REGULATION OF OSTEOCLAST DIFFERENTIATION BY TRANSCRIPTION FACTORS MITF, PU.1 AND EOS

DISSEPTION

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By
Rong Hu, M.S.

The Ohio State University
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Dissertation Committee:
Professor Michael C. Ostrowski, advisor
Professor Beth S. Lee
Professor Denis C. Guttridge
Professor Gustavo W. Leone

Approved by:

Molecular, Cellular and Developmental Biology Graduate Program
ABSTRACT

The microphthalmia-associated transcription factor (MITF), a basic helix-loop-helix leucine zipper (bHLH-Zip) transcription factor, regulates distinct target genes in several cell types including osteoclasts. Osteoclasts are terminally differentiated multinuclear cells responsible for bone resorption. CSF-1 and RANKL are two critical cytokines to induce osteoclast differentiation from bone marrow-derived precursors. In osteoclasts, MITF interacts with the Ets family transcription factor PU.1 to synergistically activate target genes like Cathepsin K (Ctsk) and Acid Phosphatase 5 (Acp5). The region of MITF required for the physical interaction with PU.1 is the bHLH-Zip domain. To finer map the interacting domain of MITF, we introduced point mutations into the loop region of MITF via in vitro site-directed mutagenesis. The properties of mutated MITF proteins were analyzed by transient transfection assays, EMSAs, and in vitro GST pulldown assays. We identified that specific amino acids including N235, D236, and W241 in the loop region of MITF are critical to mediate physical and functional interaction with PU.1.

Both MITF and PU.1 are known transcriptional activators in osteoclasts. Results presented here demonstrate that they can also act as components of repressor complexes that suppress target gene expression in committed myeloid
precursors. The direct interaction of MITF and PU.1 with the zinc-finger protein Eos, an Ikaros family member, appears to be necessary for repression of Ctsk and Acp5. In bone marrow-derived precursors treated with CSF-1 alone, Eos formed a complex with MITF and PU.1 at target gene promoters and suppressed transcription through recruitment of co-repressors CtBP and Sin3A. Eos expression was reduced during osteoclast differentiation initiated by combined CSF-1 and RANKL stimulation and Eos association with Ctsk and Acp5 promoters was significantly decreased. Subsequently, MITF and PU.1 recruited co-activators to these target promoters resulting in robust expression of target genes. Overexpression of Eos in bone marrow-derived precursors inhibited multinuclear osteoclast formation and repressed transcription of subset of osteoclast specific genes that are regulated by MITF and PU.1. This work provides a novel mechanism to account for the modulation of MITF and PU.1 activity in committed myeloid progenitors prior to the initiation of osteoclast differentiation in response to the appropriate extracellular signals.
Dedicated to my family
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VITA

July 23, 1976........................................Born in Zhejiang Province, China

June, 1998.................. B.S. in Cell Biology, Wuhan University, Wuhan, China

June, 2001......................... M.S. in Virology, Wuhan University, Wuhan, China

June, 2006.............................. M.S. in Applied Statistics, The Ohio State University

2001-present.............................. Graduate Teaching and Research Assistant, The Ohio State University

PUBLICATIONS

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FIELDS OF STUDY

Major Field: Molecular, Cellular and Developmental Biology
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abstract</td>
<td>ii</td>
</tr>
<tr>
<td>Dedication</td>
<td>iv</td>
</tr>
<tr>
<td>Acknowledgments</td>
<td>v</td>
</tr>
<tr>
<td>Vita</td>
<td>vi</td>
</tr>
<tr>
<td>List of Figures</td>
<td>xii</td>
</tr>
<tr>
<td>List of Tables</td>
<td>xv</td>
</tr>
<tr>
<td>Abbreviations</td>
<td>xvi</td>
</tr>
<tr>
<td>Chapters:</td>
<td></td>
</tr>
<tr>
<td>1. Introduction</td>
<td></td>
</tr>
<tr>
<td>1.1 The bone tissue</td>
<td>2</td>
</tr>
<tr>
<td>1.1.1 Bone matrix</td>
<td>3</td>
</tr>
<tr>
<td>1.1.2 Bone structure</td>
<td>3</td>
</tr>
<tr>
<td>1.1.3 Bone ossification</td>
<td>4</td>
</tr>
<tr>
<td>1.1.4 Bone modeling and remodeling</td>
<td>8</td>
</tr>
<tr>
<td>1.2 Cells in bone</td>
<td>11</td>
</tr>
<tr>
<td>1.2.1 Osteoblasts</td>
<td>11</td>
</tr>
<tr>
<td>1.2.2 Osteocytes</td>
<td>14</td>
</tr>
<tr>
<td>1.2.3 Osteoclasts</td>
<td>15</td>
</tr>
<tr>
<td>1.2.3.1 Morphology of osteoclast</td>
<td>15</td>
</tr>
<tr>
<td>1.2.3.2 Bone resorption by osteoclast</td>
<td>17</td>
</tr>
<tr>
<td>1.2.3.3 Enzymes important for osteoclast function</td>
<td>26</td>
</tr>
<tr>
<td>1.2.3.4 Origin and differentiation of osteoclast</td>
<td>31</td>
</tr>
<tr>
<td>1.3 Signaling pathways involved in osteoclast differentiation</td>
<td>39</td>
</tr>
<tr>
<td>1.3.1 CSF-1/c-FMS signaling</td>
<td>41</td>
</tr>
<tr>
<td>1.3.1.1 CSF-1/c-FMS required for osteoclastogenesis</td>
<td>41</td>
</tr>
<tr>
<td>1.3.1.2 Signals mediated by CSF-1/c-FMs</td>
<td>42</td>
</tr>
<tr>
<td>1.3.2 RANKL/RANK central regulators of osteoclastogenesis</td>
<td>45</td>
</tr>
<tr>
<td>1.3.2.1 The molecular triad OPG/RANKL/RANK</td>
<td>45</td>
</tr>
<tr>
<td>1.3.2.2 RANKL/RANK signaling</td>
<td>48</td>
</tr>
<tr>
<td>Section</td>
<td>Page</td>
</tr>
<tr>
<td>------------------------------------------------------------------------</td>
<td>------</td>
</tr>
<tr>
<td>1.3.3 Cross talk between CSF-1 and RANKL signaling with other signaling pathways</td>
<td>53</td>
</tr>
<tr>
<td>1.3.4 Other modulators of osteoclast function</td>
<td></td>
</tr>
<tr>
<td>1.3.4.1 Stimulatory factors</td>
<td>55</td>
</tr>
<tr>
<td>1.3.4.2 Inhibitory factors</td>
<td>58</td>
</tr>
<tr>
<td>1.4 Transcriptional control of osteoclast differentiation</td>
<td>60</td>
</tr>
<tr>
<td>1.4.1 Nuclear factor kappa B (NF-κB)</td>
<td>60</td>
</tr>
<tr>
<td>1.4.2 Activator protein-1 (AP-1)</td>
<td>63</td>
</tr>
<tr>
<td>1.4.3 PU.1</td>
<td>65</td>
</tr>
<tr>
<td>1.4.4 Nuclear factor of activated T cell c1 (NFATc1)</td>
<td>68</td>
</tr>
<tr>
<td>1.4.5 Microphthalmia-associated transcription factor (MITF)</td>
<td>69</td>
</tr>
<tr>
<td>1.4.5.1 Mitf gene and MITF protein</td>
<td>69</td>
</tr>
<tr>
<td>1.4.5.2 MITF mutations and biological role of MITF</td>
<td>76</td>
</tr>
<tr>
<td>1.4.5.3 MITF in osteoclast</td>
<td>77</td>
</tr>
<tr>
<td>1.5 Summary</td>
<td>84</td>
</tr>
<tr>
<td>2. Material and Methods</td>
<td>86</td>
</tr>
<tr>
<td>2.1 Antibodies</td>
<td>86</td>
</tr>
<tr>
<td>2.2 Plasmid constructs</td>
<td>86</td>
</tr>
<tr>
<td>2.3 DNA manipulations</td>
<td>89</td>
</tr>
<tr>
<td>2.3.1 Agarose gel electrophoresis</td>
<td>89</td>
</tr>
<tr>
<td>2.3.2 Polymerase chain reaction (PCR)</td>
<td>89</td>
</tr>
<tr>
<td>2.3.3 Small- and large-scale preparation of plasmid DNA</td>
<td>90</td>
</tr>
<tr>
<td>2.3.4 Cloning of DNA</td>
<td>91</td>
</tr>
<tr>
<td>2.3.4.1 Restriction enzyme digestion</td>
<td>91</td>
</tr>
<tr>
<td>2.3.4.2 Alkaline phosphatase reaction</td>
<td>91</td>
</tr>
<tr>
<td>2.3.4.3 DNA ligation</td>
<td>92</td>
</tr>
<tr>
<td>2.3.4.4 DNA transformation into E.coli competent cells</td>
<td>92</td>
</tr>
<tr>
<td>2.4 Cell lines and transfections</td>
<td>93</td>
</tr>
<tr>
<td>2.4.1 NIH 3T3</td>
<td>93</td>
</tr>
<tr>
<td>2.4.2 COS-7</td>
<td>95</td>
</tr>
<tr>
<td>2.4.3 Phoenix</td>
<td>96</td>
</tr>
<tr>
<td>2.5 Culture of primary murine osteoclast</td>
<td>97</td>
</tr>
<tr>
<td>2.5.1 In vitro osteoclast differentiation</td>
<td>97</td>
</tr>
<tr>
<td>2.5.2 TRAP staining of in vitro differentiated osteoclast</td>
<td>98</td>
</tr>
<tr>
<td>2.6 Retroviral transduction of primary osteoclast precursors</td>
<td>98</td>
</tr>
<tr>
<td>2.7 Expression of recombinant proteins in E.coli</td>
<td>99</td>
</tr>
<tr>
<td>2.7.1 GST fusion proteins</td>
<td>99</td>
</tr>
<tr>
<td>2.7.2 His6-fusion proteins</td>
<td>100</td>
</tr>
</tbody>
</table>
2.8 In vitro GST pulldown assays.........................................................101
2.9 Immunoprecipitation and immunoblotting.................................101
2.10 Electrophoretic mobility shift assays (EMSA)...............................103
2.11 Chromatin immunoprecipitation (ChIP) and ReChIP......................104
2.12 RNA purification and reverse transcription..................................107
2.13 Real-Time PCR and data analysis..............................................108
2.14 In vitro site-directed mutagenesis..............................................110

3. Mapping amino acids in MITF important for interaction with PU.1........112
   3.1 Introduction.............................................................................112
   3.2 Results..................................................................................114
      3.2.1 Construction of mutations in the loop region of MITF..........114
      3.2.2 Identification of mutations in MITF that lack functional
           interaction with PU.1..............................................................116
      3.2.3 DNA-binding affinities of mutated MITF proteins to the Acp5
           promoter.............................................................................118
      3.2.4 Physical interactions between mutated MITF proteins and
           PU.1..................................................................................121
   3.3 Conclusion.............................................................................123

4. Eos modulates MITF and PU.1 action on osteoclast specific genes in
   committed myeloid progenitors......................................................125
   4.1 Introduction.............................................................................125
      4.1.1 Ikaros family transcription factors......................................126
      4.1.2 Eos protein........................................................................128
      4.1.3 Gene repression mediated by Ikaros family proteins.........130
      4.1.4 Co-repressor complexes and gene repression..................132
   4.2 Results..................................................................................136
      4.2.1 Eos expression is downregulated during osteoclast
           differentiation.................................................................136
      4.2.2 Eos inhibits Acp5 and Ctsk promoter activation by MITF and
           PU.1..................................................................................138
      4.2.3 Eos binds to the Acp5 promoter sequence in vitro............139
      4.2.4 Physical interactions between Eos and PU.1.....................143
      4.2.5 Physical interactions between Eos and MITF....................146
      4.2.6 Association of Eos with Acp5 and Ctsk promoters decreases
           during osteoclast differentiation.................................150
4.2.7 Dynamic recruitment of co-repressors and co-activators to the Acp5 and Ctsk promoters during osteoclast differentiation.................................................................151

4.2.8 Eos associates with MITF, PU.1, and co-repressors in osteoclast precursors.........................................................154

4.2.9 Overexpression of Eos disrupts osteoclast differentiation.157
  4.2.9.1 Retroviral transduction of Eos in BMMs..............157
  4.2.9.2 Overexpression of Eos inhibits multinuclear osteoclast formation........................................159
  4.2.9.3 Overexpression of Eos suppresses the expression of the subset of osteoclast specific genes......161

4.3 Conclusion..............................................................................................................................................161

5. Discussion.........................................................................................................................................................165
  5.1 Collaborative action of MITF and PU.1 in osteoclast..........165
  5.2 Negative regulation of MITF and PU.1 activity by Eos........168
  5.3 Dynamic association of co-repressors and co-activators with target genes during osteoclast differentiation.................................................................172
  5.4 Potential involvement of CSF-1/RANKL signaling in Eos action..174

Bibliography.........................................................................................................................................................178
# LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Schematic view of a longitudinal section through a growing long bone</td>
<td>5</td>
</tr>
<tr>
<td>2</td>
<td>Schematic diagram of bone formation through intramembranous and endochondral ossification</td>
<td>7</td>
</tr>
<tr>
<td>3</td>
<td>Schematic illustration of bone remodeling sequences and duration of various phases of bone remodeling</td>
<td>10</td>
</tr>
<tr>
<td>4</td>
<td>Role of canonical Wnt-β-catenin signaling during lineage differentiation and maturation of osteoblasts</td>
<td>13</td>
</tr>
<tr>
<td>5</td>
<td>Transmission electron micrograph of a rat osteoclast</td>
<td>16</td>
</tr>
<tr>
<td>6</td>
<td>Schematic representation of a non-polarized and a polarized osteoclast</td>
<td>20</td>
</tr>
<tr>
<td>7</td>
<td>Membrane domains and trafficking pathways of bone-resorption osteoclast</td>
<td>21</td>
</tr>
<tr>
<td>8</td>
<td>Transcytosis pathways to remove the degradation products in osteoclasts</td>
<td>25</td>
</tr>
<tr>
<td>9</td>
<td>Osteoclasts are cells of hematopoietic origin</td>
<td>34</td>
</tr>
<tr>
<td>10</td>
<td>A schematic representation of osteoclast differentiation and activation supported by osteoblasts/stromal cells</td>
<td>40</td>
</tr>
<tr>
<td>11</td>
<td>Model for major pathways initiated by CSF-1 binding to extracellular domain of c-FMS</td>
<td>44</td>
</tr>
<tr>
<td>12</td>
<td>Schematic representation of RANKL/RANK signaling pathways</td>
<td>51</td>
</tr>
</tbody>
</table>
The differentiation pathway of osteoclast progenitors into functionally active osteoclasts and the cytokines and transcription factors required for each step of differentiation.................................................................62

Human and murine Mitf genomic organization depicted to scale and nine different Mitf isoforms identified so far.................................................................71

Schematic representation of MITF protein............................................................75

M-CSF and RANKL activate MITF and TFE3 in osteoclasts.........................83

Protein sequence comparison of bHLH domain of MITF and USF2...........115

Identification of mutated MITF proteins that lack of functional interactions with PU.1........................................................................................................117

DNA-binding affinities of mutated MITF proteins to the Acp5 promoter sequence........................................................................................................119

Cold-competition assays for mutated MITF proteins on the Acp5 promoter sequence....................................................................................................120

Physical interactions between mutated MITF proteins and PU.1.........122

Schematic representation of Eos and Ikaros.......................................................129

Mi-2 and Sin3 complexes.................................................................................134

Eos expression is downregulated during osteoclast differentiation......137

Eos represses both Acp5 and Ctsk promoter activity.......................141

Eos binds to Acp5 promoter sequence in vitro and form complex with PU.1 on the Acp5 promoter........................................................142

Physical interaction of Eos with PU.1 in vitro.................................................144

Association of Eos with PU.1 in vivo.................................................................145
Identification of Eos and MITF interacting domain………………………..148

Eos and MITF physically interact and form a ternary complex on the Acp5 promoter…………………………………………………………..149

Recruitment of Eos, MITF and PU.1 to the Acp5 and Ctsk promoter during osteoclast differentiation………………………………………152

Dynamic recruitment of co-repressors and co-activators to the Acp5 and Ctsk promoters during osteoclast differentiation…………………….155

Association of Eos, MITF and PU.1 with co-repressors on Ctsk and Acp5 promoters in osteoclast precursors………………………………………...156

Retroviral transduction of Eos in BMMs……………………………………158

Overexpression of Eos in BMMs inhibits multinuclear osteoclast formation……………………………………………………………………..160

Overexpression of Eos inhibits expression of subset of osteoclast specific genes……………………………………………………………………..162

A schematic model of Acp5 and Ctsk gene regulation by transcription factors and their co-factors during osteoclast differentiation………164
# LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Molecular triad OPG/RANKL/RANK.</td>
<td>47</td>
</tr>
<tr>
<td>2</td>
<td><em>Mitf</em> mutant alleles resulting osteopetrotic phenotype.</td>
<td>79</td>
</tr>
<tr>
<td>3</td>
<td>Primers used for subcloning.</td>
<td>88</td>
</tr>
<tr>
<td>4</td>
<td>Real-Time PCR primers for RNA analysis.</td>
<td>109</td>
</tr>
<tr>
<td>5</td>
<td>Sense strand sequences for primers used in site-directed MITF mutagenesis.</td>
<td>111</td>
</tr>
</tbody>
</table>
### ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acp5/TRAP</td>
<td>Acid phosphatase 5/Tartrate-resistant acid phosphatase</td>
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<tr>
<td>bHLH-Zip</td>
<td>Basic helix-loop-helix leucine zipper</td>
</tr>
<tr>
<td>BMMs</td>
<td>Bone marrow-derived monocyte/macrophage precursor cells</td>
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<tr>
<td>CAII</td>
<td>Carbonic anhydrase II</td>
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<tr>
<td>CBP</td>
<td>CREB binding protein</td>
</tr>
<tr>
<td>ChIP</td>
<td>Chromatin immunoprecipitation</td>
</tr>
<tr>
<td>Co-IP</td>
<td>Co-immunoprecipitation</td>
</tr>
<tr>
<td>CTR</td>
<td>Calcitonin receptor</td>
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<tr>
<td>CTSK</td>
<td>Cathepsin K</td>
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<tr>
<td>CSF-1</td>
<td>Colony stimulating factor 1</td>
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<tr>
<td>ERK</td>
<td>Extracellular-signal regulated kinase</td>
</tr>
<tr>
<td>IFN</td>
<td>Interferon</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>IKK</td>
<td>IκB kinase</td>
</tr>
<tr>
<td>ITAM</td>
<td>Immunoreceptor tyrosine-based activation motif</td>
</tr>
<tr>
<td>JNK</td>
<td>Jun-N-terminal Kinase</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
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</tr>
<tr>
<td>MAPK</td>
<td>Mitogen-activated protein kinase</td>
</tr>
<tr>
<td>MITF</td>
<td>Microphthalmia-associated transcription factor</td>
</tr>
<tr>
<td>NFATc1</td>
<td>Nuclear factor of activated T cell c1</td>
</tr>
<tr>
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<td>Nuclear factor kappa B</td>
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<tr>
<td>OCLs</td>
<td>Osteoclast-like cells</td>
</tr>
<tr>
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<td>Osteopontin</td>
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<tr>
<td>OPG</td>
<td>Osteoprotegrin</td>
</tr>
<tr>
<td>OSCAR</td>
<td>Osteoclast-associated receptor</td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphatidylinositol-3'-kinase</td>
</tr>
<tr>
<td>PTH</td>
<td>Parathyroid hormone</td>
</tr>
<tr>
<td>TGF</td>
<td>Transforming growth factor</td>
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<tr>
<td>TNF</td>
<td>Tumor necrosis factor</td>
</tr>
<tr>
<td>TRAF</td>
<td>TNF receptor-associated factor</td>
</tr>
<tr>
<td>RANK</td>
<td>Receptor activator of NF-κB</td>
</tr>
<tr>
<td>RANKL</td>
<td>Receptor activator of NF-κB ligand</td>
</tr>
</tbody>
</table>
In all higher organisms, life begins with a single cell, the newly fertilized egg. The process of development gives rise to a complex organism with many specialized cell types. Every specialized cell develops from a not yet specialized precursor, which has the potential to become particular kinds of cells, through the process termed as cell differentiation. To accomplish cell differentiation, different pathways are switched “on” and “off” to coordinately control the production of specific proteins at the correct time and in the correct amounts. Unraveling the fascinating and mysterious processes that achieve the intricate coordination has been one of the fundamental questions of modern biology.

