital to the survival of an organism are calcium homeostasis and hematopoiesis. Given the importance of both these processes, it seems likely that any type of resorptive cells were developed to support these functions in addition to skeletal maintenance. There are cells that respond to changes in mechanical loading and are responsible for the repair of microfractures, the maintenance of the bone skeleton. The study of skeletal systems has also demonstrated that the resorption of mineralized tissues
is required for normal skeletal maturation [2].

The endocrine role of bone, maintaining calcium homeostasis is essential to the survival of any organism. Bone acts as a reservoir for calcium (Ca++) and phosphate (Pi). When Ca++ and/or Pi levels in blood are low, the peptide hormone called parathyroid hormone (PTH) is released from the parathyroid glands. PTH induces tubular reabsorption of Ca++ from the kidneys and stimulates the kidneys to produce increased levels of a sterol hormone referred to as 1,25 dihydroxyvitamin D (1,25(OH)2D). 1,25(OH)2D, in turn, acts upon the small intestine to enhance Ca++ absorption, and along with PTH, stimulates osteoblasts to release a factor (called RANK-L) that binds to the surface of osteoclasts to enhance their activity. This causes an increase in osteoclast-mediated bone degradation, which in turn, releases Ca++ and Pi into the circulation. The overall effect is an increase in the levels of circulating Ca++. The secretion of PTH is controlled by the calcium-sensing receptor (CaSR), which monitors blood Ca++ levels. Accordingly, when circulating calcium levels are high, PTH secretion is inhibited, resulting in the uptake of Ca++ by the bone for storage and by the kidneys for excretion.

There are two different types of bone, cortical and cancellous. The two differ both in structure and function. Cancellous (spongy) bone has a loosely organized matrix and functions in metabolism. Cortical bone is more densely packed and the fibrils form lamellae, and it provides mechanical and protective functions [3].

The formation of the skeleton occurs two different ways, through intramembranous (direct) or endochondral (indirect) ossification.

**Intramembranous Ossification**

Intramembranous ossification occurs exclusively within bones associated with the
skin and ectoderm, such as the bones of the cranium, facial bones, clavicle and parts of the mandible. This direct method of bone ossification begins during embryonic development, with osteoprogenitor cells which differentiate into osteoblasts and then proceed to secrete bone matrix. Although it begins within an avascular environment, osteoblastic differentiation and the onset of mineralization is highly dependant upon vascularization. At first, mesenchymal precursors develop into mature secretory osteoblasts that secrete a matrix rich in collagen type I, forming irregularly shaped bone spicules. Then newly developed osteoblasts at the periphery continue to add bone to form a beam-like structure called a trabecula, creating spongy bone. As the process of bone remodeling continues, the continuous addition of matrix to the trabeculae eventually transforms the cancellous bone into a more compact form (Figure 1, [4]).

**Endochondral Ossification**

Endochondral ossification proceeds via a completely different mechanism which is much more complicated. All of the bones of the appendicular skeleton, including limbs, pectoral and pelvic girdles form through this pathway. The bones begin as cartilage elements that eventually become calcified, thus coined the indirect ossification method. In the mouse, the process starts at approximately embryonic day 12 (e12) with the formation of condensations by the vast accumulation of undifferentiated cells of mesenchymal origin. These cells differentiate into chondroblasts, which secrete collagen type II, IX and XI, to form a cartilage matrix [4]. This cartilaginous area is insulated from the surrounding mesenchyme by the perichondrium, formed by a thin membrane of flattened cells. [5] As new matrix is secreted, the cells within the center become enclosed and develop into chondrocytes. These chondrocytes proliferate and mature rapidly, becoming flat in shape, forming longitudinal columns along the axis of the developing bone [4]. These cells continue to mature, forming an enlarged cell known as a hypertrophic chondrocyte, which secretes a collagen type X matrix that gradually
Figure 1. Intramembranous bone formation. Condensation of mesenchyme is followed by vascularization and the differentiation of osteoblasts. The osteoblasts synthesize osteoid matrix which mineralizes to form bone.
becomes calcified [6]. This area is known as the growth plate. During this time, the perichondrial tissue is invaded by blood vessels and the cells on the inside of this structure differentiate into osteoblasts [4]. These periosteal osteoblasts secrete a layer of primary bone, forming the bone collar adjacent to the hypertrophic chondrocytes [5]. It is at this point that the thin perichondrial membrane covering the newly formed bone develops into a periosteum, continuing to supply the area with bone matrix to form the diaphysis, located midshaft on the bone. The cartilage matrix undergoes changes in composition within the hypertrophic zone triggering blood vessel invasion, at which point the hypertrophic chondrocytes undergo apoptosis [4]. The blood vessels transport skeletal progenitors to the interior, such as septoclasts and osteoclast precursors, which degrade the uncalcified portions of the cartilage, as well as osteoblasts [7]. The osteoblasts secrete bone matrix of the first trabeculae, establishing the primary ossification center within the metaphysis. It is within this area that the osteoclasts resorb these newly formed trabeculae to form the marrow space. This expansion of the newly formed growth plate proceeds from the articular ends known as the epiphysis to the diaphysis, resulting in longitudinal growth of the bony element, displayed in Figure 2 [4].

**Cells of Bone**

The skeleton is a complex collection of metabolically active cells residing within or near a mineralized matrix. It is composed of five different cell types, osteoblasts, osteoclasts, osteocytes, bone lining cells and chondrocytes. Osteoblasts are fully differentiated cells originating from local osteoprogenitor cells. They are responsible for the production of bone matrix and the regulation of mineralization, through secretion of type I collagen and non-collagenous proteins. Osteoclasts are large, multinucleated cells arising from hematopoietic precursor cells. They function solely in bone degradation and regulation of mineral metabolism. Osteocytes are the mature osteoblasts, which become
Figure 2. Growth plate structure. A late stage of the endochondral ossification process is shown on the left, with the bone having already developed into the distinct segments of the diaphysis, metaphysis, and epiphysis. The calcified cartilage of the primary spongiosa is illustrated. The various cell populations of the growth plate are shown at the right.
encased within the bone matrix and therefore have the capacity to sense biomechanical signals as well as to synthesize bone. The bone-lining cells are flat, inactive cells which reside within areas of bone that is not undergoing remodeling. It is believed that these cells are the precursors of osteoblasts [3]. Lastly, chondrocytes originate from mesenchymal stem cells, are located within the cartilage. They are the only cell present within the non-vascularized area and are responsible for the production and maintenance of cartilaginous matrix [4].

**Osteoblast Development and Function**

The synthesis, deposition, and mineralization of bone organic matrix are all complex processes which require the controlled expression of a number of genes [8]. The maturation sequence of osteoblasts has been divided into three consecutive phases: proliferation, extracellular matrix maturation, and mineralization [9].

Osteoblast differentiation and function are dependant upon a transcription factor called Runt domain factor 2/core binding factor α 1 (Runx2/Cbfa1) [4]. Runx2/Cbfa1 is a transcription factor of the runt family, expressed within osteoblasts as shown by the Ducy et al. group using Northern blot [10]. Studies using Runx2/Cbfa1-deficient mice demonstrated the requirement of this protein for skeletal formation, as they were completely devoid of osteoblasts, and therefore, bone matrix [11]. The developmental processes of bone and cartilage suggest that the same progenitor cell can differentiate into an osteoblast as well as a chondrocyte [8]. Grigoriadis et al. (1988) established a calvarial cell line to prove that a pluripotent progenitor cell can differentiate into many tissue-specific cells of bone, cartilage, adipose and muscle [12]. The differentiation of these progenitor cells is under the control of various tissue-specific transcription factors, Runx2/Cbfa1 being the factor specific for bone. Due to its essential role in osteoblastic bone formation, Runx2/Cbfa1 is essential for the formation of membranous as well as endochondral bone. Recently, two additional regulators of osteoblast differentiation were
identified, called osterix (*Osx*) and activating transcription factor 4 (ATF4). Briefly, *Osx* encodes a zinc finger-containing protein and is expressed specifically within osteoblasts. Mice deficient in this protein lack osteoblasts completely, thus preventing the formation of bone. The arrest in osteoblast development occurs at a later step than in Runx2/Cbfa1 null mice, indicating that Osx is located downstream of the runt family member [13].

ATF4 is a basic domain-leucine zipper (bZip) transcription factor, which forms heterodimers and homodimers to regulate many stages of osteoblast development. ATF4 is involved in the onset of differentiation, synthesis of type I collagen, gene-expression and terminal differentiation of osteoblasts. Therefore, mice deficient in ATF4 are runted [14].

Osteoblasts express multiple differentiation markers such as type I collagen and many extracellular matrix (ECM) proteins. Type I collagen is produced abundantly during the proliferative phase, and is the first recognizable marker of osteoblast differentiation. ECM proteins include osteocalcin, osteonectin, osteopontin, bone sialoprotein and proteoglycans [8]. Of these, the only major non-collagenous (secretory) matrix protein unique to osteoblasts is osteocalcin. Another well-known characteristic of osteoblast development, is high alkaline phosphatase (ALP) enzyme activity during the matrix maturation phase, which triggers bone mineralization [9].

Bone growth involves the coordination of many different cellular activities. The initiation of and speed at which they occur are dependant upon local and humoral factors whose relative concentrations and sequences of appearance vary during development. The level of bone mass reflects the balance of bone formation and resorption, which at the cellular level involves the coordinate regulation of bone forming cells (osteoblasts) and bone-resorbing cells (osteoclasts). Osteoblasts influence the differentiation of osteoclasts by expressing RANK-L, and this will be detailed later. It is important to note that skeletal maintenance is just as complicated as development of the skeleton [4].
Osteoclast Development

During the first 20 years of skeletal development, all cells are devoted to bone remodeling, where the formation of bone both precedes and exceeds its resorption. Osteoclastic activation depends upon local factors produced by all stromal cells, and is not restricted to osteoblasts [1]. The formation of osteoclasts in murine co-cultures of bone marrow and stromal cells by Takahashi et al. (1988), established that close contact between these two cell types was essential for osteoclastogenesis [15]. This system allows for the production of the two factors both necessary and sufficient for the differentiation of osteoclasts. Stromal cells produce two secreted proteins that influence the differentiation and function of osteoclasts, the polypeptide growth factor macrophage colony stimulating factor (M-CSF) and the recently identified TNF-related cytokine RANK-L in response to osteotrophic factors like vitamin D3, parathyroid hormone (PTH), and PTH related protein. These stromal-derived cytokines can be secreted locally or deposited within the bone matrix to induce the expression of genes specific to the osteoclast lineage, such as TRAP, cathepsin K (CATK), and the calcitonin receptor. Following this stage, the adult skeleton undergoes the process of remodeling, where localized resorption is succeeded by formation of bone (also known as coupling) equal in magnitude. Bone remodeling is the predominant metabolic process regulating bone structure and function during adult life [1]. These pathways will be detailed below.

Pre-osteoclasts are formed from hematopoietic stem cells located in the bone marrow and their differentiation is controlled by a signaling cascade involving several classes of transcription factors. The transcription factor PU.1 initiates the cascade. The cells enter the circulation and move towards the bone surface for conversion into active multinucleated osteoclasts[16]. This involves c-Fos expression as well as NF-κB activation, resulting in the formation of functional osteoclasts (Figure 3). Both NF-κB and c-fos are targets of the signaling cascade triggered by ligand (RANK-L) binding to its
Figure 3. Osteoclast maturation. Differentiation begins with expression of PU.1, followed by activation of c-fos and NFκB. Osteoblasts and stromal cells secrete M-CSF and RANK-L, two cytokines both necessary and sufficient for osteoclast development and activity. This results in the formation of pre-osteoclasts which then fuse to form a multinucleated osteoclast. Upon attachment to the surface, a signaling cascade initiates activation of the resorbing cell.
receptor on osteoclasts (RANK) [16, 17]. Other transcription factors involved in the control of osteoclast development are Nfatc1 and MITF. The critical role played by each of these factors has been established using genetic manipulation [13, 18].

The gene acting the earliest during osteoclastogenesis is a hematopoietic transcription factor called PU.1, which controls the cell-lineage differentiation of macrophages and osteoclasts from hematopoietic precursors. PU.1 is a member of the Ets family of transcription factors, and accordingly, contains a winged helix-turn-helix motif. Studies using a targeted inactivation of the PU.1 gene in mice demonstrate the necessity of this gene for osteoclast differentiation, since the mice develop a severe osteopetrotic phenotype due to the absence of osteoclasts. PU.1 deficient mice lack osteoclasts and macrophages, demonstrating that PU.1 controls the differentiation of these cells, and regulates the initial stages of myeloid differentiation [16].

Another key regulator of osteoclast differentiation is the proto-oncogene, c-fos, which acts later during development. C-fos is a member of the basic domain-leucine zipper (bZip) family of transcription factors, and has been shown to dimerize with Jun family members. C-fos gene expression plays an important role during osteoclast development, as it is expressed within mouse cartilage, bones and teeth. Wang et al. (1992) constructed a c-fos deletion in the mouse, and attributed the osteopetrotic phenotype to a complete absence of osteoclasts, indicating the requirement of this protein for the differentiation of monocyte precursors into osteoclasts. They noticed an increase in the number of macrophages within these mice, which suggested that c-Fos acts as a repressor of macrophage maturation, confirming that it is located downstream of PU.1 for the control of osteoclast differentiation. Furthermore, this demonstrated the vital role of c-fos during osteoclastogenesis, and ultimately bone resorption [17].

NFATc1 (NFAT2), a member of the nuclear factor of activated T cells (NFAT)
family of transcription factors, is the most strongly induced transcription factor gene
within osteoclasts following stimulation with RANK-L. NFAT2-deficient ES cell lines
were developed that were not able to differentiate further than the monocyte/macrophage
stage, as they normally would, following stimulation by RANK-L. These studies
established the essential role of NFAT2 in mediating signaling induced by RANK-L [18].

A basic helix-loop-helix-leucine zipper (bHLH-Zip) transcription factor called
microphthalmia transcription factor (MITF) is expressed at high levels in osteoclasts and
is also required for osteoclast differentiation. MITF is activated through phosphorylation
by M-CSF and RANK-L, and induces the expression of Bcl-2 and target genes essential
for osteoclast development and function. A mutation of the mi locus resulted in
differentiated osteoclasts that were incapable of bone resorption. This suggests that
MITF acts late during osteoclast development, and is more involved in the functional
activation of osteoclasts [13].

Osteoclast differentiation is also controlled by a protein secreted by the osteoblast
lineage called osteoprotegerin (OPG). Studies by Simonet et al. (1997) showed that
animals overexpressing OPG develop an osteopetrotic phenotype, and used histology to
attribute this to a large decrease in osteoclasts in the trabeculae. Therefore, it was not the
deficiency of osteoclast precursors causing this phenotype, but the differentiation of these
cells into functional osteoclasts. The Opg gene, encodes for OPG, a decoy receptor for
receptor-activator of NF-kappa B (NF-κB) ligand (RANK-L), to block its binding to
receptor-activator of NF-κB (RANK) on osteoclasts. This places OPG as a negative
regulator during the late stages of osteoclast differentiation [19].

The act of bone resorption produces factors that aid in the recruitment of
osteoblasts, which in turn, secrete factors that stimulate osteoclasts. These two cell types
work together in unison to maintain the integral structure of bone [3]. Bone remodeling
is a continuously occurring dynamic process.

**Osteoclasts and Resorption**

Osteoclasts develop from the fusion of multiple (usually 5 or 6) differentiated hematopoietic stem cells (also known as osteoclast precursors). Characteristics of mature osteoclasts include tartrate-resistant acid phosphatase activity (TRAP), calcitonin receptors, c-src activity, matrix metalloproteinase-9 (MMP-9) activity, and Cathepsin K (CATK) activity. Osteoclasts are the only cell type capable of forming resorption pits on bone and dentin slices. Osteoclast bone resorption is a complicated multistep process that begins with matrix recognition and attachment of the osteoclast, followed by polarization, formation of the sealing zone, and secretion of acids and lysosomal enzymes to the resorbing surface [20].

It has been shown that the larger the cell size, the higher the osteoclast activity, however the number of nuclei present is not a factor. Osteoclasts originate from stem cells of the hematopoietic tissues, and share a common differentiation pathway with macrophages until the final differentiation steps. The stem cells proliferate into mononuclear pre-osteoclasts within bone marrow and then move towards the mineralized tissues. At this point, multiple cells fuse to form a multinuclear precursor cell. Once this cell arrives at the bone surface, the resorption cycle is initiated by the retraction of the bone-lining cells. This action results in the exposure of the underlying osteoid which is removed by osteoclasts, so that the osteoclast can attach to the mineralized matrix. There is a highly regulated coordination of events during bone degradation called the resorption cycle [21].
Osteoclast Activation

Upon interaction with the mineralized bone extracellular matrix, the osteoclast induces clustering of the αvβ3 receptor and initiates intracellular signals. This initiation, downstream of αvβ3, causes tyrosine phosphorylation of Pyk-2 by Src, enabling binding. This triggers a signal transduction cascade involving pp60^src (Src), Pyk2/CAKβ/RAFTK (Pyk2), Cas, Crk-associated substrate (p130Cas), and c-Cbl (Cbl); causing activation of the osteoclast through regulation of actin-ring formation, osteoclast migration and bone resorption [22].

Soriano et al. (1991) created an osteopetrotic mouse by targeted disruption of the c-src gene. The product of the c-src proto-oncogene is a 60 kDa protein, Src, which belongs to the family of nonreceptor-type tyrosine kinases. Src consists of a Src-homology 2 (SH2) domain, a Src-homology 3 (SH3) domain and a catalytic domain [23]. Not long afterwards, experiments showed that osteoclasts express high levels of Src, and those deficient in this protein were unable to resorb bone due to the failure of ruffled border formation [24, 25]. These studies suggested the essential role of Src-mediated signaling in osteoclast activation and polarization. This requirement was further localized to the kinase activity of Src, as shown through experiments over-expressing Csk, the negative regulator of Src kinase. This suggested that the absence of ruffled border in the Src-mutants could be related to defective tyrosine phosphorylation of cytoskeletal proteins [26].

