COORDINATION OF CELL CYCLE AND CELL DIFFERENTIATION BY RECEPTOR ACTIVATOR OF NF-KAPPA-B LIGAND DURING OSTEOCLAST DIFFERENTIATION

DISSERTATION

Presented in Partial Fulfillment of the Requirements for The Degree
Doctor of Philosophy in the Graduate School of The Ohio State University

By
Uma Sankar

The Ohio State University
2003

Dissertation Committee: Approved by
Professor Michael C. Ostrowski, Adviser
Professor Gustavo W. Leone Adviser
Professor Natarajan Muthusamy Molecular, Cellular and Developmental Biology
Professor Russell J. Hill
ABSTRACT

Osteoclasts are bone resorbing multinuclear cells formed by the fusion of hematopoietic mononuclear precursor cells of the macrophage/monocyte lineage. Microphthalmia transcription factor (MITF) is a basic helix-loop-helix leucine zipper transcription factor that is important for the differentiation of many cell types, including osteoclasts and melanocytes. MITF regulates the expression of osteoclast-differentiation marker genes, Tartrate-resistant acid phosphatase (TRAP) and cathepsin K. Deletion in arginine 215 in the basic domain of MITF results in severe osteopetrosis in homozygous recessive mice (Mitf\textsuperscript{mi/mi}). A substitution of arginine 216, in the basic domain, with lysine results in age resolving osteopetrosis in mice in the homozygous condition (Mitf\textsuperscript{or/or}). However, mice that are homozygous recessive for a substitution of isoleucine to asparagline in the basic domain of this transcription factor (Mitf\textsuperscript{wh/wh}) do not exhibit any osteopetrosis. We identified several novel genes regulated by Mitf with potential roles in osteoclast differentiation via microarray analysis of cDNA from WT and Mitf\textsuperscript{mi/mi} osteoclasts. In particular, Eos, HOX11L2, Hematopoietic cell phosphatase (HCP) and p9 were confirmed to be expressed in lower levels in Mitf\textsuperscript{mi/mi} osteoclasts compared to the WT. We also observed that while TRAP mRNA levels were upregulated in Mitf\textsuperscript{or/or}, similar to the levels in WT, Cathepsin...
K levels were lower in both Mitf\textsuperscript{mi/mi} and Mitf\textsuperscript{or/or} osteoclasts. Thus, Mitf-regulated genes could be classified into two groups based on their expression pattern in the Mitf\textsuperscript{or/or} mutant.

Osteoclast progenitors shift from a population of actively proliferating cells to that of committed, post-mitotic mononuclear precursor cells prior to becoming multinuclear osteoclasts. We observed that the cytokine, receptor activator of NF-kB ligand (RANKL), induces wildtype (WT) osteoclast progenitors to withdraw from cell cycle within 24 hours of its application. This event coincides with elevation in p27\textsuperscript{KIP1} (via the p38 MAPK pathway) and p21\textsuperscript{CIP1} and with decreased CDK2 activity. We also observed that p27\textsuperscript{KIP1} is required by osteoclast progenitors to exit from the cell cycle in response to RANKL and that p27\textsuperscript{KIP1/-} osteoclasts express lower levels of TRAP mRNA. However, deficiency in p21\textsuperscript{CIP1} does not affect any of these phenomena. We also observed that removal of p27\textsuperscript{KIP1} or p21\textsuperscript{CIP1} alone does not affect osteoclast differentiation or function, \textit{in vitro} or \textit{in vivo}. Osteoclast progenitors from p27\textsuperscript{KIP1/-}p21\textsuperscript{CIP1/-} double knockout mice do not withdraw from cell cycle in response to RANKL and express significantly lower levels of TRAP and Cathepsin K mRNA. p27\textsuperscript{KIP1/-}p21\textsuperscript{CIP1/-} mice exhibit age resolving osteopetrosis. In addition, precursors from the double mutant mice form fewer multinuclear functional osteoclasts \textit{in vitro}. These data suggest that only p27\textsuperscript{KIP1} has a role in cell cycle withdrawal during osteoclast differentiation while both p21\textsuperscript{CIP1} and p27\textsuperscript{KIP1} might have additional, potentially redundant roles, during osteoclast differentiation.
Dedicated to my parents and to Keith, my best friend ....
ACKNOWLEDGMENTS

I would like to thank my adviser, Dr. Michael C. Ostrowski, for his guidance, support and for the helpful scientific discussions. I would also like to thank Drs. Gustavo W. Leone, Raj Muthusamy and Russell J. Hill, members of my dissertation committee, for their helpful guidance and support. In addition, I would like to thank all the past and present members of the Ostrowski laboratory for their support and discussions.

I would also like to thank Dr. Thomas Rosol for his help with the analysis of the osteopetrotic phenotype in the mutant mice. I would also like to thank Drs. Andrew Koff and Ming You for providing us with the p27^KIP1 and p21^CIP1 knockout mice. Special thanks to the staff at the ULAR facility at the Ohio State University for the help with maintaining the mouse colonies and to Evelyn at the College of Veterinary Biosciences for help with the histological sections. Also, special thanks to Krupen Patel for help with maintaining the mouse colonies and help with the histochemical analysis.

Finally, I would like to thank my family for their love and support and my husband Keith for his love and support during my graduate school career.
VITA

May 1, 1968 .............................................. Born, Trivandrum, India

1992 ..................................................... BSc. (Agriculture), Kerala
Agricultural University, India

1997 ..................................................... MS. Department of Horticulture and
Crop Sciences, The Ohio State University

PUBLICATIONS


FIELDS OF STUDY

Major Field: Molecular, Cellular and Developmental Biology
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ABBREVIATIONS

bHLHZIP- Basic helix-loop-helix-leucine zipper
BrdU-Bromodeoxy Uridine
DKO-Double knockout
M-CSF1-Macrophage colony stimulating factor 1 (also, CSF1)
Mitf-Micophthalmia-associated transcription factor
OCLs-Osteoclasts
PI-Propidium Iodide
RANKL- Receptor activator of NFκB ligand
RANK-Receptor activator of NFκB
TBA-Total bone area
TBP-Total bone perimeter
TBS-Total bone surface
TRAP-Tartrate resistant acid phosphatase
UBA-Unresorbed bone area
WT-Wild Type
CHAPTER 1

INTRODUCTION

"We have penetrated far less deeply into the regularities obtaining within the realm of living things, but deeply enough nevertheless to sense at least the rule of fixed necessity... What is still lacking here is a grasp of the connections of profound generality, but not a knowledge of order itself." Albert Einstein

Development is a fascinating, orderly yet intricate multicellular process where a complete organism arises from a single fertilized egg. In the course of development, the same genetic material present in every cell, directs each cell in a different manner so that it can grow, multiply and finally differentiate to assume a specific cell or tissue fate in a multicellular organism. Different pathways are switched “on” and “off” to co-ordinate the assumption of distinct morphologic characteristics of a fully differentiated cell. Understanding the intricacies of such a complex co-ordination of molecular and cellular events
during multicellular development has been a constant quest for generations of life scientists.

Bone is a vertebrate tissue unique in texture and composition from its evolutionary predecessors. The vertebrate skeletal system has evolved from a “gel” or “sol” support system in unicellular organisms to the “exoskeletal or dermal” structures such as fins, to the contemporary rigid vertebral column, formed initially of cartilage and later replaced with mineralized skeletal structures. In addition to serving as a mechanical support, the bone also acts as a huge reservoir of calcium and bone marrow cells in the vertebrate body.

Composed of mineralized matrix, the vertebrate bone is a living tissue containing two diverse yet interconnected cell populations, viz., the mesenchymal chondroblast and osteoblast cells that form the skeleton and the hematopoietic osteoclast cells that resorb the mineralized bone matrix (Marks and Popoff, 1988). Understanding the biology of the cells of the bone that make it a living mass is crucial to the comprehension of the biology of development, maintenance and repair of the bone tissue and to the treatment of the various disorders in the bone.

1.1 THE BONE TISSUE

The bone tissue is made up of specifically oriented type I collagen fibers and non-collagenous glycoproteins and proteoglycans termed “ground substance”. Hydroxyapatite crystals ([3Ca₃(PO₄)₂].(OH)₂) are embedded in the
collagen fibers and in the ground substance of the matrix, oriented in the same
direction as the collagen fibers. The highly ionic ground substance is thought to
play a role in the calcification of the collagenous matrix during bone formation
(Baron, 1999). The calcified bone contains living osteocytes, which are the
bone forming osteoblasts embedded in small osteocytic lacunae, inside the
calcified matrix (25,000/mm$^2$ of bone) (Baron, 1999). These features render the
bone, a developmentally complex tissue.

1.1.1 DEVELOPMENT OF BONE TISSUE

The skeleton is formed by two distinct processes of bone development:
intramembraneous ossification and endochondral ossification. Flat bones (skull
bones, scapula, mandible) form primarily via intramembraneous ossification
while long bones (femur, tibia, humerus) are formed by a combination of
intramembraneous and endochondral ossifications. An appreciation of the
structural make-up of long bones is essential to understand the types of bone
development.

1.1.2 STRUCTURE OF THE LONG BONE

The long bone has two extremities called the epiphyses, a midshaft or
the diaphysis and a growing zone in between termed the metaphysis (Figure 1).
The epiphysis is separated from the metaphysis by a layer of epiphyseal
cartilage or the growth plate. The growth plate consists of a layer of proliferating
chondroblasts and an expanding layer of cartilage that becomes increasingly calcified, remodeled and replaced with new bone as the growth phase continues. The cortical bone, which forms the external part of the long bone, is thinner in the metaphysis and epiphyses areas while it is thick and dense in the diaphysis where it encloses the bone marrow cavity. The internal space in the epiphyseal and metaphyseal areas contains thin calcified trabeculae, also called spongy or cancellous bone, which also enclose hematopoietic bone marrow. Finally, there are two main surfaces of the long bone, the external or the periosteal surface and the internal or the endosteal surface. Cortical and trabecular bones are comprised of the same cells and same matrix components. However, 90% of the cortical bone is calcified whereas only 25% of the trabeculae are calcified. The trabecular bone also contains bone marrow, blood vessels and connective tissue, indicating that it primarily plays a metabolic role whereas the cortical bone plays a mechanical and protective role (Marks and Popoff, 1988; and Baron, 1999). The long bone is formed by a combination of intramembraneous and endochondral ossifications as detailed in subsequent sections.
Figure 1. Schematic view of a longitudinal section through a growing long bone.
1.1.3 INTRAMEMBRANEOUS AND ENDOCHONDRAL OSSIFICATIONS

During intramembranous ossification, mesenchymal cells within a highly vascularized area proliferate and differentiate into osteoblasts. These osteoblasts synthesize a bone matrix, with irregularly oriented collagen fibers, delayed in calcification. Blood vessels present in the trabeculae of this “woven bone” later form the bone marrow cavity. This “woven bone” is later replaced with mature lamellar bone (Baron, 1999).

During the endochondral ossification phase of long bone formation, mesenchymal cells undergo condensation at sites that will occupy the future skeletal elements (Figure 2). These mesenchymal cells within the condensed regions undergo differentiation into chondroblasts, which secrete the cartilaginous matrix. The chondroblasts get progressively trapped in the cartilaginous matrix within lacunae and are termed chondrocytes. Once the cartilage is calcified, the chondrocytes become progressively enlarged, then undergo hypertrophy followed by apoptosis. Thereafter, the chondrocytes are restricted to a narrow zone termed the growth plate where they participate in longitudinal growth along with the osteoblasts (Marks and Popoff, 1988; Baron, 1999; Wagner and Karsenty, 2001).
Figure 2. Schematic view of stages in endochondral ossification in a developing equine long bone.
A. Patch of woven bone formation, penetration of cartilage by osteoclasts followed by vascular invasion. B. Bone marrow cavity formation by osteoclasts, secondary ossification in the epiphysis. C. Longitudinal growth by ossification of the epiphysis.
(Reproduced from McIlwraith CW. In: Developmental Orthopedic Disease. http://www.colostate.edu/depts/equine/graduate/orthopedics/questions/dod.html website)
During early long bone development, a patch of woven bone is formed around the future midshaft area of the long bone and is rapidly calcified. Multinuclear osteoclasts penetrate the cartilage by digesting the patch of calcified woven matrix and begin resorbing the calcified cartilaginous matrix. This is followed by vascular invasion of the partially resorbed cartilage to form the future bone marrow cavity. The blood vessels bring mesenchymal cells with them, which will differentiate into future osteoblasts (Figure 2).

The newly formed osteoblasts deposit woven bone to replace the unresorbed cartilage and this becomes the trabecular bone termed primary spongiosa. This woven bone undergoes further remodeling to become the secondary spongiosa. Thus, the main difference between the two types of ossification processes is that in intramembraneous ossification, there is no cartilage model and that the mesenchymal cells differentiate directly into osteoblasts without a chondroblast intermediary (Marks and Popoff, 1988; Baron, 1999; Wagner and Karsenty, 2001). In a normal adult skeleton, bone formation occurs only in areas where bone resorption had previously taken place. This timed process, termed “bone remodeling” is the major method of bone turnover after the initial period of development and growth (Figures 3 and 4 respectively). Bone formation and maintenance is made possible by the coordinated activities of the two interconnected cell populations in the bone; the mesenchymal chondrocytes and osteoblasts and the hematopoietic osteoclasts.
Figure 3. Schematic illustration of bone remodeling.
Bone remodeling sequence as occurring in trabecular bone.
Figure 4. Illustration of the duration of various phases of trabecular bone remodeling sequence.
Duration of trabecular bone remodeling calculated by histomorphometric analysis of bone biopsy samples obtained from young individuals. The different phases in the illustration are as follows: (I) osteoclastic resorption; (II) reversal of resorption; (III) preosteoblast migration and differentiation into osteoblasts; (IV) osteoblastic matrix formation and (V) mineralization of the bone matrix.
1.2 CELLS IN THE BONE

Chondrocytes, osteoblasts and osteoclasts are the three main types of cells in the bone and make the bone tissue a living mass.

1.2.1 CHONDROCYTES

Chondrocytes, that secrete the catilagenous matrix, originate from mesenchymal precursors and exist in four subpopulations during endochondral growth plate development, viz., resting, proliferating, prehypertrophic and hypertrophic populations. These stages in the life of a chondrocyte are characterized by distinct molecular markers and are controlled by a network of growth factors and signaling molecules. Fibroblast growth factor (FGF) inhibits chondrocyte proliferation via the engagement of its receptor, FGFR3. Activating mutations in FGFR3 leads to decreased bone growth in achondroplasia and hypochondroplasia in humans (Olsen, 1999; Wagner and Karsenty, 2001). The transcription factor, Sox-9 is required for early mesenchymal condensation. The parathyroid hormone related peptide (PTHrP) and the Indian hedgehog (Ihh) regulate chondrocyte maturation. Ihh secreted by prehypertrophic chondrocytes upregulates PTHrP, which in turn suppress their differentiation into hypertrophic chondrocytes, blocking endochondral bone formation (Lanske et al., 1996; Vortkamp et al., 1996; St-Jacques et al., 1999; Olsen, 1999 and Wagner and Karensty, 2001). Most recently, it was shown that the transcription factor Cbfa1, a major regulator of osteoblast differentiation, might also play a role in chondrocyte hypertrophy. Cbfa1 null mice lack hypertrophic chondrocytes in
several but not all skeletal elements (Inada et al., 1999; Kim et al., 1999; Takeda et al., 2001; Ueta et al., 2001). Finally, the hypertrophic chondrocytes secrete vascular endothelial growth factor (VEGF), which promotes vascular invasion of the calcified matrix as it is resorbed by the multinuclear osteoclast.

1.2.2 OSTEOBLASTS

Osteoblasts, the cells responsible for secretion of the bone matrix, are of mesenchymal origin and express high levels of alkaline phosphatase enzyme activity as they differentiate. Ultrastructurally, the osteoblast cells have a large nucleus, an enlarged Golgi and an extensive endoplasmic reticulum (ER). Insulin-like growth factor (IGF), transforming growth factor-β (TGF-β), FGF, platelet-derived growth factor (PDGF), parathyroid hormone (PTH) and PTHrP, 1,25(OH)2D3 (Vitamin D3), glucocorticoids and prostaglandin E2 and various cytokines regulate osteoblast proliferation and differentiation. The earliest specific marker of osteoblast differentiation is Cbfa1, which is required for osteoblast differentiation. Overexpression of Cbfa1 induces osteoblast-specific gene expression in fibroblasts and myoblasts and induces ectopic endochondral bone formation in vivo. Cbfa1 null mutant mice display a complete lack of bone formation due to defects in osteoblast differentiation (Ducy et al., 1997; Komori et al., 1997; Lian et al., 1999; Wagner and Karensty, 2001).

During endochondral bone formation and during the remodeling process in the adult bone, osteoblasts and osteoclasts work closely with each other
(Figure 3). Osteoblasts produce several cytokines that support osteoclast differentiation and survival. The constant communication between the osteoblast and osteoclasts is very important for the growth and maintenance of healthy bone, healing of fractures and for the maintenance of calcium homeostasis in the body. Disruption in this communication result in disease conditions such as osteoporosis and osteopetrosis. Osteoporosis is a metabolic bone disease characterized by low bone mass and microarchitectural deterioration of bone tissue, leading to enhanced bone fragility and a consequent increase in risk of fracture (Wasnich, 1999). Osteopetrosis is an inherited metabolic bone disorder characterized by accumulation of primary spongiosa in bone marrow cavities due to reduced or blocked osteoclast formation and function (Marks and Lane, 1976; Marks, 1987 and Nii et al., 1995).

1.2.3 OSTEOCLASTS

Osteoclasts are multinuclear cells, arising from precursors of the hematopoietic, monocyte/macrophage lineage that are primarily responsible for bone resorption. In co-ordination with osteoblasts, osteoclasts play an important role in the healthy maintenance of the bone tissue.

1.2.3.1 MORPHOLOGY OF THE OSTEOCLAST

Fully differentiated osteoclasts are giant multinuclear cells that contain between 2 and 20 nuclei and ranging in size up to 100 mm in diameter.
Osteoclasts are found attached to bone surfaces, usually located on the endosteal surface or on the periosteal surface beneath the periosteum. These are extremely rare cells on an adult bone, usually 2-3 per mm\(^3\) although it is not unusual to see up to 4 or 5 osteoclasts in the same resorptive site. However, their numbers are markedly increased in the metaphyseal areas of the growing bone.

Ultrastructurally, a typical osteoclast has abundant Golgi complexes characteristically located in the perinuclear area, numerous and pleomorphic mitochondria and a large number of lysosomes and transport vesicles loaded with lysosomal enzymes (Figure 5). The cytoplasm appears densely granular and “foamy” due to the presence of several vacuoles. The nuclei are centrally located, differ in shape from one another and have one or two nucleoli per nucleus. The most prominent ultrastructural feature of osteoclasts is the presence of ruffled borders, which are deep folding of the plasma membrane to form finger-like cytoplasmic projections in the area of the cell facing the bone surface. A ring of contractile proteins in the center of the ruffled border, termed the “sealing zone”, facilitates attachment to the bone surface, essentially “sealing off” the sub-osteoclastic bone-resorbing compartment from the rest of the microenvironment. The cytoplasm over the sealing membrane is devoid of any organelles except for free polyribosomes and abundant filamentous actin (F-actin) and is termed the “clear zone”. (Marks and Popoff, 1988; Roodman, 1996; Suda et al., 1997; Karsenty, 1999; Baron, 1999 and Teitelbaum, 2000).
Once attached to the bone surface, the osteoclast assumes an "activated" state.

**Figure 5. Section of an osteoclast stained for the lysosomal enzyme arylsufatase.**
Nuclei (n), endoplasmic reticulum (ER), sealing zone (sz; single arrow) and extracellular space between foldings of the plasma membrane (double arrows) are shown.
1.2.3.2 MECHANISM OF BONE RESORPTION BY OSTEOCLASTS

1.2.3.2.1 ATTACHMENT TO BONE SURFACE AND CONSEQUENT SIGNALING EVENTS

Attachment of osteoclasts to the bone surface is facilitated by receptors belonging to the integrin superfamily present on the sealing membrane. The heterodimeric $\alpha\beta$ integrin receptors have a characteristic arginine-glycine-aspartate (RGD) trimeric sequence that recognize the same RGD trimeric repeats in matrix proteins such as collagens, osteopontin, fibronectin, vitronectin, and thrombospondin. The predominant integrin receptor found on osteoclast plasma membrane is the $\alpha V\beta_3$ or vitronectin receptor (Marks and Popoff, 1988; Teti et al., 1991; Roodman, 1996; Suda et al., 1997; Baron, 1999 and Teitelbaum, 2000).

The major attachment molecule in osteoclasts is $\alpha V\beta_3$ integrin. Mice with targeted disruption of $\beta_3$ integrin subunit are not osteopetrotic, but develop mild osteosclerosis later in life. The osteoclasts from these mice exhibit normal polarization with mild disruption in the ruffled border, do not form actin rings and resorb bone with reduced efficiency (Ducy et al., 1997 and McHugh et al., 2000). Blocking $\alpha V\beta_3$ integrin using echistatin, a disintegrin, was shown to inhibit osteoclast migration. Moreover, $\alpha V\beta_3$ deficient mice have low levels of blood calcium and increased skeletal mass (Teitelbaum, 2000). The precise function of integrin might not be to form the tight sealing zone, but to initiate signaling events upon its engagement, that might help in the spreading and
motility of the active osteoclasts (Teitelbaum, 2000 and Duong and Rodan, 2001).

Engagement of \(\alpha V\beta_3\) integrin receptors during the formation of the sealing zone by osteoclasts has been shown to activate phosphatidyl inositol 3-kinase (PI-3K), non-receptor tyrosine kinase c-Src, adhesion-related tyrosine kinase PYK2 and the integrin-related signaling molecule p130Cas (Cas, Crk associated substrate) (Figure 6). PI-3K was shown to translocate to the cytoplasm following engagement of \(\alpha V\beta_3\) integrin receptor. Addition of Wortmannin, a potent inhibitor of PI-3K activity, inhibits osteoclast resorption in vitro (Sato et al., 1996; Nakamura et al., 1995 and Duong and Rodan, 2001). Herbimycin A, an inhibitor of tyrosine kinases was shown to inhibit osteoclast resorption in vivo and in vitro, thus demonstrating that the activity of these kinases is important for osteoclast activity (Yoneda et al., 1993 and Suda et al., 1997). Mouse deficient for c-Src exhibit severe osteopetrosis due to the inactivation of osteoclasts. The osteoclasts from these mice do not form ruffled borders or F-actin rings and fail to adhere to the bone surface (Soriano et al., 1991). Further, the tyrosine phosphorylation and kinase activities of PYK2 is markedly reduced in these osteoclasts, confirming that PYK2 acts downstream of c-Src in this pathway (Figure 6).
Figure 6. Model for $\alpha V\beta 3$ integrin-mediated signaling pathway during attachment to bone matrix by osteoclasts. (Duong et al., 2000)
In normal osteoclasts, clustering of $\alpha_v\beta_3$ integrin increases PYK2 phosphorylation leading to its tight association with c-Src and in activated osteoclasts, PYK2 localizes to the sealing zone where it interacts with the cytoskeletal molecule paxillin. Inhibiting PYK2 with antisense oligonucleotides inhibits osteoclast spreading, motility and resorption. PYK2 is thought to play a role in cytoskeletal rearrangement associated with osteoclast adhesion, spreading, motility and polarization (Duong et al., 1998 and Duong and Rodan, 2001). The signaling molecule p130$^{\text{Cas}}$ becomes highly tyrosine phosphorylated upon $\alpha_v\beta_3$ clustering and this phosphoprotein localizes to the sealing zone of activated osteoclasts (Figure 6) (Nakamura et al., 1998 and Lakkakorpi et al., 1999). Thus, activation of c-Src/PYK2/p130$^{\text{Cas}}$ via integrin engagement plays a major role in osteoclast attachment to the bone surface and its spreading and migration during bone resorption (Duong et al., 2000; Teitelbaum, 2000 and Duong and Rodan, 2001).

1.2.3.2.2 ACTIVATION OF OSTEOCLASTS

Osteoclasts become activated following attachment to the bone surface (Figure 7). Activated osteoclasts are highly polarized cells and remain so during resorption. The plasma membrane of a polarized osteoclast is characterized by the presence of two domains; the basolateral domain facing the vascular stream and the apical domain facing the bone surface to which the cell is attached (Figure 7). The nuclei and other organelles are located in the
basolateral domain while the apical domain predominantly contains lysosomes. As mentioned earlier, the polarized osteoclasts have a characteristic ruffled border, with sealing zones that help attach to the bone surface (Figure 7). The plasma membrane in the sealing zone is termed the sealing membrane. The cytoplasm over the sealing membrane is devoid of any organelles except for free polyribosomes and abundant filamentous actin (F-actin) and is termed the “clear zone”. The F-actin in the sealing zone localizes in punctuate plasma membrane protrusions called “podosomes” (Marks and Popoff, 1988; Teti et al., 1991; Roodman, 1996; Baron, 1999 and Teitelbaum, 2000).

The plasma membrane of the ruffled borders contains lysosomal proteins such as matrix metalloproteinases 9 (MMP9), cysteine proteases such as cathepsin K and phosphatases such as tartarate-resistant acid phosphatase (TRAP). It also contains electrogenic proton adenosine triphosphatase (H\(^{+}\)-ATPase) pumps and chloride channels (CIC-7) that are involved in the acidification of the sealing zone. The basolateral plasma membrane is enriched in sodium-potassium pumps (Na\(^{+}\), K\(^{+}\) ATPase), HCO\(_{3}^{-}\)/Cl\(^{-}\) exchangers, Na\(^{+}\)/Ca\(_{2}^{+}\) exchangers and Na\(^{+}\)/H\(^{+}\) exchangers that help maintain the intra-osteoclastic pH during the acidification process (Figure 8) (Marks and Popoff, 1988; Roodman, 1996; Baron, 1999 and Teitelbaum, 2000).

Following attachment to the bone and consequent activation the osteoclast cell engages in active demineralization, bone matrix degradation and resorption.
Figure 7. Schematic representation of a non polarized (A) and a polarized (B) osteoclast.
Osteoclasts are able to switch between polarized and non-polarized states based on their attachment to the bone matrix.
(Teti et al., 1991)
1.2.3.2.3 Bone Demineralization and Resorption by Osteoclasts

With the help of H^+ ATPase pumps and charge-coupled ClC-7 channels located on the ruffled borders, the osteoclast rapidly acidifies the extracellular compartment to a pH of ~4.5 by secreting H^+ and Cl^- ions across the ruffled borders. Protons for this purpose are supplied by the enzyme carbonic anhydrase II present in the cytosol; the ATP and CO_2 are supplied by the mitochondria while the HCO_3^-/Cl^- exchangers bring in the Cl^- ions. Further, cathepsin K and TRAP are also secreted into the extracellular compartment beneath the ruffled borders. The extracellular compartment essentially serves as a secondary lysosome with acidic pH and with the presence of lysosomal enzymes such as cathepsin K and MMP-9. The acid environment in the extracellular compartment leads to the releasing and dissolving of hydroxyapatite [Ca_{10}(PO_4)_6(OH)_2] crystals from the collagen matrix; the process termed demineralization. The lysosomal enzymes, which are active in the highly acidic environment, now start degrading the matrix components, the products of which are endocytosed, transported and released at the basolateral domain of the osteoclasts (Figures 8 and 9). Thus, osteoclastic bone resorption helps maintain calcium and inorganic phosphate homeostasis (Baron, 1999 and Teitelbaum, 2000).
Figure 8. Schematic representation of enzyme secretion and ion transport polarity in osteoclasts during active bone resorption.
Figure 9. Schematic illustration of vesicular movement during bone resorption by osteoclasts.
Movement of vesicular carrying cargos of degraded collagen move towards the basolateral domain for transcytosis. Latent TGF-b gets released to the basolateral extracellular space in this manner, which then inhibits bone resorption by osteoclasts.
(Mostov and Werb, 1997)
Osteoclasts from mice lacking cathepsin K, carbonic anhydrase II and H\(^{+}\)ATPase fail to resorb bone. Osteoclasts lacking H\(^{+}\)ATPase and carbonic anhydrase II fail to acidify the resorptive microenvironment and those lacking cathepsin K cannot degrade bone collagen (Sly et al., 1983; Gowen et al., 1999 and Li et al., 2000). Also, antisense oligonucleotides that inhibit carbonic anhydrase II block osteoclast bone resorption (Roodman, 1996). Kornak et al. reported that mice deficient for the ubiquitous expression of ClC–7 HCl\(^{-}\) channel exhibit severe osteopetrosis. The multinuclear osteoclasts fail to resorb bone due to their inability to acidify the bone matrix. Further, these authors also reported the presence of CLCN7 mutations in a patient with human infantile malignant osteopetrosis (Kornak et al., 2001).

1.2.3.3 STIMULATORS AND INHIBITORS OF OSTEOCLAST ACTIVITY

An understanding of the molecules that stimulate and inhibit osteoclast activity is crucial to gain insight into the regulation of osteoclast function at the molecular level.

1.2.3.3.1 STIMULATORS OF OSTEOCLAST ACTIVITY

Stimulators of osteoclast activity include systemic hormones such as vitamin D3, parathyroid hormone (PTH); several types of prostaglandins and local factors such as interleukin 1(IL-1), macrophage colony stimulating factor, (M-CSF1), receptor activator of NFkB ligand (RANKL), TGF-\(\alpha\), tumor necrosis factor (TNF) \(\alpha\) and TNF \(\beta\), IL-6 and IL-11 (Suda 1997, Mundy, 1999 and
Roodman, 1999). Metabolites of vitamin D₃ are potent stimulators of osteoclast formation, fusion and of bone resorption activity by the osteoclasts (Kurihara, 1990; Menaa et al., 1997). PTH, a systemic hormone produced by the parathyroid glands, plays an important role in the maintenance of calcium homeostasis owing to its stimulatory effects on osteoclastic bone resorption and on re-absorption of calcium by renal cells. The effects of PTH and vitamin D₃ on osteoclastic formation and activity are thought to be indirect, via their effect on osteoblasts, where they stimulate the production of M-CSF1 and RANKL (Mundy, 1999 and Roodman, 1999).

Prostaglandins, in general, are thought to potent stimulators of osteoclast differentiation and activity (Chenu et al., 1990; Suda et al., 1997; Heymann et al., 1998 and Takahashi et al., 1998). IL-1, a cytokine produced by macrophages, osteoblasts and by the osteoclasts, is a stimulator of osteoclast formation and resorption. Administration of IL-1 in vivo stimulated the formation of CFU-GM (granulocyte macrophage colony-forming unit), the earliest known osteoclast precursor, in addition to inducing hypocalcaemia (Pfeilschifter et al., 1989; Uy et al., 1995 and Roodman, 1999). M-CSF1 is required for osteoclast differentiation and plays a role in enhancing osteoclast survival, decreasing apoptosis and in osteoclast motility (Yoshida et al., 1990; Weir et al., 1993; Fuller et al., 1993 and Sundquist et al., 1995). The IL-6 and IL-11 are activators of osteoclast formation and function. Marrow stromal cells, monocyte/macrophages, osteoblasts and osteoclasts produce IL-6. IL-6 and IL-11 are thought to potentiate the stimulatory effect of PTH and PTHrP on
osteoclast resorption and maintenance of calcium homeostasis (Mundy, 1999 and Roodman, 1999). Finally, annexin II, an autocrine molecule, was shown to promote osteoclast formation from bone marrow cells and to enhance the bone resorptive capacity of osteoclasts. Annexin II is expressed on the surface of osteoclasts and inhibition of this molecule lead to an inhibition of osteoclastic resorption (Takahashi et al., 1994; Nebsitt and Horton, 1995 and Menaa et al., 1999).

1.2.3.3.2 INHIBITORS OF OSTEOCLAST ACTIVITY

Although the processes involved in the initiation of bone resorption are relatively well understood, the events that arrest them and subsequently potentiate osteoclast apoptosis are not. The inhibitory molecules of osteoclast activity include TGF-β, interferon (IFN)-α, IFN-β and IFN-γ, estrogen, calcitonin, prostaglandin E2 (PGE2), IL-4, IL-18, bisphosphonates, nitric oxide and osteoprotegrin (OPG) (Suda 1997, Mundy, 1999 and Roodman, 1999). The autocrine factor, TGF-β is a “coupling factor” that is released and activated during osteoclastic bone resorption, which then inhibits osteoclast formation and bone resorption. TGF-β also induces new bone formation by the osteoblasts by promoting their proliferation, differentiation and migration. Bone contains a large amount of latent TGF-β, which form the largest source of TGF-β in the body besides being secreted by immune cells, osteoclasts and osteoblasts (Figure 9) (Mundy, 1999 and Roodman, 1999). IFN-γ is a
multifunctional lymphokine produced by activated T-cells that inhibits
osteoclasts differentiation and osteoclastic bone resorption (Gowen and Mundy,
1986; Mundy, 1999 and Rodan, 1999). In a recent study, it was shown that IFN-
α and IFN-β negatively regulate osteoclast function, primarily by inhibiting
osteoclastogenesis. Particularly, IFN-β is induced by RANKL to function as a
feed back loop to suppress osteoclast differentiation by interfering with the
RANKL-induced expression of c-Fos, a transcription factor critical for osteoclast
differentiation. Further, mice deficient in IFNAR1, one of the IFN α/β receptor
components, exhibit osteoporosis due to enhanced number of osteoclasts. IFN
β-/- mice also exhibit osteopenia due to increased number of osteoclasts
(Takayanagi et al., 2002).

Estrogens suppress osteoclast function mainly by inhibiting osteoclast
formation, suppressing the production of bone-resorbing cytokines like IL-6 and
IL-1 and by promoting apoptosis in osteoclasts (Jilka et al., 1992; Mundy, 1999
and Kousteni et al., 2002). Recent studies have shown that estrogen inhibits
osteoclastogenesis by suppressing Jun N-terminal kinase (JNK) activation by
RANKL and JNK-mediated phosphorylation and expression of c-Jun and c-Fos
transcription factors (Shevde et al., 2000 and Srivastava et al., 2001).

Calcitonin, a systemic hormone secreted by the parathyroid follicles, is
another potent inhibitor of osteoclast activity. Interestingly, calcitonin receptor
(CTR) is one of the earliest markers present in osteoclast progenitors.
Calcitonin acts by stimulating adenylcyclase activity and cAMP accumulation,
resulting in the immobilization of osteoclasts and contraction of osteoclasts
away from the bone. Osteoclasts continuously exposed to calcitonin are capable of escaping its effects as calcitonin downregulates messenger RNA (mRNA) expression of its receptor, CTR (Mundy, 1999 and Roodman, 1999).

In conclusion, the bone resorbing multinuclear osteoclast is a highly specialized cell, with a crucial role in the healthy maintenance of the bone and also in the development of new bone tissue in the young. As mentioned earlier, several disease conditions like osteopetrosis and osteoporosis develop due to impairment in osteoclast differentiation and function. Therefore, an appreciation of osteoclast differentiation at the molecular level is pivotal towards understanding the molecular basis of the diseases of the bone.

1.2.3.4 ORIGIN AND DIFFERENTIATION OF OSTEOCLASTS

1.2.3.4.1 ORIGIN OF OSTEOCLASTS

Kölliker discovered osteoclasts in 1873. Since then, the origin of osteoclasts has been a highly debated topic, with the earliest theory being that osteoclasts and osteoblasts originated from a common mesenchymal progenitor (Young et al., 1962). Evidence for a blood-borne, extra-skeletal origin came from several elegant studies performed during early 1960s to late 1970s. Earlier newt limb and rat parabiosis experiments demonstrated that $[^{3}\text{H}]$-thymidine labeled blood monocytes and not mesenchymal cells gave rise to osteoclasts (Fischman and Hay, 1962; Göthlin and Ericsson, 1973; Buring, 1975 and Nijweide et al., 1986). Experiments with quail limbs grafted on
chorioallantoic membrane of fertilized chick egg, demonstrated that the osteoclasts formed on the quail limb grafts had nuclei that originated from the chick embryo (Kahn and Simmons, 1975; Joterau and Le Douarin, 1978 and Nijweide et al, 1986). Walker et al demonstrated that parabiotic union of the osteopetrotic mice with normal littermates could cure osteopetrosis in microphthalmic (mi) and in grey lethal (gl) mice (Walker, 1972 and Walker, 1973). Further, bone marrow transplantation was demonstrated to cure infantile and juvenile osteopetrosis in patients (Coccia et al., 1980; Sorell et al., 1981 and Nijweide et al, 1986). These studies demonstrated that osteoclast precursors are present in the bone marrow and peripheral blood and that osteoclasts might have a hematopoietic origin.

1.2.3.4.2 OSTEOCLAST PRECURSORS

Using an in vivo rat model, Baron, et al. demonstrated that prior to the formation of multinuclear osteoclasts, mononuclear cells containing non-specific esterase (NSE), an enzyme present in monocytes, increase in number locally. Further, as these mononuclear cells expressing non-specific esterase attach to the bone, they differentiate into cells containing TRAP and lose non-specific esterase activity. The presence of cells that stain for both enzymes implied that these cells were steps in a single differentiation process. TRAP positive mononuclear cells fused to form multinuclear osteoclasts in these animals (Baron et al., 1986). Work from other groups demonstrated that peripheral
blood monocytes or macrophages, when labeled with charcoal particles, thorotrast, [\(^3\)H]-thymidine or Barr bodies, formed multinucleated osteoclasts containing the respective labels (Jee and Nolan, 1963, Göthlin and Ericsson, 1973; Tinkler et al., 1981; Stanka and Bargsten, 1983 and Nijweide et al., 1986). Zambonin-Zallone et al. demonstrated that peripheral blood monocytes could fuse with purified avian osteoclasts (Zambonin-Zallone et al., 1985 and Zambonin-Zallone et al., 1984). Burger et al demonstrated that multinuclear osteoclasts were formed when bone rudiments devoid of osteoclasts were co-cultured with plasma clots containing bone marrow cells in the presence of M-CSF1, and that these osteoclasts came from monocytic precursors (Burger et al., 1982; Burger et al., 1984; Nijweide et al, 1986 and Roodman, 1999). Studies during the late 1980s demonstrated that macrophage antigens were expressed on osteoclasts and that a monoclonal antibody to macrophage and macrophage polykaryons cross-reacted with osteoclasts (Oursler et al., 1985; Athanasou et al., 1988 and Roodman, 1996). Finally, Kurihara et al. showed that osteoclasts could be formed from highly purified CFU-GM in human marrow cultures (Kurihara et al., 1990). Taken together, these data suggest that osteoclast precursors are derived from a hematopoietic, monocyte/macrophage lineage with CFU-GM being the earliest known precursor (Figure 10).

Numerous studies conducted over several years laid the groundwork for the advancement made towards appreciating several aspects of osteoclast differentiation.
Figure 10. Osteoclasts arise from a hematopoietic, monocyte-macrophage lineage with CFU-GM being the earliest precursor.

(CFC = colony forming cell; CFU = colony forming unit; capability for self renewal is shown by the arched arrow)

In a pioneering study using the rat *in vivo* model, Baron and co-workers investigated the kinetics of differentiation of osteoclast precursors. Briefly, in this study, the upper right row of molars in adult rats was extracted. The lack of antagonistic teeth led to egression of the lower right row of molars and induction of a synchronous remodeling sequence along the periosteum at the outer surface of the right limb of the mandible. The measurements in this study were based on the kinetics of the number of osteoclasts/precursors found along the periosteum as a function of time after induction of remodeling. They observed the number of NSE positive mononuclear cells increased 1 day after induction of remodeling and peaked at 2 days after induction. Between days 2 and 3 after induction, the number of NSE positive mononuclear cells decreased sharply returning to control levels by day 5. TRAP positive mononuclear cells appeared by day 1 and their numbers peaked by day 2. However, their number remained at maximum values even at 5 days after induction. The first TRAP positive multinuclear osteoclasts appeared 3 days after induction, with their numbers peaking by day 4 at an average of 16 osteoclasts per mm. These investigators also observed that at day 2, majority of the TRAP positive cells were found closer to the bone surface, majority of the NSE positive cells were found closer to the vascular layer of the periosteum and cells positive for both enzymes were found in a layer in between. These data suggested that cells expressing NSE
and TRAP were two consecutive steps in the differentiation of osteoclasts (Baron et al., 1986).

1.2.3.4.4 in vitro STUDIES OF OSTEOCLAST DIFFERENTIATION

To understand the molecular mechanisms involved in osteoclast differentiation, in vitro culture systems suitable for osteoclast differentiation needed to be developed. Testa and co-workers were the first to modify a feline bone marrow culture system to form osteoclasts (Testa et al., 1981 and Roodman, 1999). It was later shown the addition of osteopetrotic factors could modulate the formation of osteoclasts from this system and that the precursors of these cells were of a monocyte/macrophage lineage (Ibbotson et al., 1984 and Roodman, 1999). Later, Takahashi et al. demonstrated that TRAP positive multinuclear cells can be obtained from murine marrow cultures within 5-6 days when cultured in the presence of vitamin D$_3$. The earliest marker for cells in the osteoclast lineage in this system was MMP-9, whose expression preceded that of TRAP in these cells (Takahashi et al., 1988). When non-adherent mononuclear human bone marrow cells were cultured in the presence of vitamin D$_3$, osteoclasts were formed in three weeks. It was demonstrated that these mononuclear cells were proliferating during the first week of culture and differentiate and fuse to form osteoclasts during the second and third week of culture (Macdonald et al., 1987; Thavarajah et al., 1991; Kassem et al., 1991 and Roodman, 1999).
Udagawa et al. showed that osteoclasts were formed when mouse spleen cells were co-cultured with an appropriate bone marrow derived stromal cell line or mouse primary calvarial cells in the presence of dexamethasone and vitamin D$_3$. These osteoclasts expressed TRAP, CTR and show enhanced cAMP expression upon exposure to calcitonin (Udagawa et al., 1989 and Akatsu et al., 1992). Finally, cell-to-cell contact between the osteoclasts and stromal cells or the osteoblasts was shown to be absolutely critical for osteoclast differentiation in this type of system (Tamura et al., 1993 and Roodman, 1999).

1.2.3.5 OSTEOCLAST DIFFERENTIATION AND CELL CYCLE ARREST

Scheven et al. investigated the kinetics of osteoclast precursor proliferation and formation \textit{in vitro}, using metatarsal bones of embryonic mice of different ages cultured continuously in the presence of [³H]-thymidine. In 15-day old metatarsals, TRAP positive mononuclear and multinuclear osteoclasts formed mainly from proliferating, [³H]-thymidine incorporating mononuclear cells. In day 16 metatarsals, TRAP positive osteoclasts formed from equal numbers of labeled (proliferating) and unlabeled (post-mitotic) precursors. However, in day 17 metatarsals, the majority of TRAP positive multinuclear osteoclasts formed were from unlabeled precursors. This suggested that by day 17, precursor population in the metatarsals were predominantly post-mitotic.

Further, in this study, irradiation of 15 day old bones lead to a complete elimination of osteoclast precursors while that of day 16 bones only lead to a
partial inhibition as some of the post-mitotic precursors differentiated into multinuclear osteoclasts. However, irradiation of 17 or 18 day old metatarsal bones did not significantly affect the kinetics of osteoclast differentiation. Thus, in the periosteum of embryonic metatarsal bones, a shift occurs from a population composed of mainly proliferating osteoclast progenitors at day 15 post-conception, to a population composed of post-mitotic precursors on day 17. Multinuclear osteoclasts are formed on day 18 post-conception (Scheven et al., 1986).

Further, when human bone marrow co-cultures were cultured in the presence of vitamin D$_3$, it was observed that the mononuclear cells proliferate during the first week. These cells differentiate and fuse to form osteoclasts during the second and third week of culture (Macdonald et al., 1987; Thavarajah et al., 1991; Kassem et al., 1991 and Roodman, 1999). Tanaka et al. demonstrated that murine osteoclast progenitors proliferate during the first four days in co-culture while their differentiation into multinuclear osteoclasts occurred during the 5$^{th}$ and 6$^{th}$ days in co-culture. In this study, osteoclast formation was inhibited when hydroxyurea was added to the culture during the first 4 days of co-culture but not when it was added on days 5 and 6 of co-culture (Tanaka et al., 1993).

More recently, Meiyanto et al. have shown that treatment of Raw 264.7 cells with RANKL lead to the conversion of retinoblastoma (Rb) protein from cell cycle permissive hyperphosphorylated form to the cell cycle prohibiting hypophosphorylated form. They also observed the induction of p21$^{CIP1}$ cell cycle
inhibitor and a delayed entry of the cells into the S-phase of the cell cycle consequent to RANKL treatment. They also showed that hydroxyurea treatment of the cells prior to the addition of RANKL, when the cells are in a proliferation phase, leads to a block in osteoclast formation (Meiyanto et al., 2001). Okahashi et al. showed that p21\textsuperscript{CIP1} and p27\textsuperscript{KIP1} are transiently induced in primary osteoclast progenitor cells upon RANKL and TNF\textalpha treatment and that osteoclast formation in these cultures could be blocked by the addition of a combination of antisense oligonucleotides. However, these workers suggested that the cell cycle inhibitors played a role other than cell cycle inhibition during osteoclast differentiation as following RANKL addition, as they did not observe cell cycle arrest in osteoclast precursor cells (Okahashi et al., 2001).

