Inactivation of the Integrin-Linked Kinase in osteoblasts increases mineralization

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Abstract

Signals from the extracellular matrix (ECM), mediated by integrins, play critical roles in regulating gene expression, differentiation, function, and survival of osteoblasts. Following engagement with the ECM, integrin receptors signal via multiple downstream effectors, including the Integrin-Linked Kinase (ILK). The net effect of stimulating the ILK signaling cascade is to modulate gene transcription: ILK-dependent phosphorylation of the cJun transcriptional coactivator, αNAC, induces the nuclear accumulation of the coactivator and potentiates c-Jun-dependent transcription. In order to determine the role of ILK in osteoblasts, we first inactivated ILK using RNA knockdown in osteoblastic cells. In parallel, we engineered mice with specific inactivation of ILK in osteoblasts and analyzed the phenotype of the mutant animals, as well as the behavior of primary cultures of ILK-deficient osteoblasts. MC3T3-E1 preosteoblasts were stably transfected with expression plasmid vectors encoding specific ILK siRNAs or a control, unrelated siRNA. Pools of cells stably transfected with each of the expression vectors were established and analyzed. ILK protein expression was efficiently inhibited in the ILK siRNA-transfected cells. Inhibition of *Ilk* expression led to cytoplasmic retention of αNAC and increased expression of the osteoblastic differentiation markers, collagen type I, bone sialoprotein, and osteocalcin. Strikingly, ILK-deficient osteoblasts showed dramatically increased mineralization. Mice with one inactivated ILK allele ($ILK^{+/-}$) were mated with Col-I-Cre transgenic mice. Progeny from this cross (Col-I-Cre;$ILK^{+/-}$) was bred to mice homozygous for a floxed ILK allele ($ILK^{fl/fl}$) to yield mutant mice with ILK-deficient osteoblasts (Col-I -Cre;$ILK^{-/fl}$). The mutant animals thus had one ILK allele inactivated in all tissues, and both alleles disrupted in osteoblasts. Bone was harvested at intervals and analyzed using micro-CT and histomorphometry. Surprisingly, while we could document ILK inactivation in the target cells, we did not measure any significant changes in any static or dynamic histomorphometric parameter. Gene expression levels assessed using RT-qPCR were unchanged between control and mutant mice, with the exception of reduced ILK expression in bone tissue. These surprising results prompted us to evaluate

the behavior of primary osteoblasts isolated from the osteoblast-specific ILK deficient mice. When placed in culture, ILK-deficient primary osteoblasts showed increased expression of osteoblastic differentiation markers and increased mineralization, similar to the ILK-knockdown osteoblasts. These data suggest that in vivo, redundancy of function could account for the absence of an obvious osteoblast phenotype. The main difference between the tissue environment and the ex vivo cultures involves the ECM, and thus we cultured the ILK-knockdown cells on either plastic or the ECM component, type I collagen. Culture on collagen reduced the exuberant mineralization of ILK-deficient osteoblasts, and this was accompanied by increased phosphorylation of Focal Adhesion Kinase (FAK) on residue serine 722. Taken together, our data suggest that the ILK-αNAC cascade normally inhibits osteoblastic activity, and that this activity involves ECM-integrin interaction. We propose that in vivo, other signaling pathways operating downstream of ECM-engaged integrins, such as signaling through FAK, are able to compensate for the loss of ILK activity, leading to the absence of an obvious phenotype in osteoblast-specific ILK-deficient mice.

Résume

Les signaux de la matrice extracellulaire (ECM) qui sont transmis par les récepteurs intégrines sont essentiels pour contrôler l'expression génique, la différenciation, la fonction, et la viabilité des ostéoblastes. Suite à la liaison entre les intégrines et l'ECM, ces récepteurs transmettent leur signal via des messagers secondaires, incluant l'*Integrin-Linked Kinase* (ILK). La stimulation de ILK a comme effet de moduler l'expression génique : ILK est capable de phosphoryler le coactivateur transcriptionnel αNAC, ce qui induit la translocation nucléaire de ce dernier. Notre but était d'étudier le rôle de ILK dans les ostéoblastes. Premièrement, nous avons inhibé ILK en utilisant des ARN interférents dans les ostéoblastes MC3T3-E1. En parallèle, nous avons développé des souris déficientes en ILK dans les ostéoblastes seulement, et nous avons analysé le phénotype osseux ainsi que le comportement en cultures primaires des cellules ostéoblastiques déficientes en ILK. L'inhibition de ILK par interférence à l'ARN induit la rétention cytoplasmique d'αNAC, et augmente l'expression de marqueurs ostéoblastiques, tel le collagène de type I, la sialoprotéine de l'os, et l'ostéocalcine. De plus, nous avons mesuré une augmentation dramatique de la minéralisation. Des croisements précis ont permis de produire des souris dont un allèle de ILK est inactif dans tous les tissus, et l'autre allèle est inactif seulement dans les ostéoblastes (génotype : ColI-Cre; $ILK^{-/fl}$). Les os ont été récoltés à plusieurs intervalles, et analysés par μCT et histomorphométrie. Les souris déficientes en ILK dans les ostéoblastes ne présentent aucun changement des paramètres histomorphométriques statiques ou dynamiques. L'expression génique par analyse RT-qPCR ne présente aucun changement, à l'exception d'une réduction du niveau d'expression de ILK. Ces résultats nous ont conduit à évaluer le comportement des ostéoblastes déficients en ILK en cultures primaires. Celles-ci présentent une augmentation des marqueurs d'expression génique ostéoblastiques, et forment plus de dépôts de minéral que les contrôles; un comportement similaire aux cellules MC3T3-E1 qui sont déficientes en ILK. Ces données suggèrent qu'*in vivo*, il y a un mécanisme de compensation inopérant *ex vivo*. La différence principale entre les conditions de culture et celles *in vivo* est la matrice extracellulaire. Nous avons donc

cultivé les cellules MC3T3-E1 déficientes en ILK sur une matrice de collagène, et celles-ci ne produisaient pas autant de minéral que celles cultivées sur du plastique. De plus, une autre protéine, la *Focal Adhesion Kinase* (FAK), était plus phosphorylée à l'acide aminé Serine 722. Nos données suggèrent que normalement, la cascade ILK-αNAC permet d'inhiber l'activité ostéoblastique, et que cette activité est dépendante de l'interaction matrice-intégrines. Nous proposons qu'*in vivo*, d'autres signaux opèrent pour compenser la perte d'activité de ILK (en particulier FAK), expliquant le phénotype des souris qui sont déficientes en ILK dans les ostéoblastes.

Aknowledgements

This work is the culmination of much time spent by others who have had the patience and the knowledge to introduce me to this field of research. I would like to thank my supervisor, Dr. Rene St-Arnaud, for recruiting me into his laboratory and providing me with the opportunity, the guidance and the resources to achieve the project presented herein. His patience and understanding were fundamental factors to the success of this work, and his contributions to my future endeavors will continue beyond its completion. The laboratory skills and knowledge present in this research environment were also particularly insightful, and much appreciation goes to the laboratory members that have contributed their time to discuss technical and theoretical concepts. Furthermore, I would like to thank Alice Arabian for engineering the ILK-KO and ILK floxed mice used in these experiments, as well as Gerard Karsenty for engineering the collagen type 1 Cre transgenic mice. Finally, I would like to acknowledge the animal health technicians who have taken the time to maintain and care for all the mice used in these experiments.

Division of Labor

I performed the following procedures:

- Testing of the siRNA molecules;
- Design and engineering of the siRNA stable vector;
- RNA harvesting, and subsequent reverse transcription and Real-Time qPCR;
- Western blots and subsequent analysis;
- Cell culture experiments;
- Von Kossa staining and quantification;
- Immunocytochemistry and analysis;
- DNA preparation (not tail cuts) and genotyping;
- Some serum collections;
- Some tibia collections;
- Tissue embedding, slicing, and subsequent dynamic histomorphometric analysis;
- Primary cultures of calvarial osteoblasts.

Alice Arabian engineered the ILK knockout mouse as well as the ILK floxed mouse.
Gerard Karsenty contributed the Collagen type I driven Cre mouse.
Animal Health technicians maintained the mouse colony, and performed most tibia and serum collections.
Guylaine Bedard prepared all figures.

List of abbreviations

αNAC – Nascent polypeptide associated complex α

AP-1 – Activating protein 1

ATF – Activating Transcription Factor

DLx5 – Distal-less homeobox-5

DMP-1 – dentin matrix protein 1

ECM – Extracellular Matrix

ERK – Extracellular signal related kinase

FAK – Focal Adhesion Kinase

FAK – Focal adhesion kinase

FGF - Fibroblast growth factor

GSK3β - glycogen synthase 3β

HIF-1 – hypoxia inducible factor-1

ILK – Intergrin-linked kinase

ILKAP – ILK activating protein

JNK – c-Jun N-terminal kinase

MAPK – mitogen activated protein kinase

MEK – MAPK/ERK activating kinase

MEPE – matrix extracellular phosphoglycoprotein

Msx2 – msh homeobox homologue-2

mTOR – mammalian target of rapamycin

PDK -1 - Pyruvate dehydrogenase kinase 1

PI3K – Phosphoinositol-3-OH Kinase

PINCH – particularly interesting new cystein-histidine rich protein

PIP2 - Phosphatidylinositol bisphosphate

PIP3 – Phosphatidylinositol (3,4,5)-trisphosphate

PKB – Protein Kinase B

PTHrP – Parathyroid hormone related protein

RANKL - Receptor Activator for Nuclear Factor κ B Ligand

RT-qPCR – Reverse transcription quantitative polymerase chain reaction

Runx2 – runt-related-transcription factor 2

SOST - Sclerostin

Src – $pp60^{Src}$ proto-oncogene of the Rous Sarcoma Virus

VCAM-1 - Vascular cell adhesion molecule 1

VEGF – Vascular endothelial growth factor

To my parents

I. Introduction

Bone is a specialized connective tissue, and together with cartilage, makes up the skeletal system. This tissue serves three functions: (1) mechanical support for locomotion and load bearing, (2) protection of vital organs, (3) metabolic buffer for mineral ions such as calcium and phosphate. The highly structured components of bone, accompanied by its mineral and protein constituents, make it one of the few mineralized tissues of the body. As such, bone has unique cell types and regulatory mechanisms that are crucial for its formation and maintenance.

Three cell types have a predominant role in bone physiology: the osteoblast and the chondrocyte of mesenchymal origin, and the osteoclast of hemopoietic origin. Each of these cell types has its unique role in bone. Briefly, osteoblasts lay down the protein rich osteoid that will become mineralized bone, chondrocytes lay down cartilage, and osteoclasts resorb bone matrix. These three cell types, put together, can produce bone through endochondral or intramembranous ossification, and can model, remodel, and repair bone.

Far from being a static tissue, bone is continuously being remodeled: osteoclasts resorb bone matrix, followed by osteoblasts that lay down fresh osteoid. In this manner, the skeleton can maintain its integrity through time. The process of bone modeling and remodeling is highly regulated by autocrine, paracrine and endocrine systems. In addition, biomechanical forces can have a dramatic effect on the remodeling process. The homeostasis of this specialized connective tissue is dependent upon the integration of all these influences.

I.1 Bone formation during development

Two types of bone formation are evident during development: endochondral ossification (mesenchymal progenitors condense and form a cartilage template that will be replaced by bone), or intramembranous ossification (mesenchymal progenitors

condense and differentiate directly into osteoblasts that lay down osteoid). Intramembranous ossification is responsible for the development of the flat bones of the skull and the addition of bone on the periosteal surfaces of long bones. In contrast, endochondral ossification is responsible for the formation of long bones of the appendicular skeleton and the vertebrae of the axial skeleton. In addition to these two fundamentally different bone formation processes, different parts of the skeleton derive from different embryonic lineages. Both neural crest and mesoderm contribute to the skeleton. Craniofacial bones arise from the neural crest, while the mesoderm gives rise to the postcranial limb, rib, appendicular skeletons and the skull base. The facial skeleton, which includes the mandibular, maxillary, and hyoid bones come from the neural crest, while the some cranial bones come from the cephalic mesoderm. The dorsal paraxial mesoderm gives rise to somites, the sclerotome and eventually the axial skeleton, and the lateral plate mesoderm gives rise to the appendicular skeleton. Once these lineages are derived, they have been genetically programmed to give rise to the skeletal element intended, and recent molecular genetic techniques has provided insight as to the regulatory mechanisms that tightly control the process of bone formation during development (Bilezikhan J.P. 2002).

Intramembranous ossification

During intramembranous ossification, bone is directly laid down in a disorderly fashion initially, but will be remodeled with time into stronger lamellar bone. This process is particularly important in the craniofacial bones and in the process of cranial suture.

A group of mesenchymal precursor cells under the influence of local growth factors such as FGFs, Hedgehogs, PTHrP, and the transcription factors Runx2 and Osterix, will proliferate and form mesenchymal condensations. These form in highly vascularised regions of the embryonic connective tissue, and with time, the mesenchymal cells will stop proliferating and begin to differentiate into pre-osteoblasts and then into osteoblasts. These cells become highly polarized, secreting bone matrix that is not yet

calcified. The matrix formed from these osteoblasts contains large amounts of collagen in disorganized and irregular bundles. As such, this initial bone matrix will calcify in a disorderly manner, and will have a high number of terminally differentiated osteoblasts that become surrounded by the matrix, called osteocytes. This type of bone is called woven bone, and in areas of bone marrow development, blood vessels that are surrounded by this woven bone trabeculae will develop into hemopoietic bone marrow. Eventually this woven bone will be remodeled under the action of the osteoclasts that will resorb the matrix, and new osteoblasts will be recruited to lay down more organized matrix that will be much stronger. This stronger matrix is called lamellar bone (Favus 2003).

Endochondral ossification

This process of bone formation is distinct from intramembranous ossification in that it involves the initial formation of cartilaginous template, which becomes replaced by woven bone, and finally by the deposition of strong lamellar bone. This type of bone formation is particularly important in lengthening the long bone, and in the formation of the axial skeleton.

We will focus on the particularities of long bone formation, a process that begins with the formation of mesenchymal condensations in vascularised areas of the embryo, which receive local growth factors and transcription factors very similar to those discussed above, but probably in different proportions. These condensations will differentiate into pre-chondroblasts then into chondroblasts though the activity of the specific transcription factor Sox9. Chondroblasts then begin to secrete collagen type II rich matrix, and this becomes the foundation of a cartilaginous template that forms the primordial skeleton. As the chondroblasts form the cartilage, they also become entombed into their own matrix, and differentiate into chondrocytes. These chondrocytes will continue to differentiate, and at the epiphysis of the long bones, called the growth plate, they will proliferate and form isogenous groups, while continuing to secrete collagen. These chondrocytes begin to terminally differentiate into hypertrophic chondrocytes closer to the diaphysis of the bone under the influence of local factors such as PTHrP and

Indian Hedgehog that are produced by these chondrocytes in a self-regulation loop. These hypertrophic chondrocytes allow for the limb to lengthen, and will eventually undergo apoptosis (Figure 1).

At the outer ring of the cartilage template, woven bone begins to form by intramembranous ossification, this will become the periosteum, and under the influence of VEGF, new blood vessels invade and penetrate the cartilage with the help of matrix resorbing osteoclasts. These blood vessels will form the basis for the development of hemopoietic bone marrow.

At the diaphysis of the growth plate, the calcified cartilage becomes resorbed by the osteoclasts, and woven bone is then deposited by osteoblasts that are recruited from the newly formed blood vessels. This region of the growth plate is called the primary spongiosa. This woven bone undergoes resorption by the osteoclasts, and the new osteoblasts lay down stronger lamellar bone in the region of the growth plate called the secondary spongiosa.

In this manner, it is truly the interplay of these three major bone cells that allow for the longitudinal growth of the bone through development into a fully modeled adult (Favus 2003).

I.2 Bone remodeling

Once growth and modeling of the skeleton has taken place, bones constantly alter their internal structure in a process called remodeling. Remodeling is crucial for bones to maintain their integrity, and involves the coordinated action of osteoblasts and osteoclasts under the control of biomechanical forces and biochemical factors in the microenvironment. Remodeling allows for the bone to adapt to changes in mechanical forces, and repairs the cracks and fatigue damage that accumulates with use. Finally, remodeling influences the serum biochemical levels because bone is a large reservoir of calcium and phosphate, as such, it can be used to increase ionic levels in serum over a long period. Ionic regulation is not under the minute-to-minute control of bone, however, because remodeling is a very slow process, and occurs in many discriminate microenvironments (Bilezikhan J.P. 2002).

Basic multicellular unit (BMUs) is the name of the subset of cells (osteoclasts and osteoblasts) that are required for remodeling bone at any given location (Figure 2). The process begins with the origination and organization of cells that have been stimulated by systemic hormones such as parathyroid hormone, 1,25- dihydroxyvitamin D, interleukins 6 and 11, estrogen, androgens, and prostaglandins. The cells are then activated as more osteoclasts are recruited to the site, and the activation front spreads across the periosteal surface of the bone. Circulating pre-osteoblasts are recruited to the site, and bone lining cells begin to secrete RANKL. The fully mature osteoclasts resorb the bone and digest the collagen, leaving Howship's lacunae. As the osteoclasts advance, fresh osteoblasts are recruited to form new bone and fill the lacunae. All that will be left is fresh mineralized bone with new bone lining cells and osteocytes until the site becomes target to another BMU (Bilezikhan J.P. 2002).

I.3 The osteoblast lineage

Overview of osteoblast differentiation

The osteoblast is the functional cell type in bone that will produce osteoid that will mineralize with time. It is characterized by a series of genetic markers that control its differentiation from mesenchymal stem cells to terminally differentiated osteocytes or bone lining cells.

Osteoblasts are derived from the local proliferation of mesenchymal stem cells in the bone marrow or along the periosteum (Figure 3). These condensed cells are highly proliferative, and express transcription factors such are runt-related-transcription factor 2 (Runx2/Cbfa1), osterix (Osx), distal-less homeobox-5 (Dlx5), and msh homeobox homologue-2 (Msx2) (Lian *et al.* 1998; Wagner 2002). The expression of these markers allows for the mesenchymal condensations to differentiate into osteoprogenitor cells (pre-osteoblasts), driving them away from other mesenchymal cell types such as fibroblasts, adipocytes, myoblasts, and chondrocytes (Lian *et al.* 2006). Differentiation along the osteoblast lineage requires the coordinated action of two important transcription factors:

Runx2 and Osx. Mice that are deficient for either of these genes show complete lack of osteoblasts, suggesting that these two genes are crucial for the coordinate differentiation of osteoblasts (Komori *et al.* 1997; Otto *et al.* 1997; Nakashima *et al.* 2002).

Pre-osteoblasts are elongated cells that remain highly mitotic, and are often visible near mature osteoblasts. With time, committed pre-osteoblast express type I collagen and bone sialoprotein, and differentiate further into mature osteoblasts, that have a developed rough endoplasmic reticulum required for production of matrix proteins such as type I collagen, osteocalcin, osteopontin, bone sialoprotein, and enzymes required for mineralization such as alkaline phosphatase (Erlebacher *et al.* 1995; Robling *et al.* 2006). Osteoblasts are post-mitotic, cuboidal cells, that line the bone forming front and lay down unmineralised osteoid (Franz-Odendaal *et al.* 2006). The proteins that are secreted by the osteoblasts are important in determining the characteristic of the biomineralized matrix. As proteins are secreted from the osteoblasts, calcium and phosphorus ions incorporate into the matrix in a highly ordered and repetitive structure, allowing for mineralization of the matrix to occur under the control of the secreted proteins (Anderson 1989). As osteoid begins to accumulate, some of osteoblasts become trapped within their own matrix. With time, these osteoblasts generate long cytoplasmic extensions, and express E11, and become immature osteocytes (Robling *et al.* 2006). As they become more deeply embedded into the mineralizing bone, they become terminally differentiated osteocytes, expressing dentin matrix protein-1 (DMP-1), matrix extracellular phosphoglycoprotein (MEPE), and SOST. These osteocytes remain entombed in the bone. Their role in bone is still under investigation; however, it is widely postulated that they act as a sensory mechanism that can provide biomechanical stress signals and guide the repair/remodeling process) (Lian *et al.* 1998; Franz-Odendaal *et al.* 2006) (Rubin *et al.* 2006).

Pathways regulating osteoblastogenesis

The AP-1 transcription factor complex

A great variety of dimers composed of members of the Fos, Jun and ATF families of proteins constitute the transcription factor complex AP-1. While Fos proteins (c-fos, fosB, fra-1, fra-2) can only heterodimerise with members of the Jun family, the Jun proteins (c-jun, JunB, JunD) can both homodimerise and heterodimerise with Fos members to form transcriptionally active complexes. Certain members of the ATF family of proteins are also part of the AP-1 complexes. This transcriptionally active complex has been shown to have important roles in many cells types, including bone, where various knock-out mice have been generated (Wagner 2002).

In osteoblasts, AP-1 activity can be induced by transforming growth factor β, PTH, and 1,25 dihydroxy vitamin D, which are all potent regulators of osteoblast proliferation and differentiation. Expression of all Fos and Jun members are initially very high in the developing osteoblasts, but in mature osteoblasts, Fra2 and JunD become the major components of the AP-1 complex (McCabe *et al.* 1996). This suggests that they are important for the synthesis and secretion of the matrix proteins. Corroborating the role of AP-1 in increasing bone formation, the Fra-1 and ΔFosB overexpressing mice show strikingly similar phenotypes that includes increased bone formation and osteosclerosis of the skeleton. In addition, these mice show reduced adipogenesis, suggesting that these proteins are important in promoting differentiation along the osteoblast lineage (Jochum *et al.* 2000; Sabatakos *et al.* 2000).

A role for c-jun in osteoblast gene expression has recently been suggested with *in vitro* work looking at the osteocalcin gene. Osteocalcin is a terminal differentiation marker of the osteoblastic lineage. It is synthesized, secreted and deposited by the osteoblast at the time of bone mineralization. Mice deficient in osteocalcin show increased mineralization as well as endocrine disruption leading to β-cell proliferation, glucose intolerance, and insulin resistance (Lee *et al.* 2007). Thus although initially thought to be an inhibitor of mineralization, osteocalcin's role in bone is now being reevaluated. Additional work is required to determine if osteocalcin is a direct regulator of mineralization or if it is an indirect manifestation of the endocrine role of the protein on mineralization.

It has recently been shown that c-jun homodimers have the ability to bind the AP-1 site proximal to the OCN gene and activate its transcription, and that this process is dependent upon the binding of the αNAC coactivator (Akhouayri *et al.* 2007). This suggests an important role for both c-jun and αNAC in terminally differentiated osteoblasts, and maybe osteocytes.

In addition to these transcription factors, a bZip factor activating transcription factor 4 (ATF4) was recently shown to regulate the onset of osteoblasts differentiation, and was an important factor throughout the osteoblast differentiation process, regulating both collagen type I synthesis and osteocalcin activity. It has been shown to bind and regulate the osteocalcin promoter in a RSK2-dependent manner (ribosomal S6 kinase-2) (Yang *et al.* 2004). The physiological role of ATF4 is clearly important, as suggested by the ATF4 deficient mice that are small, and have low bone mass compared to littermates (Tanaka *et al.* 1998; Hettmann *et al.* 2000; Masuoka *et al.* 2002; Yang *et al.* 2004).

Thus, several signaling events will modulate the proliferation and differentiation of osteoblasts through indirectly activating or repressing the AP-1 transcription factor complex. These signaling events are mediated cell adhesion to the ECM, and then transmitted by second messengers to the nucleus to regulate gene expression.

I.4 Regulation of bone by mechanosensors

These transcriptional pathways are highly dependent upon the cell's ability to adhere and sense its external physical environment. In order to fulfill its mechanical and metabolic roles, bone must be able to integrate local and systemic signals that will determine the optimal bone mass and guide its remodeling pattern, as well as balance osteoblast bone deposition and osteoclast resorption.

While metabolic demands of the skeleton are managed largely through hormones, the structural functions of bone are set primarily by genetic determinants and then by mechanical loading. The body's ability to function properly is ultimately dependent on the mechanical properties of bone. Bone must be stiff, resilient to fatigue, and have the ability to withstand extremes of physical activity. Like many organs, it has the capacity to

adapt to its physical environment, and respond to subtle changes in functional demands, such as to be optimized for each individual's physiology. Bone cells such as the osteoclast, osteoblast and osteocyte must all be fine tuned to work in unison when remodeling the skeleton. It has been known for a long time that these cells must have some kind of framework to transduce mechanical stimuli into intracellular signaling pathways and ultimately gene transcription that reflect the needs of the microenvironment. (Rubin *et al.* 2006).

Mechanosensors on the cells surface that direct gene transcription include ion channels, connexins, and integrins. These form the basis for attachment and in conjunction to hormonal stimuli, determine the cell fate. Below we will detail on a particular cell surface receptor that has a particularly important role in osteoblasts.

I.5 Integrin Receptors

Integrins are the major cell receptor molecules that regulate cell adhesion to an extracellular matrix (ECM). Their activation is usually required for cell survival, and although they are located on the cell membrane, they have profound effects in the cytoplasm by engaging several protein complexes, and indirect influences in the nucleus where gene expression can be regulated.

These receptors are key players in transducing extracellular signal such as matrix composition and biomechanical forces to the intracellular compartment, where effector proteins can integrate this information and regulate cell survival, differentiation, migration, and proliferation. Integrins are essential for anchoring a cell to the extracellular matrix, and through partner protein interactions, can effectively link the ECM to the actin cytoskeleton of the cell.

Since their discovery, integrins have been intensely studied. Their critical role in several cell types has been clearly demonstrated by the variety of phenotypes present when one of the subunits is knocked-out. Phenotypes for these knock-outs include: complete block in preimplantation development (β1), lethal developmental defect (α4, α5, αv, β8), perinatal lethality (α3, α6, α8, β4, β8), defects in leukocyte function (αL,

αM, αE, β2, β7), inflammation (β6), hemostasis (αIIb, β6, α2), bone remodeling (β3) and angiogenesis (α1, β3) (Hynes 2002).

Structure of the integrin receptor

Integrins are heterodimeric proteins with an α and a β subunit. There are currently 18α and 8β subunits that have been characterized and these have been known to assemble into 24 αβ integrin receptors to date (Hynes 2002). The combination of one α and one β confers the specificity of the integrin receptor to a number of ECM proteins. Ligands to the integrin receptors are generally short peptide sequences of ECM proteins such as collagen, fibronectin, vitronectin, and laminin. Other ligands for integrins have been demonstrated as leukocyte specific, which will bind counter-receptors of the Ig superfamily such as VCAM-1 (Figure 4) (Hynes 2002).

Signaling through the integrin receptor is bidirectional: signals from the matrix are transmitted to the cytoplasm, while the ability of the integrin to bind to the matrix is regulated by intracellular signals, as such, the cytoplasmic domain of integrins determines the receptor's activation state (Hynes 2002). This reciprocal interaction is required for adherent cells to survive and differentiate, while neoplastic cells often loose this anchorage dependent survival signal.

Integrin function

The cytoplasmic tail of integrins is generally short and always devoid of kinase activity. In order to transduce signals to and from the intracellular milieu, integrins must cluster together and form focal adhesion complexes. These focal adhesions promote more clustering along the membrane in a positive feedback loop, and strengthen the bond to the ECM, which in turn restructures its extracellular proteins, thus forming aggregates on each side of the membrane. On the cytoplasmic tail of the integrin, protein kinases and adaptor proteins are recruited sending downstream signals to the nucleus and other cytosolic organelles. In addition, the actin cytoskeleton binds to these adaptor proteins,

and reorganized the actin network depending on the location of the focal adhesion. In a migrating cell, the leading edge will form new adhesions, as the actin network will create new protrusions in the membrane based on integrin signaling. In parallel, the rear of the cell will dissolve its current focal adhesions, and recycle the actin to the leading edge (Brakebusch *et al.* 2003).

Thus, integrins as a stand-alone protein can accomplish very little, and in order for the cell to survive, grow, migrate, and differentiate, partner proteins must be activated to transduce signals.

The role of integrins in osteoblasts

Osteoblasts are highly polarized cells that will secrete large amounts of osteoid before terminally differentiating into osteocytes, or into bone lining cells. To effectively secrete proteins and then differentiate, they are particularly dependent on their attachment to bony extracellular matrix. Bone matrix is formed mainly of hydroxyapatite crystals, but the main protein is collagen type I, accompanied by fibronectin, and laminin. All of these form a scaffold on which the osteoblast can bind via its array of integrin receptors.

In vitro models

The importance of the matrix in osteoblasts is particularly evident in studies using antibodies targeted at integrins or extracellular matrix proteins. Fibronectin is a major component of bone matrix, and has been shown to be a ligand for several αβ integrin heterodimers. *In vitro* studies using targeted antibodies to fibronectin suggest that it is required in the early process of osteoid mineralization. In addition, immature osteoblasts that are exposed to anti-fibronectin fail to fully differentiate, while maintaining the ability to attach to the matrix (Moursi *et al.* 1996). However, when mature osteoblasts are exposed to anti-fibronectin treatment, they show characteristic apoptotic features (Globus *et al.* 1998). Thus fibronectin is required for early osteoblast differentiation, and becomes

a survival factor in mature osteoblasts. These data suggest an important role for integrin signaling in osteoblastogenesis.

Conversely, when antibodies are targeted to specific integrin receptors known to be expressed in the osteoblasts, differentiation is inhibited and osteoid mineralization is reduced. Mineralisation is blocked as much 95% for the collagen type I receptor α2β1, up to 65% in the vitronectin αvβ3, up to 20% for α3β1 and α8β1, and 45% for the β1 subunit (Schneider *et al.* 2001). In addition to blocking mineralization, the differentiation of the osteoblasts is also reduced. In contrast, when the αvβ3 or αvβ5 receptors are blocked using specific antibodies, mineralization and differentiation are unaffected (Moursi *et al.* 1997). Thus, put together, the signaling pathways involved in integrin signaling can effect several different aspects of osteoblast physiology, and integrins can even have an influence on the extracellular matrix formed by the osteoblasts, emphasizing the bi-directional role of these receptors.

Signaling pathways are also dependent upon the ECM surrounding the cell. A recent study proposed separate pathways regulating mesenchymal progenitor cell differentiation based on the ECM plating. Here, they showed that while vitronectin plating was able to activate the focal adhesion kinase and the ERK pathways, plating on collagen type I only activated the ERK pathways after one week in culture. Hence, signaling through the integrin can be substrate dependent (Kundu *et al.* 2006).

In vivo model

The use of transgenic mice has also been particularly useful to the study of integrin function in an *in vivo* setting. These studies have focuses on the β1 integrin subunit, which is highly expressed on the cell surface of osteoblast and osteocytes (Gohel *et al.* 1995). It is believed to be of particular importance to this lineage because it binds to most of the ECM proteins present in bone, including collagen type I (α1β1, α2β1), and fibronectin (α3β1, α5β1, α8β1), to name a few. Thus, a dominant negative form of the β1 subunit (β1-DN) was expressed in osteoblast of mice under the control of the osteocalcin promoter, ensuring that only differentiated osteoblasts and osteocytes had the

extracellular domain of the β1 subunit removed (Zimmerman *et al.* 2000). As opposed to the *in vitro* assays, the β1-DN osteoblasts growing in their physiological environment were able to grow, differentiate, and remain attached to the bone matrix. When these cells were then taken out of their milieu, and plated in culture, they lost this ability to remain attached and detached from the surface substratum. This highlights the importance of the ECM in regulating the survival of these cells, and the local factors present *in vivo* that maintain the cells integrity that are dependent on the integrin receptor.