Investigation of the downstream modulators of c-Src identified a 120 kDa protein called Cbl. This proto-oncogene product is tyrosine-phosphorylated in response to the activation through signaling pathways such as M-CSF and c-Src, and is involved in modulating osteoclast cytoskeleton and cell motility [27]. Cbl is an adaptor protein and
ubiquitin ligase [27, 28]. Not only is the level of phosphorylated c-Cbl markedly reduced in c-Src-deficient osteoclasts, the two proteins have been shown co-localize within the membranes of intracellular vesicles in osteoclasts. Src-induced phosphorylation of Cbl regulates the binding of Cbl to several of its downstream targets. This data supports the notion that signal transduction from the cell membrane through Src tyrosine kinase is necessary for osteoclast-mediated bone resorption [23].

Pyk2 is a cytoplasmic member of the focal adhesion kinase family, with proline-rich regions within its C terminus [29, 30]. Pyk2 has been shown to localize within podosomes and the sealing some of resorbing osteoclasts, through immunofluorescence. In vitro, this protein has been shown to bind Src, Fyn, p130Cas, paxillin and Grb-2. Duong et al. (1998) demonstrated that upon integrin-mediated attachment of the osteoclast to the bone surface, Pyk2 tyrosine phosphorylation is induced, through binding of Src. Pyk2 binds the Src-homology 2 (SH2) domain of Src, which mediates its tyrosine phosphorylation [30]. Studies of Pyk2 tyrosine phosphorylation and kinase activity in Src-deficient mice displayed a marked reduction, indicating its position downstream of the c-Src signaling pathway and its involvement in actin ring formation [29].

Pyk2 was shown to co-localize with a 130 kDa protein within the actin ring of resorbing osteoclasts called p130Cas. The proline-rich regions of Pyk2 bind the Src-homology 3 domain of p130Cas, allowing for Src-mediated tyrosine phosphorylation. Osteoclasts deficient in Src do not display phosphorylation of p130Cas tyrosine residues, suggesting that it is located downstream of the Src signaling pathway and involved in organization of the cytoskeleton [31].

Following the induction of signal transduction by attachment to the bone surface, the osteoclast polarizes, giving rise to three morphologically distinct areas. These include the basolateral membrane, which is not in contact with the bone; the tight sealing zone
(clear zone), which closely associated with the bone surface; and the ruffled border, an intricately folded membrane that faces the resorbing surface. This creates an isolated resorptive microenvironment [32]. αvβ3 is responsible for transmitting the matrix-derived signals to cause reorganization of the osteoclast cytoskeleton [33]. Formation of the ruffled membrane is concomitant with reorganization of the actin cytoskeleton [34]. There is a unique organization of microfilaments within resorbing osteoclasts. This structure consists of a “double circle” of vinculin with F-actin dispersed within and is referred to as an actin ring. The sealing zone is essential for osteoclastic polarization, since it forms a diffusion barrier in order to allow the secretion of acid and lysosomal enzymes into the space beneath the ruffled membrane [35].

The osteoclast itself is activated through interaction of RANK with its ligand (RANK-L) and undergoes actin reorganization to form a tight junction between the bone surface and basal membrane to generate a compartment. This is followed by polarization of the osteoclast, which forms a snug interaction with the bone surface, as a result of cytoskeletal reorganization. The osteoclast plasma membrane forms a basolateral domain, a basal membrane and a ruffled border; encompassed by the sealing zone. This binding of the cell to the mineralized tissues creates an area isolated from the extracellular fluid where acidification takes place. During the resorptive phase, the cell organizes its fibrillar actin in a ring-like structure surrounding the bone microenvironment therefore isolating this resorptive space [36].

The ligand for receptor-activator of NFκB, RANK-L also known as osteoprotegerin ligand (OPGL), osteoclast differentiation factor (ODF) and TNF-related activation-induced cytokine (TRANCE) was identified in 1997 as a member of the tumor necrosis family (TNF) [37]. RANK-L is expressed by osteoblasts/stromal cells and induces differentiation and activation of osteoclasts through RANK [8]. This ligand has been shown to play important roles in the regulation of biological processes such as bone
homeostasis, immune function and development of mammary glands [1]. Signaling mechanisms activated by RANK in osteoclasts include: recruitment of TNF receptor-associated factor (TRAF) proteins, the cascades of mitogen-activated protein kinases (ERK, JNK and p38), the induction of phosphatidylinositol 3-kinase-dependant Akt activation and the activation of transcription factors (NFκB, AP-1 and NFAT2). [38].

The family of tumor necrosis factor (TNF) receptor-associated factors (TRAFs) consists of 6 different adaptor molecules that are responsible for the mediation of intracellular signaling through various cytokine receptors [39]. Since members of the tumor necrosis receptor family lack intrinsic enzyme activity within their intracellular domains, they compensate by recruiting adaptor proteins, through which they transduce signals. TRAF6 is the only known member involved in physiological bone development, as shown in studies by Lomaga et al. (1999) [40]. TRAF6 has been shown to play a critical role in RANK signaling within osteoclasts. This is demonstrated by the inability of osteoclasts to function normally within TRAF6 knockout mice. Mice lacking TRAF6 displayed defects in bone resorption and tooth eruption, due to abundance of dysfunctional osteoclasts, resulting in an osteopetrotic phenotype [40]. Also, TRAF6 is involved in RANK-mediated organization of the cytoskeleton and osteoclastic resorption as shown by Armstrong et al. (2002) [41].

Interaction of RANK-L with its receptor located on the osteoclast surface causes induction of TNF-associated factor 6 (TRAF6) signaling, displayed in Figure 4, which ultimately results in the activation of NF-κB [42]. Activation of TRAF6 induces inhibitory κB (IκB) kinase (IKK) activity, resulting in the phosphorylation and subsequent degradation of IκB. This allows for the translocation of NF-κB into the nucleus, where it binds to DNA target sites [38]. Miyazaki et al. (2000) have shown that NF-κB regulates osteoclast activation for bone resorption [26]. Mice deficient in the NF-κB proteins p50 and p52 are unable to form differentiated osteoclasts, demonstrating the
Figure 4. **RANK signaling pathways.** The binding of RANK-L onto RANK on osteoclast precursors activates multiple signaling pathways. These pathways involve kinases and transcription factors such as: NFκB, JNK, Src and p38 MAP kinase. Each pathway is highlighted in a different color.
critical role of this transcription factor [43].

RANK-L selectively induces the expression of nuclear factor of activated T cells-2 (NFAT2), a calcineurin- and calcium-regulated transcription factor, though both the TRAF6 and c-Fos signaling pathways. Both activating protein-1 (AP-1) and NF-κB binding sites are present within the promoter region of the NFAT2 gene [44]. The dimeric leucine-zipper containing transcription factor, AP-1, is a major target of cell growth, differentiation, and stress signaling pathways. TRAF6 has also been implicated in the regulation of NFAT2 protein expression, suggesting that the NFAT2 gene is the convergent target of the TRAF6 and c-Fos pathways. Using a transient assay, Takayanagi et al. (2002) provided evidence that the formation of a complex between NFAT2 and c-Fos is critical for the activation of genes involved in osteoclastogenesis. Therefore, NFAT2 may be the regulator of the terminal differentiation of osteoclasts, functioning downstream of RANK [18].

The activation of all three mitogen-activated protein kinase (MAPK) families, extracellular signal-related kinase (ERK), c-Jun N-terminal kinase (JNK) and the stress-activated protein kinase p38 are also activated by RANK. The Ras superfamily of GTPases, located downstream of RANK, are implicated in a number of cellular physiological processes including cell proliferation and differentiation. One of the most important signaling cascades located downstream of Ras is the MAPK cascade, which is activated through a Ras-Raf interaction, which results in the phosphorylation, and thus, activation of ERK. ERK has been shown to induce and activate c-Fos, and mice deficient in c-Fos develop osteopetrosis, caused by a failure to commit to the osteoclast lineage. This underscores the fundamental role of AP-1 in osteoclastogenesis. Neither the inhibition nor the activation of ERK affected bone resorption, indicating that it is responsible for maintaining osteoclast survival, rather than activation [26]. TRAF6 is required to activate JNK and p38 as shown by the TRAF6-deficient mice. JNK has been
shown to increase AP-1 activity by the phosphorylation of c-Jun. Stimulation of p38 results in the downstream activation of the transcriptional regulator MITF, which has been shown to control TRAP and CATK gene expression and considering that these enzymes are required by the mature osteoclast, this indicates the importance of p38 signal transduction [38].

RANK-L has also been shown to activate the anti-apoptotic serine/threonine kinase protein kinase B (Akt/PKB) through a signaling complex involving Src and TRAF6 [45]. Through its activation of multiple downstream effectors, the Akt/PKB kinase mediates the anti-apoptotic function of phosphatidylinositol 3-kinase (PI3K). Within osteoclasts, RANK has been shown to activate Akt, although indirectly, through the elevation of Src kinase activity. Stimulation using interleukin-1 (IL-1) as well as RANK-L, both induced the association of TRAF6 with c-Src, leading to the formation of a large signaling complex. This complex included both Pyk-2 and p130Cas, and resulted in the actin-ring formation and the activation of osteoclasts. This suggests that Src and PI3K may function at the same place, where adhesion mediated signaling and RANK signaling converges, allowing for coordination of actin organization and ruffled border formation to facilitate the bone resorption process [46].

The ruffled border forms the resorbing part of this cell, where there is an accumulation of acidic intracellular vesicles. Mineralized bone is made up of hydroxyapatite crystals and solubilization of these can only be carried out using a low pH. This is achieved through the use of Vacuolar-type ATPase proton pumps [32, 47]. Within this membrane, the vacuolar-type proton ATPase mediates the transport of protons into the resorption area. Cathepsin K and MMP-9 in lysosomes are also secreted to degrade the organic matrix of bone [48].

Bone degradation begins with the hydration of CO₂ into carbonic acid (H₂CO₃),
which then dissociates into protons ($H^+$) and bicarbonate ions ($HCO_3^-$). The protons are pumped through the ATPase into the extracellular space and the $HCO_3^-$ is exchanged for $Cl^-$ in an energy independent manner, and passes into the resorptive space, coupling with $H^+$ ions to form HCl. This influx of hydrochloric acid causes the pH to drop to 4.5, allowing the breakdown of mineralized bone [36].

There are other molecules present within the resorption lacunae that aid in acidification such as carbonic anhydrase II (CAII) [49]. Other molecules are present within this space, such as cathepsin K [50], and MMP-9 [51], which can degrade insoluble type I collagen and collagen fragments, respectively [50, 51].

Following resorption, the bone degradation products are removed from the area. This is accomplished by TRAP-expressing transcytotic vesicles, which generate highly destructive oxygen species. Following degradation of the mineralized area, the osteoclast detaches from the surface and migrates to a new site [52].

Studies have shown that osteoclasts have the ability to undergo several resorption cycles before they are targeted for apoptosis.

**Osteopetroses**

Osteopetrosis is a family of diseases characterized by the failure of long bones to be properly remodeled. It is a pathological bone condition, and the primary underlining mechanism involved in all forms of osteopetrosis is the failure of normal osteoclastic bone resorption. The severe increase in bone density observed can be attributed to the absence of osteoclasts or functional defects within the osteoclast. There are two main types of osteopetrosis: malignant (infantile) and benign [53, 54].
Malignant osteopetrosis is less common within patients since it is a recessive disorder, and is usually diagnosed shortly after birth. Patients exhibiting this type osteopetrosis experience abnormal bone remodeling, hematological difficulties, and narrowing of the foramen, causing neurological impairment. Most untreated patients die by the age of 4 due to severe complications, however, bone marrow transplantation in addition to medication, has been shown to efficiently treat the disease. There are more than three different genes involved in the etiology of malignant osteopetrosis; they are TCIRG1, Clcn7 and OSTM1. These genes all exist as loss-of-function mutations and are mostly involved in the acidification process. TCIRG1 encodes for a subunit of the vacuolar proton-ATPase (V-ATPase) located in the ruffled membrane of osteoclasts. Clcn7 is a gene encoding for chloride channel 7, and the protein also acts within the ruffled border. The last gene, osteopetrosis associated transmembrane protein 1 (OSTM1), is localized in the cytoplasm. Functional studies have suggested a role for OSTM1 in cytoskeletal organization or ruffled border formation, however, its exact role in bone resorption has yet to be established [53].

Benign osteopetrosis is inherited in a dominant fashion, and most patients are diagnosed as adults. Patients suffer from frequent fractures and exhibit healing problems, however the disease does not alter life expectancy. Benign osteopetrosis is generally characterized by the presence of osteosclerotic bones, mainly in the cranium. The two genes that have been identified as individually causing this type of osteopetrosis are LRP5 and Clcn7 (described previously). LRP5 is encoded by a gene for low-density lipoprotein receptor-related protein 5, which acts as an important mediator of osteoblastic function. Gain-of-function mutations of LRP5 have resulted in high bone density within patients [53].

The carbonic anhydrase II (CAII) gene is responsible for production of H+ ions necessary for acidification of the area below the ruffled membrane in osteoclasts.
Mutations in CAII results in an osteopetrosis, which is usually coupled to the development of renal tubular acidosis (dRTA) also known as Guibald-Vainsel syndrome. Clinical manifestations aside from the increase in bone density include short stature, cerebral calcifications, mental retardation, and fractures [53].

As previously mentioned, osteoclastic bone resorption is a highly regulated process involving cell differentiation, fusion, polarization, cell-surface binding, demineralization and matrix degradation [53]. It is likely, therefore, that a defect within any of these steps may cause a skeletal defect. A large number of animal models displaying osteopetrosis have been used as tools to decipher the underlying mechanisms of this bone disorder [55]. The study of osteopetrosis in mutant mice has led to significant advances in the understanding of the processes regulating bone mass [53].

Some additional examples of the mice exhibiting osteopetrosis are: the op/op mouse, mi/mi mouse, and ia/ia rat, as well as the man-made c-Src and c-fos knockout mice [23, 56-59]. The phenotype of the op/op mouse arises from an autosomal recessive inactivating mutation in the colony- stimulating factor –1 (CSF-1) gene resulting in the absence of CSF-1. This causes an impaired development of mononuclear phagocytes, characterized by a decrease in both macrophage and osteoclast number. The consequence of this severe decrease in cellularity is an occlusion of the marrow cavity. It is important to note, however, that these deficiencies are not permanent, and can usually be regulated in a CSF-1 independent manner by 35 weeks of age [59]. There is a transcription factor that was more recently identified, a gene encoding a novel basic helix-loop-helix (bHLH) protein with an important in osteoclast function. Studies using the mouse microphthalmia mutation (mi/mi), showed this phenotype to cause a mutation in the bHLH family member, resulting in an osteopetrotic phenotype. The osteoclasts were differentiated but failed to resorb bone [58]. Osteoclasts within the rats exhibiting the ia/ia genotype display an enlarged clear zone, causing poor ruffled border formation.
There is also an absence of detectable TRAP molecules within the resorptive lacunae, resulting in a defect in bone matrix resorption [56, 57].

A c-Src gene disruption yields an osteopetrotic phenotype in which the c-src tyrosine kinase is defective. Because of this, there is a reduced level of phosphorylated proteins which are necessary for formation of the ruffled border within osteoclasts [25]. This results in the presence of osteoclasts lacking the proper machinery at the surface to resorb bone. Within the c-fos knockout mice that were created, there are very few mature osteoclasts within the bone. Studies indicate an increased number of macrophages complimentary to the decrease in osteoclasts, implicating c-fos in the regulation of the differentiation of precursor cells into cells with an osteoclastic fate [23].

In contrast to osteopetrotic animal models, however, the molecular mechanisms playing a role in human osteopetrosis are almost completely restricted to osteoclast function. In fact, most of the mutations causing this disease have been identified in genes involved in acidification of the resorption lacunae [53]. This apparent lack of osteopetrosis gene mutations in other steps of bone resorption in humans could be due to the fact that they cause death before birth, and thusly, evade identification. The identification of genes involved in this disease makes it easier to perform molecular diagnostics and thus, provide treatment. Therefore, to further unravel the pathogenesis of osteopetrosis, additional genetic, cell biological and histological studies will be of major importance. This will also contribute to the understanding of the complex mechanisms regulating skeletal modeling.

**Integrins**

As previously mentioned, it has been shown that integrins play an important role in mediating osteoclast attachment to the mineralized surface [35]. Several classes of
adhesion molecules mediate cell attachment, such as integrins, cadherins, and selectins. More than 20 distinct heterodimers of the integrin family have been identified [20].

Integrins are transmembrane heterodimeric proteins made up of distinct combinations of the 16 alpha and 8 beta subunits. Generally, the integrin external domains can recognize matrix proteins through their interaction with specific amino acid motifs, causing interaction with intracellular signaling molecules. Integrins have a high capacity to bind extracellular matrix proteins, and therefore can mediate cell-surface attachment [60].

α-cytoplasmic chains are very diverse, vary in size from 120-180 kDa and β-cytoplasmic chains are somewhat conserved, and are 90-120 kDa with a high cysteine content. Both integrin subunits are transmembrane N-glycosylated glycoproteins with a large extracellular domain, a single hydrophobic transmembrane region, and a short cytoplasmic domain. Integrins act as cell surface receptors capable of transducing signals both resulting in biochemical changes within cells and in modification of integrin activity [48].

The cytoplasmic domain of these heterodimers then associates with intracellular molecules, specifically those involved in downstream signaling and organization of the cytoskeleton. Given their capacity to bind extracellular matrix proteins, integrins presented themselves as very good candidates for the mediation of osteoclast attachment to bone [48].

Teitelbaum et al. (2000) carried out extensive study to determine which integrins were expressed on the osteoclast surface and their substrate specificity. They coated tissue culture plastic with isolated bone matrix proteins such as fibronectin, collagen, vitronectin and others and placed osteoclastic cultures into the wells. Osteoclasts adhered
to proteins containing the amino acid motif arginine-glycine-aspartic acid (RGD tripeptide) and mainly recognized by the αv family of integrins, particularly αvβ3 and αvβ5. To determine which of these αv integrins were expressed by osteoclasts, they took bone marrow macrophages and supplemented them with osteoclast-specific cytokines. Cells were lysed and immunoprecipitated early in development, and then again upon the appearance of the osteoclast phenotype, using antibodies to β3 and β5 integrin. Freshly isolated bone marrow cells exclusively express αvβ5 and in mature osteoclasts, β5 is replaced by β3 [60]. It was also demonstrated that GM-CSF a proinflammatory cytokine involved in osteoclast differentiation accelerates the loss of β5 integrin. This is accomplished by degradation of β5 mRNA which simultaneously induces β3 expression via transcriptional stimulation [61].