Cell cycle, its regulation and the role of cyclin dependent kinase inhibitors in development are mentioned in great detail in Chapter 5.

A host of in vivo and in vitro studies, as mentioned in the previous sections, have resulted in the identification of several molecules and signaling pathways that regulate several aspects of osteoclast differentiation and biology. Subsequent sections will detail the roles played by several of these molecules during osteoclast differentiation.

1.2.3.6 MOLECULES INVOLVED IN OSTEOCLAST DIFFERENTIATION

1.2.3.6.1 M-CSF1(CSF-1)

It is now known that M-CSF1 and RANKL, the two important cytokines both necessary and sufficient for osteoclastogenesis, are produced by stromal
osteoblasts, macrophages, endothelial cells and T-lymphocytes (Figure 11). M-CSF1 is a secreted molecule critical for macrophage differentiation and survival. It is also required for the proliferation, differentiation and survival of osteoclast progenitors and mature osteoclasts. M-CSF1 binds to its receptor, c-FMS, present on early osteoclast precursors, monocytes and macrophages (Ugadawa et al., 1989 and Weir et al., 1993).

The importance of M-CSF1 in osteoclastogenesis was evident from examining the op/op osteopetrotic mouse mutants (Felix et al., 1990 and Wilktor-Jedrzejczak et al., 1990). The op/op mice, incapable of producing M-CSF1, exhibit severe osteopetrosis that resolves with age. It was later found that these mice have an extra thymidine insertion in the coding region of M-CSF1 gene that generated a stop codon 20 bases downstream of the point mutation, thus prematurely terminating M-CSF1 protein translation (Yoshida et al., 1990). Subsequently, it was shown that the osteopetrosis in op/op mice could be cured by administration of recombinant human M-CSF1 (Felix, 1990). Tanaka et al. demonstrated that M-CSF1 was required for proliferation and differentiation of osteoclasts. They demonstrated that when murine spleen cells were co-cultured with murine calvarial cells (osteoblasts) for six days in the presence of vitamin D3, the addition of anti-M-CSF1 or anti-c-FMS antibodies on the first four days or the last two days of co-culture, inhibited osteoclast formation. They also observed the same results when the spleen cells were co-cultured with osteoblasts from op/op mice for the first 4 days or the last two days of co-culture. Thus M-CSF1 is indispensable for both proliferation of
osteoclast progenitors and for their differentiation into multinuclear osteoclasts (Tanaka et al., 1993). Takahashi et al. showed that when co-cultures are treated sequentially with M-CSF1 followed by vitamin D$_3$, M-CSF1 allowed for the proliferation and expansion of the osteoclast precursor pool (Takahashi et al., 1991). Finally, Fuller et al. demonstrated that M-CSF1 prevented apoptosis of mature osteoclasts, enhanced their motility and inhibited bone resorption (Fuller et al., 1993). Removal of M-CSF1 producing stromal cells from a co-culture system enhanced osteoclast apoptosis (Jimi et al., 1995).

1.2.3.6.2 OPG

In 1997 and 1998, two independent groups reported that a secreted TNF receptor-like protein with no transmembrane or cytoplasmic domains, termed osteoprotegrin (OPG) or osteoclastogenesis inhibiting factor (OCIF), caused severe osteopetrosis in mice (Simonet et al., 1997 and Yasuda et al., 1998). Although OPG mRNA is expressed in a number of tissues including lung, heart, kidney, liver, stomach, intestine, brain, spinal chord, thyroid gland and bone, its major established role is to block osteoclastogenesis and activity (Simonet et al., 1997 and Yasuda et al., 1998). Mice with targeted deletion in OPG exhibit severe osteoporosis due to markedly increased number of osteoclasts and increased bone resorption. These mice also exhibit profound calcification of the large arteries (Bucay et al., 1998; Mizuno et al., 1998 and Min, 2000).
1.2.3.6.3 RANKL

Using OPG as a probe, expression libraries were screened to identify its ligand initially termed OPG ligand (OPGL) or osteoclast differentiation factor (ODF) (Lacey et al., 1998 and Yasuda et al., 1998). OPGL was identical to two previously identified members of the TNF ligand family; viz. TNF-related activation-induced cytokine (TRANCE) from T cells and receptor activator of NFκB ligand (RANKL) from dendritic cells (Wong, 1997 and Anderson et al., 1997). RANKL has been shown to exist in a 40-45 kilo Daltons (kDa) membrane-bound form and in a 30 kDa soluble form. RANKL mRNA is expressed at high levels in bone, bone marrow cells and in lymphoid cells (Lacey et al., 1999; Lum et al., 1999 and Khosla, 2001). RANKL stimulates differentiation and activation of osteoclasts and inhibits osteoclast apoptosis (Lacey et al., 1999 and Khosla, 2001). In the presence of M-CSF1, RANKL is both necessary and sufficient to support osteoclast differentiation (Lacey et al., 1998 and Yasuda et al., 1998). RANKL is expressed on activated T-lymphocytes, which can trigger osteoclastogenesis that might be the pathogenic principle towards joint destruction in rheumatoid arthritis (Kong et al., 1999b).

Mice with a targeted deletion in RANKL exhibit severe osteopetrosis, including a delay in tooth eruption due to the lack of osteoclasts, in addition to defects in early T and B lymphocyte differentiation, defects in thymic differentiation and lack of lymph nodes (Kong et al., 1999a). The osteoblasts from these mice do not support osteoclast differentiation in vitro. However the hematopoietic precursors from these mice are capable of differentiating into
multinuclear osteoclasts in the presence of recombinant RANKL and M-CSF1, indicating that the osteoclastogenesis defect in these mice stems from the inability of the osteoblastic stroma to produce RANKL (Kong et al., 1999a). RANKL is essential for the survival of mature osteoclasts in the presence of M-CSF1 (Lacey et al., 2000).

### 1.2.3.6.4 RANK

The receptor for RANKL was already known to be a TNF receptor superfamily member, TNFRSIIB, also known as receptor activator of NFκB (RANK). RANKL was first identified as a protein in dendritic cells involved with immune responses (Anderson et al., 1997). Hsu et al. showed that RANK mRNA was expressed in osteoclast progenitors, mature osteoclasts, monocytes, macrophages, T and B cells, dendritic cells and in fibroblasts, with the highest expression seen in osteoclasts within areas of active bone resorption. They also observed a limited expression of RANK mRNA in mature chondrocytes. Treatment of osteoclast progenitors with antibodies raised against the extracellular domain of RANK (RANK-Ab) lead to the formation of multinuclear osteoclasts in vitro (Hsu et al., 1999). Transgenic mice expressing the entire extracellular domain of RANK fused to human IgG-g1 driven by the apolipoprotein E gene promoter (soluble RANK-Fc fusion gene) exhibit severe osteopetrosis due to markedly reduced numbers of TRAP positive osteoclasts, similar to the phenotype seen in OPG transgenic mice (Hsu et al., 1999). Further, Hsu et al. also demonstrated that treatment of Raw 264.7 cells, a
murine myeloid cell line, with either RANKL or with RANK-Ab lead to their differentiation to multinuclear osteoclasts and to the induction of osteoclast-specific gene expression (Hsu et al., 1999).

RANK knock out mice exhibit severe osteopetrosis due to the lack of osteoclasts in addition to defects in early T and B lymphocyte differentiation and lack of peripheral lymph nodes (Li, et al., 2000). Osteoclastogenesis in RANK−/− mice can be restored by retroviral-mediated introduction of RANK cDNA into the bone marrow of these mice (Li et al., 2000). In patients with human familial expansile osteolysis, a rare autosomal disorder characterized by focal areas of increased bone remodeling, a heterozygous insertion mutation in exon 1 of RANK was shown to increase RANK-mediated NFκB activation (Theill et al., 2002). Taken together, these data suggest that RANKL-RANK engagement is required for differentiation of osteoclasts and for osteoclast activity (Figures 11 and 14).
Figure 11. Schematic illustration of osteoclast differentiation regulated by M-CSF1 and RANKL-RANK signaling. Solid arrows represent stimulants and dotted arrows represent inhibitors of osteoclast differentiation. (Theill et al., 2002)
1.2.3.6.5 TRAFs

Yeast two-hybrid studies and further in vitro and in vivo binding studies revealed that RANK interacted with tumor necrosis factor receptor-associated factors (TRAF) 1, 2, 3, 5 and 6. Mapping studies showed that multiple TRAF binding sites clustered in two distinct domains in the C-terminal domain of RANK and these binding sites were found to be important for RANK-induced of NFκB and JNK activation. TRAF6 binds to a membrane proximal region of the RANK cytoplasmic tail, between amino acids 336 and 454, while TRAFs 1-5 bind to a more C-terminal region. Elimination of TRAF6 binding sites in RANK completely inhibited the activation of NFκB and partially inhibited JNK activation. This suggests that TRAF6-mediated RANKL-RANK signaling is required for NFκB activation and that its role might be redundant for initiating JNK activation. Dominant negative forms of TRAFs 2 and 5 were able to inhibit NFκB activity (Wong et al., 1998). Thus, TRAF2, TRAF5, and TRAF6 activate JNK and NFκB pathways in response to RANK-RANKL signaling (Arch et al., 1998). Armstrong et al. have shown recently that TRAF6-mediated signaling might play a role in cytoskeletal reorganization of mature osteoclasts and in bone resorption by activated osteoclasts (Armstrong et al., 2002).

Even though TRAF2^{-/-}, TRAF3^{-/-} and TRAF5^{-/-} mice have been generated, none of these exhibit osteopetrosis. Recently, Kanazawa et al. showed that osteoclast progenitors from TRAF5^{-/-} mice were not able to differentiate into mature osteoclasts in the presence of RANKL or TNFα even though JNK and NFκB activity remained normal in these cells (Kanazawa et al., 44).
2003). Mice deficient in TRAF6 exhibit osteopetrosis with impaired tooth eruption and remodeling due to defects in osteoclast function. These mice have TRAP positive multinuclear cells at numbers similar to those in wild type (WT) mice but most of the osteoclasts are withdrawn from the bone surface. TRAF6−/− mice are deficient in IL-1, CD40 and lipopolysaccharide (LPS) mediated activation of NFκB and in IL-1-mediated JNK activation (Lomaga et al., 1999). However, a different group that also generated TRAF6−/− mice, Naito et al., reported that their TRAF6−/− mice harbor very few TRAP positive mononuclear osteoclast progenitors (Naito et al., 1999). This discrepancy might be due to the difference in the knock out strategy used by the two groups.

All TRAFs share a common stretch of amino acids at their carboxyl terminus, called the TRAF domain, that is further divided into two sub regions (Figure 12). The carboxyl-terminal TRAF-C region is highly conserved among the members of the TRAF family and has been shown to mediate the binding of the TRAF proteins to their associated receptors. The amino-terminal half of the TRAF domain, TRAF-N, is predicted to form a coiled-coil structure and has been shown to mediate both homo- and heterodimerization of the TRAF proteins. With the exception of TRAF1, all TRAFs contain an amino-terminal RING finger domain and a stretch of multiple zinc fingers. The deletion of the RING finger domain results in the generation of dominant-negative TRAF mutants, suggesting that the RING finger domain might be required to transduce downstream signaling events. Kobayashi et al. showed that the RING domain and the first zinc finger domain in TRAF are required for the full
activation of JNK and p38-mitogen activated protein kinase pathways and the zinc finger domains alone are required for the activation of NFκB (Kobayashi et al., 2001). Kobayashi et al. further demonstrated through cell rescue experiments that the RING finger domain is important for osteoclast maturation and the second and third zinc finger domains areas required for osteoclast differentiation (Figure 13) (Kobayashi et al., 2001).

Lomaga et al. deleted the exon 1 of TRAF6 eliminating the RING finger domain and leaving the zing finger domains intact to generate their TRAF6−/− mice (Lomaga et al., 1999). TRAF6 deletion made by Naito et al. eliminated part of the RING finger as well as three of the zing finger regions, thus eliminating the signal transducing ability of the molecule (Naito et al., 1999).
Figure 12. Schematic representation of the structure of the TRAF family of proteins.
(NLS = nuclear localization domain; X represents the number of Zinc fingers)
(Inoue et al., 2000)
Figure 13. Schematic representation of the functional domains of TRAF6. (Kobayashi et al., 2001)
1.2.3.6.6 NFκB

NFκB1(p50)/NFκB2(p52) double knock out mice exhibit severe osteopetrosis due to a block in osteoclast differentiation prior to the formation of TRAP positive mononuclear cells (Franzoso et al., 1997). The precise role of NFκB in osteoclastogenesis or in osteoclast activity has not been delineated. NFκB regulates IL-6 receptor through binding to NFκB response elements and activating gene expression. NFκB also directly or indirectly enhances the production of IL-1, IL-6 and TNFα by stromal cells and by macrophages in patients with rheumatoid arthritis. Estrogen receptor (ER) has been shown to interact with NFκB heterodimers and prevent them from binding to their response elements. Down regulation of this interaction leads to acceleration in bone loss as seen in post-menopausal women (Boyce et al., 1999). Treatment of isolated osteoclasts or marrow cultures with NFκB antisense oligonucleotides or with NFκB inhibitors leads to increased apoptosis of these cells indicating that NFκB might also be involved in the survival of mature osteoclasts (Boyce, 1999).

1.2.3.6.7 ACTIVATION OF JNK, p38 AND OTHER SIGNALING PATHWAYS

RANKL-RANK signaling has been shown to induce JNK and NFκB activation via TRAF6, through its binding to TGF-β-activating kinase (TAK) (Inoue et al., 2000). RANKL-RANK induced activation of JNK is important for osteoclast differentiation and activation (Jimi et al., 1999). Overexpression of a
mutant of c-Jun that cannot be phosphorylated by JNK in macrophages blocks their differentiation into osteoclasts. Estrogen has been shown to block osteoclast differentiation by inhibiting RANK-mediated JNK activation and c-Jun activation and expression (Shevde et al., 2000; Srivastava et al., 2001 and Teitelbaum, 2000).

RANKL-RANK signaling also activates the anti-apoptotic serine/threonine kinase Akt/Protein kinase B (PKB) via TRAF6 and the tyrosine kinase c-Src. Following RANK receptor engagement, TRAF6 interacts with the SH3 domain of c-Src, enhancing the kinase activity of c-Src leading to the phosphorylation of downstream molecules such as c-Cbl. Addition of c-Src inhibitors or a deficiency in c-Src, as in the case of c-Src−/− mice, abolishes RANK mediated activation of Akt/PKB. RANK can recruit TRAF6, c-Cbl and PI-3 kinase in a RANKL and c-Src-dependent manner (Wong et al., 1999 and Theill et al., 2002).

Matsumoto et al. showed that p38 MAPK pathway is activated by RANKL and TNFα during osteoclast differentiation and that the differentiation of osteoclasts in the presence of TNFα and M-CSF1, could be blocked by adding SB203580, a specific inhibitor of the p38 MAPK pathway (Matsumoto et al., 2000). These data suggests that p38 MAPK pathway plays a critical role in osteoclast differentiation. RANK/RANKL signaling through TRAF6 induces the activation of p38 MAPK pathway. Activation of this pathway is abolished in TRAF6−/− MEFs. The RING and the first zinc finger domains in TRAF are required for the full activation of p38 MAPK by RANKL-RANK signaling and

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TRAF6 accomplishes this via its interaction with TAK1 following receptor engagement (Kobayashi et al., 2001). Recent studies have shown that p38 MAPK phosphorylates microphthalmia transcription factor (Mitf), a transcription factor that plays a role in osteoclast differentiation, in response to RANKL signaling and that this event correlates with increased expression of TRAP (Mansky et al., 2002). TRAP has been shown to be a direct target of Mitf regulation (Luchin et al., 2000). Mansky et al. also showed that addition of SB203580 blocked the RANKL-mediated phosphorylation of Mitf and induction of TRAP gene expression (Mansky et al., 2002b).

In summary, RANKL-RANK signaling in the presence of M-CSF1 leads to the activation of multiple TRAFs. Activated TRAFs, via known and unknown molecules, activate JNK, p38 and NFκB signaling pathways. These pathways turn on transcription factors that in turn regulate osteoclast-specific gene expression program. These events coordinately regulate osteoclast differentiation, activation, survival and apoptosis (Figure 14).
Figure 14. RANK-RANKL signaling pathways in osteoclasts (Theill et al., 2002)
1.2.3.6.8 TNFα

TNFα has been shown to induce osteoclast formation from bone marrow or spleen cells via the engagement of its receptors p55 and p75 (Azuma et al., 2000). TNFα-induced formation of multinuclear osteoclasts was blocked completely by the addition of anti-p55 antibody and partially by the addition of anti-p75 antibody, but not by the addition of OPG (Kobayashi et al., 2000 and Azuma et al., 2000). However, Lam et al. observed based on their studies that TNFα alone is not capable of inducing osteoclast differentiation, but does so in vitro and in vivo by directly targeting macrophages within a stromal environment that expressed permissive levels of RANKL (Lam et al., 2000). Recent reports suggest that TNFα also promotes actin ring formation in mature osteoclasts and that it strongly synergized with RANKL to promote osteoclast differentiation.

Several transcription factors, including PU.1, c-Fos, NFκB and Mitf are known to regulate various aspects of osteoclast differentiation.

1.2.3.7 TRANSCRIPTIONAL CONTROL OF OSTEOCLAST DIFFERENTIATION

The Ets family transcription factor, PU.1, is expressed specifically in myeloid/monocytes, B-lymphocytes and other hematopoietic lineages. Elimination of Pu.1 leads to defects in the generation of progenitors for B and T lymphocytes, monocytes and granulocytes (Scott et al., 1994 and McKercher et al., 1996). PU.1 was earlier shown to regulate the transcription of c-fms, the receptor for C-FMS (Sherr et al., 1985) and this lead Tondravi et al. to further
examine the possibility that PU.1 might also play a role in macrophage and osteoclast differentiation. They showed that messenger RNA for PU.1 progressively increases as marrow macrophages assume the osteoclast phenotype and that PU.1 expression increased with the \textit{in vitro} induction of osteoclastogenesis by either 1,25-dihydroxyvitamin D\textsubscript{3} or dexamethasone. PU.1 knock out mice exhibit severe osteopetrosis due to a complete lack of macrophages and osteoclasts. These animals could be rescued by marrow transplantation, with complete restoration of osteoclast and macrophage differentiation, verifying that the PU.1 lesion is cell autonomous to haematopoietic cells (Tondravi et al., 1997). Thus, PU.1 is the earliest marker of osteoclast lineage as it controls the differentiation of both macrophages and osteoclasts (Figure 15).

Major components of AP-1 transcription factor complex, c-Jun, c-Fos and Fra-1 have been implicated to play a role in osteoclast differentiation. As mentioned previously, the tyrosine kinase JNK has been shown to phosphorylate c-Jun and increase its expression levels in response to RANKL, but the exact role of this transcription factor in osteoclast differentiation is not yet clear, since c-Jun knock out mice are embryonic lethal at E12.5 days (Wagner, 2002). Mice lacking c-Fos, on the other hand are viable, albeit dependent on the genetic background, but exhibit severe osteopetrosis. The lack of osteoclasts in these mice is accompanied by an increased number of macrophages (Johnson et al., 1992; Wang et al., 1992 and Grigoriadis et al., 1994). The osteopetrosis in c-Fos\textsuperscript{-/-} mice could be cured by bone marrow
transplantation implying the defect in these osteoclasts is cell autonomous (Grigoriadis., 1994). This observation suggests that c-Fos acts downstream of PU.1 in the genetic control of commitment to the osteoclast lineage by early progenitor cells (Figure 15).

Matsuo et al. showed through \textit{in vitro} retroviral mediated gene transfer that Fra-1 could rescue the differentiation phenotype in c-Fos\(^{-/-}\) osteoclasts. They further demonstrated that a Fra-1 mutant lacking the transactivation domain showed the greatest rescue activity (Matsuo et al., 2000). Besides, c-Fos and Fra-1 are upregulated in a c-Fos-dependent manner in osteoclast progenitors upon RANKL treatment (Wagner 2002). Total restoration of osteoclast differentiation could be achieved by “knocking in” \textit{fra1} gene into \textit{c-fos} locus and this rescue of osteopetrosis and osteoclast differentiation in c-Fos\(^{-/-}\) mice is dependent on the dosage of Fra-1 (Fleischmann et al., 2000). In addition, ectopic expression of Fra-1 increases osteoclast differentiation \textit{in vitro} (Owens et al., 1999 and Matsuo et al., 2000). Fra-1 overexpressing mice have osteosclerosis and increased bone formation (Jochum et al., 2000). However, Fra1 knock out mice have no osteoclast defect and Fra-1 is not required for osteoclast differentiation \textit{in vivo}, thus suggesting that Fra-1 and c-Fos might have maintained functional equivalence during vertebrate evolution (Schreiber et al., 2000 and Wagner 2002).

\textit{NF\kappa B} is another transcription factor that is involved in the genetic control of osteoclast differentiation (Figure 15). As mentioned previously, p50/p52 double knock out mice exhibit severe osteopetrosis due to a block in osteoclast
differentiation prior to the formation of TRAP positive mononuclear cells (Franzoso et al., 1997). Thus, in the genetic control of osteoclast differentiation, NFκB might act downstream of PU.1 and c-Fos (Figure 15).

The basic helix-loop-helix leucine zipper transcription factor, Mitf has been found to play a role in osteoclast differentiation, downstream of the roles played by three transcriptions described earlier, viz. PU.1, c-Fos and NFkB (Figure 15). Mice homozygous for the mutant mi allele develop severe osteopetrosis due to an inability of mononuclear osteoclast precursors, that express lower levels of TRAP, to fuse and form multinuclear osteoclasts capable of resorbing bone (Hertwig 1942 and Hodgkinson et al., 1993). The Mitf mi/mi mice carry a semi-dominant mutation in the mi allele, where one of the arginines in the basic domains of the transcription factor is deleted, rendering the protein unable to bind to DNA and causing it to act in a dominant negative fashion (Hodkinson et al., 1993 and Karsenty 2001). Interestingly a loss of function mutation at the Mitf locus, Mitf va/va does not cause osteopetrosis in the homozygous condition (Hemesath et al., 1994 and Steingrimsson et al., 1994). In addition, Mitf and PU.1 have been shown to synergistically activate TRAP promoter, in transient transfection assays, by binding to their respective binding sites on the promoter. Further, PU.1 and Mitf were found to physically interact with each other and heterozygous Mitf mi/PU.1 +/− mice developed osteopetrosis (Luchin et al., 2001). The role of Mitf in osteoclast biology has been elaborated in detail in later sections.
It was recently reported that the oncogene c-myc is upregulated when RAW 264.7 cells were induced to differentiate into osteoclasts through treatment with RANKL. Treatment of the culture with a dominant negative Myc blocked the formation of multinuclear osteoclasts and caused a reduction in TRAP and cathepsin K mRNA levels (Battaglino et al., 2002). However the role of Myc in osteoclast differentiation is not known.

1.2.3.8 MARKERS OF OSTEOCLAST DIFFERENTIATION

MMP-9, TRAP, cathepsin K, carbonic anhydrase II and CTR are the major markers of osteoclast differentiation (Figure 15). MMP-9 is the earliest marker gene expressed by osteoclast precursors. MMP-9 is a collagenase that can cleave the α2-chain of collagen types I, III, IV and V as well as gelatins (Hill et al., 1995 and Roodman, 1996). However, MMP-9 knock out mice exhibit only mild osteopetrosis that resolves with age, presumably due to a delay in chondrocyte ossification (Vu et al., 1998). The long bones of these mice have abnormal growth plates due to an expansion of the layer of hypertrophic chondrocytes in the growth plates. This phenotype could be corrected via bone marrow transplantation from WT mice (Vu et al., 1998).

TRAP is regarded as the classic marker enzyme for osteoclast differentiation (Figure 15). TRAP is expressed at high levels in differentiated osteoclasts. TRAP enzyme is present in lysosomes, Golgi, extracellular channels of the ruffled border and in the space between the osteoclasts and bone (Lucht, 1971). The potential function of TRAP in osteoclasts might be...
regulation of the biological activity of bone matrix phosphoproteins like osteopontin and bone sialoprotein via their dephosphorylation and the degradation of collagen via the formation of reactive oxygen species (Ek-Rylander et al., 1994 and Halleen et al., 1999). The physiological function of TRAP in osteoclasts is not very clear. Blocking antibodies against TRAP have been shown to block bone resorption in in vitro cultures.

Transgenic mice overexpressing TRAP driven by an SV-40 enhancer exhibit mild osteoporosis with decreased trabecular density (Zaidi et al., 1989 and Angel et al., 2000). However, mice lacking TRAP exhibit only a mild late onset osteopetrosis. These mice have an increased mineral density of the appendicular and axial skeleton, besides exhibiting abnormal endochondral ossification. The osteoclasts from these mice have moderately defective bone resorption activity in vitro (Hayman et al., 1996). More recently, Hollberg et al. showed that osteoclasts from TRAP$^{-/-}$ mice exhibited an increased relative area of ruffled borders and accumulated numerous cytoplasmic vesicles along the ruffled border and basolateral areas of the cytoplasm indicating a disturbed intracellular vesicle transport system. Since these cells expressed normal levels of cathepsin K, the authors concluded that the vesicles might not be derived from the secretory pathway (Hollberg et al., 2002). Further, Mansky et al. have demonstrated that TRAP mRNA levels are elevated about 6-8 fold in osteoclast precursors within 24 hours of RANKL treatment (Mansky et al., 2002b). TRAP expression in osteoclasts was shown to be regulated by Mitf, by binding directly
to E-box elements on TRAP promoter, and by cooperation between Mitf and PU.1 (Luchin et al., 2000 and Luchin et al., 2001).

The roles of CTR and carbonic anhydrase II in osteoclast biology have been detailed in previous sections (Figure 15). Cathepsin K is a lysosomal cysteine protease belonging to the papain family of proteases (Bromme and Okamoto, 1995). This enzyme is predominantly observed in osteoclasts and is expressed at high levels in osteoclasts from osteoarthritic hips and in giant cells from giant cell tumors of the bone (Roodman, 1996). Cathepsin K is also regarded as an important marker of osteoclast differentiation. Cathepsin K has potent endoprotease activity at acid pH and is thought to play a role in degrading type I collagen during osteoclastic bone resorption (Figure 15) (Gowen, 1999 and Karsenty, 2002). Besides cathepsin K, osteoclasts also express lower levels of cathepsins B, D and L. Cathepsins B and L are released to the extracellular matrix and degrade the bone matrix during osteoclastic bone resorption (Roodman, 1996).

Inhibitors of cathepsin K and antisense oligonucleotides against cathepsin K block bone resorption by osteoclasts *in vitro* (Votta et al., 1997 and Inui et al., 1997). Cathepsin K mRNA levels are upregulated within 3 days after treatment with RANKL (CorisIDEO et al., 2001). Cathepsin K deficiency in humans causes a condition called pycnodisostosis, characterized by short stature, osteosclerosis, unclosed cranial structures, apoplastic mandibles and by double rows of teeth (Gelb et al., 1996). Mice deficient in cathepsin K contain multinuclear osteoclasts that cannot degrade the collagen matrix. These
osteoclasts are capable of demineralization of the bone and form shallow resorption pits \textit{in vitro} (Saftig et al., 1998 and Gowen et al., 1999). Motyckova et al. demonstrated that Mitf regulates the expression of cathepsin K by directly binding to E-box elements on its promoter and that a dominant negative form of Mitf protein was able to disrupt this regulation. They also showed that cathepsin K mRNA and protein levels were lower in $\textit{Mitf}^{\text{mi/mi}}$ mutant osteoclasts and that overexpression of WT-Mitf upregulates the endogenous levels of cathepsin K in osteoclasts (Motyckova et al., 2001).

Figure 15. Factors involved in the regulation of osteoclast differentiation and activation.
In summary, the expression of the two major markers of osteoclast differentiation viz., TRAP and cathepsin K, is directly under the regulation of the basic helix-loop-helix leucine zipper (bHLH-zip) transcription factor, Mitf. As mentioned earlier, homozygous Mitf\textsuperscript{mi/mi} mice develop severe osteopetrosis due to an inability of the mononuclear precursors to fuse and form multinuclear osteoclasts. It is thus evident that Mitf might play an important role in osteoclast differentiation, prior to fusion. A host of studies, both previous and recent, have shown that Mitf might be a target of p38 MAPK signaling pathway and that it might regulate the expression of target genes in collaboration with other transcription factors like PU.1 (Luchin et al., 2001). Further work needs to be done towards identifying more Mitf-target genes involved in osteoclast differentiation, which will lead to a greater appreciation of the precise role played by this transcription factor in osteoclasts.

1.2.3.9 ROLE OF MICROPHTHALMIA TRANSCRIPTION FACTOR IN OSTEOCLAST DIFFERENTIATION

1.2.3.9.1 MITF

The microphthalmia locus, located on murine chromosome 6p, encodes for the bHLH-Zip transcription factor, Mitf (Hodgkinson et al., 1993; Hughes et al., 1993 and Sato et al., 1999). The mammalian bHLH-Zip family includes several transcription factors important for cellular processes such as cell proliferation and differentiation, like Myo-D, Max/Myc, Tfe3, TfeB, TfeC, USF and AP-4 (Hemesath et al., 1994). Mitf is most closely related to Tfe3, TfeB and
TfeC proteins and together, these proteins make up a subfamily of Mitf-related transcription factors. Tfe3 and TfeB are more ubiquitously expressed while the expression of TfeC is restricted to monocytes and macrophages (Rehli et al., 1999). The basic domain in these factors is required for binding to a canonical CANNTG ‘E-box’ sequence of DNA. The HLH and leucine zipper domains are required for protein-protein interactions (Figure 16). Mitf forms stable heterodimers with the related Tfe3, TfeB and TfeC and has been shown to bind to DNA as stable homodimers or heterodimers (Hodgkinson, 1993; Hemesath et al., 1994; Moore, 1995 and Mansky et al., 2002c).

Tfe3 and TfeC were found to collaborate with Mitf to transactivate the TRAP promoter in transient transfection experiments (Mansky et al., 2002c). Aksan and Goding showed that the T residue flanking the CATGTG E-box at the –4 position determines the efficiency of Mitf-binding to DNA. Thus, Mitf will bind preferably and more efficiently to TCATGTGA E-box elements on promoters of target genes (Aksan and Goding, 1998). Mitf contains 3 putative activation domains, two in the amino (N) terminus and one in the carboxyl (C) terminus (Figure 16) (Sato et al., 1997; Takeda et al., 2000b and Mansky et al., 2002a). In melanocytes, the first N-terminal activation domain, AD1 (between residues 115 and 132), is more efficient in activating transcription than the C-terminal domain (Sato et al., 1997). Mansky et al. demonstrated that the second activation domain in the N-terminus, AD2, between residues 140 and 185, is required for the transactivation of E-Cadherin and TRAP in osteoclasts. Further
they also found that an MITF mutant lacking AD2 was less efficient in rescuing osteoclast differentiation seen in Mitf$^{mli/mi}$ osteoclasts (Mansky et al., 2002b).

Figure 16. Schematic depiction of Mitf.
(AD = Activation domain)
The murine Mitf (which spans over 50 kilo base pairs (kbp) and the human MITF genes contain nine exons (Udono et al., 2000 and Hallson et al., 2000). The first exon exists in at least six different known divergent forms, leading to the presence of six different known isoforms of Mitf and MITF (Figure 17) (Fuse et al., 1999; Hallson et al., 2000; Udono et al., 2000 and Takeda et al., 2002). Hallson et al. have reported that the murine Mitf gene is capable of generating at least 13 different isoforms (Hallson et al., 2000). The known Mitf isoforms are Mitf-M, Mitf-A, Mitf-H, Mitf-B, Mitf-C and Mitf-D (Udono et al., 2000; Hallson et al., 2000; Goding, 2000 and Takeda et al., 2002). All MITF isoforms share the bHLH-Zip domain and the C-terminal carboxyl domains, but differ in the amino terminal region (Figure 17). The amino-terminus of Mitf-M isoform, referred to as M-domain, comprises 11 amino acid residues encoded by exon 1M (Yashumoto et al., 1998; Udono et al., 2000 and Hallson et al., 2000). In contrast, all other isoforms have extended amino termini, consisting of a unique N-terminus and a common region called B1b domain (Fuse et al., 1999; Hallson et al., 2000; Udono et al., 2000 and Takeda et al., 2002). Thus the N-terminal unique domains for Mitf-A, B, H, C and D are formed by alternative splicing of exons 1A, 1H, 1B, 1C and 1D, respectively.
Mitf-M is expressed mainly in melanocytes and melanoma cells, where it has been shown to activate melanocyte-specific promoters (Yashumoto et al., 1998). The heart-form Mitf-H is highly expressed in heart (Hodgkinson et al., 1993 and Steingrimsson et al., 1994). Mitf-H and Mitf-A are also expressed in the kidney and in many cell types including retinal pigmented epithelium (RPE) and melanocytes (Fuse et al., 1999; Goding, 2000 and Takeda et al., 2002).
Mitf-C is expressed in many cell types including RPE but is excluded from melanocytes (Fuse et al., 1999). Mitf-D is expressed in RPE, macrophages and osteoclasts but not in melanocytes or natural killer cells (Takeda et al., 2002). Mitf-M and Mitf-A isoforms were detected in primary osteoclast-like cells and were found to activate TRAP promoter by themselves and in collaboration with Tfe3 in Raw 264.7 cells (Mansky et al., 2002c).

An important feature of Mitf gene is the presence of two differentially spliced forms, the Mitf (+) and Mitf(-) isoforms that differ by the presence and absence of alternatively spliced six amino acid insert, TACIFP (Figure 16) (Hodgkinson et al., 1993; Hemesath et al., 1994; and Sato et al., 1997). The Mitf + form was found to bind to DNA 20% more efficiently than the MITF - form, suggesting that presence of the extra 6 amino acids might stabilize the basic domain/DNA complex (Hemesath et al., 1994). Both Mitf-M and Mitf-H forms were expressed as + and – versions (Hemesath et al., 1994 and Hallson et al., 2000).

1.2.3.9.2 REGULATION OF Mitf

Much of the current knowledge regarding the regulation of Mitf, both at the transcriptional and post-transcriptional level, comes from work done in melanocytes. Transcription factors that have been implicated in the transcriptional regulation of Mitf are Pax3, Sox10, cAMP responsive element binding protein (CREB) and Lef1. Mitf promoters have binding sites for each of these proteins (Goding, 2000). Waardenburg syndrome type 1, which is
phenotypically very similar to Waardenburg syndrome type 2 caused by mutations in MITF, is caused by mutations in Pax3 gene (Tassebehji et al., 1993). Also, Pax3 regulates Mitf expression in melanocytes and in melanocyte cell lines by directly binding to its promoter (Watanabe et al., 1998). Mutations in Sox10 lead to deafness and pigmentation disorders in mice and humans, similar to those caused by mutations in Mitf (Goding, 2000). Sox10 can bind to two sites within Mitf promoter and can increase Mitf expression levels by several fold (Potterf et al., 2000 and Goding, 2000). Mitf binding to E-box elements in target genes is increased in response to elevated cAMP levels (Bertolotto et al., 1996 and Bertolotto et al., 1998) and Fuse et al. have reported the presence of a cAMP responsive element in the Mitf promoter (Fuse et al., 1996). Finally, functional binding sites for the Wnt-family transcription factor Lef1 are present in the promoters of Mitf in Zebrafish, mouse and humans (Dorsky et al., 2000 and Takeda et al., 2000b).

The N-terminal activation domain in Mitf has been shown to interact with CBP (CREB binding protein) specifically through a region in CBP that is required for complex formation with adenoviral E1A, p300 and c-Fos (Sato et al., 1997). These data suggest that CBP/p300 might act as transcriptional co-factors of Mitf to drive target gene expression, at least in melanocytes. Further, it has been shown that stimulation of melanoma cells with c-Kit ligand Steel factor (SI) results in phosphorylation of Mitf by MAPK at serine 73. This S73 phosphorylation of Mitf by MAPK leads to the upregulation of the ability of Mitf to transactivate the tyrosinase pigmentation promoter (Hemesath et al., 1998).
Wu et al. showed that C-kit signaling results in the phosphorylation of Mitf by yet another kinase, viz. p90 Rsk, at serine 409. This dual phosphorylation event potentiated by c-Kit signaling leads to the targeting of Mitf by the ubiquitination pathway, which leads to its degradation (Wu et al., 2000).

Weilbaecher et al. showed that M-CSF1 induces the phosphorylation of Mitf at S73 in osteoclasts and that the expression of Mitf S73A mutant in Raw 264.7 cells decreased their ability to form multinuclear osteoclasts when treated with RANKL (Weilbaecher et al., 2001). Recently, Mansky et al. showed that Mitf is a target of the p38 MAPK pathway during osteoclast differentiation. They showed that Mitf is rapidly and persistently phosphorylated upon RANKL stimulation and that p38 MAPK phosphorylates MITF at serine 307 both in vivo and in in vitro kinase assays. Further, phosphorylation of Mitf at S307 by p38 MAPK correlated with elevated TRAP expression levels in these osteoclasts, which could be blocked by the addition of SB203580, a specific inhibitor of p38 MAPK (Mansky et al., 2002b).

1.2.3.9.3 Mitf AND OSTEOCLAST-SPECIFIC TARGET GENE EXPRESSION

As mentioned in the previous sections, it is known that Mitf regulates the expression of its target genes like TRAP, cathepsin K and E-Cadherin via directly binding to the ‘E-box’ elements on their promoters and via collaboration with other transcription factors like PU.1 (Motyckova et al., 2001, Luchin et al., 2000, Mansky et al., 2002a and Mansky et al., 2002c). TRAP and cathepsin K are important markers of osteoclast differentiation and mice knocked out for the
expression of these genes exhibit mild osteopetrosis. More recently, microarray analysis of osteoclasts from Mitf<sup>mi/mi</sup> mice by Rho et al., lead to the identification of OSCAR, a novel member of the leukocyte receptor family and collagenase 1V as potential Mitf- regulated genes in osteoclasts. Further, these workers also showed that Mitf and Pu.1 could synergistically induce OSCAR gene expression in transient transfection analysis (Rho et al., 2002 and So et al., 2003). The main limitation to this study was that the investigators using a “spotted microarray” containing a selective set of cDNAs and hence, was potentially biased. Further studies involving the comparison of gene expression profiles between wildtype (WT) and Mitf<sup>mi/mi</sup> osteoclasts via oligonucleotide micro arrays containing most of the known mouse genes and expressed tag sequences (ESTs) would help uncover novel genes that are regulated by Mitf and might play a role in osteoclast differentiation.

Consequently, much of the current knowledge about the role of Mitf in the differentiation of osteoclasts and other cells comes from the analysis of the phenotypes of the various spontaneous and irradiation mutations of the Mitf locus in several organisms.

**1.2.3.9.4 MUTATIONS IN THE Mitf LOCUS**

The original mutation in the Mitf locus was recognized 61 years ago due to its effect upon coat color in the descendents of an irradiated male mouse (Hertwig, 1942). This mutation, <i>mi</i>, is semidominantly inherited as the heterozygotes have a partial phenotype with respect to coat color. Thus, mice
heterozygous for the $mi$ mutation often have white spots on the belly, head and tail and have lesser pigmentation in the iris than the WT mice. The homozygous $Mitf^{mi/mi}$ mice have a white coat color due to a lack of melanocytes in the skin, small eyes or microphthalmia, reduced eye pigmentation due to a defect in RPE cell differentiation, retinal degeneration, early onset deafness due to a lack of melanocytes in the inner ear, reduced numbers of mast and natural killer cells and osteopetrosis due a block in osteoclast differentiation (Marks and Walker, 1981; Moore, 1995; Goding, 2000 and Steingrimsson et al., 2003). Thus, the Mitf gene product is required for the differentiation of several cell types belonging to different lineages. The human homologous gene MITF is mutated in families with Waardenburg syndrome type II (WS2) (Tassabehji et al., 1994). Patients heterozygous for dominantly inherited WS2 show varying degrees of deafness and patchy abnormal pigmentation of the hair, skin and eyes (Moore, 1995 and Goding, 2000).

More than 20 different mutants of the Mitf allele, arising spontaneously or by irradiation, have been identified in mice. Almost all of these mutants have a pigmentation phenotype of the coat, eye or the inner ear, suggesting an important role for Mitf in melanocyte development (Moore, 1995 and Steingrimsson et al., 2003). Some mutants have defects in the RPE and mast cells while very few of these have an osteoclast phenotype (Tables 1 and 2). $Mitf^{mi/mi}$ exhibits severe osteopetrosis, $Mitf^{or/or}$ exhibits a moderate osteopetrosis that resolves with age while $Mitf^{ew/ew}$, $Mitf^{dl/dl}$ and $Mitf^{crc/crc}$ exhibit very mild forms of osteopetrosis (Moore, 1995, Nii et al., 1995; Steingrimsson et al., 2002 and
Steingrimsson et al., 2003). Of these, \( \text{Mitf}^{\text{ew/ew}} \) show the mildest bone phenotype or hyperosteosis, with bony trabeculae extending into the bone marrow cavity further than in the WT, resembling the phenotype seen in Camurati-Engelmann disease (Steingrimsson et al., 2002). Interestingly, a loss of function mutant \( \text{Mitf}^{\text{vga9/vga9}} \), exhibits white coat color and \textit{microphthalmia} but does not have osteopetrosis (Moore, 1995 and Steingrimsson et al., 2002).
Table 1. Summary of the phenotypes in Mitf mutant mice. (Moore, 1995)

<table>
<thead>
<tr>
<th>Allele name (symbol)</th>
<th>Phenotype of heterozygote</th>
<th>Phenotype of homozygote</th>
<th>DNA lesion</th>
<th>In vitro DNA binding</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>homodimer</td>
<td>heterodimer</td>
</tr>
<tr>
<td>Mouse</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Semidominant</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>White (Mi&lt;sup&gt;+&lt;/sup&gt;)</td>
<td>White head spot</td>
<td>W&lt;sub&gt;1&lt;/sub&gt;: S&lt;sub&gt;1&lt;/sub&gt;; O&lt;sub&gt;1&lt;/sub&gt;; MC</td>
<td>delR215* in basic region</td>
<td>No</td>
</tr>
<tr>
<td></td>
<td></td>
<td>W&lt;sub&gt;1&lt;/sub&gt;: O</td>
<td>R18K in basic region</td>
<td>No</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Recessive</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>cloudy eyes (m)&lt;sup&gt;+&lt;/sup&gt;</td>
<td>+</td>
<td>W&lt;sub&gt;1&lt;/sub&gt;: S&lt;sub&gt;1&lt;/sub&gt; with EP, 1: cataracts</td>
<td>R265 stop (leucine zipper)</td>
<td>No</td>
</tr>
<tr>
<td></td>
<td></td>
<td>W&lt;sub&gt;1&lt;/sub&gt;: S&lt;sub&gt;1&lt;/sub&gt; eyelids never open</td>
<td>Splice defect (no exon 6)</td>
<td>No</td>
</tr>
<tr>
<td>spotted white (m)&lt;sup&gt;+&lt;/sup&gt;</td>
<td>+</td>
<td>W&lt;sub&gt;1&lt;/sub&gt;: S&lt;sub&gt;1&lt;/sub&gt; coat (white spots on back and belly; progressive depigmentation of coat, skin and eyes; t.; old mice (W&lt;sub&gt;1&lt;/sub&gt;: RD; 1))</td>
<td>D222N in helix I</td>
<td>Yes</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Human</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MITF-W1&lt;sup&gt;002&lt;/sup&gt;</td>
<td>Variable penetrance in carriers, hearing loss, reduced pigmentation of iris, white forelock, early graying</td>
<td>Unknown</td>
<td>GT to AT point mutation affecting exon 1 to exon 2</td>
<td>Splicing</td>
</tr>
<tr>
<td>MITF-W1&lt;sup&gt;026&lt;/sup&gt;</td>
<td>As for MITF-W1&lt;sup&gt;002&lt;/sup&gt;</td>
<td>Unknown</td>
<td>AG to CG point mutation affecting exon 4 to exon 5</td>
<td>Splicing</td>
</tr>
</tbody>
</table>

Abbreviations: M, microphthalmia; O, osteopetrosis; I, inner ear defect and/or hearing loss; W, white coat color; D, dilution of coat colour; EP, reduced eye pigmentation; BD, retinal degeneration; MC<sub>1</sub>, reduced mast cell numbers; C, reduced melanocyte proliferation in vitro; +, wild type; s, severe; m, mild; i, intermediate (e.g. S<sub>1</sub> indicates severe microphthalmia).

*Heterodimerization tested with TFE3 and wild-type ML.

†The protein encoded by Mi<sup>p</sup> can heterodimerize with wild-type Mi only in the presence of the six amino acid alternative exon.

‡Dominant-negative assay was the suppression of TFE3 DNA binding by the Mi<sup>p</sup> variant protein.

§The protein encoded by Mi<sup>p</sup> acts in a dominant-negative fashion only at the absence of the six amino acid alternative exon.