Histologically, the bones of the transgenic mice were abnormal compared to those of the wild-type. Although osteocyte lacunae were normal, the canaliculi that contain the cytoplasmic extensions of the osteocytes were abnormal. The cortical bone appeared thinner, and was more porous in transgenics. In addition, the cortical bone was more disorganized and the bone formation rate in these mice was reduced. The parietal bones of the skull were thinner than controls. Finally, the osteoblasts lost their polarized morphology, and seemed to have defects in either collagen type I and laminin secretion or production.

This phenotype suggests an important role for integrin signaling in osteoblasts matrix secretion and production, and ultimately in the formation and organization of the skeleton. The skeletal phenotype of the male mice was compensated for with age, and the bone grew stronger. In females, however, the bone remained thinner, and the number of osteoclasts was increased compared to wild-types. This further suggests that the role of sex hormones may play a role and influence integrin signaling pathways, and that this pathway also modulates the activation of osteoclasts.

I.6 Integrins downstream signaling

There are several important downstream binding partners to integrins, and focal adhesions can involve over 50 proteins working in unison. For the purpose of this work, we will restrict our focus to two important binding partners of integrins, and examine each pathway individually.

Focal adhesion kinase

Focal adhesion kinase (FAK) is a cytoplasmic tyrosine kinase that co-localises to sites of integrin clustering. It is a principal mediator of signals from the integrin receptor, and has the ability to recruit various proteins to the clustering site. Its binding site to the integrin is still being explored, but based on binding of FAK to peptides of the β1 subunit, it is believed to bind to integrins directly (Schaller *et al.* 1995). However, deleting the integrin binding domain of FAK does not prevent it from localizing to focal adhesions, and deleting the FAK binding region of the β3 subunit does not prevent FAK activation (Tahiliani *et al.* 1997; Shen *et al.* 1999). Therefore it is possible that *in vivo*, FAK binds integrins indirectly, through an adaptor protein such as talin and paxillin, which are able to bind integrins directly and indirectly (Brakebusch *et al.* 2003).

Upon binding to the ECM and integrin activation, FAK becomes phosphorylated at Tyr397 either directly by autophosphorylation or indirectly after Src phosphorylates Tyr576 and 577. Upon activation, FAK phosphorylates several targets that regulate cell fate such as PI-3-kinase that will then phosphorylate PKB/Akt and regulate cell survival. FAK has also been suggested to regulate cell cycle progression and proliferation by the activation of JNK. Upon FAK activation, a complex forms with the recruitment Src, $p130^{CAS}$, and Crk. This complex activates JNK that gets shuttled to the nucleus and phosphorylates the c-Jun transcription factor that binds to c-Fos and forms the AP-1 transcription complex, an important regulator of gene expression in osteoblasts and other cell types (Giancotti *et al.* 1999).

In the pre-osteoblastic cell line MC3T3-E1, FAK has been shown to regulate differentiation through two parallel pathways when these cells are plated on collagen type I. Using antisense FAK to inhibit FAK activity, the ERK-MAPK pathway was inhibited, and expression of the differentiation marker alkaline phosphatase was also reduced compared to control cells (Takeuchi *et al.* 1997). In a similar experiment, FAK was inhibited using antisense oligonucleotides, and the osteoblasts were stimulated with bone morphogenic protein 2, a treatment known to induce differentiation and matrix deposition

in the osteoblasts through the Smad pathways. In FAK deficient cells, this pathway could not be activated, and cells did not differentiate as determined by osteocalcin expression, a marker for late stage osteoblast differentiation (Tamura *et al.* 2001).

Thus, integrin signaling through FAK is one mechanism that regulates osteoblast differentiation and gene expression through several well defined pathways.

In vivo model of FAK modulation

In bone, the role of FAK has only recently been elucidated by the generation of a conditional knock-out of FAK in osteoblasts. The group of Helms *et al* found that when FAK is knocked-out in differentiated osteoblasts, the differentiation process is perturbed *in vitro*. This corroborates evidence from other groups that have used blocking anti-bodies against integrins and have completely blocked osteoblasts differentiation. However, some of the FAK null osteoblasts still differentiate, and when the skeletal phenotype is observed, there is no change in the bone architecture of mice in development or in adult mice, suggesting that FAK is not required for osteoblast differentiation *in vivo*. The group then stressed the system by inducing mono-cortical fractures to the bone of these FAK null mice, and observed delayed fracture healing and remodeling at the fracture site. To further investigate the relative contributions of osteoblasts or osteoclasts in the delayed process, they took bone fragments from FAK null mice and placed them in the fracture site of wild-type mice, and vice versa. The results showed that the osteoclasts were slower at resorbing the matrix that was produced by the FAK null osteoblasts, suggesting that FAK is potentially involved in formation of the osteoid by the osteoblast. Thus FAK is required *ex vivo*, but mechanisms exist *in vivo* to maintain normal bone homeostasis until the system becomes challenged (Kim *et al.* 2007).

Integrin-Linked Kinase

Another protein that has recently been characterized and found to impact on integrin signaling is integrin-linked kinase (ILK). This protein was identified in 1996 as being able to bind and phosphorylate the β1 integrin subunit (Hannigan *et al.* 1996). ILK plays a dual role in the cell: it has a kinase function that can phosphorylate several proteins, thereby mediate a regulatory effect on several signaling cascades; in addition it can serve as an adapter molecule that bind to proteins such as PINCH, α-parvin, β-parvin, and paxillin. As an adapter molecule, ILK and its binding partners serve to link the actin cytoskeleton to the integrin receptor and the ECM, while at the same time regulating actin polymerization. As a kinase, ILK can phosphorylate a number of targets involved in cell survival, proliferation, differentiation, and migration (Figure 5). Many of these regulatory abilities stem from its ability to indirectly regulate gene expression in several cell types, and it is increasingly seen as a therapeutic target in tumorigenic cells (Hannigan *et al.* 2005).

ILK structure/function

The gene encoding *Ilk* was mapped to the distal tip of human chromosome 11 (11p15.4/15.5). Analyses of mRNA expression and human genomic database searches indicated that only one *ILK* gene exists. The translated ILK protein is 452 amino acids in length, and comprises four amino-terminal ankyrin repeats, a central pleckstrin homology (Globus *et al.*)-like domain, and a carboxy-terminal kinase catalytic domain (Figure 6) (Hannigan *et al.* 2005).

The ankyrin repeats allow ILK to bind to the β1 integrin subunit, but this region is conserved in several proteins, and is important to facilitate protein-protein interactions. In this case, this region can also bind to PINCH, and ILKAP, the latter being a negative regulator of ILK kinase activity and signaling. The PH-like domain of ILK contains a binding site that is a likely target of phosphatidylinositol3,4,5-triphosphate (PIP3) regulation. *In vitro* studies have suggested that this PH-like domain is a mechanism by which PI3-kinase can activate ILK through PIP3, but not PIP2 (Delcommenne *et al.* 1998).

ILK signaling pathway

ILK has a very important role to play as an adapter protein to regulate the actin cytoskeleton. However, its kinase domain phosphorylates a wide range of substrates, which mediate many effects on the cell. We will focus on relevant protein pathways here that can effect cell survival, differentiation and ultimately gene expression (Figure 5).

ILK activity is stimulated by adhesion to the extracellular matrix through the integrin receptors, or by extracellular stimuli such as cytokines or growth factors in a phosphatidylinositol kinase (PI3K)-dependent manner. The main product of PI3K activity is PIP3, and this phospho-lipid acts as a second messenger to bind to the PH-like domain of ILK, thereby activating the kinase domain of the protein. This activation step has been proven to be important for the regulation of both PKB/Akt and glycogen synthase kinase 3β (GSK3β) (Delcommenne *et al.* 1998).

The activation of PKB/Akt inhibits apoptosis; therefore this step is important in cell survival. It has become clear that the activation of PKB/Akt is dependent upon the binding of PIP3 to the PH domain of PKB/Akt, thus allowing the constitutively active PDK-1 to phosphorylate threonine 308, and partially activating PKB. At the same time, PIP3 activates ILK that will phosphorylate serine 473 of PKB, rendering the molecule fully active and able to promote cell survival.

On the other hand, glycogen synthase kinase 3β (GSK3β) is involved in cell fate determination and is a regulator of gene expression. *In vitro* experiments suggest that ILK can negatively regulate GSK3β by phosphorylating this protein on Serine-9. When GSK3β is phosphorylated and inhibited by ILK, it has a direct influence on the c-Jun protein, which becomes hypophosphorylated. Under this state, c-jun exposes a DNA binding domain, and is able to form homodimers and heterodimers with other jun and fos family members, and activate the AP-1 transcription factor family. This is another very influential gene expression pathway that has been directly linked to the activation of ILK (Troussard *et al.* 1999; Troussard *et al.* 2003).

The important role of co-activators in gene expression has recently become more evident with the characterization of αNAC that functions in the cytosol to control translation and in the nucleus as a transcriptional co-activator. Recent *in vitro* data has suggested that αNAC is shuttled to the nucleus following ILK mediated phosphorylation at Serine 43, and once accumulated in the nucleus, αNAC acts as a coactivator by binding c-jun, and potentiating AP-1 activation (Quélo *et al.* 2004). Site-specific mutagenesis of this phosphoacceptor site to an alanine prevents phosphorylation of αNAC, and promotes cytoplasmic accumulation of the protein, leading to reduced AP-1 activity. Furthermore, αNAC cytoplasmic stability is regulated by GSK3β. The latter can phosphorylate αNAC on threonine 159, and target it for degradation by the 26S proteasome (Quélo *et al.* 2004). Thus ILK activity mediates direct and indirect subcellular signaling events through GSK3β, which will propagate the signal to c-jun and αNAC. These molecules combined will potently activate the AP-1 transcription factor family, and impact cell fate.

ILK signaling in bone cells

ILK signaling has been investigated in chondrocytes and osteoclasts using a targeted Cre-loxP approach. This allows for the specific excision of the *Ilk* gene in chondrocytes or osteoclasts specifically.

The Cre-loxP methodology allows for the specific ablation of any DNA fragment that is flanked by loxP DNA sequences. The Cre recombinase enzyme will excise any fragment contained within that region. Thus, by controlling the expression of Cre using a cell specific promoter, a gene can be ablated specifically in any cell type, at a specific time in the differentiation pathway, given a suitable promoter can be found.

This approach allows for the generation of a mouse strain that has ILK-deficient chondrocytes, which are important in laying down cartilage, and particularly important in the growth plate of long bones. Disruption of *Ilk* expression leads to chondrodysplasia characterized by a disorganized growth plate and dwarfism. Differentiation of the chondrocytes was normal as assessed by gene expression markers, but proliferation of early chondrocytes of the growth plate was reduced, as assessed by BrdU labeling or

proliferating cell nuclear antigen. This phenotype is accompanied by a decrease in cyclin D1 expression, a regulator of cell cycle progression (Terpstra *et al.* 2003).

The Cre-loxP methodology was also used to generate ILK-deficient osteoclasts, and these mice appeared phenotypically normal, but hostological analysis of the proxial tibia showed an increase in bone volume and trabecular thickness. *In vitro* cultures and bone sections stained for TRAP suggest that osteoclastogenesis is not affected by ILK deficiency, and that the number of osteoclast is increased. Primary cultures of osteoclasts plated on dentin and calcium phosphate discs showed reduced resorption activity, however. Serum collagen degradation fragments suggest that there is also a systemic decrease in the resorption activity in ILK-deficient mice. Thus, ILK-deficient osteoclast show normal osteoclastogenesis but abnormal activity (Dossa 2006).

Integrin signaling through ILK is important in bone cells, and this can have a profound impact on histomorphometric parameters of bone, as evidenced by ILK-deficient osteoclasts and chondrocytes.

I.7 Goal and strategy

The primary aim of our research is to determine the role of ILK in osteoblast function. Studies of integrin and FAK modulation in osteoblast suggest that this pathway can have profound effects in this cell type. Therefore we set out to test the *in vivo* and *in vitro* effects of downregulating ILK specifically in osteoblasts. In order to achieve this, we used two parallel approaches.

The first approach we used was the generation of an ILK deficient cell line derived from mouse calvaria, the MC3T3-E1 cell line. Using RNA interference, we were able to selectively inhibit ILK translation by degrading ILK mRNA, and study the cells' phenotype *in vitro*.

The second *in vivo* approach was performed on a mouse model generated using the Cre-loxP methodology. Briefly, this method involved introducing a loxP DNA sequence flanking the *Ilk* gene. This sequence has no physiological effect under normal circumstances. Thereafter, a transgene is incorporated into the germ line of these mice.

The *cre* gene is a recombinase that has the ability to excise the DNA fragment that is flanked by these loxP sites, thereby inactivating the *Ilk* gene in this case. In addition, the Cre recombinase is driven by the osteoblast specific promoter of collagen type I, which is therefore expressed in maturing osteoblasts. This method allows for the inactivation of ILK in mature osteoblasts only.

We report here that cultured ILK-deficient osteoblasts show exuberant mineralization accompanied by cytoplasmic retention of αNAC. RT-qPCR also shows that these cells have increased osteoblasts specific differentiation markers, and matrix protein production. Surprisingly, in mice with osteoblast-specific ILK inactivation, while we could document ILK inactivation in osteoblasts, we did not measure any significant changes in static or dynamic histomorphometric parameters. This suggests a redundancy of function *in vivo*. The main difference between cultured cells and the *in vivo* environment is the extracellular matrix. Therefore, we cultured cells on type I collagen matrix and observed a decrease in mineralization accompanied by an increase in FAK phosphorylation on residue Serine 722.

Taken together, our data suggests that the ILK-αNAC cascade normally inhibits osteoblastic activity, and that this activity involves ECM-integrin interaction. We propose that *in vivo*, other signaling pathways operating downstream of ECM-engaged integrins, such as signaling through FAK, are able to compensate for the loss of ILK activity, leading to the absence of an obvious phenotype in osteoblast-specific ILK-deficient mice.

II. Materials and Methods

II.1 Transfection of small interfering RNA sequences

The siRNA used in these experiments were initially identified from Ambion as pre-designed Silencer® sequences. ILK 782 is siRNA ID: 67803 (Cat: 16704), ILK 285 is siRNA ID: 159172 (Cat: 16704). Both of these siRNA as targeted at different regions of ILK (Locus ID: 16202) (Table 1). One Negative Control siRNA (Cat: 4611) is used to ensure that the siRNA vector and or transfection has no deleterious effect on the cells, and to ensure specificity of the sequence.

All three of these siRNAs were tested in culture with transient transfections before incorporating the sequences into a vector. MC3T3-E1 cells were seeded in 6 well-plates at 100,000 cells/well 24 hours before transfection. siRNA sequences at 20, 50, or 100 nM were incubated with siPORT Amine and OPTI-MEM1 for 8 hours, and then diluted by 75% with addition of OPTI-MEM1. RNA from cells was harvested 48 hours after transfection with the siRNA.

II.2 RNA isolation, and RT-Real-Time qPCR

RNA was harvested using the RNAqueous-4PCR kit (Ambion, Cat: AM1914). Briefly, cells are washed in PBS (137mM NaCl, 2.7mM KCl, 4.3mM Na2HPO4, 1.4mM KH2PO4, pH 7.3) and lysed by vortexing for several seconds in a guanidinium lysis proprietary solution. The sample is then mixed with ethanol, and applied to a silica filter cartridge that quantitatively binds mRNAs and rRNAs. The filters are then washed in proprietary solutions to removed contaminants, and the sample is treated with a DNaseI to remove any remaining DNA.

Samples were reverse transcribed using the Applied Biosystems High Capacity cDNA Reverse Transcription Kit (Cat: 4368813). The RT reaction involved a proprietary mix of RT buffer, dNTP mix, Random primers, RNase inhibitors and the MultiScribe™ Reverse Transcriptase.

To quantitatively measure the mRNA transcripts in the samples, proprietary primers were purchased from Roche Diagnostics. Each primer set is specific to one gene of interest. The cDNA that was harvested and reverse transcribed was measured by optical density, and mixed to the proprietary buffer and primers for the Real-Time reaction. In every Real-Time reaction, a reference gene is used to normalize the amount of RNA in the sample, and the target gene is expressed as a ratio to this reference gene.
The Real-Time plate reader (7500 Real-Time PCR, Roche) measured the fluorescent dyes incorporated into the primers (TaqMan, Roche), and as a result, provides a quantitative assessment of the mRNA of the gene of interest, called the Ct value. This Ct value of the target gene is then normalized to the Ct value of the reference gene ($Ct_{target} - Ct_{reference}$ = dCt). This dCt provides a relative expression in each sample for the target gene. To compare several samples, the dCt of each value is compared to a calibrator value ($dCt_{sample1} - Ct_{calibrator}$ = ddCt). This ddCt value is then expressed in terms of a relative value that reflects the exponential nature of PCR that doubles at every Ct increase (2^-ddCT = final relative expression value).

II.3 Generating Stable ILK Deficient Cells

Once the sequences identified were known to be effective, the sequences were cross-referenced to the NCBI Blast database to ensure that there was no sequence homology to other mouse genes. Using the Ambion website, and the sequences already tested, we designed the sequences to be incorporated into the vector for transfection (Table 2). These sequences have an added loop sequence that will be cleaved once expressed in the cell.

These sequences were then synthesized by AlphaDNA (Montreal) annealed with the annealing solution provided (Ambion) at 90^{o}C for 3 minutes, then at 37^{o}C for 1 hour. These were then ligated into the Silencer p3.1 H1 vector from Ambion (Cat: 5768) using the T4 DNA ligase provided for 2 hours at room temperature, transformed and amplified in XL1-Blue Supercompetent Cells (Stratagene, Cat:200236). Plasmid DNA was

harvested with the mini-preparation kit (Qiagen) and sequenced at the McGill Univeristy and Genome Quebec Innovation Centre on the 3730xl DNA Analyzer systems from Applied Biosystems platform. Cells were then plated for transfection at 120,000 cells/well in 6 well plates, incubated overnight, and transfected with 2 μg of plamid with Geneporter (Gene Therapy Systems, San Diego, CA) and αMEM (Gibco) for a total volume of 500 μl. Four hours post-transfection, the volume was doubled with the addition of αMEM with 20% FBS. Twenty-four hours post-transfection, cells were washed with PBS and cultured with αMEM, 10% FBS. Seventy-two hours post-transfection, cells were split with Trypsin-EDTA 0.25% (Gibco) for 5 to 10 minutes, and twelve hours after, were cultured with αMEM, 10% FBS and 3 μg/ml puromycin. 48 hours later, cells were cultured with αMEM, 10% FBS an 2 μg puromycin for the remainder of the experiments. Cell populations were established as pools of stably transfected clones and this represent the most frequent integration event.

Sequence Name	**Sense (5' > 3')**	**Antisense (5' > 3')**	**ILK Nucleotides targeted**
ILK 285	GCACGGAU UAAUGUGAUGAtt	UCAUCACAU UAAUCCGUGCtc	285 - 303
ILK 782	GGAAAAGC AGGGACUUCAAtt	UUGAAGACCCU GCUUUUCCtt	782 - 800

Table 1. siRNA sequences used for transient transfection of MC3T3-E1 pre-osteoblasts

	ILK 285	**ILK 782**
Sense (5' > 3')	GATCCGCACGGATTA ATGTGATGATTCAAG AGATCATCACATTAA TCCGTGCTCTTTTTTGGAAA	GATCCGGAAAAGCA GGGACTTCAATTCAAGA GATTGAAGTCCCTGCTTTT CCTTTTTTGGAAA
Antisense (5' > 3')	AGCTTTTCCAAAA AAGAGCACGGATTA	AGCTTTTCCAAAAAAG GAAAAGCAGGGACTTC

	ATGTGATGATCTCTTGAATCA TCACATTAATCCGTGCG	AATCTCTTGAATTGAAGT CCCTGCTTTTCCG
Vector	p*Silencer*3.1-H1 puro	p*Silencer*3.1-H1 puro
Promoter	H1	H1
Cell Selection	Puromycin	Puromycin

Table 2. shRNA sequences with hairpin loop that are ligated into a vector and stably expressed in MC3T3-E1 cells.

II.4 Mineralisation assay for MC3T3 pre-osteoblasts

Cells were grown to confluency, subcultured using 0.25% Trypsin (Gibco), and plated on 12 well-plates in triplicates. Cells were plated at 120,000 cells per well, grown to confluency, and then cultured for another 21 days with the addition of ascorbic acid (50 μg/ml) and β-glycerol-phosphate (5 mM), in 10% FBS and αMEM. Media was changed 2 to 3 times per week. In the case of collagen matrix plating, the wells were covered in 0.1% Collagen type I (Sigma, Cat: C8919), incubated for one hour, and washed with PBS before plating the cells.

At 21 days post confluency, cells were washed three times with PBS (described above), fixed with 10% neutral formalin for 5 minutes, washed with water, and exposed to 5% silver nitrate in water for one hour under UV light. Cells were then rinsed with water, and exposed to 5% sodium thiosulfate to stop the reaction. Plates were then photographed.

Photographs were analysed with the Bioquant Osteo II software. This analysis consisted of assigning the bone volume as the mineralised volume of the well that appears dark, as well as assigning the minimum threshold value for the intensity of the stain. This value was then divided by the total volume of the well, to give the 'BV/TV'.

II.5 Protein Isolation

Cells were grown for one week post confluency, at which point the protein was harvested. Cells were washed with PBS, and incubated for 10 minutes with shaking in RIPA lisys buffer (50 mM Tris-HCL, pH 7.4,150 mM NaCl, 1 mM EDTA, 1% Na-deoxycholate, 1 % NP-40, 1 mM PMSF, and aprotinin, elupeptin, pepstatin cocktail to 1 μl/ml (Sigma, Cat: P8340). Cell membranes were disrupted with 30 seconds exposure to a Sonic Dismembrator (Fisher Scientific) at 40% amplitude to ensure that membrane protein such as ILK are properly dissolved. Lysates were centrifuged at 12,000 rpm for 5 minutes, the supernatant was transferred to a new tube and frozen at -80^{o}C.

II.6 Protein Immunoblotting

Cells lysates were quantified with the Bio-Rad Protein assay (Bio-Rad) to determine the concentration of protein in the samples. Proteins were then mixed with 100mM DTT, and loading buffer (125mM Tris-HCl, 4% SDS, 50 % Glycerol, and bromophenol blue until visible). Samples were boiled for 5 minutes and loaded into a western blotting apparatus. This consisted of passing the proteins through two gels by electrophoresis, the first being a compression gel (4% acrylamide) and a separation gel (7.5 to 12 % acrylamide depending on protein size). All gels were loaded with Magic Mark XP (Invitrogen LC5602) or Rainbow RPN800V (General Electric Health Care) to determine protein size on the gel. Following electrophoresis for one to two hours at 60 V initially, then at 120 V in migration buffer (25 mM Tris-HCl, Ph 8.3, 190 mM Glycine, 0.5% SDS), the membrane was removed from the apparatus and the proteins were transferred onto a nitrocellulose ECL membrane (Amersham Biosciences) for one hour at 60 V in transfer buffer (25 mM Tris-HCl, 190 mM Glycine, 0.05% SDS, 20% methanol).

Once the proteins are bound to the membrane, it is incubated into a solution of TBS-T (ph 7.4, 10mM Tris-HCl, .150mM NaCl, 0.05% Tween 20) and blocked for one hour, followed by overnight incubation at 4^{o}C of the primary antibody at the concentration indicated (Table 3). Following this incubation, membranes are washed 3 times in TBS-T, then incubated for one hour at room temperature in blocking solution and the corresponding secondary horse radish peroxidase linked antibody at the indicated

concentration (Table 3). Membranes were then washed and incubated for 5 minutes in Immobilon Western (Millipore, Cat:WBLKS0100), and exposed to a chemiluminescent film (GE Healthcare) and developed.

	Bloking	Primary Antibody	Secondary Antibody
αNAC	5% powdered milk	1/200	1/4000
P-Gsk3β	5% powdered milk	1/200	1/2000
Gsk3β	5% powdered milk	1/200	1/2000
C-Jun	5% powdered milk	1/300	1/4000
P-Fak	5% BSA, 2%Horse serum	1/200	1/2000
Fak	5% BSA, 2%Horse serum	1/500	1/3000

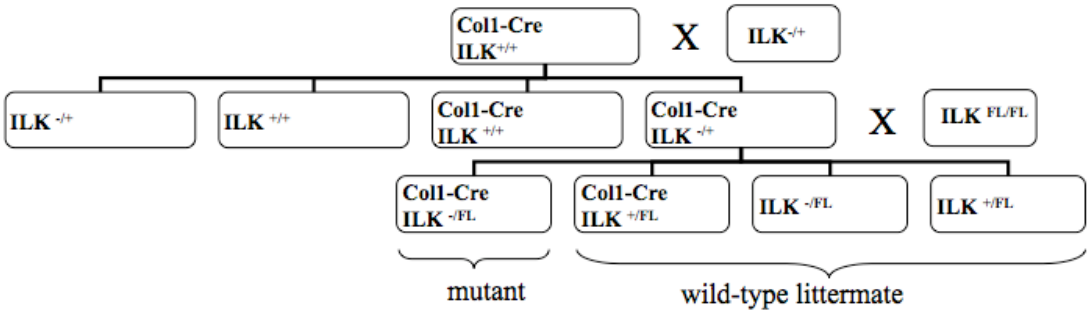
Table 3. List of antibodies with blocking solution used and dilutions.

II.7 Immunocytochemistry

Glass coverslips were placed in six well plates and gelatin (0.1 %) was used to coat the glass for one hour, then washed off. Cells were then plated at 100,000 cells per well in a six well plate and cultured for 48 hours under normal conditions (as above). Cells were washed three times with TBS, fixed with 4% paraformaldehyde for 30 minutes, washed, permeabilised with 1 % Triton-X 100, washed, then blocked with 1% BSA for 30 minutes. The primary αNAC antibody was then prepared in TBS-T with 1% BSA, and used at 1/200 dilutions for one hour at room temperature. Cells were washed three times, and incubated with the corresponding secondary FITC linked antibody in blocking solution (1% BSA) for 30 minutes at room temperature. Cells were washed, and then mounted with VectaShield mounting medium with DAPI (Vector). Photographs of the slides were taken immediately. Cells were counted on seven fields taken at 20x magnification, and based on localisation of the protein, and marked as either diffuse in the cell, preferentially nuclear, or preferentially cytoplasmic.

II.8 Mice derivation and genotyping

Conditional ILK knock-out mice were generated by breeding mice homozygous for the ILK floxed allele (flanked by LoxP sites on the kinase domain,), with mice that were heterozygous for the ILK systemic knock-out with the addition of a Cre recombinase downstream of an early Collagen type I promoter ($ilk^{fl/fl}$ x $ilk^{+/-}$ cre). As a result, ILK was conditionally knocked-out in osteoblasts of mice that expressed Cre recombinase. These mice were engineered by Alice Arabian (ILK floxed and heterozygous KO), and Gerard Karsenty (Col1-Cre mouse) before my candidature. The scheme below illustrate this breeding process.



Schematic 1: Breeding strategy for the osteoblast-specific ILK-deficient mice.

The mice derived from this breeding could have four genotype, with mendelian proportions: $ilk^{-/fl}$; $ilk^{+/fl}$; $ilk^{+/fl}$, cre; $ilk^{-/fl}$,*cre*. To determine the genotype of the mice, tail snips were taken at two weeks old (for tibia collection), or at the time of sacrifice (for primary calvaria culture). Tail snips were placed in lysis buffer (pH 8.5, 100 mM Tris-HCl, 200 mM NaCl, 5 mM EDTA, 0.2% SDS, 10 µg/ml proteinase K) at 55°C overnight, then were microcentrifuged for 10 minutes at 12,000 rpm, the supernatant was mixed with an equal volume of isopropanol, incubated for 10 minutes, and then microcentrigured for 10 minutes at 12,000 rpm. The supernatant was discarded, and the pellet was washed with 70% ethanol, air dried and resuspended in TE buffer (pH 7.7, Tris-HCl 10 mM, EDTA 0.1 mM).

Once the genomic DNA was isolated, an aliquot was taken for polymerase chain reaction (PCR) for the *ilk* genotype as well as for the presence of the Cre recombinase.

Primer sequences used were as follows:

RCRE

5'-TGAAGCATGTTTAGCTGGCCCA-3'

RCRE

5'-GACCGTACACCAAAATTTGCCTGC-3'

ILKNEOKO

5'-GACATAGCGTTGGCTACCCGTGATA-3'

ILKKO 5'

5'-GAAATGCTGATCATGCGTGGAGC-3'

PCR buffer composition:

	RCRE (µl)	ILKKO (µl)
10x PCR buffer (Invitrogen)	2.5	2.6
dNTP (2mM)	2.6	2.5
Primes 5' (5mM)	2.5	3.12
Primes 3' (5mM)	2.5	3.12
$MgCl_2$ (50mM)	2.25	0.78
DMSO	0	0.52
TaqMan	0.25	0.26
ddH_2O	11.5	12
Genomic DNA	1	1
Total reaction Volume	**25**	**26**

Amplification was done by GeneAmp PCR 9700 (Applied Biosciences) under the following conditions:

Temperature (^{o}C)	RCRE (minutes)	ILKKO (minutes)	Cycles
94	5	5	
94	0:45	0:30	35 x
57	0:45	0:30	
72	1	0:45	
72	7	7	
4	infinity	infinity	

II.9 DNA and RNA purification from calvaria

Isolation of RNA from calvaria was done for mice to analyse expression patterns at 5, 14, and 42 days old. DNA was harvested at 42 days old to assess whether the excision of ILK by Cre recombinase was efficient. Both methods are based on the same initial reagent: Trizol Reagent (Invitrogen).

Mice were sacrificed by cervical dislocation, and the butterfly shaped calvaria were dissected, the periosteum was removed with a Kimwipe, and samples were placed in RNALater (Ambion) and stored at -20^{o}C. Samples were then crushed with a mortar, placed in Trizol Reagent, and further homogenized with a Homogenizer (Brinkmann) at maximum intensity for 1 minute. Samples were stored at room temperature for 5 minutes, then centrifuged at 12,000 xg at 4^{0}C for 10 minutes. Chloroform was added to the samples, shaken by hand, then centrifuged for 15 minutes at 12,000 xg at 4^{0}C. The top aqueous phase was kept for the RNA while the bottom organic phase was kept for the DNA.

The DNA was subsequently precipitated with 100% ethanol, and centrifuged at 2,000 xg for 5 minutes at 4^{0}C. The pellet was washed twice with 0.1M sodium citrate in 10% ethanol. Finally the DNA pellet was washed with 75% ethanol, and resuspended in 50 μl of TE buffer (as described). Once purified, the DNA underwent PCR with ILK floxed primers.

The RNA was subsequently precipitated with isopropanol, centrifuged, and washed twice with 75% ethanol in DEPC water. The pellet was resuspended in nuclease free water (Ambion), and the concentration and purity of the total RNA was calculated by measuring the optical density of the sample at 260 and 280 μm.

II.10 Blood Collection and Analysis

Mice were taken at 42 days old and anesthetized with isoflurane. Blood was collected by the animal health technicians or by myself through a cardiac puncture, and the mouse was then euthanised by cervical dislocation. The blood was incubated for at least 30 minutes at room temperature, then microcentrifuged at 6,000 rpm for 6 minutes. The supernatant was collected and stored at -80^{o}C.

Blood samples were then sent for calcium, phosphate and alkaline phosphatase analysis at the McGill Animal Resources Centre.

Analysis of the collagen degradation product in the serum (Ctx) were done with the RatLaps ELISA Ctx kit (Nordic Bioscience Diagnostics A/S, Denmark). Briefly, this kit provides antibodies that detect a terminal peptide that is a product of collagen degradation that circulates in the blood. Samples are pipetted into wells that are pre-incubated with the biotinylated peptide, and probed with a primary antibody and then a secondary antibody that is conjugated to a peroxidase. A chromogenic substrate is added, and the absorbance value is measured in a spectrophotometer, and the values a compared to a standard curve of known dilutions.