Bone, the material that makes vertebrates distinct from other animals, has evolved over several hundred million years to become a remarkable tissue. Composed of mineralized matrix, bone is a dynamic connective tissue, comprising an exquisite assembly of functionally distinct cell populations required for its function and integrity: the mesenchymal osteoblasts that form the bone and the hematopoietic osteoclasts that resorb the mineralized bone matrix. Understanding the biology of the cells of the bone that make it a living tissue is
crucial to understanding the development, maintenance and repair of the bone tissue and to the treatment of the various bone-related disorders.

Our laboratory uses osteoclasts as a model to study molecular mechanisms and regulation of cell differentiation. Osteoclasts have attracted a great deal of attention among researchers because of their peculiar morphological features and unique functions. Osteoclasts are terminally differentiated cells that are capable of bone resorption (see below). It is now well established that osteoclasts play essential roles not only in normal skeletal development but also in pathological bone destruction such as in osteoporosis, Paget’s disease, rheumatoid arthritis, and bone metastasis. Therefore, regulating osteoclast differentiation or function can be an ideal therapeutic approach for such pathological conditions. For that purpose, it is important to understand the molecular events involved in osteoclast differentiation and activation. In addition to the great importance of their function in the life of an organism, osteoclasts provide us a good model for studies of cell differentiation. Understanding the molecular events implicated in the osteoclast differentiation may allow us to better understand mechanisms of cell differentiation in general.

1.1 THE BONE TISSUE

Bones provide a rigid frame work, known as the skeleton. The human adult skeleton is comprised of 213 bones and composes the largest proportion of the body’s connective tissue mass. Depending on its location, each bone supports one or more specific functions, including structural support and movement, protection of vital organs, and maintenance of mineral homeostasis.
1.1.1 Bone matrix

The bone matrix is physiologically mineralized and is unique in that it is constantly regenerated throughout life as a consequence of bone turnover. The basic building block of the bone matrix fiber network is type I collagen, which composes about 90% of organic matrix and serves as scaffolding to bind and orient other proteins. Noncollagenous proteins compose 10-15% of the total bone protein content and can be broken into several general groups: proteoglycans, glycoproteins and \(\gamma\)-carboxylated proteins. The organic matrix of bone provides elasticity and flexibility to bone and also determines its structural organization. The major component of the inorganic matrix, in the form of hydroxyapatite crystals \([\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2]\), provides mechanical rigidity and load bearing strength to the bone composite (Robey and Boskey, 2006).

1.1.2 Bone structure

There are two types of bone tissue: cortical bone (also known as compact bone) and trabecular bone (also called cancellous bone or spongy bone). Cortical bone is densely packed and makes up approximately 80% of the adult skeleton. Trabecular bone is a honeycomb-like network of interconnected trabecular plates and is surrounded by a shell of cortical bone (Figure 1). The relative proportions of the two types of bone tissue vary considerably among different skeletal sites. About 80-90% of the cortical bone is calcified; only 15-20% of the trabecular bone is calcified, the remaining volume is occupied by bone marrow, blood vessels and connective tissues. From a functional
standpoint, trabecular bone is generally considered to be more metabolically active than is cortical bone, which is best suited for its mechanical role (Dempster, 2006).

Anatomists distinguish between two main types of bone: flat bones (such as the skull, mandible, and scapula) and long bones (such as the femur, tibia, and radius). Long bones consist of a hollow tube (shaft or diaphysis), which flairs at the ends to form the cone-shaped metaphyses, the region below the growth plate, and the epiphyses, the regions above the growth plate (Figure 1). Bones have an outer fibrous sheath called the periosteum, and the inner surface, which directly contacts the bone marrow, is referred to as the endosteum (Dempster, 2006). It is well known that the skeleton serves as the body’s main repository for calcium and plays a key role in homeostasis of serum calcium concentration. Bone also serves as a rich source of growth factors and cytokines that are important for the differentiation and survival of hematopoietic stem cells (Taichman, 2005).

1.1.3 Bone ossification

The skeleton is formed by two modes of ossification: intramembranous and endochondral ossification (Figure 2). Flat bones form primarily via intramembranous ossification and long bones develop by a combination of endochondral and intramembranous ossifications. The bone formed by either intramembrous or endochondral ossification is remodeled by the osteoclasts, which are cells capable of bone resorption. During intramembranous ossification (Figure 2A), mesenchymal cells within a highly vascularized area proliferate and
Figure 1: Schematic view of a longitudinal section through a growing long bone (http://training.seer.cancer.gov/module_anatomy/unit3_4_bone_classification.html).
differentiate into osteoblasts. Osteoblasts first produce a fibrillar, non-mineralized matrix (osteonid), which later becomes mineralized and reorganized into compact bone. Osteoblasts synthesize and deposit bone matrix around themselves. When osteoblasts are embedded in matrix, they differentiate into osteocytes (Franz-Odendaal et al., 2006). Blood vessels present in the trabeculae of this “woven bone” later form the bone marrow cavity.

Most bones are formed by endochondral ossification (Figure 2B). In this process, mesenchymal condensations differentiate into chondrocytes, forming cartilaginous template prefiguring the future skeletal element. Chondrocytes in the center of this cartilage template stop proliferating, becoming prehypertrophic and mature into hypertrophic chondrocytes, which are characterized by an increase in size, vacuolization and secretion of distinct extracellular matrix. Mesenchymal cells surrounding the cartilage element flatten and form the perichondrium, a condensed multilayered tissue. Blood vessels start to invade perichondrium through osteoclast derived holes made in bony collar and the cartilage template which once devoid of blood vessels is now vascularized. Perichondrial cells that are adjacent to the middle region of the cartilage template differentiate into osteoblasts after receiving signals from hypertrophic chondrocytes (Hartmann, 2006). The osteoblasts then form a collar of compact bone around the diaphysis. At the same time, the cartilage in the center of the diaphysis begins to disintegrate due to the apoptosis of entrapped chondrocytes. Osteoblasts penetrate the disintegrating cartilage and replace it with spongy
Figure 2: Schematic diagram of bone formation through intramembranous ossification (A) (Hartmann, 2006) and endochondral ossification (B) (Gibert, 2000).
bone. After spongy bone is formed in the diaphysis, osteoclasts which are bone resorption cells, break down the newly formed bone to open up the medullary cavity. This forms a primary ossification center. Ossification continues from this center toward the ends of the bones. The cartilage in the epiphyses continues to grow and secondary ossification centers form in the epiphyses. Ossification in the epiphyses is similar to that in the diaphysis except that the spongy bone is retained instead of being broken down to form a medullary cavity (Gibert, 2000).

1.1.4 Bone modeling and remodeling

Each bone is sculpted by modeling and is constantly renewed by a process termed remodeling, beginning in embryogenesis and continuing throughout the lifetime of mammals and other animals. The term bone modeling describes the process whereby bones are shaped or reshaped by independent action of osteoblasts and osteoclasts. This occurs, for example, during growth or in the adult to change the shape of the bone in response to mechanical loads (mechanical adaptation) (Dempster, 2006). In adult humans, bone modeling occurs less often than bone remodeling, particularly in cancellous bone, but it does take place in normal subjects and may be increased in certain diseases like hypoparathyroidism and renal bone disease (Kobayashi et al., 2003; Ubara et al., 2003; Ubara et al., 2005).

The adult skeleton is always in a dynamic state and about 10% of bone in our body is replaced every year. Bone remodeling is distinguished from modeling by the fact that bone formation is tightly coupled to prior bone resorption. Bone remodeling provides a mechanism for preservation of bone
strength by replacing old fatigued bone by new mechanically sound bone, and thus plays a key role in bone maintenance and function.

Bone remodeling is achieved by the collaborative and sequential efforts of a group of bone cells that are collectively termed the bone remodeling unit (BRU) (Dempster, 2002). There are four distinct phases in the remodeling cycle: activation, resorption, reversal, and formation (Figure 3) and each phase has a different duration (Figure 3). Activation describes the initiation event that transforms a previously quiescent bone surface into a remodeling one, which involves recruitment of cells of the monocyte-macrophage lineage from the circulation (Roodman, 1999), infiltration of the bone lining cell layer, and fusion of the mononuclear cells to form multinucleated osteoclasts. The resorption phase is carried out by activated osteoclasts and ends with osteoclast apoptosis (Reddy, 2004). During reversal, the resorption lacuna is populated by mononuclear cells, osteocytes that have been liberated from the bone by osteoclasts, and preosteoblasts that are newly recruited to begin the formation phase of the cycle (Baron et al., 1980; Tran Van et al., 1982). It is during the reversal phase that all important coupling signals are sent out to summon osteoblasts into the resorption cavities to replace the bone that has been removed by osteoclasts. Without an efficient coupling mechanism, each remodeling transaction would result in net loss of bone. The exact nature of the coupling signals in currently undefined, but there are a number of hypotheses (Burr, 2002; Parfitt, 2002; Martin and Sims, 2005). Formation is a two-step process in which the osteoblasts initially synthesize the organic matrix and then
Figure 3: Schematic illustration of (A) bone remodeling sequences and (B) duration of various phases of bone remodeling (Baron, 1999). I: osteoclastic resorption; II: reversal of resorption; III: preosteobast migration and differentiation to mature osteoblast; IV: osteoblastic matrix formation; V: mineralization of bone matrix.
regulate its mineralization. The difference between the volume of bone removed by the osteoclasts and that replaced by the osteoblasts is referred to as the “bone balance”. The rate of remodeling and bone balance in each remodeling unit differs depending on anatomical location and also as a function of age and in disease state. Knowledge of the fundamental principles of remodeling provides an excellent framework for understanding age-related changes in bone structure and geometry, as well as the pathogenesis of metabolic bone diseases and the effects of drugs used to treat them (Dempster, 2006).

1.2 CELLS IN BONE

Osteoblasts, osteocytes, and osteoclasts are the three main cell types in the bone and make the bone tissue a living mass.

1.2.1 Osteoblasts

Osteoblasts, the cells responsible for secretion of the bone matrix, are of mesenchymal origin. The bone microenvironment supports a continual supply of osteoprogenitor cells. The bone marrow stroma contains cells with robust proliferation potential that can form single colonies (CFU-Fs) with the capacity to form bone, cartilage, adipocytes, and fibrous tissue (Pittenger et al., 1999; Jiang et al., 2002). These CFU-Fs are now commonly referred as mesenchymal stem cells (MSCs) and are distinguished from the hematopoietic stem cells (HSCs). Commitment of MSCs to tissue-specific cell types is orchestrated by morphogens, developmental signaling pathways, and transcriptional regulators (Aubin et al., 2006).
The canonical Wnt/β-catenin pathway plays an important role in osteoblast lineage differentiation, especially in controlling the differentiation of the common progenitors into osteoblast lineages vs. chondrogenic lineage (Hartmann, 2006). In the canonical Wnt/β-catenin pathway, binding of the extracellular Wnt cytokines to Frizzled receptors and LRP 5/6 co-receptors on cells stimulates intracellular events that prevent proteolytic degradation of β-catenin in the cytoplasm. This stable β-catenin can enter the cell nucleus and control transcription of downstream target genes (Logan and Nusse, 2004). In this case, Wnt signaling induces the expression of genes that are required for osteoblastic cell differentiation and inhibits transcription of genes needed for chondrocytic differentiation. Transcription factors Cbfa1/Runx2, Osterix, and Atf4 are critical for the osteoblast lineage (Nakashima and de Crombrugghe, 2003; Kobayashi and Kronenberg, 2005), whereas Sox9, together with its targets Sox5 and Sox6, is required for chondrogenic lineage (Figure 4) (Lefebvre et al., 2001; Akiyama et al., 2004). Multiple skeletal defects are also manifested in mice with mutations in Wnts, LRP5, or the downstream transcriptional regulator (Kato et al., 2002; Day et al., 2005; Hill et al., 2005; Holmen et al., 2005). Bone morphogenetic proteins (BMPs), BMP-2, BMP-4, and BMP-7 are potent inducers of osteogenesis in vivo and in vitro (Canalis et al., 2003) and are currently being used clinically to augment fracture repair (Seeherman and Wozney, 2005). In addition, osteoblasts respond to osteotropic hormones, including parathyroid hormone (PTH), 1,25 dihydroxyvitamin D3, and glucocorticoids, such as dexamethasone (Aubin et al., 1988; Beresford et al., 1994; Cheng et al., 1994).
Figure 4: Role of canonical Wnt-β-catenin signaling during lineage differentiation and maturation of osteoblasts (Hartmann, 2006). (A) β-catenin negatively regulates the differentiation of mesenchymal cells into a common skeletal precursor. High levels of β-catenin are required to induce genes critical for osteoblast differentiation, whereas suppress the chondrogenic potential of uncommitted progenitors. (B) Wnt signaling is also required for the expansion committed osteoprogenitors.
The active mature osteoblast on the bone surface is distinguished by its morphological and ultrastructural properties, having a large nucleus, enlarged Golgi, and extensive endoplasmic reticulum. The osteoblast expresses high level of alkaline phosphatase, one of the earliest markers of the osteoblast phenotype, and secretes type I collagen and specialized bone matrix proteins toward the bone forming front. On quiescent bone surfaces, single layers of flattened osteoblasts or bone lining cells are observed and they are in close contact with each other (Aubin et al., 2006). The temporal expression of proteins involved in extracellular matrix biosynthesis and matrix mineralization provides a panel of osteoblast phenotypic markers that reflect stages of osteoblast differentiation.

1.2.2 Osteocytes

During the terminal stage of differentiation, osteoblasts can transform into osteocytes embedded in mineralized bone matrix. Osteocytes are by far the most abundant cellular component of mammalian bone, making up to 95% of all bone cells (Marotti, 1996). Osteocytes can live for long periods, even decades in healthy human bone. A distinguishing morphological feature of osteocytes is the location of each osteocyte in lacunae and the numerous cellular extensions of filapodia processes that lie in canaliculi (Palumbo et al., 1990).

Osteocytes communicate with each other and surface osteoblasts through their cellular processes and function as strain and stress sensors, responding to physiological signals that are very important for maintaining bone structure. They form a continuum by connection at the tip of their cell processes
through gap junctions principally comprised of Connexin 43. Gap junction formation is essential for osteocyte maturation, activity, and survival (Furlan et al., 2001; Schiller et al., 2001; Plotkin et al., 2002). Thus, bone cells form a functional network within which cells at all stages of bone formation from preosteoblasts to mature osteocytes remain connected. Osteocytes have the capacity to synthesize certain matrix molecules and regulate the deposition of mineral to maintain the necessary surrounding barrier of bone fluid in their lacunae and canalicular network for diffusion of physiological elements. They also function in osteolysis and contain lysosomal vacuoles and other features of phagocytic cells (Aubin et al., 2006).

1.2.3 Osteoclasts

1.2.3.1 Morphology of the osteoclasts

Osteoclasts are giant multinuclear cells that contain 2-20 nuclei per cell and can reach a size up to 100 µm in diameter (Lucht, 1980). Osteoclasts are found on the surface of the bone and can take many shapes. They are usually rare cells in bone, except for the sites of active bone remodeling (Meunier et al., 1980).

Ultrastructurally (Figure 5), the osteoclast has abundant Golgi complexes located in the perinuclear area, numerous and pleomorphic mitochondria and a large number of lysosomes and transport vesicles loaded with lysosomal enzymes (Holtrop and King, 1977). The cytoplasm contains dense granules, and the nuclei are usually centrally located but very polymorphic
Figure 5: Transmission electron micrograph of a rat osteoclast (Mulari et al., 2003). The sealing zone (SZ) surrounds the convoluted ruffled border (RB). Adjacent to the ruffled border, a number of electron translucent vacuoles (v) are seen. N, Nucleus. White bar=1µm.
and there are normally one or two nucleoli per nucleus. The rough endoplasmic reticulum (ER) is present in small quantity and free ribosomes are abundant and can occur as single ribosomes or polyribosomes (Lucht, 1980). The most prominent ultrastructural feature of osteoclasts is the ruffled borders, which are comprised of a series of finger-like cytoplasmic projections of the plasma membrane adjacent to the bone. The ruffled border is entirely surrounded by an annular zone rich in contractile proteins and this annular zone is called the “sealing zone” to essentially seal off the sub-osteoclastic bone-resorbing compartment from the rest of the bone microenvironment (de Saint-Georges et al., 1989).

1.2.3.2 Bone resorption by osteoclast

Osteoclasts have developed efficient and unique machinery for dissolving mineral and degrading organic bone matrix. Bone resorption requires a sequence of cellular events (termed the bone resorption cycle): migration of the osteoclast to the resorption site, its attachment to bone, polarization and formation of new membrane domains, degradation of bone matrix, removal of degradation products from the resorption lacuna, and finally either apoptosis of the osteoclast or their return to the non-resorbing stage (Vaananen et al., 2000).

**Attachment of osteoclasts to the bone**

The initial step of bone resorption is the attachment of an osteoclast to the bone matrix and the process is facilitated by integrins (Teitelbaum, 2006). Integrins are transmembrane αβ heterodimeric receptors that mediate cell/cell and cell/matrix recognition (Hynes, 1992). At least five different integrins are
expressed in osteoclasts: \( \alpha_v\beta_3, \alpha_v\beta_5, \alpha_2\beta_1, \alpha_v\beta_1, \) and \( \alpha_9\beta_1 \) (Nesbitt et al., 1993; Rao et al., 2006). Several lines of evidence have shown that \( \alpha_v\beta_3 \) is the principal osteoclast integrin. \( \alpha_v\beta_3 \) is highly expressed in osteoclasts and recognizes the arginine-glycine-aspartic acid (RGD) motif. This motif is present in a number of bone-residing proteins, such as osteopontin (OPN), bone sialoprotein, and fibronectin (Flores et al., 1992; Flores et al., 1996). In addition, antibodies against \( \alpha_v\beta_3 \) and RGD-containing peptides are effective inhibitors of bone resorption both \textit{in vitro} and \textit{in vivo} (Horton et al., 1991; Fisher et al., 1993; King et al., 1994). \( \beta_3 \) knockout (\( \beta_3^{-/-} \)) mice have been generated and these mice demonstrated an age-dependent increase in bone mass, consistent with osteoclast dysfunction. Osteoclasts derived from \( \beta_3^{-/-} \) mice have a crenated appearance indicative of a cytoskeletal abnormality. These cells fail to spread, form actin rings and lack resorptive activity when placed on dentin slices (McHugh et al., 2001). Very recent results have demonstrated that a novel osteoclast integrin \( \alpha_9\beta_1 \) also regulates osteoclast formation and function (Rao et al., 2006).