The appearance of these two integrins at precise times of osteoclast development, indicate specific roles for each of them. αvβ5 has been shown to mediate cell attachment by negotiating the initial recognition of bone matrix and cell spreading, whereas αvβ3 regulates only cell attachment, as spreading is not required later during osteoclast activation [61].

The expression of αvβ3 on the surface of mature osteoclasts suggested the notion that it was involved in mediating bone resorption [33]. It was for this reason that a mouse deficient of αvβ3 integrin was constructed [33, 62].

Animals deficient in αvβ3 integrin suffer from Glanzmann’s thrombasthenia, since the β3 integrin is expressed in association with the glycoprotein IIb on platelets. This molecule is an essential clotting factor, therefore mutant mice experience a shortened life span as a result of excessive bleeding. Visually, αvβ3-deficient mice appear indistinguishable from the wild-type mice, as the shape of the knockout skeleton is unaltered. However, osteoclast function in the absence of αvβ3 is abnormal.
Osteoclasts generated *in vitro* from mice lacking αvβ3 exhibit distinct morphological and functional abnormalities. They endure difficulties in spreading, lack actin rings, and form irregular ruffled borders. The main defect seen in the ruffled border is the presence of wide and blunt folds (rather than the numerous narrow membrane protrusions). The cells are capable of initiating resorption pits, but due to these abnormalities, they are shallow and poorly defined. The cells seemed unable to form deep well-defined pits on dentin, perhaps due to a defect in cell adhesion mediated specifically by integrin αvβ3. The depth of lacunae excavated was measured to be only about 60% of what was removed by wild-type osteoclasts, illustrating that other integrins probably cannot compensate for this resorption defect [33]. McHugh *et al.* (2000) noticed an increase of 3.5 times more osteoclasts *in vivo*. β3-null marrow-derived osteoclastic cultures stained positive for TRAP, but failed to spread as shown by adhesion experiments. This confirms that this integrin is not involved in osteoclastic differentiation, as shown by previous studies of integrin expression. In contrast to the normal thin villous appearance of wild-type ruffled membranes, the structure of this organelle in β3 knockout osteoclasts consists of thick, blunted projections. The failure of mutant osteoclasts to normally generate this structure suggests that αvβ3 mediates essential matrix-derived signals [33, 62].

Osteoclasts express multiple integrins on their surface such as α2β1, αvβ1, α4β1, αvβ5, α5β1 [60]. Two others, including αvβ3 and α9β1 recognize the RGD motif, [60], [63]. Of all the integrins expressed by osteoclasts, the most abundant is the αvβ3 integrin called the vitronectin receptor, which is present in high copy number within the cell [60]. This integrin is expressed only in a limited number of cell types and the cells with the highest *in vivo* expression are osteoclasts [20]. It has been shown to bind several non-collagenous bone protein ligands such as vitronectin, osteopontin, bone sialoprotein and to some extent, fibronectin [60].
Vitronectin Receptor

Interference with integrin receptor αvβ3, also known as the vitronectin receptor, using various approaches leads to an inhibition of bone resorption both in vitro and in vivo [20]. The first evidence that this integrin may play an important role in osteoclast function was obtained when Chambers et al. (1986) inhibited bone resorption in vitro using an antibody raised against osteoclasts, and the antigen of this antibody was identified as the receptor αvβ3 [20, 64].

McHugh et al. (1999) generated αvβ3 deficient mice by targeted disruption of the β3 integrin subunit, and these mice displayed a late onset of osteopetrosis 3-6 months after birth. The cells could not spread, lacked actin rings, and had reduced resorption activity. β3-deficient osteoclasts had abnormal ruffled membranes, were unable to spread and lacked actin rings. There are more osteoclasts present within β3 knockout mice, but they were not able to resorb to their full potential. The fact that normal multinucleated cells formed in the absence of β3 excluded an essential role of this integrin in osteoclast development and precursor fusion [33].

Rescue was made possible by expression of full-length human β3 integrin. Interestingly, truncated β3, lacking a cytoplasmic tail was completely ineffective in restoring function to these osteosclerotic osteoclasts. These studies indicated the importance of the cytoplasmic domain, although short, to osteoclast function [62]. β3 integrin has been found to associate with vinculin and talin (both actin binding proteins) within the podosomes of OLCs cultured on glass [35]. This implies that there are amino acids present within the cytoplasmic tail which are essential for organization of the osteoclast cytoskeleton [62].

The functions of integrins are mediated through the recruitment of cytoplasmic
effectors. One of these is ILK, which connects integrins to the actin cytoskeleton and transduces signals from integrins into the cell [65]. Integrin signals play important roles in development, angiogenesis, infections, and many other complex physiological or pathological processes [66].

There is an abundance of signal transduction pathways operating downstream of integrin activation to regulate transcription factor activity. The main pathways that have been studied involve ERK, JNK and the activation of NFAT2. A basic overview of the MAP kinase signal transduction pathway is given here. First, the SH2-adaptor protein Shc is tyrosine-phosphorylated in response to growth factor-activated tyrosine kinases. This activated Shc then interacts with Grb2/Sem-5 through its SH2 domain, following which, Ras effector molecules such as the guanine nucleotide exchange factor, Son of Sevenless (Sos), are recruited to the plasma membrane for cellular-Ras (c-Ras) modulation. This results in the activation of c-Ras, and ultimately the MAP kinases. c-Ras is a GDP/GTP binding GTPase which mediates signal transduction and cellular-Raf (c-Raf) is a serine/threonine kinase [67].

Focal adhesion kinase (FAK) is a protein tyrosine kinase that is activated by integrin-mediated adhesion and is localized to adhesion sites. FAK is essential for organization of the cytoskeleton and cell motility, mediated through several molecular mechanisms. There are a vast number of downstream signal transduction pathways that converge on cell migration, suggesting that FAK acts as a central coordinator of these pathways (Figure 5). It has also been implicated in the activation of pathways involving ERK and JNK, regulating the cell-cycle. The importance of these pathways is demonstrated by FAK knockout mice, which not only display defects in cell motility, but also display abnormal regulation of the small GTPase Rho and mitogen-activated protein kinase (MAPK) ERK. The adaptor protein p130Cas has also been shown to bind FAK and has been linked to activation of the small GTPase Rac to promote motility [68].
Figure 5. Integrin signaling. Model of integrin-mediated signaling pathways, involving FAK, ERK, JNK, MAPK and ILK proteins. These all work to regulate transcription factor activity.
Upon indirect activation via an activated cytoplasmic or receptor tyrosine kinase, Ras interacts with Raf and causes the membrane translocation and activation of Raf. The activated Raf then tyrosine/threonine phosphorylates MAP kinase/ERK-activating kinase (MEK), which in turn, phosphorylates ERK. *In vitro* studies also suggest a link between MEK and the MAP kinases, as MEK has been shown to potentially activate ERK [67].

**Integrin-Linked Kinase**

The Integrin-linked kinase (ILK) was first identified through a yeast-two-hybrid screen using the integrin β1 cytoplasmic domain as bait. Co-immunoprecipitation with β1 in mammalian cell lysates confirmed interaction of the β1 chain with this 59 kDa protein. ILK is a serine/threonine protein kinase, with three structurally distinct domains (Figure 6, [69]). Closest to the N-terminus of ILK are 4 ankyrin-like repeats, connected to a pleckstrin homology (PH) domain, and then completed with a catalytic domain at the carboxy terminus. The catalytic domain is where ILK kinase activity is conferred [65] and has been shown to interact with the cytoplasmic domains of integrin β1, β2, and β3 subunits [70].

Hannigan *et al.* (1996) demonstrated that a β1 integrin cytoplasmic domain peptide was phosphorylated *in vitro* by ILK, one of the first indications of ILK kinase activity. The kinase activity of ILK was also demonstrated by its ability to readily phosphorylate myelin basic protein (MBP) *in vitro*. ILK was shown to colocalize in focal plaques with β1. Using antibodies to the vitronectin receptor, immunoprecipitation with ILK indicates that ILK interacts with β3 and/or β5 integrin subunits. The association of ILK with these integrin subunits, and specific regulation of its kinase activity by adhesion to an extracellular substrate suggest that ILK is a key mediator of integrin signaling [69].

The pleckstrin homology domain acts as a phosphoinositide-binding domain, and
Figure 6. ILK protein structure. The 59 kDa serine/threonine protein kinase consists of 3 domains. There are four ankyrin-like repeats, which modulate interaction with cytoplasmic and skeletal proteins, the pleckstrin homology domain that binds phosphoinositides and the kinase domain that has been shown to interact with integrin β chains.
binding of phosphatidylinositol 3,4,5-triphosphate has been shown to activate ILK kinase activity [70]. Structure-function analysis of ILK by Hannigan et al. (1996) suggest that the ankyrin repeat motif represents a protein interaction module specifying interactions with cytoplasmic or cytoskeletal proteins [69].

ILK is activated by attachment of integrins to ECM or stimulation of receptor tyrosine kinases by growth factors. The kinase activity of ILK is stimulated upon integrin engagement with the surface, as well as by growth factors and chemokines in a PI-3Kinase-dependant manner, and negatively regulated by the lipid phosphatase tumor suppressor (PTEN) as well as the PP2C protein phosphatase (ILKAP) [71].

ILK is widespread in terms of its distribution of expression in tissues, and is expressed by most mammalian cells. Its sequence is highly conserved across species, with the highest expression levels within cardiac and skeletal muscle. ILK has been shown to be highly conserved, evolutionarily among species such as human, mice, and drosophila. ILK plays a central role in the control of key signaling pathways causing the stimulation of downstream effectors, as well as activating/repressing genes involved in encoding proteins regulating cell survival, cell cycle progression, cell adhesion and ECM modification [72].

Upon integrin-mediated attachment, ILK activity is stimulated. ILK has been shown to phosphorylate downstream effectors protein kinase B/Akt (PKB) and glycogen synthase kinase 3 (GSK3), establishing a role for ILK as a kinase. Both these genes are involved in pathways that control cell proliferation, cell survival, protein translation and metabolic changes. Phosphorylation by ILK at the serine 473 site (one of the two sites required for activation) results in the activation of PKB/AKT, which then promotes cell survival by inhibiting apoptosis [70]. Delcommenne et al. (1998) also showed that a kinase-deficient form of ILK severely inhibits PKB serine-473 phosphorylation, and this
data suggests that ILK is a key effector of the phosphatidylinositol-3-kinase (PI3K)-
dependant signaling pathway [70]. Conversely, ILK-mediated phosphorylation of GSK3 at the serine 9 site causes downregulation of its kinase activity, upregulating the expression of cell cycle genes [65]. In the absence of ILK, GSK-3 remains activated and sends β-catenin for ubiquitination, following its phosphorylation. Therefore, ILK is an indirect regulator of β-catenin. Novak et al. (1998) showed that the overexpression of ILK causes the translocation of β-catenin to the nucleus, resulting in the formation of a complex with lymphoid enhancer binding factor 1 (LEF-1) [73]. This could be relevant to the transcriptional activation of mesenchymal genes in osteoblasts through the Wnt-signalling pathway [73, 74]. It is important to note that this activity is dependant upon an active kinase, because the overexpression of a kinase-deficient ILK (E359K) did not induce the nuclear localization of this protein [73].

ILK is also involved in the regulation of smooth muscle contraction in the absence of Ca^{2+}, as shown by Deng et al. (2001). They showed that ILK exercises its kinase activity by phosphorylating serine 19 and threonine 18 residues on myosin light chain (MLC); which is usually done by myosin light chain kinase (MLCK) in the presence of cytosolic Ca^{2+}. This allows for the initiation of a signaling cascade that triggers cross-bridge cycling, inhibition of myosin light chain phosphatase (MLCP) and ultimately contraction of smooth muscle. These studies also suggested a role for ILK in regulation in “non-muscle” motility, such as cell migration, chemotaxis, cytokinesis, as well as other related events [75].

ILK also plays a major role in the suppression of anoikis and therefore presents a likely therapeutic target in the treatment of breast cancer. This is due to the fact that cell lines that have become resistant to anoikis, the mechanism of apoptosis which results from the loss of cell-matrix contact, which is used to prevent oncogenic growth, display an increase in ILK activity. Studies show that treatment of these cell lines with inhibitors
of ILK kinase activity induces a significant level of anoikis [76].

Inhibition of ILK activity has been shown to suppress the growth of human carcinoma cells in the colon of SCID mice. Several different studies suggest that these phenotypes are largely attributed to the increase in ILK kinase activity and phosphorylation of downstream effectors involved in cell proliferation and survival [71, 73, 77].

Ishii et al. (2001) transiently expressed ILK in neuroblastoma cells (N1E-115) and noticed that ILK expression caused stimulation of neurite outgrowth. However, stable transfection using a kinase-deficient mutant of ILK caused inhibition of neurite outgrowth, indicating the requirement for ILK kinase activity in development of neuroblastoma cells [78].

Some investigators have suggested that ILK may not actually be a functional protein-serine/threonine kinase, based on the alignment of analogous sequences from different species. This is due to the presence of three divergent motifs (of the total eight) which are normally conserved within most active kinases. This, with the subcellular localization of ILK with integrins on focal adhesion plaques [79] and the interaction of ILK with cytoskeletal proteins suggests, rather, an adaptor protein role for ILK as part of a large complex mediating the link between integrins and actin reorganization [65, 80-82].

ILK is a focal adhesion protein that is highly involved in cell adhesion with the extracellular matrix and acts as a molecular scaffold for other proteins involved in regulation of the actin cytoskeleton [80]. These include α-parvin and paxillin (which all bind the c-terminus of ILK) as well as particularly interesting new cysteine-histidine-rich protein-1 (PINCH-1), PINCH-2 (which bind the n-terminus) [65].
The α-parvin protein, also known as calponin homology (CH) domain-containing ILK-binding protein (CH-ILKBP) and actopaxin, is 42 kDa in size, widely expressed and highly conserved [81]. It is a small member of the α-actinin superfamily, containing solely an actin-binding domain (ABD), which is formed by the CH domains, within its N-terminus. Using a GFP tag, α-parvin has been shown to co-localize with actin filaments within the invaginations of the fibroblast lamellipodia ruffles as well as focal adhesion contacts. The calponin homology domain of parvin allows for its targeting to focal adhesions. α-parvin involvement has been implicated in the process of matrix remodeling and turnover of adhesion complexes, yet the exact molecular mechanism remains to be examined. Shortly after the identification of α-parvin, homologous proteins were cloned and named β- and γ-parvins [82]. The CH-domain of mouse α-parvin displays 94% similarity with paralogous β-parvin, and 71% similarity with γ-parvin. β-parvin, or affixin, has also been shown to localize to FAs, however it inhibits ILK activity to reverse the oncogenic effects of ILK in cancer cells [83].

Paxillin is a multidomain adaptor protein, which functions as a molecular scaffold to facilitate signaling through its localization to focal adhesions (FA). Using co-immunoprecipitation, paxillin was shown to interact with the c-terminus of ILK, resulting in the recruitment of vinculin, α-actinin and other binding proteins. Paxillin contains four LIM domains within its carboxy terminus, which are responsible for its attachment to Fas and interaction with α-parvin. This α-parvin-paxillin interaction has been implicated in regulation of integrin association with the ECM as well as the organization of actin during cell adhesion and spreading. Within the NH2 domain of paxillin, there are five leucine-rich repeating sequences (LD motifs) involved in regulating cytoskeletal modeling and gene expression by binding GTPases such as Rac and Rho. Interestingly, the N-terminus of paxillin also supports the binding of several protein tyrosine kinases such as FAK, Pyk2, Csk and Src. Paxillin, FAK and Cas all function as scaffolding proteins, involved in coordinating integrin-mediated signal transduction for the regulation
of cell motility, gene expression and cell proliferation [84].

PINCH was initially isolated from a human cDNA library that was screened using antibodies to recognize senescent erythrocytes. The ILK N-terminus has been shown to bind the first PINCH LIM domain, as shown by two-hybrid studies. PINCH-binding activity, however, is dependant upon all four of the ILK-ankyrin repeats [81]. PINCH binding to ILK is crucial for ILK focal adhesion and this has been demonstrated in many species. According to studies by Hobert et al. (1999), the *C. elegans* homologue of mammalian PINCH is located within muscles of the body wall and concentrated at focal-adhesion-like muscle attachment sites (dense bodies) [85]. In Drosophila, the PINCH homologue has also been found to colocalize with integrins [72].

Studies by Nikolopoulos & Turner (2002) demonstrate that the ILK kinase-dead mutant (E359K), is unable to bind paxillin or α-parvin *in vivo*, and does not localize to FAs. This indicates that the correct subcellular localization of ILK through intact protein interaction with the paxillin-α-parvin complex is likely to impact significantly on normal ILK signaling [84].

The simultaneous interaction of two separate ILK domains result in the formation of a stable PINCH-ILK-α-parvin (PIP) complex in cells, which provide a crucial link between integrins and the actin cytoskeleton and aid in the transduction of diverse signals from the ECM to intracellular effectors. The formation of this complex is essential to the function of each of its members within most cellular processes. These interactions suggest an important adaptor protein role for ILK [72].

Yeast-two-hybrid assays confirmed the interaction of α- and β-parvin with the ILK carboxy-terminal domain [65]. Interaction with α-parvin regulates ILK activity and downstream signaling to Akt/PKB and GSK-3 [86]. Using two-hybrid and co-
immunoprecipitation assays, ILK was also shown to bind to PINCH with very high affinity [65]. This interaction regulates the localization of ILK to focal adhesion [71]. PINCH, in turn, binds with low affinity to Nck2 (also called Nck- β, Grb4), as demonstrated by yeast-two-hybrid studies [87]. Nck-2 is a member of the Nck adaptor protein family and contains one C-terminal Src-homology 2 (SH2) domain and three N-terminal Src-homology 3 (SH3) domains, through which it mediates interaction with PINCH LIM domain. It is involved in small GTPase signaling pathways as well as multiple cellular processes. Inactivation of the mouse Nck genes results in embryonic defects resembling those induced by the loss of integrin/fibronectin signaling. The interaction of Nck-2 with PINCH likely plays an important role in coupling cell-ECM adhesion to small GTPase signaling pathways, and thus regulation of actin cytoskeletal organization and motility [72].

Analysis of ILK mutants in both worms and flies demonstrate an essential kinase-independent role of this protein in coupling [88, 89].

Studies in *caenorhabditis elegans*, show that the ILK homolog, pat-4, is a component of the dense bodies in muscle and required for proper assembly. Not only is the rescue of the mutant phenotype possible with kinase-dead forms of pat-4, but Mackinnon and colleagues were unable to find any function for this protein in Wnt signaling cascades, suggesting that this ILK homolog functions primarily as an adaptor molecule [88].