II.11 Tibia collection and μCT

Mice were harvested at 5, 14 and 42 days old, and euthanised by cervical dislocation by myself or the animal health technicians. The fur of the mouse was washed with 70% ethanol, and removed to expose the tibia and femur. The tibia was cut on the distal end at the ankle, and the femur was cut distally, above the knee joint. The tibia was then placed in 4% paraformaldehyde in PBS, then incubated overnight. The samples were

then washed in PBS, and placed in 70% ethanol. The samples were taken to the McGill Bone and Periodontal Research centre for microcomputed-tomography analysis of the trabecular bone of the tibia. The instrument used was the SkyScan 1072, with a detector at 1024 x 1024 pixels, 12 bit cooled CCD –camera. This allows for high definition images of the trabuclar and cortical bone, and the bone volume / tissue volume is measured, as well as the trabecular thickness, separation, and number.

II.12 Dynamic Histomorphometry

Mice were bred as described. Seven days before sacrifice, mice were injected intraperitoneally with a solution of either calcein or demeclocycline. Mice at 14 days old received calcein (25μg/g), while mice at 42 days old received demeclocycline (30 μg/g). The calcein fluorescent dye was dissolved in 150mM NaCl, 237 mM $NaHCO_3$ in PBS (autoclaved) and filtered sterile. The demeclocycline dye was dissolved in 10% ethanol in immunoPBS. Mice were injected at 7 days and at 2 days before sacrifice. The tibia was collected and fixed as described, and dehydrated in graded ethanol baths (50%, 70%, 90%, and 100% for 1 hours, 2x each). Sampled were then washed in xylene, incubated overnight in purified methyl methacrylate (MMA) at 4^{o}C, then in 1% MMA (13% v/v di-butyl-phtalate, 1% w/v benzoyl peroxide) for two overnights at 4 ^{o}C, then in 4.5 % MMA (17% v/v di-butyl-phtalate, 4.5% w/v benzoyl peroxide) overnight at 4^{o}C. Samples were then placed in new vials, and incubated for about two weeks in the dark and at room temperature in 4.5% MMA to allow the samples to harden. The samples were oriented in such a way as to allow them to be reproducibly cut along the longitudinal axis.

The hardened samples were then cut using a microtome (Leica RM 2255), and the sections were placed in 70% ethanol on silane plus slides (Scientific Device Laboratories), and incubated overnight at 55^{o} C. Samples were then mounted with MicroKit, and photographed under fluorescent light.

Photographs were then analysed using BioQuant Osteo II imaging software that inputs the bone and tissue volumes, as well as the spacing between the fluorescent lines

that represent the bone that has been formed during the 5 day interval of injections. The software then calculates the mineral apposition rate and the bone formation rates.

II.13 Primary Culture of Osteoblasts

Mice were bred and genotyped as described, and were collected at 5 to 14 days old. They were euthanised by cervical dislocation, and the head was sprayed with 70% ethanol. The heads were then taken into the laminar flow tissue culture hood, and the butterfly shaped calvaria was dissected and placed into cold PBS with 4x antibiotic/antimicotic. The periosteum and endosteum of the calvaria was then removed microscopically, and they were washed again in cold PBS with 4x antibiotic/antimicotic. The calvaria was then minced, and placed into a digestive medium (1mg/ml Collagenase D, 2x antibiotic/antimicotic, αMEM) at 37^{o}C with shaking for 10 minutes, three times. At the last incubation, the bones were placed in αMEM with 20% FBS and 1x antibiotic/antimicotic, and allowed to grow in a culture dish for several days. The media was changed and cells were grown in αMEM with 20% FBS, 10 nM dexamethansone, 50 μg/ml ascorbic acid, 5 mM β-glycerol-phosphate, and 1x antibiotic/antimicotic.

About one week after plating, cells were split with 0.05% Trypsin-EDTA (Gibco), counted and replated at about 20,000 cells in a 24 well plate. RNA was then collected as described.

For mineralization of primary osteoblasts, cells were split about one week after the initial plating with 0.05% Trypsin-EDTA (Gibco), counted, and replated at about 90,000 cells per well in a 24 well plate, and cultured for 21 days. Mineral deposits were then quantified by Von Kossa staining as described.

II.14 Statistical analysis

All statistical analyses were done using the GraphPad Prism software. When two groups were being compared, a t-test was used, and when more than 2 groups were analysed, a one way anova with a Bonferroni post-test were used to analyze establish

significance. In cases of two variables such as genotype and time (as in experiments with primary cells and gene expression), two-way anova was used to establish significance between the genotypes over time. A $p<0.05$ was accepted as significant.

III Results

III.1 ILK-deficient MC3T3-E1 pre-osteoblasts have increased activity

The primary objective of this study was to determine the role of ILK in osteoblasts. This can be done most efficiently using cell lines. In particular, we used the pre-osteoblastic cell line MC3T3-E1. These cells are derived from mouse calvaria that have been established into a permanent cell line that adequately mimics osteoblastogenesis *in vitro* (Sudo *et al.* 1983). In conjunction to this, we used RNA interference to inhibit *Ilk* mRNA and protein expression in these cells. Briefly, RNA interference relies on a mammalian cell's defensive system that suppresses or degrades foreign nucleic acids in a highly specific manner. As such, one can transfect cells with double stranded RNA and produce a silencing effect on RNA sequences that match (Hannon *et al.* 2004).

To begin understanding the role of ILK in osteoblast, we used the pre-osteoblast cell line MC3T3-E1, and knocked down *Ilk* messenger RNA transcripts using RNA interference. To determine the best double-stranded RNA molecule, we tested several siRNAs in transient transfections. This consists of transfecting double-stranded RNA into these cells and assessing RNA transcript levels using Real-Time qPCR after 48 hours in culture. Two molecules displayed significant reduction in *Ilk* transcript levels. The ILK 782 significantly decreased ILK mRNA levels while ILK 285 had a dose dependent profile of ILK inhibition (Figure 7 A,B respectively). The selectivity of the RNA molecule was tested by transfection of a Negative Control (NC) sequence that consisted of a scrambled GAPDH sequence with no homology to known mouse genes. Transient transfection with this RNA molecule showed no significant reduction in ILK RNA transcript levels as assessed by RT-qPCR (Figure 7, C).

The ILK specific siRNA sequences were then synthesized as a complementary sequence with a loop, annealed, and cloned into a suitable expression vector to obtain stable pools of ILK-deficient cells. These cells have the advantage of allowing *Ilk* to

remain inhibited over a longer period of time, namely the three weeks that are necessary for these osteoblasts to secrete mineralizing matrix. To confirm the inhibition of ILK, we cultured cells for a week after confluency, and extracted total protein for western blotting using an anti-ILK antibody (Figure 8A). To determine the relative ratio of the protein quantification, we normalized the loading to the c-jun protein that remains unaltered by the treatment (Figure 8A). Accordingly, ILK expression levels are reduced up to 50% in the ILK 782 pool and 90% in the ILK 285 pool, as compared to the NC control protein levels (Figure 8B).

To determine the transcript levels of osteoblast specific markers, we used RT-qPCR and harvested RNA from cells at one week and two weeks post confluence. Collagen type I is expressed early in osteoblast differentiation, with bone sialoprotein expressed later. Both are extracellular proteins important in normal osteoid mineralisation. In ILK-deficient osteoblasts, these genes are upregulated when mRNA is harvested at 14 days post confluence (Figure 9 A, B). Osteocalcin is another matrix protein that is expressed in differentiated osteoblasts, and we see here that at 7 days post confluence, expression of this marker gene is higher in ILK-deficient osteoblasts (Figure 9C). These data suggest that there is an increase in bone matrix proteins secretions in ILK-deficient osteoblasts.

To confirm these findings, cells were cultured for three weeks, which is the times that is required for the osteoblasts to differentiate and produce matrix that will mineralize (Figure 10A). Both ILK-deficient cell lines produced significantly more mineralized matrix than the control, as quantified by Von Kossa staining (Figure 10B). Taken together, these data suggest that ILK has an inhibitory effect on matrix production and mineralization in osteoblasts.

To identify downstream components of integrin signaling that could influence matrix synthesis in the osteoblast, we first concentrated on effector molecules that have been shown to be regulated by ILK kinase activity. GSK3β phosphorylation on Serine 9 was assessed by western blotting, and values were normalized to the total GSK3β (Figure

11A). This residue appears to be hypophosphorylated in ILK deficient cells by 90% in ILK 782 clones and 50% in ILK 285 clones (Figure 11B). These data suggest that ILK deficiency causes GSK3β hypophosphorylation and hyperactivity, and can thus modulate downstream molecules and ultimately impact AP-1 transcriptional activity.

Integrin signaling through ILK has a direct ability to phosphorylate the αNAC coactivator on Ser43. This stabilisation step shuttles αNAC to the nucleus where is can bind the osteocalcin promoter at an AP-1 transcription site and positively regulate this specific osteoblastic gene. Immunofluorescence shows that αNAC was predominantly cytosolic in ILK deficient clones (Figure 12 A,B). Quantification of these data showed that ILK-knockdown cells have up to 50% less αNAC in the nucleus compared to the controls (Figure 12C).

These data suggest that ILK is a modulator of downstream molecules that are involved in osteoblast function. In particular, it affects on αNAC subcellular localization ultimately resulting in increased bone mineral formation.

III.2 Analysis of the mouse model

Our *in vitro* data suggested that ILK regulates αNAC sub-cellular localization to inhibit osteoblast function. To test the physiological relevance of these observations we studied osteoblast specific ILK-deficient mice obtained by crossing the previously engineered floxed *Ilk* mice (this is an *Ilk* allele flanked by loxP sites) (Figure 13) with transgenic mice expressing the Cre recombinase under the control of the osteoblast specific collagen 2.3 kb *Col1a1* promoter fragment. Thus mutant mice have one allele completely knocked-out (ILK-KO), while the other allele is floxed ($ILK^{-/fl}$) and will be excised by Cre.

In this manner, a conditional *Ilk* knock-out can be generated specifically in osteoblasts. To genotype these mice and distinguish the wild-types from the mutants, we used PCR to test for the presence of Cre recombinase (Figure 14A). The selected primers (described in Materials and Methods) yielded a 200bp amplimer. The second allele that

was knocked-out was also tested by PCR, and the primers give a 1kb diagnostic amplimer (Figure 14B). Mice with the *Ilk*-KO and the Cre genotype are the mutants that are ILK-deficient in their osteoblasts (genotype: Col1-Cre; $ILK^{-/fl}$).

To estimate the efficiency of the osteoblast specific Cre mediated excision, DNA was harvested from the osteoblast-rich calvaria. PCR using specific primers for the deletion region show that Cre does efficiently excise the kinase domain of ILK because the ILK band is reduced to 250 bp, compared to the non-excised floxed band of 2.1 kb (Figure 14C). This provides proof of concept that the Cre is efficient at excising ILK.

To gain further insight into the efficiency of the collagen I promoter that is driving the Cre expression, and to confirm the genotyping, we used RT-qPCR from RNA harvested directly from the calvaria and analysed Cre transcript levels. As expected, mice that do not show the Cre expression band by PCR have no Cre expression, as opposed to the mutant population that has elevated Cre expression levels (Figure 15).

In a final attempt to confirm that the *Ilk* gene is knocked-out, *Ilk* transcript levels were analysed from calvarial RNA harvested in mice at 5, 14 and 42 days old. In all cases, expression was reduced in the -/fl cre mutant population compared to the +/fl cre wild-type controls. Mice at 5 days post natal show a significant reduction of 70% in *Ilk* transcript levels (Figure 16A), while older mice harvested at 2 weeks and 6 weeks show reduction of 50% and 30%, respectively, compared to the wild type (Figure 16 B,C). Although *Ilk* ablation did not reach 100%, transcript levels were still reduced in the mutant population.

III.3 Characterisation of the mouse model

The preferred model based on the *in vitro* data accumulated to date is that there may be increased bone deposition in the mutant mice. Therefore, a series of experiments were designed to investigate bone formation and bone remodeling in these mice.

Blood biochemistry

Blood biochemistry can provide information regarding the bone formation and turnover using marker proteins and ions that reflect bone homeostasis. We harvested blood from 42 day old mice, and subjected it to alkaline phosphatase (ALP), calcium, and phosphorus testing. ALP is a marker of osteoblast activity and bone formation, and calcium and phosphorus are the primary mineral ions in bone, and serum levels provide information regarding mineral homeostasis. Levels of all of these markers remain unchanged in mutant mice compared to control (Figure 17). In addition to these analyses, collagen degradation products were analysed to assess the resoptive activity of osteoclasts, and no difference was observed between the mutant and wild type population (Figure 18). These data suggest that the bone homeostasis of the mutant mice is not significantly altered, and that bone turnover was not affected in the mutant mice.

Static histomorphometry

Tibia were harvested at 5, 14, and 42 days old, and were subjected to μCT computer analyses to obtain static histomorphometry parameters (Figures 19 A,B; 20 A,B; 21 A,B respectively). In all cases, the trabecular BV/TV remained unchanged between the mutant and control populations, although we observed a trend in the younger mutant mice towards increased bone volume (Figure 19C, 20C, 21C). This may be related to the previous finding that ILK transcript levels are more reduced in younger mice as opposed to the 42 day old mice.

Dynamic Histomophometry

Dynamic histomorphometric parameters provide information regarding the amount of bone that is being formed by the osteoblast over a given period of time. Mutant and control mice were injected with either calcein or demeclocycline at 14 days or at 42 days. These dyes bind to mineralizing calcium, thereby staining the bone at the bone

forming front. When the dye is given twice within a 5-day period, the bone formation rate and the mineral apposition rate can be calculated. Tibia samples were collected from the injected mice, and there was no significant difference found in either the bone forming rate or the mineral apposition rate at 2 weeks (Figure 22) or at 6 weeks (Figure 23). These data support the biochemical analysis that did not reveal any perturbation of bone turnover as assessed by alkaline phosphatase and collagen fragments in serum.

Osteoblast expression markers in the calvaria.

Calvaria are a rich source of osteoblasts, and we wanted to investigate the gene expression patterns of the osteoblasts *in vivo*. As such, we harvested RNA from calvaria of mice at 5, 14, and 42 days post natal, and assayed for the differentiation marker Runx2, and the ECM proteins collagen type I and bone sialoprotein. Expression of Runx2, involved in promoting osteoblast differentiation, remained unchanged at all time points, suggesting that the osteoblasts of these mice have a normal differentiation pattern (Figures 24A, 25A, 26A). Similarly, secretion of matrix proteins collagen type I and bone sialoprotein was normal compared to control, suggesting that these osteoblast function normally *in vivo* (Figures 24B,C, 25B,C, 26B,C). Thus *in vivo*, *Ilk* ablation did not affect osteoblast differentiation or function.

III.4 Primary osteoblasts cultures

The results obtained from osteoblast-specific ILK mutant mice appear to conflict with the *in vitro* analysis of ILK-knockdown osteoblasts. One element that could explain this discrepancy involves the different environment of the osteoblasts *in vivo* or in culture. To test this interpretation, we studied the osteoblasts from mutant mice in primary tissue culture.

Primary cells were harvested from the calvaria of mice at about one to two weeks of age, and cells were grown for up to three weeks post confluency. RNA was harvested

at confluence, and at one, two and three weeks post confluence for RT-qPCR analysis. Transcript levels of ILK were analysed as a function of time, and levels in the mutants were significantly reduced compared to the control (Figure 27). As observed previously, ILK levels were not completely ablated, simply reduced compared to the wild-type. Nevertheless, osteoblasts markers for matrix proteins are significantly elevated (Collagen I, Figure 28C) or show a trend towards increased synthesis (Bone sialoprotein) (Figure 28B). Furthermore, the transcription marker Runx2, which is crucial for the differentiation of osteoblasts, was significantly elevated in mutant cells in primary culture (Figure 28A).

Primary cells were cultured for immunofluorescence, and staining for αNAC showed that the protein was predominantly present in the cytosol of mutant cells as opposed to control cells (Figure 29 A,B). Therefore, αNAC cannot exert is transcriptional regulation of osteoblast genes in the mutant cells. The changes in the subcellular localization of αNAC were statistically significant (Figure 29C).

We cultured the *ex vivo* cells for three weeks post-confluence, thus allowing the cells to produce mineralized matrix. This was then assayed by Von Kossa staining for mineral (Figure 30A), and quantified (Figure 30B). Corroborating our observations with ILK-knockdown cells (Figure 10), the ILK-deficient cells produced almost double the amount of mineral matrix than the control (Figure 30B).

These *ex vivo* results corroborate the findings from the MC3T3-E1 cell line in which ILK was inhibited by RNAi, and suggests that when the osteoblasts are taken out of their native environment, they loose an important compensatory mechanism that is able to counteract the deficiency of ILK *in vivo*.

To address this mechanism, we must remember that ILK is a membrane protein whose role is to transmit information from the integrin receptor, itself bound to the extracellular matrix. Therefore, we hypothesized that the extracellular matrix of bone may have an important role in attenuating the phenotype that was observed *in vivo*. Furthermore, the most abundant matrix protein in bone is collagen type I, which is also capable of binding the integrin receptor. Hence, it is possible for collagen fibers in the

bone, bound to the integrin receptor, to somehow compensate for ILK signaling. To test this hypothesis, we utilized the ILK-knockdown MC3T3-E1 cells.

III.5 Collagen matrix can compensate for ILK-deficiency

To test the role of collagen type I, we cultured ILK-deficient MC3T3-E1 cells on collagen matrix or on regular plastic culture dishes, and allowed them to produce matrix for three weeks before analyzing the amount of mineral by Von Kossa staining (Figure 31). In control cells, mineralization was reduced by 25%, whereas in the ILK deficient cells, mineralization was reduced by 40% in the ILK 285 clones and 35% in the ILK 782 clones. Thus ILK deficient cells plated on collagen mineralize less than those plated on plastic. This suggests that the bone matrix that is native to the osteoblasts can compensate for the ILK-deficiency by activating parallel pathways in the osteoblast.

To identify what molecules may be involved in this compensatory pathway, we studied the role of focal adhesion kinase in osteoblasts. FAK is a membrane protein that is important in recruiting intracellular proteins to the membrane to form focal adhesions that bind the cells to the extracellular matrix (Cox *et al.* 2006). We hypothesized that on collagen matrix, FAK would be activated and compensate for the ILK deficiency.

We cultured cells for two hours on plastic or on collagen matrix and then harvested total protein and assessed the activity of FAK by investigating a phosphorylation site at Serine 722. This residue is a substrate for GSK3β activity, and phosphorylation at this site has been shown to have an inhibitory effect on FAK activity (Bianchi *et al.* 2005). In control cells plated on plastic, FAK is hyperphosphorylated on Ser722, therefore it is less active. In contrast, when cells are plated on collagen, FAK is hypophosphorylated, hence more active (Figure 32). In ILK-deficient osteoblast, this trend is reversed, with hyperactive FAK in collagen plated cells, and hypoactive FAK was detected in plastic plated cells. We propose that changes in FAK signaling upon adhesion to ECM components compensate for ILK deficiency in osteoblasts *in vivo*.

IV. Discussion

IV.1 Summary of Results

To investigate the role of ILK in osteoblasts, we first generated ILK knockdown cells using specific siRNA sequences transfected in MC3T3-E1 cells. These cells accurately mimic differentiation and proliferation of cells of the osteoblast lineage. When ILK-deficient MC3T3-E1 cells were cultured for three weeks, they showed increased expression of type I collagen, bone sialoprotein, and osteocalcin accompanied by augmented mineralization. Furthermore, these cells have increased GSK3β activity and retain αNAC in their cytoplasm.

These results contrast with the phenotype of osteoblast-specific ILK-deficient mice. We studied static and dynamic histomorphometric parameters in 5, 14, and 42 days old mice, and observed only subtle changes in these parameters. Osteoblast differentiation markers such as Runx2, bone sialoprotein and type I collagen were unaffected by ILK deficiency. Blood biochemical markers such as alkaline phosphatase, calcium and phosphate also remained unaffected. Osteoclast activity as measured by collagen fragments was not different in mutant mice.

To address these discrepancies, we cultured primary osteoblasts in conditions that were similar to those of the MC3T3-E1 cells. This lead to increased mineralization and retention of cytoplasmic αNAC. Gene expression markers such as Runx2, bone sialoprotein and type I collagen were increased over time in the primary osteoblasts. This phenotype strongly correlates with what was observed in the knockdown cells. Thus, the surprising *in vivo* results lead us to suggest the possibility of a compensatory pathway that is unique to the bone microenvironment. To test this hypothesis, we cultured the ILK-deficient MC3T3-E1 cells on collagen and observed a reduction in their exuberant mineralization. FAK activity was also investigated using the phosphoacceptor site serine 722. It appears as though this important effector kinase is hypoactive in ILK-deficient

osteoblasts compared to control cells on collagen matrix. This suggests a possible mechanism in compensating for osteoblast activity in ILK-deficient cells.

IV.2 Molecular mechanisms

ILK-deficient osteoblasts

The phenotype that we observe *in vitro* is strongly supported by two independent methodologies. The use of both RNA knock-down technology and a conditional knock-out mouse have generated a phenotype that supports the hypothesis that differentiation in ILK-deficient osteoblasts is increased and accompanied by increased mineralization *in vitro*. Any concerns about putative non-specific effects of the siRNA molecules are addressed by the use of two independent RNA sequences that both show similar phenotypes compared to a control sequence that has already been well characterized (Yu 2007). The observed *in vitro* phenotype would support further studies aimed at addressing the role in ILK signaling in other aspects of cell biology. ILK signaling has been known to have important effects on cell survival, growth and adhesion. Investigations into the kinetics of ILK-deficient osteoblasts using growth curves over 5 days has shown that these cells grow faster than control (data not shown). Studies on cell survival looking at caspase activity or mitochondrial functional could also provide interesting insight into parallel mechanisms that are influenced by ILK. A well characterized protein is Akt/PKB that is phosphorylated by ILK and has been known to be very important for cell survival (Hannigan *et al.* 2005). Another fruitful avenue of investigation is the strong possibility that cell adhesion is affected by ILK inactivation. Studies of ILK-deficient osteoclasts have shown that these have adhesion defects linked to integrin signaling (Dossa 2006).

Pathways Investigated

The results suggest a role for ILK in modulating bone formation through ECM-integrin dependent mechanisms. Cells cultured on plastic showed exuberant

mineralisation, and this could be a result of the ILK-GSK3β -AP1 axis, which can regulate osteoblastogenesis. The documented decrease in ILK expression leads to a decrease in nuclear shuttling of αNAC, probably through the established regulatory site at Serine 43. This could reduce the activity of the AP-1 transcription factor through reduced coactivation of c-jun (Akhouayri *et al.* 2005). Furthermore, GSK3β can also inhibit c-jun by phosphorylating its DNA binding domain (de Groot *et al.* 1993). Thus, cytoplasmic αNAC and increased GSK3β activity observed in ILK-deficient osteoblasts would suggest reduced c-jun mediated AP-1 transcription factor activation. This mechanism would suggest that bone formation and osteocalcin expression could be decreased, however, we observe the opposite effect in our model. Thus, other pathways must act to support the increased bone formation that we observe in the ILK-deficient mutants. Similarly, the increase in osteocalcin expression observed in the knockdown cells would suggest that other signaling pathways are activating the gene. Several other transcription factors are known to interact with the osteocalcin promoter, including Runx2, Atf4 and Dlx5 (Lian *et al.* 1998) (Yang *et al.* 2004). These transcription factors are themselves regulated by other pathways in osteoblasts, and it is likely that they may be differentially regulated in ILK-deficient osteoblasts. Future work would be needed to investigate AP-1 mediated transcription by using cells co-transfected with a luciferase reporter driven by the AP-1 promoter. Also, analyses on the pathways that are able to regulate the osteocalcin promoter using chromatin immunoprecipitation for potential modulators of this promoter in ILK-deficient cells is likely to provide insight into compensatory mechanism that lead to increased osteocalcin expression and bone formation.

αNAC can regulate integrin expression

The role of the αNAC coactivator in regulating bone formation is strengthened by recent preliminary data using RNA interference to reduce αNAC levels in MC3T3-E1 osteoblasts (Akhouayri O, St-Arnaud R, unpublished). These cells show exuberant mineralization when plated on plastic. This mirrors the behavior of the ILK-deficient osteoblasts, and suggests that increased bone formation in both these osteoblast mutant

cell lines is likely mediated by a genetic mechanism that would be normally dependent on nuclear αNAC strictly controlling bone formation. In an effort to investigate mechanisms by which αNAC can regulate gene transcription, chromatin immunoprecipitation has been done by other members of the laboratory. Briefly, this technique involves isolating chromatin and then using specific antibodies targeted at αNAC. The αNAC-chromatin complex is then precipitated, and placed on a DNA array to identify promoter sites on which αNAC is bound in order to regulate gene transcription. We found that αNAC can bind and regulate transcription of the α11 and β6 integrin subunits in MC3T3-E1 osteoblasts. Given the similarities in mineralization and αNAC subcellular localization between the ILK knockdown cells and the ILK-deficient primary osteoblasts, it is likely that similar mechanisms are acting in both these cells types. This could suggest a mechanism to explain the phenotype seen in the knock-out mice, whereby the integrins become differentially regulated by αNAC, and are no longer strongly dependent on ILK signaling *in vivo*. The integrin-ECM interaction also seems to play an increasingly important role as the osteoblasts mature, possibly due to the important interactions that the entombed osteocytes maintains with the ECM: as the osteoblasts differentiate into osteocytes, they grow cytoplasmic extensions that are thought to be important in sensing mechanical load. The observation that integrins control αNAC through ILK activity and that αNAC can also regulate integrins in late stage osteoblasts, suggest that their may be a delayed feedback mechanism operating to sensitize the osteocytes into mechanosensing cells, possibly by changing the integrin receptor proportions. As such, biomechanical stress may provide interesting insight in ILK-deficient cells, as well as αNAC deficient osteoblasts *in vivo*.

Ultrasound induces osteoblastogenesis

Recently is has been suggested that integrin signaling through ILK mediates ultrasound induced fracture healing by activating osteoblasts and chondrocytes (Hsu *et al.* 2007; Tang *et al.* 2007). In chondrocytes ultrasound increases cyclooxygenase-2 expression in an ILK/AKT/NF-kappB dependent pathway, while in osteoblasts ultrasound

induces inducible nitric oxide synthase (iNOS) in an ILK/Akt/mTOR/HIF1α dependent pathway. Thus integrin signaling mediates acoustic pressure changes to the cell and coordinates gene expression in two important mesenchymal bone cell types, albeit by different signaling pathways. The observation that the integrin-ILK/FAK-effector pathways only seem to be involved when bone is stressed suggests that downstream signaling events mediated by the integrin would be qualitative in nature: as long as the system is in homeostasis, a qualitative change in osteoblastogenesis may be unlikely to occur. In experiments with the osteoblast specific ILK-deficient model mouse, we could induce fractures and observe fracture healing properties with the induction of ultrasound. We would expect that ILK-deficient osteoblast mice would have reduced fracture healing compared to the control population. Integrin mediated ultrasound response in osteoblasts has also been suggested to be dependent on FAK, reinforcing the hypothesis that integrin signaling can mediates these qualitative changes in cell systems (Tang *et al.* 2006).

IV.3 Alternate pathways in vivo

The discrepancy observed between the *in vitro* and *in vivo* phenotypes suggest that other signaling pathways are activated in the osteoblasts to compensate for ILK inactivation *in vivo*. On plastic dishes FAK activity is increased in ILK-deficient osteoblasts. When these cells are plated on collagen they have significantly more hypoactive FAK than the wild-type cells. This is accompanied by a decrease in the mineralisation potentiation of ILK-deficient cells plated on collagen, and we hypothesize that FAK activity may contribute to the reduced mineralisation observed in the *in vitro* cultures, as well as contributing to the *in vivo* phenotype. Increased FAK activity is suggested to be accompanied by increased JNK activity, through the activation of FAK-Src-p130^{cas}-Crk (Giancotti *et al.* 1999). Activated JNK can in turn phosphorylate c-jun and promote heterodimerisation with c-fos, thereby promoting AP-1 transcription factor activity. This can be an important mechanism for osteoblast proliferation, which may be accompanied by FAK mediated stimulation of the Ras-MAP Kinase signaling cascade, which has also been shown to be an important activator of osteoblast differentiation

(Schindeler *et al.* 2006). This pathways is also involved in cell proliferation, and its dual role in osteoblasts are still being contrasted and confirmed. Our ECM data suggests that FAK may be differentially regulated *in vivo*, and this pathway may be a meaningful target to investigate as a possible compensatory mechanism.

Interestingly, the MC3T3-E1 ILK knockdown cells also show increased proliferation after 5 days in culture (data not shown), suggesting that this dual Ras-MAPK proliferation/differentiation pathway may be influenced by ILK. Pre-osteoblasts may proliferate more quickly, thereby reaching maximal confluence in culture before differentiating into mature osteoblasts and depositing bone. Although each cell may lay down the same amount of bone than controls, the ILK knockdown cells may be more numerous, and may reach confluence and differentiate earlier than the controls, thus having more time to deposit bone. *In vivo*, this mechanism could be controlled for: the pre-osteoblasts proliferate more quickly, but once the site is saturated and bone is layed down, regulatory mechanism act to prevent excess bone from being deposited. This mechanism would have no impact on bone formation rate or mineral apposition rate, and given that the current model mouse has a collagen I driven Cre, it may be acting too late to observe this *in vivo.* An earlier promoter driving Cre expression could be used to test this hypothesis, and osteoblast on the growth plate could be counted or labeled with BrdU labeling for proliferation.

Although we have restricted our analysis to the GSK3β-αNAC-cjun-AP1 pathway, the implication of other pathways such as the JNK-cfos-AP1 and FAK-Ras-MAPK pathway are both strong candidates to explain the observed increase in mineral deposition from ILK-deficient osteoblasts. Experiments investigating these signaling pathways could use the currently available AP-1 reporter assay that could be diagnostic of the AP-1 activity of the cell system. The implication that modulation of FAK activity could compensate for ILK signaling suggests that FAK can stimulate AP-1 mediated transcription independently of ILK activity and the molecular pathways involved could be investigated. Alternatively, western blots can be used to distinguish the activity of the Ras-MAPK effecter molecules such as MEK and ERK. Establishing the relative

importance of these pathways would be important to determine a primary downstream target to ILK that regulates bone formation and osteoblastogenesis.

IV.4 Phenotypic considerations

The increase in mineralisation that was observed in cultured osteoblasts was completely compensated *in vivo*. The decreased FAK activity observed in ILK-deficient osteoblasts compared to wild-type cells plated on collagen suggests a FAK dependent mechanism that could compensate intracellular signaling events. The normal bone observed in the osteoblast specific ILK-deficient mutant mice could also be a consequence of the relatively inefficient Cre mediated excision of the *Ilk* gene. mRNA levels of *Ilk* were only slightly reduced, up to 60% *ex vivo*. Thus, with about 40 % of ILK levels present in the cell, the penetrance of the Cre enzyme is quite poor. There are several reasons that this could occur: (1) the RNA samples are from a heterogenous cell population, formed mostly of osteoblasts, but also some fibroblasts that do not have *Ilk* excised and could be misleading. (2) Another reason is that the *Ilk* allele may be difficult for the Cre enzyme to excise. This is unlikely given that this allele has been excised in two other model mice, the chondrocyte and osteoclast specific knock-out mice (Terpstra *et al.* 2003; Dossa 2006). (3) Another possibility is that these cells have a fitness disadvantage compared to normal cells, and are therefore selected against. This is possible, but our knockdown cells suggest that ILK-deficient osteoblasts are able to survive normally and proliferate faster than normal (this may not be the case *in vivo*, however). (4) Finally the most likely reason is related to the promoter that drives Cre expression, which is the type I collagen fragment that is osteoblast specific. The activity of this artificial promoter most likely does not reflect the response of its natural counterpart. As such, the complete ablation of *Ilk* expression would likely require the use of a more robust promoter driving Cre. One such mouse that is currently available is the osterix promoter driven Cre, which will excise *Ilk* in early osteoblastogenesis. Another approach could be to excise *Ilk* in late osteoblastogenesis, when bone is layed down. This would provide insight into the role of ILK in osteocytes, and possibly in the

mechanosensing properties of this molecule. Such experiments could use the osteocalcin or DMP-1 promoter to drive Cre expression (Dacquin *et al.* 2002; Robling *et al.* 2006).