One of three arginines that constitute the 5' end of the basic region is deleted.
<table>
<thead>
<tr>
<th>Allele symbol</th>
<th>Phenotype Heterozygote</th>
<th>Phenotype Homozygote</th>
<th>Molecular defect</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\text{Mitf}^{mi}$</td>
<td>Iris pigment less than in wild type; occasional spots on belly, head, or tail</td>
<td>White coat; microphthalmia; incisors fail to erupt; osteopetrosis</td>
<td>Deletion of R215; basic domain</td>
</tr>
<tr>
<td>$\text{Mitf}^{Mi-or}$</td>
<td>Slight dilution of coat color; pale ears and tail; belly streak or head spot</td>
<td>White coat; microphthalmia; incisors fail to erupt; osteopetrosis</td>
<td>R216K; basic domain</td>
</tr>
<tr>
<td>$\text{Mitf}^{Mi-wh}$</td>
<td>Dilute coat color; eyes dark ruby; white spots on feet, tail, and belly</td>
<td>White coat; eyes small and slightly pigmented</td>
<td>I212N; basic domain</td>
</tr>
<tr>
<td>$\text{Mitf}^{mi-ew}$</td>
<td>Normal</td>
<td>White coat; microphthalmia; hyperosteosis</td>
<td>25 amino acid deletion; basic domain</td>
</tr>
<tr>
<td>$\text{Mitf}^{mi-vga9}$</td>
<td>Normal</td>
<td>White coat; microphthalmia</td>
<td>Insertion and deletion; regulatory</td>
</tr>
</tbody>
</table>

Table 2. Comparison of the phenotypes in Mitf alleles with mutations in the basic domain and Mitf$^{vga/vga}$. (Steingrimsson et al., 2002)
The mutations that affect bone resorption more severely, viz., Mitf<sup>mi/mi</sup> and Mitf<sup>or/or</sup> have mutations in the basic region of the Mitf gene product (Figure 18). Mitf<sup>mi/mi</sup> mutation is due to a 3 bp deletion in the N-terminus, which deletes one of the three arginines in the basic domain of the protein (ΔR215) while the Mitf<sup>or/or</sup> mutation is due to the substitution of another arginine in the same basic domain, with a lysine (R216K) (Figure 18, Table 2). Another mutation in the basic domain of Mitf, Mitf<sup>wh/wh</sup>, arising due to a conservative substitution of an isoleucine to asparagine (I212N), does not cause osteopetrosis or any defects in osteoclast differentiation (Moore, 1995). Interestingly, these mutants have the most severe coat color phenotype among all the Mitf mutants (Moore, 1995 and Steingrimsson et al., 2003). All three of these mutants are semidominantly inherited meaning that the heterozygotes also have a phenotype.

All three of these mutant proteins could not bind to DNA as homodimers. In addition, they blocked DNA binding by WT Mitf protein. Mitf<sup>mi/mi</sup> and Mitf<sup>or/or</sup>, both in the plus and minus forms, blocked DNA binding by Tfe3, suggesting that they behave as dominant negative molecules. Interestingly, only Mitf<sup>wh/wh</sup> minus form acted as a dominant negative with Tfe3 for DNA binding. The plus form of Mitf<sup>wh/wh</sup> was quite capable of binding to DNA as a heterodimer with Tfe3 (Hemesath, 1994). The basic domain of b-HLH proteins binds to DNA through an α-helix that forms an uninterrupted structure with helix 1 of the HLH domain (Fisher et al., 1991 and Ferré-D’Amaré et al., 1993). This unstable α-helix requires DNA binding to stabilize its folding (Fisher et al., 1993 and Ferré-D’Amaré et al., 1994). The arginine at position 215 in Max/DNA co-crystal
structure was shown to make a phosphate contact (Ferré-D’Amaré et al., 1993). It has been suggested that the R215 deletion in Mitf$^{mi/mi}$ would shift the basic $\alpha$-helical domain 100$^0$ relative to the HLH domain precluding DNA binding. This and the fact that R216K mutation in Mitf$^{or/or}$ could not substitute for DNA binding suggests that these arginines might form a salt bridge with another amino acid in the vicinity to stabilize the $\alpha$-helix (Hemesath et al., 1994).

Figure 18. Illustration of Mitf protein structure along with the location of mutations in Mitf$^{mi/mi}$, Mitf$^{or/or}$ and Mitf$^{wh/wh}$. 
It has long been proposed in the field that Mitf, Tfe3 and TfeC might play redundant roles in osteoclast differentiation and that Mitf<sup>mi/mi</sup> and Mitf<sup>or/or</sup> might enter into dominant negative heterodimers with Tfe3 or TfeC to manifest the osteopetrosis seen in these mutant mice. This would be a possible explanation for why mice lacking the expression of Mitf, Mitf<sup>vga9/vga9</sup>, do not exhibit osteopetrosis. To address this issue, Steingrimsson et al. generated mice that were homozygous for all possible combinations of Mitf<sup>mi/mi</sup>, Mitf<sup>vga9/vga9</sup>, and Mitf<sup>ew/ew</sup> and with Tfe3<sup>-/-</sup> and with TfeC<sup>-/-</sup>. They found that while Tfe3<sup>-/-</sup>, TfeC<sup>-/-</sup> or Mitf<sup>wh/wh</sup> do not exhibit osteopetrosis, double homozygous Tfe3<sup>-/-</sup>Mitf<sup>wh/wh</sup>, Tfe3<sup>-/-</sup>Mitf<sup>ew/ew</sup>, Tfe3<sup>-/-</sup>Mitf<sup>vga9/vga9</sup> and triple homozygous Tfe3<sup>-/-</sup>TfeC<sup>-/-</sup>Mitf<sup>wh/wh</sup> exhibit severe osteopetrosis (Table 3). However TfeC mutants did not alter the bone phenotype in homozygous Mitf, TfeB, or Tfe3 mutants (Steingrimsson et al., 2003). These data suggest that Tfe3 has a role in osteoclast differentiation, one that is redundant with Mitf.
<table>
<thead>
<tr>
<th>Genotype</th>
<th>Phenotype</th>
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<tbody>
<tr>
<td></td>
<td>Eyes</td>
</tr>
<tr>
<td>Single mutants</td>
<td></td>
</tr>
<tr>
<td>Tfe3&lt;sup&gt;3&lt;/sup&gt;/Tfe3&lt;sup&gt;3&lt;/sup&gt;</td>
<td>—</td>
</tr>
<tr>
<td>Tfe&lt;sup&gt;C&lt;/sup&gt;/Tfe&lt;sup&gt;C&lt;/sup&gt;</td>
<td>—</td>
</tr>
<tr>
<td>Tfe&lt;sup&gt;b&lt;/sup&gt;/Tfe&lt;sup&gt;b&lt;/sup&gt;</td>
<td>ND</td>
</tr>
<tr>
<td>Mitf&lt;sup&gt;m-vga9&lt;/sup&gt;/Mitf&lt;sup&gt;m-vga9&lt;/sup&gt;</td>
<td>++++</td>
</tr>
<tr>
<td>Mitf&lt;sup&gt;m&lt;/sup&gt;/Mitf&lt;sup&gt;m&lt;/sup&gt;</td>
<td>++++</td>
</tr>
<tr>
<td>Mitf&lt;sup&gt;wh&lt;/sup&gt;/Mitf&lt;sup&gt;wh&lt;/sup&gt;</td>
<td>++</td>
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<tr>
<td>Mitf&lt;sup&gt;ew&lt;/sup&gt;/Mitf&lt;sup&gt;ew&lt;/sup&gt;</td>
<td>++++</td>
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<tr>
<td>Double and triple mutants</td>
<td></td>
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<tr>
<td>Mitf&lt;sup&gt;wh&lt;/sup&gt;/Mitf&lt;sup&gt;wh&lt;/sup&gt;, Tfe&lt;sup&gt;C&lt;/sup&gt;/Tfe&lt;sup&gt;C&lt;/sup&gt;</td>
<td>++</td>
</tr>
<tr>
<td>Mitf&lt;sup&gt;wh&lt;/sup&gt;/Mitf&lt;sup&gt;wh&lt;/sup&gt;, Tfe&lt;sup&gt;3&lt;/sup&gt;/Tfe&lt;sup&gt;3&lt;/sup&gt;</td>
<td>++</td>
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<td>Mitf&lt;sup&gt;m&lt;/sup&gt;/Mitf&lt;sup&gt;m&lt;/sup&gt;, Tfe&lt;sup&gt;C&lt;/sup&gt;/Tfe&lt;sup&gt;C&lt;/sup&gt;</td>
<td>++++</td>
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<td>Mitf&lt;sup&gt;ew&lt;/sup&gt;/Mitf&lt;sup&gt;ew&lt;/sup&gt;, Tfe&lt;sup&gt;C&lt;/sup&gt;/+</td>
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<td>Mitf&lt;sup&gt;ew&lt;/sup&gt;/Mitf&lt;sup&gt;ew&lt;/sup&gt;, Tfe&lt;sup&gt;3&lt;/sup&gt;/Tfe&lt;sup&gt;3&lt;/sup&gt;</td>
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—, no change; +, affected; ++++, severely affected. ND, not determined because of embryonic lethality.

**Table 3. Summary of phenotypes of Mitf, Tfe3 and TfeC mutant mice.** (Steingrimsson et al., 2002)
Finally, the Mitf gene is evolutionarily conserved from fish, including poeciliid *Xiphophorus*, puffer fishes *Fugu rubripes* and *Tetraodon nigroviridis*, and zebra fish *Danio rerio*, to quails and from mice and rats to humans. Apart from mice and humans, Mitf mutations have also been reported in zebra fish, rats, hamsters and quail (Moutier et al., 1989; Cielinski and Marks, 1995, Hodgkinson et al., 1998; Mochii et al., 1998 and Lister et al., 1999). The quail Silver and the rat Mitf$^{mib/mib}$ mutations result in mild osteopetrosis. The quail Silver mutation results from a histidine to arginine mutation in the basic domain and a stop codon shortly after the b-Zip domain. The homozygous Silver quail mutants do exhibit severe osteopetrosis, albeit milder than that in the Mitf$^{mi/mi}$ mouse mutants, and do have fewer numbers of multinuclear osteoclasts (Mochii et al., 1998). The rat Mitf$^{mib/mib}$ mutation, which arises due a deletion comprising the 3' half of the Mitf gene (deletion of the HLH-bZip and C-terminal activation domains) and possible loss of function, results only in mild osteopetrosis that resolves with age (Moutier et al., 1989; Cielinski and Marks, 1995 and Weilbaecher et al., 1998). At birth the mutant rats exhibited extremely low numbers of poorly formed osteoclasts that lacked ruffled borders and expressed decreased levels of TRAP and carbonic anhydrase II messages. However, the osteoclastic defects and osteopetrotic phenotype in these homozygous mutant rats resolve as they age (Cielinski and Marks, 1995 and Weilbaecher et al., 1998).

In summary, Mitf appears to have a unique and essential role in osteoclast differentiation. Of all the 20 different irradiation and spontaneous
mutations of the *mi* allele, osteopetrosis appears to be the most severe in *Mitf*<sup>mi/mi</sup> and *Mitf*<sup>or/or</sup> mutant mice. However, the level of penetrance of this phenotype in these two mutants is different.

### 1.2.3.9.5 OSTEOPETROSIS IN *Mitf*<sup>mi/mi</sup> AND *Mitf*<sup>or/or</sup> MUTANTS

Histological, histochemical and radiological examinations have shown that the *Mitf*<sup>mi/mi</sup> mutant mice exhibit classical symptoms of osteopetrosis characterized by accumulation of primary spongiosa in the bone marrow cavity, shorter and denser rib and long bones and by the failure in the eruption of incisors (Figure 19). All these symptoms have been attributed to the lack of osteoclast activity as a result of defective osteoclast differentiation (Grüneberg, 1948; Moore, 1995 and Luchin et al., 2000).

Original bone marrow transplantation experiments performed on these mice demonstrated that the osteopetrosis in the *Mitf*<sup>mi/mi</sup> mice could be rescued by transplanting WT spleen or bone marrow cell suspensions into the bone marrow of the mutant mice (Walker, 1975a and Marks, 1989). Likewise, transplantation of bone marrow from *Mitf*<sup>mi/mi</sup> mice to that of lethally irradiated WT mice caused osteopetrosis in WT mice (Walker, 1975b and Marks, 1989). These experiments showed that the osteopetrosis defect in *Mitf*<sup>mi/mi</sup> mutant mice is intrinsic to osteoclasts and to its hematopoietic precursors in the bone marrow.
Figure 19. Osteopetrotic phenotype in Mitf<sup>mi/mi</sup> mice.
A: Radiological analysis of femurs from WT and Mitf<sup>mi/mi</sup> mice; B: Hematoxylin and Eosin staining of sections of femurs from WT and Mitf<sup>mi/mi</sup> mice (arrow heads indicating unresorbed trabecular bone); C: TRAP stained femur sections from WT and Mitfmi/mi mice.
(Luchin et al., 2002)
Thesingh and Scherft observed that the osteoclasts in embryonic and fetal $Mitf^{mi/mi}$ mice were predominantly mononuclear and lacked ruffled borders and a clear zone. Further, they observed that these cells exhibited many osteoclastic morphologic properties such as abundant mitochondria, positive succinic dehydrogenase and acid phosphatase reactions (Thesingh and Scherft, 1985). The osteoclasts from the mutant mice have also been shown to be able to adhere to bone surfaces, spread from surfaces, form lamellodopa and express TRAP and CTR markers (Helfrich and Mieremet, 1988 and Graves and Jilka, 1990). Further, Graves and Jilka showed that calvarial cultures from $Mitf^{mi/mi}$ mutant mice failed to form multinuclear osteoclasts and were defective in bone resorption in response to PTH stimulation. The cultures from WT cells produced multinuclear osteoclasts capable of bone resorption (Graves and Jilka, 1990). Interestingly, the occurrence of multinuclear osteoclasts and foreign body giant cells was observed in cultures of precursors derived from the bone marrow in young $Mitf^{mi/mi}$ mice and the presence of multinuclear osteoclasts, albeit in smaller numbers compared to WT, was observed in adult $Mitf^{mi/mi}$ mice (Thesingh and Scherft, 1985 and Al-Douri and Johnson, 1982).

$Microphthalmia$-Oak Ridge ($Mitf^{or/or}$) mutation was first found in the offspring of a male mouse whose spermatogonia had received $\gamma$-irradiation (Nii et al., 1995). The phenotype exhibited by $Mitf^{or/or}$ mice is very similar to those exhibited by $Mitf^{mi/mi}$ mice; white coat color, small eyes and failure of incisors to erupt or poorly formed incisors later in life (Nii et al., 1995). Radiological examination of the long bones of 2-3 day old newborn mice revealed the
presence of sclerotic lesions in the diaphyseal and metaphyseal areas of the long bone and those of 30-day old mice revealed the presence of sclerotic lesions only in the metaphyseal areas of the bone (Sankar and Ostrowski, unpublished observations).

Histological analysis of the long bone sections from Mitf<sup>pr/or</sup> mutant mice at 1, 10, 18 and 37 days of age revealed that the accumulation of unresorbed primary spongiosa at the metaphysis increased with age, with almost no accumulation seen in the long bones of 1-day old mice (Nii et al., 1995). Ultrastructurally, the osteoclasts from Mitf<sup>pr/or</sup> mutant mice were multinuclear, TRAP positive and had well-developed ruffled borders. However, the vacuoles in these osteoclasts were larger than those from the osteoclasts in WT mice (Nii et al., 1995). The difference in the osteopetrosis phenotype between Mitf<sup>mi/mi</sup> (B6C3Fe) and Mitf<sup>pr/or</sup> (C3H) mice could be in part due to a difference in their genetic backgrounds (Nii et al., 1995). The milder osteopetrosis phenotype seen in Mitf<sup>pr/or</sup> is similar to the one seen in intermediate autosomal recessive osteopetrosis seen in humans (Nii et al., 1995).

As mentioned earlier, the Mitf<sup>wh/wh</sup> mutant has no apparent defects in osteoclasts or bone resorption, even though this mutation occurs in the basic domain of Mitf also. Thus, it is very interesting that three mutations that affect the same basic domain of Mitf protein and affect its DNA binding capabilities, viz. Mitf<sup>mi/mi</sup>, Mitf<sup>pr/or</sup> and Mitf<sup>wh/wh</sup>, behave differently with respect to their effect on osteoclast differentiation and ensuing activity. Hence these mutations, Mitf<sup>mi/mi</sup>, Mitf<sup>pr/or</sup> and Mitf<sup>wh/wh</sup>, form an allelic series of Mitf mutants that could
provide crucial insights into the role of Mitf in osteoclast differentiation. Thus, genes involved in osteoclast differentiation that are regulated by Mitf, are bound to be differentially expressed in these different allelic mutants of Mitf.

1.3 CONCLUSION

Mitf is required for the differentiation of several cell types belonging to different lineages including osteoclasts and melanocytes and is evolutionarily conserved from fish to humans. Although it is evident that Mitf has a major role in osteoclast differentiation and function, the precise role played by this transcription factor in osteoclastogenesis is largely unknown. Analysis of the differential expression of osteoclast-specific genes in Mitf mutants that exhibit osteopetrosis will help identify novel Mitf-regulated genes involved in the regulation of osteoclast differentiation and activation. Gene depletion and overexpression studies would clarify the role of these novel genes in osteoclast biology, ultimately bringing the field closer towards mapping out the precise roles undertaken by Mitf during osteoclast differentiation and function.
CHAPTER 2

MATERIALS AND METHODS

2.1 DNA MANIPULATIONS

2.1.1 AGAROSE GEL ELECTROPHORESIS

Agarose gels were prepared by dissolving agarose (Fisher Scientific) in 1X Tris-acetic acid-ethylenediaminetetraacetic acid, Na salt (EDTA) (TAE) buffer (40mM Tris-acetate, 2mM Na₂EDTA·2H₂O; pH 8.0; made as 50X stock). Concentration of agarose in the gel was determined based on the size range of DNA fragments to be separated. Thus, a 2% gel was used to separate DNA fragments between 0.2-0.6 kilo bases (kb); 1.2% to separate fragments between 0.6-1kb; 1% gel to separate fragments between 1-2kb and 0.8% gel to separate DNA fragments more than 2kb in size. DNA was resuspended in DNA loading dye (0.5X Tris-boric acid-EDTA (TBE) (89.2mM Tris, 89mM boric acid, 2mM Na₂EDTA·2H₂O; pH 8.0; made as 5X stock), 16%glycerol, 100mg/ml Bromphenol Blue (BPB) and 0.4 mg/ml Xylene Cylenol; final concentration; prepared as 5X stock) and loaded onto the wells in the cast agarose gel. The
DNA fragments were separated in the presence of 1X TAE buffer at 80-120 volts (Ausubel et al., 1997).

2.1.2 POLYMERASE CHAIN REACTION (PCR)

PCR amplification from plasmid DNA was performed by using a reaction mixture containing 1pg of plasmid DNA, 0.2µM each of forward and reverse primers, 1.5µM MgCl₂, 50µM deoxy-nucleotide tri phosphates (dNTPs) (Roche), 1X PCR buffer and 1 unit (U) of DNA polymerase (Invitrogen) in a total volume of 50-100µl. PCR was performed using the following thermo cycling conditions: an initial denaturation at 95⁰C for 4 minutes followed by 30 cycles of denaturation at 94⁰C for 1 minute, annealing at 55⁰C for 30 seconds followed by extension at 72⁰C for 1.5 minutes and a final extension step at 72⁰C for 7 minutes. Reaction products were analyzed by electrophoresing 1/10th the volume of the PCR reaction on agarose gels. The PCR products were purified using QIAquick PCR-purification kit from Qiagen, in a total elution volume of 200µl.

Purified PCR fragments to be used for cloning were subject to “TA-fill in” of the 5' over-hangs with Klenow fragment of DNA polymerase I (Klenow). Briefly, 200µl of purified PCR products were incubated with 1X Klenow buffer (50mM Tris-HCl, pH 7.4; 6.5mM MgCl₂; 1mM DTT and 32µg/ml BSA); 0.033mM dTTP/dATP mix (Roche Diagnostics) and 10 U of Klenow (Roche) in a total volume of 300µl, for 1 hour at 37⁰C. Thereafter, Klenow was heat inactivated at
65°C for 10 minutes and the DNA was purified by phenol-chloroform extraction followed by precipitation using 0.3M NaOAc and ethanol.

The procedure for PCR amplification of genomic DNA for mouse genotyping is detailed in sections 2.3.3 and 2.3.4.

2.1.3 CLONING OF DNA
2.1.3.1 RESTRICTION DIGESTS

Restriction digests of DNA were conducted with commercially available endonucleases (NEB, Fermentas). The digests were performed using the appropriate buffers and under conditions recommended by the manufacturer in a total volume of 20-100 µl, depending on the amount of DNA. The products of restriction digestion were analyzed by agarose electrophoresis along with undigested controls and DNA size markers.

2.1.3.2 ALKALINE PHOSPHATASE TREATMENT AND DNA LIGATION

Linearized vector DNA was subjected to alkaline phosphatase treatment prior to ligation, to avoid self-ligation of “sticky ends”. The vector DNA was digested with the appropriate restriction enzyme or enzymes. In case of a sequential digest, the vector was digested with the first enzyme for 2 hours. A diagnostic agarose gel electrophoresis was performed along with an uncut vector control to confirm linearization. Linearized vector was incubated with alkaline phosphatase buffer (1X final concentration; Roche) and 5 µl of Calf Intestine Alkaline Phosphatase (Roche) in a total volume of 200 µl, at 37°C for
one hour. The enzyme was heat-inactivated at 65°C for 20 minutes and the DNA was purified by phenol-chloroform extraction followed by ethanol precipitation thereafter. The DNA was resuspended in an appropriate volume of Tris-EDTA (TE; 10mM Tris-HCl; pH8.0 and 0.1mM EDTA) and the second digestion was carried out. The restricted DNA fragments are separated on agarose gel electrophoresis and gel purified using QIAquick gel extraction kit, according to manufacturer’s directions.

Linearized vectors were ligated to inserts containing compatible sticky or blunt ends using a molar ratio of 1:3. The reaction was performed in the presence of 1U of T4 DNA ligase (Roche) using the buffer supplied by the manufacturer, at room temperature for 4 hours or at 14°C overnight.

2.1.3.3 PHENOL-CHLOROFORM EXTRACTION AND PRECIPITATION OF DNA USING ETHANOL

Equal volume of phenol-chloroform-isoamyl alcohol (24:24:1) (Amresco) was added to the DNA samples and extracted for 5 minutes with vortexing. The extracted sample was centrifuged at 13,000 revolutions per minute (rpm) for five minutes. The aqueous upper phase was transferred to a fresh tube and extracted with equal volume of chloroform-isoamyl alcohol (24:1) (Amresco) for 5 minutes followed by centrifugation and the clear upper phase was transferred to a fresh tube. The DNA was precipitated by adding 1/10th the volume of 3M NaOAc and 2.5 volumes of 100% ethanol (–20°C) at –80°C for 20 minutes or at –20°C overnight. DNA was centrifuged at 4°C for 20 minutes at 13,000 rpm,
washed in 1ml of 70% ethanol, air dried and resuspended in an appropriate volume of TE.

2.1.3.4 DNA TRANSFORMATION INTO ESCHERICHIA COLI (E.COLI) COMPETENT CELLS

2.1.3.4.1 PREPARATION OF E. coli COMPETENT CELLS

A small amount of inoculum from frozen glycerol stocks of DH5α or BL21(DE3)-pLys-S was streaked onto Luria broth (LB) or LB-Chloramphenicol (Chlor) agar petriplates respectively and incubated overnight at 37°C. A single colony was used to inoculate a 5ml LB or LB-Chlor liquid media and the culture was grown overnight at 37°C with shaking. Next morning, 2ml of overnight culture was used to inoculate 200ml of LB or LB-Chlor media in a 1liter flask. The culture was grown at 37°C with constant shaking until the cells reached an optical density 600 (OD₆₀₀) of 0.5 as measured using a spectrophotometer. The culture was chilled on ice for 20 minutes and centrifuged at 4°C at 4000 rpm in a Sorvall CS-3 rotor. The cell pellet was placed on ice, resuspended in ½ volume of ice-cold 5mM CaCl₂, chilled on ice for 20 minutes and centrifuged once again as earlier. The cell pellet was finally resuspended in 1/20th volume of ice-cold 50mM CaCl₂ and 15% glycerol, aliquoted into screw cap tubes @ 400µl/tube, quick frozen on dry ice and stored at -80°C until further use.
2.1.3.4.2 DNA TRANSFORMATION INTO COMPETENT CELLS

One hundred microliters of the frozen competent cells were slowly thawed on ice, mixed with either 5µg of supercoiled plasmid DNA or the entire ligation mix and incubated on ice for 30 minutes. The cells were heat shocked for 2 minutes and immediately recovered by adding 1ml of LB and shaking at 37°C for 1 hour. Ten and 100µl aliquots of the mixture were plated on LB plates with the appropriate antibody. When transforming ligation reactions, the remaining recovered cells were spun down, resuspended in a smaller volume of media and the entire mixture was plated as mentioned before. The plates were incubated at 37°C for 12-16 hours. Screening for recombinant colonies were accomplished by inoculating single colonies into overnight cultures, extracting the plasmid DNA via miniprep, restriction digestion of the DNA and analyzing the products by agarose electrophoresis.

2.1.4 PLASMID MINI AND MAXIPREPS

Single colonies of bacterial cells transformed supercoiled or ligation mixture were inoculated into 5ml cultures of LB media (from plates as mentioned above) containing the appropriate antibody and grown overnight at 37°C with shaking. One and a half milliliters of overnight culture were transferred to an Eppendorf tube and centrifuged. The process was repeated so as to obtain a cell pellet from 3ml of overnight culture. The cell pellet was resuspended in 100µl of solution P1 (50mM Tris-Cl, pH 8.0; 10mM EDTA and 100µg/ml RNaseA). Another 100µl of solution P2 (200mM NaOH and 1% SDS)
was added and the contents were mixed by gently inverting the tube 5-6 times. Lysis of the cells was allowed to proceed by incubating the contents for 15 minutes at room temperature. Thereafter, 100ml of solution P3 (3M KOAc, pH 5.2) was added, the contents were mixed as before by inverting the tube 5-6 times and the tube was incubated on ice for 20 minutes. The tubes were then centrifuged at 4°C for 30 minutes at 13,000 rpm. The clear supernatant was transferred to a fresh tube and extracted with phenol-chloroform mixture, as mentioned before. DNA was precipitated by adding 2.5 volumes of 100% ethanol (-20°C) to the supernatant followed by centrifugation of the contents at 4°C for 20 minutes at 13,000 rpm. The DNA pellet was washed with 1ml of 70% ethanol, air-dried and resuspended in 50-100µl of TE. The miniprep DNA samples were screened by restriction digestion followed by analysis via agarose electrophoresis.

Maxiprep procedure was used for large-scale preparation of plasmid DNA from recombinant colonies containing the desired plasmid constructs. Portions of the overnight cultures remaining after miniprep were diluted 1:200 into fresh 5ml cultures of LB media containing the appropriate antibodies and grown at 37°C with shaking for 8 hours. Alternatively, single colonies from a fresh plate were inoculated into 5ml cultures and grown for 8 hours as mentioned above. These cultures were further diluted 1:250 into fresh 200ml LB media with appropriate antibody and grown overnight with shaking at 37°C. Large amounts of plasmid DNA was isolated from these cultures using Qiagen Plasmid Maxi kit according to manufacturer’s directions.
2.2 PREPARATION OF RECOMBINANT RANKL AND L-CELL MEDIA CONTAINING MCSF1

Soluble recombinant RANKL was generated by amplifying a portion of the RANKL cDNA that corresponds to amino acids 158-316 containing only the C-terminal TNF domain by PCR, cloning the PCR product into a pET32b expression plasmid, expressing the recombinant protein in *Escherichia coli* followed by purification of soluble RANKL from the bacterial lysates (Lacey et al., 1998 and Motychova et al., 2001)

2.2.1 CLONING OF SOLUBLE RANKL cDNA

The soluble portion of RANKL, corresponding to amino acids 158-316, was amplified by polymerase chain reaction (PCR) from a full length RANKL cDNA clone using forward primer, 5’CGCGAGATCTACCATGGGCCATCATCA and the reverse primer, 5’CCCAAGCTTGGGGTCTATGTCTCTGAAC. The PCR products, with 5’ BglII and 3’ HindIII sites, were purified using QIAquick PCR-purification kit from Qiagen, in a total elution volume of 150µl. Purified PCR fragments were subject to “TA-fill in” of the 5’ over-hangs with Klenow. Blunt-ended PCR products were ligated into a Sma1-linearized pBSK vector (Stratagene) and the ligation mixture was transformed into *E. coli* DH5α competent cells. Recombinant colonies were screened via miniprep followed by restriction digestion with HindIII and BglIII and analysis on 1% agarose gels. Four recombinants were subject to DNA sequencing.
Large amount of recombinant DNA clone was generated by maxiprep. The PCR fragment was released by restriction digestion with HindIII and BgIII. Simultaneously, pET32b vector was digested with the same enzymes and alkaline phosphatase treated. The Hind3-BglIII digested pET32b vector and PCR product were run on 0.8% and 1% agarose-TAE gels, respectively. Gel fragments containing the vector and insert were excised and purified using QIAquick gel extraction kit. The vector and insert fragments were ligated at room temperature (RT) overnight and the entire ligation mixture was transformed into DH5α competent cells. The colonies were screened by miniprep followed by restriction digest and two recombinant DNA samples were bulked up by maxiprep.

2.2.2 EXPRESSION OF pET32B-RANKL

One hundred microliters of BL21(DE3)-pLys-S E.coli competent cells were transformed with 2µg of maxiprepped recombinant pET32b-RANKL DNA and plated on LB-ampicillin-chloramphemicol (LB-Amp-Chlor) plates. Four recombinant colonies were inoculated into 5ml LB-Amp-Chlor cultures and were grown overnight at 37°C with shaking. The next morning, 250µl of overnight cultures were diluted into fresh 50ml 2YT media and grown to an optical density (OD)₆₀₀ of 0.6 at 37°C. The cultures were divided into two tubes @ 25ml per tube. One 25ml culture was induced with 50µl of isopropyl β-D-thiogalactopyranoside (IPTG) (2mM final concentration), split into two 12.5ml portions in two 50ml conical tubes. The uninduced cultures were also divided into two
portions like mentioned above. The cultures were grown at either $30^\circ$C or at $37^\circ$C for three hours with shaking and equal amount of cells, as determined by OD$_{600}$ values, were withdrawn from the cultures every hour. The cells were centrifuged, resuspended in 100$\mu$l of 2X sodium dodecyl sulphate-poly acrylamide gel electrophoresis (SDS-PAGE) buffer (100mM Tris-HCl (pH 6.8), 6% $\beta$-ME, 4%SDS, 0.2% BPB and 20% glycerol), boiled for 5 minutes and centrifuged at 20,000 rpm at 4$^\circ$C for 20 minutes. The supernatants were frozen in a fresh tube at $-80^\circ$C until all samples were collected. The samples were run on a 12% SDS-PAGE, stained in 1% Coomassie blue solution, destained and the expressed protein products were visualized by comparing with uninduced samples.

2.2.3 LARGE SCALE PURIFICATION OF RECOMBINANT SOLUBLE RANKL

One freshly transformed recombinant pET32b-RANKL- BL21(DE3)-pLys-S colony was inoculated in 5ml of 2YT-Amp-Chlor media and grown for 8 hours with shaking at $37^\circ$C. This culture was diluted 1:200 into 100ml of 2YT-Amp-Chlor media and grown overnight at $37^\circ$C. Overnight culture was used to inoculate 10 liters of 2YT-Amp-Chlor media, grown to an OD$_{600}$ of 0.6, induced with IPTG (2mM final) and the induced culture was grown for an additional 2 hours at $37^\circ$C. The cells were spun down into a tight pellet and frozen at $-80^\circ$C. The frozen pellet was thawed on ice in the presence of 10ml of sonication buffer (50mM sodium phosphate, 300mM NaCl, 0.5% NP-40, 5mM $\beta$-ME, 10$\mu$g/ml leupeptin, 10$\mu$g/ml pepstatin, 2$\mu$g/ml aprotinin and 1mM phenylmethylsulfonyl fluoride).
fluoride (PMSF)) with occasional vortexing. An additional 15ml of sonication buffer was added to the mixture together with 14μl of RNase-free DNase1 (Roche) and the bacterial soup was sonicated six times on ice, with a 30 second pulse each time. The tubes were then rotated at RT for 30 minutes to continue lysis, centrifuged at 12,000 rpm for 30 minutes at 4°C.

While the bacterial lysates were being centrifuged, 2ml of Nickel-agarose Ni-NTA superflow beads (Qiagen) was added to a 50ml conical tube containing 20ml of sonication buffer, rotated on the wheel at 4°C for 5 minutes, centrifuged and the supernatant was discarded. The Ni-NTA beads were washed two more times in this manner. The supernatant from the centrifuged bacterial lysates was added on top of the washed Ni-NTA beads and this slurry was rotated on the wheel at 4°C for 1 hour. The lysate-Ni-NTA slurry was then slowly poured into a 1ml protein purification column (BioRad). The first 20ml of the flow through was repoured back into the column. The column was washed with 50ml of wash buffer (20mM Tris-HCl; pH 8.0), 0.5M NaCl, 10% glycerol, 30mM imidazole and 1mM PMSF. Finally, recombinant pET32b-RANKL bound to the column was eluted using 7ml of elution buffer (20mM Tris-HCl; pH 8.0), 0.5M NaCl, 10% glycerol, 500mM imidazole and freshly added 1mM PMSF. The elutions were collected in 0.5μl fractions and 10μl fractions from these samples were run on 12% SDS-PAGE to determine concentrations. Fractions containing the highest concentration of RANKL were pooled and frozen at –80°C as 10μl aliquots. The fractions were tested for their ability to support osteoclast...
differentiation on freshly isolated bone marrow cells, in the presence of M-CSF1.

**2.2.4 PREPARATION OF M-CSF**

Recombinant M-CSF1 was a gift from Chiron Corp., Emeryville, CA. Serum-free conditioned media from murine L-cells containing M-CSF1 was prepared by seeding L-cells, previously grown to confluence in Dulbecco’s modified eagle medium (DMEM) and 10% calf serum, at \(5.7 \times 10^4\) cells/ml in serum-free DMEM and growing them at 37°C for 35 days with 7% CO\(_2\) in air. The media was harvested, vacuum-filtered and stored at –80°C in the presence of 0.02% NaN\(_3\) (Stanley, 1985).

**2.3 MOUSE LINES AND GENOTYPING**

Mice used in this study were housed at the Ohio State University Laboratory Animal Resources (ULAR) facilities and were maintained in accordance with ULAR protocol.

**2.3.1 p27\(^{KIP1/-}\), p21\(^{CIP1/-}\) AND p27\(^{KIP1/-}\) p21\(^{CIP1/-}\) MOUSE LINES**

The p27\(^{KIP1/-}\) mice (C57BL/6J) were a generous gift from Dr. Andrew Koff (Kiyokawa et al., 1996) and the p21\(^{CIP1/-}\) mice (B6129SF2/J), originally made by Dr. Tyler Jacks, were a generous gift from Dr. Ming You (Brugarolas et al., 1995). Female p27\(^{KIP1/+/}\) mice are infertile. Hence, male p27\(^{KIP1/-}\) or p27\(^{KIP1/+/}\) mice were mated with female p27\(^{KIP1/+/}\) mice to generate p27\(^{KIP1/-}\) and
WT littermates for experiments. Male and female p21$^{\text{CIP1}/-}$ mice were mated to obtain offspring for experiments.

In order to generate p27$^{\text{KIP1}/-}$p21$^{\text{CIP1}/-}$ double knockout mice, the following strategy was used: male p27$^{\text{KIP1}/-}$ mice were mated with female p21$^{\text{CIP1}/-}$ mice to obtain p27$^{\text{KIP1}/+}$p21$^{\text{CIP1}/+}$ progeny. Male and female mice of this genotype were mated to generate p27$^{\text{KIP1}/+}$p21$^{\text{CIP1}/-}$ and p27$^{\text{KIP1}/-}$p21$^{\text{CIP1}/-}$ mice. Female p27$^{\text{KIP1}/+}$p21$^{\text{CIP1}/-}$ mice were then mated with p27$^{\text{KIP1}/+}$p21$^{\text{CIP1}/-}$ or p27$^{\text{KIP1}/-}$p21$^{\text{CIP1}/-}$ males to generate p27$^{\text{KIP1}/-}$p21$^{\text{CIP1}/-}$ double knock out mice.

2.3.2 Mitf$^{\text{mi/mi}}$, Mitf$^{\text{or/or}}$ AND Mitf$^{\text{wh/wh}}$ MOUSE LINES

The Mitf$^{\text{wh/wh}}$ (C57BL/6J), Mitf$^{\text{mi/mi}}$ (B6C3Fe) and Mitf$^{\text{or/or}}$ (C57BL/6J) mice were housed in the Ohio State University Laboratory Animal Resources (ULAR) facility.

2.3.3 MOUSE GENOMIC DNA EXTRACTION FROM TAIL CLIPS

Mouse genomic DNA was isolated from tail clips. Tail clips were digested overnight at 55°C in 0.5ml TE-SDS (50mM Tris-Cl; pH 8.0, 100mM EDTA and 0.5% SDS) and 1μg/ml proteinase K. Genomic DNA was extracted for 5 min in Tris-HCl buffer-saturated phenol (pH 8.0). The aqueous phase was transferred to a fresh tube and further extracted with phenol-chloroform-isoamyl alcohol (24:24:1) for 3 minutes and finally with chloroform-isoamyl alcohol for 2 minutes. The aqueous phase was transferred to a fresh tube and the genomic DNA was precipitated in the presence of 1/10th the volume of 3M NaOAc (pH
5.2) and 1 volume of 100% ethanol. The DNA was centrifuged, washed in 1ml of 70% ethanol, air-dried and resuspended in 50-100μl of TE at 37°C overnight.

### 2.3.4 GENOTYPING MOUSE LINES

The following primers were used to genotype p21<sup>CIP1-/−</sup>, p27<sup>KIP1-/−</sup>, p27<sup>KIP1+/−</sup>, p21<sup>CIP1+/−</sup>, p21<sup>CIP1+/−</sup>p27<sup>KIP1+/−</sup>, p27<sup>KIP1+/−</sup>p21<sup>CIP1+/−</sup>, p27<sup>KIP1-/−</sup>p21<sup>CIP1-/−</sup> mice (Table 6).

<table>
<thead>
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<th>Genotype</th>
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<th>Primer Sequence</th>
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<tr>
<td>p21&lt;sup&gt;CIP1&lt;/sup&gt;</td>
<td>p21-WT1</td>
<td>5’ AAG CCT TGA TTC TGA TGT GGG C 3’</td>
</tr>
<tr>
<td></td>
<td>p21-WT2</td>
<td>5’ TGA CGA AGT CAA AGT TCC ACC 3’</td>
</tr>
<tr>
<td></td>
<td>p21-Neo</td>
<td>5’ GCT ATC AGG ACA TAG CGT TGG C 3’</td>
</tr>
<tr>
<td>p27&lt;sup&gt;KIP1&lt;/sup&gt;</td>
<td>p27-KO1</td>
<td>5’ ACG TGA GAG TGT CTA ACG G 3’</td>
</tr>
<tr>
<td></td>
<td>p27-KO2</td>
<td>5’ AGT GCT TCT CCA AGT CCC 3’</td>
</tr>
<tr>
<td></td>
<td>p27-KO3</td>
<td>5’ GCG AGG ATC TCG TCG TGA C 3’</td>
</tr>
</tbody>
</table>

Table 4. Primers used for genotyping mice.

Both p21<sup>CIP1</sup> and p27<sup>KIP1</sup> genotyping were performed by PCR using genomic DNA prepared from tail clips. Briefly, for p21<sup>CIP1</sup> genotyping, 0.5μM of each of the three primers mentioned in Table 6 were incubated with 1μg of genomic DNA, 1X PCR buffer, 2mM MgCl₂, 0.2mM dNTPs (Roche) and 0.625U
of Taq polymerase (Invitrogen) in a total volume of 25μl. For \( p27^{\text{KIP1}} \) genotyping, 0.5μM of \( p27-\text{KO1} \), 0.25μM \( p27-\text{KO2} \) and 0.25μM \( p27-\text{KO3} \) were incubated in a total volume of 25μl, in the presence of 1μg of genomic DNA, 1X PCR buffer, 2mM MgCl2, 0.2mM dNTPs and 0.625U of Taq polymerase. PCR was performed under the following cycling conditions: 95°C 5 min; 40 cycles of 95°C 1 min, 60°C 1 min followed by 72°C for 3 min; and a final 10 min extension at 72°C. The products were electrophoresed on 1.2% agarose gels for \( p21^{\text{CIP1}} \) genotyping and on 2% gels for \( p27^{\text{KIP1}} \) genotyping. The WT and mutant bands for \( p21^{\text{CIP1}} \) genotyping are 900bp and 750bp respectively and those for \( p27^{\text{KIP1}} \) are 130bp and 450bp respectively (Kiyokawa et al., 1996 and Brugaralos et al., 1998).

Genotyping for \( \text{Mitf}^{\text{mi/mi}}, \text{Mitf}^{\text{or/or}} \) and \( \text{Mitf}^{\text{wh/wh}} \) mice was accomplished based on the coat color and microphthalmia phenotype of the homozygous recessive mutant mice in these backgrounds (Moore et al., 1994; Nii et al., 1995 and Steingrimsson et al., 2003).

2.4 RADIOGRAPHY AND HISTOMORPHOMETRIC ANALYSIS OF MURINE FEMURS

2.4.1 RADIOGRAPHIC EXAMINATION OF MOUSE SKELETONS

Newborn, 15-30 day old and adult mice were sacrificed and their skins and organs were removed. The mice were then fixed in 3.7% paraformaldehyde solution at 4°C for 48 hours, and transferred to 70% ethanol. Radiography was
performed on X-OMAT V film (Kodak) using a Faxitron X-ray machine (Hewlett Packard, Model 43855A) at 35 kvp for 2 min for newborn mice and 45 kvp for 2 min for 15-30 day old and adult mice. The X-rays were analyzed using a dissecting microscope attached to Magnafire™ camera and Imaging software (Olympus).

2.4.2 INFILTRATION, EMBEDDING, SECTIONING, HISTOLOGY AND HISTO MORPHOMETRIC ANALYSIS OF MOUSE FEMURS

The femurs from mutant and control mice were decalcified by incubating in 10% EDTA in 10mM Tris-HCl (pH 7.4) at 4°C overnight with 2-3 changes of the EDTA solution. The decalcified femurs were rinsed extensively in distilled water (diH₂O) and dehydrated by incubating them for 2 hours in 50% ethanol and for 4 hours in two changes of 70% ethanol at 4°C. Dehydrated femurs were infiltrated with glycol methacrylate solution (Polysciences, Inc) for 4 hours at 4°C, changing the infiltration solution every hour. The infiltrated femurs were further cleaned off of intervening muscle tissue, trimmed to lay straight on a flat surface and embedded on ice using the JB-4 embedding kit (Polysciences, Inc), according to manufacturer’s directions.

Femurs embedded in glycol methacrylate blocks were sectioned using Historange microtome (LKB Instruments, Rockville, MD). The sections (5 µ thickness) were stained for TRAP activity. Briefly, the slides were incubated at 37°C for 1 hour in a solution prepared in warm water containing 0.1M NaOAc;3H₂O (pH5.2), 0.0268M Tartrate solution (pH 5.2), 25mg of Naphthol 99
AS-BI phosphoric acid in N,N'-dimethyl formamide (Sigma) and 30µg of Fast Garnet GBC salt (Sigma) in a total volume of 50ml. TRAP-stained sections were counterstained with hematoxylin (Histology Control Systems, Inc) for 1 minute, air-dried, mounted on RapidMount (Histology Control Systems, Inc) and coverslipped. The sections were visualized using Olympus BH2 microscope and images captured using Magnafire™ camera and image analysis software (Olympus).

Histomorphometric analyses to measure total bone area, perimeter, and surface, total unresorbed bone area, and osteoclast number, area, perimeter and surface were performed using Bioquant Nova software (R&M Biometrics).

2.5 CULTURE OF PRIMARY MURINE OSTEOCLASTS

All tissue culture-coated dishes used to culture primary osteoclasts were coated for 12 hours with 0.1% gelatin made in 1X phosphate-buffered saline (PBS, pH 7.4). For cell cycle studies, Thermanox plastic coverslips (13 mm diameter; Nalgene Nunc International) were placed in 24-well dishes and coated with gelatin as mentioned before. Bone marrow and spleen cells were used as sources of hematopoietic osteoclast precursor cells. Bone marrow cells were derived from femurs of 15-30 day old and adult WT, p21<sup>CIP1-/-</sup>, p27<sup>KIP1-/-</sup>, p27<sup>KIP1+/+</sup>, p21<sup>CIP1-/-</sup>, p27<sup>KIP1+/+</sup>, p27<sup>KIP1+/-</sup>, p21<sup>CIP1-/-</sup>, p27<sup>KIP1-/-</sup>, p27<sup>KIP1-/-</sup>, p21<sup>CIP1+/-</sup>, p21<sup>CIP1-/+</sup>, Mitf<sup>+/+</sup> and Mitf<sup>+/+</sup> mice. Spleen cells were derived from newborn WT, Mitf<sup>mi/mi</sup> and Mitf<sup>or/or</sup>
mice and from 30 day-old Mit${^m/mi}$ mice. Bone marrow and spleen cells were counted and plated on petri dishes at cell densities as summarized in Table 7.