In *drosophila melanogaster*, the role of ILK in actin stabilization at integrin attachment sites in muscle was established. ILK is a component of the structure linking the cytoskeleton and the plasma membrane at sites of integrin-mediated adhesion. Loss of ILK activity causes a severe muscle defect in worms and flies, but rescue is possible by expression of kinase-dead ILK transgenes confirming ILK’s role as an adaptor.
protein, which is independent of its kinase activity [89].

According to studies of ILK in mouse skin, expression was abundant throughout the dermis, which is ECM-rich, as well as hair follicles and basal cells of the interfollicular epidermis. These studies implicate ILK as an important coordinator of the integrin and growth factor signaling pathways in the development of the skin [90].

Loss of ILK activity within chondrocytes causes skeletal growth retardations characterized by a disorganized growth plate and dwarfism, this is a direct result of abnormal chondrocyte shape and reduced proliferation in vivo [91].

These studies suggest a cell type specific and tissue-specific role of ILK in the regulation of signaling pathways.

Sequence alignment was performed that suggests that ILK is not a bona fide kinase itself, but would act as an adaptor to recruit a serine-473 kinase [92]. This rationale, based on sequence differences, is not very convincing, however, since other protein kinases have been identified with amino acid substitutions in highly conserved regions. An example of this are Mik1 and Vps15p kinases, which both lack all three glycine motifs in the GXGXXG motif [93, 94]. This evidence, combined with the evidence of the multiple systems requiring the kinase activity of ILK suggest that ILK functions not only as a true kinase but also as an adaptor protein.

Since disruption of this integrin β3-mediated signaling has such a pronounced effect on osteoclasts, we are interested in disrupting the signaling cascade further downstream of the αvβ3 integrin. The total loss of ILK expression in mice leads to peri-implantation lethality [65]. The data mentioned so far illustrates the power of cell-specific gene deletion experiments to uncover multiple functions of a given gene. In the
case of the osteoblast lineage, ILK activity has not yet been explored. The availability of mouse strains expressing Cre in osteoclasts (such as the TRAP-Cre strain), will allow for a better understanding of the roles of ILK and the pathways it regulates within this cell type.

Therefore, to disrupt ILK downstream of integrin β chains, we used the mouse strain expressing Cre late during osteoclast development. By disrupting ILK exclusively within osteoclasts, a cell abundantly expressing the αvβ3 integrin, we hoped to establish a role for ILK in integrin-mediated signaling in osteoclasts. Here, we report that mice with an osteoclast-specific inactivation of ILK suffer from a mild osteopetrotic phenotype.

**Materials and Methods**

**Strains**

Mice were kept in a 12-hour light, 12-hour dark cycle environmentally controlled barrier animal facility and were fed mouse chow and water *ad libitum*. All animal procedures were reviewed and approved by the Shriners Animal Care Committee and followed the guidelines of the Canadian Council on Animal Care. The following strains were used:
- Rosa 26 Reporter strain (R)
- TRAPCre transgenic strain (Roodman Laboratory)
- TRAPCre X Rosa 26 R
- ILK KO strain
- TRAPCre X ILK KO strain
- ILK fl/fl strain (shown in Figure 7)
- TRAPCre X ILK KO/fl strain (referred to as ILK LVO)
Figure 7. **Structure of the targeted ILK locus.** LoxP sites have been inserted within the ILK locus, flanking the entire kinase domain (exon 5-12) to allow for Cre-mediated excision of the floxed sequence. Cre-mediated excision creates a null allele.
DNA Isolation and PCR Genotyping

Mouse tails were cut at 3 weeks of age following weening from the mother. No more than one centimeter of the tail tip was placed in 0.5mL lysis buffer (100mM Tris-HCl @ pH 8.5; 5mM EDTA; 0.2% SDS; 200mM NaCl; 100μg Proteinase K/mL) and placed at 55°C, continuously agitating for several hours or overnight.

Following this, the tail digest was centrifuged at 12000 rpm for 10 minutes to sediment any indigestible material such as hairs and tissue residue. One volume of isopropanol (propan-2-ol) was added to the lysate and the samples were gently agitated until the precipitation of the DNA was completed, usually requiring several hours. The precipitate was then transferred to 100μL of TE buffer (10mM Tris-HCl; 0.1mM EDTA; *adjusted to pH 7.5). Complete dissolution of the DNA precipitate took several hours at 37°C [95].

In order for the DNA to be genotyped PCR the following screening methods have been developed.

| ILK Knockout Gene Primer Sequence | ILK neo KO 5'-GAC ATA GCG TTG GCT ACC CGT GAT A-3' | ILK KO 5'-GAA ATG CTG ATC ATG CGT GGA GC-3' |
| CRE Gene Primer Sequence | R-CRE 5' 5'-GACCGTACACCAAAAATTGGCTGC-3' | R-CRE 3' 5'-TGAAGCATGTTTAGCTGCCCA-3' |

Table 1: PCR Primers

<table>
<thead>
<tr>
<th>ILK PCR Reaction</th>
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<tr>
<td>2.50μL 10X Buffer</td>
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<tr>
<td>1.50μL 50mM MgCl₂</td>
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<tr>
<td>2.50μL ILK neo KO Primer</td>
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<tr>
<td>2.50μL ILK KO Primer</td>
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<tr>
<td>2.50μL 2mM dNTPs</td>
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<tr>
<td>0.25μL Taq DNA Polymerase</td>
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ILK PCR Program:

The tubes, each with 25μL of the reaction mixture were placed in the GeneAmp 9700 PCR System (Applied Biosystems, Foster City, California, USA) machine and programmed for first denaturing step at 94°C for 3 minutes. The template DNA was allowed to denature at 94°C for 45 seconds, next the primers were annealed at 60°C for 45 seconds to promote specific binding to the target sequence and then extended at 72°C for 1 minute; these three steps were repeated for 35 cycles, following which the samples were placed at 72°C for 10 minutes for the final extension. To finish, the samples were cooled to 4°C until ready to be removed from the machine [91].

CRE PCR Reaction

2.50μL 10X Buffer
2.25μL 50mM MgCl₂
1.50μL R-CRE 5’ Primer
1.50μL R-CRE 3’ Primer
2.50μL 2mM dNTPs
0.25μL Taq DNA Polymerase
14.0μL ddH₂O
1.0μL tail DNA

CRE PCR Program:

The tubes, each with 25μL of the reaction mixture were placed in the GeneAmp 9700 PCR System (Applied Biosystems) machine and programmed for first denaturing step at 94°C for 4 minutes. The template DNA was allowed to denature at 94°C for 45 seconds, next the primers were annealed at 62°C for 45 seconds to promote specific binding to the target sequence and then extended at 72°C for 1 minute; these three steps were repeated for 35 cycles, following which the samples were placed at 72°C for 7
minutes for the final extension. To finish, the samples were cooled to 4°C until ready to
be removed from the machine [91].

**Bone Resorption Assay**

Blood was obtained from 6-week old ILK-deficient and wild-type mice by cardiac
puncture and allowed to coagulate for 30 minutes at room temperature. This was
followed by centrifugation at 5000rpm to separate cells from the blood serum, and the
supernatant (serum) was retained. An enzyme-linked immunosorbent assay (ELISA) kit
called the RatLaps ELISA (Nordic Biosciences A/S, Chesapeake, Virginia, USA) was
used for the *in vitro* quantification of type I collagen fragments in mouse serum and
released during bone resorption.

Following the manufacturer's instructions, 100μL of Biotinylated RatLaps
Antigen was added to each well of the plate and incubated for 30 minutes at room
temperature. The wells were then washed out 5 times using the Washing Solution. Then,
20μL of each standard (6 plus the control) or 20μL of each serum sample was added to
the wells, followed by 100μL of Primary Antibody and this was incubated at 4°C
overnight. The next day, wells were rinsed 5 times with the Washing Solution. To each
well, 100μL of Peroxidase conjugated Goat anti-Rabbit IgG Antibody was added and
incubated for 1 hour at room temperature. Wells were rinsed again using the Washing
Solution, followed by addition of 100μL of chromogenic Substrate Solution to each well
for 15 minutes. In order to stop the color reaction, 100μL of the Stopping Solution was
added to each well and absorbance was measured using an Elx 808 Spectrophotometer
(Fisher Scientific Company, Ottawa, Ontario, CA) at a wavelength of 450nm.
Embedding and Sectioning

Sucrose OCT

Femurs were removed from e15.5-16.5 TRAPCre x Rosa 26 R mice and fixed for 1 hour using 4% paraformaldehyde (PFA). They were then placed in a solution containing two parts 20% sucrose in phosphate buffered saline (PBS) and one part OCT Compound Tissue Tek (Electron Microscopy Sciences, Hatfield, PA, USA) for 1 hour at 4°C. This step was repeated two more times, with the last one carried out on ice under vacuum. The samples were then placed in disposable vinyl specimen molds called cryomolds and covered with the previous solution over dry ice. They were kept at -20°C until ready for use, usually 2-5 days (adapted from [96]).

Cryosectioning

A Bright 5040 cryostat microtome (Bright Instrument Company Limited, Huntington, England) with a disposable Magnacut knife blade (Bright Instrument Company Limited) was used to cut the frozen OCT-embedded samples. Sections were cut at a thickness of 8µm and collected onto Silane Plus slides (Fisher) slides. Specimens were kept in a cryostat until all samples were cut, and stored at -80°C until ready for use. Samples were brought to room temperature and the OCT compound was removed by rinsing slides in PBS, just before staining (adapted from [96]).

Paraffin

Tibiae were dissected from 6-week old ILKΔc mice and fixed in 4% PFA overnight at 4°C. After a 30 minute rinse in PBS, the samples were placed in a
constantly shaking solution of ImmunoCal (Decal Corporation, Tallman, New York, USA), a formic acid bone decalcifier, for 2 weeks. The bones were then rinsed in PBS twice for one hour each time. This was followed by dehydration in a graded ethanol series from 50% to 100%. The dehydrated tibiae were placed in xylene twice, for 30 minutes each time. The xylene was switched to a solution containing half xylene, half VIP Processing/Embedding Medium (Electron Microscopy Sciences, Hatfield, Pennsylvania, USA) and left overnight at room temperature to allow infiltration of the paraplast. The xylene:paraplast mixture was melted and removed. This was replaced by 100% paraplast in the vials three times for one hour each time. Following this, the plastic mold was filled with 100% paraplast on a hot plate at 60°C and the sample placed inside. The support cassette was placed on top and allowed to cool overnight on the cold plate. Samples were stored at 4°C until ready for use [97].

Paraffin Sectioning and Deparaffinization

A Leica RM2255 Microtome (Leica Microsystems, Richmond Hill, Ontario, CA) with an S35 disposable Microtome Blade (Fisher Scientific) was used to cut the ice-cooled samples embedded in paraplast. Sections were cut at 7μm thickness and placed in a 37°C water bath prior to collection onto Silane Plus Plus slides (Fisher Scientific). Sections were heat-fixed using a slide warmer (Lab-line Instrument Inc., Melrose Park, IL, USA) overnight, and then deparaffinized by immersion in histology grade xylene four times for 2 minutes each. Following this, the sections were re-hydrated using a graded ethanol series decreasing from 100% to 70%, finishing with a rinse in dH₂O [91, 97]. Sections were ready for staining at this point.
Methyl Methacrylate Embedding

Tibiae were dissected from 6-week old ILK\(\Delta\) mice and fixed in 4% PFA overnight at 4°C. Following a 30 minute rinse in PBS, the samples were dehydrated in a graded ethanol series increasing from 50% to 100%. The dehydrated bones were placed in fresh xylene three times, each for 40 minutes. The samples were then immersed in increasing concentrations of purified MMA (starting from 0%, 1%, 4.5%) using each concentration twice overnight at 4°C. During the last addition of 4.5% MMA the vials were replaced with new ones and set aside, completely still to allow polymerization at room temperature. Polymerization of the MMA took a period of 5 days [98-100].

**Methyl Methacrylate Purification:** Using a 1-liter separation flask we mixed 500 mL of MMA with 250 mL 1N NaOH, mixed vigorously and allowed to sit for several minutes. The non-aqueous phase was decanted, and 250mL of fresh NaOH was added again. This step was repeated 3-4 times until the non-aqueous phase turned clear. In order to eliminate the NaOH, these steps were repeated with 250mL of deionized water (dH\(2\)O) three times. We added 150 g of CaCl\(2\) into an empty tinted bottle and poured in 500 mL of the MMA solution to get rid of the dH\(2\)O. After leaving this at 4°C overnight, the MMA solution was filtered three times with 2 pieces of Whatman paper each time to eliminate CaCl\(2\) suspension. The purified MMA solution was kept at 4°C in the dark until ready for use due to its sensitivity to light. Different concentrations of 1% and 4.5% benzoyl peroxide were prepared using purified MMA mixed with dibutyl phthalate in a ratio of (17:83). These solutions were filtered again before use [98].

MMA Sectioning and Deplasticization

A Bone Band Saw (Mar-Med Incorporated, Cleveland, Ohio, USA) equipped with a 1/8” wide diamond blade (Mar-Med Inc.) was used to trim the samples embedded in MMA. A Leica RM2255 Microtome (Leica Microsystems) with a 16cm/d (HM) blade
was used to slice the blocks. Sections were collected at a thickness of 5μm on Silane Plus slides (Fisher Scientific) and fixed onto the slides using 70% ethanol and rolling a plastic cover on top. Slides were tightly clamped together, with empty slides in between, (16 slides per clamp) and placed in a 55°C oven to dry overnight. Next, the plastic pieces were carefully removed from the slides, making effort not to disturb the samples. Slides were soaked in ethylene glycol monoethyl ether acetate (EGMA) four times for 15 minutes each to remove the hardened MMA. Slides were allowed to dry in the fume hood for 2.5 hours at room temperature and then stored away from the light until ready for use [99, 100].

Staining

**Lac Z**

Following removal of OCT compound, slides were covered by a β-galactosidase Staining Solution (2mM MgCl₂, 4mM K₃Fe(CN)₆, 4mM K₄Fe(CN)₆, 0.01% tween-20; 0.4mg/mL X-galactosidase in DMF) within a humid chamber protected from the light for 14 hours at 37°C. Sections were rinsed in PBS and counterstained with 0.1% Safranin O for 30 seconds. Slides were rinsed again in PBS and mounted using GVA mount, sealing the edges with NUDE Hard as Nails nail polish (Sally Hansen, Uniondale, New York, USA) [101].

**Tartrate Resistant Acid Phosphatase (TRAP)**

*Paraffin Sections:* 12mg of Napthol AS-BI phosphate (Sigma-Aldrich, Oakville, Ontario, CA) was combined with 0.75mL N, N-dimethylformamide and allowed to dissolve. To this we added 75 mL of 0.2M acetate buffer (pH 5.0), 105mg Fast Red Violet LB salt (Sigma), and 180μL 10% MgCl₂. This solution, called Complete
Burnstone’s media, was then filtered into an acid washed coplin jar, and brought to 37°C in a water bath. Once at this temperature, 0.87g of sodium tartrate dihydrate (Sigma) was added to form the TRAP staining solution. Sections were immersed into the stain for 2.5 minutes at 37°C and then placed under running cold water for 30 minutes. The samples were allowed to dry at room temperature and then mounted using GVA Mount (Zymed Laboratories Inc., South San Francisco, CA, USA), and the edges sealed using NUDE Hard as Nails nail polish (Sally Hansen) [102].

**Primary Cultures:** Following the manufacturer’s instructions, Technical Bulletin #445 for TRAP Staining of Osteoclasts on BD BioCoat Osteologic Discs was used. To prepare the TRAP Buffer (pH 5.0) we added 50mL of 0.1M acetate buffer, 10mL of 0.3M sodium tartrate dihydrate, 1mL 10mg/mL Napthol AS-MX Phosphate (Sigma), and 100µL triton x-100 to 38.9mL of deionized water. This buffer was prewarmed to 37°C in a water bath, following which 3mg of Fast Red Violet LB was added to form the TRAP staining solution. After fixation using 10% glutaraldehyde for 15 minutes at 37°C, the cells were rinsed with PBS twice. The surface of the cells was then covered with 300µL of the TRAP stain for 10 minutes at 37°C. Once finished, the cells were rinsed with PBS and ready for immediate examination [103-105].

**Goldner**

Following deplasticization, MMA sections were re-hydrated in a graded ethanol series decreasing from 70% to 40% and then immersed into dH₂O for one minute. Immediately following, slides were placed in a solution of Weigert’s Hematoxyline (Fisher Scientific) for 25 minutes, and then rinsed for 10 minutes in running dH₂O. The next staining solution used was Acid Fuschin-ponceau (Mallinckrodt Baker, Inc., Phillipsburg, New Jersey, USA) for 30 minutes, followed by a quick rinse in 1% acetic acid. The slides were then placed in a solution of Orange G (Fisher Scientific) for 8
minutes and again rinsed quickly in 1% acetic acid. Lastly, the slides were immersed in a Light Green SF solution (Fisher Scientific) for 40 minutes and dipped into 1% acetic acid to clear excess stain. Samples were placed immediately in a mixture of tertiary butanol and xylene, increasing to solutions of xylene alone for 10 seconds each. Slides were mounted using MicroKitt (M.E.C.A. Ltee, Montreal, Quebec, CA) and allowed to dry overnight in the fume hood before viewing (modified from [99, 100, 106]).

**Von Kossa**

Following the manufacturer’s instructions, Technical Bulletin #444 for von Kossa Staining of Osteoclast Resorption on BD BioCoat Osteologic Discs was used. After plating primary osteoclastic cultures on BD BioCoat OSTEOLOGIC analysis discs (BD Biosciences, Franklin Lakes, New Jersey, USA) for a period of 9 days, the primary cells were washed away using bleach. The discs were washed in dH2O three times and then stained for 30 minutes using a 5% Silver Nitrate (Merck & Co. Incorporated, Whitehouse Station, New Jersey, USA) solution. Discs were rinsed again three times using dH2O to wash out excess silver nitrate to prevent a black precipitate from forming. The stain was developed using a mixture of 5% sodium carbonate in a 25% formalin solution for 1 minute. After washing three times with dH2O, stained discs were fixed using 5% sodium thiosulphate for 2 minutes, and were ready for viewing [107, 108].