Phenotypes of related mouse models

Studies to investigate the role of integrin signaling in late osteoblastogenesis have been performed and suggest an important role for integrin biomechanical sensing in osteocytes. Using an osteocalcin driven dominant-negative form of the β1 integrin subunit that lacks the extracellular tail of the receptor, this construct effectively inhibits β1 in mature osteoblasts and osteocytes. To determine the mechanical impact of this mutation, adult mice underwent mechanical unloading, such that the hindlimbs of the mice are suspended by the tail for one week, thus preventing any load from being sensed by the osteocytes (Iwaniec *et al.* 2005). The transgenic mice are characterized as having lower cancellous bone mass at the femoral metaphysis and lumbar body, reduced curvature of the proximal tibia, and decreased bone strength and stiffness of the femoral diaphysis. This dramatic phenotype suggest an important role for integrin signaling downstream of the β1 subunit. The use of the β1-DN has the advantage of being able to completely ablate all integrin mediated signaling within the cell. Therefore, unlike mouse models that focus on FAK or ILK signaling pathways, compensation and differential pathways along the integrin pathways are eliminated in the β1-DN mouse model. Nevertheless, ILK is likely an important component of mechanical regulation in the cells, and experiments focusing on the mechanical loading may be envisaged if a robust ILK-deficient osteoblast specific mouse model can be generated.

The β1-DN mouse model does seem to behave quite differently from the ILK-deficient osteoblasts, if only that the cells display impaired adhesion to collagen matrix (Globus *et al.* 2005). In growing mice, there are also differences in bone mass and strength, which are reduced compared to wild-type. The behavior of this mouse model suggest that this phenotype may be particularly severe compared to what may be expected from ILK knockout osteoblasts. The MC3T3-E1 cells that we have generated showed nearly complete ablation of *Ilk* expression. Although we did not perform an adhesion

assay on plastic or collagen, ILK-deficient cells remained attached to the surface substratum when exposed to type I collagen and remained able to differentiate and produce mineral matrix. This suggests that osteoblast attachment requires this extracellular tail of the β1 subunit, and that downstream signaling partners act as secondary transduction pathways that can modulate the response in a more qualitative manner. Alternatively, the reduced mineral deposition observed in the ILK-deficient osteoblasts plated on collagen could be secondary to an adhesion defect, similar to the β1-DN mouse model, and an adhesion assay would be required to rule out this possibility.

The presented phenotype is supported by the FAK osteoblasts-specific knock-out mouse, where there was no morphological phenotype. This reflects our model, where there is no *in vivo* change in bone morphology, emphasizing the likelihood that integrin signaling is likely redundant in function and can be compensated for downstream of the β1 subunit. The FAK conditional knock-out mouse model needed to be stressed by using mono-cortical fractures to induce a qualitative change in fracture repair, and this was accompanied by a possible change in the osteoblast-produced osteoid. As such, experiments in the *Ilk* conditional knock-out could also investigate the effect of inducing fractures and observing parameters involved in fracture healing or using distraction osteogenesis to observe parameters involved in new bone formation at a fracture site.

The finding that the matrix produced can be modulated by integrin-mediated events in the FAK model mouse seems particularly surprising, and raises the question as to the quality of the matrix produced by ILK-deficient osteoblasts. We have used the Von Kossa method to study mineral formation based on the property of the silver nitrate to bind to calcium that is present in the bone, and stain black when exposed to light. A more detailed analysis of the phenotype could include examining the architecture of the mineral phase and extracellular matrix composition and organization in these mice at the ultrastructural level using transmission electron microscopy to determine crystal size and morphology, as well as organic-inorganic relationships (McKee *et al.* 1995). Additional methods of mineral analysis could include X-ray microanalysis for elemental composition and X-ray diffraction techniques for mineral characterization (Landis 1996).

Female sex steroid hormones have also been suggested to play a role in integrin signaling. In the β1 dominant negative adult mice, parietal bone mass was reduced in 35 day old mice, but by 90 days, the males had compensated for the differences, but the females retained their low bone mass, and had increased staining for osteoclast activity in the growth plate (Zimmerman *et al.* 2000). This implies a protective effect of testosterone in male mice, or a causative effect of estrogen/progesterone in female mice, and this can impact integrin signaling. As such, a study investigating the respective roles of these steroid hormones could be envisaged by ovariectomising females and castrating males of the osteoblast conditional knock-out mice at a young age, and observing the impact on bone formation and bone mass, as well as biomechanical properties of the bone.

IV.5 Conclusions

Our findings support a role for ILK in osteoblastogenesis. The surprising phenotype of the conditional knock-out mouse raises questions as to the natural environmental cues *in vivo* that require ILK signaling, given that integrins are required *in vivo* for normal osteogenesis. Tissue culture conditions allow us to dissect a possible role for ILK signaling, and even a moderate decrease as observed in the primary cells provide an observable increase in bone formation. Reconciling both these findings will require more work using a more robust mouse model, and possibly with different temporally regulated promoter sequences driving Cre expression.

Inhibiting the ILK-αNAC cascade seems to have evolved compensatory mechanisms over the course of evolution to prevent dramatic changes to cell and systemic physiology in the natural environment. When these cells are taken out of their normal milieu and placed in culture, these evolutionary compensation mechanisms appear to fail, leading to the phenotype that we observe. Targeting downstream of ILK, a site-specific mutation of αNAC is currently being engineered that would prevents its nuclear shuttling by ILK. This molecule is less likely to have compensatory mechanisms and the characterization of this model mouse will remain an interesting endeavor to explore.

References

Akhouayri, O., I. Quélo and R. St-Arnaud (2005). "Sequence-specific DNA binding by the alphaNAC coactivator is required for potentiation of c-Jun-dependent transcription of the osteocalcin gene." Mol Cell Biol **25**(9): 3452-60.

Akhouayri, O. and R. St -Arnaud (2007). "Differential mechanisms of transcriptional regulation of the mouse osteocalcin gene by Jun family members." Calcif Tissue Int **80**(2): 123-31.

Anderson, H. C. (1989). "Mechanism of mineral formation in bone." Lab Invest **60**(3): 320-30.

Bianchi, M., S. De Lucchini, O. Marin, D. L. Turner, S. K. Hanks and E. Villa-Moruzzi (2005). "Regulation of FAK Ser-722 phosphorylation and kinase activity by GSK3 and PP1 during cell spreading and migration." Biochem J **391**(Pt 2): 359-70.

Bilezikhan J.P., R. L. G., Rodan G.A. (2002). "Principles of Bone Biology." Academic Press **1**.

Brakebusch, C. and R. Fässler (2003). "The integrin-actin connection, an eternal love affair." EMBO J **22**(10): 2324-33.

Cox, B. D., M. Natarajan, M. R. Stettner and C. L. Gladson (2006). "New concepts regarding focal adhesion kinase promotion of cell migration and proliferation." J Cell Biochem **99**(1): 35-52.

Dacquin, R., M. Starbuck, T. Schinke and G. Karsenty (2002). "Mouse alpha1(I)-collagen promoter is the best known promoter to drive efficient Cre recombinase expression in osteoblast." Dev Dyn **224**(2): 245-51.

de Groot, R. P., J. Auwerx, M. Bourouis and P. Sassone-Corsi (1993). "Negative regulation of Jun/AP-1: conserved function of glycogen synthase kinase 3 and the Drosophila kinase shaggy." Oncogene **8**(4): 841-7.

Delcommenne, M., C. Tan, V. Gray, L. Rue, J. Woodgett and S. Dedhar (1998). "Phosphoinositide-3-OH kinase-dependent regulation of glycogen synthase kinase 3 and protein kinase B/AKT by the integrin-linked kinase." Proc Natl Acad Sci USA **95**(19): 11211-6.

Dossa, T. (2006). "Osteoclast-specific inactivation of the Integrin-Linked Kinase (ILK) inhibits bone resorption." McGill University, Department of Human Genetics.

Erlebacher, A., E. H. Filvaroff, S. E. Gitelman and R. Derynck (1995). "Toward a molecular understanding of skeletal development." Cell **80**(3): 371-8.

Favus, M. J. (2003). "Primer on the Metabolic Bone Diseases and Disorders of Mineral Metabolism." American Society for Bone and Mineral Research.

Franz-Odendaal, T. A., B. K. Hall and P. E. Witten (2006). "Buried alive: how osteoblasts become osteocytes." Dev Dyn **235**(1): 176-90.

Giancotti, F. G. and E. Ruoslahti (1999). "Integrin signaling." Science **285**(5430): 1028-32.

Globus, R. K., D. Amblard, Y. Nishimura, U. T. Iwaniec, J. B. Kim, E. A. Almeida, C. H. Damsky, T. J. Wronski and M. C. van der Meulen (2005). "Skeletal phenotype of

growing transgenic mice that express a function-perturbing form of beta1 integrin in osteoblasts." Calcif Tissue Int **76**(1): 39-49.

Globus, R. K., S. B. Doty, J. C. Lull, E. Holmuhamedov, M. J. Humphries and C. H. Damsky (1998). "Fibronectin is a survival factor for differentiated osteoblasts." J Cell Sci **111 (Pt 10)**: 1385-93.

Gohel, A. R., A. R. Hand and G. A. Gronowicz (1995). "Immunogold localization of beta 1-integrin in bone: effect of glucocorticoids and insulin-like growth factor I on integrins and osteocyte formation." J Histochem Cytochem **43**(11): 1085-96.

Hannigan, G., A. A. Troussard and S. Dedhar (2005). "Integrin-linked kinase: a cancer therapeutic target unique among its ILK." Nat Rev Cancer **5**(1): 51-63.

Hannigan, G. E., C. Leung-Hagesteijn, L. Fitz-Gibbon, M. G. Coppolino, G. Radeva, J. Filmus, J. C. Bell and S. Dedhar (1996). "Regulation of cell adhesion and anchorage-dependent growth by a new beta 1-integrin-linked protein kinase." Nature **379**(6560): 91-6.

Hannon, G. J. and J. J. Rossi (2004). "Unlocking the potential of the human genome with RNA interference." Nature **431**(7006): 371-8.

Hettmann, T., K. Barton and J. M. Leiden (2000). "Microphthalmia due to p53-mediated apoptosis of anterior lens epithelial cells in mice lacking the CREB-2 transcription factor." Dev Biol **222**(1): 110-23.

Hsu, H. C., Y. C. Fong, C. S. Chang, C. J. Hsu, S. F. Hsu, J. G. Lin, W. M. Fu, R. S. Yang and C. H. Tang (2007). "Ultrasound induces cyclooxygenase-2 expression through integrin, integrin-linked kinase, Akt, NF-kappaB and p300 pathway in human chondrocytes." Cell Signal **19**(11): 2317-28.

Hynes, R. O. (2002). "Integrins: bidirectional, allosteric signaling machines." Cell **110**(6): 673-87.

Iwaniec, U. T., T. J. Wronski, D. Amblard, Y. Nishimura and e. al. (2005). "Effects of disrupted β1-integrin function on the skeletal response to short-term hindlimb unloading …." Journal of Applied Physiology.

Jochum, W., J. P. David, C. Elliott, A. Wutz, H. Plenk, K. Matsuo and E. F. Wagner (2000). "Increased bone formation and osteosclerosis in mice overexpressing the transcription factor Fra-1." Nat Med **6**(9): 980-4.

Kim, J. B., P. Leucht, C. A. Luppen, Y. J. Park, H. E. Beggs, C. H. Damsky and J. A. Helms (2007). "Reconciling the roles of FAK in osteoblast differentiation, osteoclast remodeling, and bone regeneration." Bone **41**(1): 39-51.

Komori, T., H. Yagi, S. Nomura, A. Yamaguchi, K. Sasaki, K. Deguchi, Y. Shimizu, R. T. Bronson, Y. H. Gao, M. Inada, M. Sato, R. Okamoto, Y. Kitamura, S. Yoshiki and T. Kishimoto (1997). "Targeted disruption of Cbfa1 results in a complete lack of bone formation owing to maturational arrest of osteoblasts." Cell **89**(5): 755-64.

Kundu, A. K. and A. J. Putnam (2006). "Vitronectin and collagen I differentially regulate osteogenesis in mesenchymal stem cells." Biochem Biophys Res Commun **347**(1): 347-57.

Landis, W. J. (1996). "Mineral characterization in calcifying tissues: atomic, molecular and macromolecular perspectives." Connect Tissue Res **34**(4): 239-46.

Lee, N. K., H. Sowa, E. Hinoi, M. Ferron, J. D. Ahn, C. Confavreux, R. Dacquin, P. J. Mee, M. D. McKee, D. Y. Jung, Z. Zhang, J. K. Kim, F. Mauvais-Jarvis, P. Ducy

and G. Karsenty (2007). "Endocrine regulation of energy metabolism by the skeleton." Cell **130**(3): 456-69.

Lian, J. B., G. S. Stein, A. Javed, A. J. van Wijnen, J. L. Stein, M. Montecino, M. Q. Hassan, T. Gaur, C. J. Lengner and D. W. Young (2006). "Networks and hubs for the transcriptional control of osteoblastogenesis." Reviews in endocrine & metabolic disorders **7**(1-2): 1-16.

Lian, J. B., G. S. Stein, J. L. Stein and A. J. van Wijnen (1998). "Osteocalcin gene promoter: unlocking the secrets for regulation of osteoblast growth and differentiation." J Cell Biochem Suppl **30-31**: 62-72.

Masuoka, H. C. and T. M. Townes (2002). "Targeted disruption of the activating transcription factor 4 gene results in severe fetal anemia in mice." Blood **99**(3): 736-45.

McCabe, L. R., C. Banerjee, R. Kundu, R. J. Harrison, P. R. Dobner, J. L. Stein, J. B. Lian and G. S. Stein (1996). "Developmental expression and activities of specific fos and jun proteins are functionally related to osteoblast maturation: role of Fra-2 and Jun D during differentiation." Endocrinology **137**(10): 4398-408.

McKee, M. D. and A. Nanci (1995). "Postembedding colloidal-gold immunocytochemistry of noncollagenous extracellular matrix proteins in mineralized tissues." Microsc Res Tech **31**(1): 44-62.

Moursi, A. M., C. H. Damsky, J. Lull, D. Zimmerman, S. B. Doty, S. Aota and R. K. Globus (1996). "Fibronectin regulates calvarial osteoblast differentiation." J Cell Sci **109 (Pt 6)**: 1369-80.

Moursi, A. M., R. K. Globus and C. H. Damsky (1997). "Interactions between integrin receptors and fibronectin are required for calvarial osteoblast differentiation in vitro." J Cell Sci **110 (Pt 18)**: 2187-96.

Nakashima, K., X. Zhou, G. Kunkel, Z. Zhang, J. M. Deng, R. R. Behringer and B. de Crombrugghe (2002). "The novel zinc finger-containing transcription factor osterix is required for osteoblast differentiation and bone formation." Cell **108**(1): 17-29.

Otto, F., A. P. Thornell, T. Crompton, A. Denzel, K. C. Gilmour, I. R. Rosewell, G. W. Stamp, R. S. Beddington, S. Mundlos, B. R. Olsen, P. B. Selby and M. J. Owen (1997). "Cbfa1, a candidate gene for cleidocranial dysplasia syndrome, is essential for osteoblast differentiation and bone development." Cell **89**(5): 765-71.

Quélo, I., O. Akhouayri, J. Prud'homme and R. St -Arnaud (2004). "GSK3 beta-dependent phosphorylation of the alpha NAC coactivator regulates its nuclear translocation and proteasome-mediated degradation." Biochemistry **43**(10): 2906-14.

Quélo, I., C. Gauthier, G. E. Hannigan, S. Dedhar and R. St -Arnaud (2004). "Integrin-linked kinase regulates the nuclear entry of the c-Jun coactivator alpha-NAC and its coactivation potency." J Biol Chem **279**(42): 43893-9.

Robling, A. G., A. B. Castillo and C. H. Turner (2006). "Biomechanical and molecular regulation of bone remodeling." Annual review of biomedical engineering **8**: 455-98.

Rubin, J., C. Rubin and C. R. Jacobs (2006). "Molecular pathways mediating mechanical signaling in bone." Gene **367**: 1-16.

Sabatakos, G., N. A. Sims, J. Chen, K. Aoki, M. B. Kelz, M. Amling, Y. Bouali, K. Mukhopadhyay, K. Ford, E. J. Nestler and R. Baron (2000). "Overexpression of DeltaFosB transcription factor(s) increases bone formation and inhibits adipogenesis." Nat Med **6**(9): 985-90.

Schaller, M. D., C. A. Otey, J. D. Hildebrand and J. T. Parsons (1995). "Focal adhesion kinase and paxillin bind to peptides mimicking beta integrin cytoplasmic domains." J Cell Biol **130**(5): 1181-7.

Schindeler, A. and D. G. Little (2006). "Ras-MAPK signaling in osteogenic differentiation: friend or foe?" J Bone Miner Res **21**(9): 1331-8.

Schneider, G. B., R. Zaharias and C. Stanford (2001). "Osteoblast integrin adhesion and signaling regulate mineralization." J Dent Res **80**(6): 1540-4.

Shen, Y. and M. D. Schaller (1999). "Focal adhesion targeting: the critical determinant of FAK regulation and substrate phosphorylation." Mol Biol Cell **10**(8): 2507-18.

Sudo, H., H. A. Kodama, Y. Amagai, S. Yamamoto and S. Kasai (1983). "In vitro differentiation and calcification in a new clonal osteogenic cell line derived from newborn mouse calvaria." J Cell Biol **96**(1): 191-8.

Tahiliani, P. D., L. Singh, K. L. Auer and S. E. LaFlamme (1997). "The role of conserved amino acid motifs within the integrin beta3 cytoplasmic domain in triggering focal adhesion kinase phosphorylation." J Biol Chem **272**(12): 7892-8.

Takeuchi, Y., M. Suzawa, T. Kikuchi, E. Nishida, T. Fujita and T. Matsumoto (1997). "Differentiation and transforming growth factor-beta receptor down-regulation by collagen-alpha2beta1 integrin interaction is mediated by focal adhesion kinase and its downstream signals in murine osteoblastic cells." J Biol Chem **272**(46): 29309-16.

Tamura, Y., Y. Takeuchi, M. Suzawa, S. Fukumoto, M. Kato, K. Miyazono and T. Fujita (2001). "Focal adhesion kinase activity is required for bone morphogenetic protein--Smad1 signaling and osteoblastic differentiation in murine MC3T3-E1 cells." J Bone Miner Res **16**(10): 1772-9.

Tanaka, T., T. Tsujimura, K. Takeda, A. Sugihara, A. Maekawa, N. Terada, N. Yoshida and S. Akira (1998). "Targeted disruption of ATF4 discloses its essential role in the formation of eye lens fibres." Genes Cells **3**(12): 801-10.

Tang, C. H., D. Y. Lu, T. W. Tan, W. M. Fu and R. S. Yang (2007). "Ultrasound induces hypoxia-inducible factor-1 activation and inducible nitric-oxide synthase expression through the integrin/integrin-linked kinase/Akt/mammalian target of rapamycin pathway in osteoblasts." J Biol Chem **282**(35): 25406-15.

Tang, C. H., R. S. Yang, T. H. Huang, D. Y. Lu, W. J. Chuang, T. F. Huang and W. M. Fu (2006). "Ultrasound stimulates cyclooxygenase-2 expression and increases bone formation through integrin, focal adhesion kinase, phosphatidylinositol 3-kinase, and Akt pathway in osteoblasts." Mol Pharmacol **69**(6): 2047-57.

Terpstra, L., J. Prud'homme, A. Arabian, S. Takeda, G. Karsenty, S. Dedhar and R. St -Arnaud (2003). "Reduced chondrocyte proliferation and chondrodysplasia in mice lacking the integrin-linked kinase in chondrocytes." J Cell Biol **162**(1): 139-48.

Troussard, A. A., N. M. Mawji, C. Ong, A. Mui, R. St -Arnaud and S. Dedhar (2003). "Conditional knock-out of integrin-linked kinase demonstrates an essential role in protein kinase B/Akt activation." J Biol Chem **278**(25): 22374-8.

Troussard, A. A., C. Tan, T. N. Yoganathan and S. Dedhar (1999). "Cell-extracellular matrix interactions stimulate the AP-1 transcription factor in an integrin-linked kinase- and glycogen synthase kinase 3-dependent manner." Mol Cell Biol **19**(11): 7420-7.

Wagner, E. F. (2002). "Functions of AP1 (Fos/Jun) in bone development." Ann Rheum Dis **61 Suppl 2**: ii40-2.

Yang, X., K. Matsuda, P. Bialek, S. Jacquot, H. C. Masuoka, T. Schinke, L. Li, S. Brancorsini, P. Sassone-Corsi, T. M. Townes, A. Hanauer and G. Karsenty (2004). "ATF4 is a substrate of RSK2 and an essential regulator of osteoblast biology; implication for Coffin-Lowry Syndrome." Cell **117**(3): 387-98.

Yu, V. (2007). "Factor Inhibiting ATF4-mediated Transcription is a Novel Leucine Zipper Transcriptional Repressor that Regulates Bone Mass." McGill University, Department of Human Genetics.

Zimmerman, D., F. Jin, P. Leboy, S. Hardy and C. H. Damsky (2000). "Impaired bone formation in transgenic mice resulting from altered integrin function in osteoblasts." Dev Biol **220**(1): 2-15.

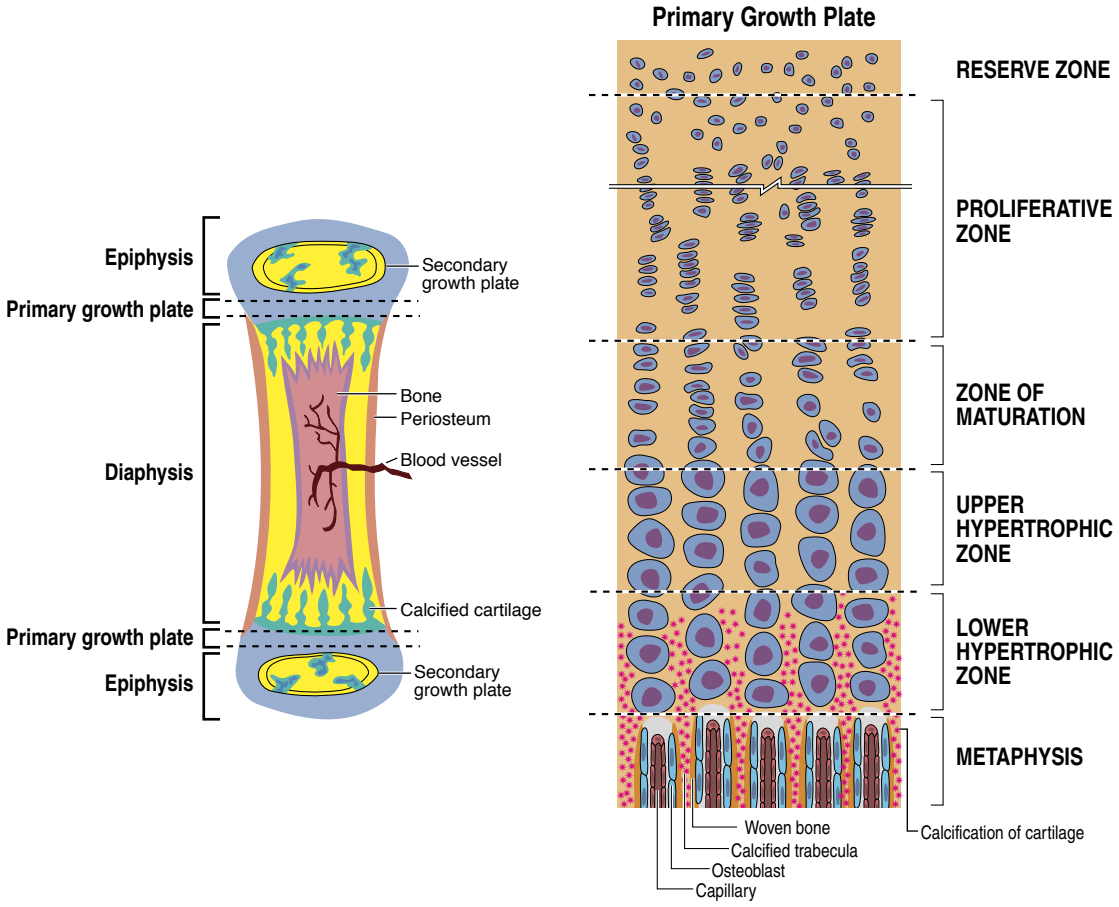


Figure 1: Schematic of bone development through endochondral ossification

Chondrocytes form isogenous groups in the proliferative zone of the growth plate. They will mature and differentiate into hypertrophic chondrocytes, thereby lengthening the epiphysis of the limb. Closer to the diaphysis, the cartilage surrounding the hypertrophic chondrocytes calcifies, and becomes resorbed. The invading blood vessels bring in precursor cells that will become osteoblasts and lay down new bone.

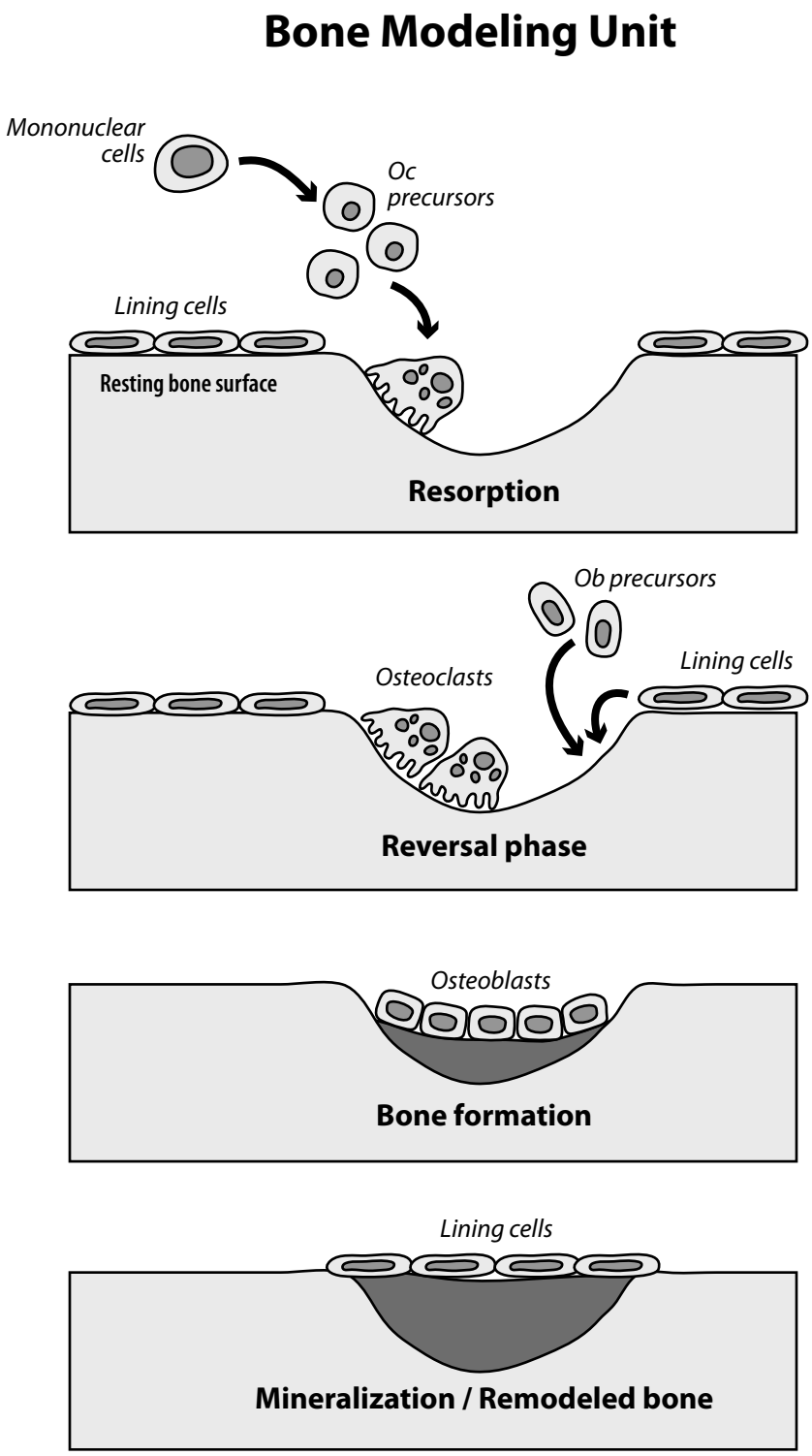


Figure 2: Schematic of a bone modeling unit (BMU)
Under the influence of local and systemic factors, mononuclear cells from the vasculature migrate to the bone and differentiate into osteoclasts. These cells will resorb the bone at a local site, and recruit pre-osteoblasts to differentiate into osteoblasts and form new bone.