Hematopoietic precursor cells were cultured for three days in DMEM media containing 10% heat inactivated fetal bovine serum and 50ng/ml recombinant MCSF1. The cells were then either maintained in the same concentration of MCSF1 (proliferation media) or switched to a combination of 25ng/ml of MCSF1 and 50ng/ml of RANKL (differentiation media I). The media was changed every two days.

<table>
<thead>
<tr>
<th>Culture dishes used</th>
<th>Surface area (Sq.cm)</th>
<th>Number of cells per ml per well</th>
<th>Total volume of media</th>
<th>Type of experiments conducted</th>
</tr>
</thead>
<tbody>
<tr>
<td>24-well</td>
<td>2</td>
<td>$0.5 \times 10^6$</td>
<td>1ml</td>
<td>BrdU-labeling</td>
</tr>
<tr>
<td>6-well</td>
<td>10</td>
<td>$0.89 \times 10^6$</td>
<td>3ml</td>
<td>RNA and protein analyses</td>
</tr>
<tr>
<td>60mm</td>
<td>20</td>
<td>$1.34 \times 10^6$</td>
<td>4ml</td>
<td>Protein, Immunoprecipitation, kinase assays</td>
</tr>
<tr>
<td>100mm square (non-coated)</td>
<td>56</td>
<td>$1.5 \times 10^6$</td>
<td>12 ml</td>
<td>Differentiation and resorption pit assays</td>
</tr>
</tbody>
</table>

Table 5. Summary of surface areas of various tissue culture dishes, respective osteoclast precursor cell densities and the type of experiments conducted.
For differentiation and calcium phosphate resorption pit assays, bone marrow or spleen cells were placed in non-tissue culture coated petri dishes in DMEM media containing 10% fetal bovine serum and 3% L-cell media. L-cell media containing MCSF1 was prepared as previously mentioned. After 3 days, the adherent cells were harvested, counted and plated in the presence of 2% L-cell media and 50ng/ml of RANKL at a density of $5 \times 10^4$ cells/well in a 24-well dish for formation of multinuclear osteoclasts or at a density of $1 \times 10^4$ cells/well on a 16-well BD Biocoat Osteologic multitest slide coated with calcium phosphate (BD Biosciences) for resorption pit assays. In both cases, L-cell media concentration was lowered to 1% on the 2nd day and thereafter the cells were maintained in 1% L-cell media and 50ng/ml of RANKL (differentiation media II). The media was changed every two days.

2.6 OSTEOCLAST DIFFERENTIATION AND FUNCTIONAL ASSAYS

2.6.1 TRAP STAINING OF in vitro DIFFERENTIATED OSTEOCLASTS

Osteoclast precursors plated on 24-well dishes in differentiation media II as mentioned in section 2.5 were maintained for 9 days, with the media being replaced every two days. After 9 days, osteoclasts were washed twice with PBS, fixed in 3.7% paraformaldehyde (Polysciences, Inc) in 1X PBS at 4°C for 15 min and washed twice with PBS. Differentiated osteoclasts were assayed for TRAP activity, as mentioned in section 2.4.2, for one hour at 37°C and counterstained with 10μM bis-benzamide (Sigma) for 5 minutes at room temperature.
The number of multinuclear osteoclasts was estimated using IX51 Olympus inverted microscope and images were captured using Magnafire™ camera and image analysis software (Olympus).

2.6.2 CALCIUM PHOSPHATE RESORPTION PIT FUNCTIONAL ASSAYS

Osteoclast precursors plated on BD Biocoat Osteologic 16-well multitest slides in differentiation media II as mentioned in section 2.5 were maintained for 11 days, with the media being replaced every two days. The wells in the multitest slides are coated with a sub-micron synthetic calcium phosphate thin film, that serve as bone-like resorption surface for differentiated osteoclasts. After 12 days in differentiation media II, osteoclasts were removed by incubation in sodium hypochlorite (6% bleach, 5.2% NaCl) solution for 5 minutes at room temperature. The wells were washed thrice using alkaline-buffered water (pH 7.0) for 5 minutes each. The slides were air-dried, visualized using IX51 Olympus inverted microscope and images were captured using Magnafire™ camera and image analysis software (Olympus). The area, number and perimeter of the pits were measured using Bioquant Nova software (R&M Biometrics).

2.7 BRDU LABELING AND CELL CYCLE ANALYSIS

Osteoclast precursor cells were first treated with 0.4µM 5-Fluoro-2’deoxy Uridine to block endogenous thymidine synthesis. The cells were then pulsed
for 30 min with 10μM BromodeoxyUridine (BrdU) (BD Biosciences). BrdU labeled cells were washed with PBS and fixed in 70% ethanol (-20°C) for 30 minutes at room temperature. Fixed cells were stored at –20°C until cells from all time points were collected. Ethanol was removed from the wells and coverslips with cells were airdried for 15 minutes. The cells were incubated for 2 min in 0.07N NaOH and neutralized with two 15 min washes in 1X PBS (pH 8.5). The cells were blocked for 5 min in a blocking buffer containing 1% BSA, 0.5% Tween 20 and 2% normal goat serum in 1X PBS and then incubated with 1:50 dilution of αBrdU antibody (BD Biosciences) in blocking buffer for 1 hour at room temperature. The cells were washed thrice with 1X PBS, incubated with 1:200 dilution fluorescein (FITC) conjugated secondary antibody (Jackson Immuno Research Laboratories, Inc.) for 1 hour followed by three more washes in 1X PBS. Finally, the cells were incubated in 0.04 μg/ml of propidium iodide (PI) for 1 minute, washed twice with water and visualized using fluorescent microscopy. An average of 4000 cells were counted for each time point in each experiment. Percentage of cells positive for BrdU over the total number of PI-positive cells was determined.

2.8 PREPARATION OF RNA, cDNA AND REAL-TIME PCR

Total RNA was prepared using Trizol (Life Technologies) according to manufacturer’s protocol. To make the cDNA, 1 μg of RNA was incubated with 1X cDNA buffer, 1mM dNTPs, 20U of RNase inhibitor, 24U of Reverse
Transcriptase (all from Roche) and 0.5 µg random hexamers (Promega), in a total volume of 20 µl, at 50°C for 60 min and the reaction was frozen at –20°C until further use. The primers and Taqman probes for Real-Time PCR were purchased from Applied Biosystems and their sequences are summarized in Table 6.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer/Probe</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>TRAP</td>
<td>Forward</td>
<td>5’ GAT CTC CAA GCG CTG GAA CTT</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>5’ CAG TTA TGT TTG TAC GTG GAA TTT TGA</td>
</tr>
<tr>
<td></td>
<td>Taqman probe</td>
<td>5’ 6-Fam-CCC AGC CCT TAC TAC CGT TTG CGC-Tamara</td>
</tr>
<tr>
<td>CathepsinK</td>
<td>Forward</td>
<td>5’ ACC CAG TGG GAG CTA TGG AA</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>5’ TCC CAA ATT AAA CGC CGA GA</td>
</tr>
<tr>
<td></td>
<td>Taqman probe</td>
<td>5’ 6-Fam-CAT CCA CCT TGC TGT TAT ACT GCT TCT GGT GA-Tamara</td>
</tr>
<tr>
<td>c-FMS</td>
<td>Forward</td>
<td>5’ CTG GAA TAA TCT GAC CTT TGA GCT C</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>5’ CGT CAC AGA ACA GGA CAT CAG AG</td>
</tr>
<tr>
<td></td>
<td>Taqman probe</td>
<td>5’ 6-Fam-CCT GCG ATA TCC CCC AGA GGT CAG TG-Tamara</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 6: Primers and Taqman probes used for Real Time PCR analysis.
GAPDH and 18S primers and probes were purchased as kits from Applied Biosystems and were used according to manufacturer’s protocol. For Taqman Real-Time PCR assays with TRAP, cathepsin K and c-FMS, 1X Taqman Universal PCR mix (Applied Biosystems), 2µL of the reverse transcription (RT) reaction, 0.25µM each of the forward and reverse primers and 0.24 µM of the probe in a total volume of 25µl was used.

Real Time PCR analysis was also used to verify the MITF-target genes identified via micro-array analysis. The primers used in this analysis are listed in Table 7. The reaction mixture consisted of 1X SYBR® Green PCR master mix (Applied Biosystems), 0.3µM of each primer and 2µl of the RT reaction in a total volume of 25µl.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Eos</td>
<td>Forward</td>
<td>5' CTC AGT GCC AAC TCC ATC AA</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>5' GCC TAA GGG CTC TGA CAA TG</td>
</tr>
<tr>
<td>HOX11L2</td>
<td>Forward</td>
<td>5' GTC TGC CGC CTC CAC TTG GT</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>5' CGC CAC CCA AGC GTA AGA AG</td>
</tr>
<tr>
<td>HCP</td>
<td>Forward</td>
<td>5’ CCA GGA TGG TGA GGT GGT TT</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>5’ GGG AGA AGT CAC CCT GGT TCT T</td>
</tr>
<tr>
<td>P9</td>
<td>Forward</td>
<td>5’ CAG AGA TGA CAA CAT GTT CCA GAT TGG</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>5’ GCT GGC TCC ATT GTT CCA TGT</td>
</tr>
</tbody>
</table>

Table 7. Primers used for verification of potential Mitf-target genes by Real Time PCR analysis
Real Time PCR conditions were as follows: step 1 50\(^0\)C 2 min, step 2 95\(^0\)C 10 min, step 3 95\(^0\)C 15 sec, step 4 60\(^0\)C 1 min with 40 cycles of steps 3 and 4. The signal was detected at step 4. Real Time PCR was performed in either the Cepheid Smart Cycler System (Cepheid) or the ABI PRISM 7700 sequence detection system (Applied Biosystems) and the cycle threshold \((C_T)\) values were analyzed on a spreadsheet. \(C_T\) value was determined by analyzing the 2\(^{nd}\) derivative of the growth curve and the fold induction was calculated as the difference in \(C_T\) values \((\Delta C_T)\) between control and RANKL treated samples raised to the power of 2 \((2^{\Delta C_T})\). These were further normalized against \(\Delta C_T\) values for GAPDH or 18S.

2.9 PREPARATION OF RNA, cDNA, cRNA AND MICROARRAY ANALYSIS

2.9.1 PREPARATION OF RNA AND cDNA

Total RNA was prepared using Trizol (Life Technologies) according to manufacturer’s protocol. The RNA isolated using Trizol was resuspended in 100\(\mu\)l of DEPC H\(_2\)O and subjected to a second clean-up using Qiagen RNeasy Total RNA isolation kit according to manufacturer’s directions. The purified RNA was eluted in 30ml of DEPC H\(_2\)O and the concentration was measured using a spectrophotometer.

cDNA synthesis was performed with 5.0-8.0\(\mu\)g of total RNA using the Superscript Choice system (Life Technologies). Total RNA was incubated with 100pmol of \(T_7\)-(dT)\(_{24}\) primer (5’ GGC CAG TGA ATT GTA ATA CGA CTC ACT
ATA GGG AGG CGG; 100pm/µl; Genset Corp) and DEPC H₂O in a total volume of 11µl at 70°C for 10 minutes, followed by a quick centrifugation. The tube was quick chilled on ice. The mixture was incubated with 1X first strand buffer, 10mM DTT and 500µM dNTP at 42°C for 2 minutes. One microliter of SSII Reverse transcriptase (200U) was added immediately, mixed well and the mixture incubated at 42°C for 1 hour.

The first strand reaction was placed on ice and the contents of the tube quickly spun to bring down the condensation on the sides of the tube. The first strand reaction was incubated with 1X second strand buffer, 200mM dNTPs 10U of E. coli DNA ligase, 40U of E. coli DNA polymerase I and 2U of E. coli RNase H in a final volume of 150µl at 16°C for 2 hours. At the end of the incubation period, 10U of T4 DNA polymerase was added to the tube and the mixture was further incubated at 16°C for 5 minutes. The cDNA reaction was stopped by adding 10µl of 0.5M EDTA, purified by phenol-chloroform (Ambion) extraction followed by ethanol precipitation using 3.75M NH₄OAc and 100% ethanol. The cDNA pellet was washed in 1ml of 80% ethanol, air-dried and resuspended in 12µl of DEPC H₂O.

2.9.2 PREPARATION OF cRNA AND MICROARRAY HYBRIDIZATION

Biotin-labeled cRNA was made from freshly synthesized cDNA using BioArray HighYield RNA Transcript labeling kit (ENZO Diagnostics, Inc) according to manufacturer’s protocol. The cRNA was purified with Qiagen RNeasy Total RNA isolation kit and the concentration was determined using
spectrophotometer. The A260/A280 ratio of the cRNA must be more than 2.0. Further, the quality of the cRNA was verified by performing 1% TAE electrophoresis. The cRNA was fragmented using a buffer containing 200mM Tris-Acetate, 500mM Potassium Acetate and 150mM Magnesium Acetate in a total volume of 40µl at 94°C for 35 min. This reaction was split equally into two tubes. And frozen at –80°C until use. The fragmented cRNA from one tube was hybridized on the Affymetrix GeneChip® Test array to assure quality of the cRNA and further hybridized to Affymetrix GeneChip® Murine Genome MG-U74Av2 (version 2) probe array. The MG-U74Av2 array contains a glass slide coated with 16 pairs of oligonucleotide probes (25mer) towards ~6000 functionally characterized genes and an additional ~6000 ESTs. Micro array data were analyzed using the DNA-CHIP Analyzer software (Li et al., 2001).

2.10 IMMUNOBLOTTING, IMMUNOPRECIPITATION AND KINASE ASSAYS

2.10.1 SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS (SDS-PAGE) AND IMMUNOBLOTTING

Whole cell extracts were prepared by lysing osteoclast precursors in 2X SDS-PAGE buffer, boiled for 10 minutes, centrifuged at 20,000 rpm for 20 minutes and the supernatant was frozen at –80°C until all time points were collected. The proteins were separated on 12% or 15% SDS-PAGE. The upper stacking gel was composed of 0.125M Tris-HCl (pH 6.8), 0.1%SDS, 4% 29:1 acrylamide-bisacrylamide and the lower resolving gel was composed of 0.375M
Tris-HCl (pH 8.8), 0.1% SDS and 12-15% 29:1 acrylamide-bisacrylamide. The lysates were boiled for five minutes, loaded onto the gels, electrophoresed in a buffer containing 25mM Tris, 190mM glycine and 0.1% SDS. SDS-PAGE was performed using a Mini-PROTEAN II apparatus (BioRad) at 60V until the proteins entered the resolving gel and at 80V thereafter.

Proteins separated via SDS-PAGE were transferred onto OPTITRAN nitrocellulose membranes (Schleicher and Schuell) in the presence of a transfer buffer containing 25mM Tris, 190mM glycine and 20% methanol using the Mini-PROTEAN II apparatus (BioRad). The nitrocellulose membranes were blocked in 5% dry milk solution made in wash buffer containing 50mM Tris-HCl (pH 7.4) and 200mM NaCl at 4°C overnight. The membrane was probed with polyclonal antibodies using dilutions and conditions as summarized in Table 10.
Table 8. Summary of antibodies and conditions used in this study.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Dilution</th>
<th>Secondary antibody</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>p27&lt;sup&gt;KIP1&lt;/sup&gt;(C-19)</td>
<td>1:500</td>
<td>Rabbit polyclonal</td>
<td>1:1000</td>
</tr>
<tr>
<td>p21&lt;sup&gt;CIP1&lt;/sup&gt;(M-19)</td>
<td>1:1000</td>
<td>Rabbit polyclonal</td>
<td>1:1500</td>
</tr>
<tr>
<td>p18&lt;sup&gt;INK4C&lt;/sup&gt;(M-20)</td>
<td>1:1000</td>
<td>Rabbit polyclonal</td>
<td>1:1500</td>
</tr>
<tr>
<td>p19&lt;sup&gt;INK4D&lt;/sup&gt;(M-167)</td>
<td>1:1000</td>
<td>Rabbit polyclonal</td>
<td>1:1500</td>
</tr>
<tr>
<td>CDK2(M2)</td>
<td>1:1000</td>
<td>Rabbit polyclonal</td>
<td>1:1500</td>
</tr>
<tr>
<td>ERK1</td>
<td>1:1000</td>
<td>Rabbit polyclonal</td>
<td>1:1500</td>
</tr>
</tbody>
</table>

All antibodies were purchased from Santa Cruz Biotechnology.

Incubations with primary and secondary antibodies were performed for 1 hour at room temperature. The blots were washed 4 times following incubation with each antibody in wash buffer containing 0.1% Tween-20. ERK1 antibody was used as a loading control. Signals were detected using LumiGLO luminescent substrate (KPL) and the signal intensity was determined using a Lumi-Imager (Roche).

To perform signaling experiments, 10μM SB203580 or 40μM PD98059 (both from Calbiochem) or 10μM LY294002 (AG Scientific) were added to the cells together with RANKL and whole cell lysates were prepared at the indicated time points.
**2.10.2 IMMUNOPRECIPITATION AND KINASE ASSAYS**

Equal number of cells from each sample were lysed in Tween lysis buffer (TLB) containing 50mM HEPES (pH 8), 1mM EDTA, 2.5mM EGTA, 150 mM NaCl, 0.1% Tween 20, 1mM NaF, 0.1mM sodium orthovanadate, 25mM β-glycerophosphate, 100µg/ml PMSF and 10µg/ml each of aprotinin and leupeptin. The supernatant was incubated with 5µg of αCDK2 antibody 1 hour at 4°C. Then, 20µl of rProtein G agarose beads (50% slurry, Life Technologies) was added and the incubation continued for 3 more hours. The immunocomplex bound on the beads was washed six times in TLB, thrice in wash buffer containing 20mM Tris-HCl (pH 8), 100mM NaCl, 1mM EDTA and 0.5% NP-40 and once in kinase buffer containing 50 mM Tris-HCl (pH 7.5), 10mM MgCl₂ and 1mM DTT. Kinase activity in the immunoprecipitate was assayed by adding kinase buffer, 5µg of Histone H1 (Roche), 1µM ATP, 5µCi of \(^{32}\)PATP (3000Ci/mmole) in a final volume of 50µl and incubating at 30°C for 30 minutes. The reactions were run on 12% SDS-PAGE, blotted onto nitrocellulose membrane and visualized by autoradiography. The blots were subsequently probed with αCDK2 antibody.
CHAPTER 3

IDENTIFICATION OF NOVEL Mitf-REGULATED OSTEOCLAST-SPECIFIC GENES VIA COMPARATIVE MICROARRAY ANALYSIS

3.1 INTRODUCTION

Osteoporosis and osteopetrosis are among the bone diseases that result from the disruption of constant communication that takes place between two interconnected cell populations of the vertebrate bone, viz., mesenchymal osteoblasts that secrete the bone matrix and hematopoietic osteoclasts that resorb the bone matrix (Teitelbaum, 2000). Osteoclasts are bone-resorbing, tartarate-resistant acid phosphatase (TRAP) positive multinuclear cells that differentiate from mononuclear cells of hematopoietic, macrophage/monocyte origin (Roodman, 1997). Osteoblasts secrete two cytokines, macrophage colony stimulating factor (M-CSF) and receptor activator of NFκB ligand (RANKL), which are both necessary and sufficient for osteoclast differentiation. Binding of RANKL to its receptor RANK present on osteoclasts, initiates a cascade of signaling events, including the activation of mitogen activated
protein kinase (MAPK), Src kinase, Jun kinase (JNK), phosphatidylinositol-3 (PI-3) kinase and the p38 MAPK pathways that lead to the activation of several transcription factors including NFκB, microphthalmia transcription factor (Mitf), PU.1 and c-Fos. These events lead to the activation of osteoclast marker genes like TRAP and cathepsin K and ultimately to the differentiation of multinuclear osteoclasts from mononuclear precursors. Inhibition of the p38 MAPK pathway by the drug SB203580 blocks the formation of multinuclear cells from mononuclear precursor cells (Matsumoto et al., 2000). The p38 MAPK pathway has been shown to target Mitf in osteoclasts, leading to its activation via phosphorylation and to a concomitant increase in TRAP expression. Furthermore, inhibition of p38 MAPK pathway causes a reduction in upregulation of TRAP gene expression by RANKL (Mansky et al., 2002).

Mitf is a basic helix-loop-helix leucine zipper transcription factor (bHLH-Zip) encoded by the microphthalmia (mi) locus (Hodgkinson et al., 1993). Mitf is closely related to the bHLH-Zip transcription factors Tfe3, TfeB and TfeC and these proteins make up a subfamily of Mitf-related transcription factors. Mitf forms stable heterodimers with the related Tfe3, TfeB and TfeC and has been shown to bind to DNA as stable homodimers or heterodimers (Moore, 1995 and Mansky et al., 2002a). The basic domain of Mitf binds to “TCATGTGA” E-box elements on promoters of target genes (Aksan and Goding, 1998). Mitf regulates the expression of TRAP, cathepsin K and E-Cadherin in osteoclasts by directly binding to the E-box elements in their promoters (Luchin et al., 2000; Motyckova et al., 2001 and Mansky et al., 2002). In addition, Tfe3 and TfeC
collaborate with Mitf to transactivate the TRAP promoter (Mansky et al., 2002b). Tfe3 has been shown to play redundant roles with Mitf during osteoclast differentiation (Steingrimsson et al., 2002). In addition, Mitf and PU.1 have been shown to bind to each other and synergistically activate TRAP and osteoclast associated receptor (OSCAR) promoter by directly binding to their respective binding sites on these promoters (Luchin et al., 2001 and Kim et al., 2002).

Mitf is required for the differentiation of several cell types belonging to different lineages including osteoclasts and melanocytes and is evolutionarily conserved from fish to humans. Human MITF is mutated in families with Waardenburg syndrome type II (WS2) and patients heterozygous for WS2 show varying degrees of deafness and patchy abnormal pigmentation of the hair, skin and eyes (Moore, 1995 and Goding, 2000). More than 20 different mutants of the Mitf allele have been identified in mice. A semi-dominant mutation in the mi allele, Mitf<sup>mi/mi</sup>, caused by the deletion of arginine (R215) in the basic domain of Mitf, renders the protein unable to bind to DNA and causes it to act in a dominant negative fashion (Hodkinson et al., 1993 and Karsenty 2001). The homozygous Mitf<sup>mi/mi</sup> mice exhibit white coat color, microphthalmia, reduced eye pigmentation, retinal degeneration, early onset deafness, reduced numbers of mast and natural killer cells and severe osteopetrosis (Moore, 1995 and Goding, 2000). Mitf<sup>mi/mi</sup> mice develop severe osteopetrosis due to an inability of mononuclear osteoclast precursors to fuse and form multinuclear osteoclasts capable of resorbing bone (Hodgkinson et al., 1993). In addition, Mitf<sup>mi/mi</sup> osteoclasts express lower levels of TRAP and cathepsin K (Luchin et al., 2000
and Motyckova et al., 2001). In addition, Mitf<sup>r/o</sup>r mice harboring a substitution of R216 in the basic domain with lysine develop osteopetrosis that improves with age (Nii et al., 1995). Mutant Mitf<sup>r/o</sup>r proteins are also incapable of binding DNA and act as dominant negative proteins, similar to Mitf<sup>mi/mi</sup> (Moore, 1995).

Although it is evident that Mitf has a major role in osteoclast differentiation and function, the precise role played by this transcription factor in osteoclastogenesis is largely unknown. Genes that are directly or indirectly regulated by Mitf during osteoclast differentiation are bound to be down-regulated in Mitf<sup>mi/mi</sup> osteoclasts. Comparison of the differential expression of osteoclast-specific genes in Mitf<sup>mi/mi</sup> and wildtype (WT) osteoclasts, might result in the identification of novel Mitf-regulated genes and help gather further insights into the precise role of Mitf during osteoclast differentiation.

To test the hypothesis that Mitf regulates the expression of several genes that are involved in osteoclast differentiation, we performed a comparative analysis of the gene expression patterns between WT and Mitf<sup>mi/mi</sup> osteoclast precursors during RANKL-mediated in vitro differentiation, using Affymetrix GeneChip® oligonucleotide microarrays and identified several genes that are down-regulated in Mitf<sup>mi/mi</sup> osteoclasts. The differential expression of four of the novel Mitf-regulated genes, hematopoietic cell phosphatase (HCP), Eos, HOX11L2 and p9, in Mitf<sup>mi/mi</sup> osteoclasts was confirmed via real-time PCR.
3.2 RESULTS

3.2.1 OSTEOPETROSIS IN Mitf<sup>mi/mi</sup> MICE IS DUE TO THE REDUCED ABILITY OF OSTEOCLAST PRECURSORS TO FORM FUNCTIONAL MULTINUCLEAR OSTEOCLASTS CAPABLE OF BONE RESORPTION

Radiographic analysis of the long bones from newborn and 30-day old WT and Mitf<sup>mi/mi</sup> mice indicate the presence of shorter denser long bones with the accumulation of primary spongiosa in the metaphysis of femurs in Mitf<sup>mi/mi</sup> mice (Fig. 20Ai). Histomorphometric analysis of TRAP-stained femur sections revealed that the percentage of unresorbed trabecular bone area (UBA) relative to the total bone area (TBA) in WT was 5.4±1.9 in newborn and 4.2±0.5 in 30-day old animals (Fig. 20B). In contrast, the percentage of UBA in Mitf<sup>mi/mi</sup> femurs was 20.8±4.8 in newborn and 15.1±3.5 in 30-day old animals (Fig. 20B). The total number of osteoclasts (# of OCL) per unit bone perimeter (UBP) was 12±0.5 and 2.7±0.2 in newborn and 30-day old WT and Mitf<sup>mi/mi</sup> femurs. Percentage of total osteoclast surfaces relative to total bone surface (TBS; both trabecular and cortical surfaces) was 54.2±4 and 20.1±0.4 in newborn and 30-day old WT mice (Fig. 20C). However, the percentage of total osteoclast surfaces relative to TBS was 5.9±1.5 and 6.2±0.4 respectively in similar age groups of Mitf<sup>mi/mi</sup> mice (Fig. 20C). In general, numerous intensely TRAP-positive osteoclasts with ruffled borders were found attached to the bone surface in newborn and 30-day old from WT femurs. In contrast, femurs from newborn and 30-day old Mitf<sup>mi/mi</sup> mice had significantly fewer osteoclasts that were smaller and stained weakly for TRAP (Fig. 20Aii).
Precursors from newborn and 30-day old Mitf<sup>mi/mi</sup> mice formed none to very few multinuclear osteoclasts, compared to those from the WT (Fig. 20Hi). Precursors from newborn WT mice formed 2±1.1 multinuclear osteoclasts with 6-12 nuclei and 108±12.2 osteoclasts with 3-5 nuclei, while those from newborn Mitf<sup>mi/mi</sup> mice formed only 13±1.1 osteoclasts with 3-5 nuclei and none with 6 or more nuclei (Table 9). Precursors from 30-day old WT mice formed 30±11.1 osteoclasts with more than 12 nuclei, 58±6.7 osteoclasts with 6-12 nuclei and 582±18.9 osteoclasts with 3-5 nuclei, while those from 30-day old Mitf<sup>mi/mi</sup> mice formed only 34±3.3 osteoclasts with 3-5 nuclei (Table 9). Overall, all of the osteoclasts formed by the Mitf<sup>mi/mi</sup> precursors contained only 3-5 nuclei and almost all these had only 3 nuclei in them. To investigate whether Mitf<sup>mi/mi</sup> osteoclasts were capable of forming resorption pits, WT and Mitf<sup>mi/mi</sup> precursors were plated on calcium phosphate coated surfaces and the number, area and area distribution of the pits were measured using the Bioquant Nova software (Fig. 20Hii; Table 10). As seen from the table, while WT precursors formed numerous pits with area range of 100-60,000 and 100-1,000,000 square pixels in case of those newborn and 30-day old WT mice respectively, Mitf<sup>mi/mi</sup> osteoclasts formed very few pits with much smaller area range: 100-2500 for newborn and 100-5000 for 30-day old (Table 10).

Next, we compared TRAP and cathepsin K gene expression levels in newborn and 30-day old WT and Mitf<sup>mi/mi</sup> osteoclasts. TRAP levels were only induced to 4.1±0.6 and 2.5±0.4 fold in newborn Mitf<sup>mi/mi</sup> osteoclasts while it was
induced 7.4±0.1 and 10.3±1.3 fold in newborn WT osteoclasts, at 24 and 72 hours after adding RANKL (Fig. 20D). Similarly, at the indicated time points, TRAP was induced 8.4±0.7 and 11.5±2 fold in 30-day old WT osteoclasts and 2.8±0.5 and 2.8±0.6 fold in 30-day old Mitf<sup>mi/mi</sup> osteoclasts (Fig. 20E). At 72 hours after treatment with RANKL, cathepsin K was induced 4.9±0.4 and 9.3±0.5 fold in WT osteoclasts from newborn and 30-day mice, while it was only induced 2.4±0.3 and 1.8±1.56 fold by Mitf<sup>mi/mi</sup> osteoclasts from similar age groups (Figs. 20F and 20G). Taken together, these data present quantitative evidence that Mitf<sup>mi/mi</sup> mice exhibit severe osteopetrosis and that Mitf<sup>mi/mi</sup> mononuclear precursors are compromised in their ability to form functional multinuclear osteoclasts expressing normal levels of TRAP and cathepsin K, indicating that Mitf plays a significant role during osteoclast differentiation.
Figure 20. Mitf<sup>mi/mi</sup> mice exhibit osteopetrosis; their osteoclast precursors do not form functional multinuclear osteoclasts and express lower levels of TRAP and cathepsin K. Ai: Radiographic examination of femurs from mice.
of indicated genotypes and at indicated age groups; Aii: TRAP-stained (purple stain) sections of femur from these mice at 20X magnification with arrow heads indicating osteoclasts; B: Histomorphometric analysis of the TRAP-stained femur sections (shown in Aii) indicating the % unresorbed trabecular bone area over the total bone area (%UBA/TBA) (n=3) and C: % osteoclast (OCL) surfaces over the total bone surface (n=3); E and F: Fold induction of TRAP and G and H: Fold induction of cathepsin K in WT and $Mitf^{mi/mi}$ osteoclasts from newborn and 30-day old mice, respectively; Hi: Resorption pits and Hii: TRAP-stained differentiated osteoclasts from WT and $Mitf^{mi/mi}$ mice at indicated age groups.

<table>
<thead>
<tr>
<th>Quantitation of Multinuclear OCLs (# of Cells/Square mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NEWBORN</td>
</tr>
<tr>
<td>---------</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>&gt;12 nuclei</td>
</tr>
<tr>
<td>6-12 nuclei</td>
</tr>
<tr>
<td>3-5 nuclei</td>
</tr>
</tbody>
</table>
| Average of 2 experiments is shown |}

Table 9. Table indicating the number of TRAP-positive multinuclear osteoclasts, classified according to the number of nuclei present, formed by osteoclast precursors from WT and $Mitf^{mi/mi}$ mice.
Table 10. Number, total resorption pit area and the area range distribution of the resorption pits formed by osteoclast precursors from WT and Mitf^{mi/mi} mice.

<table>
<thead>
<tr>
<th>AREA DISTRIBUTION (Square Pixels)</th>
<th>NEWBORN</th>
<th>30-DAY OLD</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>WT</td>
<td>mi/mi</td>
</tr>
<tr>
<td>&lt;100</td>
<td>80</td>
<td>11</td>
</tr>
<tr>
<td>100-250</td>
<td>58</td>
<td>7</td>
</tr>
<tr>
<td>250-500</td>
<td>51</td>
<td>2</td>
</tr>
<tr>
<td>500-1000</td>
<td>49</td>
<td>2</td>
</tr>
<tr>
<td>1000-2500</td>
<td>50</td>
<td>1</td>
</tr>
<tr>
<td>2500-5000</td>
<td>27</td>
<td>0</td>
</tr>
<tr>
<td>5000-10000</td>
<td>27</td>
<td>0</td>
</tr>
<tr>
<td>10000-20000</td>
<td>15</td>
<td>0</td>
</tr>
<tr>
<td>20000-40000</td>
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<td>0</td>
</tr>
<tr>
<td>40000-60000</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>60000-80000</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>80000-100000</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>100000-250000</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>250000-500000</td>
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<td>0</td>
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<td>0</td>
</tr>
<tr>
<td>1500000-2000000</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Representative of 3 independent experiments is shown.
3.2.2 DIFFERENTIAL EXPRESSION OF GENES BETWEEN WT AND Mitf<sup>mi/mi</sup>
DURING OSTEOCLAST DIFFERENTIATION

To investigate the precise role of Mitf during osteoclast differentiation, we performed microarray analysis of the gene expression patterns in WT and Mitf<sup>mi/mi</sup> osteoclast precursors at 0, 24 and 72 hours after adding RANKL. Further, the microarray-based gene expression patterns between WT and the mutant osteoclasts were compared to identify genes that are differentially regulated in Mitf<sup>mi/mi</sup> during osteoclastogenesis.

Microarray analysis was performed using the DNA-CHIP Analyzer software (Li et al., 2001). A total of two experiments were performed and the probe intensities from each chip were normalized to the respective baseline chips. First, the gene expression patterns in WT osteoclasts, between 0 and 24 hours, 0 and 72 hours and 24 and 72 hours after RANKL treatment, were compared within individual experiments. Using model-based standard errors, a 95% confidence interval for difference in expression for each gene was constructed. Genes from two experiments with a fold difference of 2 or more were combined using their geometric mean. Further, a simple permutation method was applied to the above dataset to eliminate false discovery. For this, we considered two permutations obtained by changing the WT/Mitf<sup>mi/mi</sup> labels of chips in each experiment and applied the above procedure to the permuted dataset. A low false discovery rate of 5% was detected. Our data indicated that, in Wt osteoclast precursors, 362 genes were upregulated at 24 hours and 191 genes were upregulated at 72 hours after adding RANKL, relative to their
respective expression levels before adding RANKL (0 hour) (Table 11). Similarly, at 72 hours after treatment with RANKL, 141 genes were upregulated relative to their expression levels at 24 hours after adding RANKL (Table 11). Concurrently, relative to the expression levels before adding RANKL, 357 genes were downregulated at 24 hours and 50 genes were downregulated at 72 hours after adding RANKL. Also, 114 genes were downregulated at 72 hours compared to their expression at 24 hours after RANKL treatment (Table 11). These data suggest that differential expression in majority of the genes occurs during the first 24 hours of treatment with RANKL.

<table>
<thead>
<tr>
<th></th>
<th>0 Hour</th>
<th>24 hours</th>
<th>72 hours</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 hour</td>
<td>--</td>
<td>362</td>
<td>191</td>
</tr>
<tr>
<td>24 hours</td>
<td>357</td>
<td>--</td>
<td>141</td>
</tr>
<tr>
<td>72 hours</td>
<td>50</td>
<td>114</td>
<td>--</td>
</tr>
</tbody>
</table>

Table 11. Summary of the number of genes differentially expressed between 0 and 24 hours, 0 and 72 hours and 24 and 72 hours, after RANKL treatment. Each entry represents the number of genes overexpressed at the time point indicated in each column relative to the time point indicated in each row.
In order to identify novel genes involved in osteoclast differentiation, we first performed a comparison of gene expression patterns in WT osteoclasts, at the indicated time points. The expression profiles of a total of 891 expressed in WT osteoclasts were grouped into 5 clusters using the K-mean clustering method. Several novel genes, whose expression levels changed at the indicated time points only in the WT osteoclasts, were identified using this approach. For example, two clusters contained a total of 122 genes that were upregulated in WT osteoclasts, at 24 hours after treatment with RANKL. Overall, the data indicates that largest fold differences occurred for early response genes, i.e., genes whose expression levels changed within the first 24 hours of treatment with RANKL (Tables 12-17). Information regarding the top 20 genes in each class, whose expression levels recorded the highest change, is summarized in tables 12-17.
Table 12. Representative list of genes upregulated within 24 hours of RANKL treatment in WT osteoclasts.

<table>
<thead>
<tr>
<th>Probe set</th>
<th>GeneBank</th>
<th>Gene name</th>
<th>Fold change</th>
</tr>
</thead>
<tbody>
<tr>
<td>162073_r_at</td>
<td>AV326267</td>
<td>protein tyrosine phosphatase, receptor type, L</td>
<td>65.3</td>
</tr>
<tr>
<td>162376_r_at</td>
<td>AV243403</td>
<td>expressed sequence C77604</td>
<td>34.3</td>
</tr>
<tr>
<td>96305_at</td>
<td>AI845854</td>
<td>N-methylpurine-DNA glycosylase</td>
<td>33</td>
</tr>
<tr>
<td>94857_at</td>
<td>U10420</td>
<td>N-methylpurine-DNA glycosylase</td>
<td>31.2</td>
</tr>
<tr>
<td>162211_r_at</td>
<td>AV379320</td>
<td>hyaluronidase 2</td>
<td>28.8</td>
</tr>
<tr>
<td>93228_at</td>
<td>U25691</td>
<td>helicase, lymphoid specific</td>
<td>27.1</td>
</tr>
<tr>
<td>161034_at</td>
<td>AA067557</td>
<td>phospholipase A2, group X</td>
<td>25.2</td>
</tr>
<tr>
<td>95062_at</td>
<td>AB026997</td>
<td>Calpastatin</td>
<td>25.2</td>
</tr>
<tr>
<td>161008_at</td>
<td>AI428936</td>
<td>expressed sequence AI428936</td>
<td>23.3</td>
</tr>
<tr>
<td>93952_r_at</td>
<td>AA409629</td>
<td>expressed sequence AA409629</td>
<td>18.2</td>
</tr>
<tr>
<td>161608_r_at</td>
<td>AV348858</td>
<td>hyaluronidase 2</td>
<td>17.9</td>
</tr>
<tr>
<td>95696_at</td>
<td>AI840882</td>
<td>Thioredoxin-like 2</td>
<td>17.4</td>
</tr>
<tr>
<td>99635_at</td>
<td>AI845183</td>
<td>DNA segment, Chr 6, Wayne State University 147, expressed</td>
<td>17.4</td>
</tr>
<tr>
<td>94330_at</td>
<td>AA710564</td>
<td>RIKEN cDNA 0610033B02 gene</td>
<td>16.8</td>
</tr>
<tr>
<td>94077_f_at</td>
<td>D31717</td>
<td>ribophorin II</td>
<td>16.5</td>
</tr>
<tr>
<td>101540_at</td>
<td>AF069519</td>
<td>thymine DNA glycosylase</td>
<td>15.4</td>
</tr>
<tr>
<td>97779_at</td>
<td>X78545</td>
<td>mast cell protease 8</td>
<td>14.8</td>
</tr>
<tr>
<td>100407_at</td>
<td>L38580</td>
<td>Galanin</td>
<td>14.6</td>
</tr>
<tr>
<td>161095_i_at</td>
<td>D49429</td>
<td>RAD21 homolog (S. pombe)</td>
<td>14.1</td>
</tr>
<tr>
<td>94856_r_at</td>
<td>AI842362</td>
<td>DNA segment, Chr 4, ERATO Doi 13, expressed</td>
<td>13.1</td>
</tr>
</tbody>
</table>
Table 13. Representative list of genes **downregulated within 24 hours of RANKL treatment in WT osteoclasts**.
Table 14. Representative list of genes upregulated within 72 hours of RANKL treatment in WT osteoclasts.

<table>
<thead>
<tr>
<th>Probe set</th>
<th>GeneBank</th>
<th>Gene name</th>
<th>Fold change</th>
</tr>
</thead>
<tbody>
<tr>
<td>162073_r_at</td>
<td>AV326267</td>
<td>calpastatin</td>
<td>59.6</td>
</tr>
<tr>
<td>162211_r_at</td>
<td>AV379320</td>
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<td>29.5</td>
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<td>95062_at</td>
<td>AB026997</td>
<td>helicase, lymphoid specific</td>
<td>17.9</td>
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<td>93226_at</td>
<td>U25691</td>
<td>N-methylpurine-DNA glycosylase</td>
<td>16.8</td>
</tr>
<tr>
<td>94857_at</td>
<td>U10420</td>
<td>expressed sequence C77604</td>
<td>16.1</td>
</tr>
<tr>
<td>95306_at</td>
<td>AB45684</td>
<td>thioredoxin-like 2</td>
<td>11.5</td>
</tr>
<tr>
<td>95369_at</td>
<td>AB403882</td>
<td>Galanin</td>
<td>10.9</td>
</tr>
<tr>
<td>100407_at</td>
<td>L38680</td>
<td>mast cell protease 8</td>
<td>10.8</td>
</tr>
<tr>
<td>93777_at</td>
<td>X78545</td>
<td>phospholipase A2, group X</td>
<td>9.41</td>
</tr>
<tr>
<td>161034_at</td>
<td>AA007557</td>
<td>G protein-coupled receptor 56</td>
<td>8.66</td>
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<tr>
<td>102767_at</td>
<td>AB41664</td>
<td>DNA segment, Chr 2, Wayne State University 23 gene</td>
<td>8.32</td>
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<tr>
<td>100706_f_at</td>
<td>AA407367</td>
<td>RIKEN cDNA 1500009C23 gene</td>
<td>7.8</td>
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<tr>
<td>100528_at</td>
<td>AB62278</td>
<td>DNA segment, Chr 6, Wayne State University 147 gene</td>
<td>7.48</td>
</tr>
<tr>
<td>99635_at</td>
<td>AB45183</td>
<td>RIKEN cDNA 3110005M08 gene</td>
<td>6.76</td>
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<tr>
<td>92577_f_at</td>
<td>AW047116</td>
<td>thymine DNA glycosylase</td>
<td>6.61</td>
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<tr>
<td>101540_at</td>
<td>AF069519</td>
<td>hyaluronidase 2</td>
<td>6.55</td>
</tr>
<tr>
<td>101608_r_at</td>
<td>AV348558</td>
<td>protein tyrosine phosphatase, receptor type, L</td>
<td>6.5</td>
</tr>
<tr>
<td>162376_r_at</td>
<td>AV243403</td>
<td>expressed sequence A428936</td>
<td>6.2</td>
</tr>
<tr>
<td>181008_at</td>
<td>AWA25870</td>
<td>phosphatidylcholine transfer protein</td>
<td>5.91</td>
</tr>
<tr>
<td>101173_at</td>
<td>Z50024</td>
<td></td>
<td>5.89</td>
</tr>
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</table>
Table 15. Representative list of genes downregulated within 72 hours of RANKL treatment in WT osteoclasts.

<table>
<thead>
<tr>
<th>Probe set</th>
<th>GeneBank</th>
<th>Gene name</th>
<th>Fold change</th>
</tr>
</thead>
<tbody>
<tr>
<td>160925_at</td>
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<tr>
<td>161457_at</td>
<td>AV297156</td>
<td>cell division cycle 2 homolog (S. pombe)-like 2</td>
<td>6.96</td>
</tr>
<tr>
<td>100684_at</td>
<td>U92794</td>
<td>protein kinase C substrate 80K-H</td>
<td>5.87</td>
</tr>
<tr>
<td>93941_at</td>
<td>X81683</td>
<td>Brachyury</td>
<td>3.74</td>
</tr>
<tr>
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<td>RIKEN cDNA 1700023011 gene</td>
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<td>101883_at</td>
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<td>3.12</td>
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<td>AW124813</td>
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<td>AF018952</td>
<td>aquaporin 8</td>
<td>2.87</td>
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<td>D85232</td>
<td>lymphocyte antigen 6 complex, locus C</td>
<td>2.51</td>
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<td></td>
<td>2.46</td>
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<tr>
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<td>solute carrier family 30 (zinc transporter), member 4</td>
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<td>2.38</td>
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<td>RIKEN cDNA 2310032K15 gene</td>
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<td>AW047032</td>
<td>protein (peptidyl-prolyl cis/trans isomerase) NIMA-interacting1</td>
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<td>161776_at</td>
<td>AV346275</td>
<td>matrilin 2</td>
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<td>AW121930</td>
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<tr>
<td>99801_at</td>
<td>X70514</td>
<td>Nodal</td>
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Table 16. Representative list of genes upregulated at 72 hours compared to 24 hours after RANKL treatment in WT osteoclasts.