The cytoskeletal abnormalities present in \( \beta_3 \)-deficient osteoclasts have been suggested to result from insufficient Rho GTPase activation in response to growth factors such as macrophage colony-stimulating factor (M-CSF, also called CSF-1) (Faccio et al., 2003). It is well established that the Rho family of GTPase, which include RhoA, Rac, and CDC42, plays an important role in mediating cytoskeletal organization (Machesky and Hall, 1997). Guanine nucleotide exchange factors (GEFs) are mediators of Rho GTPases, stimulating exchange of GDP for GTP (Schmidt and Hall, 2002). Notably, Vav3, a member of Vav
family of Rho GEFs, has been shown to be essential for stimulating osteoclast activation. Vav3-deficient mice have increased bone mass and are protected from bone loss induced by systemic bone resorption stimuli such as PTH and RANKL. In addition, Vav3-deficient osteoclasts show defective actin cytoskeleton organization, polarization, spreading, and resorptive activity resulting from impaired signaling downstream of the CSF-1 receptor (C-FMS) and αvβ3 integrin (Faccio et al., 2005).

**Polarization of resorbing osteoclasts**

Osteoclasts become activated following attachment to the bone surface. Activated osteoclasts are highly polarized cells and remain so during the resorption process (Figure 6) (Teti et al., 1991). The resorption function is dependent on the organization of the actin cytoskeleton into the sealing zone, a unique structure only seen in resorbing osteoclasts. 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” (Lakkakorpi and Vaananen, 1991; Teti et al., 1991; Jurdic et al., 2006). Calcitonin, a peptide known to inhibit osteoclast resorption, induces a rapid destruction of the actin ring, which supports the importance of this unique structure for osteoclast function (Lakkakorpi and Vaananen, 1990).

Resorbing osteoclasts contain not only the sealing zone but also other membrane domains: a ruffled border, a functional secretory domain and a basolateral membrane (Figure 7). The ruffled border is a resorbing organelle,
Figure 6: Schematic representation of a non-polarized (A) and a polarized (B) osteoclast (Teti et al., 1991).
Figure 7: Membrane domains and trafficking pathways of bone-resorption osteoclast (Vaaninen et al., 2000). BL, basolateral domain; FSD, functional secretory domain (pink); SZ, sealing zone (green); RB, ruffled border (black); RL, resorption lacuna (white). Brown vesicles illustrate vesicular pathways from the trans-Golgi network and the basolateral membrane to RB, and yellow vesicles illustrate the transcytotic route from the RB to the FSD. Vesicular pathways from the trans-Golgi network to the apical (black vesicles) and basolateral (blue vesicles) membrane domains are shown. HA, haemagglutinin; VSV-G, vesicular stomatitis virus G protein.
and it is formed by fusion of intracellular acidic vesicles with the region of plasma membrane. During this fusion process much internal membrane is transferred, and forms long, finger-like projections that penetrate the bone matrix (Vaananen et al., 2000). The ruffled border is unique to osteoclasts and the characteristics of the ruffled border do not match that of any other plasma membrane domain described. Recent results indicate that the functional secretory domain may function as a site for exocytosis of resorbed and transcytosed matrix-degradation products (Nesbitt and Horton, 1997; Salo et al., 1997).

**Bone demineralization and resorption in the resorption lacuna**

Bone matrix degradation involves dissolution of the inorganic component, crystalline hydroxyapatite, and proteolytic cleavage of organic component, mostly type I collagen. During this process, tightly packed hydroxyapatite crystals must be dissolved first to allow proteases access to the organic matrix. It is now generally accepted that the dissolution of mineral occurs in the resorption lacuna with a very acidic environment (Baron et al., 1985). The resorption lacuna is an extracellular space between the ruffled border membrane and the bone matrix, and is sealed from the extracellular fluid by the sealing zone. The low pH (in the range of 4.5-4.9 compared to 7.3-7.5 pH range of the bone fluid) in the lacuna is achieved by the action of ATP-dependent vacuolar proton pumps (V-ATPase) both at the ruffled border membrane and in intracellular vacuoles (Blair et al., 1989; Vaananen et al., 1990; Wang et al., 1992).
A number of functional studies have demonstrated the importance of the V-ATPase in the process of bone resorption. Inhibition of osteoclast proton transport by bafilomycin A1 (a specific inhibitor of bone V-ATPase), or knock-down of V-ATPase expression by antisense oligonucleotides abolishes bone resorption in isolated osteoclast culture (Sundquist et al., 1990; Laitala and Vaananen, 1994). The recent finding that V-ATPase at the ruffled border contains cell-specific subunits has further encouraged development of resorption inhibitors that inhibit the osteoclast proton pump (Hernando et al., 1995; van Hille et al., 1995; Li et al., 1996; Visentin et al., 2000). oc/oc mutant mice, which develop severe lethal osteopetrosis, were found to bear a 1.6kb deletion in 5' part of the 116 kDa subunit of V-ATPase (Scimeca et al., 2000). Osteoclast-like cells derived from oc/oc mice display an abnormal localization of V-ATPase (Nakamura et al., 1997). In addition, targeted deletion of this subunit of V-ATPase in mice resulted in severe osteopetrosis and lack of osteoclast–mediated extracellular acidification (Li et al., 1999). In humans, mutations in the OC116 gene, which is the homologue of the mouse 116 kDa subunit and encodes the a3 subunit of human V-ATPase, result in the condition termed infantile malignant osteopetrosis, characterized by severe osteopetrosis at very early ages (Kornak et al., 2000).

V-ATPase does not generate protons on its own, but requires a supply of protons produced by cytoplasmic carbonic anhydrase II, which is highly expressed in osteoclasts from various species (Gay and Mueller, 1974; Zheng et al., 1993). Carbonic anhydrase II (CA II) catalyzes the reversible hydration of
CO₂ (CO₂+H₂O => HCO₃⁻+H⁺) (Sly et al., 1991). Excess cytoplasmic bicarbonate is removed via the chloride-bicarbonate exchanger located in the basolateral membrane (Hall and Chambers, 1989). Accordingly, a high number of chloride channels are present at the ruffled border, which allow a flow of chloride anions into the resorption lacuna to maintain electroneutrality (Schlesinger et al., 1997).

After solubilization of the mineral phase, several enzymes are secreted by osteoclasts to degrade the organic matrix. Although the exact sequence of events at the resorption lacuna is still obscure, acid phosphatase 5 (Acp5), also known as Tartrate Resistant Acid Phosphatase (TRAP), and two major classes of proteolytic enzymes, lysosomal cysteine proteinases (Cathepsins) and matrix metalloproteinases (MMPs) have been shown to play important roles in the resorption process (see below).

**Removal of degradation products from the resorption lacuna**

After matrix degradation, the degradation products are removed from the resorption lacuna through a transcytotic vesicular pathway (Figure 8). Microscopic studies showed that the degraded bone proteins, as well as inorganic bone matrix components, were endocytosed through the ruffled border and then transcytosed in vesicles across the osteoclast to the functional secretory domain, where they are liberated into the extracellular space (Nesbitt and Horton, 1997; Palokangas et al., 1997; Salo et al., 1997; Mulari et al., 2003). However, the characteristics of transcytotic vesicles and the signaling events involved in the transcytosis remain to be defined.
Figure 8: Transcytosis pathways to remove the degradation products in osteoclasts (Mostov and Werb, 1997).
1.2.3.3 Enzymes important for osteoclast function

In addition to morphological features, osteoclasts also contain high levels of several enzymes that distinguish them from other cell types. Cathepsin K (CTSK), acid phosphatase 5 (ACP5), matrix metalloproteinase 9 (MMP-9), and carbonic anhydrase II (CA II) are regarded as marker enzymes of osteoclast differentiation and activity. The expressions of these marker enzymes are greatly induced during osteoclast differentiation and activation.

**Cathepsin K (CTSK)**

Cathepsin K, a member of the cathepsin family of lysosomal cystein proteinase, plays a critical role in normal bone resorption. Indeed, cathepsin K is the major weapon in the armamentarium of the osteoclast. Cathepsin K is secreted by bone-resorbing osteoclasts and activated in an acidic bone microenvironment to efficiently degrade type I collagen, the most abundant extracellular protein in bone matrix (Inaoka et al., 1995). Cathepsin K is preferentially and highly expressed in the osteoclasts from all species that have been studied (Tezuka et al., 1994b; Inaoka et al., 1995; Yamaza et al., 1998; Xia et al., 1999). During mouse embryogenesis, cathepsin K expression is restricted to osteoclasts at sites of active cartilage and bone modeling, whereas osteoblasts and osteocytes do not express either the transcript or the protein (Dodds et al., 1998). Besides high level of cathepsin K, osteoclasts also contain lower levels of cathespin B, D, L (Goto et al., 1994).

A number of studies have suggested the essential role of cathepsin K in bone resorption. Nonsense, missense, and stop codon mutations in the
cathepsin K gene have been identified in patients with pycnodysostosis, an autosomal recessive osteochondrodysplasia characterized by short stature and osteosclerosis (Gelb et al., 1996; Johnson et al., 1996; Hou et al., 1999). Cathespsin K knock out mice develop osteopetrosis and display features characteristic of pycnodysostosis. In these mice, bone demineralization by osteoclasts is intact, whereas bone matrix degradation is markedly diminished. Furthermore, osteoclasts isolated from cathepsin K null mice exhibit impaired bone resorption in vitro (Saftig et al., 1998; Gowen et al., 1999). Cathepsin K specific inhibitors markedly reduce bone resorption by osteoclasts in vitro and in vivo (Votta et al., 1997; Xia et al., 1999; Stroup et al., 2001). Since cathepsin K plays such an important role in the bone degradation and appears to be a limiting step in osteoclastic bone resorption, modulation of its expression and activity are very attractive goals to treat bone-related disorders caused by excess bone resorption. Indeed, a number of pharmaceutical firms are actively developing inhibitors of cathepsin K enzymatic activity and are conducting clinical trials to use these agents to treat osteoporosis.

**Acid phosphatase 5 (ACP5)/TRAP**

Acid phosphatase 5 (ACP5), also known as Tartrate-Resistant Acid Phosphatase (TRAP), is highly expressed in osteoclasts and regarded as the classic marker enzyme for osteoclast differentiation. ACP5 enzyme is present in lysosomes, Golgi apparatus, extracellular channels of the ruffled border and the resorption lacunae (Lucht, 1971; Minkin, 1982). Previous studies have demonstrated the importance of ACP5 in bone resorption. Mice lacking ACP5
showed disrupted endochondral ossification with decreased resorptive activity of osteoclast (Hayman et al., 1996). Osteoclasts derived from these mice exhibited altered ruffled borders and disturbed intracellular vesicle transport (Hollberg et al., 2002), Transgenic mice overexpressing ACP5 were associated with mild osteoporosis with increased bone turnover (Angel et al., 2000). In addition, treatment with antibodies against ACP5 reduced bone resorption activity of osteoclast in vitro (Zaidi et al., 1989; Moonga et al., 1990).

The precise role of ACP5 in osteoclast is not fully understood yet and different functions have been suggested for ACP5. A potential function of ACP5 in the osteoclast might be regulation of the biological activity of bone matrix phosphoproteins like osteopontin and bone sialoprotein by their dephosphorylation (Ek-Rylander et al., 1994; Suter et al., 2001; Andersson et al., 2003). ACP5 has also been shown to generate reactive oxygen species (ROS) capable of degrading collagen and other bone matrix proteins. This activity, together with the co-localization of ACP5 and collagen fragments in transcytotic vesicles, suggests a role of ACP5 in further destruction of matrix-degradation products in the transcytotic vesicles (Halleen et al., 1999). Very recently, it has been reported that cathepsin K increases ACP5 catalytic activity through excising the repressive loop domain of ACP5 (Ljusberg et al., 2005).

Apart from osteoclasts, some reports suggest ACP5 expression may be found in other cells including osteoblasts, osteocytes, bone marrow macrophages, and dendritic cells (Bianco et al., 1987; Bianco et al., 1988; Ek-Rylander et al., 1991; Hayman et al., 2000; Hayman et al., 2001). Interestingly,
not all osteoblasts appear to contain ACP5 activity and the highest activity was found in osteoblasts in close vicinity to the osteoclasts or their precursors, suggesting that ACP5 activity in osteoblasts might be regulated by osteoclasts (Yamamoto and Nagai, 1998; Perez-Amodio et al., 2004). In addition, osteoblastic ACP5 differs from the osteoclastic enzyme, including a distinct pI (isoelectric point) and substrates (Yamamoto and Nagai, 1998; Lau and Baylink, 2003). Contrary to ACP5 in osteoclasts, which is secreted by bone-resorbing osteoclasts, osteoblastic ACP5 is an intracellular enzyme. ACP5 is endocytosed and subsequently inactivated by osteoblast-like cells (Perez-Amodio et al., 2005; Perez-Amodio et al., 2006), thus providing a possible mechanism to control ACP5 activity in osteoblasts to prevent excess bone matrix degradation.

**Matrix Metalloproteinase 9 (MMP-9)**

Another marker of osteoclasts is MMP-9, also known as gelatinase B (Okada et al., 1995). MMP-9 can cleave the $\alpha_2$-chain of type I collagen and collagen types III, IV, and V, as well as gelatins. High levels of MMP-9 are found in osteoclasts from various species studied (Reponen et al., 1994; Tezuka et al., 1994a; Wucherpfennig et al., 1994; Okada et al., 1995). Expression of other MMPs were also detected in osteoclasts, though at a much lower level than MMP-9 (Kusano et al., 1998). Selective inhibitors of gelatinase A and B inhibited interleukin-1 (IL-1) stimulated bone resorption by isolated rat osteoclasts (Hill et al., 1995). Expression of MMP-9 in mouse calvaria culture was upregulated by IL-1 and IL-6, cytokines that are known to stimulate bone resorption (Kusano et al., 1998). However, MMP-9 knock out mice exhibit only mild osteopetrosis that
resolves with age and this phenotype could be corrected by bone marrow transplantation from wild type mice. Growth plates of the long bones in MMP-9 deficient mice had an abnormal expansion of the layer of hypertrophic chondrocytes, which presumably resulted in delayed endochondral ossification (Vu et al., 1998). It was also shown that the resorption activity in the osteoclasts from MMP-9 null mice was comparable to that of wild type mice, but MMP-9 deficiency resulted in the delayed invasion of osteoclasts into calcified cartilage (Engsig et al., 2000).

**Carbonic anhydrase II (CA II)**

Among 13 known carbonic anhydrases (CAs), CA II is the one with highest catalytic activity and is expressed at high levels in osteoclasts (Gay and Mueller, 1974; Baird et al., 1997). In addition, it was shown that active osteoclasts express higher levels of CA II than resting osteoclasts (Asotra et al., 1994).

As mentioned in previous sections (Chapter 1.2.3.2), CA II plays an important role in bone resorption by supplying protons that are transported into the resorption lacuna to achieve a very acidic microenvironment (Sly et al., 1991; Lehenkari et al., 1998). Inhibition of CA II activity or expression resulted in decreased bone resorption by osteoclasts *in vitro* (Minkin and Jennings, 1972; Laitala and Vaananen, 1994). CA II deficiency in human is a rare autosomal recessive disorder of osteopetrosis with renal tubular acidosis and brain calcification (Sly et al., 1983). Bone marrow transplantation corrects osteopetrosis in the CA II deficiency syndrome (McMahon et al., 2001).
Surprisingly, CA II null mice do not exhibit an osteopetrotic phenotype (Lewis et al., 1988). This could be explained by the compensation of the lack of CA II by other members of CAs or by differences in bone metabolism between mouse and human.

1.2.3.4 Origin and differentiation of osteoclasts

Hematopoietic origin of the osteoclast

Since the first description of osteoclasts by Albert Kölliker in 1873 (Kölliker, 1873), the origin of osteoclasts has been a highly debated topic, with the earliest theory of both osteoclasts and osteoblasts from a common mesenchymal progenitor (Young, 1962). It is now well established that osteoclasts are of hematopoietic origin. This conclusion was reached mainly from several decisive investigations conducted during the 1960s and 1970s.

Early studies utilizing $[^{3}H]$-thymidine labeling of cells showed that blood monocytes, but not mesenchymal cells, fused to form multinuclear osteoclasts in newts and rats (Fischman and Hay, 1962; Gothlin and Ericsson, 1973). These studies were followed by the chick-quail chimera experiments, in which osteoclast-free bone rudiments of quails were grafted onto the chorioallantoic membrane of chicken embryos. It was found that majority of the osteoclasts appearing on the bone rudiments were of chick origin, whereas all of the osteoblasts in the rudiments were of quail origin (Kahn and Simmons, 1975), thus confirming the extraskeletal origin of osteoclasts. Further evidence that osteoclasts are derived from hematopoietic origin has come from the pioneering
work by Walker using osteopetrotic mice. These original experiments demonstrated that osteopetrotic features in the gray lethal (gl), microphthalmia (mi) mutant mice and ia rats could be cured after its parabiotic union to a normal littermate (Walker, 1972; Walker, 1973; Marks, 1976). These findings suggest that during parabiosis, progenitors of osteoclasts were recruited from the normal littermate via the blood stream. Walker further reported that transplantation of bone marrow or spleen cells from gl/gl and mi/mi mice into irradiated normal animals resulted in the emergence of osteopetrosis in donor mice, on the other hand, transplantation of spleen or bone marrow cells from normal mice could restore bone resorption in osteopetrotic gl/gl and mi/mi mice (Walker, 1975b; Walker, 1975a; Walker, 1975c). Subsequently, it was shown that transfer of bone marrow cells or spleen cells from beige (a non-osteopetrotic mutation) mice to mi/mi mice led to new osteoclast formation all with giant lysosomal inclusions, typical of osteoclasts from beige donors (Ash et al., 1980). In humans, bone marrow transplantation can correct infantile malignant osteopetrosis, juvenile osteopetrosis and osteopetrosis due to carbonic anhydrase II deficiency (Coccia et al., 1980; Sorell et al., 1981; McMahon et al., 2001). All these results provide convincing evidence that osteoclasts are cells of hematopoietic origin.

**Osteoclast precursors**

The overwhelming majority of studies demonstrate that the osteoclast precursor is in the monocyte-macrophage lineage. Using an *in vivo* model of osteoclast differentiation, Baron et al. demonstrated by histological techniques that mononuclear cells containing non-specific esterase (NSE), an enzyme
present in monocytes, increased in number locally and attached to the bone surface. As they differentiated, these cells started to express Acp5/TRAP, a marker enzyme of osteoclasts, and eventually lost their non-specific esterase activity and formed multinucleated osteoclasts. The presence of cells that were stained for both enzymes implied that these cells were steps in a single differentiation process (Baron et al., 1986).