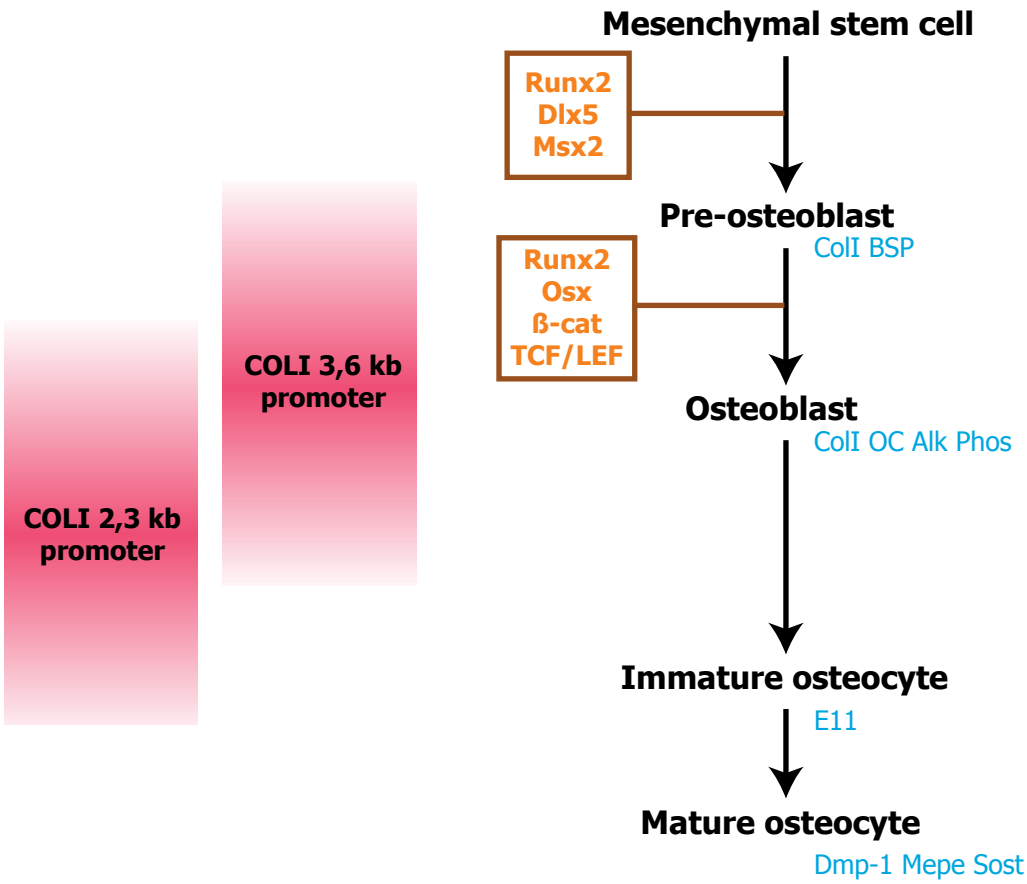


Figure 3: Representation of Osteoblast differentiation
Osteoblasts are of mesenchymal origin. Differentiation along the osteoblast lineage is dependent upon the timely expression and action of transcription factors (shown in orange box). These will in turn promote differentiation of the osteoblasts and activate transcription of proteins important for matrix secretions and mineralization (marked in blue). Two important collagen type I promoter fragments are known to be specific to the osteoblast lineage and expressed at a specific time in the differentiation sequence (marked in red). *Adapted from Robling A.G., 2006*

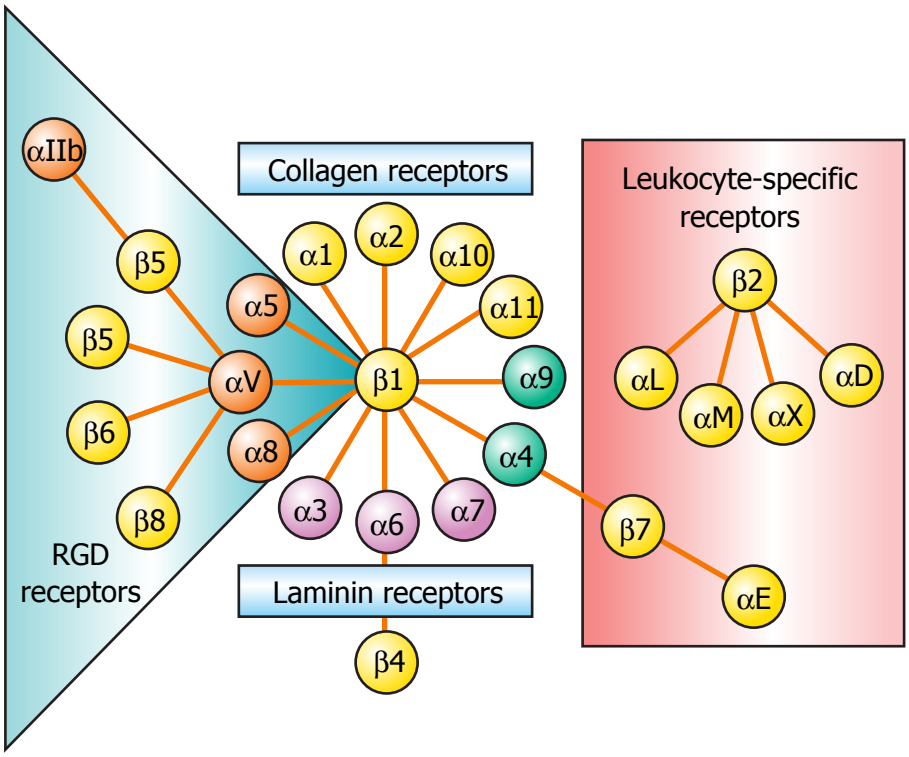


Figure 4: Integrin heterodimers and their respective matrix specificities
Integrins are formed by the dimerization of an α and a β subunit. These are differentially expressed in cell types, and in the osteoblasts the α1 subunit is particularly important. This subunit can heterodimerise with several β subunits thereby mediating adhesion to several extracellular matrices such as collagen and laminin. This is a schematic, and not a comprehensive review of all combinations. *Adapted from Hynes R.O., 2002*

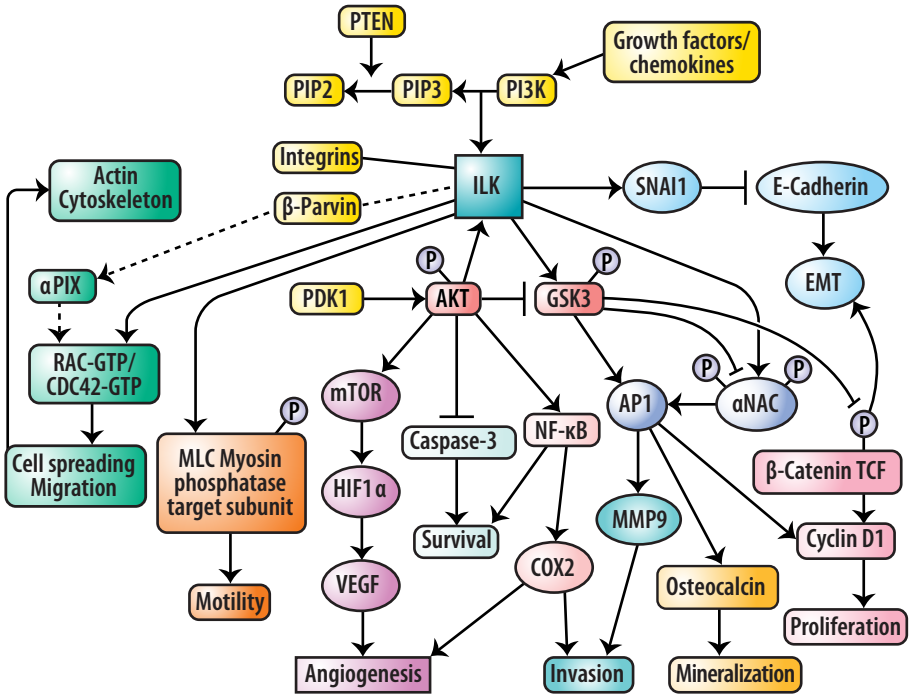


Figure 5: Signaling pathways downstream of the Integrin-Linked Kinase
ILK signaling affects several aspects of cell biology such as migration, motility, angiogenesis, invasion, proliferation and mineralization. These effects are mediated by adhesion to an ECM, and through integrin activation, ILK signals though several effector proteins to mediate effects on gene transcription in several cell types. Of particular interest is the activation of αNAC, which can mediate AP-1 transcription and effect mineralization through the activity of osteocalcin. *Adapted from Hannigan G., 2005*

Figure 6: Structure of the Integrin-Linked Kinase
ILK has three basic domans: a kinase domain, a pleckstrin homology domain, and four ankyrin repeats. The kinase domain allows for ILK to be an important signaling molecule in the cytosol, while the entire molecule has the ability to bind other proteins and act as an adapter to reorganize the cells cytoskeleton. *Adapted from Hannigan G., 2005*

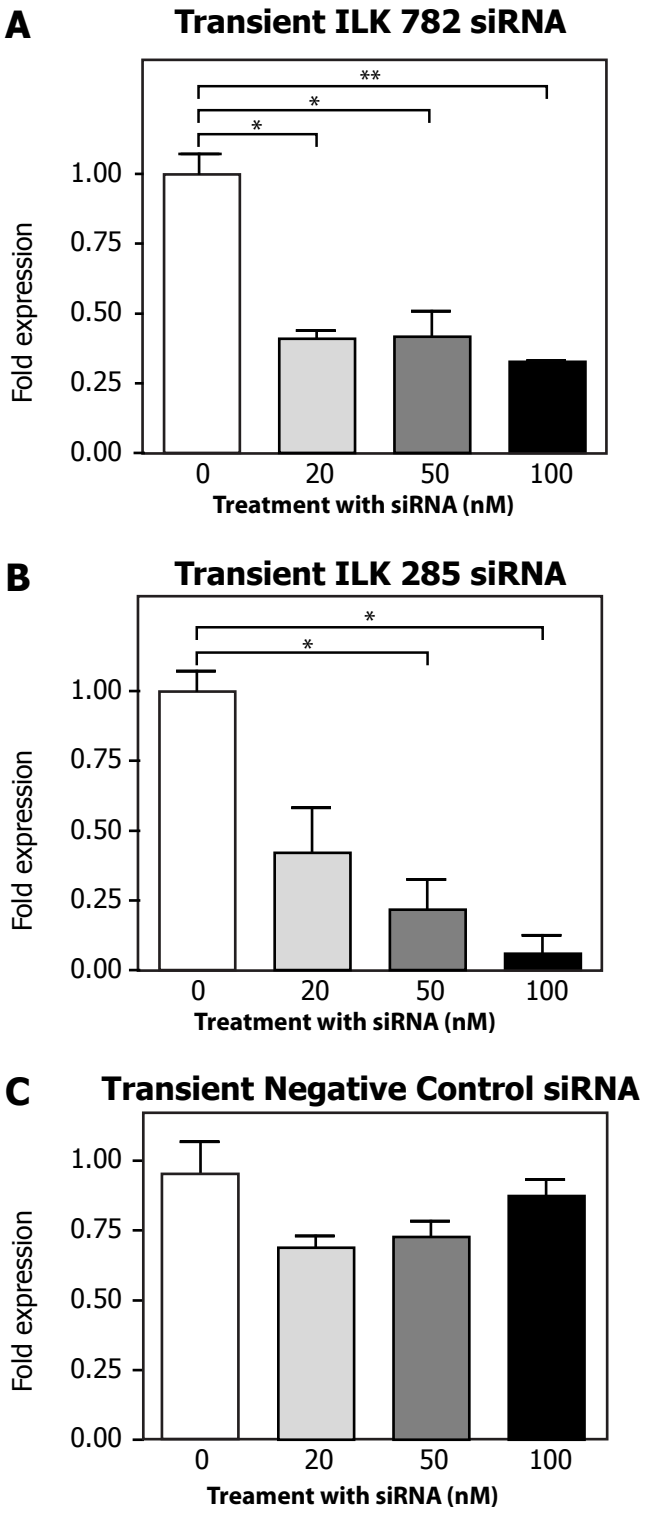


Figure 7: ILK is transiently inhibited by small interfering RNA molecules. MC3T3-E1 cells were transfected with small interfering RNA molecules targeted to a short fragment of the ILK RNA sequence. These sequences were complimentary to two different regions of ILK, and both are effective at degrading ILK RNA in a dose dependent manner (A, B). The control used is the negative control (NC) sequence, which consists of a scrambled GAPDH molecule (C). *, $p<0.05$; **, $p<0.01$

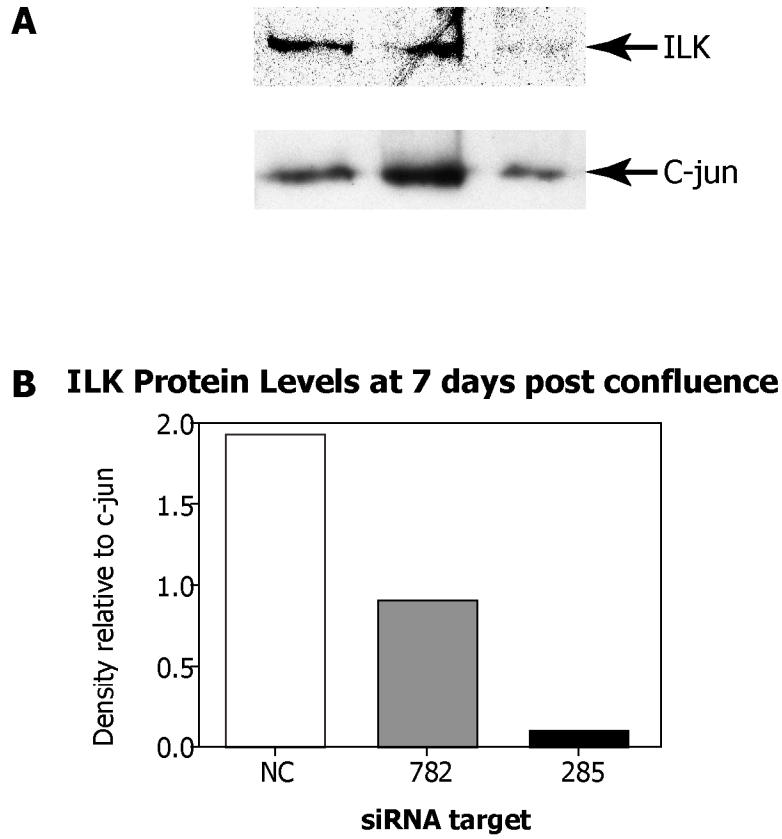


Figure 8: ILK is inhibited in the MC3T3-E1 osteoblastic cell line
Stably transfected pools were harvested for protein, and assessed for ILK protein expression by western blotting (**A**). Relative expression is shown as a density ratio and is normalized to the constitutively expressed c-jun protein (**B**).

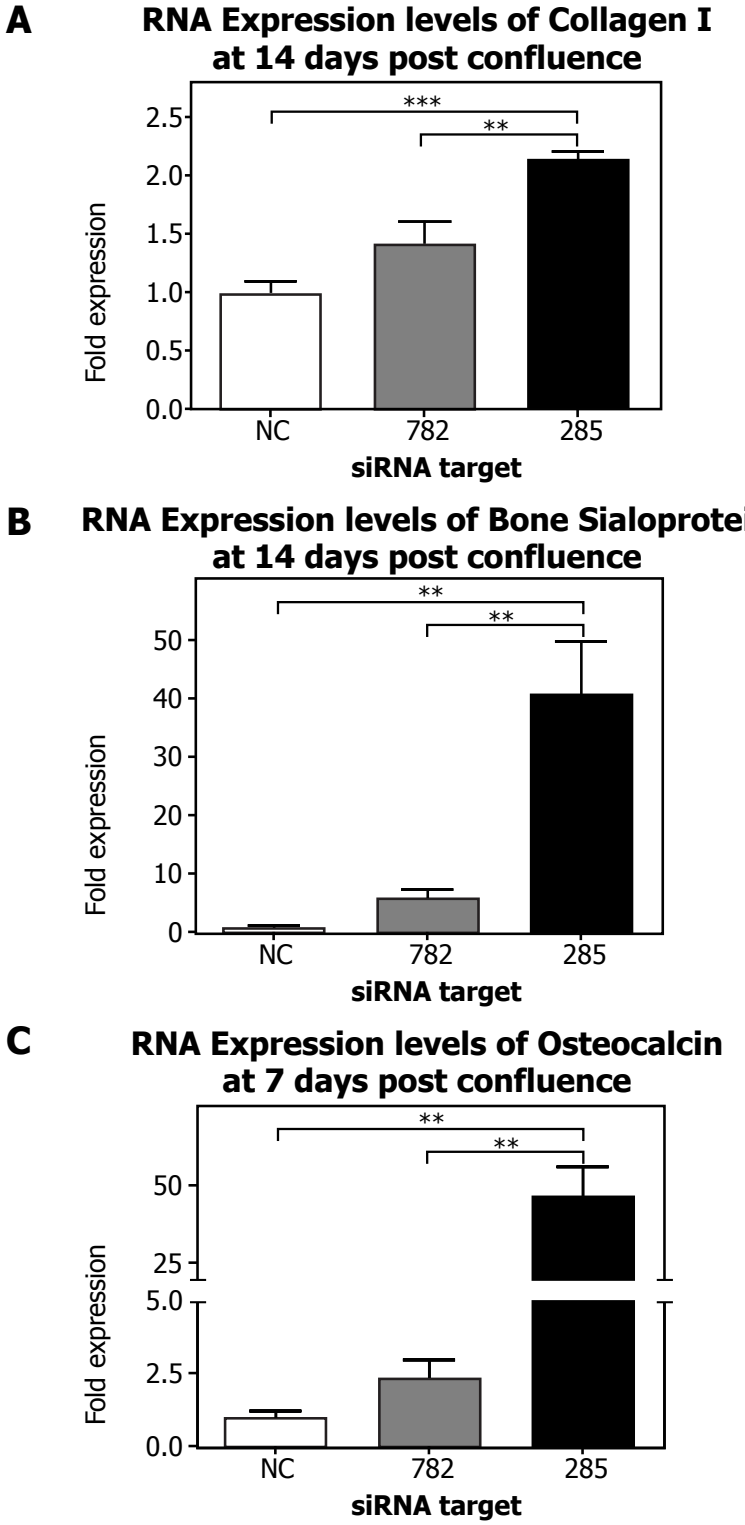
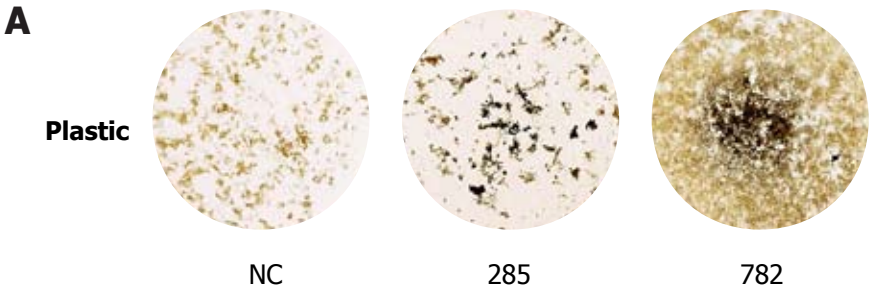


Figure 9: Expression profile of ILK-deficient osteoblasts

ILK-deficient MC3T3-E1 pre-osteoblasts were isolated and RNA was harvested at 7 and 14 days post confluence. Expression of osteocalcin, a marker of differentiated osteoblasts, is significantly increased in ILK-deficient osteoblasts compared to the negative control (C). Secretion of the matrix proteins collagen type I (A) and bone sialoprotein (B) is also increased at 14 days in ILK-deficient osteoblasts. **, $p<0.05$; ***, $p<0.001$



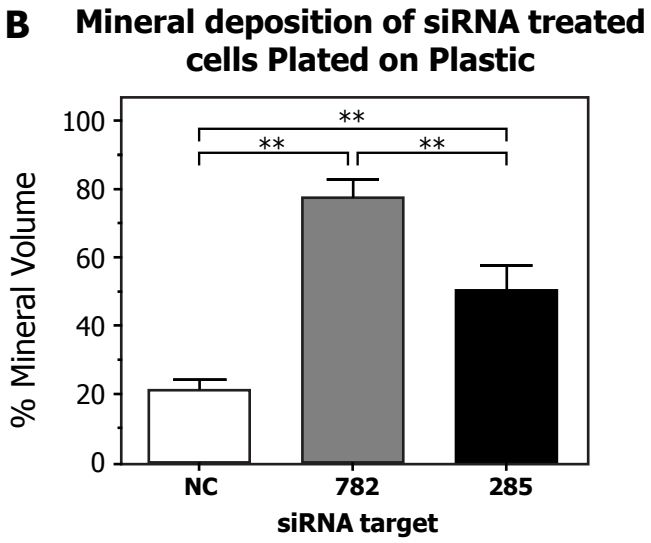
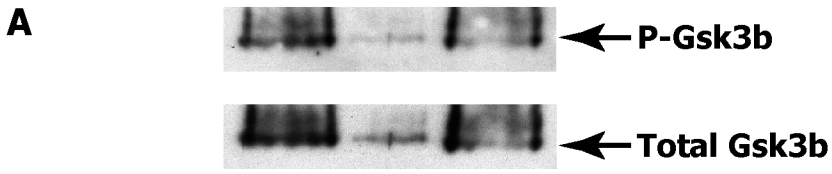


Figure 10: ILK siRNA treated cells deposit more mineral than control
(**A**) ILK-deficient MC3T3-E1 osteoblasts were grown for three weeks in medium containing ascorbic acid and ß-glycerol-phosphate to enable them to differentiate and produce mineralizing matrix (**B**). Quantification of the mineral volume that is standardized to the well size was measured using image analysis software, and ILK-deficient osteoblasts lay down more mineral matrix then the NC control. **, $p<0.01$



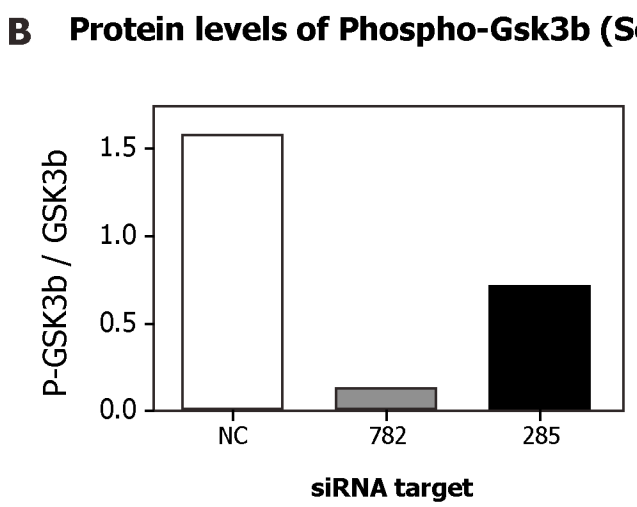


Figure 11: GSK3ß phosphorylation is decreased by reduced ILK levels in pre-osteoblasts

Proteins were isolated from ILK-deficient MC3T3 osteoblasts after one week in culture, and the activation level of GSK3ß was assessed using antibodies directed at Ser 9-phospho-GSK3ß (**A**). When levels of phosphorylation are quantified, we observed a reduced inhibition of GSK3ß in the ILK-deficient osteoblasts (**B**).

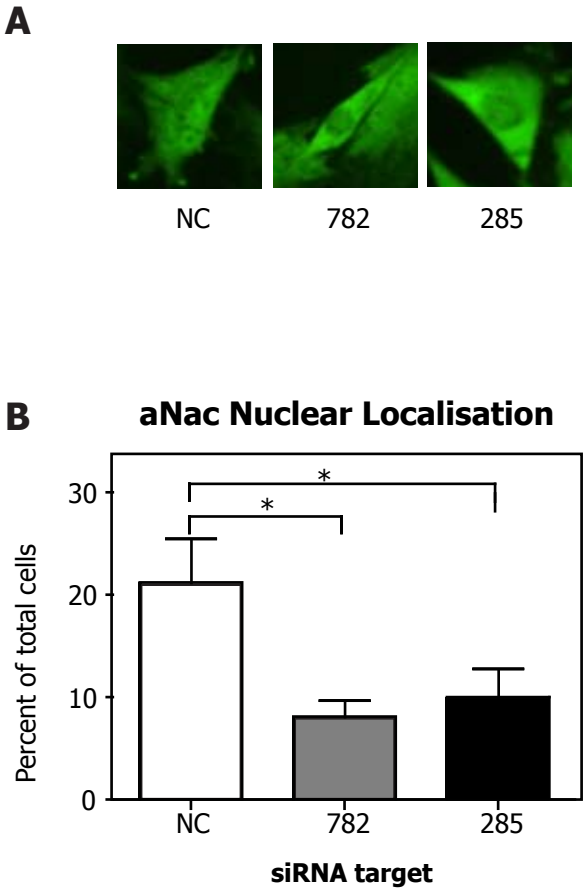


Figure 12: αNAC preferentially localises to the cytosol in ILK deficient osteoblasts

Immunofluorescence was used to assess the subcellular localization of αNAC (**A**). Cells were counted, and αNAC appears to be present in the nucleus in only about half as many cells as in the NC controls (**B**). *, $p<0.05$

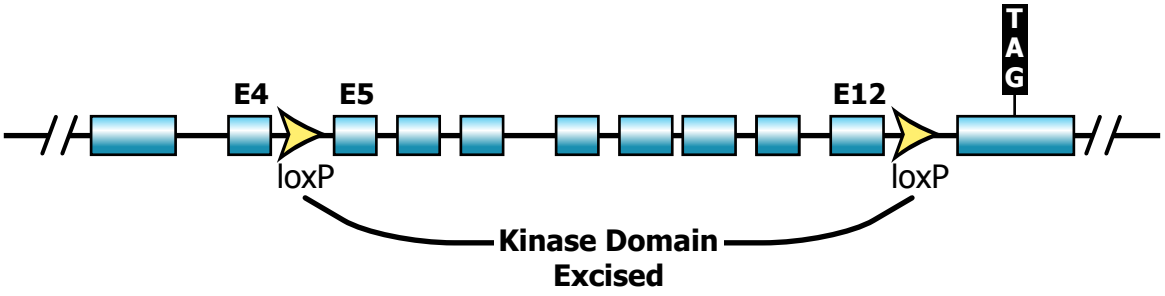


Figure 13: Targetting vector used for deletion of *Ilk* kinase domain by Cre recombinase

Schematic representation of the loxP sites (yellow triangle) that flank the kinase domain of *Ilk*. Under the control of the Collagen type I promoter, Cre recombinase will be expressed in differentiating osteoblasts. This recombinase will excise the *Ilk* Kinase domain encoded by the exons located between these two loxP sites. **E**, exon; **TAG**, stop codon

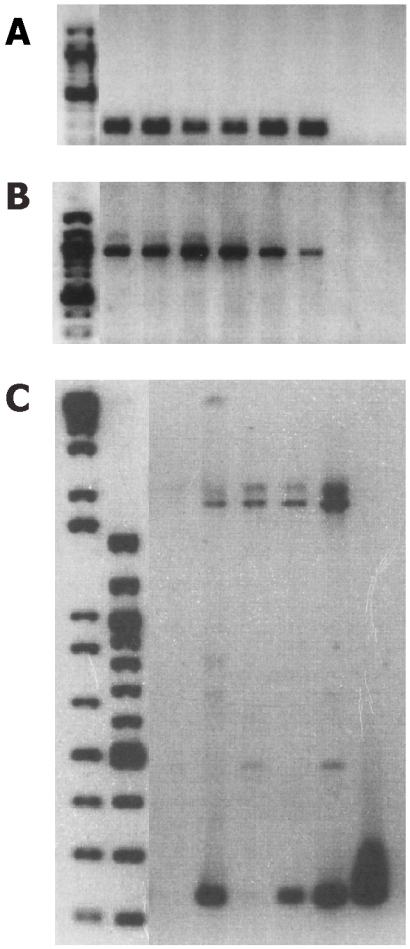


Figure 14: Representative example of genotyping
Genomic DNA harvested from the tail was used to determine the genotype of the mice. Primers for the presence of the Cre recombinase were used (**A**), and primers for the ILK knock-out allele were also used (**B**), yielding a characteristic 200bp and 1kb amplimers, respectively. Mice positive for both are -/fl cre, therefore have ILK-deficient osteoblasts. Calvarial DNA was harvested to assess the excision efficiency of Cre, and primers specific for the excised ilk kinase domain show the excised band at 250 bp, compared to the wild-type or floxed allele at 1.9 and 2.1 kb, respectively (**C**).

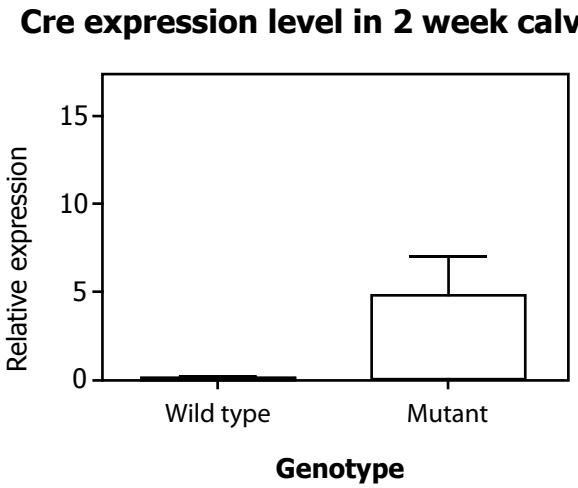


Figure 15: Cre expression levels in murine calvaria
Calvarial RNA was harvested and assayed by RT-qPCR to determine Cre recombinase expression in mutant (-/fl cre) and control populations (-/fl).