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<thead>
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<th>Probe set</th>
<th>GeneBank</th>
<th>Gene name</th>
<th>Fold change</th>
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<td>A835098</td>
<td>transcription elongation factor A (SII), 2</td>
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<td>162247_r_at</td>
<td>AV333838</td>
<td>DNA segment, Chr 1, ERATO Doi 854, expressed</td>
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<td>101914_at</td>
<td>A845484</td>
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<td>5.29</td>
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<td>A8850972</td>
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<td>4.65</td>
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<td>AW123834</td>
<td>adaptor protein complex AP-1, gamma 1 subunit</td>
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<td>Y17860</td>
<td>ganglioside-induced differentiation-associated-protein1</td>
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<td>101538_i_at</td>
<td>AW226939</td>
<td>carboxylesterase 3</td>
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<td>98833_at</td>
<td>AW048468</td>
<td></td>
<td>3.62</td>
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<td>RIKEN cDNA 1700062C23 gene</td>
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<td>92681_at</td>
<td>AJ243608</td>
<td>melanoma antigen, family L, 2</td>
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<td>101216_at</td>
<td>R75193</td>
<td></td>
<td>3.24</td>
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<tr>
<td>101282_at</td>
<td>AF054507</td>
<td>G protein-coupled receptor 44</td>
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<td>D43963</td>
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<td>AV240231</td>
<td>Fibromodulin</td>
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<td>L43668</td>
<td></td>
<td>3.08</td>
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<td>160898_at</td>
<td>AB021860</td>
<td>activator of basal transcription</td>
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<td>97935_at</td>
<td>A842970</td>
<td>RIKEN cDNA 4121402002 gene</td>
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<td>104248_at</td>
<td>AW227650</td>
<td>RIKEN cDNA 0610038P07 gene</td>
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<td>103446_at</td>
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<td>RIKEN cDNA 9130009C22 gene</td>
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<td>Probe set</td>
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<td>Gene name</td>
<td>Fold change</td>
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<tr>
<td>94741_at</td>
<td>AB008911</td>
<td>testicular serine protease 2</td>
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<td>AV243403</td>
<td>protein tyrosine phosphatase, receptor type, L</td>
<td>5.54</td>
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<td>93952_r_at</td>
<td>AA409629</td>
<td>expressed sequence AA409629</td>
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<td>93661_at</td>
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<td>zeta-chain (TCR) associated protein kinase (70kD)</td>
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<td>98619_at</td>
<td>AW121709</td>
<td>thymidylate kinase</td>
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<tr>
<td>161095_l_at</td>
<td>D49429</td>
<td>RAD21 homolog (S. pombe)</td>
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<tr>
<td>92780_f_at</td>
<td>M90535</td>
<td></td>
<td>4.27</td>
</tr>
<tr>
<td>162223_f_at</td>
<td>AV241808</td>
<td>ATPase, Ca++ transporting, cardiac muscle, fast twitch 1</td>
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<tr>
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<td>DNA segment, Chr 8, ERATO Dol 89, expressed</td>
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<td>AW125870</td>
<td>expressed sequence AI429936</td>
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<tr>
<td>161609_at</td>
<td>AV348162</td>
<td>regulator of G-protein signaling 16</td>
<td>3.79</td>
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<tr>
<td>160211_at</td>
<td>AI848885</td>
<td>DNA segment, Chr 17, Wayne State University 94, expressed</td>
<td>3.76</td>
</tr>
<tr>
<td>161833_r_at</td>
<td>AV309794</td>
<td>42 KD cGMP-dependent protein kinase anchoring protein</td>
<td>3.65</td>
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<tr>
<td>94818_at</td>
<td>AW047223</td>
<td>BTB (POZ) domain containing 1</td>
<td>3.58</td>
</tr>
<tr>
<td>161642_f_at</td>
<td>AV067633</td>
<td>regenerating islet-derived 3 alpha</td>
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<td>93657_at</td>
<td>U87620</td>
<td></td>
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<td>162146_r_at</td>
<td>AV343395</td>
<td>RIKEN cDNA 4933424M23 gene</td>
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<td>104464_s_at</td>
<td>AI642389</td>
<td>expressed sequence AI173274</td>
<td>3.13</td>
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<td>161523_r_at</td>
<td>AV225659</td>
<td>calmodulin 2</td>
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<td>92467_at</td>
<td>AJ132433</td>
<td>RIKEN cDNA E130201N18 gene</td>
<td>3.07</td>
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</table>

Table 17. Representative list of genes downregulated at 72 hours in comparison to 24 hours after RANKL treatment in WT osteoclasts.
To further identify genes that are more directly regulated by Mitf, we compared the differential expression of osteoclast-specific genes between WT and Mitf<sup>mi/mi</sup> at the indicated time points. Overall, we detected a differential expression of 1.5 fold or higher for 51 genes, between osteoclasts from WT and Mitf<sup>mi/mi</sup> (Tables 18-21). Our data indicated that the expression levels of several transcription factors including zinc finger homeobox 1a (HOX 1a) and signaling molecules including protein tyrosine phosphatase receptor type L, vitamin D receptor and T-cell receptor beta and DNA replication molecules including lymphoid specific helicase are down regulated in Mitf<sup>mi/mi</sup> osteoclasts compared to WT within 24 hours of adding RANKL (Table 18). Similarly, Homebox11-like2 (HOX11L2), Ikaros family member zinc finger transcription factor Eos, single strand DNA binding factor and RNA polymerase II co-activator, p9, hematopoietic cell phosphatase (HCP) are among the several genes downregulated in Mitf<sup>mi/mi</sup> osteoclasts when compared to the WT at 72 hours after adding RANKL (Table 19). Tables 20 and 21 represent classes of genes that are upregulated at 24 and 72 hours of RANKL treatment in Mitf<sup>mi/mi</sup> osteoclasts compared to the WT.
Table 18. Representative list of the genes downregulated in Mitf<sup>mi/mi</sup> osteoclasts at 24 hours after RANKL treatment in comparison with WT.
Table 19. Representative list of the genes downregulated in $Mitf^{mim/mi}$ osteoclasts at 72 hours after RANKL treatment in comparison with WT.

<table>
<thead>
<tr>
<th>Probe set</th>
<th>GeneBank</th>
<th>Gene name</th>
<th>Fold change</th>
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</thead>
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<tr>
<td>98072_r_at</td>
<td>M6902</td>
<td>hematopoietic cell phosphatase</td>
<td>4.58</td>
</tr>
<tr>
<td>103582_r_at</td>
<td>AI153412</td>
<td>Guanine nucleotide binding protein, alpha inhibiting 1</td>
<td>3.37</td>
</tr>
<tr>
<td>92718_at</td>
<td>D68232</td>
<td>Lymphocyte antigen 6 complex, locus C</td>
<td>2.77</td>
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<tr>
<td>92877_at</td>
<td>U36778</td>
<td>Spleen tyrosine kinase</td>
<td>2.63</td>
</tr>
<tr>
<td>102337_a_at</td>
<td>AJ223301</td>
<td>Homeobox 11-like 2 (HOX11L2)</td>
<td>2.45</td>
</tr>
<tr>
<td>96038_at</td>
<td>AW226939</td>
<td>Carboxylesterase 3</td>
<td>2.41</td>
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<tr>
<td>93321_at</td>
<td>AI849263</td>
<td>NADH dehydrogenase (ubiquinone) 1, subcomplex unknown, 1</td>
<td>2.35</td>
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<td>99413_at</td>
<td>J05479</td>
<td>Protein phophatase 3, catalytic subunit, alpha isoform</td>
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<td>104179_at</td>
<td>Y18723</td>
<td>Protease, serine, 18</td>
<td>2.09</td>
</tr>
<tr>
<td>104407_at</td>
<td>AJ603133</td>
<td>Origin recognition complex subunit 1-homologue (S.cerevisiae)</td>
<td>2.08</td>
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<tr>
<td>101980_at</td>
<td>J03750</td>
<td>Mouse single stranded DNA binding protein p9; RNA polymerase II transcriptional coactivator</td>
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<tr>
<td>98277_at</td>
<td>AB017516</td>
<td>Eos protein; zinc finger protein family</td>
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<td>160388_at</td>
<td>C78441</td>
<td>Expressed sequence C78441</td>
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<td>101465_at</td>
<td>AV324433</td>
<td>Expressed sequence AV316828</td>
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<td>97173_f_at</td>
<td>AW260202</td>
<td>RIKEN cDNA 2810025E10</td>
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<td>102794_at</td>
<td>AI840598</td>
<td>RIKEN cDNA 2310005G07</td>
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Table 20. Representative list of the genes upregulated in \textit{Mitf}^{mi/mi} osteoclasts at 24 hours after RANKL treatment in comparison with WT.

<table>
<thead>
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</tr>
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<td>AV375661</td>
<td>Glycolipid transfer protein</td>
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<td>98066_r_at</td>
<td>AL009226</td>
<td>Bromodomain containing Ring finger protein</td>
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<tr>
<td>983903_at</td>
<td>X79131</td>
<td>IB3/6 polypeptide</td>
</tr>
<tr>
<td>99473_at</td>
<td>AI847822</td>
<td>EST clone</td>
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</tbody>
</table>

Table 21. Representative list of the genes upregulated in \textit{Mitf}^{mi/mi} osteoclasts at 72 hours after RANKL treatment in comparison with WT.

<table>
<thead>
<tr>
<th>Probe set</th>
<th>GeneBank</th>
<th>Gene name</th>
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<tbody>
<tr>
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<td>AV122897</td>
<td>Frizzled homologue 4</td>
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<tr>
<td>96001_at</td>
<td>AB028920</td>
<td>Mus musculus AKAP96 mRNA</td>
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<tr>
<td>97870_s_at</td>
<td>AA798624</td>
<td>ERO1 like protein</td>
</tr>
<tr>
<td>98140_at</td>
<td>X69961</td>
<td>Cadherin 1</td>
</tr>
<tr>
<td>98303_at</td>
<td>X79131</td>
<td>IB3/6 polypeptide</td>
</tr>
<tr>
<td>98871_at</td>
<td>X98362</td>
<td>Mouse homolog of human ocular albinism 1 (Nettie ship-Falls)</td>
</tr>
<tr>
<td>99925_f_at</td>
<td>AI835567</td>
<td>microtubule based movement protein</td>
</tr>
</tbody>
</table>
To verify the differential gene expression observed via microarray analysis, we performed real-time PCR analysis on four genes, viz., Ikaros family member Eos, HOX11L2, HCP/SHP-1 and p9, which were downregulated in \( \text{Mitf}^{mi/mi} \) osteoclasts at 72 hours after RANKL treatment, in comparison to WT. Our data indicated that osteoclasts from new born WT and \( \text{Mitf}^{mi/mi} \) mice expressed Eos at 2.8±0.6 and 0.9±0.2 fold respectively; HCP at 2±0.1 and 0.6±0.3 fold respectively; HOX11L2 at 2.7±0.5 and 0.9±0.4 fold respectively and p9 at 1.6±0.4 and 0.6±0.2 fold respectively (Figs. 21A-D). Similarly, osteoclasts from 30-day old WT and \( \text{Mitf}^{mi/mi} \) mice expressed Eos at 4.8±0.7 and 1.4±0.1 fold respectively; HCP at 3.4±0.6 and 0.9±0.1 fold respectively; HOX11L2 at 13±1.4 and 5.5±0.4 fold respectively and p9 at 7±0.8 and 1.4±0.2 fold respectively (Figs. 21E-H). These data confirm the results from the comparative microarray analysis that the expression levels of Eos, HOX11L2, HCP and p9 are downregulated in \( \text{Mitf}^{mi/mi} \) osteoclasts and that Mitf directly might regulate the expression of these genes.
Figure 21. Eos, HCP, HOX11L2 and p9 are expressed at lower levels in osteoclasts from both newborn and 30-day old Mitf<sup>mi/mi</sup> mice compared to WT. The values represent fold induction levels in Wt and Mitf<sup>mi/mi</sup> osteoclasts from newborn or 30-day old mice, as indicated, calculated via real-time PCR analysis. A and E: Fold induction of Eos; B and F: Fold induction of HCP; C and G: Fold induction of HOX11L2 and D and H: Fold induction of p9.
3.3 DISCUSSION

The bHLH-Zip transcription factor, Mitf is evolutionarily conserved from fish to quail to mice to humans with important roles in the differentiation of many cell types including osteoclasts. Deletion in one of the arginines in the basic domain of Mitf results in the Mitf$^{mi/mi}$ mutant protein that is unable to bind to DNA as a homodimer or as a heterodimer with WT-Mitf or with family members such as Tfe3. Thus, the Mitf$^{mi/mi}$ mutant protein might act as a dominant negative. Furthermore, the Mitf$^{mi/mi}$ mice harboring this mutation exhibit severe osteopetrosis owing to a decreased ability of the mononuclear precursor cells to fuse and form multinuclear osteoclasts. In this study, we have compared the osteopetrotic phenotypes in newborn and 30-day old Mitf$^{mi/mi}$ mice using several parameters including careful histomorphometric analysis of long bones, in vitro differentiation and functional assays and analysis of TRAP and cathepsin K expression levels in these osteoclasts. As evident from this study, Mitf$^{mi/mi}$ mice possess significantly higher percentage of unresorbed trabeculae in their long bones and have significantly reduced numbers of multinuclear osteoclasts with significantly lower TRAP activity. Precursors from Mitf$^{mi/mi}$ mice formed very few multinuclear cells in vitro and these cells seldom possessed more than 3-4 nuclei. In contrast, precursors from WT mice were capable of forming numerous multinuclear osteoclasts that contained 6-12 or more nuclei. Osteoclast precursors from Mitf$^{mi/mi}$ mice were defective in forming resorption pits on calcium phosphate-coated surfaces. In addition, Mitf$^{mi/mi}$ osteoclast precursors expressed significantly lower levels of TRAP and Cathepsin K mRNAs in
response to RANKL, in comparison to the WT. Taken together, these data suggest that Mitf plays a major role in osteoclast biology including differentiation and function. A plethora of work from several laboratories has shown that Mitf directly regulates the expression of several osteoclast-specific genes including TRAP, E-Cadherin, Cathepsin K, Osteopontin, Collagenase IV and OSCAR (Luchin et al., 2000, Motyckova et al., 2001, Mansky et al., 2002a, Rho et al., 2002 and So et al., 2003). Mice depleted for the expression of cathepsin K and TRAP exhibit osteopetrosis, albeit in a milder form. However, the precise aspects of osteoclast differentiation regulated by Mitf and whether Mitf regulates the expression of additional osteoclast-specific genes is hitherto greatly unknown. Towards achieving this goal, we undertook an extensive analysis of the differential expression of Mitf-regulated osteoclast-specific genes in WT and Mitf\(^{mi/mi}\) osteoclast precursors via microarray analysis using oligonucleotide Affymetrix GeneChip® arrays comprising of a total of ~12,000 murine genes.

We have identified 891 novel genes, whose expression levels change during osteoclast differentiation. In particular, the expression levels of 51 novel genes are different between WT and Mitf\(^{mi/mi}\) osteoclasts. These included several transcription factors including HOX1a, HOX11L2, EOS, p9; signaling molecules including HCP, protein tyrosine phosphatase receptor type L, vitamin D receptor, GABA receptor, calmodulin and T-cell receptor beta; DNA replication molecules including lymphoid specific helicase; several proteins involved in cell metabolism including phospholipase A2; proteins involved in vesicular trafficking including phosphatidyl inositol binding clathrin assembly assembly...
protein and ATPases including cardiac muscle Ca\textsuperscript{2+} transporting fast twitch ATPase. We confirmed via real-time PCR analysis that the expression levels of Eos, HCP, HOX11L2 and p9 are significantly lower in osteoclast precursors from both newborn and 30-day old Mitf\textsuperscript{\textit{mi/mi}} osteoclasts when compared with WT. Eos belongs to the Ikaros family of Zinc finger transcriptional repressors with potential roles in neuronal and B cell differentiation (Honma et al., 1999; Perdomo et al., 2000 Kirstetter et al., 2002 and Dumortier et al., 2003). HCP is also known as Src homology 2 domain-containing phosphatase 1 (SHP-1) and is predominantly expressed in hematopoietic cells. The moth-eaten viable Mev/Mev mice harboring a hypomorphic mutation in the kinase domain of SHP-1 exhibit severe osteoporosis due to the presence of an increased number of functional osteoclasts. Hence HCP is considered to be a negative regulator of osteoclast differentiation and function (Aoki et al., 1999 and Umeda et al., 1999). HOX11L2, a homeobox-domain containing transcription factor, is an oncoprotein that is activated in T cell acute lymphoblastic leukemia (Ferrando et al., 2002). Hox11 paralogous genes have been implicated in kidney development (Wellik et al., 2002). Finally, p9 is a single stranded DNA binding protein expressed in several mammalian cell lines and whose biological function is not known (Ballard et al., 1988). It is unknown whether Eos, HOX11L2 and p9 possess any roles in osteoclast differentiation and function. Finally, we have preliminary evidence that Eos, an Ikaros family zinc finger transcriptional repressor, might be capable of repressing the activation of TRAP and Cathepsin K promoters by Mitf and PU.1 in transient transfection.
experiments, suggesting the existence of a transcriptional feed-back mechanism for Mitf during osteoclast differentiation.

In conclusion, we have identified 51 novel genes, including several transcription factors and signaling molecules that are regulated by Mitf with potential roles in osteoclast biology. Reduced expression of Eos, HOX11L2, HCP and p9 in \( Mitf^{mi/mi} \) osteoclasts was further verified independently. Careful functional characterization via gene depletion and over-expression studies of the novel genes identified through this study will help reveal the roles of these genes in osteoclast biology. Further, investigating whether these genes are under the direct regulation of Mitf in osteoclasts would contribute towards unearthing the specific roles played by Mitf during osteoclast differentiation, function and survival.
4.1 INTRODUCTION

Osteoclasts are multinuclear cells of hematopoietic origin that are primarily responsible for bone resorption. Osteopetrosis is an inherited metabolic bone disorder characterized by the accumulation of primary spongiosa in bone marrow cavities and by the failure of incisors to erupt as a consequence of reduced or blocked osteoclast formation and function (Marks and Walker, 1976; Marks, 1987 and Nii et al., 1995). In mice, osteopetrosis is observed in several irradiation-induced and spontaneous mutants including microphthalmia (mi), osteopetrosis (op/op) and gray-lethal (gl) (Grünberg, 1935, Grünberg, 1948 and Marks, 1976).

The microphthalmia locus, located on murine chromosome 6p, encodes for a basic helix-loop-helix leucine zipper (bHLH-Zip) transcription factor called
microphthalmia transcription factor (Mitf) (Hodgkinson et al., 1993). Mitf is closely related to its family members, Tfe3, TfeB and TfeC bHLH-Zip transcription factors (Hodgkinson et al., 1993). Mitf binds to 'TCANNTGA' E-box elements on promoters of target genes such as tartarate resistant acid phosphatase (TRAP), cathepsin K and E-Cadherin as homodimers or as heterodimers with Mitf-family members, to drive target gene expression [Askan and Goding, 1998; Luchin et al., 2000; Motyckova et al., 2001, Mansky et al., 2000a; Mansky et al., 2000b). The human orthologue, MITF, is mutated in families with Waardenburg syndrome type II (WS2) (Tassabehji et al., 1994).

Mitf<sup>mi/mi</sup> mutant mice exhibit severe osteopetrosis; osteoclasts from Mitf<sup>mi/mi</sup> mice do not fuse and express lower levels of TRAP and cathepsin K (Luchin et al., 2000, Motyckova et al., 2001 and Moore, 1995). Mitf<sup>mi/mi</sup> mutation is due to a 3 base pair deletion in the N-terminus, which deletes one of the four arginines in the basic domain of the protein (ΔR215) (Moore, 1995). Mitf<sup>or/or</sup> mice exhibit white coat color and small eyes. In addition, the incisors either fail to erupt in younger animals or are poorly formed later in life (Nii et al., 1995). Mitf<sup>or/or</sup> mice exhibit accumulation of primary spongiosa in the metaphysis of long bones at 10 to 37 days of age; a condition that improves, but does not become completely resolved, with age (Nii et al., 1995). Mitf<sup>or/or</sup> mutation is due to the substitution of an arginine in the basic domain with a lysine (R216K) (Moore, 1995). The Mitf<sup>wh/wh</sup> mutation occurring due to a substitution of an isoleucine to asparagine (I212N) in the basic domain of Mitf does not result in osteopetrosis or any other defects in osteoclast differentiation (Moore, 1995).
Interestingly, these three basic domain mutations cause the most severe coat color phenotype among all Mitf mutations (Moore, 1995 and Steingrimsson et al., 2003). $Mitf^{mi/mi}$ and $Mitf^{or/or}$ proteins are incapable of binding to DNA as homo or heterodimers and act as dominant negative molecules blocking DNA binding by WT-Mitf and Tfe3 (Hemesath et al., 1994). The plus form of $Mitf^{wh/wh}$, containing an alternatively spliced six amino acid insert, is capable of binding to DNA as a homodimer or as a heterodimer with Tfe3 (Hemesath et al., 1994). Recent data have suggested that Mitf and Tfe3 play redundant roles in osteoclast differentiation (Steingrimsson et al., 2002).

Three mutations in the basic domain of Mitf protein, viz. $Mitf^{mi/mi}$, $Mitf^{or/or}$ and $Mitf^{wh/wh}$, behave very differently with respect to their effect on osteoclast differentiation and ensuing activity. Thus, $Mitf^{mi/mi}$, $Mitf^{or/or}$ and $Mitf^{wh/wh}$, form an allelic series of Mitf mutants that could provide crucial insights into the role of Mitf in osteoclast differentiation. Genes regulated by Mitf that are involved in osteoclast differentiation might be differentially expressed in $Mitf^{mi/mi}$, $Mitf^{or/or}$ and $Mitf^{wh/wh}$ mutants and at different ages, raising the possibility that some genes might be more important than others in osteoclast differentiation.

We hypothesized that the level of expression of Mitf-regulated genes in $Mitf^{mi/mi}$, $Mitf^{or/or}$ and $Mitf^{wh/wh}$ osteoclasts determines the severity of osteopetrosis in these mutants. To test this hypothesis, we examined the expression levels of TRAP and cathepsin K; two genes regulated by Mitf, in osteoclast precursors from new born and 30-day old $Mitf^{mi/mi}$, $Mitf^{or/or}$ and $Mitf^{wh/wh}$ mutants. Further, to correlate changes in gene expression patterns with
osteopetrosis, we performed careful histomorphometric analysis of TRAP-stained femur sections and in vitro differentiation and functional assays with osteoclast precursors derived from these mutants. Finally, we examined the expression patterns of four novel Mitf-regulated genes, potentially involved in osteoclast differentiation, that were identified via microarray analysis of osteoclast precursors from Mitf<sup>mi/mi</sup> mutants, in these mutant osteoclasts (Sankar et al., manuscript in preparation). Our data indicates that genes regulated by Mitf, including TRAP and cathepsin K, may be categorized into two classes based on the importance of their expression patterns and the potential significance of their roles during osteoclast differentiation.

4.2 RESULTS

4.2.1 OSTEOCLAST PRECURSORS FROM NEW BORN AND 30-DAY OLD Mitf<sup>or/or</sup> MUTANTS EXPRESS LOWER LEVELS OF CATHEPSIN K AND NORMAL LEVELS OF TRAP COMPARED TO WT

To investigate whether the expression patterns of osteoclast-specific genes that are regulated by Mitf are different in Mitf<sup>mi/mi</sup>, Mitf<sup>or/or</sup> and Mitf<sup>wh/wh</sup> mutants and whether the expression of these genes vary with age, we first examined the expression levels of TRAP and cathepsin K in osteoclast precursors from new born and 30-day old mutant mice, at 24 and 72 hours after treatment with receptor activator of NFκB ligand (RANKL), by quantitative real-time PCR. Our results indicate that TRAP expression levels in osteoclast precursors from new born WT, Mitf<sup>wh/wh</sup> and Mitf<sup>or/or</sup> mice were 7.4±0.1, 7.8±0.3
and 7.3±1 fold respectively at 24 hours and 10.3±1.3, 9.3±0.1 and 6±0.4 fold respectively, at 72 hours after adding RANKL (Fig. 22A). However, osteoclasts from newborn Mitf\textsuperscript{mi/mi} mice expressed 4.1±0.6 and 2.5±0.4 fold TRAP mRNA at 24 and 72 hours after treatment with RANKL, respectively (Fig. 22A). Similarly, TRAP expression levels in osteoclast precursors from 30-day old WT, Mitf\textsuperscript{wh/wh} and Mitf\textsuperscript{or/or} mutants were 8.4±0.7, 10.8±0.3 and 14.9±2.2 fold respectively, at 24 hours and 11.5±2, 12.5±1.1 and 8.7±1.5 fold respectively, at 72 hours after adding RANKL (Fig. 22C). In contrast, TRAP expression levels in osteoclasts from 30-day old Mitf\textsuperscript{mi/mi} mice were 2.8±0.5 and 2.8±0.6 fold respectively, at the indicated time points (Fig. 22C).

Cathepsin K expression levels in osteoclasts from newborn WT and Mitf\textsuperscript{wh/wh} mice were 3.1±0.5 ad 3.5±0.4 fold respectively, at 24 hours and 4.9±0.4 and 4.6±0.1 fold respectively, at 72 hours after adding RANKL (Fig. 22B). Similarly, cathepsin K was expressed 2.9±0.9 and 2.6±0.3 fold respectively, at 24 hours and 9.3±0.5 and 7.2±1.5 fold respectively, at 72 hours after adding RANKL in osteoclasts from 30-day old WT and Mitf\textsuperscript{wh/wh} mice (Fig. 22D). In contrast, cathepsin K levels were 1.5±0.9 and 1.9±0.7 fold respectively, at 24 hours and 2.4±0.3 and 2.4±0.4 fold respectively, at 72 hours after adding RANKL, in osteoclasts from newborn Mitf\textsuperscript{mi/mi} and Mitf\textsuperscript{or/or} mice (Fig. 22B). Also, in osteoclasts from 30-day old Mitf\textsuperscript{mi/mi} and Mitf\textsuperscript{or/or} mice, cathepsin K levels were 1±0.53 and 1.2±0.1 fold respectively, at 24 hours and 1.8±1.56 and 3.3±1.5 fold respectively, at 72 hours after adding RANKL (Fig. 22D). We
further examined the levels of TRAP and cathepsin K in osteoclast precursors from adult (3 month old) Mitf<sup>or/or</sup> mice. Our results indicate that TRAP expression levels were 5.5±0.3 and 4.7±0.1 fold and cathepsin K levels were 1.1±0.3 and 0.9±0.5 fold respectively at 24 and 72 hours after adding RANKL. Taken together, these results indicate that Mitf<sup>or/or</sup> osteoclasts express TRAP mRNA at levels similar to those expressed by WT and Mitf<sup>wh/wh</sup> osteoclasts. The level of TRAP expression in the mutants does not change with age. However, cathepsin K expression levels are significantly lower in Mitf<sup>or/or</sup> mutants compared to WT and Mitf<sup>wh/wh</sup> at all time points and age groups. As expected, Mitf<sup>mi/mi</sup> osteoclasts expressed significantly lower levels of TRAP and cathepsin K at all ages, compared to those in WT osteoclasts.
Figure 22. mi-Oakridge (mi-OR) mutants, both newborn and 30-day old, express lower levels of Cathepsin K mRNA but normal levels of TRAP mRNA. Quantitative Taqman Real-Time PCR assay was performed on cDNA prepared from osteoclasts of newborn and 30-day old WT, Mitf<sup>wh/wh</sup> (mi-White), Mitf<sup>mi/mi</sup> (mi/mi) and Mitf<sup>or/or</sup> (mi-OR) mice against TRAP, Cathepsin K, c-FMS and GAPDH. Comparison of fold induction in newborn mice and 30-day old mice for: A and D: TRAP; B and D: Cathepsin K; and C and F: c-FMS at 24 and 72 hours after RANKL treatment is shown. All values were first normalized to GAPDH levels and then expressed as fold induction over CSF1-only controls (0 hour time point). An average of 3 experiments is shown.
4.2.2 OSTEOCLASTS FROM NEW BORN AND 30 DAY OLD Mitf<sup>mi/mi</sup> AND Mitf<sup>or/or</sup> MICE EXPRESS LOWER LEVELS OF Eos, HOX11L2, HCP AND p9 GENES

We identified several Mitf-regulated genes that are expressed at lower levels in osteoclasts from Mitf<sup>mi/mi</sup> mice compared to those from WT mice, at 72 hours after adding RANKL, by microarray analysis (Chapter 3). Out of these, four genes viz., Ikaros family zinc finger transcriptional repressor Eos, homeobox protein HOX11L2, hematopoietic cell phosphatase (HCP) and single strand DNA-binding protein p9, were further verified independently by quantitative real-time PCR (Figure 21). We examined the expression patterns of these four genes in osteoclast precursors from new born and 30-day old WT, Mitf<sup>wh/wh</sup>, Mitf<sup>mi/mi</sup> and Mitf<sup>or/or</sup> mice, at 72 hours after RANKL treatment, by real-time PCR. Our data indicated that osteoclasts from new born WT, Mitf<sup>wh/wh</sup>, Mitf<sup>mi/mi</sup> and Mitf<sup>or/or</sup> mice expressed Eos at 2.8±0.6, 1.7±0.1, 0.9±0.2 and 1.2±0.1 fold respectively; HCP at 2±0.1, 1.5±0.4, 0.6±0.3 and 0.7±0 fold respectively; HOX11L2 at 2.7±0.5, 2.4±0.1, 0.9±0.4 and 1±0.3 fold respectively and p9 at 1.6±0.4, 2±0.4, 0.6±0.2 and 0.7±0.1 fold respectively (Figs. 23A-D). Similarly, osteoclasts from 30-day old WT, Mitf<sup>wh/wh</sup>, Mitf<sup>mi/mi</sup> and Mitf<sup>or/or</sup> mice expressed Eos at 4.8±0.7, 3.0±0.2, 1.4±0.1 and 1.8±0.1 fold respectively; HCP at 3.4±0.6, 2.8±0.3, 0.9±0.1 and 1.3±0.1 fold respectively; HOX11L2 at 13±1.4, 6.5±0.3, 5.5±0.4 and 2±0.04 fold respectively and p9 at 7±0.8, 5.6±0.3, 1.4±0.2 and 2.3±0.03 fold respectively (Figs. 23E-H). The expression levels of these genes in osteoclasts from adult Mitf<sup>or/or</sup> mice were also significantly lower than those in
WT osteoclasts (data not shown). These data indicate that similar to cathepsin K, the expression levels of Eos, HOX11L2, HCP and p9 are significantly lower in osteoclasts from Mitf<sup>p<sub>rior</sub></sup> mice at all age groups.
Figure 23. Eos, HOX11L2, HCP and p9 are down-regulated in osteoclasts derived from both Mitf^{or/} and Mitf^{mi/mi} new-born and 30-day old mice. Quantitative Real-Time PCR assay was performed on cDNA prepared from osteoclasts of newborn WT, Mitf^{wh/wh} (mi-White), Mitf^{mi/mi} (mi/mi) and Mitf^{or/or} (mi-OR) mice at 0 and 72 hours after adding RANKL. Fold induction at 72 hours in osteoclasts from newborn and 30-day old mice for A and E: EOS; B and F: HCP; C and G: HOX11L2 and D and H: p9 are shown. All values were first normalized to GAPDH levels and then expressed as fold induction over 0 hour controls. An average of 3 experiments is shown.
4.2.3 Mitf<sup>pr/or</sup> MICE ARE OSTEOPETROTIC; BUT THE PHENOTYPE IS Milder COMPARED TO Mitf<sup>mi/mi</sup>

In order to correlate the osteopetrotic phenotype with the results from the gene expression analysis, we performed radiographic and histomorphometric analysis of TRAP-stained femur sections from newborn and 30-day old WT, Mitf<sup>wh/wh</sup>, Mitf<sup>mi/mi</sup> and Mitf<sup>pr/or</sup> mice. Radiographic examination revealed that sclerotic lesions were present primarily in the diaphysis of the newborn Mitf<sup>mi/mi</sup> and Mitf<sup>pr/or</sup> and 30-day old Mitf<sup>mi/mi</sup> femurs (Figs. 24A and 25A). In contrast, the lesions presented shifted towards the distal metaphysis of the 30-day old Mitf<sup>pr/or</sup> femurs (Fig. 25A). Similarly, histomorphological analysis of TRAP stained sections revealed that unresorbed endochondral trabeculae were present mainly in the diaphysis of newborn Mitf<sup>mi/mi</sup> and Mitf<sup>pr/or</sup> and 30-day old Mitf<sup>mi/mi</sup> femurs and in the distal metaphysis of 30-day old Mitf<sup>pr/or</sup> femurs (Figs. 24B and 25B). Histomorphometric analysis revealed that the percentage of unresorbed bone area (UBA) relative to the total bone area (TBA) was 5.4±1.9, 5.8±0.3, 20.8±4.8 and 14.6±0.5 respectively, in newborn WT, Mitf<sup>wh/wh</sup>, Mitf<sup>mi/mi</sup> and Mitf<sup>pr/or</sup> femurs (Fig. 24D) and 4.2±0.5, 4.5±0.2, 15.1±3.5 and 9.7±1.1 respectively, in 30-day old WT, Mitf<sup>wh/wh</sup>, Mitf<sup>mi/mi</sup> and Mitf<sup>pr/or</sup> femurs (Fig. 25D). The total number of osteoclasts (# of OCL) per unit bone perimeter (UBP) was 12±0.5, 11.5±2, 4±0.2 and 6.3±0.5 respectively, in newborn WT, Mitf<sup>wh/wh</sup>, Mitf<sup>mi/mi</sup> and Mitf<sup>pr/or</sup> femurs and 2.7±0, 1.7±0.1, 0.6±0 and 1±0 respectively, in 30-day old WT, Mitf<sup>wh/wh</sup>, Mitf<sup>mi/mi</sup> and Mitf<sup>pr/or</sup> femurs (Figs. 24E and 25E). Percentage of total osteoclast surface relative to total bone surface (TBS; both
trabecular and cortical surfaces) was 54.2 ± 4, 55.3 ± 5.9 ± 1.5 and 20.1 ± 0.1 respectively, in newborn WT, Mitf<sup>wh/wh</sup>, Mitf<sup>mi/mi</sup> and Mitf<sup>or/or</sup> femurs and 20.1 ± 0.4, 26.9 ± 0.8, 6.2 ± 0.4 and 14.2 ± 1.76 respectively, in 30-day old WT, Mitf<sup>wh/wh</sup>, Mitf<sup>mi/mi</sup> and Mitf<sup>or/or</sup> femurs (Figs. 24F and 25F). Overall, numerous, intensely TRAP-positive osteoclasts with ruffled borders were found attached to the trabecular surface in newborn and 30-day old from WT and Mitf<sup>wh/wh</sup> femurs. In contrast, femurs from newborn and 30-day old Mitf<sup>mi/mi</sup> mice had significantly fewer osteoclasts that stained weakly for TRAP and these osteoclasts were much smaller in size compared to WT (Figs. 24C and 25C). Osteoclasts in both newborn and 30-day old Mitf<sup>or/or</sup> femurs were similar in size to the WT osteoclasts. However, the number of osteoclasts and the total osteoclast surface in Mitf<sup>or/or</sup> were significantly smaller than WT but slightly higher than that in Mitf<sup>mi/mi</sup>. Similarly, the percentage of UBA in Mitf<sup>or/or</sup> femurs was lower than WT but higher than that seen in Mitf<sup>mi/mi</sup> femurs. Taken together, these data add quantitative evidence to the observation that Mitf<sup>or/or</sup> mice do exhibit osteopetrosis that is milder than that seen in Mitf<sup>mi/mi</sup>.
Figure 24. Osteopetrosis in newborn $Mitf^{or/or}$ mice is intermediate between WT and $Mitf^{mi/mi}$. A: Radiography of long bones from newborn WT, $Mitf^{wh/wh}$ (mi-White), $Mitf^{mi/mi}$ (mi/mi) and $Mitf^{or/or}$ (mi-OR) mice. B: TRAP and hematoxylin stained femur sections from these mice at 10X magnification and arrowheads represent unresorbed endochondral trabeculae in A and B. C: 20X magnification showing differences in the numbers and sizes of the osteoclasts (arrowheads) among the different genotypes. Histomorphometric analysis of D: % of unresorbed trabecular area (UBA) over the total bone area (TBA); E: number (#) of osteoclasts (OCL) over the total bone perimeter and F: % of total OCL surface over the total bone surface (TBS).
Figure 25. Osteopetrosis in 30-day old $\text{Mitf}^{or/or}$ mice shifts to the distal metaphysis although the number and size of the osteoclasts remain intermediate between WT and $\text{Mitf}^{mi/mi}$. Radiography of long bones from 30-day old WT, $\text{Mitf}^{wh/wh}$ (mi-White), $\text{Mitf}^{mi/mi}$ (mi/mi) and $\text{Mitf}^{or/or}$ (mi-OR) mice. B: TRAP and hematoxylin stained femur sections from these mice at 10X magnification and arrowheads represent unresorbed endochondral trabeculae in A and B. C: 20X magnification showing differences in the numbers and sizes of the osteoclasts (arrowheads) among the different genotypes. Histomorphometric analysis of D: % of unresorbed trabecular area (UBA) over the total bone area (TBA); E: number (#) of osteoclasts (OCL) over the total bone perimeter and F: % of total OCL surface over the total bone surface (TBS).
4.2.4 OSTEOCLAST PRECURSORS FROM Mitf<sup>mi/mi</sup> AND NEWBORN Mitf<sup>or/or</sup> MICE FORM VERY FEW FUNCTIONAL, MULTINUCLEAR OSTEOCLASTS in vitro

As mentioned earlier, femurs from Mitf<sup>or/or</sup> mice contained multinuclear osteoclasts albeit in significantly fewer numbers. In addition, osteopetrosis in Mitf<sup>or/or</sup> mice is milder than that in Mitf<sup>mi/mi</sup> and the condition improves with age (Nii et al., 1995). To investigate whether the osteoclast precursors from newborn and 30-day old mice Mitf<sup>or/or</sup> were capable of forming functional, multinuclear osteoclasts in vitro, we plated osteoclast precursors from newborn and 30-day old WT, Mitf<sup>wh/wh</sup>, Mitf<sup>mi/mi</sup> and Mitf<sup>or/or</sup> mice in gelatin-coated dishes in differentiation media-I for the formation of multinuclear osteoclasts (Figs. 26Ai and 26Bi) or in calcium phosphate coated BD Biocoat osteologic multitest slides (BD Biosciences) for the formation of resorption pits by functional osteoclasts (Figs. 26Aii and 26Bii). Our results indicate that precursors from newborn and 30-day old Mitf<sup>mi/mi</sup> and Mitf<sup>or/or</sup> mice were not capable of forming any multinuclear osteoclasts with more than 5 nuclei and very few multinuclear osteoclasts with 3-5 nuclei (Table 22). Interestingly, precursors from 30-day old Mitf<sup>or/or</sup> mice formed multinuclear osteoclasts with 3-5 nuclei at numbers higher than Mitf<sup>mi/mi</sup> (116±5.6 vs. 34±3.3) but significantly lower than those formed by WT precursors (582±18.9) (Table 22).

Osteoclast precursors from newborn Mitf<sup>wh/wh</sup> mice formed the highest number of pits (521) with the largest range of surface area (100 – 250,000 sq. pixels) while those from newborn WT mice formed 359 pits with an area range
of 100 – 60,000 sq. pixels, in a typical representative experiment (Table 23). Similarly, precursors from 30-day old WT and $Mitf^{wh/wh}$ mice formed 1643 and 874 pits with a wide range of surface area (Table 23). In contrast, precursors from newborn and 30-day old $Mitf^{mi/mi}$ and $Mitf^{or/or}$ mice formed fewer numbers of pits with smaller range of surface area (Table 23). However, precursors from 30-day old $Mitf^{or/or}$ mice formed resorption pits at slightly higher numbers (80) than those formed by $Mitf^{mi/mi}$ precursors (45) and these pits had surface area in the range of 100-100,000 sq. pixels (Table 23). Taken together, these results indicate that although precursors from older $Mitf^{or/or}$ mice are capable of forming functional, multinuclear osteoclasts at numbers slightly larger than those from $Mitf^{mi/mi}$, $Mitf^{or/or}$ osteoclast precursors are fundamentally defective in osteoclast differentiation and function.
Figure 26. Osteoclast precursors from newborn and 30-day old $Mitf^{mi/mi}$ and newborn $Mitf^{or/or}$ form very few multinuclear osteoclasts or resorption pits during in vitro differentiation and functional assays. However, 30-day old $Mitf^{or/or}$ osteoclasts do form larger resorption pits at slightly higher numbers than the newborn osteoclasts. Precursors from new-born and 30-day old WT, $Mitf^{wh/wh}$ (mi-White), $Mitf^{mi/mi}$ (mi/mi) and $Mitf^{or/or}$ (mi-OR) were plated either for formation of multinuclear cells or plated on calcium phosphate slides for formation of resorption pits. Ai: Assay for formation of multinuclear cells by newborn precursors; Aii: Resorption pit assay by newborn osteoclasts; Bi: Assay for formation of multinuclear cells by 30-day old precursors; Bii: Resorption pit assay by 30-day old osteoclasts;
Table 22. Table indicating the number of TRAP-positive multinuclear osteoclasts, classified according to the number of nuclei present, formed by osteoclast precursors from WT, Mitf<sub>wh/wh</sub> (mi<sup>WH</sup>), Mitf<sub>mi/mi</sub> (mi/mi) and Mitf<sub>or/or</sub> (mi<sup>OR</sup>) mice.

<table>
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<th>New-Born</th>
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<td></td>
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<td>mi/mi</td>
<td>mi&lt;sup&gt;OR&lt;/sup&gt;</td>
<td>WT</td>
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<td>6-12 nuclei</td>
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<td>7±1.11</td>
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<td>58±6.67</td>
<td>23±1.11</td>
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<td>3-5 nuclei</td>
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<td>13±1.11</td>
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<td>681±26.68</td>
<td>34±3.33</td>
<td>116±5.56</td>
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Average of 2 experiments is shown.
Table 24. Number, total resorption pit area and the area range distribution of the resorption pits formed by osteoclast precursors from WT, *Mitf<sup>wh/wh</sup>* (mi<sup>WH</sup>), *Mitf<sup>mi/mi</sup>* (mi/mi) and *Mitf<sup>or/or</sup>* (mi<sup>OR</sup>) mice.

<table>
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<th>AREA DISTRIBUTION (Square Pixels)</th>
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<td>mi/mi</td>
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4.3 DISCUSSION

\(Mitf^{mi/mi}, Mitf^{or/or}\) and \(Mitf^{wh/wh}\), form an allelic series of Mitf mutants, arising from mutations in the basic domain of the Mitf protein, which behave very differently with respect to osteoclast differentiation. The results from the work presented here and from previous studies indicate that while \(Mitf^{mi/mi}\) mice harbor mostly mononuclear osteoclasts, \(Mitf^{or/or}\) mice do contain multinuclear osteoclasts. However, both \(Mitf^{mi/mi}\) and \(Mitf^{or/or}\) contain fewer numbers of osteoclasts than WT. Furthermore, the number and size of osteoclasts and the percentage of unresorbed bone area in \(Mitf^{or/or}\) mice are intermediate between those in newborn and 30-day old \(Mitf^{mi/mi}\) and WT. The accumulation of primary spongiosa shifts from diaphysis in newborns to the distal metaphysis in 30-day old \(Mitf^{or/or}\) mice. Precursors from 30-day old mice formed functional, multinuclear osteoclasts at numbers slightly higher than those formed by \(Mitf^{mi/mi}\) and newborn \(Mitf^{or/or}\) precursors, but significantly lower than those formed by WT precursors from age-matched mice. Thus, osteopetrosis in \(Mitf^{or/or}\) mice improves with age, but does not become completely resolved. The correlative Mitf-regulated gene expression data indicates that while \(Mitf^{or/or}\) mice express TRAP at levels similar to WT, they express cathepsin K, HCP, HOX11L2, Eos and p9 at levels that are significantly lower than those expressed by WT. As mentioned earlier, HCP, HOX11L2, Eos and p9 were identified via microarray analysis as Mitf-regulated genes with potential roles in osteoclast differentiation.
Eos belongs to the Ikaros family of Zinc finger transcriptional repressors (Honma et al., 1999 and Perdomo et al., 2000). Eos is implicated in having a role in neuronal development and the Ikaros family has been found to have roles in neuronal development, B cell differentiation and in neutrophil differentiation (Honma et al., 1999, Kirstetter et al., 2002 and Dumortier et al., 2003). HCP, also known as Src homology 2 domain-containing phosphatase 1 (SHP-1) is predominantly expressed in hematopoietic cells and has been implicated to be a negative regulator of osteoclast differentiation and function (Aoki et al., 1999 and Umeda et al., 1999). HOX11L2, a homeobox-domain containing transcription factor, is an oncoprotein that is activated in T cell acute lymphoblastic leukemia (Ferrando et al., 2002). Hox11 paralogous genes have been implicated in kidney development (Wellik et al., 2002). Finally, p9 is a single stranded DNA binding protein expressed in several mammalian cell lines and whose biological function is not known (Ballard et al., 1988). Whether any of the newly identified Mitf-regulated genes, barring HCP, are involved in osteoclast differentiation and function needs to be investigated.