Several other studies have demonstrated that peripheral blood monocytes or macrophages, when labeled with charcoal particles, thorotrast, $[^3H]$-thymidine, or Barr bodies, gave rise to multinucleated osteoclasts containing the respective labels (Jee and Nolan, 1963; Gothlin and Ericsson, 1973; Tinkler et al., 1981; Stanka and Bargsten, 1983). It was also reported that peripheral blood monocytes could fuse with purified avian osteoclasts (Zambonin Zallone et al., 1984; Zambonin Zallone and Teti, 1985). In addition, purified avian monocytes, but not mature macrophages, could fuse with osteoclasts in vitro (Marchisio et al., 1984). Burger et al. were the first group to demonstrate the formation of osteoclasts from marrow cells in vitro. In their studies, multinuclear osteoclasts formed when fetal bone rudiments lacking osteoclasts were cocultured with plasma clots in the presence CSF-1, therefore, identifying the precursor for these osteoclasts as a cell in the monocytic lineage (Burger et al., 1982). More recent studies illustrated that a monoclonal antibody recognizing macrophages and macrophage polykaryones also cross-reacted with osteoclasts and macrophage antigens were also expressed on osteoclasts (Oursler et al., 1985; Athanasou et al., 1988). Finally, Kurihara et al. have shown that
Figure 9: Osteoclasts are cells of hematopoietic origin.
osteoclast-like cells formed in human marrow cultures using cells derived from highly purified population of CFU-GM, the granulocyte-macrophage progenitor (Kurihara et al., 1990b). Another group has also shown that murine marrow enriched for CFU-GM gave rise to osteoclasts (Schneider and Relfson, 1988). Taken together, these data strongly support that osteoclast precursors are derived from cells in monocyte-macrophage lineage, possibly with CFU-GM as the earliest recognizable progenitor (Figure 9).

**In vivo studies of osteoclast differentiation**

As mentioned previously, Baron and co-workers developed an *in vivo* model to investigate the kinetics of osteoclast formation in the rat in which the formation of multinucleated osteoclasts was induced at a predictable site (Baron et al., 1986). In this study, bone resorption was induced by extracting the upper right row of molars in adult rats. The lack of opposing teeth induced bone remodeling along the periosteum of the mandible. The animals were then sacrificed in groups, every 24 h, and the mandibles were analyzed. It was observed that the number of mononuclear cells expressing non-specific esterase (NSE), an enzyme present in monocytes, increased 1 day after induction of bone remodeling and peaked at 2 days after induction. Between 2 days and 3 days after induction, the number of NSE positive mononuclear cells markedly decreased and returned to the control levels by 5 days. TRAP positive mononuclear cells appeared after 1 day and their numbers peaked by day 2 and remained at maximum levels even at 5 days after induction. The first TRAP
positive multinuclear osteoclasts occurred after 3 days of induction. These results indicated that by day 3 the precursors stopped proliferating and started to fuse into multinuclear cells. It was also observed that at day 2, the majority of cells positive for TRAP were found closer to the bone surface, while most of the NSE positive cells were closer to the vascular layer of the periosteum. Cells positive for both were in a layer in between, suggesting expressing NSE and TRAP were sequential events during osteoclast formation (Baron et al., 1986).

The in vivo development of osteoclasts was studied in embryonic mouse metatarsal bones (Scheven et al., 1986). This study showed that TRAP positive mononuclear cells were absent in day 15 embryonic bone and appeared in the periosteum on embryonic day 17, followed by an increase in the number of TRAP positive multinuclear osteoclasts and a corresponding decrease in the number of mononuclear TRAP-expressing cells. On day 18, TRAP positive multinuclear osteoclasts invaded the bone rudiment and started to resorb the calcified cartilage matrix, resulting in the formation of the bone marrow cavity (Scheven et al., 1986).

Uy et al. have also reported an in vivo model to examine the mechanism of action of osteotropic factors such as IL-1 and PTH on various stages of osteoclast formation (Uy et al., 1995a; Uy et al., 1995b). In this model system, mice were either injected with a factor or implanted with Chinese hamster ovary cells that have been transfected with the cDNA for the factor and constitutively expressed the factor of interest.
These *in vivo* studies have provided important insights into the sequence of osteoclast differentiation and the site of various cytokines involved in normal bone remodeling. However, these techniques are extremely tedious, and it is difficult to isolate cells from these preparations for additional studies.

**In vitro model systems for osteoclast differentiation**

Progress in understanding the molecular events that occur during osteoclast differentiation and osteoclastic bone resorption has been difficult because osteoclasts are few in number, are extremely difficult to isolate because they are embedded in a calcified matrix, and are fragile, and may not viable after isolation from bone. Though osteoclasts were discovered in 1873, only within the last decade have the molecular details of osteoclast differentiation begun to emerge. Along with mouse genetic approaches, primary cell culture systems to efficiently induce osteoclast differentiation *in vitro* play a key role in unraveling the molecular mechanisms involved in osteoclast differentiation and function.

Testa and coworkers were the first to use feline bone marrow cultured in α-minimum essential medium with horse serum in the absence of any osteotropic factors to form osteoclast-like cells (Testa et al., 1981). Ibbotson et al. further characterized this culture system and demonstrated that the precursors for these cells were cells in the monocyte-macrophage lineage and osteotropic factors, such as vitamin D3, PTH and prostaglandin E2 (PGE$_2$), could enhance formation of these osteoclast-like cells in co-culture system (Ibbotson et al., 1984). Since those initial studies, marrow cells from baboons (Roodman et al., 1985), humans
(MacDonald et al., 1987), rabbits (Fuller and Chambers, 1987) and mice (Takahashi et al., 1988b) have been used for examination of osteoclast-like cells formation from their progenitor cells.

Takahashi et al. demonstrated that osteoclast-like cells can be obtained from mouse marrow culture within 5-6 days in the presence of vitamin D$_3$ or PTH. Interestingly, more than 90 % of TRAP positive mononuclear cell clusters and multinuclear cells were found near colonies of alkaline phosphatase (a marker of osteoblast) positive cells, indicating osteoblasts might play an important role in osteoclast formation (Takahashi et al., 1988b). They also showed that numerous osteoclast-like cells formed in a co-culture system with mouse spleen cells and osteoblastic cells isolated from mouse calvariae in the presence of vitamin D$_3$. Neither the same co-cultures without the vitamin D$_3$ nor isolated cultures containing either spleen cells or osteoblastic cells with the vitamin D$_3$ produced TRAP-positive cells. In addition, when spleen cells and osteoblastic cells were cultured separately from each other by a membrane filter (0.45 micron), no TRAP-positive cells were formed (Takahashi et al., 1988a). These results indicate osteoblasts and cell-cell contact with osteoblasts are required for the differentiation of osteoclast progenitors in splenic tissues into multinucleated osteoclasts. Udagawa et al. also used mouse spleen cells as a source of osteoclast precursors and demonstrated that they could form multinuclear cells when co-cultured with an appropriate bone marrow-derived stromal cell line (MC3T3-G2-PA6 or ST2) or with primary mouse calvarial cells in the presence of dexamethasone and vitamin D$_3$ (Udagawa et al., 1989). These data further
confirm that cell-cell contact with stromal cells or osteoblasts is absolutely required for osteoclastogenesis.

Subsequently, a host of *in vivo* and *in vitro* studies have led to the identification of two factors produced by osteoblasts or by stromal cells that are necessary and sufficient to induce osteoclast formation (Figure 10): CSF-1 (Wiktor-Jedrzejczak et al., 1990; Yoshida et al., 1990) and RANKL (Lacey et al., 1998; Yasuda et al., 1998b). The following sections will detail the roles of these molecules and signaling pathways that regulate osteoclast differentiation.

### 1.3 SIGNALING PATHWAYS INVOLVED IN OSTEOCLAST DIFFERENTIATION

How signaling events are synchronized to produce specific changes in gene expression and differentiation is crucial to understanding osteoclast biology. Osteoclast-specific genes switch from “off” to “on” or vice versa during the process of differentiation. These changes in gene expression are connected directly to receptors through intracellular signaling mechanisms. Osteoclast differentiation requires tyrosine kinase-family and tumor necrosis-family receptors, c-FMS and receptor activator of nuclear factor-κB (RANK) signaling. CSF-1 and RANKL, the ligands for these receptors, are necessary and sufficient for *in vitro* differentiation; however additional signaling events via immune-related tyrosine kinase modules have also been shown to be of importance for osteoclast differentiation.
Figure 10: A schematic representation of osteoclast differentiation and activation supported by osteoblasts/stromal cells (Suda et al., 1999). Osteoblasts/stromal cells provide CSF-1 and RANKL, which are two cytokines necessary and sufficient for osteoclast differentiation.
1.3.1 CSF-1/c-FMS signaling

1.3.1.1 CSF-1 and c-FMS are required for osteoclast differentiation

Colony-stimulating factor-1 (CSF-1) was initially identified as the growth factor-stimulating production of macrophage colonies from bone marrow cells in vitro and is a physiological growth factor for monocyte-macrophage lineage (Stanley et al., 1978; Stanley et al., 1983). The critical role of CSF-1 in osteoclastogenesis was established by examining the osteopetrotic (op/op) mice (Yoshida et al., 1990; Wiktor-Jedrzejczak et al., 1991) and toothless (tl/tl) mutant rats (Dobbins et al., 2002; Van Wesenbeeck et al., 2002). The op/op mice are unable to produce functional CSF-1 because of a frameshift mutation in the Csf-1 gene and exhibit severe osteopetrosis due to deficiency of osteoclasts (Yoshida et al., 1990; Takahashi et al., 1991a). The osteopetrosis in op/op mice can be cured by administration of exogenous CSF-1 (Felix et al., 1990; Kodama et al., 1991), confirming the essential role of this cytokine in the differentiation of osteoclasts. The biological effects of CSF-1 are mediated by its homodimeric receptor c-FMS, which is the cellular homologue of the feline transforming virus v-FMS and predominantly expressed in cells of the monocyte-macrophage lineage (Byrne et al., 1981; Sherr et al., 1985; McKinstry et al., 1997). The functional linkage between CSF-1 and its sole receptor is established by the fact that targeted disruption of c-fms expression in mice exhibits the same major phenotypes as the op/op mice (Dai et al., 2002).

CSF-1 is indispensable for both proliferation and differentiation of osteoclast precursors (Tanaka et al., 1993). When murine spleen cells were co-
cultured with osteoblastic cells for six days in the presence of vitamin D$_3$, addition of antibody against CSF-1 or c-FMS, either during the first 4 days or the last 2 days of culture, inhibited osteoclast-like multinucleated cells formation (Tanaka et al., 1993). Marrow culture experiments have shown that if the cultures are treated sequentially with CSF-1 followed by vitamin D$_3$, CSF-1 can induce the proliferation and expansion of the osteoclast precursor pool (Takahashi et al., 1991b).

In addition to its direct effects on osteoclast precursors, CSF-1 may also affect mature osteoclasts. c-FMS, the receptor for CSF-1, is expressed on mature osteoclasts (Weir et al., 1993). Fuller and co-workers have also identified a role for CSF-1 in maintaining the survival and chemotactic behavior of mature osteoclasts. In their studies, CSF-1 prevented apoptosis of osteoclasts, enhanced osteoclast motility, and inhibited bone resorption (Fuller et al., 1993).

As mentioned earlier, CSF-1 is produced by murine stromal cells as well as calvarial osteoblasts and is the factor responsible for their effects on osteoclast formation in co-culture systems. In addition, removal of CSF-1 producing stromal cells from the co-culture system enhanced osteoclast apoptosis (Jimi et al., 1995).

1.3.1.2 Signals mediated by CSF-1/c-FMS during osteoclast differentiation

CSF-1 is required for osteoclast differentiation in vivo as well as in vitro (see above). However, the exact signaling pathways and mechanisms mediated by CSF-1 during osteoclast differentiation remain obscure. A great body of
knowledge about CSF-1 signaling is obtained from the context of macrophage growth and differentiation. It’s likely that some of the signaling pathways involved in macrophage biology are also essential for osteoclast differentiation and function.

C-FMS, the sole receptor for CSF-1, is a member of the tyrosine kinase receptor family. Binding of CSF-1 to the extracellular domain of the c-FMS leads to receptor autophosphorylation on specific tyrosine residues that serve as anchoring sites for signaling molecules with SH2 domains (Figure 11). Activated c-FMS plays a pivotal role in the assembly of the specific multimeric complexes that are involved in cell proliferation, survival, differentiation, and cytoskeletal reorganization (Yeung et al., 1998; Kanagasundaram et al., 1999; Bourette and Rohrschneider, 2000; Ross, 2006). This massive macromolecular complex assembled on activated c-FMS also suggests the downstream signals may be very complicated. PI3K/Akt pathway and Ras/Raf/Mek/Erk1/2 are well established transducers of CSF-1 signaling in macrophage proliferation (Pixley and Stanley, 2004), though their exact roles in osteoclast differentiation remain to be further defined. c-Src also appears to be a mediator of CSF-1 signaling pathway in osteoclast, as evidenced by the fact that Src knock out mice exhibit osteopetrosis (Insogna et al., 1997; Miyazaki et al., 2004). CSF-1 treatment induced tyrosine phosphorylation of c-Src in mouse osteoclasts (Insogna et al., 1997), as well as direct association with engineered c-FMS receptor in fibroblasts (Miyazaki et al., 2004).
Figure 11: Model for major pathways initiated by CSF-1 binding to extracellular domain of c-FMS (Ross and Teitelbaum, 2005).
Additionally, phospholipase C gamma (PLCγ) may also mediate c-FMS signals. Nakamura et al. demonstrated that a PLC inhibitor blocks cell adhesion, CSF-1 mediated cell spreading, and αvβ3 integrin-mediated cytoskeletal reorganization in prefusion osteoclasts derived from c-Src deficient mice, possibly via PLCγ (Nakamura et al., 2001). Very recently, it has been reported that CSF-1 induces stable interaction of c-FMS with αvβ3 integrin in osteoclasts (Elsegood et al., 2006), suggesting integrin signaling and CSF-1 signaling might collaborate to regulate osteoclast function. However, the nature of this interaction remains to be clarified.

1.3.2 RANKL/RANK: central regulators of osteoclast differentiation

1.3.2.1 The molecular triad OPG/RANKL/RANK

The discovery of the molecular triad RANKL/RANK/OPG has revolutionized our understanding of the processes underlying osteoclast formation and activation (Table 1). In 1997 and 1998, two groups independently reported that a secreted tumor necrosis factor (TNF)-related protein with no transmembrane or cytoplasmic domains, termed osteoprotegrin (OPG) or osteoclastogenesis inhibiting factor (OCIF) is responsible for the marked osteopetrosis phenotype when overexpressed in transgenic mice. Complementary experiments revealed that this osteopetrosis is associated with a decrease in osteoclastogenesis and in osteoclast activation (Simonet et al., 1997; Yasuda et al., 1998a). Conversely, OPG deficient mice exhibit severe
osteoporosis (Mizuno et al., 1998), confirming its inhibitory role in osteoclast differentiation and function.

Soon after the identification of OPG, the Amgen group and Yasuda et al. further identified its ligand (Lacey et al., 1998; Yasuda et al., 1998b), initially called OPG ligand (OPGL) or osteoclast differentiation factor (ODF), and now receptor activator of NF-κB ligand (RANKL) as the most commonly used terminology. As Anderson et al. had already identified RANK as the receptor for RANKL (Anderson et al., 1997), the third protagonist of the triad has easily taken its place in bone biology. RANK is a transmembrane protein with a large C-intracytoplasmic domain and an N-extracellular domain and possesses cysteine-rich domains involved in its multimerization. Lacking a cytoplasmic domain, OPG appears to be the decoy receptor of RANKL, thus a natural antagonist of RANKL to abrogate the effect of RANKL on osteoclastogenesis.

RANKL mRNA is expressed at high levels in bone marrow cells and in lymphoid cells. RANKL has been shown to exist in a 40-45 kDa membrane-bound form and in a 30 kDa soluble form (Khosla, 2001). The soluble RANKL can result from the shedding of its membrane form by the metalloprotease-disintegrin TNF-α converting enzyme (Lum et al., 1999). Soluble RANKL was demonstrated to be able to directly bind to the surface of osteoclasts (Lacey et al., 1998; Yasuda et al., 1998b). Both soluble and membrane forms of RANKL expressed by osteoblasts exert their activity through binding to the RANK receptor on osteoclasts (Hsu et al., 1999).
### Table 1: Molecular triad OPG/RANKL/RANK (Suda et al., 1999).

<table>
<thead>
<tr>
<th>Structure</th>
<th>Proposed Nomenclature</th>
<th>Other Nomenclatures</th>
</tr>
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<tbody>
<tr>
<td><strong>Ligand</strong></td>
<td></td>
<td></td>
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<tr>
<td>Extracellular domain</td>
<td>RANKL</td>
<td>ODF</td>
</tr>
<tr>
<td>Receptor activator of NF-κB ligand</td>
<td>OPGL</td>
<td>Osteoprotegerin ligand</td>
</tr>
<tr>
<td>TRANCE</td>
<td>Osteoclast differentiation factor</td>
<td></td>
</tr>
<tr>
<td><strong>Receptor</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Extracellular domain</td>
<td>RANK</td>
<td>OCIF</td>
</tr>
<tr>
<td>Receptor activator of NF-κB</td>
<td>Osteoprotegerin inhibitory factor</td>
<td></td>
</tr>
<tr>
<td><strong>Decoy receptor</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>OPG</td>
<td>TR1</td>
<td>TNF receptor-like molecule 1</td>
</tr>
<tr>
<td>Osteoprotegerin</td>
<td></td>
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</table>
Targeted disruption of RANKL in mice results in a lack of osteoclasts and severe osteopetrosis. The osteoblasts from these mice do not support osteoclast differentiation \textit{in vitro}. However the hematopoietic precursors from these mice are capable of differentiating into multinuclear osteoclasts in the presence of recombinant RANKL and CSF-1, indicating that the osteoclastogenesis defect in RANKL null mice stems from the inability of the osteoblastic/stromal cells to produce RANKL (Kong et al., 1999; Kim et al., 2000). Similar to RANKL deficient mice, RANK knockout mice also develop profound osteopetrosis and lack TRAP positive osteoclast precursors (Dougall et al., 1999; Li et al., 2000). Bone marrow transplantation from wild type mice and retrovirus-mediated introduction of RANK were able to restore osteoclastogenesis in RANK null mice (Dougall et al., 1999; Li et al., 2000). A combination of recombinant soluble RANKL and CSF-1 is sufficient for osteoclast formation from spleen or bone marrow cells and osteoclastic-specific genes expression, which allows osteoclast differentiation \textit{in vitro} in the absence of osteoblastic cells (Lacey et al., 1998; Quinn et al., 1998).

1.3.2.2 RANKL/RANK signaling

The binding of RANKL to the extracellular RANK domain leads to RANK oligomerization and initiation of a series of signaling pathways essential for the differentiation and activation of osteoclasts (Figure 12). The initial step in RANK signaling is formation of a complex with the TNF receptor-associated factor (TRAF) adaptor proteins through the cytoplasmic domain of RANK. Among the six members of the TRAF family, only TRAF6 appears to be critical to osteoclast
differentiation and activation, though TRAF2 and TRAF5 have also been shown to bind RANK (Kim et al., 1999a; Naito et al., 1999; Armstrong et al., 2002; Fuller et al., 2002). TRAF6-deficient mice exhibit severe osteopetrosis, along with defects in osteoclast formation (Lomaga et al., 1999; Naito et al., 1999). RANKL stimulation results in rapid activation of NF-κB signaling, as well as the mitogen-activated protein kinases (MAPK) cascades such as extracellular-signal regulated kinase (ERK), Jun-N-terminal kinase (JNK), and p38 stress kinase pathway. Such activation was severely abrogated in precursor cells from TRAF6 null mice and can be restored by retroviral-mediated introduction of wild type TRAF6 into the TRAF6 null cells (Kobayashi et al., 2001; Wei et al., 2001). Moreover, the phosphatidylinositol-3'-kinase (PI3K)/Akt pathways are also targets of TRAF6 that promote osteoclast survival through the mammalian target of rapamycin (mTOR) (Gingery et al., 2003; Glantschnig et al., 2003).