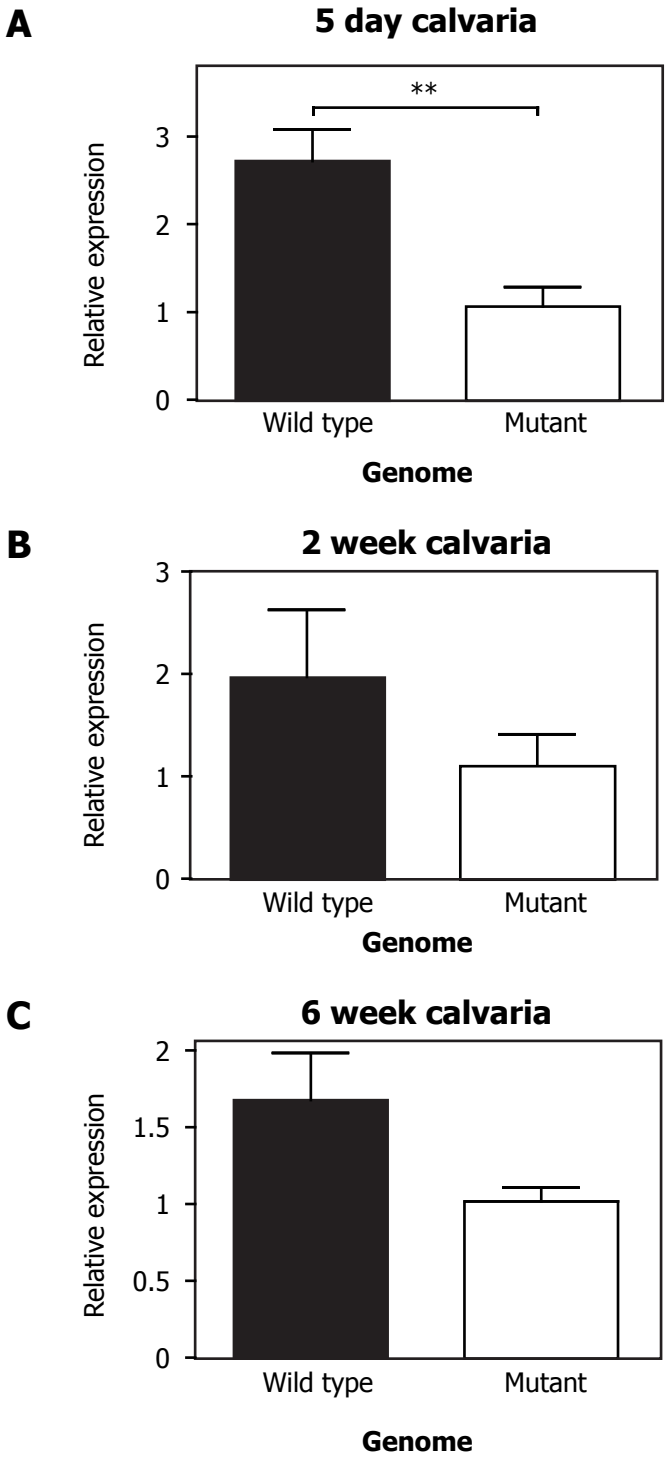


Figure 16: *Ilk* expression levels in murine calvaria
Calvarial RNA was harvested because of its relatively high number of osteoblasts and osteocytes. *Ilk* transcript levels were assessed by RT-qPCR, and they were significantly reduced in 5 day old mice by 60% (**A**). *Ilk* transcripts in older mice were reduced by 50% and 40% in 2 and 6 week old mice, respectively, although these did not reach significance (**B, C**). **, $p<0.01$

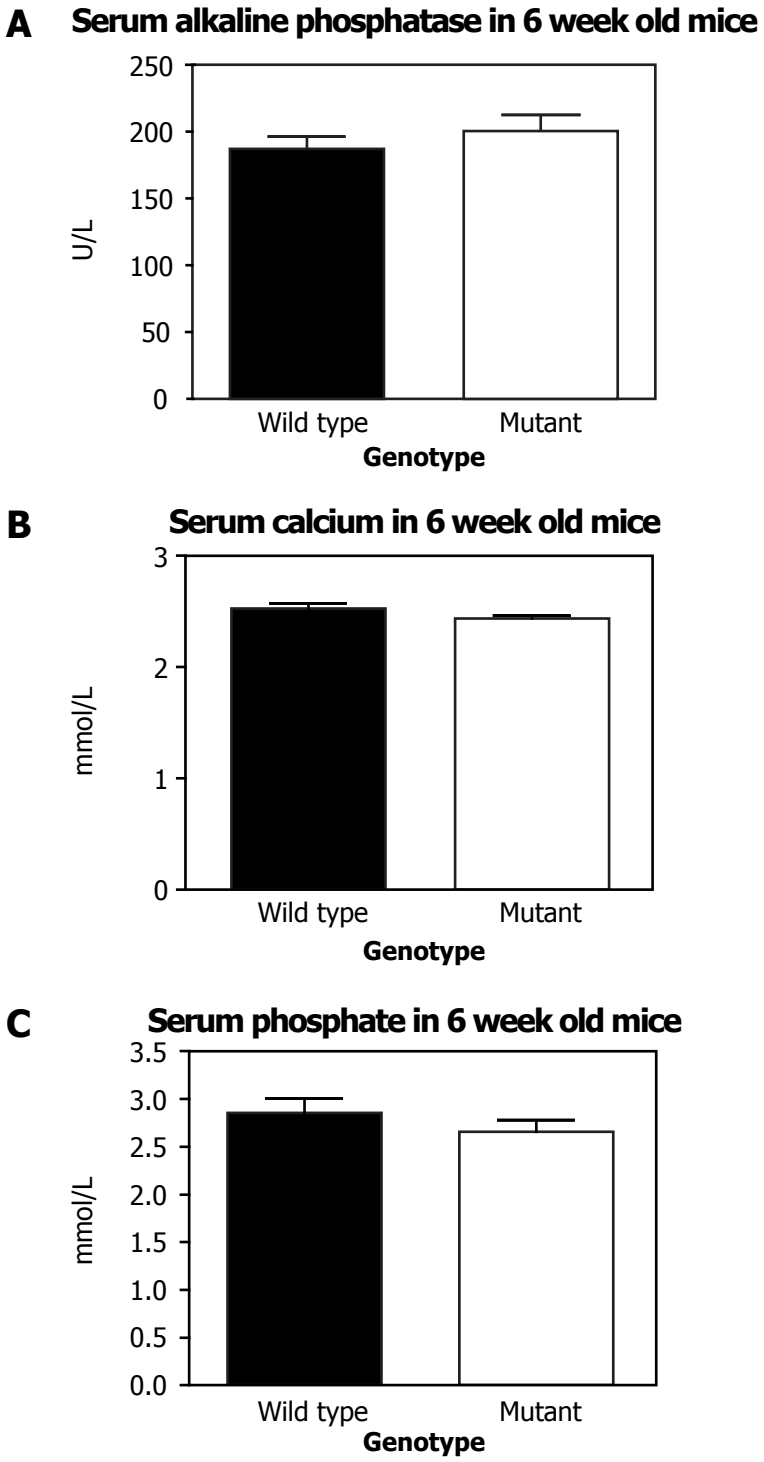


Figure 17: Blood biochemistry of 42 day old osteoblast-specific ILK-deficient mice

Serum levels of the bone formation marker alkaline phosphatase remain unchanged in adult mice (**A**). Levels of the mineral ions calcium and phosphate also remain unchanged in adult mice when compared to the control (**B, C**)

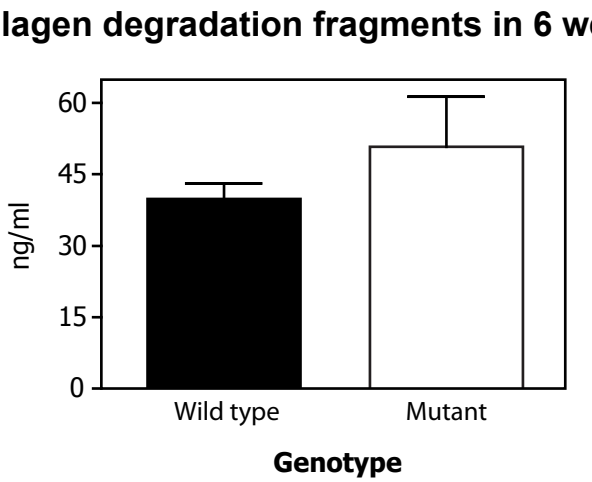


Figure 18: Collagen degradation fragments in 42 day old osteoblast-speciic ILK-deficient mice

Collagen degradation fragments in the serum of adult mice are a marker of bone resorption. In mice that have ILK-deficient osteoblasts, there was no increase in the amount of collagen degradation when compared to control litter mates.

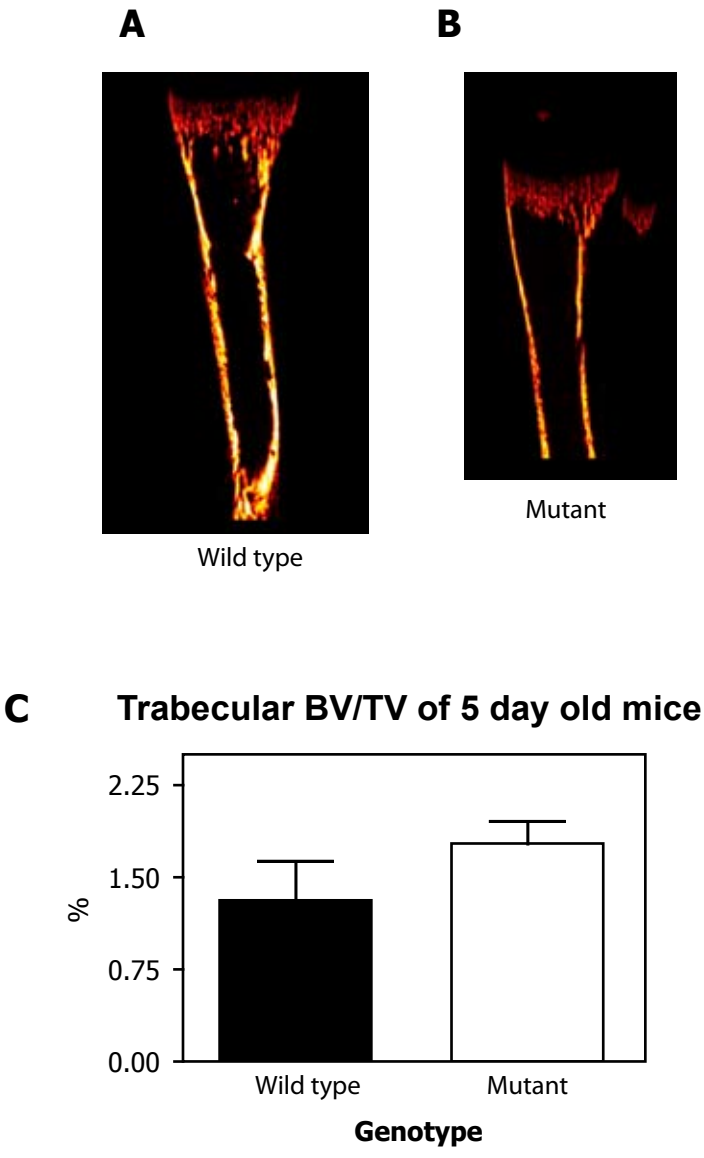
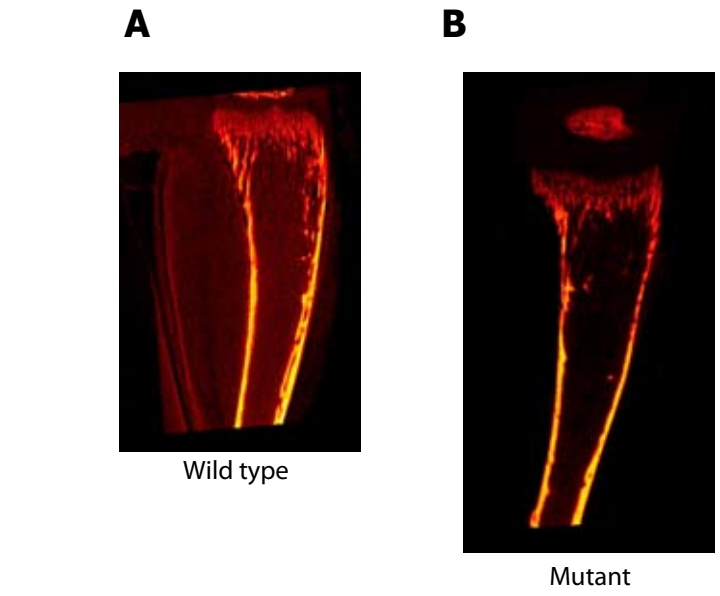


Figure 19: Static histomorphometry of 5 day old tibia
Tibia were collected in 5 day old control and mutant mice and quantitatively analysed with µCT for trabecular bone volume relative to the tissue volume (**A, B**). Although there is a slight trend towards increased BV/TV in the mutant, this trend was not statistically significant (**C**).



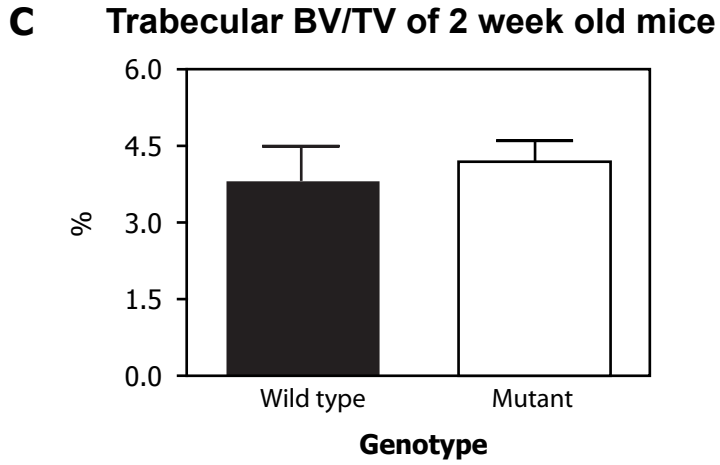
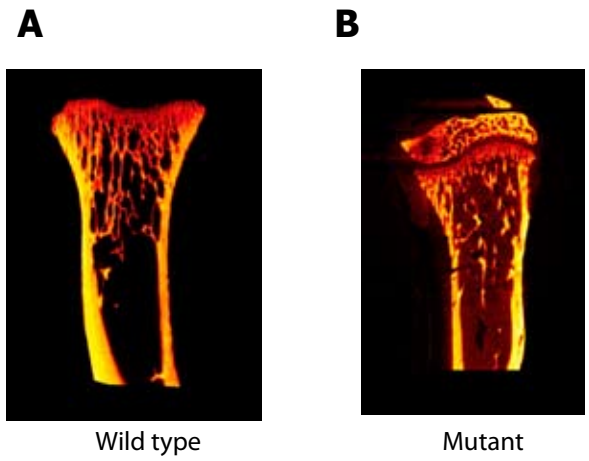


Figure 20: Static histomorphotery of tibia of 14 day old mice
Tibia were collected in 14 day old control and mutant mice and quantitatively analysed with µCT for trabecular bone volume relative to the tissue volume (**A, B**). Quantitave analysis shows no difference between the wild type and mutant (**C**).



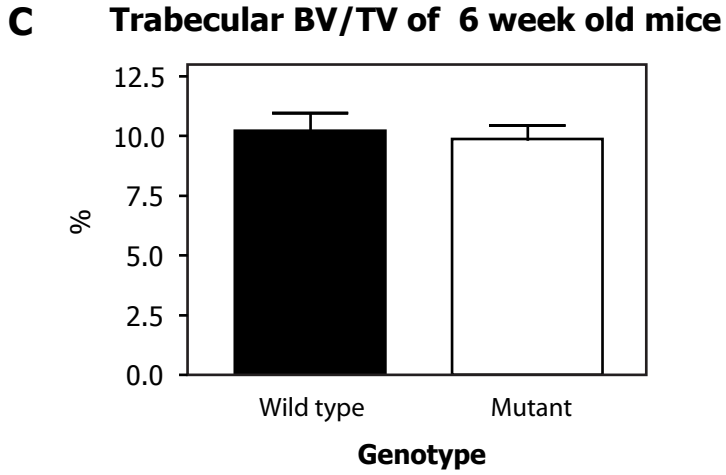


Figure 21: Static histomophometry of tibia of 42 day old mice
Tibia were collected in 42 day old control and mutant mice and quantitatively analysed with µCT for trabecular bone volume relative to the tissue volume (**A, B**). Quantitative analysis shows no difference in the BV/TV between the mutant and control (**C**).

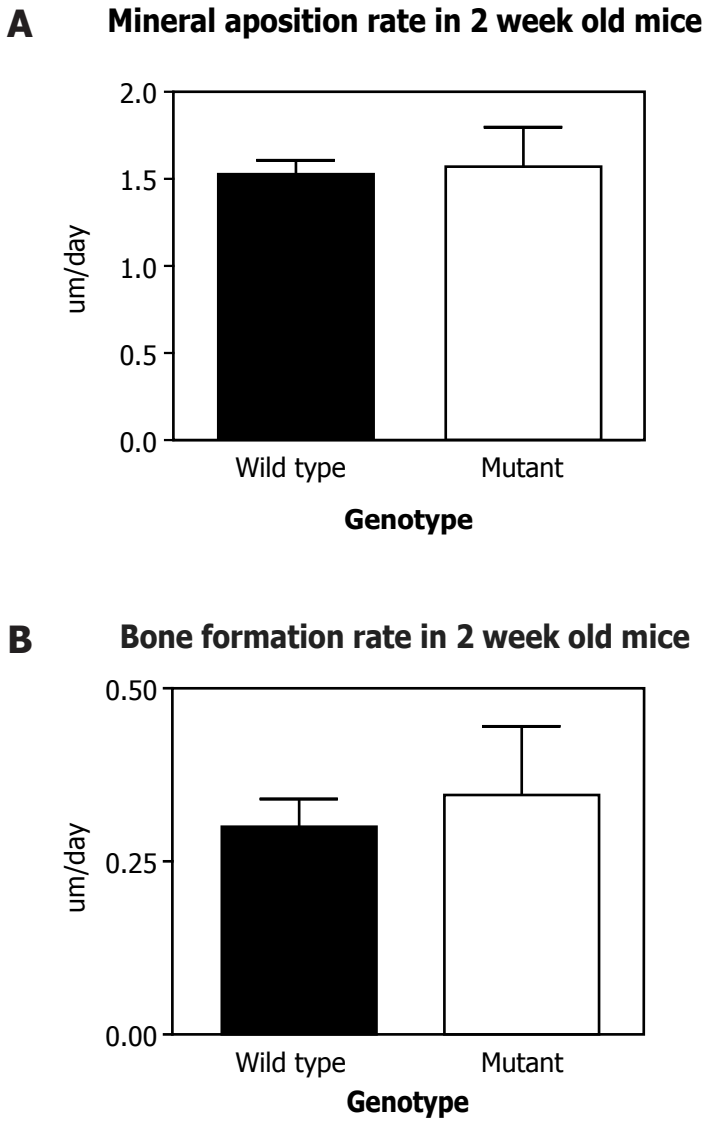


Figure 22: Dynamic Histomorphonetry of 14 day old murine tibia
Mutant and wild type mice were injected twice with calcein florescent label five days apart to mark the bone forming front on the two injection days. Analysis of mineral apposition rate (MAR) and bone formation rate (BFR) are based on the distance between the fluorescent labels. Not difference was observed in the MAR of the BFR between mutant and wild-type mice (**A, B**).

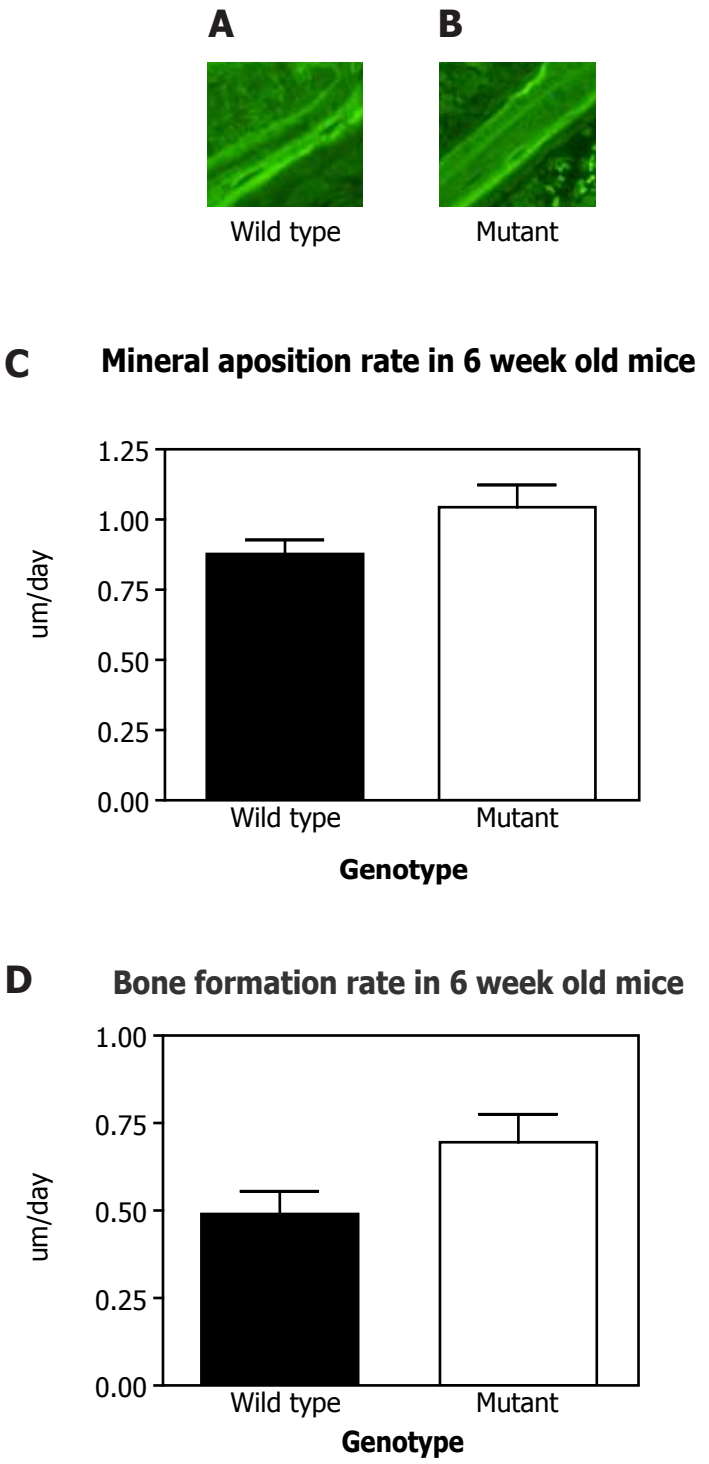


Figure 23: Dynamic Histomorphometry of 42 day old murine tibia
Mutant and wild type mice were injected twice with calcein florescent label five days apart to mark the bone forming front on the two injection days (**A, B**). Analysis of mineral apposition rate (MAR) and bone formation rate (BFR) are based on the distance between the fluorescent labels. Not difference was observed in the MAR of the BFR between mutant and wild-type mice (**C, D**).

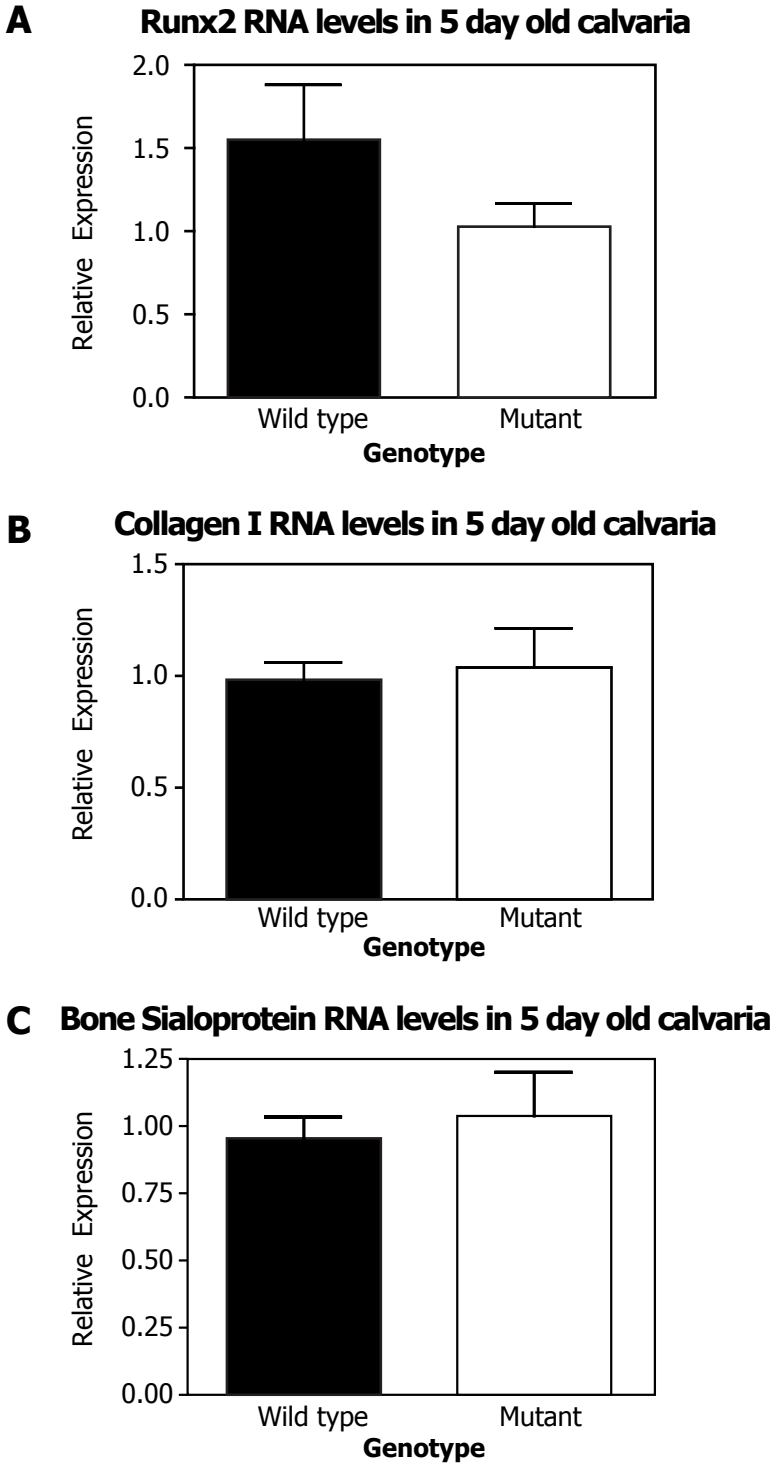


Figure 24: Expression profile of 5 day old calvaria
Calvaria were dissected from 5 day old wild type and osteoblast-specific ILK-deficient mice, and RNA was harvested and reverse transcribed for quantitative PCR. Transcript levels of the transcription factor Runx2 remained unchanged in the mutants (**A**), and expression of matrix proteins such as collagen type I and bone sialoprotein also remained unaffected by deletion of ILK (**B, C**).

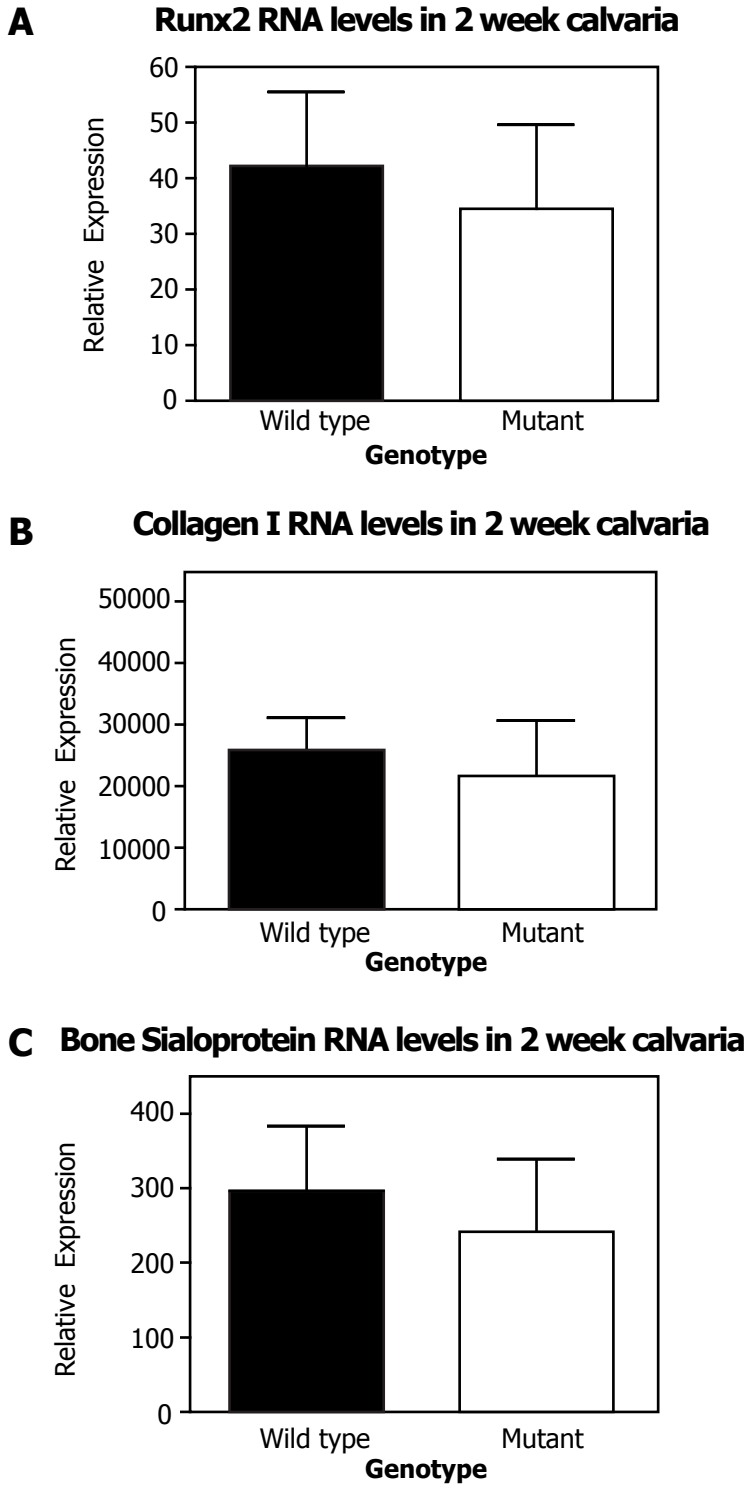


Figure 25: Calvaria Expression markers at 14 day old

Calvaria were dissected from 14 day old wild type and osteoblast-specific ILK-deficient mice, and RNA was harvested and reverse transcribed for quantitative PCR. Transcript levels of the transcription factor Runx2 remain unchanged in the mutants (**A**), and expression of matrix proteins such as collagen type I and bone sialoprotein are also unaffected by deletion of ILK (**B, C**).

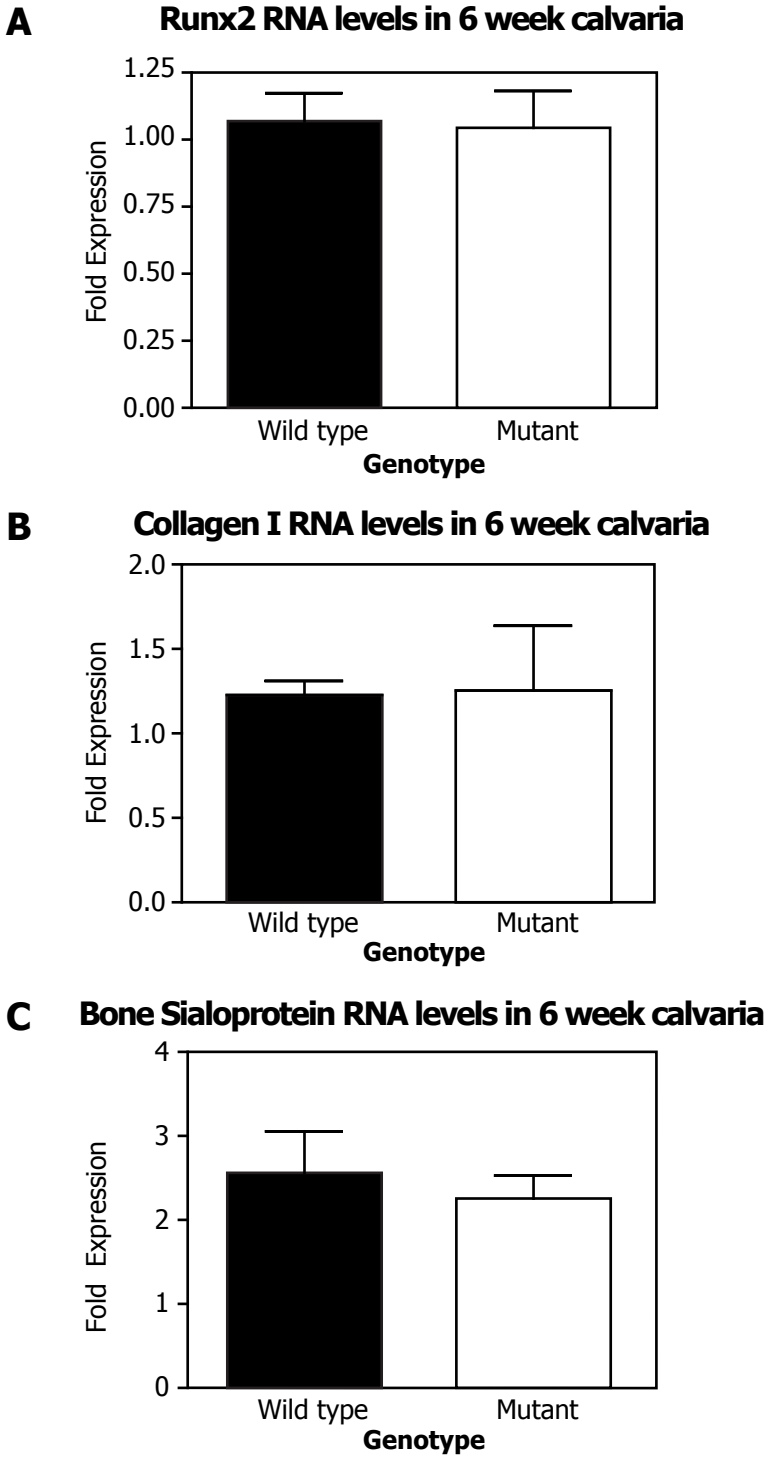


Figure 26: Calvaria Expression markers at 42 day old

Calvaria were dissected from 42 day old wild type and osteoblast-specific ILK-deficient mice, and RNA was harvested and reverse transcribed for quantitative PCR. Transcript levels of the transcription factor Runx2 remain unchanged in the mutants (**A**), and expression of matrix proteins such as collagen type I and bone sialoprotein also remained unaffected by deletion of ILK (**B, C**).