TRAP is an early response gene during osteoclast differentiation, whose expression is upregulated 6-7 fold within 30 minutes of adding RANKL Mansky et al., 2002a). The precise role played by TRAP in osteoclast differentiation is unclear, though its overexpression leads to mild osteoporosis and its removal leads to mild, late onset osteopetrosis in transgenic mice due to moderately defective bone resorption by osteoclasts (Hayman et al., 1996). In contrast, cathepsin K is a late response gene in osteoclasts, whose expression is
significantly upregulated only by 72 hours after treatment with RANKL. Mice deficient in cathepsin K exhibit osteopetrosis due to the inability of the multinuclear osteoclasts to degrade the collagen matrix of the bone (Saftig et al., 1998 and Gowen et al., 1999). It is not known whether TRAP and cathepsin K have any roles during osteoclast differentiation per se. Mitf<sup>pr</sup> osteoclasts express lower levels of cathepsin K and precursors from Mitf<sup>pr</sup> mice form very few functional, multinuclear osteoclasts in vitro. This suggests that the late response gene cathepsin K, might be important for osteoclast differentiation and function, by itself or in combination with other Mitf-regulated genes like Eos, HOX11L2, HCP and p9. The fact that TRAP is expressed at WT levels in Mitf<sup>pr</sup> osteoclasts, suggests that the expression of early response genes like TRAP might not be very critical for osteoclast differentiation and function. This suggestion is supported by the milder phenotype of TRAP knockout mice. Thus, it is possible to propose that Mitf-regulated genes could be categorized into two classes based on the significance of their roles during osteoclast differentiation and function: class 1 consisting of early response genes similar to TRAP that might be less critical and class 2 consisting of late response genes like cathepsin K, HOX11L2, HCP, Eos and p9, which might be more critical for osteoclast differentiation and function.

Mitf<sup>pr</sup> and Mitf<sup>mi/mi</sup> proteins inhibit DNA binding by WT-Mitf and Tfe3 and consequently act as dominant negative proteins. The expression levels of TRAP and cathepsin K, two genes that are directly regulated by Mitf, are different in Mitf<sup>pr</sup> osteoclasts, raising the possibility that a modifier is enabling
transcription from TRAP promoter in Mitf\textsuperscript{\text{or/or}} osteoclasts. Recent reports have demonstrated that Mitf and PU.1 are capable of activating TRAP promoter in a synergistic manner by directly binding to their respective binding sites and that Mitf\textsuperscript{\text{mi/mi}} blocks the transactivation of TRAP by PU.1 (Luchin et al., 2001). This raises the possibility that PU.1 might be the modifier in Mitf\textsuperscript{\text{or/or}} osteoclasts that enables the transcriptional activation of TRAP promoter. This hypothesis would be based on the assumption that Mitf\textsuperscript{\text{or/or}} might not act as a strong dominant negative with PU.1 as Mitf\textsuperscript{\text{mi/mi}} does. In this context, it would be interesting to investigate whether Mitf and PU.1 co-operate to bring about cathepsin K expression in osteoclasts.

In conclusion, the data from this study indicates that Mitf\textsuperscript{\text{or/or}} osteoclasts express TRAP at levels similar to WT but express lower levels of cathepsin K, Eos, HOX11L2, HCP and p9 proteins. Mitf\textsuperscript{\text{or/or}} mice exhibit osteopetrosis that improves with age and osteoclasts from these mice form fewer functional multinuclear osteoclasts \textit{in vitro}. Taking the gene expression, phenotypic and \textit{in vitro} functional data together, our results indicate that Mitf regulated osteoclast-specific genes might be categorized into two classes; one comprised of early response genes like TRAP and the other comprised of late responsive genes like cathepsin K.
CHAPTER 5

RANKL CO-ORDINATES CELL CYCLE WITHDRAWAL WITH OSTEOCLAST DIFFERENTIATION VIA THE CDK INHIBITORS, \( p27^{\text{KIP1}} \) AND \( p21^{\text{CIP1}} \)

5.1 INTRODUCTION

Bone development and maintenance in vertebrate animals are carried out by the coordinated action of two distinct cell types, osteoblasts and osteoclasts (Roodman, 1999). Osteoblasts are a mesenchymal cell type that form bone. Mononuclear precursors of myeloid origin fuse to form multinuclear osteoclasts capable of bone resorption (Roodman, 1999). In response to developmental or homeostatic signals, osteoblasts produce two factors, macrophage colony stimulating factor1 (CSF1) and receptor activator of NF\( \kappa \)B ligand (RANKL) that in combination are necessary and sufficient for osteoclast differentiation (Lacey et al., 1998; Yasuda et al., 1998; Takahashi et al., 1999; Kong et al., 1999; Khosla 2001 and Theill et al., 2002). RANKL binds to its receptor RANK, a member of the TNF-receptor super family, to effect osteoclast

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differentiation (Hsu et al., 1999; Lei et al., 2000). Soluble RANKL, in the presence of CSF1, induces the *in vitro* formation of multinuclear osteoclasts from macrophage/monocyte precursor cells. CSF1 is essential for the proliferation of the precursor cells and for the survival of differentiated osteoclasts (Tanaka et al., 1993, Weilbacher et al., 2000). Mice with targeted deletions in RANKL or RANK exhibit osteopetrosis due to the lack of differentiated osteoclasts and CSF1 mutant *op/op* mice exhibit an age-resolving osteopetrosis (Felix et al., 1990; Lacey et al., 1998; Kong et al., 1999 and Li et al., 2000).

Previous studies have indicated that between days 15 and 17 of embryonic metatarsal development in mice, osteoclast progenitors shift from a population of actively proliferating cells to that of committed, post-mitotic mononuclear precursor cells. These precursors start to fuse on day 18 to become multinuclear osteoclasts (Scheven et al., 1986 and Taylor et al., 1989). Osteoclast progenitors grown in co-culture with osteoblasts actively proliferate for the first 4 days in co-culture. However, 80% of these precursors withdraw from cell cycle by day 5 in co-culture (Tanaka et al., 1993). Thus, osteoclast progenitors appear to be postmitotic at the time of fusion to become tartrate-resistant acid phosphatase (TRAP) positive differentiated osteoclasts. However, the mechanism by which osteoclast precursors exit the cell cycle remains unknown.

In general, the progenitor cells must cease proliferating and enter into a state of quiescence to achieve the specialized characteristics of a fully
differentiated cell. Proliferation in mammalian cells is controlled primarily by events that lead to the activation and inactivation of cyclin-dependent kinases (CDKs) (Sherr and Roberts, 1995; Sherr and Roberts, 1999).

5.1.1 MAMMALIAN CELL CYCLE AND ITS REGULATION

5.1.1.1 THE CELL CYCLE

The cell cycle is a universal phenomenon conserved throughout evolution and is required for the growth and development of all multicellular organisms (Nurse, 2000). The mammalian cell cycle consists mainly of DNA synthesis (S) and mitosis (M) phases with a gap before the S phase called G1 and another gap before the M phase called G2 (Figure 27) (Mitchison, 1971 and Nurse, 2000). When growth factors are removed, cultured differentiated cells withdraw from cell cycle, enter into a “quiescent” stage (G0) with an unduplicated DNA content, where macromolecular synthesis is reduced (Sherr, 1994). Growth factors, mitogens, differentiation signals and temporal and special cues govern the G1 phase at the end of which a decision is made by the cells to commence DNA replication. In yeast, the G1 phase is used by the cells to grow to a critical size before initiating DNA replication and thus is governed by the presence of appropriate nutrients in the growth medium (Sherr, 1993 and Sherr, 1994). Events that occur during G1, stimulated by growth factors, mitogens and other growth stimulants, are geared towards the passage of a critical restriction point at the G1-S boundary of the cell cycle and thereby committing the cells to enter the S phase. Once the cells have passed this
critical restriction point, they do not require growth factor stimulations to complete DNA synthesis and undergo mitosis. Instead they rely on temporal downstream mechanisms in place in the cells to initiate DNA synthesis, replicate DNA, repair mistakes in DNA synthesis or repair DNA damage and undergo mitosis (Sherr, 1994). However the existence of a gap of 1-3 hours for initiation of DNA synthesis after the passage of the restriction point by the cells suggests that commitment to enter the S phase and the actual onset of DNA replication are separate events (Sherr and Roberts, 1996).
At the onset of the S phase, replication complexes are “loaded” on to chromosome associated origin recognition complexes (ORCs), a key step of which is the loading of mini chromosome maintenance (Mcm) proteins onto the chromatin. The Mcm proteins “license” DNA replication, ensuring that the same DNA is not replicated twice during a single S phase. Once the replication complexes are formed and activated, a series of replication forks form along the
DNA, commencing the replication of DNA. In mitosis or M phase, centrosomes duplicate and separate. This is followed by the formation of spindle poles with microtubules emanating from them. At the same time, chromosomes duplicate and condense. Kinetochores at the centromeres of sister chromatids attach to the microtubules emanating from the spindle poles; with one sister kinetochore attached to microtubules from one pole and the other attached to those from the opposite pole. At this time, the cohesion between the sister chromatids gets lost and they are pulled apart to the opposite poles and become nuclei of two daughter cells formed by cytokinesis (Nicklas, 1997; Nasmyth, 1999 and Nurse, 2000). Thus, it is important that the gap phases G1 and G2 serve as check points before the assumption of S and M phases. Of these, the regulation of G1 is more complex as it is under the control of several external stimuli.

5.1.1.2 CYCLIN-DEPENDENT KINASE COMPLEXES

Cyclin-dependent kinases (CDKs) play crucial roles during all aspects of the cell cycle. The CDKs exist in a complex consisting of a catalytic protein kinase subunit and a cyclin subunit. In budding and fission yeasts, a single CDK, termed Cdc28 in *Saccharomyces cerevisiae* and Cdc2 in *Schizosaccharomyces pombe*, is responsible for initiating all cell cycle transitions (Nurse, 1994, Sherr, 1994, Roberts, 1999, Nurse, 2000 and Coqueret, 2002). In mammals, the CDKs have evolved into small gene families whose individual members appear to control each phase or sub-phase of the cell cycle (Roberts, 1999 and Nurse, 2000). Also, specificity for each CDK is
achieved via its partnering with a specific cyclin at the appropriate phase, which depends on the timing of expression of each cyclin and the amount of a particular cyclin bound to the CDK. Consequently, CDK is activated by a CDK activating kinase (CAK) in the T-loop (Roberts, 1999). The G1 phase is regulated by CDK4 and CDK6 partnered with the D-type cyclins and the G1-S transition is governed by cyclin E-CDK2 complexes. Hence, the D and E-type cyclins are termed G1 cyclins. Cyclin A-CDK2 complexes regulate the S phase and the cyclin B-CDC2 complexes mainly regulate the M phase.

Cyclin-D1 deficient mice are viable but have several developmental defects, including decreased proliferation of mammary epithelial cells and retinal cells and a neurological abnormality (Sicinski et al., 1995). In order to delineate the roles of cyclins D1 and E during the G1 phase, Geng et al. used a knock-in mouse model where the cyclin D1 locus was replaced with cyclin E coding exons (Geng et al., 1999). These mice do not express any cyclin D1 but express cyclin E from a cyclin D1 promoter, in addition to the endogenous cyclin E. Thus, these mice express cyclin E in place of cyclin D1 at the appropriate time and space. This misexpression of cyclin E in place of cyclin D1 rescued the cyclin D1 knock out mice of all their developmental abnormalities. These data suggested that cyclin D1 is dispensable for cell cycle progression and that cyclin E could activate downstream steps in the cell cycle, bypassing the roles performed by cyclin D1 (Geng et al., 1999; Roberts, 1999 and Coqueret, 2002).
Two key regulatory processes that “run” this cycle “engine” are transcription and proteolysis. Genes required for the onset of S phase are transcribed during the G1 phase, mainly by the E2F family of transcription factors (Nurse, 2000 and Coqueret, 2002). Controlled expression and proteolysis of cyclins is the other main mechanism of regulating the CDK activity. Thus the cyclin A levels become detectable at the G1-S transition point. Similarly, cyclin B accumulates in the cytoplasm and enters the nucleus before the nuclear membrane breakdown. The A and B cyclins are not required for the entry into S phase and disruption of cyclin A function can inhibit chromosome replication (Pines and Hunter, 1991, Girard et al., 1991, Fang and Newport, 1991; Pagano et al., 1992; Zindy et al., 1992 and Sherr, 1993). The high levels of CDK activity present during the G2 phase has been shown to block the initiation of a further S phase in cells, ensuring that only one S phase exists in each cell cycle. When CDK activity falls as a result of cyclin proteolysis when the cell exits mitosis, the block over S phase initiation becomes relieved. CDK activity should be low in order for the replication complexes to be able to load on to the ORCs in the chromatin (Hayles, 1994, Fisher and Nurse, 1996 and Nurse, 2000).

5.1.1.2.1 PHOSPHORYLATION OF RETINOBLASTOMA PROTEIN BY G1 CYCLIN-CDKS

The main target of the G1 cyclin-CDK complexes (Cyclin D1, D2 and D3-CDK4/6 and cyclin E/CDK2) is the retinoblastoma (Rb) protein (Ewen et al.,
1993; Sherr, 1994 and Sherr, 1996). Rb is a central component of a repression complex, comprised of histone deacetylases, histone methylases and Swi/Snf remodeling complexes, that represses the transcription of many genes required for the G1-S transition and during S phase (Flemington et al., 1993; Helin et al., 1993; Brehm et al., 1998; Luo et al., 1998; Magnaghi-Jaulin et al., 1998; Nielson et al., 2001 and Coqueret, 2002). In a non- or hypophosphorylated form, Rb actively represses transcription of E2F-responsive genes like cyclin E in particular (Weintraub et al., 1992; Weintraub et al., 1995 and Bremner et al., 1995). This mechanism ensures that genes required in the S-phase are repressed during early G1 and are accessible only during the late G1/S transition phase.

Rb has at least 16 consensus sites for phosphorylation by CDKs and has been shown to undergo successive phosphorylation by cyclinD/CDK4 and cyclin E/CDK2 complexes during G1 (Sherr, 1996 and Coqueret, 2002). During early G1, extra-cellular growth factor and mitogen signals stimulate the formation of cyclin D-CDK4/6 complexes, which phosphorylate Rb at multiple sites, relieving its repression of E2F. Free E2F activates the transcription of several target genes including cyclin E, which complexes with CDK2. Cyclin E/CDK2 complexes now hyperphosphorylate Rb at multiple sites leading to further activation of E2F (Figure 23). Thus, the primary role of cyclin D/CDK4/6 seems to be to regulate the activation of cyclinE/CDK2 complexes (Geng et al., 1999).
5.1.1.2 REGULATION OF G1-S TRANSITION DURING CELL CYCLE

Fully differentiated cells seldom proliferate and rapidly proliferating cells seldom undergo differentiation, although these processes are not mutually exclusive (Coffman and Studzinski, 1999). The decision whether to undergo DNA synthesis or to arrest in G1 and commit to differentiation has to be taken before the cells pass the restriction point before the G1-S transition. Proteins that inhibit CDK activity during G1 are responsible for regulating this decision by the cells. These proteins, termed cyclin dependent kinase inhibitors (CDKIs), are responsible for inhibiting the cell cycle before the G1-S transition and also at G2 in lieu of DNA damage and other harmful cellular conditions that will make cell cycle progression harmful (Sherr and Roberts, 1999 and Coffman and Studzinski, 1999).

5.1.1.3 CDK INHIBITORS

Two main families of CDKIs are known to date: the INK4 (inhibitors of CDKs) family comprising of p16\textsuperscript{INK4a}, p15\textsuperscript{INK4b}, p18\textsuperscript{INK4c} and p19\textsuperscript{INK4D} and the Cip/Kip family comprising of p2\textsuperscript{1CIP1}, p2\textsuperscript{7KIP1} and p5\textsuperscript{7KIP2} (Serrano et al., 1993; Hannon and Beach, 1994; Guan et al., 1994; Hirai et al., 1995; Chan et al., 1995; Hirai et al., 1995; Harper et al., 1993; Noda et al., 1994; el-Deiry et al., 1993; Koff et al., 1993; Polyak et al., 1994a; Polyak et al., 1994b; Lee et al., 1995 and Matsuoka et al., 1995).
The INK4 family members possess multiple ankyrin repeats, with p16\textsuperscript{INK4a} and p15\textsuperscript{INK4b} containing 4 repeats and p18\textsuperscript{INK4C} and p19\textsuperscript{INK4D} containing five repeats (Luh et al., 1997, Kalus et al., 1997; Venkataramani et al., 1998; Baumgartner et al., 1998; Byeon et al., 1998; Renner et al., and Li et al., 1999). Ankyrin repeats are structural motifs that enter into protein-protein interactions (Bork, 1993). The INK4 proteins bind specifically to CDK4 and CDK6 precluding their ability to bind to ATP and to cyclin (Figures 31 and 32) (Luh et al., 1997, Kalus et al., 1997; Venkataramani et al., 1998; Baumgartner et al., 1998; Byeon et al., 1998; Renner et al; Li et al., 1999; Russo et al., 1998; Brotherton et al., 1998; Endicott et al., 1999 and Pavletich et al., 1999). The INK4s can also bind to cyclinD-CDK4/6 complexes to form ternary complexes. However, binary complexes comprising of CDK4/6 and INK4 are more abundant in vivo than the ternary complex of cyclin D-CDK4/6-INK and binding of INK4s to the cyclin-CDK complexes weaken the affinity of cyclins for CDK. Thus INK4 binding to cyclin-CDK complexes leads to eventual dissociation of cyclin from these complexes (Jeffrey et al., 2000).

The INK4 family members bind to CDK4/6 and block the ability of cyclinD-CDK4/6 complexes to phosphorylate Rb during G1 (Figure 29). The highly specific activity of INK4 towards the early G1 cyclins suggests that their activity is dependent on the presence of Rb. Thus, in the absence of Rb, cyclin E levels become elevated and cyclin D-CDK4/6 complexes do not inhibit S.
phase entry in cells (Koh et al., 1995; Lukas et al., 1995; Medema et al., 1995 and Vidal and Koff, 2000).

5.1.1.3.2 CIP/KIP FAMILY

In contrast to the INK4s, the Cip/Kip family members are broader in their preference of CDKs in that they can bind to cyclin D, E, A and B containing CDK complexes. They have been shown to preferentially inhibit CDK2-containing complexes, presumably in a stoichiometric fashion, both in vivo and in vitro (Soos et al., 1996; Blain et al., 1997; Cheng et al., 1999 and Sherr and Roberts, 1999). The family members, p21\textsuperscript{CIP1}, p27\textsuperscript{KIP1} and p57\textsuperscript{KIP2} all share a common N-terminal CDK inhibitory domain that is capable of interacting with both cyclin and CDKs (Figure 28) (Russo et al., 1996a; Pavletich, 1999). Besides the CDK binding domain, p21\textsuperscript{CIP1} also contains a PCNA binding domain in its C-terminus. The C-terminus of p21\textsuperscript{CIP1} was found to bind to PCNA and inhibit its ability to promote DNA synthesis but not DNA repair activity (Waga et al., 1994 and Flores-Rozas et al., 1994). p27\textsuperscript{KIP1} and p57\textsuperscript{KIP2} share a common QT (glutamate-threonine) domain, which might have a role in nuclear localization (Koff et al., 1993, Polyak et al., 1994a; Polyak et al., 1994b; Lee et al., 1995; Matsuoka et al., 1995 and Bhuiyan et al., 1999). In addition, p57 contains a unique PAPA (proline-alanine) domain and an acidic domain in the middle of the molecule (Lee et al., 1995 and Matsuoka et al., 1993).
The Cip/Kip proteins, $p21^{\text{CIP1}}$ and $p27^{\text{KIP1}}$, are stoichiometric inhibitors of cyclin/CDK2 complexes (Figure 29). At low concentrations, $p21^{\text{CIP1}}$ binds to cyclin/CDK2 complexes but does not inhibit CDK2-dependent kinase activity. However, increase in the amount of $p21^{\text{CIP1}}$ in this complex causes inhibition of the CDK2 kinase activity (Zhang et al., 1994). Both $p21^{\text{CIP1}}$ and $p27^{\text{KIP1}}$ are thought to be required for the formation of cyclin D-CDK4/6 complexes. Mouse embryonic fibroblasts (MEFs) that lack both $p27^{\text{KIP1}}$ and $p21^{\text{CIP1}}$ fail to assemble
detectable amounts of cyclin D-CDK4/6 complexes. Besides, these MEFs express cyclin D at much reduced levels compared to WT MEFs and are unable to direct cyclin D to the nucleus. Restoration of the CDK1 function in these MEFs via ectopic expression was able to correct all these deficiencies (Cheng et al., 1999).

Work from several laboratories suggests that besides phosphorylating Rb, the other main role of Cyclin D-CDK4/6 complexes during G1 is to sequester p27\(^{KIP1}\) so that active cyclin E-CDK2 complexes could facilitate S phase progression (Figure 30). For example, the addition of Cyclin-CDK4 to extracts from quiescent mink lung epithelial Mv1Lu cells reverses the ability of p27\(^{KIP1}\) to inhibit CDK2 activity (Polyak et al., 1994). Further, when Mv1Lu cells enter G1, p27\(^{KIP1}\) exists in a complex with cyclin D-CDK4 and following treatment with TGF-\(\beta\), p15\(^{INK4B}\) rapidly associates with cyclin D-CDK4 complexes, “freeing” p27\(^{KIP1}\) to interact with and inhibit CDK2 complexes (Reynisdottir et al., 1995).
Figure 29. G1-S restriction point control by CDKIs.
D = cyclin D; E = cyclin E; K4 = CDK4 and 2 = CDK2)
(Sherr, 1996)

Figure 30. Interactions of Cip/Kip family with G1 cyclin-CDKs during the regulation of G1-S transition.
(Sherr and Roberts, 1999)
Cells from CDK4\(^{-/-}\) mice exhibit a delay in reentry into S phase when released from quiescence. However, cells from CDK4\(^{-/-}\) p27\(^{KIP1-/-}\) mice do not exhibit such a delay, suggesting that p27\(^{KIP1}\) acts downstream of CDK4 and that one of the major roles of cyclinD/CDK4 is to inactivate p27\(^{KIP1}\), as mentioned previously (Tsutsui et al., 1999). Further, as mentioned earlier, cyclin D1\(^{-/-}\) mice display several developmental abnormalities including hypoplastic retinas and have mammary glands that fail to undergo normal lobulo alveolar development during pregnancy (Sicinski et al., 1995 and Fantl et al., 1995). However mice in which both cyclin D1 and p27\(^{KIP1}\) are deleted undergo normal development (Geng et al., 2000). It should be noted that both p27\(^{KIP1-/-}\) and cyclin D1\(^{-/-}\) p27\(^{KIP1-/-}\) mice exhibit elevated levels of CDK2 kinase activity (Nakayama et al., 1996; Fero et al., 1996 and Geng et al., 2000). Sugimoto et al. recently reported the presence of a residual cyclin D-CDK4 activity in MEFs lacking both p21\(^{CIP1}\) and p27\(^{KIP1}\), suggesting that there might be additional mechanisms that regulate the assembly of cyclinD-CDK4 complexes (Sugimoto et al., 2002). The increase in the amount of p27\(^{KIP1}\) associated with cyclin E-CDK2 is associated with growth arrest in several cell lines (Koff and Polyak, 1995).

The regulation of p27\(^{KIP1}\) is mainly at the translational and post-translational levels and very rarely at the transcriptional level as p21\(^{CIP1}\) (Roberts et al., 1994; Pagano et al., 1995; Agrawal et al., 1996 and Hengst and Reed, 1996). Phosphorylation of p27\(^{KIP1}\) on threonine 187 (T187) by cyclin E-CDK2 complexes leads to its recognition and ubiquitination by the ubiquitin ligase SKP2 and to its degradation by the 26S proteosome (Pagano et al.,

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1995; Scheffner et al., 1999 and Shwab and Tyers, 2001). This ubiquitination and degradation of p27<sub>KIP1</sub> upon phosphorylation was promoted by Myc-enhanced expression of Cul1 gene, which encodes a critical component of the ubiquitin ligase, SKP2 and independently by a protein called Jab1 (O’Hagan et al., 2000; Tomoda et al., 1999 and Scheffner, 1999). Equilibrium between loosely and tightly bound states of cyclin E-CDK2 complexes with p27<sub>KIP1</sub> might depend on the ambient ATP concentration, thus determining whether p27<sub>KIP1</sub> is an inhibitor of cyclin E-CDK2 or its substrate. This is also dependent on p27<sub>KIP1</sub>-cyclin E-CDK2 stoichiometry, which in turn depends on mitogenic signals (Sheaff et al., 1997). Vlach et al. demonstrated that p27<sub>KIP1</sub> must exist in a cyclin E-bound non-inhibitory confirmation in order to become a substrate for CDK2 (Vlach et al., 1997).

In pre-B cell line, Ba/F3, IL-3-mediated activation of PI-3 kinase has been shown to inhibit the Forkhead transcription factor FKHR-L1. FKHR-L1 has been shown to elevate p27Kip1 mRNA expression via enhancing p27<sub>KIP1</sub> promoter activity in response to IL-1 (Dijkers et al., 2000). Levels of p27<sub>KIP1</sub> mRNA were elevated in response to IL-6 in IL-6-responsive melanoma cells mediated by the transcription factor, signal transducers and activators of transcription 3 (STAT3) (Kortylewski et al., 1999).

5.1.1.4 MECHANISM OF CDK INHIBITION BY CDKIs

Monomeric CDK consists of a β-sheet rich N-terminal lobe (N-lobe), a large α-helix rich C-terminal lobe (C-lobe) and a deep cleft at the junction of
these two lobes which is the site of ATP binding and catalysis. In addition, CDK has a unique α-helix with a unique PSTAIRE (one-letter amino acid code) sequence and a characteristic T-loop that contains several activating phosphorylation sites (Figure 31) (De Bond et al., 1993 and Pavletich, 1999). Cyclin binds to one side of the catalytic cleft, interacts with both lobes forming a continuous protein-protein interface (Figure 32) (Jeffrey et al., 1995). Cyclin also makes a key contact with the PSTAIRE sequence, besides making contacts with the T-loop. Cyclin moves the PSTAIRE helix into the catalytic cleft and rotates it by $90^\circ$. It also changes the T-loop structure and position. Cyclin binding brings a catalytic glutamate side chain that is part of the PSTAIRE helix of the CDK, into the catalytic cleft and positions it such that this glutamate along with a lysine, aspartate and a magnesium ion could co-ordinate and position ATP atoms for catalysis. Cyclin binding also moves the T-loop away from the catalytic cleft entrance, opening up the catalytic cleft entrance and allowing the activation of the CDK by CAKs by phosphorylating residues on the T-loop (Figure 32) (Jeffrey et al., 1995 and Pavletich, 1999). Phosphorylated T-loop undergoes further conformational change, completing the reorganization of the substrate-binding site started by the cyclins (Russo et al., 1996b).

Mechanism of inhibition of CDKs by the INK4 family was revealed following the resolution of the structures of CDK6-p16$^{\text{INK4A}}$ and CDK4-p19$^{\text{INK4D}}$ (Russo et al., 1998 and Brotherton et al., 1998). INK4s bind next to the catalytic cleft, opposite to but not overlapping with cyclin binding sites, contacting both N and C lobes and forming a continuous interface (Figures 31 and 32). The
residues in the 2\textsuperscript{nd} and 3\textsuperscript{rd} ankyrin repeats participate in this interaction. The interaction with INK4 by the N and C lobes results in a 15\degree rotation of the two lobes around an axis perpendicular to that of the catalytic cleft. This leads to the misalignment of the N and C lobes and the PSTAIRE helix with respect to cyclin binding (Figure 26) (Russo et al., 1998; Brotherton et al., 1998; Jeffrey et al., 2000 and Pavletich, 2000).

Russo et al. showed that p27\textsuperscript{KIP1} is capable of binding to both cyclin A and CDK2 in a phosphorylated cyclin A-CDK2 complex (Figure 31) (Russo et al., 1996a). p27 inserts a 3\textsubscript{10}-helix into the catalytic cleft of the CDK and thereby inhibiting the kinase. The p27 helix mimics the ATP substrate, both in position and in the residues it contacts inside the catalytic cleft (Russo et al., 1996b and Pavletich, 1999). Another mechanism of inhibition of p27 is by changing the shape of the N lobe of catalytic cleft by “flattening the \(\beta\)-sheet” whereby the catalytic cleft loses many of its ATP binding residues (Russo et al., 1996b and Pavletich, 1999).
Figure 31. Inhibition of cyclin-CDK complexes by CDKIs. (Pavletich, 1999)

Figure 32. Schematic illustration of conformational changes in CDK upon INK4-binding and cyclin binding. (Jeffrey et al., 2000)
5.1.1.5 EXPRESSION PATTERNS OF CDKIs DURING DEVELOPMENT

5.1.1.5.1 INK4 EXPRESSION DURING DIFFERENTIATION AND DEVELOPMENT

INK4 proteins are differentially expressed during development. Both \(p18^{\text{INK4C}}\) and \(p19^{\text{INK4D}}\) are expressed during embryonic development while \(p16^{\text{INK4A}}\) expression is restricted to the lung and spleen of older mice with increased mRNA expression detected during aging (Zindy et al., 1997). Expression of \(p16^{\text{INK4A}}\) and \(p15^{\text{INK4B}}\) were induced when mouse embryos were cultured as MEFs. However, the levels of \(p16^{\text{INK4A}}\) and \(p18^{\text{INK4C}}\) expression increased as the cells approached senescence. Thus, \(p18^{\text{INK4C}}\) and \(p19^{\text{INK4D}}\) might regulate pre- and post-natal development while \(p16\) might play a role mainly during cellular senescence (Zindy et al., 1997). Also, \(p15^{\text{INK4B}}\) levels are upregulated during TGF-\(\beta\) mediated growth arrest (Hannon and Beach, 1994; Robson et al., 1999; Vidal and Koff, 2000 and Seoane et al., 2001). The \(p16^{\text{INK4A}}\) gene was independently isolated as a candidate tumor suppressor gene located at chromosomal position 9p21, which is deleted in many human tumors and is linked to hereditary predisposition to melanoma (Kamb et al., 1994; Noburi et al., 1994 and Serrano, 1997).

Mice expressing an unstable truncated version of \(p16^{\text{INK4A}}\) develop normally. A small percentage of animals that develop B-cell lymphomas after a long latency (Krimpenfort et al., 2001 and Ortega et al., 2002). Mice that are homozygous for a mutation that deletes exon 1\(\alpha\) of \(p16^{\text{INK4A}}\) develop tumors at a higher rate (25%) by one year of age (Sharpless et al., 2001 and Ortega et
null mice develop normally and develop tumors at very low rate (about 10%) after a long latency period (Latres et al., 2000 and Ortega et al., 2002). $p^{15iNK4B}$ mice develop normally, but develop pituitary tumors with high penetrance and exhibit gigantism and widespread organomegaly (Franklin et al., 1998). These phenotypes of $p^{18iNK4C/-}$ is very similar to those exhibited by $p^{27KIP1/-}$ mice (Fero et al., 1996; Nakayama et al., 1996 and Kiyokawa et al., 1996). Finally, the loss of $p^{19iNK4D}$ does not cause any obvious phenotypes in mice, except testicular atrophy. However, these mice appear to be fertile. Testicular atrophy is associated with increased apoptosis of the germ cells, which correlates with the high expression levels of $p^{19iNK4D}$ in these cells (Zindy et al., 2000).

Mice lacking both $p^{18iNK4C}$ and $p^{19iNK4D}$ exhibit male infertility owing to a severe defect in the production of mature sperm cells due to a delayed exit of spermatogonia from the cell cycle (Zindy et al., 2001). However ablation of both $p^{15iNK4B}$ and $p^{18iNK4C}$ does not result in an overtly severe phenotype than seen in $p^{18iNK4C/-}$ mice (Latres et al., 2000). Except for the fact that the expression of $p^{15iNK4B}$ is elevated in response to TGF-β, the manner in which the abundance of the INK4 family is regulated is not well understood (Seoane et al., 2001).
5.1.1.5.2 CIP/KIP EXPRESSION DURING DEVELOPMENT

5.1.1.5.2.1 Roles of p21\textsuperscript{CIP1} during differentiation and development

p21\textsuperscript{CIP1} was identified by independent groups using multiple approaches: as a CDK-interacting protein in a yeast two-hybrid system, as a growth inhibitor from p53-activated cells or biochemically purified as a cyclin-CDK2 binding protein or as a growth inhibitor from senescent cells or (Harper et al., 1993, el-Diery et al., 1993; Xiong et al., 1993; Gu et al., 1993 and Noda et al., 1994). p21\textsuperscript{CIP1} has been shown to be transcriptionally induced by p53 and is the primary mediator of p53-mediated cell cycle arrest following DNA damage (el-Diery et al., 1993; Dulic et al., 1994 and Sheaff et al., 1997).

MyoD was shown to induce p21\textsuperscript{CIP1} expression in terminally differentiated muscle cells and forced expression of p21\textsuperscript{CIP1} causes differentiation of myoblasts. This activity of p21\textsuperscript{CIP1} was not dependent on p53-mediated activation of p21\textsuperscript{CIP1} (Halevy et al., 1995 and Skapek et al., 1995). However, mice homozygous for a targeted deletion in p21\textsuperscript{CIP1} undergo normal development, including muscle development (Deng et al., 1995). MEFs from p21\textsuperscript{CIP1/-} mice fail to undergo G1 checkpoint arrest in response to DNA damage (Deng et al., 1995 and Brugarolas et al., 1995). In addition, p21\textsuperscript{CIP1} is capable of binding to PCNA, a subunit of DNA polymerase \(\delta\) and inhibiting its effect on initiating DNA replication but not on DNA damage repair (Zhang et al., 1993 and Luo et al., 1995). This is consistent with the observation that p21 is expressed in cells undergoing proliferation (Vidal and Koff, 2000 and Engel et al., 2003).
Keratinocytes from p21\textsuperscript{CIP1-/-} mice exhibit altered and prolonged S phase and have decreased expression of a subset of differentiation markers (Missero et al., 1996). Further, transcriptional activation of p21\textsuperscript{CIP1} by Vitamin D\textsubscript{3} has been shown to induce the differentiation of the myelomonocytic cell line, U937 and p21\textsuperscript{CIP1} was later shown to be a target of HOXA10 in differentiating myelomonocytes (Liu et al., 1996 and Bromleigh and Freedman, 2000). Brugarolas et al. demonstrated that p21\textsuperscript{CIP1} is required for proliferation control in MEFs from Rb\textsuperscript{-/-} mice since these MEFs can undergo anchorage-independent growth in the absence of p21\textsuperscript{CIP1}. In addition, Rb\textsuperscript{-/-}p21\textsuperscript{CIP1-/-} mice have a higher incidence of tumors than Rb\textsuperscript{-/-} mice (Brugaralos et al., 1998). Kramer et al. reported that iron causes an increase of p21\textsuperscript{CIP1} during the macrophage/monocyte differentiation of HL60 leukemia cell lines (Kramer et al., 2001). Cell cycle exit by differentiating megakaryocytes correlated with the p53-independent expression of p21\textsuperscript{CIP1} (Taniguchi et al., 1999 and Baccini et al., 2001).

High amounts of activated Raf causes a G1 cell cycle arrest through the upregulation of p21\textsuperscript{CIP1} while moderate levels of Raf induces cell cycle progression by elevating cyclin D expression (Sewing et al., 1997). Similarly, when signaling through Ras-related Rho GTPase is inhibited, constitutively active Ras induces the expression of p21\textsuperscript{CIP1} and facilitates a G1 arrest. Conversely, when Rho is active, it suppresses p21\textsuperscript{CIP1} mRNA expression and facilitates the induction of S phase (Olsen et al., 1998). Further, the proto-oncogene c-Myc has been shown to induce transcriptional repression of p21\textsuperscript{CIP1}
in growth arrested human keratinocytes as well as in colon cancer cell lines (Claassen and Hann, 2000 and Seoane et al., 2002). Finally, Cheng et al. demonstrated that the quiescent status of hematopoietic stem cells is maintained by \( \text{p21}^{\text{CIP1}} \) and that in its absence, increased cell cycling by the stem cells leads to their exhaustion (Cheng et al., 2000). Thus, the regulation of \( \text{p21}^{\text{CIP1}} \) during cell cycle is thought to be mainly at the transcriptional level.

**5.1.1.5.2.2 Role of \( \text{p57}^{\text{KIP2}} \) in Differentiation and Development**

Two independent groups cloned \( \text{p57}^{\text{KIP2}} \) simultaneously, looking for proteins with homology to \( \text{p21}^{\text{CIP1}} \) and \( \text{p27}^{\text{KIP1}} \) (Lee et al., 1995 and Matsuoka et al., 1995). The \( \text{p57}^{\text{KIP2}} \) locus is paternally imprinted, completely in mice and partially in humans, and only the maternal allele is expressed. Missense or frame shift mutations in \( \text{p57}^{\text{KIP2}} \) have been associated with Beckwith-Wiedemann syndrome, a genetic defect characterized by prenatal organ overgrowth and predisposition to embryonic tumors, including Wilms' tumor and rhabdomyosarcoma (Yan et al., 1997 and Zhang et al., 1997). Homozygous mice with a targeted deletion for \( \text{p57}^{\text{KIP2}} \) exhibit neo-natal lethality and major developmental defects including cleft palate, gastrointestinal abnormalities ranging from inflated GI tract to the loss of jejunum and ileum due to increased apoptosis of cells in these tissues. These mice have short limbs due to abnormal endochondral ossification caused by delayed cell cycle exit during chondrocyte differentiation (Yan et al., 1997 and Zhang et al., 1997). In addition, the \( \text{p57}^{\text{KIP2}} \) knock out mice exhibit abdominal muscle defects, renal
medullary dysplasia, adrenal cortical hyperplasia and cytomegaly and lens cell hyperproliferation and apoptosis. Thus p57 is definitely required for embryonic organ development in mice.

Rat calvarial osteoblasts show elevated expression of p57\textsuperscript{KIP2} and p57\textsuperscript{+/−} mice have altered proliferation and differentiation in several mesenchymal cell types including muscle and cartilage (Zhang et al., 1997; Urano et al., 1999 and Nishimori et al., 2001). Although, p27\textsuperscript{KIP1} and P57\textsuperscript{KIP2} are co-expressed in many organs during murine development, they have been shown to regulate the proliferation and differentiation of different cell types within an organ (Table 25). Dyer and Cepko have reported that in the embryonic retina, where both p27\textsuperscript{KIP1} and p57\textsuperscript{KIP2} are expressed, each regulates the proliferation of distinct retinal progenitor cell populations (Dyer and Cepko, 2001). Similarly, in the adrenal gland, p27\textsuperscript{KIP1} is expressed only in the adrenal medulla and p57\textsuperscript{KIP2} is expressed only in the adrenal cortex and their expressions are mutually exclusive (Nakayama and Nakayama, 1998). The expression of p57\textsuperscript{KIP2} has been shown to increase during C2C12 myoblast differentiation (Reynaud et al., 1999). MyoD is a basic-HLH transcription factor that is expressed in proliferating myoblasts prior to terminal differentiation and is a key mediator of myoblast differentiation. In proliferating myoblasts, MyoD is phosphorylated by CDK2, which leads to its degradation. Reynaud et al. demonstrated that the forced expression of p57\textsuperscript{KIP2} in C2C12 myoblasts leads to the hypophosphorylation and consequent stabilization of MyoD. The N-terminal CDK-inhibitory domain in p57\textsuperscript{KIP2} was sufficient for the stabilization of MyoD, by
directly binding to MyoD and thereby preventing cyclin E-CDK2 complex from phosphorylating it (Reynaud et al., 1999 and Reynaud et al., 2000). Further, in transient transfections, transactivation of the MyoD-responsive mouse muscle creatinase promoter by MyoD was enhanced by p57^Kip2, p27^Kip1 and p21^Cip1 but not by p16^INK4A (Reynaud et al., 1999 and Reynaud et al., 2000). p57^Kip2 has been shown to be degraded through the ubiquitin proteosome pathway in osteoblastic cells stimulated to proliferation by TGF-β and this proteolysis is mediated through Smad-mediated transcription (Urano et al., 1999 and Nishimori et al., 2001).

<table>
<thead>
<tr>
<th>Tissue</th>
<th>p21^Cip1</th>
<th>p27^Kip1</th>
<th>p57^Kip2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brain</td>
<td>−^a</td>
<td>+ (postmitotic neuron)</td>
<td>+ (postmitotic neuron)</td>
</tr>
<tr>
<td>Eye</td>
<td>−^?</td>
<td>+ (retina, lens)</td>
<td>+ (lens)</td>
</tr>
<tr>
<td>Thymus</td>
<td>−^?</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>Heart</td>
<td>−^a</td>
<td>+ (muscle, endothelium)</td>
<td>+ (endothelium)</td>
</tr>
<tr>
<td>Lung</td>
<td>−^a</td>
<td>+ (bronchus)</td>
<td>+ (bronchus)</td>
</tr>
<tr>
<td>Intestine</td>
<td>−^a</td>
<td>+ (villus)</td>
<td>+ (villus)</td>
</tr>
<tr>
<td>Liver</td>
<td>−^a</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Kidney</td>
<td>−^a</td>
<td>+ (podocyte, mesenchyma)</td>
<td>+ (podocyte, mesenchyma)</td>
</tr>
<tr>
<td>Adrenal gland</td>
<td>−^?</td>
<td>+ (medulla)</td>
<td>+ (cortex)</td>
</tr>
<tr>
<td>Muscle</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Cartilage</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Skin</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

* Tissues in which expression was observed in adult.

**Table 24. Expression of Cip/Kip members during murine development.** (Nakayama and Nakayama, 1998)
5.1.1.5.2.3 ROLE OF p27\textsuperscript{KIP1} IN G1 ARREST, DIFFERENTIATION AND DEVELOPMENT

In the early 1990s, Koff et al. observed that TGF\(\beta\) and cell-cell contacts growth arrested the cells by preventing the activation of cyclin E-CDK2 complexes (Koff et al., 1993). This CDK2 inhibitory factor was later identified as p27\textsuperscript{KIP1}; cloned by two independent groups and was shown to be able to bind to cyclin D-CDK4, cyclin E-CDK2 and cyclin A-CDK2 complexes and inhibit their activity (Polyak et al., 1994a; Polyak et al., 1994b and Toyoshima and Hunter, 1994). These authors demonstrated that p27\textsuperscript{KIP1} is primarily responsible for the regulation of CDK activity in response to extracellular stimuli and that the forced expression of p27\textsuperscript{KIP1} in cells leads to cell cycle withdrawal (Polyak et al., 1994b and Toyoshima and Hunter, 1994). Coats et al. demonstrated that inhibition of p27\textsuperscript{KIP1} using antisense oligonucleotides lead to the failure of serum-starved immortalized fibroblasts to undergo cell cycle arrest (Coats et al., 1996).

Three independent groups reported that p27\textsuperscript{KIP1/-} mice exhibit enhanced growth with increased size in the tissues that express p27\textsuperscript{KIP1} at the highest levels, viz., thymus, pituitary, adrenal glands and gonadal organs, owing to an increase in cell numbers due to increased proliferation (Nakayama et al., 1996; Kiyokawa et al., 1996 and Fero et al., 1996). In addition, these mice also exhibit disorganization of the normal cellular monolayer pattern in the retinal epithelium and female infertility due to the impairment in the ovarian luteal cell differentiation (Nakayama et al., 1996). Besides, these mice develop nodular hyperplasia in the intermediate lobe of the pituitary and increased thymic
hyperplasia associated with enhanced CDK2 kinase activity in the p27 null thymocytes and increased proliferation by the T-lymphocytes. The p27<sup>−/−</sup> mice develop spontaneous pituitary tumors, similar to Rb<sup>−/−</sup> mice (Nakayama et al., 1996; Kiyokawa et al., 1996 and Fero et al., 1996). Cells from p27<sup>−/−</sup> mice remained responsive to hormonal stimuli, suggesting that the proliferation and subsequent developmental defects in p27 knock out mice are cell autonomous (Nakayama et al., 1996; Kiyokawa et al., 1996 and Fero et al., 1996).

p27<sup>KIP1</sup> has been shown to be involved in differentiation-related cell cycle arrest in several different cell types (Sgambato et al., 2000). In HL-60 leukemia cells undergoing monocytic differentiation in response to Vitamin D<sub>3</sub>, a transient increase in p21<sup>CIP1</sup> during the initial 24-48 hours is followed by a more sustained elevation in p27<sup>KIP1</sup> levels and this mediates a definitive cell cycle arrest during monocyte differentiation (Wang et al., 1996; Hengst and Reed, 1996; Wand et al., 1997 and Koffman and Studzinski, 1999). Also, p27<sup>KIP1</sup> expression is enhanced during the vitamin D<sub>3</sub>-induced differentiation of neuroblastoma cells (Kranenburg et al., 1995 and Sgambato et al., 2000). In the absence of p27<sup>KIP1</sup>, the luteal cells of the ovary undergo differentiation without withdrawing from cell cycle, thus uncoupling differentiation and growth arrest (Tong et al., 1998). This leads to female infertility in these mice due to an inability both to release eggs during estrous and also to support the implantation of the embryos (Fero et al., 1996; Kiyokawa et al., 1996 and Nakayama et al., 1996). The levels of p27<sup>KIP1</sup> have been reported to increase and lead to cell cycle arrest during the differentiation of central glial-4 cells into astrocytes (Harvat et al., 1998).
U937 myelomonocytic cell line, ectopic expression of p27\textsuperscript{KIP1} leads to both cell cycle arrest and induction of macrophage-specific markers (Liu et al., 1996). Similarly, ectopic expression of p27\textsuperscript{KIP1} enhances the sensitivity to differentiation by HT29 colon cancer cell line and enhances the differentiation of HL-60 cell lines (Yamamoto et al., 1999 and Zhou et al., 1999). In keratinocytes, differentiation and expression of keratin 1 can be blocked by the ectopic expression of antisense oligonucleotides to p27\textsuperscript{KIP1} (Hauser et al., 1997 and Sgambato et al., 2000).