Similar to TRAF6, NF-κB is critical for osteoclastogenesis, as revealed by NF-κB knock out mice (Iotsova et al., 1997). NF-κB proteins reside in the cytoplasm of nonstimulated cells through association with the inhibitors of NF-κB (IκBs) but rapidly enter the nucleus upon cell stimulation and this process is called NF-κB activation (Ghosh and Karin, 2002). The classic NF-κB signaling pathway involves activation of the IκB kinase (IKK) complex that phosphorylates the IκBs and targets them to ubiquitin-dependent degradation (Ghosh and Karin, 2002). Events leading to NF-κB activation via TRAF6 include the serine/threonine kinase TAK1 (TGFβ activated kinase 1), TAK1 binding protein 2 (TAB2), phosphorylation of the NF-κB-inducing kinase (NIK) and IκB kinases (IKKs)
(Theoleyre et al., 2004). TAK1, along with its binding protein TAB2, have been detected in activated receptor complexes (Lee et al., 2002; Mizukami et al., 2002). Dominant-interfering mutant forms of TAK1 inhibit RANKL-mediated activation of IKKα/β, suggesting that TAK1 is important in activation of NF-κB (Yamamoto et al., 2002).

The IKK complex consists of two catalytic subunits, IKKα and IKKβ, and a regulatory subunit, IKKγ/NEMO (Ghosh and Karin, 2002). A small peptide inhibitor of IKK, which prevents the association of IKKα and IKKβ with IKKγ, was shown to block osteoclastogenesis and prevent inflammation induced bone loss in mice (Jimi et al., 2004). More recently, Ruocco et al. demonstrated by using IKKβ conditional knockout mice an essential role for this IKK-subunit in osteoclast differentiation and survival, both in vitro and in vivo. Loss of IKKβ in hematopoietic cells prevents inflammation-induced bone loss (Ruocco et al., 2005). Although IKKα is required for RANKL-induced osteoclast formation in vitro (Novack et al., 2003), the mutations that prevents IKKα activation by its upstream kinase NIK have no effect on osteoclastogenesis in vivo (Ruocco et al., 2005). These results are consistent with the absence of osteopetrotic phenotype in mice either deficent of NIK (Nik−/−) (Shinkura et al., 1999) or bearing a point mutation in the Nik gene that prevents NIK activation (Novack et al., 2003). However, it was observed that Nik−/− osteoclast precursors do not respond to RANKL in an in vitro differentiation system that is devoid of osteoblasts (Novack et al., 2003).
Figure 12: Schematic representation of RANKL/RANK signaling pathways (Theoleyre et al., 2004).
One of the MAPK cascades activated by RANKL is the JNK pathway, which leads to activation of AP-1 proteins (Darnay et al., 1998; David et al., 2002). In addition, TAK1 and the MAPK-related kinase MKK7 are required for JNK activation by RANKL (Yamamoto et al., 2002). Estrogen has been shown to block osteoclast differentiation by inhibiting RANK-mediated JNK activation as well as c-Jun activation and expression (Shevde et al., 2000; Srivastava et al., 2001). p38 is another MAPK involved in mediating key signals induced by RANKL and apparently activated via phosphorylation by MKK6 (Matsumoto et al., 2000a; Matsumoto et al., 2000b; Li et al., 2002). The RING and the first zinc finger domains in TRAF6 are required for the full activation of p38 MAPK by RANKL/RANK signaling (Kobayashi et al., 2001). Stimulation of p38 results in the downstream activation of the transcription factor MITF (Mansky et al., 2002b), which is critical for multinuclear osteoclast formation and osteoclast specific gene expression (see MITF section). Mansky et al. also showed that addition of SB203580 (p38 MAPK specific inhibitor) blocked the RANKL-mediated phosphorylation of MITF and induction of TRAP gene expression (Mansky et al., 2002b), indicating the importance of signals through p38 cascade in osteoclast function. The ERK-1 kinase is also activated by RANK signaling, and seems to be regulated upstream by activation of MEK1 (Hotokezaka et al., 2002).

The Src kinase, that is required for osteoclast activation, has also been shown to bind to TRAF6 and to allow RANK-mediated signaling to proceed through PI3K/Akt (Wong et al., 1999). It is well known that PI3K and Akt act downstream of Src to induce cell survival, cytoskeletal rearrangements and
motility. The lipid phosphatase SHIP negatively regulates PI3K/Akt signaling and SHIP- deficient mice have osteoporosis (Takeshita et al., 2002).

1.3.3 Cross talk between CSF-1 and RANKL signaling with other signaling pathways

Though it is well accepted that CSF-1 and RANKL are necessary and sufficient for the osteoclast differentiation, recent results have also provide evidence of their interactions with other signaling pathways. An example of a positive cross talk is demonstrated by the effect of TNF-α on osteoclast differentiation. TNF-α can induce osteoclast formation and activation in concert with RANKL via the TNF receptor and TRAF2 and/or TRAF6 (Komine et al., 2001; Zhang et al., 2001). However, TNF-α alone is not sufficient to promote osteoclast precursor differentiation and can only act on cells simultaneously stimulated, or primed, with RANKL (Lam et al., 2000). It is also suggested that TNF-α can induces CSF-1 expression in vivo and CSF-1 actually plays a role in TNF-α induced osteoclastogenesis (Kitaura et al., 2005).

Signaling cross talk between RANKL and interferon (IFN) pathways provides an important example of negative cross talk that limits osteoclast differentiation and function. IFN-γ produced by T-cell strongly suppresses osteoclastogenesis by interfering with the RANKL signaling pathway through downregulating TRAF6, whereas RANKL itself induces IFN-γ in osteoclasts, thus providing a negative feedback loop during osteoclastogenesis (Takayanagi et al., 2000). IFN-β represents another negative feedback of RANKL-induced osteoclastogenesis (Takayanagi et al., 2002b). Hayashi et al. also demonstrated
that IFN-β endogenously produced in osteoclast progenitors suppressed the differentiation towards osteoclasts induced by RANKL and CSF-1 (Hayashi et al., 2002). These data suggest the complex and intimate cross talk between inhibitory and stimulatory cytokines produced by the immune system in osteoclastogenesis. In a commentary article, Arron and Choi (Arron and Choi, 2000) coined the term “osteoimmunology” to describe this new interdisciplinary field of bone biology and immunology.

Very recently, Koga el al. revealed that the immunoreceptor tyrosine-based activation motif (ITAM)-harboring adaptor FcRγ and DAP12 are essential to deliver signals in concert with RANK to activate calcium signaling through PLCγ in osteoclast precursor (Koga et al., 2004). In both humans and mice, loss of the DAP12 signaling causes defective osteoclast differentiation in vitro, but only mildly affected bone development and osteoclastogenesis in vivo (Cella et al., 2003; Kaifu et al., 2003; Humphrey et al., 2004). Similarly, mice deficient for FcRγ show no significant difference in osteoclast development compared to wild type mice. However, mice deficient for both FcRγ and DAP12 exhibit severe osteopetrosis due to defective osteoclast differentiation (Koga et al., 2004; Mocsai et al., 2004). In the absence of RANKL, the stimulation of these ITAM containing proteins alone could not induce osteoclast differentiation, suggesting these ITAM signals provide co-stimulatory signals that are necessary, but not sufficient, for osteoclastogenesis. These co-stimulatory signals might provide additional mechanisms to fine-tune osteoclast differentiation in response to cues provided by the bone microenvironment.
1.3.4 Other modulators of osteoclast differentiation and activity

Besides the key molecules detailed in the previous sections, there are a number of systematic and local factors can also regulate osteoclast formation and activity. These factors may directly act upon osteoclast differentiation and activity, or through accessory cells in the bone microenvironment such as osteoblast/stromal cells and lymphocytes.

1.3.4.1 Stimulatory factors

Factors that enhance osteoclast activity include systemic hormones such as vitamin D3, parathyroid hormone (PTH), and local factors such as interleukin 1 (IL-1), IL-6 and IL-11, tumor necrosis factor (TNF)-α, TNF-β, transforming growth factor α (TGF-α), and TGF-β (Roodman, 1999).

Vitamin D3 is a well known bone resorption factor to stimulate osteoclast formation and bone resorption activity. 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 (Uy et al., 1995b). The effects of PTH and vitamin D3 on osteoclastic formation and activity are thought to be indirect, via their effect on the osteoblast, where they stimulate the production of CSF-1 and RANKL (McSheehy and Chambers, 1986; Roodman, 1999).

IL-1 is a cytokine produced by macrophages and marrow stromal cells that can stimulate bone resorption in vitro and in vivo. IL-1 induces bone resorption and osteoclast-like cells formation in murine and human marrow cultures (Gowen et al., 1983; Pfeilschifter et al., 1989). Uy et al. used an in vivo
model to examine the systemic effects of IL-1 on the different stages of osteoclast development and found IL-1 enhanced the growth and differentiation of CFU-GM, the earliest identifiable osteoclast precursor (Uy et al., 1995a). It also increased the number of more committed mononuclear osteoclast precursors and stimulated mature osteoclasts to resorb bone (Uy et al., 1995a), indicating that IL-1 affects all stages of osteoclast development. Both IL-6 and IL-11 have been shown to induce osteoclast formation in vitro (Kurihara et al., 1990a; Girasole et al., 1994). However, IL-6 does not appear, by itself, to be a potent osteotropic factor in murine systems in vivo. Instead, IL-6 potentiates the effects of other hormones, such as PTH-related protein (PTHrP), on calcium homeostasis and osteoclastic bone resorption in vivo (de la Mata et al., 1995). The effects of IL-11 appear to be mediated by inducing RANKL expression on osteoclasts and marrow stromal cells (Yasuda et al., 1998b).

Both TNF-α and TNF-β markedly stimulate the formation of osteoclast-like cells in human marrow cultures (Pfeilschifter et al., 1989), and can also affect the activity of mature osteoclasts (Thomson et al., 1987). TNFs potentiate the effects of IL-1 on osteoclast formation and appear to stimulate both proliferation and differentiation of osteoclast precursors, and also may be involved in the pathogenesis of hypercalcemia of malignancy (Fried et al., 1989; Yoneda et al., 1991).

TGF-α, a polypeptide produced by several solid tumors associated with the hypercalcemia of malignancy, can stimulate osteoclastic bone resorption in murine organ cultures (Yates et al., 1992). TGF-α is a proliferative factor that
stimulates the growth of early osteoclast precursors, but by itself it has no colony-stimulating factor-like activity (Takahashi et al., 1986).

TGF-β is secreted by bone cells as a latent complex and stored in the extracellular matrix (ECM). Bone contains very high levels of latent TGF-β and is the largest source of TGF-β in the body. The active form of TGF-β is likely released following osteoclastic bone resorption. Therefore, TGF-β has long been proposed as one of the key factors involved in coupling bone formation to previous bone resorption (Mundy, 1991). There is strong evidence that TGF-β modulates the various events of osteoclast development, including recruitment of osteoclast precursors to the bone environment, differentiation to the mature osteoclast, bone resorption, and osteoclast apoptosis (Janssens et al., 2005). However, the exact role of TGF-β in osteoclasts appears to be very complicated and both stimulatory and inhibitory effects of TGF-β on osteoclast formation and function have been reported, suggesting that it acts via more than one mechanism. TGF-β has been shown to synergize RANKL-induced osteoclast-like cells (OCLs) formation from isolated murine bone marrow precursors and monocytes (Fuller et al., 2000). TGF-β also induces OCLs formation from the cultures of RAW 264.7 cells and human monocytes (Itonaga et al., 2004). In contrast, in murine osteoblast/marrow co-cultures, TGF-β inhibits osteoclast formation by both increasing OPG production and reducing RANKL expression and possibly by induction of other inhibitors in osteoblasts (Quinn et al., 2001). TGF-β inhibits both the proliferation and fusion of human osteoclast precursors (Chenu et al., 1988). Depending on the concentration of TGF-β used in the...
studies, TGF-β has inhibiting and stimulating effects on osteoclastic bone resorption in fetal mouse bone organ cultures (Dieudonne et al., 1991). Therefore, several important parameters must be taken into account when evaluating these studies, such as cell differentiation stage, cell density, TGF-β concentration, the presence of osteoblasts/stromal cells, and other culture conditions. In the last few years, a general model for the bidirectional action of TGF-β on osteoclastogenesis has emerged: TGF-β inhibits osteoclast formation in co-cultures at high concentrations, while stimulating it in isolated bone marrow precursor cultures (Janssens et al., 2005).

1.3.4.2 Inhibitory factors

Besides OPG and IFNs (see above), other potent osteoclast inhibitory factors include calcitonin, sex steroids, and bisphosphonates (Roodman, 1999).

Calcitonin, a systemic peptide hormone, is a potent inhibitor of osteoclast activity. Calcitonin receptor (CTR) is expressed only by osteoclasts and is one of the earliest markers in osteoclast progenitors (Lee et al., 1995). Calcitonin acts on osteoclasts by stimulating cAMP accumulation, which results in immobilization of the osteoclast and contraction of the osteoclast away from the bone surface (Gorn et al., 1995). Osteoclasts continuously exposed to calcitonin can escape the effects of calcitonin (Wada et al., 1997). This might be explained in part by the downregulation of CTR mRNA expression by calcitonin on committed osteoclast precursors and mature osteoclasts (Lee et al., 1995).
Estrogen is one of the major inhibitors of osteoclast formation. The deleterious effects on the skeleton due to ovariectomy or menopause have long been appreciated. The exact mechanism responsible for the increased bone turnover with estrogen deficiency is still a matter of passionate debate. Estrogen has been implicated in inhibiting osteoclast formation mainly by suppressing the production of bone-resorbing cytokine and by promoting apoptosis in osteoclasts (Zallone, 2006). The classical receptors for estrogens (ER) are present in osteoclasts, osteoblasts/stromal cells, and their progenitors (Couse and Korach, 1999), indicating that the effects of sex steroids on bone are complex and mediated, at least in part, directly on the osteoclasts.

Bisphosphonates are stable carbon-substituted pyrophosphate analogues that are proven and effective inhibitors of osteoclast-mediated bone resorption. The mechanism of the inhibitory effects of bisphosphonates on bone resorption is still not completely understood. A possible mechanism that has been proposed is that bisphosphonates cause apoptosis when the osteoclasts are exposed to them during bone resorption. These compounds are currently widely used for the treatment of osteoporosis and osteolysis (Rogers, 2003). Since those bisphosphonates used clinically have a high affinity for bone matrix where they persist long term, this class of drugs has turned out to be an effective treatment for increased bone resorption in several indications (Quinn and Gillespie, 2005).
1.4 TRANSCRIPTIONAL CONTROL OF OSTEOCLAST DIFFERENTIATION

Regulation of gene expression by transcription factors is one of the major mechanisms for controlling cell- or tissue-specific functions. Examination of a variety of spontaneous and engineered mutant mice with bone defects, caused by loss or impairment of osteoclast function, has allowed the identification of specific transcription factors essential for osteoclast development and has provided valuable insights into the functions of these transcription factors in physiological settings. These transcription factors include NF-κB, AP-1, PU.1, MITF, and NFATc1 (Figure 13).

1.4.1 Nuclear factor-kappa B (NF-κB)

Mammalian NF-κB is a family of dimeric transcription factors that include p50/NF-κB1, p52/NF-κB2, RelA/p65, c-Rel/Rel, and RelB. All these proteins share a “Rel homology domain” that encodes a DNA binding motif, the dimerization interface, and a nuclear localization sequence. Three members, RelA, RelB, and c-Rel are synthesized as mature proteins, whereas p50 and p52 are generated by proteolytic processing from p105 and p100 precursors, respectively. RelA, RelB, and c-Rel, but not p50 and p52, also contain transcriptional activation domains unique to each of these proteins. p50 and p52 are highly homologous and are usually coexpressed, and they are the most frequent partners of the tranactivating family members RelA, c-Rel and RelB. The activity of NF-κB is controlled by its shuttling from the cytoplasm to the nucleus in
response to cell stimulation. In unstimulated cells, NF-κB dimmers are predominantly retained in the cytoplasm by association with the inhibitory IκB proteins (Baldwin, 1996). RANKL activates NF-κB in osteoclast precursors, in large part via the activation of the IκB kinase (IKK) complex, which phosphorylates IκBs at two N-terminal serine residues, leading to their ubiquitination and degradation by the 26S proteosome. This process allows NF-κB dimmers to translocate to the nucleus, where they bind to κB DNA elements to induce target gene expression (Wei et al., 2001; Jimi and Ghosh, 2005).

The critical role of NF-κB in osteoclastogenesis is demonstrated by the p50/p52 double-knockout mice (Franzoso et al., 1997; Iotsova et al., 1997). Deficiency in either p50 or p52 has no developmental defects. In contrast, p50/p52 double-knockout mice exhibit severe osteopetrosis due to defect in osteoclast survival or differentiation, suggesting the redundant functions of p50 and p52 proteins in this cell lineage. The osteopetrotic phenotype in p50/p52 double-knockout mice could be corrected by bone marrow transplantation, whereas co-culture of spleen cells from these mice with normal osteoblasts could not rescue osteoclast differentiation, indicating the hematopoietic components was impaired in these mice (Franzoso et al., 1997; Iotsova et al., 1997). TRAP positive multinucleated osteoclasts, as well as their TRAP positive mononuclear precursors were absent in p50/p52 double-knockout mice, suggesting that p50 or p52 is required for TRAP positive precursor formation (Franzoso et al., 1997). However, the formation of osteoclast progenitors expressing RANK is independent of p50 and p52 (Xing et al., 2002). One possibility to account for
Figure 13: The differentiation pathway of osteoclast progenitors into functionally active osteoclasts and the cytokines and transcription factors required for each step of differentiation.
these results may be that NF-κB factors are required for osteoclast survival as well as osteoclast differentiation, as suggested by analysis of the IkB kinase (IKK) knockout mice (Ruocco et al., 2005).

By using IKKβ conditional knockout mice, Ruocco et al. demonstrated an essential role for IKKβ in osteoclast survival and differentiation, both in vitro and in vivo. IKKβ-deficient mice develop severe osteopetrosis due to defective osteoclast formation. IKKβ-deficient osteoclast progenitors do not form osteoclasts in vitro in response to RANKL or when co-cultured with osteoblasts. In addition, IKKβ-deficient progenitors are extremely sensitive to TNF-α and undergo extensive apoptosis. Deletion of TNFR1 (TNF receptor 1) rescues IKKβ-deficient osteoclast progenitors from TNF-α-induced apoptosis (Ruocco et al., 2005), indicating that one of the mechanisms by which IKKβ-dependent NF-κB activation contributes to osteoclastogenesis is through prevention of TNF-α-induced apoptosis of osteoclast progenitors. However the loss of TNFR1 does not prevent osteopetrosis in IKKβ-deficient mice and Ikkβ−/−;Tnfr1−/− progenitors can not give rise to fully differentiated osteoclasts (Ruocco et al., 2005). Thus, in addition to the prevention of TNF-α-induced apoptosis of osteoclast progenitors, IKKβ-dependent NF-κB activation is required for terminal osteoclast differentiation.