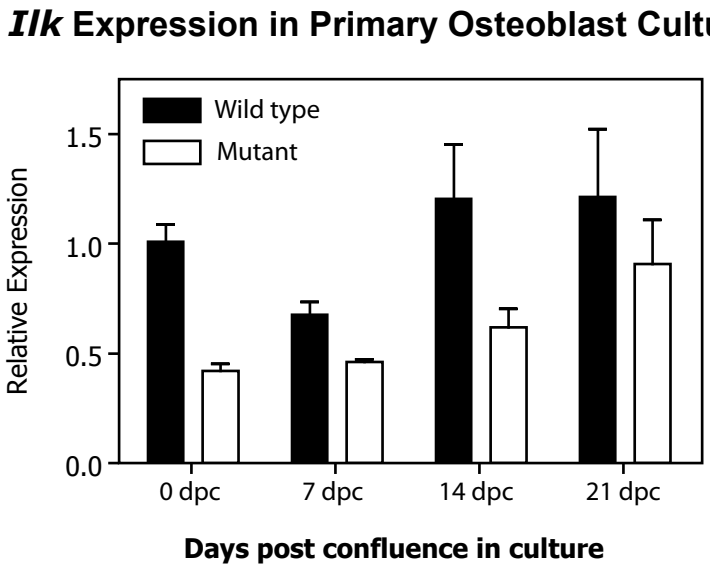


Figure 27: Expression levels of *Ilk* in cultured primary osteoblasts
Primary osteoblast cells were harvested from calvaria of young wild type and osteoblast-specific *Ilk*-deficient mice and grown in culture. Cells were harvested at 0, 7, 14, and 21 days post confluence for RNA, and transcript levels were analysed using RT-qPCR. Transcript levels of *Ilk* were significantly reduced in the mutants ($p<0.01$, anova test), but *Ilk* was not completely ablated.

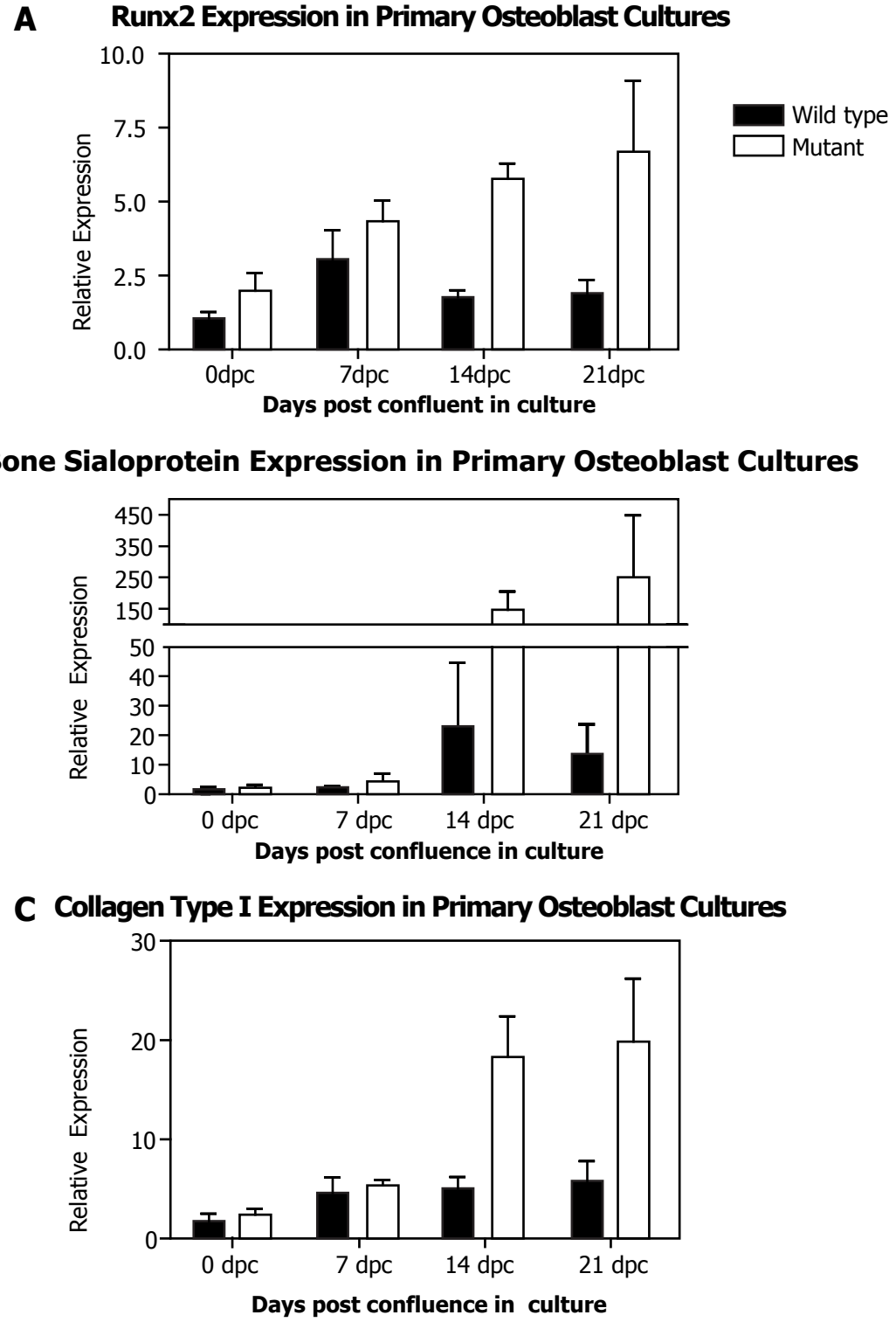


Figure 28: Expression levels of cutlured primary osteoblasts

RNA was harvested from cultured primary osteoblasts from wild type and osteoblast-specific ILK-deficient mice at 0, 7, 14, and 21 days post confluency, and transcript levels were assessed by RT-qPCR. ILK-deficient mutant osteoblasts express significantly higher levels of the transcription factor Runx2 (**A**, $p<0.01$, anova). Production of matrix proteins such as bone sialoprotein are elevated in mutants (**B**, $p>0.05$), and levels collagen type I are significantly increased (**C**, $p<0.01$, anova).

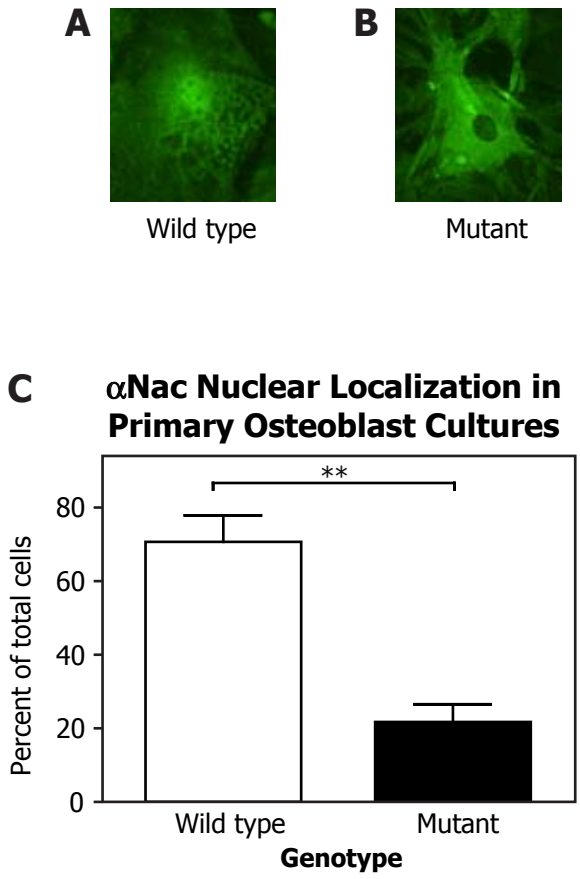


Figure 29: Immunofluorescence of primary osteoblasts stainned for αNAC

Primary osteoblasts were cultured and stained for the αNAC transcriptional coactivator (**A, B**). Cells were counted and analysed for the subcellular localization of αNAC. In mutant cells, the nuclear αNAC is reduced by 70% (**C**). **, p<0,01

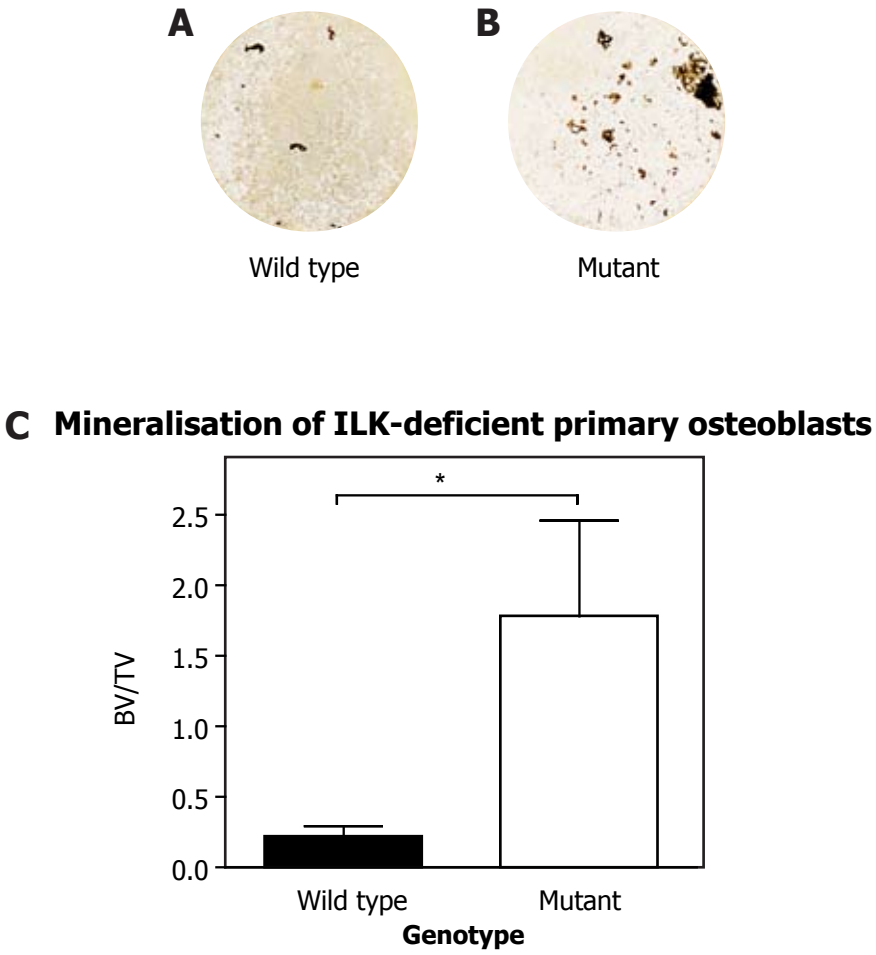
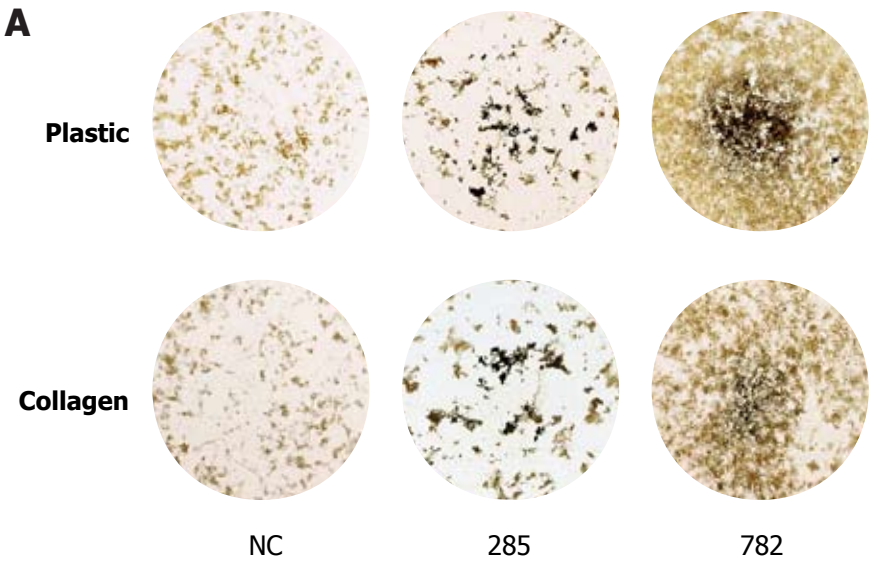


Figure 30: ILK-deficient primary osteoblasts mineralise more than control cells.

Wild type and ILK deficient primary osteoblasts were grown in culture for three weeks, and mineral deposition was quantified by Von Kossa staining in mutant and control populations (**A, B**). Quantification of bone volume using BioQuant image analysis software shows that mutant mice have almost double the mineral deposition than the control (**C**). *, $p<0,05$



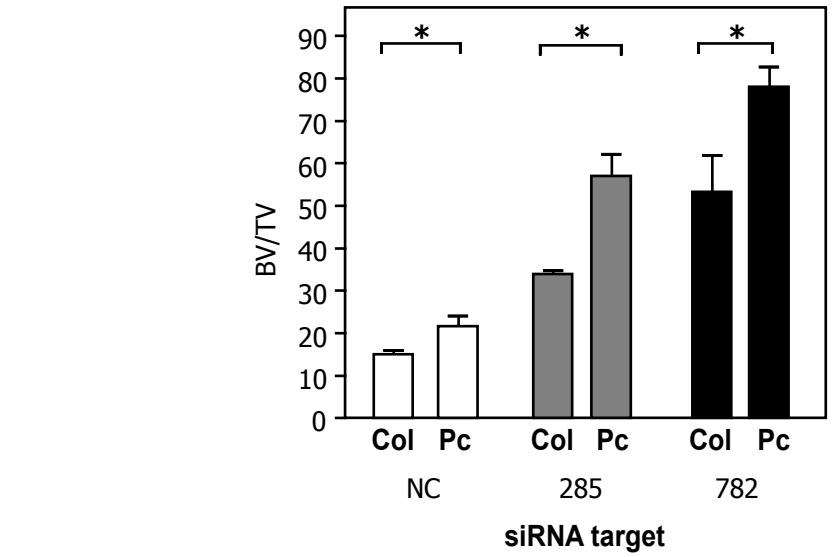


Figure 31: Mineralisation of ILK-deficient osteoblasts on collagen matrix
MC3T3-E1 pre-osteoblasts were cultured for three weeks on regular plastic dishes or on collagen coated dishes. Mineral content was assessed by Von Kossa staining (**A**). Both ILK-deficient clones (ILK285, ILK782) mineralized more than control (NC) on regular plastic (**B**). Collagen coated dishes inhibited mineralization from 25 to 40% in all cases (**B**). *, $p<0,05$

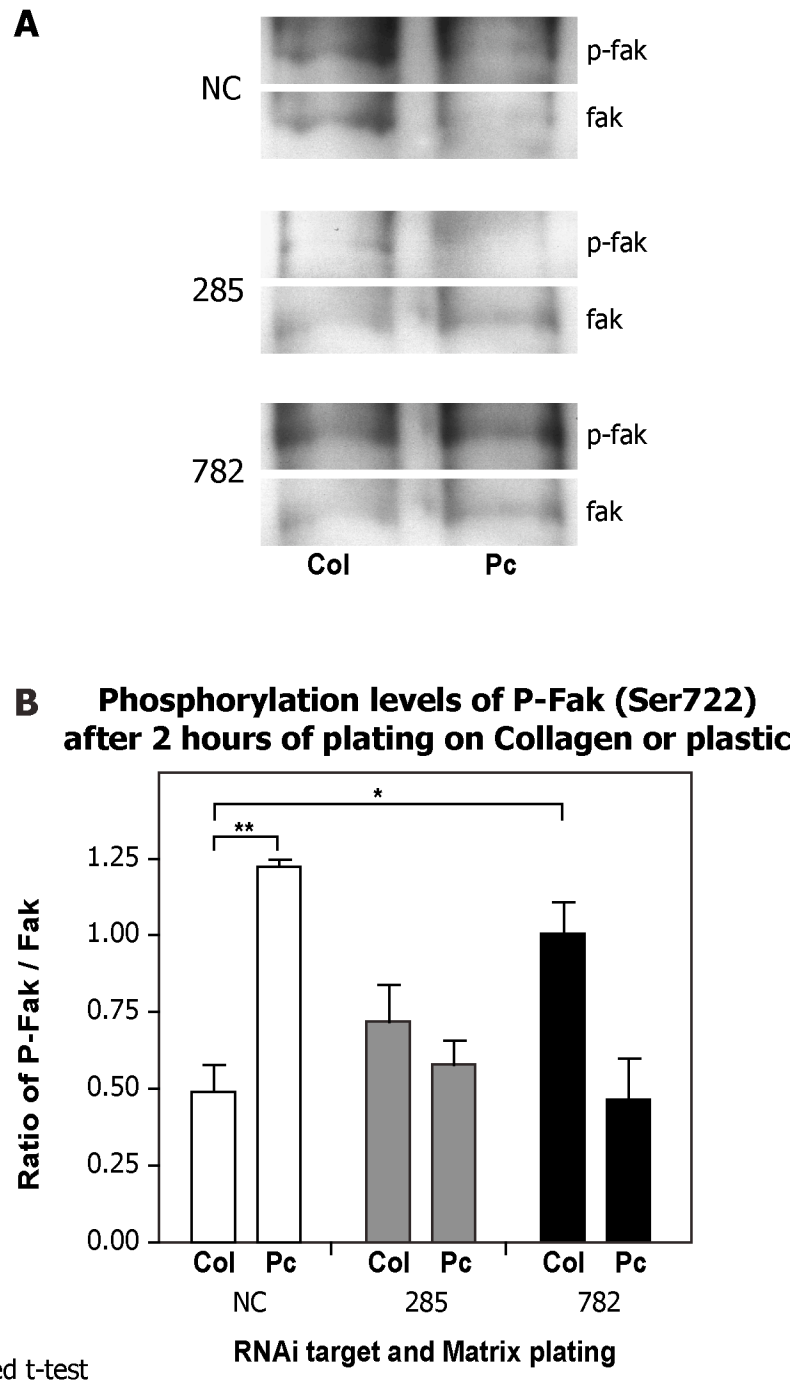


Figure 32: Phosphorylation levels of FAK when cells are plated on collagen or plastic

FAK activity levels were assayed by western blotting using antibodies targeted at phosphorylated FAK at serine 722, and the levels are normalized to total FAK expression. ILK-deficient clones and the negative control were plated for two hours before protein was extracted and probed for FAK (**A**). In ILK deficient cells, Ser722 is hyperphosphorylated when cells are plated on type I collagen, as opposed to control, where Ser722 is hypophosphorylated (**B**). *, $p<0.05$; **, $p<0.01$

Guidelines for completing the form are available at www.mcgill.ca/research/compliance/animal/forms

McGill University
Animal Use Protocol – Research

For Office Use Only:
Protocol #: 4125
Approval End Date: MAY 31, 2008
Facility Committee: SH

Title: Gene expression in bone cells (4): role of the integrin-linked kinase (ILK) during development
(must match the title of the funding source application)

☐ **New Application** ☒ **Renewal of Protocol #** 4125 ☐ **Pilot** **Category** *(see section 11)*: C

1. Investigator Data:

Principal Investigator: Dr. René St-Arnaud **Phone #:** 514-282-7155
Unit/Department: Genetics Unit, Shriners Hospital for Children **Fax#:** 514-842-5581
Address: 1529 Cedar Ave, Mtl (QC) H3G 1A6 **Email:** rst-arnaud@shriners.mcgill.ca

2. Emergency Contacts: Two people must be designated to handle emergencies.

Name: Mia Esser **Work #:** 514-842-5964 ext.3305 **Emergency #:** (514) 684-7590
Name: René St-Arnaud **Work #:** 514-282-7155 **Emergency #:** (514) 748-9129

3. Funding Source:

External ☒
Source (s): Shriners of North America
Peer Reviewed for the project proposed in this Animal Use Protocol: ☒YES ☐NO**
Status : ☒ Awarded ☐ Pending
Funding period: Jan 2005-Jan 2008

Internal ☐
Source (s): _____
Peer Reviewed: ☐ YES ☐ NO**
Status: ☐ Awarded ☐ Pending
Funding period: _____

For Office Use Only:

ACTION	✔	DATE
CCs		
DB	✔	May 31/07
APPROVED		

** All projects that have not been peer reviewed for scientific merit by the funding source require 2 Peer Review Forms to be completed e.g. Projects funded from industrial sources. Peer Review Form available at www.mcgill.ca/research/compliance/animal/forms

Proposed Start Date of Animal Use (d/m/y): _____ **or ongoing** ☒
Expected Date of Completion of Animal Use (d/m/y): _____ **or ongoing** ☒

Investigator's Statement: The information in this application is exact and complete. I assure that all care and use of animals in this proposal will be in accordance with the guidelines and policies of the Canadian Council on Animal Care and those of McGill University. I shall request the Animal Care Committee's approval prior to any deviations from this protocol as approved. I understand that this approval is valid for one year and must be approved on an annual basis.

Principal Investigator's signature: [signature] **Date:** 24-05-2007

Approved by:

Chair, Facility Animal Care Committee:	[signature] for R. St-Arnaud	**Date:** June 20, 2007
University Veterinarian:	[signature]	**Date:** June 19, 2007
Chair, Ethics Subcommittee (as per UACC policy):	—	**Date:** —
Approved Animal Use	**Beginning:** June 1, 2007	**Ending:** MAY 31, 2008

☑ This protocol has been approved with the modifications noted in Section 13.

☐ **Renewal requires submission of full Animal Use Protocol form**

2 8 JUIN 2007

4. Research 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). Indicate if Principal Investigator is not handling animals. 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 *www.animalcare.mcgill.ca* for details. Each person listed in this section must sign to indicate that s/he has read this protocol. *(Space will expand as needed)*

Name	Classification	Animal Related Training Information		Occupational Health Program *	Signature
		UACC on-line Theory course	Workshops + others		
René St-Arnaud	P.I.	March 2004	N/A-will not handle animals	Shriners Hospital	
Mia Esser	AHT	N/A	July 2004	Shriners Hospital	
Louise Marineau	AHT	N/A	July 2004	Shriners Hospital	
Nathalie Guevremont	AHT	N/A	January 2007	Shriners Hospital	
Jad El-Hoss	Grad. Student	November 2006	December 2005	Shriners Hospital	

*** Indicate for each person, if participating in the local Occupational Health Program, see *www.mcgill.ca/research/compliance/animal/occupational* for details.**

5. Summary (in language that will be understood by members of the general public)

5 a) AIMS AND BENEFITS: Describe, in a short paragraph, the overall aim of the study and its potential benefit to human/animal health or to the advancement of scientific knowledge.

Cells attach to the extracellular matrix surrounding them via integrin receptors. Upon binding, integrin receptors signal through many intermediates to regulate cell adhesion, differentiation, survival, activity, etc. One such effector intermediate is the Integrin-Linked Kinase or ILK. We want to understand the role of ILK in the differentiation and function of the cell types of bone, the osteoblast and the osteoclast. For this purpose, we have produced a strain of mice in which the ILK gene can be specifically inactivated in particular cell types. Our preliminary data show that removing ILK from osteoblasts increases their activity, suggesting that ILK normally inhibits osteoblast function. On the other hand, ILK seems important for normal osteoclast function, as inactivating the gene in osteoclasts decreases the activity of the cell. Thus one can imagine that drugs that would inhibit ILK activity, which are in pre-clinical development, would be beneficial for patients with low bone mass, such as osteoporotic adults or children treated with anti-inflammatory glucocorticoids, as they would stimulate bone formation and reduce bone resorption at the same time, leading to net increases in bone mass.

5 b) SPECIFIC OBJECTIVES OF THE STUDY: Summarize in point form the primary objectives of this study.

1-Characterize the phenotype of osteoblast-specific ILK knockout mice;
2-Characterize the phenotype of primary osteoblast cultures from osteoblast-specific ILK knockout mice;
3-Characterize the phenotype of osteoclast-specific ILK knockout mice;
4-Breed the ILK knockout mice with the beta3-integrin knockout mice to demonstrate that they interact at the genetic level;
5-Characterize the phenotype of primary osteoclast cultures from osteoclast-specific ILK knockout mice.

5 c) Indicate if and how the current goals differ from those in last year's application.

These specific objectives are an extension of the previous aims as we are progressing in our analyses. For example, specific objective 4 was initiated once we found that the osteoclast-specific ILK knockout mice have a mild osteopetrotic phenotype similar to the phenotype of the beta3-integrin knockout mice, suggesting that they act within the same signaling cascade.

5 d) List the section / subsection numbers where significant changes have been made

Section 7c: additional strains
Section 10: ovariectomy.

5 e) KEYWORDS: Using keywords only, list the procedures used on animals (e.g. anaesthesia, breeding colony, injection IP, gavage, drug administration, major survival surgery, euthanasia by exsanguination, behavioural studies). For a more complete list of suggested keywords refer to Appendix 1 of the Guidelines (www.mcgill.ca/research/compliance/animal/forms).

anesthesia; breeding colony; survival surgery; IP injections; euthanasia for tissue collection.

6. Animals Use data for CCAC

6 a) Purpose of Animal Use (Check most appropriate one):

1. ☒ **Studies of a fundamental nature/basic research**
2. ☐ **Studies for medical purposes relating to human/animal diseases/disorders**
3. ☐ **Regulatory testing**
4. ☐ **Development of products/appliances for human/veterinary medicine**
5. **If for Teaching, use the Animal Use Protocol form for Teaching (www.mcgill.ca/research/compliance/animal/forms)**

6 b) Will field studies be conducted? NO ☒ YES ☐ If yes, complete "Field Study Form"
Will the project involve genetically altering animals? NO ☒ YES ☐ If yes, complete SOP #5 or #6
Will the project involve breeding animals? NO ☐ YES ☒ If breeding transgenics or knockouts, complete SOP#4

7. Animal Data

7 a) Please justify the need for live animals versus alternate methods (e.g. tissue culture, computer simulation)

Knockout strains allow to study the role of a particular gene product in the context of the physiology of a whole animal. No tissue culture system can reproduce these analyses.

7 b) Describe the characteristics of the animal species selected that justifies its use in the proposed study (consider characteristics such as body size, species, strain, data from previous studies or unique anatomic/physiological features)

The knockout technology is only available in mice. Mice breed well and rapidly, thus allowing the study to be completed in a reasonable amount of time.

7 c) Description of animals

Quality Control Assurance: To prevent introduction of infectious diseases into animal facilities, a health status report or veterinary inspection certificate may be required prior to receiving animals from all non-commercial sources or from commercial sources whose animal health status is unknown or questionable. Quarantine and further testing may be required for these animals.

If more than 6 columns are needed, please attach another page

	Sp/strain 1	Sp/strain 2	Sp/strain 3	Sp/strain 4	Sp/strain 5	Sp/strain 6
Species	mouse	mouse	mouse	mouse	mouse	mouse
Supplier/Source	breeding	breeding	breeding	breeding	breeding	breeding
Strain	ILK KO	ILK floxed	beta3-integrin KO	Col 1-Cre Tg	Osx 1-Cre Tg	TRAP-Cre Tg
Sex	m/f	m/f	m/f	m/f	m/f	m/f
Age/Wt	up to 6 months	up to 6 months	up to 6 months	up to 6 months	up to 6 months	up to 6 months
# To be purchased	N/A	N/A	N/A	N/A	N/A	N/A
# Produced by in-house breeding	180	180	180	180	180	180
# Other (e.g.field studies)	N/A	N/A	N/A	N/A	N/A	N/A
#needed at one time	60	60	60	60	60	60
# per cage	female + pups adults: 5/cage	female + pups adults: 5/cage	female + pups adults: 5/cage	female + pups adults: 5/cage	female + pups adults: 5/cage	female + pups adults: 5/cage
TOTAL# /YEAR	**180**	**180**	**180**	**180**	**180**	**180**

Additional table to add to 'Description of animals' in full Animal Use Protocol form as well as Renewal form

	Sp/strain 7	Sp/strain 8	Sp/strain 9	Sp/strain 10	Sp/strain 11	Sp/strain 12
Species	mouse	Mouse	mouse	mouse		
Supplier/Source	breeding	Breeding	Breeding	breeding		
Strain	Col 1-Cre-ILK floxed	Osx 1-Cre-ILK floxed	TRAP-Cre-ILK floxed	ILK-beta3 integrin compound mutants		
Sex	m/f	m/f	m/f	m/f		
Age/Wt	Up to 1 year	Up to 1 year	Up to 1 year	Up to 1 year		
# To be purchased	N/A	N/A	N/A	N/A		
# Produced by in-house breeding	180	180	180	180		
# Other (e.g.field studies)	N/A	N/A	N/A	N/A		
#needed at one time	60	60	60	60		
# per cage	female + pups adults: 5/cage	female + pups adults: 5/cage	female + pups adults: 5/cage	female + pups adults: 5/cage		
TOTAL# /YEAR	**180**	**180**	**180**	**180**		

Additional table to add to 'Description of animals' in full Animal Use Protocol form as well as Renewal form

	Sp/strain 13	Sp/strain 14	Sp/strain 15	Sp/strain 16	Sp/strain 17	Sp/strain 18
Species						
Supplier/Source						
Strain						
Sex						
Age/Wt						
# To be purchased						
# Produced by in-house breeding						
# Other (e.g.field studies)						
#needed at one time						
# per cage						
TOTAL# /YEAR						

7 d) Explanation of Animal Usage: 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 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.
(Space will expand as needed)

All mice will be maintained as breeding colonies, from which experimental animals will be obtained. A breeding colony for mice in active experimental use contains approximately, on average, minimally 60 mice at one time, including active breeders, reserve stud males, males and females whose breeding success is under evaluation, pre-weaning litters and post-weaning juveniles awaiting screening. The minimum flow-through time is 10-15 weeks (gestation, weaning, screening); since three complete cycles can be completed in one year, the number of mice in one colony can be estimated at 3 x 60 = 180 mice/year.

8. Animal Husbandry and Care

8 a) If projects involves non-standard cages, diet and/or handling, please specify

N/A

8 b) Is there any component to the proposed procedures which will result in immunosuppression or decreased immune function (e.g. stress, radiation, steroids, chemotherapeutics, genetic modification of the immune system)?

NO ☒ YES ☐ **if yes, specify:**

8 c) Indicate area(s) where animal use procedures will be conducted:

Building: Shriners Hospital for Children **Room:** Procedures room

Indicate area(s) all facilities where animals will be housed:

Building: Shriners Hospital for Children **Room:** 306

If animal housing and animal use are in different locations, briefly describe procedures for transporting animals:

9. Standard Operating Procedures (SOPs)

Complete this section if you plan to use any of the UACC SOPs listed below. IT IS UACC POLICY THAT THESE SOPS BE USED WHEN APPLICABLE. Any proposed variation of the SOPs must be described and justified. The Standard Operating Procedures can be found at the UACC website at www.mcgill.ca/research/compliance/animal/procedures . The completed and signed SOP form must be attached to the protocol.

Check all SOPs that will be used:

Blood Collection UACC#1	☐	**Collection of Amphibian Oocytes UACC#9**	☐
Anaesthesia in rodents UACC#2	☐	**Rodent Survival Surgery UACC#10**	☒
Analgesia in rodents UACC#3	☐	**Anaesthesia & Analgesia Neonatal Rodents UACC#11**	☐
Breeding transgenics/knockouts UACC#4	☒	**Stereotaxic Survival Surgery in Rodents UACC#12**	☐
Transgenic Generation UACC#5	☐	**Field Studies Form**	☐
Knockout/in Generation UACC#6	☐	**Phenotype Disclosure Form**	☒
Production of Monoclonal Antibodies UACC#7	☐	**Other, specify:**	☐
Production of Polyclonal Antibodies UACC#8	☐		☐

10. Description of Procedures

10 a) : IF A PROCEDURE IS COVERED BY AN SOP, WRITE "AS PER SOP", NO FURTHER DETAIL IS REQUIRED.

FOR EACH EXPERIMENTAL GROUP, DESCRIBE ALL PROCEDURES AND TECHNIQUES, WHICH ARE NOT PART OF THE SOPs, IN THE ORDER IN WHICH THEY WILL BE PERFORMED – surgical procedures, immunizations, behavioural tests, immobilization and restraint, food/water deprivation, requirements for post-operative care, sample collection, substance administration, special monitoring, etc Appendix 2 of the Guidelines (www.mcgill.ca/research/compliance/animal/forms) provides a sample list of points that should be addressed in this section.