Accumulation of p27\textsuperscript{KIP1} correlates with cell cycle withdrawal and differentiation of oligodendrocyte progenitors (O-2A) (Casaccia-Bonnefil et al., 1997). Further, only a fraction of O2-A cells from p27\textsuperscript{KIP1}\textsuperscript{-/-} mice undergo successful differentiation compared to WT controls (Casaccia-Bonnefil et al., 1997). These data suggest that p27\textsuperscript{KIP1} plays an important role in the decision to withdraw from cell cycle prior to differentiation by oligodendrocyte progenitors. Further, ectopic expression of p27\textsuperscript{KIP1} in oligodendrocyte progenitor cells results in cell cycle arrest, even in the presence of strong mitogens. However these cells did not display the conventional oligodendrocyte differentiation markers (Tikoo et al., 1998). Further, Ghiani et al. demonstrated that β-adrenergic and glutamate receptor activation inhibits oligodendrocyte proliferation through the elevation in p21\textsuperscript{CIP1} and p27\textsuperscript{KIP1}. They also observed that while p27\textsuperscript{KIP1} levels alone cause cell cycle arrest in these progenitors, this by itself is not sufficient to induce oligodendrocyte differentiation (Ghiani et al., 1999). A counting or an intrinsic timer mechanism has been proposed to exist in oligodendrocyte
progenitors to coordinate the number of cell divisions with differentiation (Temple and Raff, 1986). Studies on embryonic gliogenesis in $p27^{KIP1-/-}$ mice by Casaccia-Bonnefil identified the presence of an increased number of embryonic glial and neonatal oligodendrocyte progenitors, which differentiate into oligodendrocytes without any delay when compared with WT controls (Casaccia-Bonnefil et al., 1999). These data suggested the existence of two different mechanisms of growth arrest: a $p27^{KIP1}$-mediated mechanism of growth arrest during the early stages of gliogenesis and a $p27$-independent mechanism of withdrawal from cell cycle and differentiation (Casaccia-Bonnefil et al., 1999). Finally, Zezula et al. reported that while both $p27^{KIP1}$ and $p21^{CIP1}$ are both required for oligodendrocyte differentiation, only $p27^{KIP1}$ is necessary for withdrawal from cell cycle prior to differentiation. $p21^{CIP1}$ was required for the establishment of the differentiation program following growth arrest (Zezula et al., 2001). During early animal life, loss of $p21^{CIP1}$ leads to hypomyelination in the cerebellum, suggesting that the loss of $p21^{CIP1}$ delayed myelination in the cerebellum (Zezula et al., 2001). Thus, in this model, $p21^{CIP1}$ and $p27^{KIP1}$ appear to play non-redundant roles during oligodendrocyte differentiation.

In rat calvarial osteoblasts, $p27^{KIP1}$ is upregulated in the post-proliferative population while $p21^{CIP1}$ is present in mainly proliferating progenitors (Drissi et al., 1999). Osteoblasts from $p27^{KIP1-/-}$ mice proliferate faster but undergo differentiation, presumably due to the increased $p21^{CIP1}$ levels. In addition, the bone marrow of $p27^{KIP1-/-}$ mice exhibit hypercellularity compared to WT mice (Drissi et al., 1999). $p27^{KIP1}$ has been shown to be an essential component of
cell cycle withdrawal during retinal histogenesis and the developmental expression pattern of p27\textsuperscript{KIP1} has been shown to correspond with the generation of several classes of retinal cell populations (Levine et al., 2000). Similarly, elevated p27\textsuperscript{KIP1} levels have been implicated in cell cycle withdrawal and differentiation of several cell types including primary erythroblasts, melanoma cell lines, thymus and hepatoma cells and retinoblasts (Hsieh et al., 2000; Henriet et al., 2000; Kolluri et al., 1999; Levine et al., 2000 and Ohnuma et al., 1999). p27\textsuperscript{KIP1} is needed for mammary gland development in a dose-dependent manner and it has been shown to regulate cyclin D-CDK4 activity during mammary gland development (Muraoka et al., 2001). In summary, p27\textsuperscript{KIP1} is associated with differentiation-induced cell cycle and differentiation of several cell types.

Recently, the Xenopus p27\textsuperscript{Xic1} was shown to function beyond cell cycle withdrawal to promote muscle differentiation and neuronal differentiation in Xenopus laevis (Vernon et al., 2003 and Vernon and Philpott, 2003). In two elegant experiments, these authors showed that p27\textsuperscript{Xic1} is expressed in developing myotome and that the ablation in p27\textsuperscript{Xic1} prevents muscle differentiation in Xenopus. Further, they also demonstrated that p27\textsuperscript{Xic1} synergizes with MyoD to promote muscle differentiation in Xenopus. A region in the N-terminal domain of p27\textsuperscript{Xic1} is sufficient for the promotion of myogenesis in Xenopus and this activity of p27\textsuperscript{Xic1} is separable from its ability to induce cell cycle withdrawal in this system (Vernon and Philpott, 2003). Similarly, Vernon et al. showed that p27\textsuperscript{Xic1} is highly expressed in neuronal progenitors and is
necessary for early neurogenesis in *Xenopus*. Ablation of p27\textsuperscript{Xic1} prevented the formation of primary neurons while its overexpression promoted the formation of primary neurons. These authors also demonstrated p27\textsuperscript{Xic1} might promote neurogenesis by stabilizing the bHLH protein, neurogenin via its N-terminal domain and that the neurogenesis function of p27\textsuperscript{Xic1} is separable from its cell cycle withdrawal function (Vernon et al., 2003).

**5.1.1.5.2.4 Functional collaborations between Cip/Kip family members during development**

The absence of any severe developmental phenotypes in most of the CDKI knock out mice except in case of p57\textsuperscript{-/-} mice, suggests that either the CDKIs have redundant roles during differentiation and development or that some other compensatory mechanism exists that compensates for the loss of individual or even multiple CDKIs. As mentioned earlier, the Cip/Kip inhibitors have been shown to collaborate in the differentiation of a number of cell types (Nakayama and Nakayama, 1998 and Vidal and Koff, 2000). Results from mice in which both p21\textsuperscript{CIP1} and p57\textsuperscript{KIP2} have been deleted show that these proteins play redundant roles in regulating the differentiation of skeletal muscle and alveoli in the lungs (Table 26) (Zhang et al., 1999). Mice lacking both p21\textsuperscript{CIP1} and p57\textsuperscript{KIP2} fail to form myotubes, display increased proliferation and apoptotic rates of myoblasts and display endoreplication in residual myotubes (Zhang et al., 1999). Expression patterns of myogenin, a transcription factor involved in myogenesis, p21\textsuperscript{CIP1} and p57\textsuperscript{KIP2} are parallel but independent during
myogenesis, suggesting that these proteins co-ordinate to induce cell cycle withdrawal and to induce a muscle differentiation-specific program of gene expression to initiate the terminal differentiation of myoblasts (Zhang et al., 1999). Similarly, studies conducted with mice lacking both p27\(^{\text{KIP1}}\) and p57\(^{\text{KIP2}}\) show that these CDKIs play redundant roles to regulate the cell cycle exit and differentiation of lens epithelial cells and placental trophoblasts (Table 26) (Zhang et al., 1998). An example for a compensatory mechanism operating in the absence of more than one CDKI could be drawn from the work of Coats et al., where p130, a member of the Rb-family, was shown to inhibit cyclin E-CDK2 in MEFs lacking both p21\(^{\text{CIP1}}\) and p27\(^{\text{KIP1}}\) (Coats et al., 1999).
<table>
<thead>
<tr>
<th>Mouse strain</th>
<th>Phenotypic consequences</th>
</tr>
</thead>
<tbody>
<tr>
<td>p21 −/−</td>
<td>No evident phenotype. MEFs show defective G1 checkpoint</td>
</tr>
<tr>
<td>p57 −/−</td>
<td>Some embryonic and neonatal lethality. Altered cell proliferation and apoptosis in several tissues.</td>
</tr>
<tr>
<td>p16 −/−</td>
<td>Normal development. General tumor predisposition.</td>
</tr>
<tr>
<td>p15 −/−</td>
<td>Viable. No tumor predisposition</td>
</tr>
<tr>
<td>p18 −/−</td>
<td>Gigantism and organomegaly. Pituitary hyperplasia.</td>
</tr>
<tr>
<td>p19 −/−</td>
<td>Testicular atrophy.</td>
</tr>
<tr>
<td>p15 −/− p18 −/−</td>
<td>Infertile animals.</td>
</tr>
<tr>
<td>p18 −/− p27 −/−</td>
<td>Increased organomegaly. Earlier onset on pituitary adenomas.</td>
</tr>
<tr>
<td>p19 −/− p27 −/−</td>
<td>Postnatal lethality at day 18. Ectopic neuronal divisions and apoptosis leading to neurological defects.</td>
</tr>
</tbody>
</table>

**Table 25. Phenotypes of mouse strains lacking one or more of the CDKIs.**

(Vidal and Koff, 2000)
Phenotypic data from knock out mouse models have led Vidal and Koff to propose three models of redundant relationships between cell-cycle inhibitors (Figure 33).

- **Pure Redundancy:** Here hypothetical CDKIs, A and B perform overlapping roles in controlling growth arrest and the absence of either A or B alone has no phenotypic consequence as the other CDKI would be able to achieve cell cycle arrest by itself.

- **Compensatory Redundancy:** In this scenario, CDKIs A and B perform different roles normally. However, in the absence of A, B might be able to compensate for the loss of A.

- **Phenotypic Redundancy:** Here, CDKIs A and B control parallel but different pathways in the regulation of cell cycle withdrawal. Thus in the absence of A, growth arrest might still be achieved via B which would still control the processes that it regulates (Vidal and Koff, 2000).
Figure 33. Models to explain redundancy in the action of CDKIs during the regulation of cell cycle progression.
(Vidal and Koff, 2000)
5.1.1.6 CONCLUSION

A plethora of studies performed by earlier workers, as detailed in previous sections, suggest that osteoclast precursors proliferate during the initial stages of development through the earliest lineages and become post-mitotic at the time of expression of TRAP and fusion into multinuclear osteoclasts. However, it remains unclear whether RANKL stimulates the osteoclast precursors to withdraw from cell cycle and if so, which downstream pathways and molecules are involved in this process. Withdrawal from cell cycle prior to differentiation of osteoclast progenitors must occur before the G1-S restriction point.

Cell cycle exit in G1 phase of the cell cycle is achieved through the action of the CDKIs. Consequently, the CDKIs play key roles in coordinating cell cycle withdrawal with differentiation in many different tissue and cell systems (reviewed in Zhou and Skoultchi, 2001). In addition to controlling cell proliferation, there is evidence to suggest that CDKIs may play a more direct role in cell differentiation. In particular, genetic studies in mice indicate that p21\textsuperscript{CIP1}, p57\textsuperscript{KIP2} and p27\textsuperscript{KIP1} alone or in combination play a direct role in differentiation (Zhou and Skoultchi, 2001). For example, in mice deficient for both p21\textsuperscript{CIP1} and p57\textsuperscript{KIP2}, terminal differentiation of skeletal muscle is affected in a manner similar to knockout of the myogenin gene, suggesting that these CDKIs might regulate myogenin activity during terminal muscle differentiation (Zhang et al, 1999). This effect may be indirect, through modulation of the activity of the Rb tumor suppressor, which in turn is required for myogenin
function (Zhang et al., 1999). Studies in Xenopus suggest a direct role for the 
p27\textsuperscript{KIP1} orthologue p27\textsuperscript{Xic1} in both neuronal and muscle differentiation through 
modulation of bHLH transcription factor stability (Vernon et al., 2003; Vernon 
and Philpott, 2003). Further, these studies suggest that different domains of 
p27\textsuperscript{Xic1} are required for cell cycle withdrawal and for promoting bHLH factor 
stability. These studies might be interesting in relation to osteoclasts 
differentiation and cell cycle arrest.

As evident from previous chapters, Mitf is a bHLH-zip factor with an 
important role in osteoclast differentiation and osteoclasts from Mitf\textsuperscript{mi/mi} mice are 
predominantly mononuclear. However, it is not known whether Mitf plays any 
role in cell cycle exit by osteoclast precursors prior to differentiation or whether 
any of the CDKIs regulate the activity of Mitf during differentiation. Thus, it will 
be interesting to know whether RANKL facilitates the cell cycle withdrawal by 
osteoclast progenitors via the upregulation or stabilization of any of the CDKIs 
and whether Mitf regulates this event. Consequently, the next question would 
be whether a delay or a block in differentiation-related cell cycle withdrawal in 
osteoclast precursors will inhibit differentiation. These questions are best 
addressed in an \textit{in vivo} context utilizing mouse mutants.

The hypothesis for this study is that RANKL co-ordinates cell cycle 
withdrawal with osteoclast differentiation through elevation in CDKI expression 
levels.

In this study, we examined the role of CDKIs in the regulation of 
differentiation-associated cell cycle arrest by osteoclast progenitors and the role
of RANKL in the co-ordination of this event. Our results indicate that RANKL causes an elevation of both $p27^{KIP1}$ and $p21^{CIP1}$ levels during osteoclast differentiation. Further, we also examined the role of Mitf in the regulation of cell cycle exit and possibly in the elevation of CDKI differentiating osteoclasts. Our studies indicate that $Mitf^{mi/mi}$ osteoclasts are delayed in withdrawing from the cell cycle in both co-culture with osteoblasts and when treated with recombinant RANKL and TNF$\alpha$. However, RANKL is capable of inducing the expression of $p27^{KIP1}$ in $Mitf^{mi/mi}$ osteoclasts, implying that elevation in CDKI levels by RANKL does not occur via Mitf. $p27^{KIP1}$ is required for cell cycle withdrawal prior to differentiation. Mice lacking both $p27^{KIP1}$ and $p21^{CIP1}$ proteins exhibit osteopetrosis and have fewer osteoclasts with lower TRAP activity and with abnormal cell morphology. RANKL-dependent differentiation of osteoclast progenitors $in vitro$ from $p27^{KIP1-/-}p21^{CIP1-/-}$ mice is impaired and the expression of the osteoclast differentiation markers TRAP and cathepsin K is affected. These data suggest that $p21^{CIP1}$ and $p27^{KIP1}$ might play redundant roles during osteoclast differentiation and that these cell cycle regulators may play a key role in RANKL mediated osteoclast differentiation that is independent of their role in cell cycle regulation.
5.2 RESULTS

5.2.1 RANKL CAUSES OSTEOCLAST PROGENITOR CELLS TO WITHDRAW FROM THE CELL CYCLE

To determine whether RANKL co-ordinates cell cycle withdrawal during osteoclast differentiation, we pulse labeled macrophage/monocyte precursors from 15-20 day old wild-type (WT) mice with BromodeoxyUridine (BrdU), at 0, 24, 48 and 72 hours in proliferation or in differentiation media (Fig. 34A, data not shown). A total of 4000 cells were counted at each time point. Our results indicate that the percentage of cells in the S-phase of the cell cycle, as indicated by BrdU incorporation, decreased from 40.9%±1.3% (n=3) before adding RANKL (time point 0) to 13.5%±1.2% (n=3) by 24 hours and to 6.3%±2% (n=3) by 48 hours of adding RANKL (Fig. 34C). Less than 5% of the cells were still in the cell cycle at 72 hours after adding RANKL (data not shown). In contrast, 37%±0.9% (n=3) and 35%±1.2% (n=3) of the cells were BrdU-positive at 24 and 48 hours of growth in proliferation media (Fig. 34A). This indicates that cell cycle withdrawal by osteoclast progenitors is a specific effect of RANKL treatment. In addition, when WT cells are treated with TNFα, the number of cells in S-phase decreases to 16±2% and to 12.2±2.5% by 24 and 48 hours, respectively (Fig. 34D). These results suggest that RANKL induces the actively proliferating osteoclast precursors of the macrophage/monocyte lineage to exit the cell cycle, possibly prior to the initiation of the differentiation program.
Figure 34. **RANKL stimulates osteoclast precursors to exit cell cycle.** WT osteoclast precursors grown in proliferation media or differentiation media-I were pulse-labeled with BrdU at the indicated time points. Panels Ai and Aii show representative images of the propidium iodide (PI)-positive cells and BrdU-positive cells, respectively, at indicated time points and treatments. (B) Percentage of cells positive for BrdU relative to the total number of PI-positive cells at 0, 24 and 48 hours in proliferation media. (C) Percentage of cells positive for BrdU relative to the total number of PI-positive cells at 0, 24 and 48 hours after RANKL treatment. (D) Percentage of cells positive for BrdU relative to the total number of PI-positive cells after TNFα treatment. A total of 4000 cells were counted in each treatment and the average of three experiments is shown.
5.2.2 **MITF<sup>mi/mi</sup> OSTEOCLASTS ARE DELAYED IN WITHDRAWAL FROM THE CELL CYCLE**

Cell cycle withdrawal is considered to be a prerequisite for terminal differentiation of cells. Osteoclast precursors have been shown to be post-mitotic prior to fusing to become multinuclear osteoclasts (Scheven et al., 1986 and Tanaka et al., 1993). Since **MITF<sup>mi/mi</sup>** osteoclasts remained predominantly mononuclear, we hypothesized that **MITF<sup>mi/mi</sup>** osteoclasts are delayed in differentiation-related cell cycle withdrawal and that RANKL coordinates cell cycle withdrawal via Mitf. We examined the kinetics of cell cycle withdrawal by **MITF<sup>mi/mi</sup>** osteoclasts in co-culture with osteoblasts and when grown in the presence of recombinant CSF1, RANKL and TNF<sub>α</sub>. Our results indicate that when grown in co-culture, the percentage of BrdU positive WT cells decrease from 49.34±4.52% on day 4 to 22.51±2.31% on day 5 and to 11.26±2.11% on day 6 and to 5.3±0.95% on day 7 (Figure 35A). In contrast, the percentage of BrdU positive **MITF<sup>mi/mi</sup>** osteoclasts was 67.4±4.66% on day 4, 63.6±4.4 on day 5, 33.8±3.44 on day 6 and 11.6±1.16 on day 7, respectively (Figure 35A). Thus, it is evident from these data that **MITF<sup>mi/mi</sup>** osteoclasts are delayed in cell cycle withdrawal by two days when grown in co-culture. In addition, when these cells were grown in the presence of recombinant CSF1, RANKL and TNF<sub>α</sub>, percentage of WT cells in the S-phase of cell cycle decreased from 40.9±1.3% at 0 time point (before adding RANKL and TNF<sub>α</sub>) to 11.15±2.6% at 24 hours to 14±2.8% at 48 hours. There were only 3.7±0.4% cells that were BrdU positive.
at 96 hours after adding the differentiation factors (Figure 35B). The WT cells
do not withdraw from cell cycle in a similar fashion, if maintained in CSF1 alone
(Figure 35C). In contrast, the percentage of BrdU positive cells in the Mitf\textsuperscript{mi/mi}
population was 48.15±4.5% at 0 hours, 38.28±4.6% at 24 hours, 35.03±4.3% at
48 hours and 14.4±6.2% at 72 hours and 3.85±1.2% at 96 hours of adding
RANKL and TNF\textsubscript{α} (Figure 35B). These data suggest that Mitf\textsuperscript{mi/mi} osteoclasts
are delayed by two days in withdrawal from cell cycle when grown in co-culture
or in the presence of recombinant RANKL and TNF\textsubscript{α}.
Figure 35. Mitf$^{mi/mi}$ osteoclasts are delayed in withdrawing from the cell cycle during differentiation, but Mitf does not mediate the RANKL-induced elevation in p27KIP1 levels. A: % of BrdU labeled cells in WT and Mitf$^{mi/mi}$ osteoclasts in co-culture with osteoblasts (n=2); B: % of WT and Mitf$^{mi/mi}$ osteoclasts in the S-phase of cell cycle when treated with RANKL and TNFα (n=3); C: % of WT and Mitf$^{mi/mi}$ osteoclasts in the S-phase of cell cycle when treated with CSF1 only (n=3); D: Western blot analysis of expression of p27KIP1, ERK1 and p19INK4D in WT and Mitf$^{mi/mi}$ osteoclasts at indicated time points and treatments.
5.2.3 ELEVATED EXPRESSION OF $p27^{kip1}$ AND $p21^{cip1}$ AND LOWERED CDK2 ACTIVITY COINCIDE WITH RANKL-INDUCED CELL CYCLE WITHDRAWAL AND ELEVATION IN $p27^{kip1}$ IS NOT REGULATED BY Mitf

The decision by cells to quit proliferation and commit to differentiation is governed mainly by the activities of INK4 and Cip/Kip-families of CDKIs, based on their cellular stoichiometry (Sherr and Roberts, 1995). To determine whether the RANKL-coordinated cell cycle withdrawal by osteoclast progenitors occurred via CDKIs, we examined the expression of various CDKIs in osteoclast progenitor cells at 12 and 24 hours after the addition of RANKL alone, or a combination of RANKL and TNF$\alpha$, by western blot. Our results show that $p27^{kip1}$ levels are elevated by 8.3±1.52 ($n=2$) fold in 12 hours after adding RANKL and by 12.2±0.8 ($n=2$) fold in 24 hours after adding RANKL and TNF$\alpha$, when compared to time point 0 (Fig. 36A). We also found that $p21^{cip1}$ levels are also elevated in the osteoclast progenitors by 2.2±0.1 ($n=2$) fold in 24 hours after adding RANKL and by 3.7±0.4 ($n=2$) fold in 24 hours after adding RANKL and TNF$\alpha$, when compared to time point 0 (Fig. 36A). However, the expression levels of $p18^{INK4c}$ or $p19^{INK4d}$ remained unchanged in the osteoclast precursors following treatment with RANKL and/or TNF$\alpha$ (Fig. 36A). ERK1 was used as a loading control in these experiments. We also confirmed by immunohistochemistry that the levels of $p27^{kip1}$ and $p21^{cip1}$ are elevated in osteoclasts at day 5, when grown in co-culture with osteoblasts (data not shown). These results indicate that the elevation in $p27^{kip1}$ and $p21^{cip1}$ protein...
levels within 12 and 24 hours of treatment with RANKL coincides with the withdrawal from cell cycle by osteoclast progenitors.

In order to arrest cells in G1, p27\textsuperscript{kip1} and p21\textsuperscript{cip1} must bind to CDK2-cyclinE complexes thereby inactivating CDK2 and preventing it from phosphorylating retinoblastoma (Rb) (Sherr and Roberts, 1995). To investigate whether elevation in p27\textsuperscript{kip1} and p21\textsuperscript{cip1} levels coincides with decreased CDK2 activity, we immunoprecipitated active CDK2 complexes from osteoclast progenitors grown in either proliferation or differentiation media-I and subjected them to kinase assays in the presence of \textsuperscript{\gamma}\textsuperscript{32P}ATP with recombinant Histone H1 as the substrate. CDK2 kinase activity was considerably diminished within 24 hours after adding RANKL, as indicated by the diminished phosphorylation of Histone H1 (Fig. 36B). Similar amounts of CDK2 protein were present in the immunocomplexes from cells grown in proliferative and differentiation media (Fig. 36B). Taken together, these results indicate that RANKL stimulates the up-regulation of p27\textsuperscript{kip1} and p21\textsuperscript{cip1} levels, which in turn inactivate CDK2 and induce osteoclast progenitors to withdraw from cell cycle.

Further, we wanted to investigate whether Mitf regulates the elevation in p27KIP1 levels. We examined the expression of p27\textsuperscript{kip1} in WT and Mitf\textsuperscript{mi/mi} osteoclasts, grown in the presence of RANKL alone or together with TNF\textalpha, by western blot analysis. Our data indicated that p27\textsuperscript{kip1} is expressed in Mitf\textsuperscript{mi/mi} osteoclasts at levels similar to those WT osteoclasts in the presence of RANKL alone or together with TNF\textalpha (Figure 35D). In other words, RANKL caused an elevation in p27\textsuperscript{kip1} expression levels in Mitf\textsuperscript{mi/mi} osteoclasts similar to that seen.
in WT osteoclasts. These results were repeated in subsequent experiments. Thus, we concluded from these data that Mitf does not coordinate the elevation in $p27^{kip1}$ levels caused by RANKL.
Figure 36. RANKL causes elevation in p27\textsuperscript{KIP1} and p21\textsuperscript{CIP1} protein levels and lower CDK2 activity in osteoclast precursors and RANKL-mediated elevation in p27\textsuperscript{KIP1} protein levels may involve the p38 MAPK pathway. A: Western blot analysis of extracts prepared from osteoclast precursors at 0, 12 and 24 hours after treatment with RANKL +/- TNF\textalpha or at 24 hours in proliferation media. Blots probed with antibodies against p27\textsuperscript{KIP1}, p21\textsuperscript{CIP1}, p18\textsuperscript{INK4C} and ERK1 are shown. ERK1 was used as a loading control. B: Kinase assay performed on \textalpha CDK2 immunoprecipitates with Histone H1 as the substrate. The blot was probed with \textalpha CDK2 antibody to verify the total amount of CDK2 present in the immunoprecipitates. C: Western blot analysis was performed on extracts prepared from 2.67*10\textsuperscript{6} osteoclast precursors treated with RANKL alone or RANKL in the presence of p38 MAPK inhibitor SB203580 (10\textmu M), or MEKK inhibitor PD98059 (40\textmu M), or PI3-kinase inhibitor LY294002 (10\textmu M), at indicated time points. The blots were probed with \textalpha p27\textsuperscript{KIP1} and \textalpha ERK1 antibodies.
5.2.4 RANKL-INDUCED ELEVATION IN p27KIP1 LEVELS OCCURS VIA THE p38 MAPK PATHWAY

The RANKL-RANK signaling cascade activates several signaling pathways including mitogen-activated protein kinase (MAPK), Src kinase and phosphatidyl inositol 3 (PI 3) kinase pathways during osteoclast differentiation. It is known through inhibitor-based studies that the p38 MAPK pathway is involved in the differentiation of bone marrow-derived precursors into osteoclasts (Matsumoto et al., 2000a and Matsumoto et al., 2000b) and in mediating osteoclast-specific gene expression (Mansky et al., 2002). Understanding which downstream pathway is used by RANKL to elevate the levels of p27KIP1 and p21CIP1 is a key step towards identifying the precise molecules responsible for these events. We treated the osteoclast precursor cells either with RANKL alone or RANKL in the presence of p38 MAPK inhibitor SB203580 (10µM) or MEK inhibitor PD98059 (40µM) or PI 3-kinase inhibitor LY294002 (10µM) and examined the levels of p27KIP1 in extracts made from these cells at 12 and 24 hours after these treatments (Fig. 36C). The p38 MAPK inhibitor SB203580 was able to block the up-regulation of p27KIP1 protein levels normally induced by RANKL within 12 hours of its treatment (compare lanes 1, 2 and 4 in Fig. 36C). The levels of p27KIP1 were also lower at 24 hours of treatment with a combination of RANKL and SB203580 than in cells treated with RANKL alone (lanes 3 and 5, Fig. 36C). However, neither PD98059 nor LY294002 was able to block the RANKL-induced elevation in p27KIP1 levels in the osteoclast progenitors (compare lanes 2, 3 with lanes 6, 7 and 8, Fig. 36C).
In contrast, the levels of p21<sup>CIP1</sup> were not changed by any of the drugs used in this experiment (data not shown). These results suggest that RANKL, via the p38 MAPK pathway increases the levels of the CDKI p27<sup>KIP1</sup>, which in turn induces the osteoclast progenitors to switch from proliferation to differentiation.

### 5.2.5 Osteoclast progenitors deficient in p27<sup>KIP1</sup> are defective in withdrawal from the cell cycle in response to RANKL

To investigate if a deficiency in either p27<sup>KIP1</sup> or p21<sup>CIP1</sup> might perturb the RANKL-coordinated decision by osteoclast progenitor cells to withdraw from cell cycle prior to differentiation, we examined the effect of RANKL treatment on cell cycle exit by osteoclast progenitor cells from p27<sup>KIP1</sup>−/− and p21<sup>CIP1</sup>−/− mice. Equal numbers of bone marrow cells were cultured for 3 days in the proliferation media. The osteoclast precursors of macrophage lineage were then either maintained in proliferation media or switched to differentiation media-I and pulse labeled with BrdU at the indicated time points to determine the percentage of cycling cells (Fig 37).

The percentage of WT cells undergoing DNA synthesis decreased from 44.4%±4.8% (n=3) at time point 0 to 10.5%±1.52% (n=3) at 24 hours and to 3.06%±0.55% (n=3) at 48 hours after adding RANKL (Fig. 37A). Similarly, when these cells were treated with TNFα the percentage of cells in the S-phase decreased to 19±4% and 9±3% by 24 and 48 hours, respectively (Fig. 37B and 37E). This indicates that 90% of the osteoclast progenitors withdraw from the
cell cycle within 24 hours after RANKL or TNFα. In contrast, WT cells do not withdraw from the cell cycle at 24, 48 or 72 hours of growth in proliferation media (Fig. 36C, data not shown). These cells begin to exit the cell cycle by 96 hours in the proliferation media, as only 19.05%±0.21% (n=3) are BrdU positive at this time point. The percentage of p21<sup>CIP1-/-</sup> cells in the S-phase decrease from 47%±2.83% (n=3) at zero, to 16.5%±0.71% (n=3) and 5.92%±4.12% (n=3), respectively at 24 and 48 hours after RANKL treatment (Fig. 37D).

Similarly, in response to TNFα the percentage cells in S-phase decreased to 19.4±0.9 and 6.6±6.4 respectively (Fig. 37E). This indicates that these cells withdraw from the cell cycle in response to RANKL or TNFα treatment, similar to WT cells. Also, this response is specific to RANKL treatment, as p21<sup>CIP1-/-</sup> osteoclast progenitors do not withdraw from cell cycle if maintained in proliferative media for 48 hours (Fig. 37F).

In case of osteoclast progenitor cells from a p27<sup>KIP1-/-</sup> background, there were 50.48%±3.22% (n=3) BrdU positive cells before the addition of RANKL. At 24 and 48 hours after the addition of RANKL, there were 25.4%±3.13% (n=3) and 30.8%±0.78% (n=3) of p27<sup>KIP1-/-</sup> osteoclast progenitors cells in the S-phase (Fig. 37A). Also, there were 42.98%±2.9% (n=3) p27<sup>KIP1-/-</sup> cells in the S-phase even at 72 hours after RANKL treatment. The percentage of BrdU positive cells in the p27<sup>KIP1-/-</sup> background decreased to 13.89%±1.35% (n=3) by 120 hours after adding RANKL, indicating that they do start withdrawing from the cell cycle with time. Additionally, percentage of p27<sup>KIP1-/-</sup> cells in the S-phase, when
maintained in TNFα was 39.7±3.4% and 29.4±3.6% at 24 and 48 hours respectively (Fig. 37E). As observed with WT cells, p27<sup>KIP1</sup>−/− cells maintained in the proliferation media continued to proliferate for 72 hours and withdrew from cell cycle by 96 hours (Fig. 37C and data not shown). Only 14.25±5.81% (n=3) of p27<sup>KIP1</sup>−/− cells were BrdU positive at 96 hours in proliferative media. These results indicate that the decision to withdraw from cell cycle following RANKL treatment is perturbed in osteoclast progenitors from p27<sup>KIP1</sup>−/− mice. In other words, p27<sup>KIP1</sup>−/− osteoclast progenitors might be defective in responding to RANKL with respect to cell cycle withdrawal and that p27<sup>KIP1</sup> is required for RANKL-mediated cell cycle withdrawal during osteoclast differentiation.
Figure 37. RANKL-induced cell cycle withdrawal is blocked in p27\textsuperscript{KIP1/-} OCL precursors but occurs in p21\textsuperscript{CIP1/-} osteoclast precursors. Osteoclast precursors from p27\textsuperscript{KIP1/-} (p27KO), p21\textsuperscript{CIP1/-} (p21KO) and WT littermates were grown in proliferation or differentiation media were pulse labeled with BrdU at the indicated time points. Percentage of BrdU-positive cells relative to total number of PI-positive cells of WT and p27KO or WT and p21KO cells grown in A and D: CSF1+RANKL; B and E: CSF1+TNF\textalpha{} and C and F: in proliferation media, at the indicated time points is shown. A total of 4000 cells were counted for each time point and the average for three experiments is shown.
5.2.6 OSTEOCLAST PRECURSORS FROM p27KIP1-/- MICE EXPRESS LOWER LEVELS OF TRAP AND CATHEPSIN K BUT DIFFERENTIATE INTO MULTINUCLEAR OSTEOCLASTS CAPABLE OF BONE RESORPTION

To investigate if the deficiency in cell cycle withdrawal by p27KIP1-/- osteoclast progenitors translated into an inability to undergo terminal differentiation into multinuclear osteoclasts and whether p21CIP1-/- osteoclast progenitors are compromised for differentiation into osteoclasts even though p21CIP1 is not required for cell cycle withdrawal, we used four complimentary approaches. First, we used quantitative real-time PCR to analyze the level of expression of osteoclast specific markers, TRAP and cathepsin K, in osteoclast progenitors from p27KIP1-/-, p21CIP1-/- and WT mice. Secondly, we asked whether p27KIP1-/- and p21CIP1-/- progenitors are capable of differentiating into multinuclear osteoclasts in vitro. Thirdly, we examined if the osteoclasts from these mice were capable of forming resorption pits on calcium phosphate coated surfaces. Finally, we examined the long bones from postnatal day 2-3 day old p27KIP1-/- and p21CIP1-/- mice by radiography followed by histomorphometric analysis of the TRAP-stained femur sections.

TRAP mRNA levels were up regulated by 7±0.65 fold (n=2) in WT and 8.03±1.03 fold (n=2) in p21CIP1-/- osteoclasts within 24 hours and by 10.97±0.49 fold (n=2) in WT and 10.66±0.37 fold (n=2) in p21CIP1-/- osteoclasts within 72 hours after adding RANKL (Fig. 38A and 38C). However, TRAP mRNA levels were only 4.56±0.51 fold (n=2) and 3.76±0.91 fold (n=2) at 24 and 72 hours in differentiation media in p27KIP1-/- osteoclasts (Fig. 38A). Thus, WT osteoclasts
express 2.75 times more TRAP mRNA than p27KIP1-/− osteoclasts at 72 hours after adding RANKL. Similarly, cathepsin K mRNA levels are elevated by 2.41±0.17 fold (n=2) in WT and by 4±1.15 fold (n=2) in p21CIP1-/− osteoclasts at 24 hours and by 4.27±0.15 fold (n=2) in WT and 6.08±0.03 fold in p21CIP1-/− cells at 72 hours of shifting to differentiation media (Fig. 38B and 38D). However, at 24 and 72 hours after adding RANKL, the levels of expression of cathepsin K were only 1.85±0.17 fold (n=2) and 1.72±0.23 fold (n=2) respectively, in p27KIP1-/− osteoclast progenitor cells (Fig. 38C). Thus, cathepsin K mRNA is expressed 3-fold higher in WT than in p27KIP1-/− osteoclasts at 72 hours in differentiation media. These data suggest that at least two key markers of osteoclast differentiation, TRAP and cathepsin K, are down regulated in osteoclasts deficient for p27KIP1 but not in those deficient in p21CIP1.

To investigate whether lower osteoclast-specific gene expression levels translated into a defect in osteoclast differentiation and function, we examined whether progenitors from p27KIP1-/− and p21CIP1-/− mice could differentiate into multinuclear cells in vitro and whether they could form resorption pits on calcium phosphate coated surfaces (BD Biocoat Osteologic slides) (Fig. 42; Tables 26 and 27). The multinuclear cells were counted and classified into 3 groups based on the number of nuclei per osteoclast. We found that osteoclast precursors from both p21CIP1-/− and p27KIP1-/− backgrounds formed multinuclear osteoclasts and that osteoclast progenitors from p27KIP1-/− background formed multinuclear cells at numbers slightly lower than those from the WT (Table 26). The resorption pits formed by osteoclasts from all three backgrounds were
counted and pit areas in square (sq.) pixels were measured using Bioquant Nova software. Osteoclast precursors from p27<sup>KIP1</sup>−/− and p21<sup>CIP1</sup>−/− mice were capable of forming functional osteoclasts that can form resorption pits (Fig. 42). The number of pits formed by p27<sup>KIP1</sup>−/− osteoclasts was approximately half the number of pits formed by WT (Table 27). The majority of the pits formed by p27<sup>KIP1</sup>−/− osteoclast progenitors fell within an area range of 20-1000 sq. pixels. In contrast, the majority of pits formed by WT osteoclasts had a much larger range of area viz., 20-10,000 sq. pixels and the majority of pits formed by p21<sup>CIP1</sup>−/− osteoclasts had an area range of 20-2500 sq. pixels (Table 27). However, osteoclasts from p27<sup>KIP1</sup>−/− and p21<sup>CIP1</sup>−/− mice formed larger pits of 40,000-100,000 sq. pixels (Table 27).

Radiographic examination of the long bones from 3-4 day old p27<sup>KIP1</sup>−/− and p21<sup>CIP1</sup>−/− mice revealed that these mice did not have osteopetrosis (Fig.40A). We also examined the sections of the femurs from 3-4 day old p27<sup>KIP1</sup>−/− and p21<sup>CIP1</sup>−/− mice for the presence of TRAP positive multinuclear osteoclasts (Fig.40B). TRAP-positive osteoclasts were present on the bone surface in both mutants, at numbers similar to those in the WT mice at the same age (Fig.40B). Taken together, these data indicate that deficiency in p27<sup>KIP1</sup> or p21<sup>CIP1</sup> by themselves in mice does not affect osteoclast differentiation and function either in vitro or in vivo.
Figure 38. TRAP and Cathepsin K mRNA levels are significantly lower in p27KIP1-/- osteoclast precursors. A: Comparison of fold induction of TRAP at 24 and 72 hours, in p27KIP1-/- against WT; B: Comparison of fold induction of Cathepsin K at 24 and 72 hours, in p27KIP1-/- against WT; C: Comparison of fold induction of TRAP at 24 and 72 hours, in p21KIP1-/- against WT and D: Comparison of fold induction of Cathepsin K at 24 and 72 hours, in p21KIP1-/- against WT is shown. All values, determined by a quantitative real-time PCR assays, were first normalized to GAPDH levels and then expressed as fold induction over CSF1-only controls (0 hour time point). The average of 2 experiments is shown.
In light of these data, we wanted to investigate if the removal of both p27\textsuperscript{KIP1} and p21\textsuperscript{CIP1} has an effect on cell cycle withdrawal by osteoclast precursors, on osteoclast-specific gene expression and on osteoclast differentiation and function \textit{in vivo} and \textit{in vitro}. First, we examined whether osteoclasts from p27p21 double knock out (p27\textsuperscript{KIP1/-}p21\textsuperscript{CIP1/-}) mice were defective in RANKL-induced cell cycle withdrawal. By analyzing the number of BrdU positive cells, we found in \textit{in vitro} assays that the percentage of cells in the S-phase in WT decreased from 40.13\%±0.34\% (n=3) at zero to 2.64\%±1.52\% (n=3) at 24 hours and to 2.18\%±0.55\% (n=3) at 48 hours after the addition of RANKL (Fig. 39A). In contrast, at zero, 24 and 48 after adding RANKL, there were 56.77\%±2.46\% (n=3), 23.18\%±0.96\% (n=3) and 41.34\%±5.58\% (n=3) cells that were BrdU positive in osteoclast progenitors from p27\textsuperscript{KIP1/-}p21\textsuperscript{CIP1/-} background (Fig. 39A). Even at 72 hours after adding RANKL, there were 42.98\%±2.9\% (n=3) BrdU positive cells in the p27\textsuperscript{KIP1/-}p21\textsuperscript{CIP1/-} population (data not shown). When cells were maintained in proliferation media, 38.03\%±3.14\% (n=3) at 24 hours and 32.98\%±1.38\% (n=3) at 48 hours in WT were BrdU positive, compared to 55.1\%±1.67\% (n=3) at 24 and 48.95\%±7.64\% (n=3) at 48 hours in the double mutant (Fig. 39B). We conclude from these data that p27\textsuperscript{KIP1} is critical for the RANKL-mediated cell
cycle withdrawal by osteoclast progenitors and that the removal of p21\textsuperscript{CIP1} in a p27\textsuperscript{KIP1} null background does not add to this effect.

Next, we investigated whether p27\textsuperscript{KIP1/-}p21\textsuperscript{CIP1/-} osteoclasts expressed lower levels of TRAP and cathepsin K. TRAP mRNA expression levels, as measured by quantitative real-time PCR, were elevated in WT osteoclasts by 6.86±0.94 \((n=2)\) fold at 24 and 12.85±1.94 fold at 72 hours after adding RANKL (Fig. 39C). In contrast TRAP expression levels were only at 2.48±1.7 fold at 24 and 1.11±0.48 fold at 72 hours in RANKL in p27\textsuperscript{KIP1/-}p21\textsuperscript{CIP1/-} cells (Fig. 39C). Similarly, cathepsin K was induced 1.68±0.12 fold at 24 hours and 6.65±1.34 fold at 72 hours in RANKL in WT whereas its expression was down regulated to 0.68±0.19 fold at 24 and to 0.96±1.16 fold at 72 hours in RANKL (Fig. 39D). In other words, at 72 hours after adding RANKL, TRAP mRNA levels were 11.5 fold and cathepsin K levels were 6.9 fold higher in WT than in p27\textsuperscript{KIP1/-}p21\textsuperscript{CIP1/-} osteoclasts. These results indicate that removal of both p27\textsuperscript{KIP1} and p21\textsuperscript{CIP1} highly compromises osteoclast-specific marker gene expression and that the elimination of p21\textsuperscript{CIP1} exacerbates the already low TRAP gene expression levels observed in a p27\textsuperscript{KIP1/-} background.
Figure 39. Osteoclast precursors from p27<sup>KIP1</sup>-/-p21<sup>CIP1</sup>-/- mice do not exit the cell cycle in the presence of RANKL and express significantly lower levels of TRAP and Cathepsin K mRNA. Osteoclast precursors from p27<sup>KIP1</sup>-/-p21<sup>CIP1</sup>-/- (p27p21DKO) and WT littermates were grown in the either proliferation or differentiation media and pulse labeled with BrdU at the indicated time points. Percentage of cells positive for BrdU incorporation over the total number of cells (PI-positive) is shown. (A) Percentage of BrdU-positive cells in p27<sup>KIP1</sup>-/- osteoclast progenitors grown in differentiation media at the indicated time points in comparison to WT cells (B) Percentage of BrdU-positive cells in p27<sup>KIP1</sup>-/- osteoclast progenitors grown in proliferation media at the indicated time points in comparison to WT cells. A total of 4000 cells were counted at each time point and the average for three experiments is shown. (C) Comparison of fold induction of TRAP mRNA in osteoclast precursors from p27p21DKO and WT cultured in differentiation media. (D) Comparison of fold induction of Cathepsin K mRNA in osteoclast precursors from p27p21DKO and WT cultured in differentiation media. An average of 3 experiments is shown.
5.2.8 OSTEOPETROSIS IN p27\textsuperscript{KIP1-/-}p21\textsuperscript{CIP1-/-} NEWBORN MICE DUE TO LOWER NUMBERS OF MULTINUCLEAR OSTEOCLASTS WITH LOWER TRAP ACTIVITY AND ABNORMAL MORPHOLOGY

To understand if the removal of both p27\textsuperscript{KIP1} and p21\textsuperscript{CIP1} affects osteoclast differentiation and function \textit{in vivo}, we examined the long bones of 3-4 day old p27\textsuperscript{KIP1-/-}p21\textsuperscript{CIP1-/-} pups and their WT littermates by radiography. Out of 35 newborn double null pups examined, 20 (57%) had sclerotic lesions in the femur diaphysis, a classic phenotype of osteopetrosis (Fig. 40A). Femur sections from WT and osteopetrotic mice were stained for TRAP activity and counter-stained with hematoxylin. We observed that p27\textsuperscript{KIP1-/-}p21\textsuperscript{CIP1-/-} mice had considerably more unresorbed trabecular bone, a hallmark of osteopetrosis, than their WT littermates (Fig. 40B, arrowheads). We performed careful histomorphometric analysis on the sections using the Bioquant Nova Software. Based on the percentage of unresorbed bone area (UBA) in the femur diaphysis, the double mutants were classified into three categories of osteopetrosis. Out of the 20 osteopetrotic double nulls, 11 had 55.33%±11% UBA (severe); 4 had 34.15%±2.6% UBA (moderate) and the remaining five had 20.18%±5.32% UBA (mild) (Fig. 40C). The WT femurs at this age had 15.77%±6.22% UBA (n=7) and p27\textsuperscript{KIP1-/-} femurs had 10.56%±0.08% (n=4) UBA (Fig. 40C). We observed that multinuclear osteoclasts were present in p27\textsuperscript{KIP1-/-}p21\textsuperscript{CIP1-/-} mice and were similar in size to those in WT mice. However, the number of osteoclasts was considerably lower in all three classes of double mutants. The percentage of total osteoclast surfaces present on the total bone...
surface (TBS; both cortical and trabecular bone surfaces) was 65.46% ± 10.34% in WT and 48.4% ± 2.64% in p27\textsuperscript{KIP1\textminus/\textminus} (Fig. 40D). However, the percentage of osteoclast surfaces on TBS was only 17.88% ± 1.5% in the severe, 20.79% ± 4.09% in the moderate and 32.92% ± 9.97% in the mild class of p27\textsuperscript{KIP1\textminus/\textminus}p21\textsuperscript{CIP1\textminus/\textminus} (Fig. 40D). The number of osteoclasts per unit total bone perimeter was 7 ± 1.5 in the severe, 7 ± 2 in the moderate and 8 ± 1 in the mild class of the double mutant and it was 18 ± 5 in the WT and 14.04 ± 1.55 in p27\textsuperscript{KIP1\textminus/\textminus} (Fig. 40E). Thus, the WT had twice the number, area and surface of osteoclasts compared to the p27\textsuperscript{KIP1\textminus/\textminus}p21\textsuperscript{CIP1\textminus/\textminus} mice.