1.4.2 Activator protein-1 (AP-1)

AP-1 is a dimeric transcription factor composed of members of the Jun proteins (c-Jun, JunB, JunD); the Fos proteins (c-Fos, FosB, Fra-1, Fra-2,
ΔFosB); and ATF proteins. The most common form of AP-1 is composed of c-Jun and c-Fos. AP-1 proteins are key regulator of bone development (Wagner and Matsuo, 2003). The critical role of c-Fos in osteoclastogenesis has been well documented. The lack of c-Fos results in pleiotropic defects, including osteopetrosis due to the absence of osteoclasts (Johnson et al., 1992; Grigoriadis et al., 1994). c-Fos deficiency also causes a lineage shift between osteoclasts and macrophages that resulted in increased numbers of bone marrow macrophages (Grigoriadis et al., 1994), suggesting a role of c-Fos in osteoclast-macrophage lineage determination in vivo.

Fra-1 (Fos-related antigen-1, also known as Fosl1) shows high homology with c-Fos in the leucine zipper and DNA binding domains, and the DNA binding specificity of Fra-1/c-Jun heterodimers is indistinguishable from c-Fos/c-Jun heterodimers (Cohen et al., 1989). Genetic studies have provided convincing evidence that Fra-1 compensates for the loss of c-Fos in osteoclastogenesis in vitro and in vivo. Overexpression of Fra-1 by retroviral gene transduction in vitro or in c-Fos null mice was able to rescue c-Fos dependent osteoclast differentiation (Matsuo et al., 2000). In addition, knock-in mice that express Fra-1 in place of c-Fos could rescue the bone pathology of c-Fos deficient mice, although a single knock-in allele is insufficient to rescue the osteopetrosis (Fleischmann et al., 2000). RANKL signaling induces transcription of Fra-1 in a c-Fos dependent manner, suggesting that Fra-1 itself a transcriptional target of c-Fos during osteoclast differentiation (Matsuo et al., 2000).
The early embryonic lethality in mice lacking Jun family proteins such as c-Jun and JunB has long prevented the study of Jun functions in osteoclasts. Recently, Wagner and colleagues generated monocytes lacking c-Jun or JunB using a conditional gene-targeting approach. They demonstrated that the deficiency of JunB in mice leads to a considerable decrease in osteoclast formation (David et al., 2002; Kenner et al., 2004).

c-Fos is a very short-lived protein that is subject to turnover by multiple degradation pathways (Acquaviva et al., 2002). The stability of c-Fos protein is mainly regulated by the ubiquitin-proteasome system in osteoclast progenitors and c-Fos as well as c-Jun proteins gradually accumulate upon RANKL signaling (Ito et al., 2005). It is well known that phosphorylation of the N-terminal activation domain of Jun by Jun-N-terminal-kinases (JNKs) modulates the transcription activity of AP-1 proteins. RANKL signaling specifically activates JNK1, but not JNK2, and this activation is required for efficient osteoclast differentiation *in vitro* (David et al., 2002).

**1.4.3 PU.1**

PU.1, a member of the Ets-family of transcription factors, is essential for the differentiation of multiple hematopoietic lineages (McKercher et al., 1996). The development of both osteoclasts and macrophages is arrested in mice bearing targeted PU.1 disruption, while the commitment to the myeloid lineage still occurs (Tondravi et al., 1997; Henkel et al., 1999), suggesting that PU.1 is important for the differentiation of monocyte-macrophage lineage cells. PU.1
appears to function earlier than NF-κB during osteoclastogenesis, since the development of macrophages occurs in mice deficient in both the p50 and p52 of NF-κB (Franzoso et al., 1997). The severe osteopetrotic phenotype in PU.1 null mice can be corrected by bone marrow transplantation, indicating that the PU.1 lesion is intrinsic to hematopoietic cells (Tondravi et al., 1997).

The molecular mechanism of PU.1-regulated osteoclastogenesis has yet to be explored. PU.1 has been shown to regulate several lineage-specific cytokine receptor genes including c-FMS and RANK (Zhang et al., 1994; Singh et al., 1999; Kwon et al., 2005). PU.1 binds and transactivates the c-FMS promoter (Reddy et al., 1994; Zhang et al., 1994). PU.1−/− progenitors fail to express RANK gene and reconstitution of PU.1 in these cells induce RANK expression (Kwon et al., 2005). In addition to the essential role of PU.1 in the early stages of osteoclast precursor development, recent studies have revealed PU.1 is also involved in the transcriptional control of osteoclast-specific genes such as TRAP, Cathepsin K, and OSCAR (osteoclast-associated receptor) (Luchin et al., 2001; So et al., 2003; Matsumoto et al., 2004). Regulation of these osteoclast-specific genes by PU.1 involves interactions with other transcription factors. PU.1 has been reported to form heterodimeric complexes with a broad range of transcription factor partners including MITF, c-Jun, c-Fos, NFATc1, NF-κB, C/EBPδ, and the interferon regulatory factor (IRF) family members such as interferon consensus sequence binding protein (ICSBP) and interferon regulatory factor 4 (IRF-4) (Pongubala et al., 1993; Nagulapalli et al., 1995; Bassuk et al., 1997; Behre et al., 1999; Luchin et al., 2001). Furthermore, PU.1
has been reported to interact with the histone acetylase CREB binding protein (CBP), implicating PU.1 in chromatin remodeling (Yamamoto et al., 1999). In addition, Nikolajczyk et al. reported that the presence of PU.1 increased chromatin accessibility in vitro and in vivo (Nikolajczyk et al., 1999). These studies provide evidence that PU.1 may be involved in determining transcriptionally active chromatin structure.

In addition to interaction with different transcription factor partners, the transcriptional activity of PU.1 is also regulated by its protein modifications. A range of studies have been reported that PU.1 phosphorylation is critical for the transcriptional activity of this factor. PU.1 is phosphorylated at several Ser residues by protein kinase CK2, specifically at Ser residues 41, 45, 132, 133, and 148. Phosphorylation of Ser-148 is required to enhance protein-protein interaction with either ICSBP or IRF-4 (Pongubala et al., 1993). Bacterial lipopolysaccharide (LPS) can induce phosphorylation of PU.1 at Ser-148 and thus increase the transactivation function of PU.1 in macrophages (Lodie et al., 1997). Furthermore, there is evidence that Ser-41 and -45 are critical for macrophage proliferation in response to CSF-1 (Celada et al., 1996). Although the contribution of PU.1 phosphorylation in osteoclast differentiation remains to be determined, it is likely that distinct phosphorylation of PU.1 events may dictate the function of PU.1 in inducible gene expression, possibly by changing its transcriptional potential and affecting the affinity for interaction partners during different stages of osteoclastogenesis.
1.4.4 Nuclear Factor of Activated T cell c1 (NFATc1)

NFATc1 (also termed NFAT2) was demonstrated to be strongly induced following RANKL stimulation in both RAW 264.7 cells and primary bone marrow-derived monocyte/macrophage precursor cells (BMMs) (Ishida et al., 2002; Takayanagi et al., 2002a). The essential role of NFATc1 in osteoclastogenesis was revealed by the observations that NFATc1 deficient embryonic stem cells can not differentiate into osteoclasts and ectopic expression of NFATc1 induces BMMs to undergo osteoclastogenesis in the absence of RANKL signaling (Takayanagi et al., 2002a). The induction of NFATc1 mRNA by RANKL was abolished in the cells lacking c-Fos and the overexpression of a constitutively active form of NFATc1 could rescue the defect in osteoclastogenesis in c-Fos deficient precursors, suggesting NFATc1 is a transcriptional target of c-Fos. During the terminal differentiation stage, NFATc1 was proposed to cooperate with c-Fos to synergistically activate osteoclast-specific genes such as TRAP and calcitonin-receptor (Takayanagi et al., 2002a; Matsuo et al., 2004).

It is well established that the activity of NFATc1 is regulated by the calcium-regulated phosphatase calcineurin (Crabtree and Olson, 2002). Following RANKL stimulation, calcineurin is activated and dephosphorylates NFATc1 resulting in translocation of active NFATc1 into the nuclei. Inhibition of NFATc1 activity by calcineurin inhibitors such as cyclosporin A and FK506 suppresses osteoclastogenesis in RAW 264.7 cells and primary bone marrow precursor cells (Ishida et al., 2002; Takayanagi et al., 2002a). FK506-mediated inhibition of NFATc1 activity also abolishes NFATc1 mRNA induction via RANKL,
indicating that NFATc1 may auto-regulate its own expression and that sustained NFATc1 activation is indispensable for osteoclast differentiation (Takayanagi et al., 2002a).

More recently, NFATc1 has been reported to synergistically activate TRAP, OSCAR and Cathepsin K gene expression together with MITF and PU.1, another two transcription factors known to be critical for osteoclast differentiation (Matsumoto et al., 2004; Kim et al., 2005a; Kim et al., 2005b). RANKL-induced NFATc1 forms a complex with PU.1 in nuclei of osteoclasts following the nuclear accumulation of NFATc1 (Matsumoto et al., 2004), indicating NFATc1 phosphorylation by the activated p38 MAP kinase may be important for its activity. Collectively, recent results have demonstrated that NFATc1 plays an integral role in terminal differentiation of osteoclasts and NFATc1 may be a key player in terminal transcriptional program during RANKL-induced osteoclast differentiation.

1.4.5 Microphthalmia-Associated Transcription Factor (MITF)

1.4.5.1 *Mitf* gene and MITF protein

*Mitf* gene and expression of multiple *Mitf* isoforms

The murine *MITF* gene was independently cloned by two groups in 1993 (Hodgkinson et al., 1993; Hughes et al., 1993), and was shown to encode a basic helix-loop-helix leucine zipper (bHLH-Zip) transcription factor (Hodgkinson et al., 1993). The *Mitf* gene is conserved in all vertebrate species studied to date, including human, mouse, rat, hamster, quail, chicken and *zebrafish* (Tachibana,
In addition, *Mitf* or *Mitf*-like gene has been described in several invertebrate species including *C. elegans* and *Drosophila*. The basic domain, as well as helix-loop-helix domain, is highly conserved in all species (Steingrimsson et al., 2004).

The *Mitf* gene contains nine exons (Udono et al., 2000). The human *Mitf* gene spans 229kb and the murine *Mitf* spans about 214 kb from the beginning of exon 1A to the end of exon 9/3' UTR (Hershey and Fisher, 2005). The *Mitf* gene has a multiple promoter that directs the initiation of specific MITF isoforms that differ in their first one or two exons, which are spliced to the common downstream exons 2 through 9 (Figure 14). Nine isoforms of MITF have been identified to date (Levy et al., 2006); all these proteins share the bHLH-Zip domains, but differ in the amino terminal region (Figure 14). *Mitf-M* directs expression of exon 1M, which is directly spliced to exons 2-9 without exon 1B1b. In contrast, all other isoforms have extended amino termini, consisting of a common region 1B1b domain and a unique N-terminus. Thus alternative splicing of exon 1 region generate nine isoforms: *Mitf-A* (Amae et al., 1998), *Mitf-J* (Hershey and Fisher, 2005), *Mitf-C* (Fuse et al., 1999), *Mitf-Mc* (Takemoto et al., 2002), *Mitf-E* (Oboki et al., 2002), *Mitf-H* (Steingrimsson et al., 1994), *Mitf-D* (Takeda et al., 2002), *Mitf-B* (Udono et al., 2000), *Mitf-M* (Hodgkinson et al., 1993; Tassabehji et al., 1994). In addition, a few internal splice variants have been described in the *Mitf* gene (Steingrimsson et al., 1994; Hallsson et al., 2000). One alternative mouse transcript results in two different transcripts that contain (+ isoform) or lack (- isoform) 18bps in exon 6 (Steingrimsson et al., 1994). These
Figure 14: (A) Human and murine Mitf genomic organization depicted to scale (Hershey and Fisher, 2005) and nine different Mitf isoforms identified so far (Levy et al., 2006). In (B), exons are marked by a black line above. All isoforms share: transactivation domain (TAD) and bHLH-Zip domains.
18 bps encode a 6-amino acid domain just upstream of the basic region (Figure 14) and the presence of these 6-amino acid has been shown to stabilize the MITF/DNA complex (Hemesath et al., 1994).

The expression patterns of these isoforms range from tissue-restricted to ubiquitous expression. Studies have suggested tissue-restricted expression of several isoforms: Mitf-M in melanocytes and melanoma cells (Amae et al., 1998; Fuse et al., 1999; Takemoto et al., 2002), as well as in primary murine osteoclasts (Mansky et al., 2002c), Mitf-E in cultured spleen mast cells (Oboki et al., 2002), and Mitf-Mc in primary bone marrow-derived mast cells and mast cell lines (Takemoto et al., 2002). Mitf-C is expressed in RPE cells, but not in melanoma cells (Fuse et al., 1999). Other isoforms (A, B, D, H, J) are more widely expressed in multiple cell types (Steingrimsson et al., 1994; Amae et al., 1998; Takeda et al., 2002; Hershey and Fisher, 2005). In osteoclasts, both Mitf-A and Mitf-M isoforms were detected in primary murine osteoclast-like cells and found to activate TRAP promoter to the same extent either by itself or in collaboration with TFE3 (Mansky et al., 2002c). More recently, using isoform-specific RT-PCR, Hershey et al. demonstrated that human osteoclasts express the A-, E-, H, B- and J-isoforms and murine osteoclasts express the A-, C-, E-, H-, D-, B-, and J-isoforms (Hershey and Fisher, 2005). Therefore, no strictly osteoclast-specific isoform is apparent. At present, very little is known about the functional differences between these alternative MITF proteins. The significance of these multiple MITF isoforms remains to be explored and such diversity may
be necessary for cell- or tissue-specific regulation of target gene expression by MITF.

**MITF and its family proteins**

MITF defines the MiT transcription factor family, which also includes the closely related TFE3, TFEB, and TFEC. MITF binds to a canonical sequence TCATGTG (called M-box) as a homodimer or heterodimer with its family members (Lowings et al., 1992; Yavuzer and Goding, 1994). TFEC appears to be restricted to cells of macrophage/monocytic lineage (Rehli et al., 1999b), while TFE3 and TFEB are more-ubiquitously expressed (Rehli et al., 1999a). Similar to MITF, both TFE3 and TFEB contain the N-terminal activation domains and function as activators of gene expression (Beckmann et al., 1990; Verastegui et al., 2000). TFEC cDNAs isolated from mice and rats do not contain the activation domain corresponding to AD1 in MITF (Zhao et al., 1993; Rehli et al., 1999b), and TFEC has been shown to interfere with TFE3-dependent transcription activation (Zhao et al., 1993). However, TFEC has also been shown to collaborate with MITF to activate the TRAP promoter in transient transfection assays (Mansky et al., 2002c), suggesting that other transcription activation domains may be present in TFEC. All four MiT members share an identical basic region and very similar HLH and Zip region; the sequences are quite divergent outside these domains. The basic domain in these factors is required for DNA sequence recognition, while HLH-Zip domains participate in protein dimerization (Hemesath et al., 1994; Moore, 1995).
In addition to bHLH-Zip region, several important functional domains have been identified in MITF (Figure 15). Four different activation domains have been identified in MITF protein (Sato et al., 1997; Takeda et al., 2000; Mansky et al., 2002a; Saito et al., 2003). In 1997, Sato et al. identified a potent N-terminal activation domain in MITF (AD1 domain) which involves amino acids 114-132 of MITF (Sato et al., 1997) that includes an amphipathic helix previously shown to be important for TFE3-mediated activation (Beckmann et al., 1990). This region of MITF is also highly conserved with a region of adenovirus E1A known to be essential for binding the CBP/p300 transcription cofactor. Sato et al. also demonstrated that the N-terminus of MITF (amino acids 1-198) interacts with CBP and co-transfection of a vector expressing CBP fused to the VP16 activation domain potentiates the ability of MITF to activate transcription, suggesting that transcription activation by MITF is achieved at least in part by recruitment of co-activator CBP (Sato et al., 1997). Mansky et al. demonstrated that the N-terminus of MITF contains a second activation domain (AD2, amino acids 140-185) which is required for the transactivation of E-cadherin and TRAP by MITF in osteoclasts (Mansky et al., 2002a). Amino acids 324-369 of MITF (AD3) represents an activation domain that confers a modest activation potential (Takeda et al., 2000). More recently, a domain encoded by the alternative 5' exon A (AD4) has been implicated in transcription activation (Saito et al., 2003). However the effect of this region on transcription is rather mild and it may be involved in tissue-specific activation.
Figure 15: Schematic representation of MITF protein (Steingrimsson et al., 2004). In (A), the various functional domains and suggested posttranslational modification sites are shown in the Mitf-a protein. In (B), the locations of mutations in the MITF protein are indicated.
1.4.5.2 MITF mutations and biological roles of MITF

The first mouse Mitf mutation (mi allele) was discovered and described by Paula Hertwig more than 60 years ago. She found white animals with small eyes among the progeny of mice treated with X rays and these mice were characterized by the absence of pigment from fur and eyes, skeletal abnormalities (osteopetrosis) and infertility (Hertwig, 1942b; Hertwig, 1942a; Gruneberg, 1948). Since the initial discovery by Hertwig, more than 20 alleles have been identified at the MITF locus (Figure 15) (Steingrimsson et al., 2004), and these mutant alleles have provided a powerful tool to dissect the biological function of MITF protein. Subsequent investigations have demonstrated that Mitf mutations affect several different cell types, including osteoclasts, mast cells, melanocytes, and retinal pigmented epithelial (RPE) cells (Moore, 1995; Steingrimsson et al., 2004). All Mitf mutations affect melanocytes to a varying degree, while some also affect RPE cells resulting in unpigmented or hypopigmented eyes that are smaller than normal (hence the name microphthalmia). A number of alleles also affect osteoclasts resulting in osteopetrosis or hyperosteosis (Steingrimsson et al., 2004), and the role of MITF in the osteoclast will be discussed in great detail below.

Mitf mutations in humans produce the autosomal dominant conditions Waardenburg Syndrome Type 2a (Tassabehji et al., 1994) and Tiez Syndrome (Amiel et al., 1998), characterized by sensorineural deafness (likely due to defective cochlear melanocytes), a white hair forelock and hypopigmentation of
the iris and skin (Price and Fisher, 2001). Mutations and/or aberrant expression of several MITF family member genes have also been reported in human cancer, including melanoma (MITF) (King et al., 2001), papillary renal cell carcinoma (TFE3, TFEB) (Clark et al., 1997; Heimann et al., 2001; Davis et al., 2003), and alveolar soft part sarcoma (TFE3) (Ladanyi et al., 2001). Mice bearing germline knockouts of TFE3 and TFEC do not exhibit recognizable phenotypes, whereas germline TFEB homozygous deficiency is associated with embryonic lethality due to placental insufficiency (Steingrimsson et al., 1998).