**Toludine Blue**

After plating primary osteoclasts on 200μm thick dentin slices obtained from walrus tusk (a generous gift from an Inuit artist), cells were washed out by sonication in a 0.25M ammonium hydroxide solution. Dentin slices were rubbed gently with cotton and then washed with dH2O. A mixture of 1% Toludine Blue O (Fisher Scientific) in a 1% solution of sodium tetraborate was used to stain the dentin for 2-3 minutes. Dentin was
rinsed in dH₂O to remove excess stain and ready for immediate viewing [109].

**Histomorphometry**

Images of Goldner stained, undecalcified, MMA-embedded tibial sections were obtained using a Leica DC300F digital camera (Leica Microsystems) connected to a Leica HC DMR Microscope (Leica Microsystems) and quantitatively analyzed using Bioquant NovaPrime software (Bioquant Image Analysis Corporation, Nashville, Tennessee, USA). Parameters measured within the metaphyseal area of the proximal tibia included, but were not restricted to: bone volume per tissue volume (BV/TV; percentage), trabecular number (Tb.N/mm) trabecular thickness (Tb.Th; microns), bone surface (BS; microns), and trabecular separation (Tb.Sp; microns). Statistical analysis was by Student T test (and nonparametric test). P < 0.05 was accepted as significant; error bars represent the S.E.M [110, 111].

**Electron Microscopy Studies**

Mice were anaesthetized with 50mg/kg Sodium Pentobarbital (Somnitol, Somnitol, MTC Pharmaceuticals, Cambridge, ON); intraperitoneally and exsanguinated by perfusion with lactated Ringer’s solution (Hospira, Montreal, Quebec, CA) into the left ventricle for 20 seconds while the right atrium was opened. The Ringer’s was replaced by 5% glutaraldehyde (Canemco, St.Laurent, Quebec, CA) in 0.1M sodium cacodylate, pH 7.2 and the perfusion continued for 15 minutes at room temperature. Tibiae were dissected in a puddle of fixative and fixation continued on the entire epiphyses 24 hours at 4°C. Fresh fix was applied for another 24 hours at 4°C. Tibiae were then cut in half, rinsed three times for 20 minutes each in 0.1M sodium cacodylate at pH 7.2 with 5% sucrose. Tissue was post-fixed in potassium reduced 1% osmium tetroxide (Canemco) for 2 hours at 4°C and dehydrated in a graded acetone series and
embedded in JEMbed epoxy resin (Canemco) [112].

One-micron thick sections were cut on a Reichert-Jung Ultracut microtome, mounted on gelatin-coated slides and stained with 1% toludine blue. Sections were examined with a Leica DMRB microscope and photographed using a coolsnap digital camera. Ultrathin sections were cut on the Reichert-Jung Ultracut microtome, mounted on copper grids, counterstained with uranyl acetate and lead citrate and examined on a Philips 400 transmission electron microscope at 80kV [112].

**RNA Extraction**

RNA was isolated from primary osteoclastic cultures generated from femur bone marrow using TRizol Reagent (Invitrogen Canada Incorporated, Burlington, Ontario, CA), following the manufacturer’s protocol for isolation of RNA. Cells were lysed within the culture dish by addition of 1mL of TRizol Reagent per 10cm² dish, and passage several times through the pipette. The homogenized samples were incubated for 5 minutes at room temperature to permit complete dissociation of nucleoprotein complexes. Next, 2 mL of chloroform (per 1mL of TRizol Reagent) was added to each sample in a 12mm×75mm polypropylene Sterile Culture Tube (Simport, Beloeil, Quebec, CA). The tubes were shaken vigorously by hand for 15 seconds, followed by incubation at room temperature for 3 minutes. The samples were centrifuged at a maximum speed of 12,000xg in a Sorvall RC 5B Plus Centrifuge for 15 minutes at 4°C. RNA remained in the colorless upper aqueous phase (about 60%) that forms, and is transferred to a fresh tube to be precipitated by mixing with 0.5mL isopropyl alcohol per 1mL TRizol Reagent used for the initial homogenization. Samples were then incubated for 10 minutes at room temperature followed by centrifugation at a maximum speed of 12,000xg at 4°C for 10 minutes. The supernatant was removed and the RNA pellet (clear in color) was washed with 1mL 75%. Sample was mixed by vortexing and then centrifuged at a speed of
7500xg at 4°C for 5 minutes. Once the ethanol was removed, the RNA pellet was air-dried briefly and redissolved in 20μL of RNase-free water, by placing the sample on ice for 1 hour.

Reverse Transcription and Real-Time PCR

Reverse Transcription

Reverse transcription of RNA to single-stranded cDNA was carried out using the High-Capacity cDNA Archive Kit (Applied Biosystems), following the protocol supplied by the manufacturer. The master mix was prepared manually and then loaded into the cDNA archive reaction plate. To make a total reaction volume of 50μL the 2X RT master mix consisted of the following:

10μL 10X Reverse Transcription Buffer
4μL 25X dNTPs
10μL Random primers
5μL MultiScribe Reverse Transcriptase
21μL Nuclease-free H₂O

For each reaction, we added 50μL of the 2X RT master mix to 50μL of the RNA sample (1μg diluted in Nuclease-free H₂O). The plate was covered and briefly centrifuged using a Sorvall RT7 Centrifuge, to spin down contents and eliminate air bubbles. The plate was placed into the GeneAmp PCR System 9700 Thermal Cycler (Applied Biosystems), and reaction volume was set at 100μL and programmed as follows:

Step 1: 25°C for 10 minutes
Step 2: 37°C for 120 minutes
Step 3: 4°C for ∞

The newly constructed cDNA was placed at –20°C until ready for the Real-Time PCR reaction.

Real-Time PCR

Real-Time PCR was carried out using the Assay-on-demand reagents (Applied Biosystems), following the manufacturer’s protocol for gene expression products. For each gene that was assayed, 12.5μL of the Universal Master Mix provided was added to 1.25μL of the Target Assay Mix (ILK, Integrin alpha V, Integrin alpha 9, Integrin beta 3, and Integrin beta 1). To this we added 11.25μL of the template, consisting of 5μL of the cDNA product (from RT reaction) and 6.25μL of nuclease-free water, to make a 25μL reaction. The wells were covered using optical adhesive covers (Applied Biosystems) and briefly centrifuged using a Sorvall RT7 centrifuge at a speed of 2000rpm to spin down contents and eliminate air bubbles. The reaction plate was placed into the 7500 Real Time PCR System Sequence Detector (Applied Biosystems) and the default PCR thermal cycling conditions were used.

Step 1 (AmpErase UNG Activation) 50°C for 2 minutes
Step 2 (AmpliTaq Gold Enzyme Activation) 95°C for 10 minutes
Step 3* (Denaturation) 95°C for 15 seconds
Step 4* (Annealing/Extension) 60°C for 1 minute
*Steps 3 & 4 repeated for 40 cycles.

Osteoclast Cultures
Femurs were excised under aseptic conditions from 22-day old mice, and allowed to soak in cold PBS containing 2% penicillin/streptomycin/fungizone for 30 minutes. Within culture hood, sterile scissors were used to remove epiphyses from each femur. Carefully, a 22-gauge needle with a 10 mL syringe filled with osteoclast culture medium* was inserted into the end of the bone shaft to flush the marrow into a 50mL Falcon tube (Sarstedt Incorporated, Newton, North Carolina, USA).

*The Osteoclast Culture Medium consisted of the following:

**Minimal Alpha Essential Medium (pH 7)**

- Fetal Bovine Serum 10%
- Penicillin/Streptomycin & Fungizone 1%
- Dexamethasone $10^{-6}$M
- Glutamine 2%

The cells were aspirated using the syringe and expelled through the needle to obtain a single cell suspension. Cells were plated at a density of $10^6$ cells/mL in a P60 plate. Every 48 hours, the wells were washed gently with warm PBS to remove non-adherent cells and fresh media containing 50ng/mL of Macrophage-Colony Stimulating Factor (M-CSF) and Receptor-Activator of NFκB Ligand (RANK-L) was added, for a total culture period of 9 days (adapted from [113]).

**Resorption Assay**

**Osteologic Bone Cell Culture System**

Cells were cultured onto the substrate following the instructions provided by the manufacturer for Osteoclast Culture Preparation. Cells were plated at a density of $10^6$ cells/mL in a 24-well plate, each well containing a synthetic calcium phosphate disc.
obtained from the Osteologic Bone Cell Culture System (BD Biosciences). The wells were placed at 37°C in a Thermo Forma Series II Water Jacketed CO₂ incubator with 5% CO₂, and 100% humidity for 24 hours. Every 48 hours, cultures were gently washed using PBS and fresh media containing cytokines M-CSF and RANK-L was added. The media was kept at a pH of 7 to avoid alteration of the Osteologic substrate. In preliminary experiments, the resorption assay was also carried out in the presence or absence of the ILK-inhibitor, QLT0267 [114].

**Dentin**

A 2x1x1 cm block of walrus tusk (a generous gift from an Inuit artist) was cut into 200μm thick slices and then following sterilization in 70% EtOH, under aseptic conditions, split into 4 pieces. Dentin slices were placed in a 96-well plate and incubated in Osteoclast Culture Medium at 37°C, with 5% CO₂, and 100% humidity in a Thermo Forma Series II Water Jacketed CO₂ incubator for 24 hours. Primary osteoclastic cells were plated at a density of 10⁶ cells/mL onto the dentin pieces. Every 48 hours, the cells were gently washed with warm PBS to remove non-adherent cells and new Osteoclast Culture Medium with the appropriate cytokines (M-CSF and RANK-L) was added. The culture period on dentin lasted 9 days (adapted from [109]).

**Protein Extraction**

Protein was isolated from primary osteoclastic cultures generated from femur bone marrow using TRIzol Reagent (Invitrogen), following the manufacturer’s instructions for protein preparation. Cells were lysed within the culture dish by addition of 1mL of TRIzol Reagent per 10cm² dish, and passing this several times through the pipette. The homogenized samples were incubated for 5 minutes at room temperature to permit complete dissociation of nucleoprotein complexes. Next, 2 mL of chloroform (per
1mL of TRIzol Reagent) was added to each sample in a 12mmx75mm polypropylene Sterile Culture Tube (Simport). The tubes were shaken vigorously by hand for 15 seconds, followed by incubation at room temperature for 3 minutes. The samples were centrifuged at a maximum speed of 12,000xg in a Sorvall RC 5B Plus Centrifuge for 15 minutes at 4°C. At this point, the aqueous phase was removed from the tube and the DNA was precipitated from the interphase and organic phase with 0.3mL EtOH.

Samples were mixed by inversion and stored for 2-3 minutes, before sedimentation by centrifugation at 4°C at a speed of 2,000xg for 5 minutes. The phenol-ethanol supernatant was transferred to a fresh tube and protein precipitated using 0.8mL isopropyl alcohol. Samples were set aside for 10 minutes and sedimented at a speed of 12,000xg for 10 minutes at 4°C. The supernatant was discarded and the protein pellet washed with 2mL of 0.3M guanidine hydrochloride in 95% EtOH 3 times. During each wash cycle, the protein pellet was stored in the wash solution for 20 minutes at room temperature and centrifuged at 7,500xg for 5 minutes at 4°C. These steps were then repeated during the final wash with EtOH, and then the pellet vacuum dried. For each sample, 50μL of 1% sodium dodecyl sulphate (SDS) and 1μL protease inhibitor, was added, and left at 50°C for 1 hour to dissolve the pellet, followed by storage at −20°C until use.

Protein Concentration: Bradford Assay

Standards were prepared by dissolving 5mg of bovine albumin fraction V (BSA) into 5mL of dH2O, and creating five 800μL standards from the 1mg/mL stock (20, 10, 5, 2.5, and 0μg/mL concentrations). For 100μL of each sample, 200μL of Bio-Rad Protein Dye (Bio-Rad Laboratories, Hercules, California, USA) was placed into each quartz cuvette and absorbance was measured at 595nm with a Beckman DU-40 Spectrophotometer (Beckman Coulter, Mississauga, Ontario, California, USA). After
calculation of the regression factor of standard curve, protein concentration was measured using the spectrophotometer and normalized according to the curve [115].

**Western Blot and Immunodetection**

A 10% separation gel (4.85mL H₂O; 2.5mL Tris 1.5M (pH 8.8); 0.1mL SDS 10%; 2.5mL Acrylamide /Bisacrylamide 40%; 50µL Ammonium Persulfate 10%; 5µL TEMED) was prepared and allowed to polymerize between the glass panels of the western blot apparatus. Following aspiration of excess water formed during polymerization, the 4% stacking gel (3.7mL H₂O; 625µL Tris 0.5M (pH 6.8); 50µL SDS 10%; 500µL Acrylamide/Bisacrylamide 40%; 25µL Ammonium Persulfate 10%; 5µL TEMED) was added and allowed to polymerize with a plastic comb to create separate wells. The comb was removed and the wells rinsed with water to remove bubbles.

The gel was mounted onto the migrating apparatus, which was then filled with a 1X migration buffer solution. 5mL of 2X loading buffer containing 100mM fresh DTT was added to 25µg of each protein sample. Each sample was boiled for 3 minutes and were then placed immediately on ice before loading onto gel. Also, 5µL of rainbow ladder (Amersham) was mixed with 5µL of 2X loading buffer and loaded onto the gel as a molecular weight marker. The gel was migrated at 60 volts (V) for 30 minutes in an electrophoresis gel box (BioRad). Once the samples had migrated to the end of the stacking gel, the voltage was increased to 140 V for 1 hour (adapted from [116, 117]).

The nitrocellulose membrane (Amersham) was soaked in water for 5 minutes followed by immersion in transfer buffer (25mM Tris in water; 190mM Glycine in water; 0.05% SDS; 20% methanol) for 5 minutes; the gel was also soaked in transfer buffer. Transfer apparatus was set up with the nitrocellulose membrane against the gel inside sandwich, filled with transfer buffer, to allow protein transfer. The apparatus was run at
60V for 1 hour, within a bucket of ice. The membrane was then washed for 1 hour in a blocking solution of 5% skim milk (Nestlé; North York, Ontario, CA) and 2% horse serum (Vector Labs) in TBS. Following washing with TBS, primary antibody (p-Akt1/2/3 (Ser 473) R Antibody or Akt1 (B-1) Antibody) was used at a concentration of 1:500 or 1:1000, respectively, overnight. The next day, following washing with TBS 3 times, secondary antibody (anti-rabbit or anti-mouse, respectively) conjugated to Horseradish peroxidase (Amersham) was added for 1 hour at room temperature at a concentration of 1:5000 in 10mL of blocking solution. Detection was performed using the ECL Western Blotting Detection Reagents (Amersham). Membrane was washed again 3 times and then placed into an exposure cassette with Kodak film for 5 minutes (adapted from [116, 117]).

**Results**

**Characterization of TRAP-Cre Phenotype**

In order to confirm the ability of the TRAP-Cre transgene to induce osteoclast-specific recombination, a reporter gene was used. A β-galactosidase gene reporter has been integrated into a ubiquitously expressed Rosa26 gene locus using homologous recombination. Expression of the β-galactosidase gene occurs upon Cre-mediated excision, which can be detected by LacZ staining. TRAP-Cre mice were mated with the ROSA26 reporter strain and examined for tissue specific recombination. Tibias from 22-day old TRAPCre X ROSA26 R mice were isolated, stained using β-galactosidase, and embedded in VIP Processing/Embedding Medium (Electron Microscopy Sciences). Slides with 6μm thick tissue slices were visualized using a high-powered light microscope.

As expected, lacZ staining was seen mainly near the ends of trabeculae in large, multinucleated cells (Figure 8). To confirm that these stained cells were osteoclasts, we
performed an assay to detect TRAP activity on consecutive sections from the same sample. The corresponding cells stained red, as seen in Figure 9. This confirmed that the TRAP-Cre reporter strain allowed for the osteoclast-specific recombination of a gene flanked with lox p sites.

**Breeding Strategy**

The experimental animals were obtained following two consecutive stages of mating. A mouse with one ILK-allele that has been inactivated using homologous recombination was first mated with a mouse expressing a transgenic Cre-recombinase under the control of an osteoclast-specific promoter (TRAP). The resulting offspring carrying one ILK-knockout allele as well as the TRAPCre transgene was subsequently mated with floxed-ILK mice as shown in Figure 10. Finally, the study mice will have one ILK allele knocked-out in all tissues and have abolished any ILK activity within osteoclasts (genotype: TRAPCre; ILK fl/-, hereafter referred to as ILK Δ/-).

**ILK inactivation**

**Range of ILK inhibition**

As an indirect mean of assessing the efficiency of CRE-mediated excision, RNA was carefully isolated from the primary osteoclast cultures derived from femur bone marrow cells using the TRIzol method. Following reverse transcription of the RNA, Real-Time PCR was conducted using an ILK Taqman assay with the β-actin Taqman assay acting as an endogenous control. Analysis of 8 individual ILK-deficient mice indicated a 62-94% range of inhibition of ILK expression, with a mean of 80% inhibition,
Figure 8. TRAP-Cre-mediated excision at the Rosa26 R locus as detected through lacZ staining in osteoclasts/large multinucleated cells. Sagittal section of the proximal femur obtained from a TRAPCre X ROSA mouse at e15.5-16.5. Bone was fixed, embedded in OCT and stained using LacZ method. Successful Cre-mediated excision results in the expression of β-galactosidase causing the blue staining, indicated by circles.
Figure 9. TRAP stained osteoclasts. Sagittal section of the proximal tibia obtained from a TRAPCre X Rosa26R mouse at e15.5-16.5. Bone was fixed, embedded in paraffin wax and stained for TRAP expression. Osteoclasts express TRAP granules within their cytoplasm and stain red, indicated by circles.
Figure 10. Breeding chart for ILK\(^{A/-}\). In order to attain minimal ILK expression, the first stage allowed for the incorporation of the knockout-ILK allele, and this was followed by introduction of the previously engineered targeted-ILK allele (floxed). We expected progeny to display normal Mendelian distribution (25% each).
as shown in Figure 11. These results suggest that the floxed ILK allele was efficiently deleted by the Cre recombinase in osteoclastic cells.

**Gross Phenotype**

Mice with an osteoclast-specific inactivation of ILK appeared phenotypically normal. Mice were collected at 3 weeks and 6 weeks of age, but no significant difference in weight was observed in the study mice versus their wild-type littermate controls (Figure 12). There were no apparent physical defects, and mice experienced normal tooth eruption.