Strains 1, 2, 3, 4, 5, 6: breeding as per SOP UACC #4. Hemizygous transgenics, heterozygous and homozygous floxed animals will be used for breeding until six months of age. Wild-type animals and retired breeders will be sacrificed by CO2 asphyxia.

Strains 7, 8, 9, 10: breeding as per SOP UACC #4. Also, control animals that are not used for phenotype analysis will be sacrificed in a CO2 chamber. For phenotype analysis, animals will be sacrificed at intervals (Embryonic day (E) 12.5, E14.5, E16.5, newborn, 3 weeks, 6 weeks, 3 months, 6 months, 1 year) to obtain bone tissue for analysis of the phenotype. To determine if osteoblast proliferation is increased or reduced in the bones of mutant animals, animals will be injected with 50 micrograms/gram BW of BrdU (Bromodeoxy Uridine, a nucleotide analog) i.p. (solution prepared at 50 micrograms/0.1 ml; newborns will receive less than 0.5 ml i.p. and pre-pubertal and adult animals will receive less than 3 ml i.p.) one hour prior to sacrifice. For embryos, the gestating female will be injected with the BrdU solution one hour prior to sacrifice. For analysis of mineral apposition rate and bone formation rate, animals will receive 30 micrograms of calcein/g body weigth in 0.1 ml PBS i.p. seven days and two days prior to sacrifice.

Ovariectomy of 6 week-old female mice (strains 7, 8, 9, 10):
If needed to magnify or induce a phenotype manifestation, female adult animals from strain 7-10 may be ovariectomized: anesthesia is induced by isoflurane inhalation (1 to 5%) to effect. After the onset of anesthesia, the lumbar dorsum is shaved bilaterally and the exposed skin prepared for aseptic surgery (a 10% povidone-iodine scrub followed by a 70% alcohol wipe). For each ovary a 3/4 cm dorsal flank incision penetrating the abdominal cavity is made. The parovarian fatty tissue is identified and retracted. The exposed ovary and associated oviduct are severed and removed. Hemostasis is achieved by hemostat pressure for 1-2 minutes. Rarely, a ligature (5-0 absorbable suture) around the severed ovarian vasculature is required to maintain hemostasis. The incision is closed (5-0 absorbable suture). One dose of buprenorphine (0.05 mg/kg in 0.1 ml i.m.) will be administered pre-surgery (30 min pre-op) and as required post-op for analgesia. Groups of 6 animals (mutant and wild-type littermates) will be ovariectomized and sacrificed 4 weeks post-surgery.

Reference:
Cariton A. Eddy. 1986. Experimental Surgery of the Genital system, p.191. In William I. Gay and James E. Heavner (ed.) Methods of Animal Experimentation: Vol 7; Research Surgery and Care of the research Animal; Part B Surgical approaches to organ systems. Academic Press, Inc., Orlando, Florida.

10 b) Experimental endpoint – for each experimental group indicate survival time

Strains 1-6: animals will be sacrificed at 6 months of age.

Strains 7-10: animals will be sacrificed at intervals (Embryonic day (E) 12.5, E14.5, E16.5, newborn, 3 weeks, 6 weeks, 3 months, 6 months, 1 year). Ovariectomized animals will be sacrificed at 4 weeks post-surgery.

10 c) Clinical endpoint – describe the conditions, complications, and criteria (e.g. >20% weight loss, maximum tumour size, vocalizing, lack of grooming) that would lead to euthanasia of an animal before the expected completion of the experiment (specify per species and project if multiple projects involved)

Animals will be sacrificed without delay if signs of distress appear: weight loss (>20%), inability to feed, paralysis, vocalizing, lack of grooming, infection of the surgical site post-ovariectomy, etc.

Frequency of monitoring: daily.

10 d) Specify person(s) who will be responsible for animal monitoring and post-procedural care *(must also be listed in section 4)*

Name: Mia Esser | **Phone #:** 514-842-5964 ext. 3305 (work)
514-484-7590 (home)

10 e) Pre-Anesthetic/Anaesthetic/Analgesic Agents: List all drugs that will be used to minimize pain, distress or discomfort. *(Table will expand as needed)*

Species	Agent	Dosage (mg/kg)	Total volume(ml) per administration	Route	Frequency/Duration
mouse (strains 7-10)	Isoflurane	To effect	1 to 5 %	inhalation	during surgery
	Buprenorphin	0.05 mg/kg	0.1 ml	i.m.	once, pre-surgery

10 f) Administration of ALL other substances: List all non-anaesthetic agents under study in the experimental component of the protocol, including but not limited to drugs, infectious agents, viruses. *(Table will expand as needed)*

Species	Agent	Dosage (mg/kg)	Total volume(ml) per administration	Route	Frequency/Duration
Mouse (strains 7-10)	BrdU (nucleotide analog)	50 µg/g bw	0.5 ml newborns; < 3 mls adults	i.p.	once; 1 h prior to sacrifice
	Calcein	30 mg/g bw	0.1 ml	i.p.	twice: 7 days and 2 days Before sacrifice

10 g) Method of Euthanasia

Specify Species	
	☐ **Anaesthetic overdose, list agent/dose/route:**
	☐ **Exsanguination with anaesthesia, list agent/dose/route:**
	☐ **Decapitation without anaesthesia *** ☐ **Decapitation with anesthesia, list agent/dose/route (including CO_2):**
	☐ **Cervical dislocation without anaesthesia *** ☐ **Cervical dislocation with anaesthesia, list agent/dose/route (including CO_2):**
Mouse, strains 1-10	☒ **CO_2 chamber only**
	☐ **Other, specify:**
	☐ **Not applicable, explain:**

*** For physical method of euthanasia without anaesthesia, please justify:**

11. Category of Invasiveness: B ☐ C ☒ D ☐ E ☐

Categories of Invasiveness (from the CCAC *Categories of Invasiveness in Animal Experiments*). Please refer to this document for a more detailed description of categories.

Category A: Studies or experiments on most invertebrates or no entire living material.

Category B: Studies or experiments causing little or no discomfort or stress. ***These might include holding animals captive, injection, percutaneous blood sampling, accepted euthanasia for tissue harvest, acute non-survival experiments in which the animals are completely anaesthetized.***

Category C: Studies or experiments involving minor stress or pain of short duration. ***These might include cannulation or catheterizations of blood vessels or body cavities under anaesthesia, minor surgery under anaesthesia, such as biopsy; short periods of restraint, overnight food and/or water deprivation which exceed periods of abstinence in nature; behavioural experiments on conscious animals that involve short-term stressful restraint.***

Category D: Studies or experiments that involve moderate to severe distress or discomfort. ***These might include major surgery under anaesthesia with subsequent recovery, prolonged (several hours or more) periods of physical restraint; induction of behavioural stresses, immunization with complete Freund's adjuvant, application of noxious stimuli, procedures that produce pain, production of transgenics (in accordance with University policy).***

Category E: Procedures that involve inflicting severe pain, near, at or above the pain threshold of unanaesthetized, conscious animals. ***Not confined to but may include exposure to noxious stimuli or agents whose effects are unknown; exposure to drugs or chemicals at levels that (may) markedly impair physiological systems and which cause death, severe pain or extreme distress or physical trauma on unanaesthetized animals.*** **According to University policy, E level studies are not permitted.**

12. Potential Hazards to Personnel and Animals It is the responsibility of the investigator to obtain the necessary Biohazard and/or Radiation Safety permits before this protocol is submitted for review. A copy of these certificates must be attached, if applicable.

No hazardous materials will be used in this study: ☒

12 a) Indicate which of the following will be used in animals:

☐ Toxic chemicals ☐ Radioisotopes ☐ Carcinogens
☐ Infectious agents (includes vectors) ☐ Transplantable tumours and/or tissues

12 b) Complete the following table for each agent to be used (use additional page as required):

Agent name			
Dosage			
Route of administration			
Frequency of administration			
Duration of administration			
Number of animals involved			
Survival time after administration			

12 c) After administration the animals will be housed in:

☐ **the animal care facility** ☐ **laboratory under supervision of laboratory personnel**

Please note that cages must be appropriately labeled at all times.

12 d) Describe potential health risk (s) to humans or animals:

12 e) Describe measures that will be used to reduce risk to the environment and all project and animal facility personnel:

12 f) If using cell lines, have they been tested?

☐ **Yes If yes, What human and/or animal pathogens have been tested?**

☐ **No If no, justify:**

13. Reviewer's Comments and Modifications (to be completed by ACC only): The Animal Care Committee has made the following modification(s) to this animal use procedure protocol during the review process. Please make these changes to your copy and comply with the recommended changes as a condition of approval.

- Specify strains to be ovariectomized (section 10a)
- Provide analgesia as required post-op (section 10a + SOP #10).

MCGILL UNIVERSITY
UNIVERSITY ANIMAL CARE COMMITTEE

UACC Standard Operating Procedure # 4 October 2005 version

TRANSGENIC OR KNOCKOUT / IN MOUSE BREEDING

1. INTRODUCTION

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2. INFORMATION REQUIRED

2.1 Species/strain(s) ***(must refer to the Sp/strain column # of the table in "Description of animals" section in main protocol):*** mouse: ILK KO, strain #1

2.2 Transgenic? ☐	**Knockout?** ☒	**Knock/in?** ☐
Gene inserted (include promoter & gene): _____	**Gene deleted:** ILK	**Gene deleted initially:** _____
Target tissue: _____	**Target tissue:** all	**Gene inserted:** _____
Ancillary tissue: _____	**Ancillary tissue:** _____	**Target tissue:** _____
		Ancillary tissue: _____

2.3 Supplier: ***Please include a complete address, contact person, phone, fax and email information. Note that a Certificate of health must be available to FACC personnel PRIOR to animal arrival.***

1. **Commercial:** _____
2. **Academic:** Strain was engineered in my lab.

2.4 Phenotype: ***Include any trait included in a published article or reported to you by the originating investigator that may affect either the breeding, physical ability of the animal to move, eat or drink, or result in a decreased lifespan.***

1. **Heterozygotes:** none.
2. **Homozygotes:** embryonic lethal at E3.5 (implantation).

2.5 Clinical endpoint - General clinical endpoint criteria signs apply.

Supply criteria specific to phenotype: _____

Include plans to monitor or alleviate this distress: N/A.

Frequency of monitoring: daily.

2.6 Are there changes to this SOP indicated in the AUP form? ☐YES ☒NO
If yes, specify changes: _____

2.7 PI Signature: _____ **Date:** 24-05-2007

MCGILL UNIVERSITY
UNIVERSITY ANIMAL CARE COMMITTEE

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2. INFORMATION REQUIRED

2.1 Species/strain(s) ***(must refer to the Sp/strain column # of the table in "Description of animals" section in main protocol):*** mouse: ILK floxed, strain #2

2.2 Transgenic? ☐	**Knockout?** ☒	**Knock/In?** ☐
Gene inserted (include promoter & gene): _____	**Gene deleted:** ILK	**Gene deleted initially:** _____
Target tissue: _____	**Target tissue:** all	**Gene inserted:** _____
Ancillary tissue: _____	**Ancillary tissue:** _____	**Target tissue:** _____
		Ancillary tissue: _____

2.3 Supplier: ***Please include a complete address, contact person, phone, fax and email information. Note that a Certificate of health must be available to FACC personnel PRIOR to animal arrival.***

1. **Commercial:** _____
2. **Academic:** Strain was engineered in my lab.

2.4 Phenotype: ***Include any trait included in a published article or reported to you by the originating investigator that may affect either the breeding, physical ability of the animal to move, eat or drink, or result in a decreased lifespan.***

1. **Heterozygotes:** none.
2. **Homozygotes:** none.

2.5 Clinical endpoint - General clinical endpoint criteria signs apply.

Supply criteria specific to phenotype: _____

Include plans to monitor or alleviate this distress: N/A.

Frequency of monitoring: daily.

2.6 Are there changes to this SOP indicated in the AUP form? ☐YES ☒NO
If yes, specify changes: _____

2.7 PI Signature: _____ **Date:** 24-05-2007

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2. INFORMATION REQUIRED

2.1 Species/strain(s) ***(must refer to the Sp/strain column # of the table in "Description of animals" section in main protocol):*** mouse: beta 3-integrin KO; strain #3

2.2 Transgenic? ☐	**Knockout?** ☒	**Knock/In?** ☐
Gene inserted (include promoter & gene): _____	**Gene deleted:** beta 3 integrin	**Gene deleted initially:** _____
Target tissue: _____	**Target tissue:** bone	**Gene inserted:** _____
Ancillary tissue: _____	**Ancillary tissue:** _____	**Target tissue:** _____
		Ancillary tissue: _____

2.3 Supplier: ***Please include a complete address, contact person, phone, fax and email information. Note that a Certificate of health must be available to FACC personnel PRIOR to animal arrival.***

1. **Commercial:** _____
2. **Academic:** Strain was obtained from Dr. F. Patrick Ross, Washington University, St.Louis

2.4 Phenotype: ***Include any trait included in a published article or reported to you by the originating investigator that may affect either the breeding, physical ability of the animal to move, eat or drink, or result in a decreased lifespan.***

1. **Heterozygotes:** none.
2. **Homozygotes:** mild osteopetrosis.

2.5 Clinical endpoint - General clinical endpoint criteria signs apply.

Supply criteria specific to phenotype: _____

Include plans to monitor or alleviate this distress: N/A; the phenotype does not cause distress in the animals.

Frequency of monitoring: daily.

2.6 Are there changes to this SOP indicated in the AUP form? ☐YES ☒NO
If yes, specify changes: _____

2.7 PI Signature: _____ **Date:** 24-05-2007

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2. INFORMATION REQUIRED

2.1 Species/strain(s) ***(must refer to the Sp/strain column # of the table in "Description of animals" section in main protocol):*** mouse; Col 1-Cre; strain # 4

2.2 Transgenic? ☒	**Knockout?** ☐	**Knock/In?** ☐
Gene inserted (include promoter & gene): Cre recombinase under the control of the collagen type I promoter **Target tissue:** bone **Ancillary tissue:** osteoblasts	**Gene deleted:** _____ **Target tissue:** _____ **Ancillary tissue:** _____	**Gene deleted initially:** _____ **Gene inserted:** _____ **Target tissue:** _____ **Ancillary tissue:** _____

2.3 Supplier: ***Please include a complete address, contact person, phone, fax and email information. Note that a Certificate of health must be available to FACC personnel PRIOR to animal arrival.***

1. **Commercial:** _____
2. **Academic:** obtained from Dr. Gérard Karsenty, Columbia University, New York.

2.4 Phenotype: ***Include any trait included in a published article or reported to you by the originating investigator that may affect either the breeding, physical ability of the animal to move, eat or drink, or result in a decreased lifespan.***

1. **Heterozygotes:** none.
2. **Homozygotes:** none.

2.5 Clinical endpoint - General clinical endpoint criteria signs apply.

Supply criteria specific to phenotype: _____

Include plans to monitor or alleviate this distress: not applicable.

Frequency of monitoring: daily.

2.6 Are there changes to this SOP indicated in the AUP form? ☐YES ☒NO

If yes, specify changes: _____

2.7 PI Signature: _____ **Date:** 24-05-2007

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2. INFORMATION REQUIRED

2.1 Species/strain(s) ***(must refer to the Sp/strain column # of the table in "Description of animals" section in main protocol):*** mouse; Osx 1-Cre; strain # 5

2.2 Transgenic? ☒	**Knockout?** ☐	**Knock/in?** ☐
Gene inserted (include promoter & gene): Cre recombinase under the control of the Osterix I promoter	**Gene deleted:** _____	**Gene deleted initially:** _____
Target tissue: bone	**Target tissue:** _____	**Gene inserted:** _____
Ancillary tissue: osteoblasts	**Ancillary tissue:** _____	**Target tissue:** _____
		Ancillary tissue: _____

2.3 Supplier: ***Please include a complete address, contact person, phone, fax and email information. Note that a Certificate of health must be available to FACC personnel PRIOR to animal arrival.***

1. **Commercial:** _____
2. **Academic:** obtained from Dr. Andrew McMahon, Harvard University, Boston.

2.4 Phenotype: ***Include any trait included in a published article or reported to you by the originating investigator that may affect either the breeding, physical ability of the animal to move, eat or drink, or result in a decreased lifespan.***

1. **Heterozygotes:** none.
2. **Homozygotes:** none.

2.5 Clinical endpoint - General clinical endpoint criteria signs apply.

Supply criteria specific to phenotype: _____

Include plans to monitor or alleviate this distress: not applicable.

Frequency of monitoring: daily.

2.6 Are there changes to this SOP indicated in the AUP form? ☐YES ☒NO

If yes, specify changes: _____

2.7 PI Signature: _____ **Date:** 24-05-2007

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2. INFORMATION REQUIRED

2.1 Species/strain(s) ***(must refer to the Sp/strain column # of the table in "Description of animals" section in main protocol):*** mouse: TRAP-Cre; strain # 6

2.2 Transgenic? ☒	**Knockout?** ☐	**Knock/in?** ☐
Gene inserted (include promoter & gene): Cre recombinase under the control of the Tartrate-resistant acid phosphatase promoter **Target tissue:** bone **Ancillary tissue:** osteoclasts	**Gene deleted:** _____ **Target tissue:** _____ **Ancillary tissue:** _____	**Gene deleted initially:** _____ **Gene inserted:** _____ **Target tissue:** _____ **Ancillary tissue:** _____

2.3 Supplier: ***Please include a complete address, contact person, phone, fax and email information. Note that a Certificate of health must be available to FACC personnel PRIOR to animal arrival.***

1. **Commercial:** _____
2. **Academic:** obtained from Dr. David Roodman, University of Pittsburgh, Pittsburgh.

2.4 Phenotype: ***Include any trait included in a published article or reported to you by the originating investigator that may affect either the breeding, physical ability of the animal to move, eat or drink, or result in a decreased lifespan.***

1. **Heterozygotes:** none.
2. **Homozygotes:** none.

2.5 Clinical endpoint - General clinical endpoint criteria signs apply.

Supply criteria specific to phenotype: _____

Include plans to monitor or alleviate this distress: not applicable.

Frequency of monitoring: daily.

2.6 Are there changes to this SOP indicated in the AUP form? ☐YES ☒NO

If yes, specify changes: _____

2.7 PI Signature: _____ **Date:** 24-05-2007

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TRANSGENIC OR KNOCKOUT / IN MOUSE BREEDING

1. INTRODUCTION

Standard Operating Procedures (SOPs) provide a detailed description of commonly used procedures. SOPs offer investigators an alternative to writing detailed procedures on their protocol forms. Any deviation from the approved procedures must be clearly described and justified in the Animal Use Protocol form (AUP). Approval of the protocol indicates approval of the deviation from the SOP for that project only. A signed SOP form (pages up to signature only) must be attached to the AUP form. The relevant SOP number must be referred to in section 9 of the AUP.

2. INFORMATION REQUIRED

2.1 Species/strain(s) ***(must refer to the Sp/strain column # of the table in "Description of animals" section in main protocol):*** mouse: Col 1-Cre-ILK floxed; strain # 7

2.2 Transgenic? ☐	**Knockout?** ☒	**Knock/in?** ☐
Gene inserted (include promoter & gene): _____	**Gene deleted:** ILK	**Gene deleted initially:** _____
Target tissue: _____	**Target tissue:** bone	**Gene inserted:** _____
Ancillary tissue: _____	**Ancillary tissue:** osteoblast	**Target tissue:** _____
		Ancillary tissue: _____

2.3 Supplier: ***Please include a complete address, contact person, phone, fax and email information. Note that a Certificate of health must be available to FACC personnel PRIOR to animal arrival.***

1. **Commercial:** _____
2. **Academic:** Strain was engineered in my lab.

2.4 Phenotype: ***Include any trait included in a published article or reported to you by the originating investigator that may affect either the breeding, physical ability of the animal to move, eat or drink, or result in a decreased lifespan.***

1. **Heterozygotes:** none.
2. **Homozygotes:** no apparent phenotype in adult animals.

2.5 Clinical endpoint - General clinical endpoint criteria signs apply.

Supply criteria specific to phenotype: _____

Include plans to monitor or alleviate this distress: N/A.

Frequency of monitoring: daily.

2.6 Are there changes to this SOP indicated in the AUP form? ☐YES ☒NO
If yes, specify changes: _____

2.7 PI Signature: _____ **Date:** 24-05-2007

MCGILL UNIVERSITY
UNIVERSITY ANIMAL CARE COMMITTEE

UACC Standard Operating Procedure # 4 October 2005 version

TRANSGENIC OR KNOCKOUT / IN MOUSE BREEDING

1. INTRODUCTION

Standard Operating Procedures (SOPs) provide a detailed description of commonly used procedures. SOPs offer investigators an alternative to writing detailed procedures on their protocol forms. Any deviation from the approved procedures must be clearly described and justified in the Animal Use Protocol form (AUP). Approval of the protocol indicates approval of the deviation from the SOP for that project only. A signed SOP form (pages up to signature only) must be attached to the AUP form. The relevant SOP number must be referred to in section 9 of the AUP.

2. INFORMATION REQUIRED

2.1 Species/strain(s) ***(must refer to the Sp/strain column # of the table in "Description of animals" section in main protocol):*** mouse: Osx-1-Cre-ILK floxed; strain # 8

2.2 Transgenic? ☐	**Knockout?** ☒	**Knock/in?** ☐
Gene inserted (include promoter & gene): _____	**Gene deleted:** ILK	**Gene deleted initially:** _____
Target tissue: _____	**Target tissue:** bone	**Gene inserted:** _____
Ancillary tissue: _____	**Ancillary tissue:** osteoblast	**Target tissue:** _____
		Ancillary tissue: _____

2.3 Supplier: ***Please include a complete address, contact person, phone, fax and email information. Note that a Certificate of health must be available to FACC personnel PRIOR to animal arrival.***

1. **Commercial:** _____
2. **Academic:** Strain was engineered in my lab.

2.4 Phenotype: ***Include any trait included in a published article or reported to you by the originating investigator that may affect either the breeding, physical ability of the animal to move, eat or drink, or result in a decreased lifespan.***

1. **Heterozygotes:** none.
2. **Homozygotes:** unknown; strain will be bred in the future; phenotype disclosure form will be submitted upon characterization of a phenotype..

2.5 Clinical endpoint - General clinical endpoint criteria signs apply.

Supply criteria specific to phenotype: _____

Include plans to monitor or alleviate this distress: N/A.

Frequency of monitoring: daily.

2.6 Are there changes to this SOP indicated in the AUP form? ☐YES ☒NO
If yes, specify changes: _____

2.7 PI Signature: _____ **Date:** 24-05-2007

MCGILL UNIVERSITY
UNIVERSITY ANIMAL CARE COMMITTEE

UACC Standard Operating Procedure # 4 October 2005 version

TRANSGENIC OR KNOCKOUT / IN MOUSE BREEDING

1. INTRODUCTION

Standard Operating Procedures (SOPs) provide a detailed description of commonly used procedures. SOPs offer investigators an alternative to writing detailed procedures on their protocol forms. Any deviation from the approved procedures must be clearly described and justified in the Animal Use Protocol form (AUP). Approval of the protocol indicates approval of the deviation from the SOP for that project only. A signed SOP form (pages up to signature only) must be attached to the AUP form. The relevant SOP number must be referred to in section 9 of the AUP.

2. INFORMATION REQUIRED

2.1 Species/strain(s) ***(must refer to the Sp/strain column # of the table in "Description of animals" section in main protocol):*** mouse: TRAP-Cre-ILK floxed; strain # 9

2.2 Transgenic? ☐	**Knockout?** ☒	**Knock/in?** ☐
Gene inserted (include promoter & gene): _____	**Gene deleted:** ILK	**Gene deleted initially:** _____
Target tissue: _____	**Target tissue:** bone	**Gene inserted:** _____
Ancillary tissue: _____	**Ancillary tissue:** osteoclast	**Target tissue:** _____
		Ancillary tissue: _____

2.3 Supplier: ***Please include a complete address, contact person, phone, fax and email information. Note that a Certificate of health must be available to FACC personnel PRIOR to animal arrival.***

1. **Commercial:** _____
2. **Academic:** Strain was engineered in my lab.

2.4 Phenotype: ***Include any trait included in a published article or reported to you by the originating investigator that may affect either the breeding, physical ability of the animal to move, eat or drink, or result in a decreased lifespan.***

1. **Heterozygotes:** none.
2. **Homozygotes:** mild osteopetrosis.

2.5 Clinical endpoint - General clinical endpoint criteria signs apply.

Supply criteria specific to phenotype: _____

Include plans to monitor or alleviate this distress: N/A; the phenotype does not cause distress in the animals.

Frequency of monitoring: daily.

2.6 Are there changes to this SOP indicated in the AUP form? ☐YES ☒NO
If yes, specify changes: _____

2.7 PI Signature: _____ **Date:** 24-05-2007

MCGILL UNIVERSITY
UNIVERSITY ANIMAL CARE COMMITTEE

UACC Standard Operating Procedure # 4 October 2005 version

TRANSGENIC OR KNOCKOUT / IN MOUSE BREEDING

1. INTRODUCTION

Standard Operating Procedures (SOPs) provide a detailed description of commonly used procedures. SOPs offer investigators an alternative to writing detailed procedures on their protocol forms. Any deviation from the approved procedures must be clearly described and justified in the Animal Use Protocol form (AUP). Approval of the protocol indicates approval of the deviation from the SOP for that project only. A signed SOP form (pages up to signature only) must be attached to the AUP form. The relevant SOP number must be referred to in section 9 of the AUP.

2. INFORMATION REQUIRED

2.1 Species/strain(s) ***(must refer to the Sp/strain column # of the table in "Description of animals" section in main protocol):*** mouse: ILK-beta3 integrin compound mutant; strain # 10

2.2 Transgenic? ☐	**Knockout?** ☒	**Knock/in?** ☐
Gene inserted (include promoter & gene): _____	**Gene deleted:** ILK and beta 3 integrin	**Gene deleted initially:** _____
Target tissue: _____	**Target tissue:** bone	**Gene inserted:** _____
Ancillary tissue: _____	**Ancillary tissue:** osteoclast	**Target tissue:** _____
		Ancillary tissue: _____

2.3 Supplier: ***Please include a complete address, contact person, phone, fax and email information. Note that a Certificate of health must be available to FACC personnel PRIOR to animal arrival.***

1. **Commercial:** _____
2. **Academic:** Strain will be bred in my lab.

2.4 Phenotype: ***Include any trait included in a published article or reported to you by the originating investigator that may affect either the breeding, physical ability of the animal to move, eat or drink, or result in a decreased lifespan.***

1. **Heterozygotes:** unknown; strain will be bred in the future; phenotype disclosure form will be submitted upon characterization of a phenotype.
2. **Homozygotes:** not applicable.

2.5 Clinical endpoint - General clinical endpoint criteria signs apply.

Supply criteria specific to phenotype: _____

Include plans to monitor or alleviate this distress: N/A.

Frequency of monitoring: daily.

2.6 Are there changes to this SOP indicated in the AUP form? ☐YES ☒NO
If yes, specify changes: _____

2.7 PI Signature: _____ **Date:** 24-05-2007

MCGILL UNIVERSITY
UNIVERSITY ANIMAL CARE COMMITTEE

UACC Standard Operating Procedure # 10 October 2005 version

RODENT SURGERY

1. INTRODUCTION

Standard Operating Procedures (SOPs) provide a detailed description of commonly used procedures. SOPs offer investigators an alternative to writing detailed procedures on their protocol forms. Any deviation from the approved procedures must be clearly described and justified in the Animal Use Protocol form (AUP). Approval of the protocol indicates approval of the deviation from the SOP for that project only. A signed SOP cover page must be attached to the Animal Use Protocol form. The relevant SOP number must be referred to in the Procedures section.

2. INFORMATION REQUIRED

2.1 **Species/strain(s):** ***(must refer to the Sp/strain column # of the table in "Description of animals" section in main protocol)***
Mouse: strains 7-10

2.2 ☒ **Survival surgery** ☐ **Non-survival surgery**

2.3 **Anaesthesia chosen:**

Procedure:	Agent:	Dose:	Route:	Re-administration
ovariectomy	Isoflurane	1-5% to effect	inhalation	to effect

2.4 **Details and expected duration of specific surgical procedure must be in the procedure section of the main protocol** ***(section 10a of full protocol form; section 6b of renewal form)***

2.5 **Intraoperative and /or post-operative analgesia** ***(for all non-survival surgeries, intraoperative analgesia may be required, however, there is no need for post operative analgesia)*:**

Procedure:	Agent:	Dose:	Route:	Re-administration
ovariectomy	buprenorphine	0.05 mg/kg	i.m.	one dose 30 min pre-surgery; as required post-op.

2.6 **Clinical endpoint –**
Clinical signs of distress requiring euthanasia: more than 20% weight loss, lack of grooming, vocalizing, ulceration, infection and surgery specific:
Immediate: hypothermia, recovery from anesthesia
In recovery: wound healing, infection, ulceration at wound site.
Add additional endpoint criteria that would be specific to the procedures:

Attention must be given to wound healing, sutures, hypothermia and recovery from anaesthesia.

Frequency of monitoring: daily

2.7 **Are there changes to this SOP indicated in the AUP form?** ☐YES ☒NO
If yes, specify changes: _____

2.8 **PI Signature:** _____ **Date:** 20 June 2007

PLEASE ATTACH ONLY THIS SIGNED COVER SHEET TO THE BACK OF EACH RELEVANT AUP FOR ANIMAL CARE COMMITTEE APPROVAL.

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

2. **Species**: mouse

   Alteration: ILK KO

   Background strain: C57Bl6

   Target Tissue: all

   DNA construct injected:

   Ancillary Tissue:

   Enhancer/promoter:

   Transgene:

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.

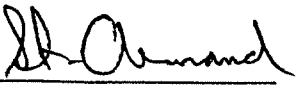
   B. Homozygotes: embryonic lethality at E3.5 (implantation)

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

   Include plans to monitor or alleviate this distress: not applicable.

   Frequency of monitoring: daily.

5. **Indicate how or if this observed phenotype will alter your Category of Invasiveness.**
   Not applicable.

6. **Signature:**  **Date:** 24-05-2007

Approved April 28, 1999
Revised November 2003

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

2. **Species**: mouse

   Alteration: beta 3 integrin KO

   Background strain: C57Bl6

   Target Tissue: all

   DNA construct injected:

   Ancillary Tissue:

   Enhancer/promoter:

   Transgene:

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.**

   Include plans to monitor or alleviate this distress: not applicable. The phenotype does not cause distress in the mice.

   Frequency of monitoring: daily.

5. **Indicate how or if this observed phenotype will alter your Category of Invasiveness.**
   Not applicable.

6. **Signature:** _____ **Date:** 24-05-2007

Approved April 28, 1999
Revised November 2003

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

2. Species: mouse

Alteration: TRAP-Cre ILK floxed

Background strain: C57Bl6

Target Tissue: bone: osteoclasts.

DNA construct injected:

Ancillary Tissue:

Enhancer/promoter:

Transgene:

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.

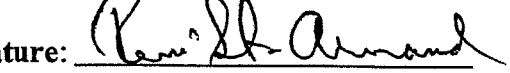
B. Homozygotes: Mild osteopetrosis.

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

Include plans to monitor or alleviate this distress: not applicable. The phenotype does not cause distress in the mice.

Frequency of monitoring: daily.

5. Indicate how or if this observed phenotype will alter your Category of Invasiveness.
Not applicable.

6. Signature:  **Date:** 24-05-2017

Approved April 28, 1999
Revised November 2003