1.4.5.3 MITF in osteoclast

*Mitf* mutant alleles with osteopetrotic phenotype

As mentioned before, osteopetrosis is one of the phenotypes associated with a few recessive *Mitf* mutant alleles, including *mi, mi-or, mi-di, mi-crc* (Table 2). Mice homozygous for the first identified allele *mi* (*mi/mi* mice) display the most severe osteopetrosis: the bone marrow cavities are filled with calcified cartilage and spongiosa spicules, teeth fail to erupt due to the lack of resorption of the interfering bone, and bones are shorter than the bones of the wild-type animals (Gruneberg, 1948; Murphy, 1973; Al-Douri and Johnson, 1987; Symons et al., 1989). Osteopetrosis may result either from increased bone formation or decreased bone resorption. Studies have demonstrated that *mi/mi* animals have similar bone formation rates, but lower bone resorption rates compared to wild type animals (Wong et al., 1983). In addition, *mi/mi* animals fail to resorb bone in response to physiologic stimuli (Raisz et al., 1977). Thus the osteopetrosis in
*mi/mi* is due to disrupted bone resorption, and therefore, due to a cell autonomous defect in osteoclasts. A number of studies have also demonstrated that the osteopetrosis in *mi/mi* animals can be corrected by the transfusion of the wild type hematopoietic precursor (Walker, 1975b; Walker, 1975a; Walker, 1975c). These pioneering investigations not only identify the hematopoietic origin of osteoclasts, but also demonstrate that the defect is intrinsic to osteoclasts, and not due to an abnormal bone microenvironment. The molecular lesion of the *mi* mutation has been revealed and it is a deletion of one of the four arginines in the basic domain, which results in the inability of the mutant protein to bind DNA either as homodimer or as a heterodimer with TFE proteins (Hemesath et al., 1994), indicating the transcription activity of MITF family proteins is indispensable for osteoclast development.

*mi oakridge* (*Mitf*<sup>mi-or</sup>) is another mutation in the DNA-binding domain. Similar to the *mi* protein, *Mitf*<sup>mi-or</sup> protein is unable to bind DNA as a homodimer or heterodimer (Hemesath et al., 1994). However, *Mitf*<sup>mi-or</sup>/ *Mitf*<sup>mi-or</sup> animals exhibit milder osteopetrosis, which improves with age (Nii et al., 1995). *mi defective iris* (*Mitf*<sup>mi-di</sup>) is a nonsense mutation which results in the deletion of Zip domain and C-terminal portion of MITF and *Mitf*<sup>mi-di</sup> also unable to bind DNA as homodimer or heterodimer (Hemesath et al., 1994). *Mitf*<sup>mi-di</sup>/ *Mitf*<sup>mi-di</sup> mice only exhibit slight osteopetrosis (West et al., 1985).

One peculiar feature is that osteopetrosis is only seen with a few strong recessive *Mitf* alleles discussed above and does not appear to be a loss-of-function phenotype. The transgene insertion mutation *Mitf*<sup>mi-vga9</sup> severely reduces
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<th>Reference</th>
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</thead>
<tbody>
<tr>
<td>microphthalmia</td>
<td>Mitf&lt;sup&gt;mi&lt;/sup&gt;</td>
<td>Iris pigment less than in wild type; spots on belly, head and tail</td>
<td>White coat, eyes small and red; incisor fail to erupt; severe osteopetrosis</td>
<td>Deletion of R216 in basic domain</td>
<td>(Hertwig, 1942a) (Hodgkinsson et al., 1993)</td>
</tr>
<tr>
<td>Oak ridge</td>
<td>Mitf&lt;sup&gt;mi-or&lt;/sup&gt;</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 in basic domain</td>
<td>(Steingrims son et al., 1994)</td>
</tr>
<tr>
<td>Defective iris</td>
<td>Mitf&lt;sup&gt;mi-di&lt;/sup&gt;</td>
<td>Slight reduced choroida pigment</td>
<td>White coat, microphthalmia; mildly defective bone resorption</td>
<td>R263 STOP</td>
<td>(West et al., 1985)</td>
</tr>
<tr>
<td>Clinical research center</td>
<td>Mitf&lt;sup&gt;mi-crc&lt;/sup&gt;</td>
<td>Iris pigment reduced; belly and tail spots</td>
<td>White coat, eyes red and small; tooth eruption failure; osteopetrosis</td>
<td>unknown</td>
<td>(Hetherington, 1976)</td>
</tr>
<tr>
<td>VGA-9</td>
<td>Mitf&lt;sup&gt;mi-vga9&lt;/sup&gt;</td>
<td>normal</td>
<td>White coat, eyes red and small; inner ear defects</td>
<td>Transgene insertion and 882bp deletion</td>
<td>(Tachibana et al., 1992)</td>
</tr>
</tbody>
</table>

Table 2: *Mitf* mutant alleles resulting osteopetrotic phenotype.
expression of the *Mitf* gene yet does not cause osteopetrosis (Hodgkinson et al., 1993; Steingrimsson et al., 2002), also suggesting that other proteins, such as TFEs, might be able to compensate its function during osteoclast development. Indeed, elegant genetic studies have demonstrated functional and genetic redundancy between MITF and TFE3 in the development of the osteoclast lineage (Steingrimsson et al., 2002). As mentioned above, neither Vga9/Vga9 nor *Tfe3* null mice display osteopetrotic phenotype, however, the combined loss of these two genes results in severe osteopetrosis.

**MITF and osteoclast-specific target gene expression**

Ultrastructural analyses show that osteoclasts from *mi/mi* mice are smaller than normal and lack ruffled borders. In addition, mononuclear osteoclasts can be detected in *mi/mi* mice but these cells are not able to form multinucleated osteoclasts, and are defective in bone resorption (Holtrop et al., 1981; Thesingh and Scherft, 1985). In spite of the striking bone phenotype of *mi/mi* mice, it was only very recently that MITF target genes in osteoclast started to be revealed. However, the role of MITF in osteoclast differentiation and the molecular mechanisms of this regulation by MITF are still largely unknown. Several lines of evidence suggest that TRAP is a target of MITF. TRAP mRNA expression is greatly diminished in OCLs derived from *mi/mi* mice. The TRAP promoter contains a highly conserved M-box sequence and the integrity of this motif is required for MITF binding to the TRAP promoter and transactivation of TRAP promoter (Luchin et al., 2000). MITF has also been shown to activate
other osteoclast-specific genes including cathepsin K, OSCAR, Clcn7 (chloride channel 7), and Ostm1 (osteopetrosis-associated transmembrane protein 1) (So et al., 2003; Matsumoto et al., 2004; Meadows et al., 2006), indicating MITF plays a critical role in regulating osteoclast-specific gene expression.

Recent studies have suggested that MITF cooperates with other transcription factors to regulate target gene expression. MITF has been shown to act with PU.1 to synergistically induce TRAP, cathepsin K, and OSCAR gene expression in luciferase assays (Luchin et al., 2001; So et al., 2003). Physical association between MITF and PU.1 has been demonstrated using overexpressed or recombinant proteins, also in primary cells (Luchin et al., 2001). Genetic interaction between MITF and PU.1 is also revealed based on the observation of approximately 25% of double heterozygotes (Mitf<sup>mi/+;PU.1</sup><sup>+/−</sup>) exhibiting age-resolving osteopetrosis compared to none in PU.1 heterozygotes (Luchin et al., 2001). Very recently, the interaction of two MITF alleles, Mitf<sup>mi</sup> and Mitf<sup>vga</sup> and a PU.1 null allele were studied (Sharma et al., 2006b). While Mitf<sup>mi/+</sup> heterozygous mice have no bone phenotype, lowering the dose of wild-type MITF by introducing the hypomorphic Mitf<sup>vga</sup> allele resulted in severe osteopetrosis in about 38% of Mitf<sup>mi/vga</sup> mice. Furthermore, 100% of Mitf<sup>mi/vga</sup>/PU.1<sup>+/−</sup> mice were found to have a severe osteopetrotic phenotype throughout their lifetime. Cells from Mitf<sup>mi/vga</sup>/PU.1<sup>+/−</sup> mice failed to visibly differentiate and expressed very low levels of Acp5 and Ctsk in response to CSF-1/RANKL stimulation (Sharma et al., 2006b). These results demonstrated that genetic interactions between MITF and PU.1 mutant alleles result in impaired
osteoclast differentiation and osteopetrosis. Considering MITF is expressed in and is essential for development of several cell types including osteoclasts, mast cells, melanocytes, retinal pigmented epithelial (RPE) cells, cooperation between MITF and hematopoietic-restricted transcription factor PU.1 provide a mechanism for MITF to regulate different sets of target genes in distinct cell types.

Besides the mode of association with other factors, the activation potential of MITF is also regulated post-translationally. In osteoclasts, both CSF-1 and RANKL signaling pathways have been shown to play a role in modulating MITF activity (Figure 16) (Weilbaecher et al., 2001; Mansky et al., 2002b). CSF-1 stimulation in OCLs induces MAPK/Erk1/2 mediated phosphorylation of MITF at serine 73, triggering the recruitment of the p300/CBP co-activator. Furthermore, a mutation of the consensus MAPK at serine 73 is deficient in forming multinuclear osteoclasts in a RAW264.7 cell culture system (Weilbaecher et al., 2001).

RANKL signaling has also been reported to trigger phosphorylation of MITF at serine 307 via p38 MAPK pathway and a constitutively active MKK6 (a specific activator of p38) can greatly enhance MITF activation on the TRAP promoter (Mansky et al., 2002b). In melanocytes, c-Kit stimulation triggers dual phosphorylation of MITF at Ser-73 and Ser-409, which up-regulate transactivation potential of MITF yet simultaneously target MITF for ubiquitin-dependent proteolysis (Wu et al., 2000). Xu et al. reported the association between MITF and the ubiquitin conjugating enzyme hUBC9 and further
Figure 16: M-CSF and RANKL activate MITF and TFE3 in osteoclasts (Hershey and Fisher, 2004). M-CSF signaling leads to phosphorylation on Ser 73 and RANKL signaling results in phosphorylation on Ser 307 in MITF protein.
identified MITF Lys-201 as a potential ubiquitination site (Xu et al., 2000). In addition, MITF is modified by small ubiquitin-like modifier (SUMO), with Lys-182 and Lys-316 identified as major sumoylation sites in MITF. Interestingly, MITF with double lys-182/316-arg mutations, although not altering dimerization, DNA binding, stability, or nuclear localization, showed a significant increase in the transcriptional stimulation of promoters containing multiple but not single MITF binding sites (Miller et al., 2005; Murakami and Arnheiter, 2005). Thus, these findings identify sumoylation as a post-translational modification that affects MITF transcriptional activity in a manner dependent on the promoter elements present in MITF target genes and suggest that sumoylation may regulate the cooperation of individual MITF molecules on promoter DNA.

1.5 SUMMARY

Bone is a rigid yet dynamic organ that is continuously renewed through a process termed remodeling. Bone remodeling is the predominant metabolic process maintaining bone structure and function during adult life, with the key participant being the osteoclast. Remarkable progress has been made during the past decade in the field of osteoclast research with the development of suitable in vitro models and application of molecular biology and genetic technology. A number of molecules that play essential role in osteoclast differentiation or function have been identified and several therapeutic agents have been developed targeting these molecules and their signal transduction pathways.
Transcription factor MITF appears to have a unique and essential role in osteoclast differentiation, as demonstrated by the severe osteopetrosic phenotype in mice homozygous for several mutant MITF alleles such as *mi*. In osteoclast, MITF is required for the expression of several osteoclast specific genes including *Acp5* and *Ctsk*. Although it is evident that MITF plays a crucial role in osteoclast differentiation, the precise role played by this transcription factor in regulating osteoclastogenesis and osteoclast specific genes expression is still largely unkown. More intriguingly, MITF is required for the differentiation of several cell types unrelated to osteoclasts and regulates a set of different target genes in different cell types. Further elucidation of molecular events involved in target gene expression regulated by MITF will allow us not only to better understand the transcriptional regulation of osteoclast differentiation, but also to better understand the mechanisms of cell differentiation in general.
CHAPTER 2

MATERIAL AND METHODS

2.1 ANTIBODIES

The peptide SSGDSSLEKDSL (corresponding to amino acids 8-19 of mouse Eos), was used to make specific antibody against Eos in rabbits (QCB/Biosource, Hopkinton, MA). Antibodies against MITF and PU.1 were previously described (Mansky et al., 2002a). Anti-BRG1 antibody was described elsewhere (Wang et al., 2005). Anti-CBP antibody was a kind gift from Marc R Montminy, San Diego, CA. Other commercially available antibodies include: Flag M2 and HA (Sigma-Aldrich, St. Louis, MO), His_{6}, GST, mSin3A, HDAC1, CtBP, and Mi-2 (Santa Cruz Biotechnology Inc, Santa Cruz, CA), Histone H3 (Upstate Cell Signaling, Charlottesville, VA).

2.2 PLASMID CONSTRUCTS

Mammalian expression vector pCDNA3-Flag-Eos, pCDNA-Flag-Eos (50-230) and bacterial expression vector pGEX-GST-Eos (101-230) were kindly provided by Merlin Crossley, Sydney, Australia and were described earlier (Perdomo et al., 2000). The luciferase reporter constructs pGL2-Acp5 and pGL2-
Ctsk, expression vector pECE-MITF and pECE-PU.1 used for 3T3 cell transfections have been described previously (Luchin et al., 2001). DNA constructs for Flag-tagged MITF and truncation mutants were recently described (Bronisz et al., 2006). Bacterial expression vectors for GST-tagged MITF and His-tagged PU.1 were previously described (Luchin et al., 2001).

To generate pEBG-GST-Eos and deletion constructs, full-length or various truncations of Eos cDNA were PCR amplified from pCDNA3-Flag-Eos plasmid using primers containing Bgl II site. The respective PCR products were digested by Bgl II restriction enzyme and cloned into the BamH I site of the mammalian GST-expression vector pEBG. The oligonucleotides used were (Table 3): Eos (F)-Bgl II and Eos-(R)-Bgl II (for full length); Eos-(F)-Bgl II and Eos-230-(R)-Bgl II (for aa 1-230); Eos-50-(F)-Bgl II and Eos-230-(R)-Bgl II (for aa 50-230); Eos-101-(F)-Bgl II and Eos-230-(R)-Bgl II (for aa 101-230); Eos-231-(F)-Bgl II and Eos-(R)-Bgl II (for aa 231-532). MSCV-FlagEos-IRES-GFP vector was constructed by inserting Flag-tagged Eos cDNA (amplified by Flag-Eos-(F)-Xho I and Eos-(R)-Xho I primers from pCDNA3-Flag-Eos plasmid) into Xho I-digested MSCV-IRES-GFP vector.

To generate the vector encoding HA-tagged PU.1 (DBD), primers PU.1-161-(F)-BamH I and PU.1-272-(R)-BamH I (Table 3) were used to PCR amplify the DNA binding domain of PU.1 and digested PCR products were then cloned into pCGN vector cut by BamHI. All of the above amplified sequences were verified by DNA sequencing.
<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>PU.1-161-(F)-BamH I</td>
<td>5’-CGCGGATCCCTTCTGCACGGGGAGACAG-3’</td>
</tr>
<tr>
<td>PU.1-272-(R)-BamH I</td>
<td>5’-CGCGGATCCCTATCAGTGGGGCGGGGAGCG-3’</td>
</tr>
<tr>
<td>Flag-Eos-(F)-Xho I</td>
<td>5’-CCGCTCGAGATGGACTACAAGGACG-3’</td>
</tr>
<tr>
<td>Eos-(R)-Xho I</td>
<td>5’-CCGCTCGAGCTAGCCACCTTATGCT-3’</td>
</tr>
<tr>
<td>Eos-(F)-Bgl II</td>
<td>5’-GCGAAGATCTATGGAATCCTTTATTTTG-3’</td>
</tr>
<tr>
<td>Eos-(R)-Bgl II</td>
<td>5’-GAAGATCTCTAGCCACCTTATGCT-3’</td>
</tr>
<tr>
<td>Eos-50-(F)-Bgl II</td>
<td>5’-GCGAAGATCTATGTACAGCGATGAGGAG-3’</td>
</tr>
<tr>
<td>Eos-101-(F)-Bgl II</td>
<td>5’-GAAGATCTCTGCGCAATGGCAAGGC-3’</td>
</tr>
<tr>
<td>Eos-231-(F)-Bgl II</td>
<td>5’-GAAGATCTTTGGCTGGCAACCAG-3’</td>
</tr>
<tr>
<td>Eos-230-(R)-Bgl II</td>
<td>5’-GAAGATCTAGCTTGGGCTTCAGTG-3’</td>
</tr>
</tbody>
</table>

Table 3: Primers used for subcloning.
2.3 DNA MANIPULATIONS

2.3.1 Agarose gel electrophoresis

Agarose gels were prepared by melting agarose (Fisher Scientific) in 1×TAE buffer (40mM Tris-acetate, 2mM Na₂EDTA; 2H₂O; pH 8.0; made as 50×stock) by boiling. The concentration of agarose in the gel was determined based on the size range of DNA fragments to be separated. A 2% gel was used to separate DNA fragments between 0.1-0.6 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 (0.5×TBE (89.2mM Tris; 89mM boric acid; 2mM EDTA, pH 8.0), 16% glycerol, 100mg/ml Bromphenol Blue and 0.4 mg/ml Xylene Cylenol; prepared as 5× stock). The DNA fragments were separated at 80-120 V in 1×TAE buffer.

2.3.2 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 dNTPs (Roche, Indianapolis, IN), 1× PCR buffer and 1 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 95°C for 4 min followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 30 sec followed by extension at 72°C for 1.5 min and a final extension step at 72°C for 7 min. Reaction products were analyzed by agarose gel electrophoresis. The PCR products were purified using QIAquick PCR-puriﬁcation kit (Qiagen, Valencia, CA), according to manufacturer’s instruction.
2.3.3 Small- and large-scale preparation of plasmid DNA from bacteria

**Small-scale preparation of plasmid DNA**

Bacterial single colony was inoculated into 5ml LB media containing the appropriate antibiotic and grown overnight at 37°C with shaking. Overnight cultures were transferred to a microfuge tube and centrifuged. Plasmid DNA was purified by QIAprep Spin Miniprep Kit (Qiagen) following the manufacturer’s instructions. Briefly, the cell pellet was resuspended in 250μl of buffer P1 supplemented with RNaseA. Cells were lysed and DNA was denatured in 250μl of buffer P2, by gently inverting the tube 5-6 times. Lysis of the cells was not allowed to proceed more than 5 min at room temperature. The lysate was neutralized by adding 350μl of buffer N3, the contents were mixed by inverting the tube 5-6 times and centrifuged for 10 min at 13,000 rpm. The clear supernatant containing renatured plasmid was transferred to QIAprep column and centrifuged for 30-60 sec. The QIAprep spin column was washed by adding 750μl of buffer PE and centrifuging for 30-60 sec. The flow-through was discarded and the column was centrifuged for additional 1 min to remove the residual wash buffer. The bound DNA was eluted from the column by 50μl of buffer EB or H₂O.

**Large-scale preparation of plasmid DNA**

Maxiprep procedure was used for large-scale preparation of plasmid DNA. A fresh single colony was inoculated into 5ml LB containing the appropriate antibiotic and grown at 37°C with shaking for 8 hr. 1ml culture was further diluted into fresh 250ml LB media with appropriate antibiotic and grown overnight with
shaking at 37°C. Cells were pelleted down at 4°C for 15 min at 4,000 rpm in the Sorvall CS-3 rotor. Large amounts of plasmid DNA were isolated from resulting cell pellets using Qiagen Plasmid Maxi kit (Qiagen) according to manufacturer’s directions.

2.3.4 Cloning of DNA

2.3.4.1 Restriction enzyme digestion

Restriction digests of DNA were conducted with commercially available endonucleases (NEB, Fermentas). The digestions 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.