Upon closer examination of the limbs, we noticed a slight shortening of the humerus in mutant animals. Both right and left humeri of 6-week old study mice were measured lengthwise using an Electronic Digital Caliper (General Tools, New York, NY, USA) and compared to wild type littermates (Figure 13). We calculated a decrease of 12% overall in humerus length within the ILK-deficient animals.

**Bone Phenotype**

Analysis of Golder-stained proximal tibial sections revealed an apparent increase in the number and size of the bone trabeculae at the metaphysis in mice with an osteoclast-specific ILK mutation (Figure 14). This increase was confirmed upon histomorphometric analysis. We measured an overall 24% increase in the volume of bone within mice lacking ILK, as seen in Figure 15 A. More specifically, we found the number of trabeculae within the primary ossification center to remain the same (Figure 15 B), while the average thickness of each spicule to increase by 44% compared to wild-type animals (Figure 15 C). This data suggests that the inactivation of ILK within osteoclasts may affect bone turnover and lead to a mild osteopetrosis phenotype.
Figure 11. ILK expression in wild-type and ILK$^{\Delta/-}$ mice. The average expression of ILK, as an indirect evaluation of Cre-excision efficiency was measured in RNA samples isolated from primary osteoclastic cultures of ILK$^{\Delta/-}$ mice. Analysis was done using Real-Time RT PCR, and control cells were arbitrarily assigned a value of 1. Bars represent the mean ± SE of measured data from eight animals of each genotype.
Figure 12. Comparison of average weight between wild-type and ILKΔ/− mice. (A) male and (B) female mice were weighed at 6 weeks of age. No significant change noted. Bars represent the mean ± SE of measured data from animals of each genotype.
Figure 13. Comparison of humerus length between wild-type and ILK\(^{\Delta/-}\) mice. The average length of humerus was measured at 6 weeks of age. Both the right and left humerus of each mouse was measured. Bars represent the mean ± SE of measured data from four ILK\(^{\Delta/-}\) mice and five wild-type littermates. **, p < 0.001
Figure 14. Increased bone volume in ILKΔ- mice. Goldner-stained, undecalciﬁed, section obtained from the proximal tibia of a 6-week old (A) wild-type and (B) ILKΔ- mouse. The ILKΔ- tibia displays an apparent increase in mineralized volume compared to its wild-type littermate.
Figure 15. Histomorphometric analysis of proximal tibial sections from wild-type and ILK\(^{Δ-}\) mice. Quatitative histomorphometry was performed on the Goldner-stained, undecalcified, proximal sections of mouse tibia. Parameters measured included (A) bone volume per tissue volume (BV/TV), (B) trabecular number (Tb.N.), and (C) trabecular thickness (Tb.Th.). Analysis was carried out using Bioquant NovaPrime software, bars represent the mean ± SE of measured data from six ILK\(^{Δ-}\) mice and five wild-type littermates. *, p < 0.05; **, p < 0.001
Osteoclast Number

Examination of TRAP stained paraffin sections suggested an increase in osteoclast number within the growth plate of ILK-deficient mice (Figure 16). Histomorphometric analysis confirmed an increase of 35% as shown in Figure 17.

Ruffled Border formation

Upon electron microscopy studies of the osteoclasts within the growth plate, we noticed a slight structural difference in osteoclast morphology within mice lacking ILK. These cells appeared to have some difficulty in developing the ruffled border, as seen in Figure 18. It appears that there is a less-developed ruffling, that is, the invaginations of the membrane do not seem to be as defined as they are within the wild-type osteoclasts. Since the proper formation of this organelle is compulsory for efficient resorption, this may perhaps give some insight to the cause of the higher mineral tissue content detected within the mutant mice.

Serum C-telopeptide Concentration

Another method used to assess the effect of ILK-inactivation on osteoclast resorption potential is by using an enzyme-linked immunosorbent assay (ELISA). When triple collagen helix is cleaved, the degradation products are released into the blood (C-terminal telopeptide) as well as the urine (N becomes calcified -terminal telopeptide). The RatLaps ELISA kit (Roche Diagnostics, Laval, Quebec, CA) was used to measure the concentration of deoxypiridinoline crosslinks present and detected a 37% decrease of this degradation product in the serum of ILK-deficient mice (Figure 19). These findings remain consistent with the increase in mineralized tissues measured by histomorphometry.
Figure 16. Terminal osteoclast differentiation in ILKΔ/Δ mice. Paraffin-embedded sections obtained from 6-week old (A) wild-type and (B) ILKΔ/Δ proximal tibia, stained for TRAP.
Figure 17. Increased osteoclastogenesis in bone sections from ILKΔ/ mice. Quantification of cells stained positive for TRAP from five individual ILKΔ/ and wild type tibial sections. Bars represent the mean ± SE of measured data from six ILKΔ/ mice and five wild type mice. ***, p < 0.0001
Figure 18. Impaired ruffled border formation in ILK<sup>Δ/-</sup> mice. Images of the osteoclast ruffled border present within tibiae of (A) wild-type and (B) ILK<sup>Δ/-</sup> mice, captured by a transmission electron microscope.
Figure 19. Decreased osteoclast activity in ILKΔl- mice. Concentration of Dpd crosslinks circulating within the blood of ILKΔl- mice and their wild-type littermates was measured by ELISA. Bars represent the mean ± SE of measured data from seven ILKΔl-mice and fourteen wild-type mice. **, p < 0.001
within mice lacking ILK.

**Osteoclastogenesis**

**Differentiation**

TRAP staining of primary cultures revealed a 100% homogenous population of osteoclast-like cells, apparent in Figure 20. Since TRAP is a marker expressed late in osteoclast development, this demonstrates that marrow cells of the ILK-deficient bones were able to fully differentiate into osteoclast-like cells (OLCs) with no apparent difficulty. Upon quantitative comparison, we calculated a 12% increase in the number of OLCs in the cultures lacking ILK (Figure 21). Combined with the data presented in Figure 17 that has been previously described, these results demonstrate that ILK inactivation does not affect osteoclastogenesis, either *in vivo* or *in vitro*. The fact that normal multinucleated osteoclasts form in the absence of ILK excluded an essential role of this molecule in osteoclast development and precursor fusion.

**Osteoclast Activity**

In order to determine if the OLCs displayed osteoclastic activity *in vitro*, a resorption assay on mineralized matrix was performed. This assay is used to confirm that the OLCs were functional bone-resorbing osteoclasts.

**Synthetic Calcium Phosphate**

Following a 9 day culture period on a synthetic calcium phosphate substrate (BD Biosciences), analysis of resorption revealed a significant decrease in osteoclast activity within osteoclasts lacking ILK, as seen in Figure 22. We observed a significantly low
Figure 20. Osteoclast differentiation *in vitro*. Osteoclasts generated from bone marrow cells were placed in culture for 9 days and then stained for TRAP activity, obtained from (A) wild-type and (B) ILK<sup>Δc</sup> femurs.
Figure 21. Increased osteoclastogenesis in the absence of ILK in vitro. Quantification of cells stained positive for TRAP from individual ILK\(^{\Delta/-}\) and wild type stem cell-derived osteoclastic cultures. Bars represent the mean ± SE of measured data from four ILK\(^{\Delta/-}\) mice and eight wild-type littermates. *, p < 0.05
Figure 22. Decreased pit formation by ILK$^{Δr}$ osteoclasts on a synthetic substrate. Osteoclastic cells derived from (A) wild-type and (B) ILK$^{Δr}$ femur bone marrow were plated on synthetic calcium phosphate discs obtained from the BD BioCoat Osteologic Culture System. Following a 9-day culture period, cells were washed away and discs were stained using von Kossa method.
number of pits formed on this substrate by ILK-deficient osteoclasts, and the number was almost doubled by wild-type cells. Also, for wild type cells the average size of each pit was much bigger that those formed by mutant osteoclasts. Quantification of the resorbed surfaces by histomorphometry revealed an 87% decrease in osteoclastic activity within ILK-lacking cells, as compared to wild-type cell cultures (Figure 23).

Dentin

The most readily accepted test of osteoclast resorption activity is carried on dentin. It is for that reason that we repeated the resorption assay on dentin slices obtained from walrus tusk as seen in Figure 24. Following a culture period of 9 days under the conditions previously described, it was apparent that there was a decrease in osteoclastic resorption activity, although less pronounced. The pits formed on the dentin by ILK-deficient osteoclasts were fewer in number and the resorption tracks were not well demarcated, as compared to those made by wild-type cells. Quantification of the resorbed surfaces by histomorphometry displayed a 45% decrease in osteoclastic resorption activity within cells lacking ILK (Figure 25). This decrease in activity could explain the increase in bone volume detected within these mice.

Signaling

Upstream signaling components

Expression of integrin receptors acting upstream of ILK within osteoclasts was monitored in order to confirm that ILK inactivation did not perturb the level of expression of upstream signaling molecules. We were interested in two molecules; the αvβ3 integrin and the α9β1 integrin, both of which are expressed on the surface of osteoclasts and heavily involved in osteoclast-mediated bone resorption [33, 63]. A Real-
Figure 23. Decreased activity of ILK$^{Δ-}$ osteoclasts on a synthetic substrate. Histomorphometry was used to quantify osteoclastic resorption of the synthetic calcium phosphate substrate, as an indirect evaluation of osteoclast activity. Bars represent the mean ± SE of measured data from four ILK$^{Δ-}$ mice and eight wild-type littermates. *, p < 0.05
Figure 24. Decreased pit formation by ILKΔ− osteoclasts on dentin. Osteoclastic cells derived from (A) wild-type and (B) ILKΔ− femur bone marrow were plated on dentin obtained from walrus tusk. Following a 9-day culture period, cells were washed away and discs were stained using Toludine blue. Image processing included whole mount image channel filtering to remove noise and whole image adjustment of brightness, contrast, color balance, and sharpening.
Figure 25. Decreased activity of ILK^{Δ/-} osteoclasts on dentin. Histomorphometry was used to quantify osteoclastic resorption of the dentin slices, as an indirect evaluation of osteoclast activity. Bars represent the mean ± SE of measured data from five animals of each genotype. ***, p < 0.0001
Time PCR assay was carried out using the αv, α9, β3, and β1Taqman assays, and in each experiment, we observed no change in the expression of any of these upstream signaling components within the ILK-deficient osteoclasts as compared to wild type cells (Figure 26). This confirms that signaling pathways upstream of ILK were not affected by the phenotype.

**Downstream Targets**

With the knowledge that the signaling pathway was not disrupted upstream, we began our investigation downstream of ILK. To begin to look at downstream signaling, protein was isolated from primary osteoclastic cultures derived from femur marrow cells using the TRIzol method. One of the proteins of interest to us was PKB/Akt, a molecule responsible regulation of apoptosis and directly phosphorylated by ILK. Phosphorylation of PKB/Akt causes activation, which results in the down-regulation of apoptosis. Preliminary western blot analysis, shown in Figure 27, indicates normal levels of Akt protein, yet diminished levels of phosphorylated Akt at the serine 473-site within the ILK-deficient mice, as compared to wild-type littermates. This data would suggest that one would observe an increase in the apoptosis within cells lacking ILK. This seems unlikely however, due to the increase in osteoclast number previously documented.

**ILK inhibitors**

ILK has been shown function both as an adaptor protein, forming complexes with cytoskeletal protein as well as a kinase, involved in downstream effector signaling. We were interested in determining which of these roles was disrupted within ILK-deficient mice. In order to do this, we tested the effect of inactivating the kinase function of ILK on osteoclastic activity. Wild-type cells were treated with QLT0267 [114], a potent inhibitor of ILK-kinase activity, at a concentration of 20μM, and incubated as previously
Figure 26. Normal integrin expression in ILK^{Δ/Δ} osteoclasts. Graph displaying the relative expression of the multiple integrins located upstream of ILK, in RNA samples isolated from primary osteoclastic cultures of ILK^{Δ/Δ} mice. For each of the integrins, gene expression is displayed relative to a value of 1 in the wild-type. Analysis was done using Real-Time RT PCR, and bars represent the mean ± SE of measured data from eight ILK^{Δ/Δ} animals. (p > 0.05; not significant)
Figure 27. Decreased phosphorylation of PKB/Akt in ILK\(^{Δ/−}\) osteoclasts. Protein was isolated from primary cultures of osteoclasts derived from ILK\(^{Δ/−}\) and wild-type mouse femur marrow cells. Analysis was performed using western blot and followed by immunodetection. PKB/Akt 75-80 kDa; PKB/Akt 90 kDa
described for a period of 9 days. We then measured changes in resorption on dentin as compared to untreated cells. Using histomorphometry software we calculated no significant changes in resorption (Figure 28). Since we have detected a decrease in resorption activity within our osteoclasts lacking ILK, this data suggests that it may not be the kinase function of ILK that is disrupted within the mutant animals.

Discussion

The aim of this project was to determine the effect of inactivating ILK in osteoclasts. We were interested in examining the effect of osteoclast-specific ILK ablation on the bone organ as a whole. To do this we studied bone at the cellular level, specifically looking at osteoclasts.

Targeted ILK-inactivation in osteoclasts allows mice to grow normally, and by external examination they are indistinguishable from their wild-type counterparts. However, upon closer analysis, we notice that the lack of ILK impacts skeletal modeling. The abundance of osteoclasts in the mice deficient of ILK establishes that the resorptive defect within this mutant arises from diminished bone degradation by the mature cell and not from arrested osteoclastogenesis. We have detected an obvious decrease in osteoclast activity. We also noted an increase in osteoclast number both in vitro and in vivo, within mice lacking ILK. Preliminary studies using EM indicate the presence of similar irregularities in the formation of the ruffled membrane, such as fewer but wider folds.

ILK Mutant Phenotype in Detail

The Rosa 26 is a reporter strain, whose ubiquitously expressed Rosa 26 gene locus was modified by integration of a β-galactosidase gene as well as insertion of a floxed stopper fragment (loxP sites). Upon excision of these sites by a Cre-recombinase,
**Figure 28. Effect of ILK-kinase inhibition on resorption.** Osteoclasts generated from bone marrow cells of wild-type femurs were plated onto synthetic calcium phosphate discs. Cells were treated with QLT01267 at a concentration of 20μM for a period of 9-days and resorption activity was measured and compared to untreated cells following von Kossa staining. Bars represent the mean ± SE of measured data from three animals of each group.
the expression of β-galactosidase is restored, and detectable through lacZ staining. Also, until this time, the TRAP-Cre transgenic strain had not been previously tested. Use of the Rosa 26 R in combination with the TRAP-Cre strain was very efficient in confirming that the TRAP-Cre transgene allowed for recombination specifically within osteoclasts, as displayed by lacZ staining. It also demonstrated that the osteoclast specific Cre-transgene was capable of excising the floxed loci within the Rosa 26 gene locus.

To obtain tissue specific deletions of a particular gene, the Cre-loxP system is commonly used. Although this strategy has been used previously by several groups, removal of the loxP-flanked sequences by Cre is not 100% efficient [118].

We used Real-Time reverse-transcription PCR as an indirect mean to assess the efficiency of TRAPCre-mediated ILK excision. We assayed 8 individual samples of RNA isolated from pure osteoclast cultures to measure the range of ILK-inhibition. Real-Time RT PCR is generally the most sensitive test of gene expression [119] and confirmed that this Cre-mediated osteoclast-specific excision was not 100% penetrant. For some study animals, ILK expression was as high as 38%, as compared to others where it was merely 6%.

This suggests that some animals were more severely affected by the phenotype than others. Overall, we measured an average of 20% of ILK expression within our study group. An 80% decrease in expression would still be significant enough to prevent full activity in osteoclasts and most likely to detect any changes in bone morphology.

An alternate method of measuring the efficiency of Cre-mediated excision would be through the use of quantitative PCR. Once DNA is isolated and quantified from primary osteoclast cultures, primers designed to flank the regions adjacent to the ILK loxP excision sites would be used for PCR. Following amplification, the samples will be
loaded on an agarose gel and then band intensity would be quantified using the phosphorImager.

There is another way to confirm excision efficiency and that would be to use immunohistochemistry, on tissue samples and an antibody for ILK, since it is being excised. The loss of ILK expression, as indicated by a decrease in staining, is a good confirmation that the excision is taking place within mutant animals. This method of analysis, however, is poorly quantifiable. Another experiment that is measurable is to carry out immunocytochemistry on primary osteoclastic cultures. Here, we would also use an antibody for ILK and can count the number of cells that still express this gene. The relative numbers would give an indirect measure of the efficiency of Cre-mediated excision.

Lastly, one can also measure the amounts of relative ILK protein levels by performing a Western blot assay on protein isolated from primary osteoclastic cultures. This would then be probed with the same ILK antibody and band intensity can be quantified using the phosphorImager.

Mouse breeding involved a two-stage procedure in order to ensure that ILK expression within osteoclasts was (minimal) the lowest that could be attainable. Due to the fact that Cre alone does not have a specific efficiency of excision, we incorporated the knockout ILK allele. A heterozygote animal carrying an inactivated ILK allele and an osteoclast-specific Cre (TRAP) was mated with a homozygous ILK-floxed mouse to ensure that the highest level of ILK-inactivation within osteoclasts was attained.

From a superficial (physical) perspective, the study mice appeared phenotypically normal. They experienced no problems with tooth eruption or within daily activities. In order to determine if there were any significant changes to the skeletal morphology, mice
were weighed at 6 weeks of age. This period marks the peak of bone formation in these animals, and we detected no significant differences in weight.

We did notice a slight difference in the sizes of humeri and proceeded to measure individual lengths. The 12% decrease measured in humerus length was our first indication the mutation may have some effect on bone morphology.