Overall, the osteoclasts in the double mutants exhibited considerably weaker staining for TRAP activity compared to those in WT (Fig 41A and 41B; compare arrowheads). The double mutant osteoclasts had none to very small amount of TRAP around the surface of the cell lining the bone whereas the WT osteoclasts had large amounts of TRAP around the same area. Unlike WT osteolcasts, which had intense TRAP staining all over the cytoplasm, the mutant osteoclasts had large areas of cytoplasm devoid of any TRAP staining. The small amount of TRAP staining present in the double mutant cells was more granular in nature. In addition, the osteoclasts from the double mutants exhibited abnormal cell morphology. These osteoclasts were heavily vacuolated compared to WT osteoclasts and most of these vacuoles were localized around the nucleus (Fig. 41B, small arrows). Taken together, these data suggest that p27\textsuperscript{KIP1} and p21\textsuperscript{CIP1} might play redundant roles in the differentiation of
osteoclasts and that elimination of both of these molecules affects osteoclast differentiation and function \textit{in vivo} in the long bones of neonatal mice.

We also examined the long bones of 15-20 day old WT and p27$^{\text{KIP1/-}}$ p21$^{\text{CIP1/-}}$ mice. Radiographic examination revealed that there were no sclerotic lesions in the long bones of the double null mice (data not shown). The femur sections from the double null and WT mice at this age possessed TRAP positive multinuclear osteoclasts of similar numbers and sizes, especially in the metaphysis area (data not shown). We also performed histomorphometric analysis of the TRAP stained femur sections from p27$^{\text{KIP1/-}}$ p21$^{\text{CIP1/-}}$ mice. WT femurs from 15-20 day old mice possessed 4.25$\pm$0.3 UBA, 17.9$\pm$0.55% total osteoclast surface/TBS and 3.62$\pm$0.31 osteoclasts per unit bone perimeter while femurs from p27$^{\text{KIP1/-}}$ p21$^{\text{CIP1/-}}$ mice possessed 5.44$\pm$0.184 UBA, 16.96$\pm$0.44% total osteoclast surface/TBS and 2.5$\pm$0.2 osteoclasts per unit bone perimeter (data not shown). These data indicate that the osteopetrosis observed in the new born, resolves with age. In other words, p27$^{\text{KIP1/-}}$ p21$^{\text{CIP1/-}}$ mice exhibit osteopetrosis only during the growth phase of the developing bone and not during the maintenance phase.
Figure 40. The osteopetrosis phenotype is differentially penetrant in postnatal 3-day old p27\textsuperscript{KIP1-/-}p21\textsuperscript{CIP1-/-} mice. A: Radiographic analysis of the long bones of 3-day old WT, p27\textsuperscript{KIP1-/-}, p21\textsuperscript{CIP1-/-} and p27\textsuperscript{KIP1-/-}p21\textsuperscript{CIP1-/-} (p27p21DKO) mice. White arrowheads indicate sclerotic lesions in the femur diaphysis of p27p21DKO mice. B: The sections of femurs stained for TRAP activity and counter stained with hematoxylin. Solid black arrowheads indicate regions of unresorbed bone in sections from p27p21DKO mice. Histomorphometric analysis was performed on the TRAP-stained sections from WT, p27\textsuperscript{KIP1-/-} and p27p21DKO mice using Bioquant Nova Software. C: The percentage (%) of unresorbed trabecular bone area (UBA) over the total bone area (TBA) in WT, p27\textsuperscript{KIP1-/-} and p27p21DKO mice. Note that the p27p21DKO were classified into three groups based on the % of UBA as indicated in the graph. D: % Total osteoclast (OCL) surface over the total bone surface (TBS) in the indicated genotypes. E: Number (#) of OCLs over unit bone perimeter (UBP) in sections from WT, p27\textsuperscript{KIP1-/-} and p27p21DKO mice.
Figure 41. Osteoclasts from $p27^{KIP1/-}p21^{CIP1/-}$ mice show abnormal morphology and stain very weakly for TRAP. Higher (20X) magnification of the TRAP stained femur sections from $p27^{KIP1/-}p21^{CIP1/-}$ (p27p21DKO) (B) and WT (A) mice, are shown here. Osteoclasts from $p27^{KIP1/-}p21^{CIP1/-}$ background show very weak staining for TRAP compared to WT as indicated by the black arrowheads and they have a higher number of vacuoles especially around the nuclei (smaller arrows).
In order to investigate whether osteoclast progenitors from \( p27^{kip1/-} \) \( p21^{cip1/-} \) mice were capable of forming functional multinuclear cells in vitro, we plated progenitor cells from WT and \( p27^{kip1/-} \) \( p21^{cip1/-} \) mice, in differentiation media-I, either on gelatin coated dishes to test for the formation of TRAP positive multinuclear osteoclasts or on calcium phosphate coated wells (BD Biocoat Osteologic slides) to test for the formation of resorption pits (Fig. 42A, 42B). In general, we observed that the progenitors from the double null mutants did form TRAP positive multinuclear cells and that they were capable of forming resorption pits on calcium phosphate coated surfaces. However, they formed fewer numbers of TRAP positive multinuclear cells and resorption pits compared to WT and the size of the pits were smaller compared to those formed by the WT (Tables 26 and 27). The number of TRAP positive multinuclear osteoclasts in \( p27^{kip1/-} \) \( p21^{cip1/-} \) were \( 2 \pm 1.11 \) (more than 12 nuclei), \( 38 \pm 1.16 \) (6-12 nuclei) and \( 98 \pm 23.33 \) (3-5 nuclei) \((n=3)\) and those in the WT were \( 15 \pm 3.33 \), \( 66 \pm 17.8 \) and \( 267 \pm 37.8 \) \((n=3)\), respectively, in each class (Table 26). The majority of the pits formed by the double null osteoclasts were within an area range of 20-500 sq. pixels while those made by the WT osteoclasts were within an area range of 20-20,000 sq. pixels (Table 27). Also, the largest pit area formed by the double null osteoclasts was in the range of 40,000-60,000 sq. pixels whereas the largest pit formed by WT osteoclasts was in the 100,000-150,000 sq. pixel range (Table 27). These data indicate that removal of
p27\textsuperscript{KIP1} and p21\textsuperscript{CIP1} in mice affects osteoclast differentiation and that this defect is cell autonomous to the osteoclasts.

Figure 42. Precursors from p27\textsuperscript{KIP1/-} p21\textsuperscript{CIP1/-} mice form fewer TRAP positive multinuclear osteoclasts and form fewer and smaller resorption pits during \textit{in vitro} differentiation and functional assays. A: Osteoclast precursors from p27\textsuperscript{KIP1/-} (p27KO), p21\textsuperscript{CIP1/-} (p21KO), p27\textsuperscript{KIP1/-} p21\textsuperscript{CIP1/-} (p27p21DKO) and WT littermates were plated on gelatin-coated dishes in differentiation media-II for the formation of multinucleated osteoclasts and the cells were fixed and stained for TRAP activity. The number of osteoclasts with 3-5, 6-12 or more than 12 nuclei was estimated using standard light microscopy (Table 26). B: Osteoclast precursors from indicated genotypes were plated on calcium phosphate coated 16-well BD Biocoat Osteologic Multitest slides (BD Biosciences) for formation of resorption pits by functional osteoclasts. The number and area of the resorption pits were calculated using light microscopy and the Bioquant Nova Software (Table 27).
<table>
<thead>
<tr>
<th>Nuclei Count</th>
<th>WT</th>
<th>p21KO</th>
<th>p27KO</th>
<th>p27p21DKO</th>
</tr>
</thead>
<tbody>
<tr>
<td>&gt;12 nuclei</td>
<td>15±3.33</td>
<td>12±3.33</td>
<td>11±1.11</td>
<td>2±1.11</td>
</tr>
<tr>
<td>6-12 nuclei</td>
<td>66±17.8</td>
<td>44±2.22</td>
<td>29±1.11</td>
<td>38±1.16</td>
</tr>
<tr>
<td>3-5 nuclei</td>
<td>267±37.8</td>
<td>229±24.5</td>
<td>176±20</td>
<td>98±23.33</td>
</tr>
</tbody>
</table>

Average of 3 experiments is shown.

Table 26. Table indicating the number of TRAP-positive multinuclear osteoclasts, classified according to the number of nuclei present, formed by osteoclast precursors from p27$^{kip1/-}$ (p27KO), p21$^{cip1/-}$ (p21KO), p27$^{kip1/-}$/p21$^{cip1/-}$ (p27p21DKO) and WT littermates.
Table 27. Number, total resorption pit area and the area range distribution of the resorption pits formed by osteoclast precursors from p27<sup>KIP1</sup>−/− (p27KO), p21<sup>CIP1</sup>−/− (p21KO), p27<sup>KIP1</sup>−/−p21<sup>CIP1</sup>−/− (p27p21DKO) and WT littermates.

<table>
<thead>
<tr>
<th>AREA DISTRIBUTION (Sq. Pixels)</th>
<th>WT</th>
<th>p21KO</th>
<th>p27KO</th>
<th>p27p27DKO</th>
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<tr>
<td>TOTAL # OF PITS</td>
<td>615</td>
<td>582</td>
<td>528</td>
<td>299</td>
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<td>&lt;100</td>
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<td>31</td>
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<td>59</td>
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<tr>
<td>250-500</td>
<td>106</td>
<td>122</td>
<td>91</td>
<td>96</td>
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<tr>
<td>500-1000</td>
<td>111</td>
<td>118</td>
<td>124</td>
<td>46</td>
</tr>
<tr>
<td>1000-2500</td>
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<td>46</td>
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<td>1</td>
</tr>
<tr>
<td>10000-20000</td>
<td>16</td>
<td>11</td>
<td>4</td>
<td>1</td>
</tr>
<tr>
<td>20000-40000</td>
<td>12</td>
<td>4</td>
<td>3</td>
<td>1</td>
</tr>
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<td>40000-60000</td>
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<td>0</td>
</tr>
</tbody>
</table>

Data from 3 individual experiments for each genotype is shown above.
5.3 DISCUSSION

The results from this study indicate that upregulation of p27\textsuperscript{KIP1} (via the p38 MAPK pathway) and p21\textsuperscript{CIP1} by RANKL causes the osteoclast precursor cells to withdraw from cell cycle and commit to the activation of differentiation-specific programs. The fact that p27\textsuperscript{KIP1}\textsuperscript{-/-} osteoclast precursors are impaired in exiting cell cycle in response to RANKL clearly establishes an important role for p27\textsuperscript{KIP1} in the commitment to differentiation-related cell cycle withdrawal by osteoclast precursors. Actively proliferating cells withdraw from the cell cycle, after a certain number of proliferative cycles, in response to either culture conditions or intrinsic timer mechanisms (Sherr and DePinho, 2000). Accordingly, osteoclast progenitors from all three genetic backgrounds withdrew from cell cycle by 96 hours when grown in proliferative media. Likewise, progenitor cells from p27\textsuperscript{KIP1}\textsuperscript{-/-} mice started to withdraw from cell cycle by 120 hours in differentiation media. It is highly probable that this does not occur in response to RANKL. In other words, osteoclast progenitors from p27\textsuperscript{KIP1}\textsuperscript{-/-} mice might inherently be defective in responding to RANKL with regard to exiting the cell cycle prior to committing to differentiation. However, p21\textsuperscript{CIP1} does not appear to be required for RANKL-induced commitment by osteoclast progenitors to withdraw from the cell cycle. Even though, p21\textsuperscript{CIP1} levels are elevated in osteoclasts in response to RANKL treatment, its deficiency alone does not affect cell cycle withdrawal or osteoclast-specific gene expression.
Neither \textit{p27^{KIP1-$\text{-}/-}$} nor \textit{p21^{CIP1-$\text{-}/-}$} mice exhibit osteopetrosis and the precursors from these mice formed functional multinuclear osteoclasts \textit{in vitro}. \textit{p27^{KIP1}} is required for the differentiation-related cell cycle withdrawal of several cell types. However, mice lacking \textit{p27^{KIP1}} do undergo normal organ development although their organs are enlarged in size due to enhanced cell proliferation (Fero et al., 1996; Kiyokawa et al., 1996; Nakayama et al., 1996 and). It is possible that osteoclast precursors in \textit{p27^{KIP1-$\text{-}/-}$} mice might undergo additional rounds of proliferation until an unknown redundant mechanism, most likely another CDKI, compensates for the loss of \textit{p27^{KIP1}}. Though \textit{p21^{CIP1}} is normally not required for differentiation-related cell cycle withdrawal in osteoclasts, it could compensate for \textit{p27^{KIP1}} in its absence. It is known that the Cip/Kip family members play redundant roles during differentiation and development (Zhang et al., 1998., Zhang et al., 1999 and Vidal and Koff, 2000).

Mice lacking both \textit{p27^{KIP1}} and \textit{p21^{CIP1}} exhibit age resolving osteopetrosis and the precursors from these mice form functional multinuclear osteoclasts \textit{in vitro} in significantly lower numbers. While \textit{p27^{KIP1}} is primarily responsible for cell cycle withdrawal prior to differentiation, it might have additional roles in other aspects of the differentiation program. Our data indicates that \textit{p27^{KIP1}} might have a role in the regulation of differentiation-specific gene expression during osteoclast differentiation. Also, it appears that \textit{p21^{CIP1}} has a role in osteoclast differentiation, analogous to its role during oligodendrocyte differentiation (Zezula et al, 2001). One possible mechanism is that \textit{p27^{KIP1}} and \textit{p21^{CIP1}} might normally perform different, non-overlapping roles during osteoclast 236
differentiation. However, in the absence of one, the other protein might be able to compensate. But the differentiation program might become severely impaired in the absence of both proteins. Thus, while p27\(^{\text{KIP1-/-}}\) or p21\(^{\text{CIP1-/-}}\) mice do not exhibit any defects in osteoclast differentiation, the double knock out p27\(^{\text{KIP1-/-}}\) p21\(^{\text{CIP1-/-}}\) neo-natal mice do exhibit impairment in osteoclast differentiation. This suggests that these two proteins exhibit functional redundancy during osteoclast differentiation, probably at the level of regulation of marker gene expression via collaboration with one or more transcription factors, e.g., microphthalmia-associated transcription factor (MITF), that regulate osteoclast differentiation.

Osteopetrosis in p27\(^{\text{KIP1-/-}}\) p21\(^{\text{CIP1-/-}}\) mice resolves with age. Age-resolving osteopetrosis is observed in other osteopetrotic mutants like the op/op and Mitf\(^{or/or}\) mice (Felix et al., 1990a; Felix et al., 1990b; Moore, 1995; Nii et al., 1995 and Roodman, 1999). It is possible that different mechanisms, including those involving p27\(^{\text{KIP1}}\) and p21\(^{\text{CIP1}}\), might operate towards coupling cell cycle withdrawal with osteoclast differentiation during the growth and the steady state phases of long bone development in mice. These pathways could operate in parallel, or compensate in an age-dependent fashion, thus explaining the milder phenotype exhibited by the double mutants.

Blocking p38 MAPK activity using the drug SB203580 inhibited the up regulation of p27\(^{\text{KIP1}}\) by RANKL. In contrast, the levels of p21\(^{\text{CIP1}}\) were not changed by any of the drugs used in this experiment. It is possible that the up-regulation of p21\(^{\text{CIP1}}\) levels occurs via one of the other pathways activated by
RANKL-RANK signaling but not addressed in this study. Blocking the p38 MAPK signaling pathway in bone marrow derived osteoclast cells using SB203580 inhibits the formation of multinuclear osteoclasts (Matsumoto et al., 2000a and Matsumoto et al., 2000b). It will be very interesting to identify the mechanism downstream of p38 MAPK pathway that leads to the RANKL-mediated elevation in p27 levels in osteoclast precursors and to examine if osteoclast progenitors treated with SB203580 are defective in differentiation-related cell cycle withdrawal. Recently, it was shown that Mitf is phosphorylated by p38 MAPK in osteoclast precursors and that the inhibition of this pathway in osteoclasts by treatment with SB203580 correlates with decreased TRAP expression levels (Mansky et al., 2002). Mitf regulates target genes like TRAP and cathepsin K by directly binding to a 7-base pair conserved sequence TCANGTG, on the promoters of these genes (Luchin et al., 2000 and Motyckova et al., 2001). Moreover, mice homozygous for the Mitf$^{mi/mi}$ mutation, which results from the deletion of one of the four arginines in the basic domain of Mitf, exhibit severe osteopetrosis (Hodgkinson et al., 1993 and Moore, 1995). Osteoclast precursors from these mutant mice are incapable of fusing into multinuclear osteoclasts and exhibit low levels of TRAP and cathepsin K (Luchin et al., 2000 and Motyckova et al., 2001).

Osteoclasts from p27$^{KIP1-/-}$ mice express low levels of TRAP and cathepsin K levels leading to the speculation that Mitf might regulate p27$^{KIP1}$ expression. However, this does not seem to be the case as even though Mitf$^{mi/mi}$ osteoclasts are delayed in withdrawal from cell cycle during
differentiation, $p27^{\text{KIP1}}$ levels are elevated in $\text{Mitf}^{\text{mi/mi}}$ precursors in response to RANKL (Figure 35). But, the similarity of the phenotypes in osteoclast precursor cells in which p38 MAPK pathway is blocked and in the osteoclasts from $\text{Mitf}^{\text{mi/mi}}$ mice and the fact that $p27^{\text{KIP1}/-}$ osteoclasts express low levels of TRAP and cathepsin K levels and that $\text{Mitf}^{\text{mi/mi}}$ osteoclasts are delayed in withdrawal from cell cycle during differentiation raises the possibility that $p27^{\text{KIP1}}$ might play a role upstream of Mitf, but downstream of p38 MAPK. This has precedence in the roles of $p27^{\text{Xic1}}$ during neuronal and muscular development in *Xenopus laevis* (Vernon et al., 2003 and Vernon and Philpott, 2003). During neuronal and muscle development in *Xenopus* embryos, $p27^{\text{Xic1}}$ is thought to play roles in differentiation that are different from its traditional role of achieving cell cycle arrest. In *Xenopus*, $p27^{\text{Xic1}}$ was shown to stabilize neurogenin to promote neurogenesis and to synergize with MyoD to promote muscle differentiation (Vernon et al., 2003 and Vernon and Philpott, 2003). Furthermore, $p57^{\text{KIP2}}$ has been shown to bind to and stabilize MyoD in C2C12 myoblasts during myogenic differentiation (Reynaud et al., 2000). It is possible that $p27^{\text{KIP1}}$ might play a role in osteoclast differentiation by regulating the activity of Mitf and that this role might be independent of its role in promoting differentiation-related cell cycle arrest.

In conclusion, our data indicates that $p27^{\text{KIP1}}$ is required for RANKL-mediated cell cycle withdrawal and for osteoclast-specific marker gene expression but its removal alone does not affect osteoclast differentiation and function. On the other hand, $p21^{\text{CIP1}}$ does not play a role in cell cycle withdrawal...
or contribute to TRAP and cathepsin K expression by itself and its removal alone does not affect osteoclast differentiation. However, removal of both p21\textsuperscript{CIP1} and p27\textsuperscript{KIP1} in mice affects osteoclast differentiation and function, at least in the early growth phase of long bone development, suggesting that p21\textsuperscript{CIP1} does play a role (possibly redundant with p27\textsuperscript{KIP1}) in osteoclast differentiation, independent of cell cycle withdrawal.
CHAPTER 6

DISCUSSION AND SIGNIFICANCE

6.1 DISCUSSION

Osteoclasts are bone resorbing multinuclear cells that arise from a hematopoietic monocyte/macrophage lineage and osteoblasts are bone-forming cells that arise from a mesenchymal lineage. Osteoclasts and osteoblasts exist in constant communication with each other to bring about growth and remodeling events that lead to the healthy maintenance of the vertebrate bone. Osteoblasts produce two cytokines, viz., RANKL and M-CSF1, two key molecules necessary and sufficient for osteoclast differentiation. RANKL-RANK signaling leads to the initiation of signaling cascades and downstream transcription events resulting in the differentiation of TRAP-positive, multinuclear osteoclasts from mononuclear precursors. Osteopetrosis and osteoporosis are among the common bone disorders arising from disruption of communication between osteoclasts and osteoblasts.

Mif is a bHLH-Zip transcription factor that is important for the differentiation of several cell types including osteoclasts. Previous studies have
established that Mitf is activated by phosphorylation by the p38 MAPK pathway following RANKL-RANK engagement during osteoclast differentiation and that this coincides with elevated expression of TRAP mRNA. Mitf is known to directly regulate the expression of TRAP, cathepsin K, osteopontin, E-cadherin and OSCAR in osteoclasts. Mitf<sup>mi/mi</sup> mice exhibit severe osteopetrosis due to an inability of the mononuclear precursor cells to fuse to become multinuclear osteoclasts. In addition, Mitf<sup>or/or</sup> mice exhibit severe osteopetrosis that partially resolves with age. However, Mitf<sup>wh/wh</sup> mice, also bearing a mutation in the basic domain of the protein, do not exhibit any osteoclast defect. Though all three mutant proteins are unable to bind to DNA as homo- or heterodimers, only Mitf<sup>mi/mi</sup> and Mitf<sup>or/or</sup> proteins behave as dominant negatives with WT-Mitf and with Tfe3 with respect to DNA binding. Tfe3 acts redundantly with Mitf in osteoclasts.

To further understand the precise role of Mitf in osteoclast differentiation and biology, we conducted comparative microarray analysis of the gene expression patterns in osteoclast precursors from WT and Mitf<sup>mi/mi</sup> mice as they differentiate following treatment with RANKL and M-CSF1. Our hypothesis was that Mitf regulates the expression of several genes involved in osteoclast differentiation. We identified 891 novel genes whose expression levels are different during osteoclast differentiation. In particular, the expression levels of 51 novel genes are different between WT and Mitf<sup>mi/mi</sup> mice. Table 28 shows a representative list of genes that are down regulated in Mitf<sup>mi/mi</sup> osteoclasts at 72 hours after adding RANKL, in comparison to WT. The differential expression of
Eos, HOX11L2, HCP and p9 were verified using quantitative real-time PCR. Of these, only HCP (SHP1) is known to have any role in osteoclast biology as a partial loss of its kinase activity leads to osteopetrosis in mutant mice. Mice homozygous for a kinase domain mutation in SHP-1 develop severe osteoporosis.

<table>
<thead>
<tr>
<th>Gene function</th>
<th>Gene name</th>
</tr>
</thead>
<tbody>
<tr>
<td>Transcription Factors</td>
<td>HOX11L2, HOX1a, Eos, p9</td>
</tr>
<tr>
<td>Signaling Molecules</td>
<td>HCP (SHP1), Spleen tyrosine kinase (SYK1), Calcineurin, Guanine nucleotide binding protein-α, Lymphocyte antigen 6 complex, locus C, Serine protease, Calmodulin, T-cell receptor-β</td>
</tr>
<tr>
<td>Cell Metabolism</td>
<td>Carboxyl esterase (Ces3), NADH dehydrogenase (Ubiquinone), Phospholipase A2</td>
</tr>
<tr>
<td>DNA replication</td>
<td>ORC1, Lymphoid specific helicase</td>
</tr>
<tr>
<td>ATPase</td>
<td>Ca2+ transporting fast twitch ATPase</td>
</tr>
<tr>
<td>Vesicular Trafficking</td>
<td>Phosphatidyl inositol binding clathrin assembly protein</td>
</tr>
</tbody>
</table>

Table 28. Representative list of genes whose expression is lower in Mitf<sup>mi/mi</sup> osteoclasts at 72 hours after adding RANKL.
The next step in this study is to verify whether the expression levels of the remaining genes identified in this study are lower in Mitf<sup>mi/mi</sup> osteoclast precursors by further real-time PCR analysis. Even after the differential expression of these genes in the mutant osteoclasts is confirmed, many questions still remain to be answered:

1) Whether these genes are directly regulated by Mitf:

a. Analysis of the promoter sequences of these genes for the presence of Mitf-binding E-box sites.

b. Whether Mitf will bind to these sites in electrophoretic mobility shift assays (EMSA) and chromosome immunoprecipitation (ChIP) assays.

c. Whether Mitf will transactivate these promoters in transient transfection assays.

2) Whether Mitf regulates the expression of these genes in coordination with other osteoclast-specific transcription factors like PU.1, as in the case of TRAP promoter. This can be addressed by the examination of Mitf containing immunocomplexes, obtained by ChIP assays on these promoters, for the presence of other transcription factors.

3) Whether Mitf-regulation of any of the genes is indirect, and if so, what is the mechanism of regulation. For example, one or more of the newly identified genes might be regulated by a transcription factor similar to HOX11L2 or a signaling molecule similar to HCP, which in turn might be regulated by Mitf. Analysis of the promoter sequences
of these genes might reveal binding sites for one or more Mitf-regulated transcription factors. This can be followed up with promoter-deletion studies to confirm regulation by these transcription factors. The other method is to identify proteins present in the immunocomplexes on specific regions of these promoters, obtained by ChIP assays using antibodies against acetylated histones. Such proteins could be identified by peptide sequencing of the components of the immunocomplexes. Though cumbersome, this approach might lead to the identification of novel regulators (potential Mitf-targets) of the genes that are indirectly regulated by Mitf.

4) Another method of testing whether Mitf regulates these genes, is to over express WT-Mitf in Mitf<sup>mi/mi</sup> osteoclasts by retroviral-based vectors and measure the expression levels of these novel genes by real-time PCR.

5) Whether any newly identified transcription factor or signaling molecule co-operate with or regulate the activity of Mitf. For example, HCP or a repressor similar to Eos might bind to Mitf and activate or repress it. This can be addressed via immunoprecipitation and in vitro binding assays, followed by transient and stable transfection assays with the putative co-activator/repressor, Mitf and the TRAP promoter.

6) The most important question would be whether any of these genes have roles in osteoclast differentiation and biology. Retroviral or adenoviral-mediated overexpression of these genes and depletion of
these genes by RNA interference or by blocking the activity of these
genes with inhibitors, if available, in RAW 264.7 cells and if possible
in primary osteoclast precursors, would uncover the precise roles of
these novel genes in osteoclast differentiation. Based on the
phenotype of the Mitf^{mi/mi} mice, it could be predicted that osteoclasts
in which one or more of these genes are inhibited might have defects
at the fusion step during osteoclast differentiation. If successful, these
studies could be extended into \textit{in vivo} gene knock out or over-
expression studies in mice.

Our studies on the comparison of Mitf-regulated gene expression in
Mitf^{mi/mi}, Mitf^{or/or} and Mitf^{wh/wh} alleles of Mitf indicate that Mitf^{or/or} osteoclasts
express TRAP at levels similar to WT but express lower levels of cathepsin K,
Eos, HOX11L2, HCP and p9 proteins. Mitf^{or/or} mice exhibit osteopetrosis that
partially improves with age and osteoclasts from these mice form fewer
functional multinuclear osteoclasts \textit{in vitro}. Taking the gene expression,
phenotypic and the \textit{in vitro} functional data together, our results suggest that Mitf
regulated osteoclast-specific genes might be categorized into two classes
based on their expression in Mitf^{or/or} osteoclasts; one comprised of early
response genes including TRAP and the other comprised of late responsive
genes including cathepsin K. Thus, it is possible to speculate, based on the
phenotypic and gene expression data from this work that the WT-level of
expression of early-response genes including TRAP, might contribute to the
resolution of the osteopetrotic phenotype in Mitf^{or/or} mice.
In this context, it is important to understand which proteins are associated with the transcription machinery on the promoters of TRAP and cathepsin K genes in Mitf\textsuperscript{\textit{prior}} osteoclasts. Performing CHIP assay using antibodies against Mitf, PU.1 and Tfe3 on these promoters, in nuclear extracts from Mitf\textsuperscript{\textit{prior}} osteoclasts, can test this. It is already known that Mitf\textsuperscript{\textit{mi/mi}} protein is capable of inhibiting activation of TRAP promoter by PU.1. It is however possible that Mitf\textsuperscript{\textit{prior}} does not act as a dominant negative with a modifier like PU.1 on TRAP promoter, thereby leading to the WT-level expression of TRAP in Mitf\textsuperscript{\textit{prior}} osteoclasts. This question can be addressed by transient and stable transfection studies in RAW264 cells using TRAP promoter attached to a readable reporter gene along with PU.1, Mitf and Mitf\textsuperscript{\textit{prior}} proteins.

Cathepsin K, Eos, HCP, HOX11L2 and p9 are expressed at lower levels in Mitf\textsuperscript{\textit{prior}} osteoclasts. Motyckova et al., showed that of the four consensus E-box sequences on cathepsin K promoter, the 1\textsuperscript{st} and 2\textsuperscript{nd} are the most important; the 3\textsuperscript{rd} is of intermediate importance and the 4\textsuperscript{th} is the least important with respect to transactivation by Mitf and Tfe3 (Motyckova et al., 2001). Even though a consensus site for PU.1 binding (GGAA) exists on the cathepsin K promoter, 15 bp upstream of the 2\textsuperscript{nd} consensus E-box, it is not known whether Mitf and PU.1 co-operate for the activation of cathepsin K promoter. It is possible that PU.1 does not have a role in the transactivation of cathepsin K, thus explaining for the low cathepsin K expression levels in the mutant osteoclasts. In this context, one has to assume that Mitf\textsuperscript{\textit{prior}} acts as a dominant negative with Tfe3 on cathepsin K promoter. These hypotheses can be verified.
by ChIP assays with Tfe3 antibody on nuclear extracts from Mitf<sup>o/o</sup> osteoclasts and by investigating for the presence of Mitf<sup>o/o</sup> and PU.1 proteins in these complexes on cathepsin K promoter. Here again, transient and stable transfection studies with cathepsin K promoter, Tfe3, Mitf<sup>o/o</sup> and PU.1 might verify whether (a) PU.1 can transactivate cathepsin K and (b) whether Mitf<sup>o/o</sup> is capable of acting as a dominant negative with Tfe3 or PU.1 in this context. These studies should also be repeated in the case of the promoters of the newly identified genes whose expression levels are lower in Mitf<sup>o/o</sup> osteoclasts. Additionally, the hypothesis that Mitf<sup>o/o</sup> acts as a dominant negative on late-response genes including cathepsin K could be tested by overexpressing Mitf<sup>o/o</sup> protein in WT osteoclasts or in RAW 264.7 cells and measuring the expression levels of these genes.

Our studies on the coordination of cell cycle arrest and osteoclast differentiation by RANKL revealed that Mitf<sup>mi/mi</sup> osteoclasts are delayed in cell cycle exit, both in co-culture with osteoblasts and in response to TNFα, compared to WT. We also observed that RANKL causes the upregulation of p21<sup>CIP1</sup> and p27<sup>KIP1</sup> and a coordinated inhibition of CDK2 activity in osteoclast precursors. However, our results indicated that RANKL caused an elevation in p27<sup>KIP1</sup> levels in Mitf<sup>mi/mi</sup> osteoclasts. One plausible explanation for this finding is that p27<sup>KIP1</sup> might not be regulated by Mitf during osteoclast differentiation. On the other hand, it is well known that Tfe3 might play a redundant role with Mitf during osteoclast differentiation. Thus, it is possible that Tfe3 is responsible for the elevation in p27<sup>KIP1</sup> levels in Mitf<sup>mi/mi</sup> osteoclasts. In addition, we observed
that the upregulation of $p27^{\text{KIP1}}$ by RANKL is mediated via the p38 MAPK pathway. Further, our results indicate that $p27^{\text{KIP1}}$ is required by the osteoclast progenitors to withdraw from cell cycle in response to RANKL, prior to differentiation. Even though $p21^{\text{CIP1}}$ levels are elevated in osteoclasts in response to RANKL treatment, its deficiency alone does not affect cell cycle withdrawal or osteoclast-specific gene expression.

We observed that $p27^{\text{KIP1}-/-}p21^{\text{CIP1}-/-}$ mice exhibit age resolving osteopetrosis and the precursors from these mice form functional multinuclear osteoclasts in vitro in significantly lower numbers. The osteopetrotic phenotype in the double knockout mice suggests that $p27^{\text{KIP1}}$ and $p21^{\text{CIP1}}$ might have redundant roles during osteoclast differentiation. One possible mechanism is that $p27^{\text{KIP1}}$ and $p21^{\text{CIP1}}$ might normally perform different, non-overlapping roles during osteoclast differentiation. However, in the absence of one, the other protein might be able to compensate. But the differentiation program might become severely impaired in the absence of both proteins. Thus, while single knock out $p27^{\text{KIP1}-/-}$ or $p21^{\text{CIP1}-/-}$ mice do not exhibit any defects in osteoclast differentiation, the double knock out $p27^{\text{KIP1}-/-}p21^{\text{CIP1}-/-}$ neonatal mice do exhibit impairment in osteoclast differentiation. This suggests that these two proteins exhibit functional redundancy during osteoclast differentiation, probably at the level of regulation of marker gene expression via collaboration with one or more transcription factors that regulate osteoclast differentiation.

As mentioned earlier, $p27^{\text{KIP1}}$ up regulation by RANKL is mediated via the p38 MAPK pathway. Hence, it will be very interesting to identify the
mechanism downstream of p38 MAPK pathway that leads to the RANKL-mediated elevation in p27\textsuperscript{KIP1} levels in osteoclast precursors and to examine if osteoclast progenitors treated with SB203580 are defective in differentiation-related cell cycle withdrawal. TRAP and cathepsin K are expressed at significantly lower levels in p27\textsuperscript{KIP1}\textsuperscript{-/-} and in the double mutant osteoclasts. p38 MAPK, an activator of Mitf, mediates the up-regulation of p27\textsuperscript{KIP1} levels in osteoclasts and Mitf directly regulates the expression of TRAP and cathepsin K. Furthermore, the osteopetrotic phenotype in the p27\textsuperscript{KIP1}\textsuperscript{-/-}p21\textsuperscript{CIP1} double knock out mice is very similar to the mild osteopetrosis observed in TRAP and cathepsin K knock out mice and the age-resolving osteopetrosis seen in Mitf\textsuperscript{+/+} mice (refer chapter 1). Interestingly, in all these mutants, the eruption of teeth and the intramembranous ossification processes of flat bone formation are not affected and their osteoclasts exhibit abnormal cell morphology. Taken together, these data suggest that there might be a connection between Mitf and p27\textsuperscript{KIP1} during osteoclast differentiation and that Mitf or its family member Tfe3 could be the downstream effector downstream of p38 MAPK pathway that leads to the RANKL-mediated elevation in p27\textsuperscript{KIP1} levels in osteoclast precursors.

In *Xenopus*, p27\textsuperscript{Xic1} was shown to stabilize neurogenin to promote neurogenesis and to synergize with MyoD to promote muscle differentiation. Furthermore, p57\textsuperscript{KIP2} has been shown to bind to and stabilize MyoD in C2C12 myoblasts during myogenic differentiation. Therefore, analogous to these systems, it is possible that p27\textsuperscript{KIP1} might play a role in osteoclast differentiation.
by regulating the activity of Mitf and that this role might be independent of its role in promoting differentiation-related cell cycle arrest.

In nutshell, it is possible that Mitf and its family members act either upstream or downstream of $p27^{KIP1}$ or both during osteoclast differentiation. Figure 43 illustrates a model that can be proposed based on the conclusions and speculations drawn from this study. Thus, we propose that $p27^{KIP1}$ regulates cell cycle withdrawal by osteoclast precursors, but both $p27^{KIP1}$ and $p21^{CIP1}$ play redundant roles in the differentiation of osteoclasts. It is our speculation that Mitf and Tfe3 might play redundant roles in the p38-mediated elevation in expression levels in differentiating osteoclasts and that Mitf might also have an additional role downstream of $p27^{KIP1}$, analogous to its role in the neuronal and muscle differentiation in Xenopus.
Figure 43. Proposed model for the role of Mitf in the regulation of RANKL-coordinated cell cycle withdrawal and cell differentiation, via the CDKIs p27\textsuperscript{KIP1} and p21\textsuperscript{CIP1}, during osteoclast differentiation.

Several key experiments can be done to test these speculations.

1) Levels of total and phosphorylated Mitf protein (using the phospho-Mitf antibody that specifically recognizes the p38 MAPK phosphorylation site on Mitf) levels in WT and p27\textsuperscript{KIP1}\textsuperscript{-/-} p21\textsuperscript{CIP1}\textsuperscript{-/-} osteoclasts during differentiation could be compared via western blot analysis of the nuclear extracts.
2) Physical interactions between p27\textsuperscript{KIP1} and Mitf, analogous to the mechanism in \textit{Xenopus}, could be tested via \textit{in vitro} binding assays and via immunoprecipitation assays. If they bind to each other, domains in p27\textsuperscript{KIP1} required for the interaction could be mapped by further \textit{in vitro} binding assays.

3) Transient transfection assays could be used to test whether different domains of p27\textsuperscript{KIP1} are able to enhance the transactivation of TRAP promoter by Mitf.

4) Whether p27\textsuperscript{KIP1} acts upstream of Mitf during osteoclast differentiation could be tested by the overexpression of Mitf in p27\textsuperscript{KIP1}\textsuperscript{-/-} and in the p27\textsuperscript{KIP1}\textsuperscript{-/-}p21\textsuperscript{CIP1}\textsuperscript{-/-} DKO osteoclasts and verifying whether this has any effect on the expression levels of TRAP and cathepsin K and on the formation of functional, multinuclear osteoclasts by these cells.

5) It is possible that Mitf and its family members regulate p27\textsuperscript{KIP1} expression, either directly or indirectly, in osteoclasts. Thus, p27\textsuperscript{KIP1} expression levels might be up regulated in Mitf\textsuperscript{milmi} osteoclasts via the action of Tfe3. This could be verified by investigating whether osteoclast precursors from Mitf\textsuperscript{vgavgatfe3\textsuperscript{-/-}} mice, which exhibit severe osteopetrosis, express p27\textsuperscript{KIP1} and whether they are deficient in RANKL-induced cell cycle withdrawal prior to differentiation. If these osteoclasts do not express p27\textsuperscript{KIP1} and do not exit the cell cycle in response to RANKL, then it might imply that Mitf family of transcription factors regulate the expression of p27\textsuperscript{KIP1} expression in osteoclasts. This could be further
confirmed if the over expression of $p27^{KIP1}$ in $Mitf^{vga/vga}$ $Tfe3^{-/-}$ mice osteoclast precursors commits them to differentiation-related cell cycle exit in response to RANKL and rescues the defects in cell differentiation in in vitro differentiation and functional assays.

6) Finally, it is possible that Mitf, $p27^{KIP1}$ and $p21^{CIP1}$ are regulating the same events during osteoclast differentiation. The $Mitf^{vga/vga}$ mice have no osteoclast phenotype, presumably due to the redundant role played by Tfe3. It will be very interesting in this context to investigate whether $Mitf^{vga/vga}$ $p27^{KIP1-/-}$ and/or $Mitf^{vga/vga}$ $p27^{KIP1-/-}$ $p21^{CIP1-/-}$ mice develop osteopetrosis and whether the osteoclast precursors from these mice form functional, multinuclear osteoclasts in vitro.

Osteopetrosis in $p27^{KIP1-/-}$ $p21^{CIP1-/-}$ mice resolves with age. It is possible that different mechanisms, including those involving $p27^{KIP1}$ and $p21^{CIP1}$, might operate, towards coupling cell cycle withdrawal with osteoclast differentiation, during the growth and the steady state phases of long bone development in mice. These mechanisms or modifier proteins could compensate in an age-dependent manner to bring about osteoclast differentiation and differentiation-mediated cell cycle arrest in the older double mutant mice. If this is the case, such a modifier(s) should be present in CDK2 immunoprecipitates from osteoclasts from adult double knock out mice. Further, to identify genes and proteins that are responsible for the rescue of the osteopetrotic phenotype in older double knock out mice, comparisons of transcription and translation profiles of osteoclasts from newborn, 15-day old and adult double knock out
mice could be made using cDNA microarray or protein microarrays. These findings might shed light on novel, parallel mechanisms that might operate to affect osteoclast differentiation and to couple cell cycle withdrawal and differentiation in osteoclasts.

6.2 SIGNIFICANCE

Mitf is an evolutionarily conserved bHLH-ZIP transcription factor with key roles in the differentiation of several cell types including melanocytes and osteoclasts. The role of Mitf in melanocyte development is rather well established. Our hypothesis, based on the osteopetrotic phenotype of the Mitf\textsuperscript{mi/mi} and Mitf\textsuperscript{or/or} mice, was that Mitf regulates the expression of several genes involved in the fusion of mononuclear precursors to form multinuclear osteoclasts. Through this work, we have identified several novel genes that might be directly or indirectly regulated by Mitf during osteoclast differentiation. We have confirmed the differential expression of four of these genes in Mitf\textsuperscript{mi/mi} osteoclasts. Additional experiments need to be performed to investigate whether these genes have important roles in osteoclast differentiation. If these genes do have important roles in osteoclast differentiation and biology and if their expression is directly or indirectly regulated by Mitf, then the findings from this study would pave the way towards delineating the precise roles played by Mitf during osteoclast differentiation. Moreover, understanding the transcriptional regulation of osteoclast differentiation, specifically by Mitf, would
help make giant strides towards understanding the molecular basis of several bone diseases such as osteoporosis and osteopetrosis and in the identification of potential drug targets towards the treatment of these diseases.

Our results from comparative osteoclast-specific gene expression studies on Mitf<sup>mi/mi</sup>, Mitf<sup>or/or</sup> and Mitf<sup>wh/wh</sup> osteoclasts indicate that Mitf regulated osteoclast-specific genes might be categorized into two classes; one comprising of early response genes including TRAP and the other comprising of late responsive genes including cathepsin K, Eos, HOX11L2, HCP and p9. These findings, for the first time, indicate that late response genes including cathepsin K might be more important to osteoclast biology than early response genes like TRAP. Thus, our findings imply the possibility that the expression of early response genes including TRAP in Mitf<sup>or/or</sup> osteoclasts at levels comparable to that in the WT, might explain the mechanism by which the osteopetrosis in Mitf<sup>or/or</sup> mice becomes resolved. We have also added quantitative evidence to the observation that osteopetrotic phenotype in Mitf<sup>or/or</sup> mice is milder than that seen in Mitf<sup>mi/mi</sup> mice. These findings also contribute greatly towards appreciation of the role played by Mitf, and more importantly, the role played by the genes regulated by Mitf in osteoclast differentiation.

Finally, we have identified for the first time that that p27<sup>KIP1</sup> is essential for osteoclasts to withdraw from cell cycle in response to RANKL and that the elimination of both p27<sup>KIP1</sup> and p21<sup>CIP1</sup> leads to osteopetrotic phenotype in neonatal mice. Further, we also have identified that p38 MAPK pathway might mediate the RANKL-induced elevation in p27<sup>KIP1</sup> levels during osteoclast
differentiation. It is known that RANKL-RANK signaling initiates the activation of several signaling pathways that have roles in osteoclast differentiation. However, the precise role of these pathways in the regulation of the several events occurring during osteoclast differentiation remains unclear. The results from this study indicates that one of the possible roles of the p38 MAPK pathway, activated by RANKL signaling, is to co-ordinate the differentiation-related cell cycle withdrawal in osteoclast precursors. If confirmed by future studies, then this finding would make a substantial contribution towards understanding the specific roles of p38 MAPK pathway in osteoclast differentiation.

The presence of higher than normal numbers of bone-resorbing osteoclasts results in osteoporosis, a common and serious disorder occurring in post-menopausal women. Results from this study have identified one key mechanism: the requirement of p27$^{\text{KIP}}$ and p21$^{\text{CIP}}$ in the coordination of osteoclast cell cycle exit and differentiation and in osteoclast differentiation per se. Identification of one or more downstream effector proteins that are up regulated or activated or down regulated by the action of p27$^{\text{KIP}}$ and p21$^{\text{CIP}}$ during osteoclast differentiation, via future work, could serve as a basis to the development of pharmacological drugs that could block or enhance the activity of such key effector proteins. Such drugs could help in the “slowing down” of the differentiation of precursors into osteoclasts in an osteoporotic individual, thus lowering the number of functional osteoclasts in these patients. Thus, this finding might contribute to the development of novel drugs that could be used to
control osteoporotic bone destruction by the osteoclasts in post-menopausal women.

In conclusion, the results from these three pieces of work have made several novel contributions towards understanding the molecular basis by which RANKL regulates the processes of osteoclast differentiation and differentiation-related cell cycle exit. Together, these findings bring the field of osteoclast biology one step closer towards understanding the precise role of some of the pathways initiated by RANKL during osteoclast differentiation.


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