Upon analysis of the tissue sections of the proximal tibia, we observe a specific staining pattern due to the Goldner technique used. Within these bone samples the mineralized tissue stains green and bone marrow and osteoid, red. Initial observation of the ILK-deficient bone suggests that there is more mineralized tissue present. Histomorphometry software allowed for the confirmation of this apparent increase, to measure 24% more bone as compared to a wild-type metaphysis. Now this can be attributed to multiple factors; such as quantity of trabecular spicules, width of individual bone spicules, and the spacing between each of them. Measurement of all parameters revealed the cause of the increase to be a direct result of an increase in trabecular thickness. Trabecular spicules within the metaphysis of mice lacking ILK were 30% thicker than those of wild-type mice. So, accordingly, we measured an equivalent decrease in the separation between individual trabeculae. This increase in mineral tissue observed within ILK-deficient mice implicates a mild form of osteopetrosis. Considering the targeted inactivation of ILK within osteoclasts, this cell is likely involved in causing this phenotype. There are two possibilities in this regard; there may not be enough fully differentiated osteoclasts present or the osteoclasts are strong in number, but may not be properly able to resorb the bone at a normal rate. Alternate causes could be endocrine dysfunction or an increase in osteoblast activity. By measuring circulating levels of Ca\(^{++}\), Pi and alkaline phosphatase, we can determine if the observed osteopetrosis is secondary to a defect in mineral homeostasis. In order to measure the effect of the mutation on osteoblast proliferation or apoptosis, the Proliferating Cell Nuclear Antigen (PCNA)
staining kit can be used to stain paraffin-embedded bone sections to measure the rate of proliferation; and a TUNNEL assay can be used to quantify apoptosis. To assess any change in the activity of osteoblasts, alkaline phosphatase levels in protein isolated from 7-day old osteoblast cultures generated from calvaria could be measured. In addition to this, alizarin red could be used to stain the nodules formed within these primary cultures after 14 days, and then quantified using histomorphometry software. As previously mentioned, however, changes in osteoblast differentiation or activity are unlikely to be involved in the observed phenotype.

Hematopoietic stem cells develop into pre-osteoclasts, many of which then fuse to form a fully differentiated osteoclast. We were interested in examining the progress of osteoclast differentiation to identify any perturbations within ILK-deficient mice.

Once the osteoclast is fully differentiated, TRAP granules used within the resorption process form within the cell. These granules can be stained using sodium tartrate to allow for visualization of cells in culture. Since the appearance of TRAP molecules is confined to the final stages of osteoclast development, and our primary osteoclast cultures stain positively for this enzyme, we are convinced that ILK is not required for osteoclastogenesis in vitro. However, it may affect the activity of these cells.

Osteoclasts were also stained by TRAP in vivo for easy identification. Quantification of cells within the bone confirmed the presence of differentiated osteoclasts, more abundant in mutant than in wild-type cells. This finding disproved our first theory of the ILK-deficient mice having less osteoclasts present to resorb the bone.

This suggested that perhaps it was not the presence of bone-resorbing cells that was responsible for the phenotype but the activity of these cells. We set out to determine the cause for the increase in mineralized tissues.
Circulating Serum Levels

Type I collagen accounts for more than 90% of the organic matrix of bone [120]. During maintenance of the skeleton, bone matrix is constantly being degraded and this process releases type I collagen fragments into the circulation. A decrease in osteoclast activity would be expected to lead to a reduction in circulating degradation products.

The RatLaps ELISA kit was used to detect the rate of bone metabolism in ILK-deficient mice as compared to normal mice. We were able to measure a decreased level (37% less) of C-terminal telopeptide circulating within the blood of mice lacking ILK. By measuring the concentration of collagen fragments in the blood serum, we have an indirect measure of the activity of these osteoclasts within ILK deficient mice. Our results are consistent with the finding of an increase in mineralized bone as a direct result of reduced osteoclastic resorption, supported by a decrease in bone degradation products found within the blood stream. An alternate assay for the measurement in vivo osteoclast activity is by detection of the N-terminal telopeptide levels within the urine excreted by these ILK-deficient mice [121].

Osteoclastic Resorption

The most convincing assay to directly test osteoclast activity is substrate resorption. At first, we tested osteoclast activity using synthetic calcium phosphate discs. Ceramic biomaterial substrates are now considered a comparable alternative to biological substrates, such as dentin, for the direct assessment of osteoclast activity [122].

The system uses a resorbable artificial bone analog in the form of small calcium phosphate films on transparent quartz substrates. Tests have shown that the resorption lacunae formed by osteoclasts on these discs are similar in morphology, although larger
than their equivalents on biologic hard tissue slices [123]. This suggests that when quantifying osteoclastic resorption using this substrate the results will display a more striking phenotype than would result from the use of dentin slices.

We calculated an activity level of only 13% within osteoclasts deficient for ILK, using these synthetic calcium phosphate discs. This degree of inhibition may seem too important to cause the relatively mild phenotype that we observe and could be associated with the previously documented data regarding resorption lacunas on the synthetic discs appearing larger than their equivalents on dentin [123].

An alternate possibility is that attachment to the synthetic substrate is hindered due to a defect in adhesion. A way to test this is by performing an adhesion assay on the ILK-deficient osteoclasts. Following the isolation of osteoclasts generated from marrow cells, they can be plated on coverslips coated by vitronectin, washed after 30 minutes to remove the non-adherent cells, and the relative number of cells remaining attached to the substrate can quantified to detect any adhesion defects.

We also tested the osteoclasts on dentin slices obtained from walrus tusk, and measured 55% activity. This was carried out on several different samples of ILK-deficient cells and suggested a less drastic osteoclast phenotype.

One interesting thing to consider is that we are detecting a decrease in osteoclast activity yet we have detected more osteoclasts within the bone. The ILK-deficient bones could likely be recruiting more osteoclasts to compensate for the decrease in resorption, therefore resulting in a greater number of osteoclasts within mice lacking ILK. Also, this could explain why we are seeing a more significant increase in the number of cells in vivo versus in vitro studies, as the rest of the organism is not present within cell culture systems.
Ruffled Membrane

Transmission Electron microscopy allows for the ultrastructural examination of cell morphology. Specialized structures such as cell membranes and other organelles can be visualized for abnormalities using this high-powered tool.

As previously described, upon attachment to the surface, the osteoclast undergoes polarization of its membranes. This includes formation of the ruffled border, which is essential for efficient bone resorption. Any perturbations in the formation of this organelle may have a severe effect on osteoclastic activity.

Intricate formation of the membrane is essential for normal osteoclastic function. As the membrane polarizes, it forms multiple folds or invaginations adjacent to the bone surface, allowing for the efficient trafficking of intracellular vesicles containing enzymes to aid in the resorption process.

With the help of our collaborators, we examined the ruffled border within osteoclasts lacking ILK. We noticed a structural abnormality within the ruffled membrane of an osteoclast located in a mutant mouse tibia. The invaginations of the border were markedly wider and fewer in number. The invaginations did not appear to penetrate deep into the bone matrix. This could be due to the fact that this osteoclast was not resorbing bone at the time the tissue was fixed or that there is, in fact, a defect in the formation of this membrane. This apparent decrease in the folding perhaps made it difficult for the cell to transport acidic vesicles into the resorption lacunae for acidification. This, if it be the case, would result in the impediment of osteoclasts to properly resorb mineralized bone. This preliminary result needs to be confirmed, however.
In order to study actin formation, adherent marrow cells will be gently placed onto substrate coated glass slides with a fine brush, and treated with rhodamine phalloidin following permeabilization. Then, using a confocal microscope, the distribution of F-actin can be assessed. Such experiments have been planned and will be executed in the near future.

**ILK and Integrins**

We did not expect that the inactivation of ILK within osteoclasts would have any effect upon upstream signaling pathways. It was however, necessary to confirm this hypothesis. The most convincing test is to measure the expression level of components located upstream of ILK within osteoclasts.

ILK has been shown to interact with integrin-β chains [60]. The most abundant integrin expressed on the osteoclast surface is the αvβ3 integrin and was demonstrated to play a role in resorption, so it makes sense to measure any changes in its expression level. Another integrin expressed by osteoclasts is the α9β1 integrin and has recently been implicated in osteoclast-mediated resorption and was another likely candidate to test expression levels upstream of ILK ([60], Roodman, personal communication)

Results from the Real-Time PCR assay confirmed our original hypothesis that the phenotype had no effect on the expression of integrins on the cell surface and therefore on upstream signaling pathways.

An alternate means of measuring the effect of this mutation on upstream signaling would be through the application of Western blot assay. Protein isolated from the primary osteoclastic cultures can be analyzed using this method. Testing protein levels is
a more accurate measurement, due to the possibility of post-translational modifications as well as protein turnover. The best approach is to measure the relative levels of αv, α9, β1, and β3 protein within these cells, by probing with individual antibodies for αv, α9, β1, and β3, and then quantifying band intensity using a phosphorImager.

**Genetic Link Between β3 and ILK**

Animals lacking the αvβ3 integrin display an osteosclerotic phenotype as a result of dysfunctional osteoclasts. β3-deficient osteoclasts lack actin rings, have abnormal ruffled membranes and are unable to spread. McHugh et al. (2000) noticed an increase of 3.5 times more osteoclasts *in vivo*. This confirms that αvβ3 is not involved in the differentiation of osteoclasts, as shown by previous studies of integrin expression, but rather their activity[33].

Mice deficient in ILK display a mildly osteopetrotic phenotype. There is an obvious increase in bone volume, which is complemented by a decrease in bone degradation product within the circulation. We have measured a 2-fold increase in the number of TRAP-positive osteoclasts *in vivo*, as well as some effect on ruffled border formation. This mutation perturbs osteoclastic bone resorption, however the cause of this has not been determined. In this regard, failure to efficiently resorb bone and possibly form a normal ruffled membrane implicates that our mutant mice will likely display a defect in actin-mediated cytoskeletal organization. The formation and organization of actin filaments within the ILK-deficient osteoclasts has yet to be explored.

Given the similarities of these two phenotypes, it would seem likely that these two molecules are functioning within a common pathway. Also, ILK was shown to bind to the β3 integrin [70]. In order to determine if ILK is located within the same signaling
pathway as integrin β3, future experiments could involve construction of a compound heterozygote. Mice carrying a single allele inactivation of either ILK or β3 within osteoclasts do not display a phenotype ([33], data not shown). By creating a cross in which one allele of both ILK and β3 are inactivated, we would be able to examine the resulting phenotype. We expect that the compound heterozygote will display a phenotype more severe than both the ILK- and β3- heterozygote mice, as a result of gene dosage. If we observe such a severity in phenotype, we have genetic proof that these two proteins are located within the same pathway. This is the gold standard of verification.

However, if the double heterozygote does not result in a more severe phenotype, this may be attributed to three different circumstances. Firstly, the two proteins may not be located within the same pathway. There may not be gene dosage occurring within combination, or there is a possibility of the presence of compensatory mechanisms. Therefore, a redundancy of function mechanism is in play, due to the presence of multiple effectors which regulate the same pathway.

**ILK Kinase Activity**

It is still not yet known if it is the kinase function of ILK or the scaffolding function (adaptor protein function), which is disrupted within the ILK-deficient mice. In order to determine this, we have commercially obtained an osteoclast precursor cell line, with bone-resorbing capability. These cells will be further treated with QLT0267, a potent inhibitor of ILK kinase activity, and monitored for changes in resorption. If the treatment of the cells with the inhibitor causes a reduction in osteoclast-mediated resorption, this would strongly suggest that the kinase function of ILK is required for normal osteoclast activity.

The experiments using QLT0267, while suggesting that the inhibition of ILK
kinase activity does not affect osteoclast function, are far from conclusive. First, we have limited experience with the precursor cell line. Second, we did not perform dose-response studies using the inhibitor in these cells. Thus, the relative contribution of the ILK kinase activity remains uncertain, but could be further tested as suggested below.

We could then attempt a rescue of the ILK-deficient osteoclasts described within this study with the expression of the constitutively active (S343D) ILK as well as kinase-dead dominant negative ILK (E359K) cDNA, using retroviral transfection. This experiment would allow us to determine which role of ILK is disturbed within the ILK-deficient osteoclasts. If rescue of the phenotype is possible with the kinase-dead ILK, then we have proof that the scaffolding function of ILK is perturbed within the mice. On the other hand, if rescue is not possible with expression of kinase-dead ILK, then we can conclude that it is indeed, the kinase role of ILK, which is disrupted within the ILK-deficient osteoclasts. This result would support our previous findings, however, since we have detected a loss of kinase activity on PKB/Akt using western blot.

Possible Mechanisms

PINCH & Paxillin

PINCH is responsible for the subcellular compartmentation of ILK. In order to prove this, Li et al. (1999) mutated ankyrin repeats of ILK to cause loss of binding to PINCH, and found that ILK was unable to localize to focal adhesions on its own [79].

PINCH and ILK participate in processes that are fundamental to the cell, such as cell-ECM interactions and intracellular signal transduction pathways regulating cell proliferation and growth. It is important to recognize that any slight changes, such as alterations in expression or activities, might contribute to the pathogenesis of diseases
such as cancer, which involve perturbations in cell proliferation and cell-ECM interactions [81]. With this in mind, it is also important to consider that ILK has been shown to function as an adaptor protein, in addition to a functional kinase, and that our studies have mainly supported the kinase role of ILK.

In order to test if there is a mechanism in play involving PINCH, one approach would be to disrupt the PINCH-binding domain of ILK (ankyrin repeats) and retrovirally transfect this disrupted ILK cDNA into ILK-deficient osteoclasts. The cells could then be cultured onto synthetic calcium phosphate or dentin to assess their resorption activity. If the phenotype is rescued, this would exclude a role of PINCH in ILK-dependant osteoclast activity.

Paxillin has been shown to interact with ILK and α-parvin, and this complex has been implicated in the regulation of actin organization during cell adhesion. This further supports the adaptor function of ILK, however the kinase activity was required for the binding interaction [84].

**Parvins**

It has been shown that α-parvin is required for recruitment of ILK to focal adhesions[124]. α-parvin has also been shown to stimulate GSK-3 phosphorylation on Serine 9, and phosphorylation of PKB/Akt on Serine 473, both of which previously have been shown to be regulated by ILK [70, 86]. Recent studies in *Drosophila*, *C. elegans* and mouse have demonstrated that ILK null mutants display significant inhibition of integrin-related cell adhesion and cytoskeletal organization. Such studies support a role for ILK in regulating cell adhesion [88, 89].

Attwell *et al.* (2003) used an ILK kinase assay on both soluble and cytoskeletal
fractions of cells in culture in order to demonstrate that ILK activity is dependent upon its subcellular localization. They also showed that reducing ILK kinase activity or downregulating ILK expression resulted in the inhibition of cell attachment and migration. It also caused the decreased localization of ILK-binding partners, such as paxillin, and α-parvin to focal adhesions. α-parvin is responsible for recruiting ILK in its active form, to focal adhesion complexes, where it then participates in downstream signaling events. Such events involve the stimulation of PKB/Akt and GSK-3 phosphorylation; and thus provides a link between ILK-mediated cytoskeletal organization and signaling [86].

Considering we have detected a possible change in phosphorylation of a downstream effector, not associated with these cytoskeletal proteins, this is a good indication that the mutation does not affect the signaling pathways involving PINCH or paxillin, however it could implicate the α-parvin protein. Perhaps one method to determine if the localization of α-parvin is altered and perhaps contributing to the phenotype observed in the ILK-deficient mice, is to perform an immunofluorescence experiment to visualize α-parvin within these cells.

PKB/Akt

Our investigation into downstream signaling began with the effector PKB/Akt which ILK phosphorylates at one of the two sites required for activation. Since we did not detect phosphorylation of PKB/Akt at the serine 473 site, it would be safe to assume that the molecule remains inactive within ILK-deficient mice. This means that inactivated PKB/Akt allows programmed cell death to occur. Perhaps there are more osteoclasts within the mutant mice that are signaled for apoptosis but from our osteoclast quantification experiments, this seems unlikely.
To address the apparent discrepancy between measured osteoclast numbers and reduced Akt phosphorylation levels, measurement of apoptosis levels in wild-type and mutant bones using a TUNNEL assay is necessary. The most convincing method of determining the rate of apoptosis would be to perform a TUNNEL Assay on tissue sections. We predict an increase in apoptosis in ILK-deficient osteoclasts. The increase in osteoclast numbers could then be due to a compensatory increase in the recruitment of osteoclasts following a reduction in resorption activity, as was previously shown for β3 integrin-deficient mice or rats treated with an inhibitor of bone resorption [33, 125]. However, regardless of the results of this assay, it is important to consider that this protein, PKB/Akt, could also be phosphorylated and activated through effector pathways parallel to the Integrin-Linked Kinase pathway, and still function normally as a down-regulator of apoptosis.

Rho Family of GTPases

Members of the Rho family of GTPases such as RhoA, Rac, and Cdc42 are involved in mediating cytoskeletal organization. Within epithelial cells, ILK has been shown to activate Rac and Cdc42, inducing dramatic reorganization of the actin cytoskeleton and cell spreading on fibronectin [126]. The binding of M-CSF to the surface of osteoclasts results in Rac activation, however, this is mediated through the β3 integrin [66]. Since ILK is located downstream of β3, examination of ILK-inactivation on cytoskeletal organization may suggest the involvement of Rho GTPases in the mutant phenotype.

α-NAC

The alpha chain of nascent polypeptide-associated complex (α-NAC) is a transcriptional co-activator, which indirectly regulates gene transcription.
osteoblasts, ILK-mediated phosphorylation of α-NAC translocates it to the nucleus. The expression of α-NAC has not yet been reported within osteoclasts. By crossing the TRAP-Cre transgenic strain with the NAC homozygous floxed strain, we can assess the effects of NAC-inactivation within osteoclasts [115].

**β-catenin and GSK-3**

β-catenin is required early in development of the osteoblastic lineage, by mesenchymal cells for the differentiation of osteoblasts. It acts upstream of the Runx2 transcription factor. Later on, in differentiated osteoblasts, β-catenin is required to control osteoclast differentiation through osteoblastic gene expression. It has been implicated as a key player in the canonical Wnt signaling pathway. The complete loss of β-catenin results in embryonic lethality [127]. The conditional inactivation of β-catenin within osteoblasts results in an osteopenic phenotype, displaying high osteoclastic resorption and low bone mass [74].

Previous studies have indicated the importance of ILK in the regulation of GSK-3 kinase activity, and its subsequent regulation of β-catenin phosphorylation [71, 74]. Normally, ILK mediates phosphorylation of GSK-3, resulting in its inactivation. This allows for the translocation of β-catenin to the nucleus, and formation of a transcriptional activation complex. In the absence of ILK, however, the activity of GSK-3 kinase would be upregulated and cause the phosphorylation of β-catenin, its ubiquitination and subsequent degradation [74, 127].

Glass II and colleagues (2005) generated a mouse strain expressing a constitutively active form of β-catenin within osteoblasts and this resulted in an osteopetrotic phenotype. This is due to the fact that the mutations of the β-catenin N-terminus prevent its phosphorylation by GSK3β and its subsequent degradation via
ubiquitination. As a result, the mice displayed high expression of OPG, a well known inhibitor of osteoclastogenesis, as well as translocation of β-catenin to the nucleus for interaction with LEF-1. The formation of the β-catenin/LEF-1 complex enhances the activity of mesenchymal gene expression. It was demonstrated that the expression of TCF proteins, specifically Tcf-1 and Tcf-4 in conjunction with β-catenin, act as negative regulators of osteoclast differentiation [74].

We are interested in determining if signaling through the β-catenin pathway is affected within the ILK-deficient mice. There are many different experiments that can be done to test this. The best experiment would be to cross mice expressing a stabilized form of β-catenin with the ILK-deficient mice used within our study. From this, two possibilities can arise. There may be no change in the phenotype, and this would prove that the β-catenin pathway is not involved in the observed phenotype. The other outcome would result in a rescue of the phenotype displayed within mice lacking ILK. If the expression of a constitutively active β-catenin is able to rescue the resorption defect documented within our study, then we will have proof that the β-catenin signaling pathway is perturbed within the ILK deficient osteoclasts.

If ILK signaling is indeed mediated through β-catenin, then an interesting experiment would be to cross the TRAP-Cre transgenic strain with the β-catenin floxed (conditionally inactivated) mice. It would be interesting to see if this combination results in a phenotype similar to that of mice expressing an osteoclast specific inactivation of ILK. It is important to note, however, that any rescue experiment of the osteoclast ILK-deficient phenotype, in which β-catenin is removed from the nucleus (by conditional inactivation, for example), would not be an informative experiment. This is because in the absence of ILK, GSK-3 sends β-catenin for degradation, preventing its translocation to the nucleus. Therefore, it will not be very informative.
The studies presented in this thesis represent the basis of description of the phenotype of the ILK-deficient mice. There is no doubt that the addition of a few mechanistic studies, as suggested in this section, will confirm our findings and allow for publication of these results.
Summary and perspectives

The phenotype that we have described here is a novel finding. We have shown that the targeted inactivation of ILK in osteoclasts causes a defect in bone resorption. Osteoclastogenesis was not disturbed, but osteoclast activity was significantly inhibited. We suggest that the PKB/Akt signaling pathway is disrupted within these mice. Further experimentation is required to confirm which additional pathway(s) is perturbed within the osteoclast-specific ILK deficient mice.
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McGill University Animal Care Committee

RENEWAL of Animal Use Protocol

For: Research X Teaching □ project

René St-Arnaud

Principal Investigator:

Functional genomics of cartilage: molecular approaches to identify novel diagnostic and therapeutic targets and Gene expression in bone cells (4): role of the integrin-linked kinase (ILK) during development

Protocol Title:

Genetics Unit, Shriners Hospital for Children

Unit, Dept. & Address:

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Level: B

Funding source: CAN-NCE

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1. Personnel and Qualifications

List the names of the Principal Investigator and of all individuals who will be in contact with animals in this study and their employment classification (investigator, technician, research assistant, undergraduate/graduate student, fellow). If an undergraduate student is involved, the role of the student and the supervision received must be described. Training is mandatory for all personnel listed here. Refer to \( \text{space will expand as needed} \) for details. Each person listed in this section must sign.

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<th>Name</th>
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<th>Animal Related Training Information</th>
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<td>René St-Arnaud</td>
<td>P.I.</td>
<td>Advanced level theory course</td>
<td>Shriners Hospital</td>
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<td>Louise Marineau</td>
<td>AHT</td>
<td>Mouse Workshop</td>
<td>Shriners Hospital</td>
<td></td>
</tr>
<tr>
<td>Judith Cowan</td>
<td>AHT</td>
<td>Mouse Workshop</td>
<td>Shriners Hospital</td>
<td></td>
</tr>
<tr>
<td>Tanya Dossa</td>
<td>Graduate student</td>
<td>Workshop + theory course</td>
<td>Shriners Hospital</td>
<td></td>
</tr>
</tbody>
</table>

* Indicate for each person, if participating in the local OHP Program, see http://www.mcgill.ca/rgo/animal/occupational/ for details.

2. Approval Signatures

Approved by:

Principal Investigator/ Course Director

Date: 12 April 2005

Chair, Facility Animal Care Committee

Date: May 25, 2005

CC Veterinarian

Date: May 12, 2005

Airperson, Ethics Subcommittee

Date: 2006

Approved Animal Use Period

Start: June 1, 2005

End: May 30, 2006
Integrins allow cells to adhere to the components of the extracellular matrix. Adhesion initiates a cascade of signals that can modulate migration, growth, or differentiation. The integrin-linked kinase (ILK) is a molecule involved in the transmission of the signal following adhesion. We have found that ILK is expressed in bone cells where it can modify proteins involved in the control of gene expression. We have engineered strains of mice that cannot express ILK in bone cells. These will allow us to study the role of ILK in bone.

If creating genetically modified animals or new combinations of genetic modifications, complete and attach a Phenotype Disclosure form (http://www.animal.ca/rgo/animal/forms/).

The procedures are the same as the original protocol: YES ☑ NO ☐

If NO, complete the following:
Detail new procedures that are different from section 10a of the original protocol (include a copy of the entire revised procedure section 10a of the original protocol with the changes and/or new procedures in CAPS):

Include here ALL procedures except transgenic procedures, including the ones described in the original protocol as well as new and changed procedures in CAPS (was section 10a in main protocol); Please only attach SOPs related to new and changed procedures to this renewal form.

Endpoints

a) For B and C level of invasiveness
The procedures are the same as the original protocol: YES ☑ NO ☐

If NO, supply new endpoints that are different from the original protocol:

b) For D level of invasiveness
Include here ALL endpoints, including the ones described in the original protocol as well as new...
8. Hazards (Check here if none are used: X)

a) Are the hazards different from original protocol? (infectious, radioactive, toxic, carcinogen, tumours)

YES □  NO □  if yes, supply details (material, risks, precautions):

b) Have the cell lines been tested for human and animal pathogens? YES: □  NO: □  None used: X

9. Description of Animals to be used in the coming year (only)

<table>
<thead>
<tr>
<th>Species</th>
<th>Supplier/Source</th>
<th>Strain</th>
<th>Sex</th>
<th>Age/Wt</th>
<th># Produced for breeding</th>
<th># Other for field study</th>
<th>Total # ofAnimals</th>
</tr>
</thead>
<tbody>
<tr>
<td>mouse</td>
<td>breeding</td>
<td>ILK-floxed</td>
<td>m/f</td>
<td>up to 6 months</td>
<td>48</td>
<td>48</td>
<td>96</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Col I-Cre</td>
<td>m/f</td>
<td>up to 6 months</td>
<td>48</td>
<td>48</td>
<td>96</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Tg</td>
<td>m/f</td>
<td>up to 1 year</td>
<td>192</td>
<td>N/A</td>
<td>192</td>
</tr>
</tbody>
</table>

10. Justification of Animal Numbers:

Based on the experimental objectives of the project, describe the number of animals required for one year. Include information on experimental and control groups, per group, and factors related to breeding, specify how many animals are used, number of litters produced, and how many offspring are used in experimental procedures. The arithmetic reasoning for the total number of animals for each column in the table above is calculated and should be made clear.

1) Projected numbers of animals are based on one litter/2 months, 8 animals/litter: 6 x 8 = 48. These numbers should be sufficient to maintain the strain in our animal facility as well as to allow mating to Cre transgenic mice.

2) These animals will be bred to the ILK 'floxed' mice in order to specifically inactivate ILK in particular cells (osteoblasts). Projected numbers of animals are based on one litter/2 months, 8 animals/litter: 6 x 8 = 48. These numbers should be sufficient to maintain the strain in our animal facility as well as to allow mating to ILK 'floxed' mice.

3) These animals will be bred to the ILK 'floxed' mice in order to specifically inactivate ILK in cartilage. Projected numbers of animals are based on one litter/2 months, 8 animals/litter: 6 x 8 = 48. These numbers should be sufficient to maintain the strain in our animal facility as well as to allow mating to ILK 'floxed' mice.
Submit to your local Facility Animal Care Committee. Please note that after two renewals, a full protocol needs to be submitted.

This approval does not imply that space will be made available. If a major increase of space needs is anticipated, please contact the appropriate animal facility manager.

<table>
<thead>
<tr>
<th>Sex</th>
<th>m/f</th>
<th>m/f</th>
<th>m/f</th>
<th>m/f</th>
<th>m/f</th>
<th>m/f</th>
</tr>
</thead>
<tbody>
<tr>
<td>Strain</td>
<td>TRAP-Cre-ILK floxed</td>
<td>TRAP-Cre-ILK floxed</td>
<td>TRAP-Cre-ILK floxed</td>
<td>TRAP-Cre-ILK floxed</td>
<td>TRAP-Cre-ILK floxed</td>
<td>TRAP-Cre-ILK floxed</td>
</tr>
<tr>
<td>Age/We</td>
<td>up to 1 year</td>
<td>up to 1 year</td>
<td>up to 1 year</td>
<td>up to 1 year</td>
<td>up to 1 year</td>
<td>up to 1 year</td>
</tr>
<tr>
<td># To be purchased</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td># Produced by in-house breeding</td>
<td>192</td>
<td>192</td>
<td>192</td>
<td>192</td>
<td>192</td>
<td>192</td>
</tr>
<tr>
<td># Other (e.g., field studies)</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>TOTAL # IP ARAB</td>
<td>192</td>
<td>192</td>
<td>192</td>
<td>192</td>
<td>192</td>
<td>192</td>
</tr>
</tbody>
</table>

10. Justification of Animal Numbers:

Based on the experimental objectives of the project, describe the number of animals required for one year. Include information on experimental and control groups, sex per group, and failure rates. For breeding, specify how many adults are used, number of offspring produced, and how many offspring are used in experimental procedures. The arithmetic explaining how the total of animals for each column in the table above is calculated should be made clear.
(4) These animals will be bred to the ILK 'floxed' mice in order to specifically inactivate in osteoclasts. The expected number of animals are based on one litter/2 months, 8 animals/litter: 6 x 8 = 48. These numbers should be sufficient to maintain the strain in our animal facility as well as to allow mating to ILK 'floxed' mice.

(5) These animals are the offspring of the crosses between the ILK-floxed and Col I-Cre mice. Projected numbers of animals are based on two litters/month, 8 animals/litter: 2 x 12 x 8 = 192.

(6) These animals are the offspring of the crosses between the ILK-floxed and Col II-Cre mice. Projected numbers of animals are based on one litter/month, 8 animals/litter: 12 x 8 = 96. Our analysis of this strain is more advanced, which is why we require less animals than for strains (5) and (7).

(7) These animals are the offspring of the crosses between the ILK-floxed and TRAP-Cre mice. Projected numbers of animals are based on two litters/month, 8 animals/litter: 2 x 12 x 8 = 192.

Submit to your local Facility Animal Care Committee. Please note that after two renewals, a full protocol needs to be submitted.

This approval does not imply that space will be made available. If a major increase of space needs is anticipated, please contact the appropriate animal facility manager.

- Mano listing requested corrections is attached.

- Corrections made and verified.
TRANSGENE AND KNOCKOUT/KNOCKIN: PHENOTYPE DISCLOSURE FORM

Once a phenotype has manifested itself, this form must be completed and a copy sent to the Animal Facility supervisor and the FACC Chair.

<table>
<thead>
<tr>
<th>1. Protocol #</th>
<th>4125-strain 5</th>
</tr>
</thead>
<tbody>
<tr>
<td>2. Species:</td>
<td>mouse</td>
</tr>
<tr>
<td>Alteration:</td>
<td>ILK K.O.</td>
</tr>
<tr>
<td>Background strain:</td>
<td>129 Sv</td>
</tr>
<tr>
<td>Target Tissue:</td>
<td>Bone: osteoblasts</td>
</tr>
<tr>
<td>DNA construct injected:</td>
<td></td>
</tr>
<tr>
<td>Ancillary Tissue:</td>
<td></td>
</tr>
<tr>
<td>Enhancer/promoter:</td>
<td>Osteoblast-specific fragment of type I collagen promoter</td>
</tr>
<tr>
<td>Transgene:</td>
<td>Cre recombinase</td>
</tr>
</tbody>
</table>

3. Observed phenotype- Include any trait that has affected the breeding, physical ability of the animal to move, eat, drink or result in a decreased lifespan.

A. Heterozygotes: None

B. Homozygotes: Mild: slightly wider growth plate and increased bone turnover

4. Distress- Include information detailing whether the observed phenotype will decrease or increase distress in the animal.

No distress accompanies this phenotype

Include plans to monitor or alleviate this distress:

Frequency of monitoring: daily

5. Indicate how or if this observed phenotype will alter your Category of Invasiveness.

No impact on the Category of Invasiveness

6. Signature: Date: 12 April 2005
McGILL UNIVERSITY
UNIVERSITY ANIMAL CARE COMMITTEE

TRANSGENE AND KNOCKOUT/KNOCKIN: PHENOTYPE DISCLOSURE FORM

Once a phenotype has manifested itself, this form must be completed and a copy sent to the Animal Facility supervisor and the FACC Chair.

1. Protocol # 4125-strain 6

2. Species: mouse
   Alteration: ILK K.O.
   Background strain: 129 Sv
   Target Tissue: Cartilage: chondrocytes
   DNA construct injected:
   Ancillary Tissue:
   Enhancer/promoter: Type II collagen promoter
   Transgene: Cre recombinase

3. Observed phenotype- Include any trait that has affected the breeding, physical ability of the animal to move, eat, drink or result in a decreased lifespan.
   A. Heterozygotes: None
   B. Homozygotes: Dwarfism

4. Distress- Include information detailing whether the observed phenotype will decrease or increase distress in the animal.
   No distress accompanies this phenotype
   Include plans to monitor or alleviate this distress:
   Frequency of monitoring: daily

5. Indicate how or if this observed phenotype will alter your Category of Invasiveness.
   No impact on Category of Invasiveness

6. Signature: ___________  Date: ___________

Approved April 28, 1999
Revised November 2003

120
McGILL UNIVERSITY
UNIVERSITY ANIMAL CARE COMMITTEE

TRANSGENE AND KNOCKOUT/KNOCKIN: PHENOTYPE DISCLOSURE FORM

Once a phenotype has manifested itself, this form must be completed and a copy sent to the Animal Facility supervisor and the FACC Chair.

1. Protocol # 4125-strain 7

2. Species: mouse Alteration: ILK K.O.
   Background strain: 129 Sv Target Tissue: Bone: osteoclasts
   DNA construct injected: Ancillary Tissue:
   Enhancer/promoter: TRAP
   Transgene: Cre recombinase

3. Observed phenotype- Include any trait that has affected the breeding, physical ability of the animal to move, eat, drink or result in a decreased lifespan.
   A. Heterozygotes: None
   B. Homozygotes: Mild osteopetrosis

4. Distress- Include information detailing whether the observed phenotype will decrease or increase distress in the animal.
   No distress accompanies this phenotype
   Include plans to monitor or alleviate this distress:
   Frequency of monitoring: daily

5. Indicate how or if this observed phenotype will alter your Category of Invasiveness.
   No impact on the Category of Invasiveness

6. Signature: 
   Date: 12 April 2005

Approved April 28, 1999
Revised November 2003
Memo

To: Dr. St-Arnaud

From: Shriners Animal Care Committee

Date: 5/27/2005

Re: Renewal, Project # 4125

The Shriners Animal Care Committee has reviewed the above protocol.

The following modifications are requested:

✓ 1. p.1 insert home and business numbers for Emergency Contact Personnel. The new home number for L.M. is 450-227-9643.

✓ 2. Insert signature of Tanya Dossa.

Please make the necessary corrections and forward the corrected application to the chairperson of the Shriners Animal Care Committee.
McGill University Animal Care Committee
AMENDMENT to Animal Use Protocol
www.mcgill.ca/rgo/animal/

Principal Investigator: René St-Arnaud
Protocol Title: Functional genomics of cartilage: molecular approaches to identify novel diagnostic and therapeutic targets, and Gene expression in bone cells (4): role of the integrin-linked kinase (ILK) during development
Unit, Dept. & Address: Genetics Unit, Shriners Hospital for Children 1529 Cedar Avenue, Montreal H3G 1A6
Email: rst-armaud@shriners.mcgill.ca

1. ADDITIONAL ANIMALS REQUESTED: (justify additional animal numbers in box 5 below).

<table>
<thead>
<tr>
<th>Species</th>
<th>Strain</th>
<th>Supplier/Source</th>
<th># Animals</th>
<th># By Breeding</th>
<th>Age</th>
<th>Sex</th>
<th>Weight</th>
<th># Needed at One Time</th>
<th>#/Cage</th>
<th>Total Per Year</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse</td>
<td>beta3-integrin KO</td>
<td>Dr. Teitelbaum</td>
<td>96</td>
<td>Up to 1 year</td>
<td>m/f</td>
<td>30 g</td>
<td>16</td>
<td>4</td>
<td>96</td>
<td></td>
</tr>
</tbody>
</table>

2. ADDITIONAL PERSONNEL: If an undergraduate student is involved, the role of the student and the supervision received must be described. Training is mandatory for all personnel listed. Refer to www.animalcare.mcgill.ca for details. Each person listed in this section must sign to indicate that s/he has read the main protocol.

Name | Classification | Animal Related Training | Occupational Health & Safety Program | Signature
---|----------------|-------------------------|-------------------------------------|--------

3. CHANGE IN FUNDING SOURCE AND/OR TITLE (title of grant must appear on the cover page of the animal protocol)

4. OTHER: (including housing, procedure, anaesthetic/analgesic, problems anticipated) If additional procedures, specify how many and which animals are to be used.

5. WHY ARE THESE AMENDMENTS NECESSARY? If requesting additional animals, justify the numbers

The ILK kinase acts downstream from the beta integrins. Deleting one copy of the ILK gene has no consequences. Similarly, deleting one copy of the beta3-integrin gene has no effect. Since the two genes act in a common cascade, we hypothesize that removing one copy of each gene in the same animals (compound heterozygote) should affect tissues where both genes act in common, such as the osteoclast. We want to breed the beta3-integrin knockout strain with the ILK KO strain to produce these compound heterozygote animals. The hypothesized phenotype is a mild osteopetrosis (increased bone mass due to a reduction in the activity of the osteoclasts). This is not a stressful phenotype for the animals.

Projected number of animals are based on one litter/month, 8 animals/litter: 12 x 8 = 96.

Approval Signatures:
Principal Investigator/ Course Director
Chair, Facility Animal Care Committee
CC Veterinarian
Chairperson, Ethics Subcommittee

Form revised January 2004

Note: the above modifications are valid until the expiration date of the main protocol.