2.3.4.2 Alkaline phosphatase reaction

Linearized vector DNA was subjected to alkaline phosphatase treatment prior to ligation, to avoid self-ligation of “sticky ends”. Linearized vector was incubated with 5 μl of Calf Intestine Alkaline Phosphatase (Roche) and supplied alkaline phosphatase buffer in a final volume of 200 μl, at 37°C for 2 hr. The enzyme was heat-inactivated at 65°C for 20 min and the DNA was purified by phenol-chloroform extraction, ethanol precipitation, and resuspended in an appropriate volume of TE (10 mM Tris-HCl, pH8.0; 0.1 mM EDTA) or H2O. The restricted DNA fragments were separated on agarose gel electrophoresis and gel purified using QIAquick gel extraction kit, according to manufacturer’s directions.
2.3.4.3 DNA ligation

Both linearized vector and restricted insert DNA fragments were gel-purified before the ligation reaction using QIAQuick gel extraction kit. For DNA ligation reaction, about 100-250ng of linearized vector DNA was used based on a molar ratio of 1:3 (vector to insert ratio). 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 hr or at 16°C overnight.

2.3.4.4 DNA transformation into E. coli competent cells

Preparation of competent cells

A small amount of frozen glycerol stocks of E.coli DH5α or BL21 (DE3)-pLys-S strain was streaked onto LB agar Petri plates and incubated overnight at 37°C. A single colony was used to inoculate a 5ml LB 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 media in a 1liter flask. The culture was grown at 37°C with constant shaking until the cells reached an optical density 600 (OD$_{600}$) of 0.3-0.5 as measured using a spectrophotometer. The culture was chilled on ice for 20 min and centrifuged at 4°C at 4000 rpm in a Sorvall CS-3 rotor. The cell pellet was placed on ice, resuspended in 1/2 volume of ice-cold 5mM CaCl$_2$, chilled on ice for 20 min and centrifuged once again as earlier. The cell pellet was finally resuspended in 1/20 volume of ice-cold 50mM CaCl$_2$ and 15% glycerol, aliquoted into sterile 1.5 ml microfuge tubes, quick frozen on dry ice and stored at -80°C until further use.
**DNA transformation**

100 µl of the frozen competent cells were slowly thawed on ice, mixed with either 0.1-1 µg of supercoiled plasmid DNA or 10 µl of ligation reaction and incubated on ice for 30 min. The cells were heat shocked for 90 sec at 42°C and immediately recovered by adding 1ml of LB and shaking at 37°C for 1 hr. When transforming plasmid DNA, 1/10 of the culture was plated on LB plates with the appropriate antibody added. When transforming ligation reactions, the cultured 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 hr (Sambrook and Russel, 2001). 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.

### 2.4 CELL LINES AND TRANSFECTION

#### 2.4.1 NIH 3T3

NIH 3T3 cells were maintained in Dulbecco's modified eagle medium (DMEM) containing 5% Calf Serum, 4mM L-glutamine, 50 u/ml streptomycin at 37°C in 5% CO₂. Cells were plated at 0.5×10⁶/100 mm dish and passed every two days. For passage, cells were lifted by trypsinization (0.5% trypsin; 0.2% EDTA in PBS) for 3-5 min.

NIH 3T3 cells were transfected using the Calcium-Phosphate precipitation method. Cells were plated at 1×10⁶/60 mm dish 24 h prior to
transfection. DNA in the total amount of 5 µg for a 60 mm dish was combined in sterile H₂O in the total volume of 375 µl. 125 µl of 1 M CaCl₂ was added to DNA and the DNA/CaCl₂ solution was then added dropwise to 0.5 ml of 2×HBS (28 mM NaCl; 50 mM HEPES; 1.5 mM Na₂HPO₄; pH 7.1). The reaction mix was incubated at room temperature for 30-45 min to allow precipitation and was then added to cells (0.5 ml/dish). The precipitate was removed in 12-16 h by washing twice with TBS (50 mM Tris-HCl, pH 7.5; 150 mM NaCl) and cells were then maintained in normal growth media for 24-36 h before harvest by freezing-thawing in PBS.

Luciferase activity was measured by adding 100 µl of luciferase reaction buffer (20 mM Tricine; 0.1 mM EDTA; 33.3 mM DTT; 2.67 mM MgSO₄; 1.07 mM (MgCO₃)₄Mg(OH)₂·5H₂O; 270 µM Coenzyme A (Li₃); 470 µM Luciferin (Promega); 530 µM ATP) to 5-20 µl of lysates of transfected cells, using the Lumat LB 9507 luminometer (EG&G Berthold).

Protein concentration assays were performed using Bio-Rad Protein Assay Reagent (Bio-Rad). Briefly, 2.5-10 µl of lysate was added to the reagent. The amount of the extract added was selected for each lysate to fall within the calibration curve linear range. For calibration curve, 0, 1, 2, 4, 6, 8, 10, 12 µl of 1 mg/ml Bovine Serum Albumin (BSA) was added to 1 ml of the reagent. 200 µl/well plate (Corning Inc.) and absorbance was measured in EIA Reader Model 2550 (Bio-Rad) plate at 600 nm. Measurements were performed in duplicates for calibration curve and in triplicates for cell lysates.
2.4.2 COS-7

COS-7 cells were maintained in Dulbecco’s modified eagle medium (DMEM) containing 10% Calf Serum, 4mM L-glutamine, 50 u/ml streptomycin at 37 °C in 5% CO₂. Cells were plated at a density of 1.5-2×10⁶/100 mm dish and passed every two days. For passage, cells were lifted by trypsinization.

The day before transfection, COS-7 cells were plated at a density of 1×10⁶/60 mm so that they were 60-80% confluent the day of transfection. COS-7 cells were transfected using Lipofectamine and Plus reagent (Invitrogen) according to the manufacturer’s instructions. Briefly, 3 µg DNA was diluted into 250 µl serum free DMEM media and mixed with 8 µl Plus reagent, incubating at room temperature for 15 min. In a second microfuge tube, 10 µl Lipofectamine reagent was diluted into 250 µl serum free DMEM and mixed. Combined material from two tubes were mixed and incubated for another 15 min at room temperature. Then the DNA-Plus-Lipofectamine Reagent complexes were added to the cells with 2 ml fresh medium without serum and mixed into the medium gently. Cells were then incubated at 37 °C in 5% CO₂ for 3 hr. After 3 hr incubation, appropriate amount of fresh medium containing serum was added to the cells to bring the final concentration to that of normal growth medium. Cells were harvested at 24 hr post transfection.

2.4.3 Phoenix

Phoenix packaging cell line (Garry Nolan, Standford University) were maintained in Dulbecco’s modified eagle medium (DMEM) containing 10% Fetal
Bovine Serum, 4mM L-glutamine, 50 u/ml streptomycin at 37°C in 5% CO₂. Cells were plated at a density of 2.5-4×10⁶/100 mm dish and passed every two days. For passage, cells were lifted by trypsinization for about 1-2 min. Unlike NIH 3T3 cells, Phoenix cells are much less adherent thus handled very gently when washing and changing medium.

Phoenix cells were transfected using a Calcium-Phosphate precipitation method based on Nolan’s protocol (http://www.stanford.edu/group/nolan/). Cells were plated at 2×10⁶/60 mm dish 24 hr prior to transfection. The dish should be approximately 80% confluent just before transfection. DNA in the total amount of 6-10µg for 60 mm dish was combined in sterile H₂O in the total volume of 375 µl. 125 µl of 1 M CaCl₂ was added to DNA. 0.5 ml of 2×HBS (28 mM NaCl; 10mM KCl; 50mM HEPES; 1.5 mM Na₂HPO₄; pH 7.1) was added to DNA/CaCl₂ solution by bubbling. Reaction mix was immediately (within 1-2 min) added to cells (0.5 ml/dish). The plates were gently rocked to insure uniform mixing and returned to the incubator. The next day (24 hr after transfection), the transfection medium was removed and replaced with the fresh growth medium. Transfection efficiency could be evaluated by GFP expression if cells were transfected with a vector expressing GFP (usually more than 70-80% cells expressing GFP 24 hr post transfection). Retroviral supernatants were harvested 48 hr and 72 hr post-transfection and filtered through a 45µM filter to remove living cells. If the retroviral supernatant is to be used within several hours, kept on ice until it is used. For long term storage, retroviral supernatants were snap frozen (resulting
in a minimal loss of viral titer) and stored at -70°C. The frozen samples were thawed by warming for a minimal period of time at 37°C.

2.5 CULTURE OF PRIMARY MURINE OSTEOCLAST

Mice (C57BL/6) used in this study were housed at the Ohio State University Laboratory Animal Resources (ULAR) facilities and were maintained in accordance with IACUC guidelines.

2.5.1 In vitro osteoclast differentiation

All tissue culture-coated dishes used to culture primary osteoclasts were coated for 12 hr with 0.1% gelatin made in 1×phosphate-buffered saline (PBS, pH 7.4). Bone marrow was used as a source of hematopoietic osteoclast precursor cells. Bone marrow cells were derived from femurs of 4 weeks old wild type mice. Bone marrow cells were plated on plastic dishes and were cultured for three days in DMEM medium containing 10% heat inactivated fetal bovine serum and 100ng/ml recombinant CSF-1 (Roche). After 3 days, the non-adherent cells were harvested, counted and plated to the tissue-culture dish at a density of $3 \times 10^6$ cells/100mm dish. The cells were then either maintained in the same concentration of CSF-1 (proliferation medium) or switched to a combination of 50ng/ml of CSF-1 and 100ng/ml of RANKL (Roche) (differentiation medium) for the formation of multinuclear osteoclasts. The medium was changed every two days.
2.5.2 TRAP staining of *in vitro* differentiated osteoclasts

After 3 days of combined CSF-1 and RANKL stimulation, 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 using Leukocyte Acid Phosphatase Kit (Sigma), according to the manufacturer's instruction. The number of TRAP positive multinuclear osteoclasts was counted using IX51 Olympus inverted microscope and images were captured using Olympus camera and Magnafire™ software.

2.6 RETROVIRAL TRANSDUCTION OF PRIMARY OSTEOCLAST PRECURSORS

Retroviral supernatants harvested from Phoenix cells mock-transfected (mock control), or transfected with MSCV-IRES-GFP vector (empty vector control), or transfected with MSCV-FlagEos-IRES-GFP construct were used to transduce primary osteoclast precursors. After 3 days CSF-1 stimulation, bone marrow derived osteoclast precursors were transferred into 12-well plates at a density of 0.2×10^6 /well and resuspended using 2 ml retroviral supernatant supplemented with polybrene (4 μg /ml) and CSF-1 (50ng/ml). The plates were centrifuged at 1600rpm for 60 min at room temperature and kept in tissue culture incubator for additional 12 hr. Then retroviral supernatant was removed and transduced cells were grown in normal growth medium containing CSF-1 for 24 hr to allow the expression of gene of interest. The cells were then treated with
combined CSF-1 and RANKL to initiate osteoclast differentiation. The viral titers were determined by infecting 3T3 cells using serial dilution and supernatant of equal viral titers were used in each experiment. Transduction efficiency was monitored by the percentage of GFP-expressing cells under the microscope and western blot for protein expression.

2.7 EXPRESSION OF RECOMBINANT PROTEINS IN E.COLI

2.7.1 GST-fusion proteins

Recombinant GST fusion proteins including full length MITF, MITF(199-298), MITF(199-298) bearing various mutations, and Eos (101-230) were expressed in BL21(DE3) cells via pGEX2T vectors and purified as previously described (Luchin et al., 2001). Briefly, BL21(DE3) cells were grown from a freshly transformed colony in 3 ml YTA media (16 g/L tryptone; 10 g/L yeast extract; 5g/L NaCl; 1 mg/ml ampicillin) at 37°C to OD$_{600}$=0.6-0.8. The 3 ml culture was inoculated into 250 ml YTA and grown at 37°C to OD$_{600}$=0.6-0.8. IPTG was then added at a final concentration of 0.1 mM to induce protein expression at 30°C for 2-6 hr. For GST-Eos (101-230) induction, ZnSO$_4$ (final 0.01 mM) was also added in addition to IPTG. Bacteria were collected and resuspended in 5 ml cold PBS (140 mM NaCl; 2.7 mM KCl; 10 mM Na$_2$HPO$_4$; 1.8 mM KH$_2$PO$_4$, pH 7.4) containing protease inhibitors (10 μg/ml aprotinin; 10 μg/ml leupeptin; 10 μg/ml antipain). The cell lysates were prepared by sonication (5-7 times, 12 sec bursts) and centrifuged 15,000rpm for 20 min. The supernatant was then incubated with Glutathione-Sepharose beads (GE Healthcare) for 2 hr at 4°C.
The beads were washed three times with PBS and the bound proteins were eluted three times with 125 μl of freshly made elution buffer (10 mM reduced glutathione in 50 mM Tris-HCl, pH 8.0) for 15 min at 4°C with occasional rock. Pooled eluates were aliquated and stored at –70°C. A fraction of recombinant proteins were run on 12 % SDS-PAGE to determine protein quality and concentration.

### 2.7.2 His<sub>6</sub>-fusion proteins

Recombinant His fusion proteins including full length PU.1, various PU.1 deletions, and Eos (231-532) were expressed in BL21(DE3) cells via pET-32a vectors and purified as previously described (Luchin et al., 2001). Briefly, BL21(DE3) cells were grown from a freshly transformed colony in 3 ml YTA media (16 g/L tryptone; 10 g/L yeast extract; 5g/L NaCl; 1 mg/ml ampicillin) at 37°C to OD<sub>600</sub>=0.6-0.8. The 3 ml culture was inoculated into 250 ml YTA and grown at 37°C to OD<sub>600</sub>=0.6-0.8. IPTG was then added at a final concentration of 1 mM to induce protein expression at 30°C for 2-4 hr. For His-Eos (231-532) induction, ZnSO<sub>4</sub> (final 0.01 mM) was also added in addition to IPTG. Bacteria were collected and resuspended in 5 ml lysis buffer (20 mM Tris-HCl, pH 8.0; 500 mM NaCl; 5 mM imidazole) containing protease inhibitors. Cell lysates were prepared by sonication at 4°C and centrifuge. The supernatant was incubated with Ni-NTA superflow beads (Qiagen) for 1-2 hr at 4°C. The beads were washed twice with wash buffer (20 mM Tris-HCl, pH 8.0; 500 mM NaCl; 50 mM imidazole) and the bound proteins were eluted three times with 125 μl of elution buffer (10 mM reduced glutathione in 50 mM Tris-HCl, pH 8.0).
buffer (20 mM Tris-HCl, pH 8.0; 500 mM NaCl; 500 mM imidazole) for 15 min at 4°C with occasional rock. The eluants were aliquoted and stored at –70°C. A fraction of recombinant proteins were run on 12 % SDS-PAGE to determine protein quality and concentration.

2.8  **IN VITRO GST PULL DOWN ASSAYS**

*In vitro* GST pull down assays were performed as previously described (Luchin et al., 2001). Briefly, 1 µg of the control GST or GST-fusion bait protein was incubated with 20 µl glutathione-sepharose beads (GE Healthcare) in 300 µl binding buffer (50 mM Tris-HCl, pH 7.6; 150 mM NaCl; 0.5% NP-40) for 1 hr at 4°C on a rotator wheel. 1 µg of His$_6$ fusion test protein was then added to the reaction and incubated for another 2 hr at 4°C. Beads were washed 5 times with 1 ml binding buffer. The bound proteins were resolved on SDS-PAGE and analyzed by western blot using antibody against His$_6$.

2.9  **IMMUNOPRECIPITATION AND IMMUNOBLOTTING**

24 hr post-transfection, COS-7 cells were washed with ice-cold PBS and resuspended in cell lysis buffer (50mM Tris-HCl, pH 7.5; 100mM NaCl; 0.5 mM dithiothreitol (DTT); 1mM EDTA; 1mM EGTA; 50 mM β-glycerophosphate; 1% Triton X-100; protease inhibitor supplemented before use). After incubation on ice for 30 min, cell lysates were centrifuged at 12,000 rpm for 20 min and the resulting supernatant was pre-cleared with 20 µl of protein G Gamma Bind Plus Sepharose (GE Healthcare) for 40 min at 4 °C. A fraction of cell lysate was used as a whole cell lysate control in the following western blot. Pre-cleared lysates
were incubated with fresh protein G beads and monoclonal anti-Flag antibody for 2-3 hr at 4 °C. For GST pull down assays, cell lysates were incubated with glutathione-sepharose beads (GE Healthcare). The bound beads were washed twice with lysis buffer, twice with lysis buffer containing 0.5 M LiCl, and twice with PBS. The proteins were eluted directly in SDS sample buffer and resolved by SDS-PAGE and analyzed by western blot.

**SDS-polyacrylamide gel electrophoresis (SDS-PAGE)**

Proteins were separated on discontinuous polyacrylamide gel electrophoresis. The upper stacking gel was composed of 0.125 M Tris-HCl (pH 6.8), 0.1% SDS, 4% 29:1 acrylamide-bisacrylamide. The lower resolving gel was composed of 0.375 M Tris-HCl (pH 8.8), 0.1% SDS, 8-12% 29:1 acrylamide-bisacrylamide. Proteins were resuspended in SDS sample buffer (62.5 mM Tris-HCl, pH6.8; 10% glycerol; 2% SDS; 1% β-mercaptoethanol (v/v); 0.0005% Bromphenol blue; prepared as 2×stock), boiled for 5 min and electrophoresed using a Mini-PROTEAN II apparatus (Bio-Rad) in the running buffer (25 mM Tris, 190 mM glycine, 0.1% SDS) at 80 V until the samples entered the resolving gel and then at 100 V.

**Immunoblotting**

Proteins separated via SDS-PAGE were transferred onto a nitrocellulose membrane (Bio-Rad) using Mini-PROTEAN II apparatus (Bio-Rad) in the transfer buffer (25 mM Tris; 190 mM glycine; 20% Methanol). The membranes were blocked in the TBS (50 mM Tris-HCl, pH 7.6; 200 mM NaCl) containing 5% dry milk at room temperature for 30-60 min. The membranes were then incubated
with appropriate antibody at room temperature for 1-2 hr in TBS containing 2-5% dry milk. Following antibody incubation, the membranes were washed 4-6 times (each 10 min) using TBST (TBS containing 0.1% tween-20). The signals were visualized by enhanced chemiluminescence (Pierce, Rockford, IL).

2.10 ELECTROPHORETIC MOBILITY SHIFT ASSAYS (EMSA)

Bacterially expressed recombinant proteins were incubated in the reaction buffer (10mM HEPES, pH 7.9; 50mM NaCl; 0.1mM DTT; 1mM EDTA; 1mg/ml BSA; 12% glycerol; 2 U/ml poly dI/dC) in the total 20 μl for 10 min at room temperature. \(10^5\) cpm double-stranded \(\gamma^{32P}\) end-labeled oligonucleotides (see below for Polynucleotide kinase reaction and Purification of oligonucleotides) were added to the reaction and incubated for additional 20 min at room temperature. For cold competition assay, appropriate amounts of unlabeled competitor were added to the reaction and incubated for 3 min at room temperature prior to the addition of labeled probes. Reactions were electrophoresed on 5% non-denaturing polyacrylamide gel in 0.25×TBE at 100 V and visualized by autoradiography. The sense strand oligonucleotide, representing the mouse TRAP proximal sequences, was 5’-TTCTGGGGAAGTCCAGTGCTCACATGACCCA-3’. The two region of the potential Eos site were mutated respectively (M1, GGAA to TTTT; M2, GTCC to CAAA).
Polynucleotide kinase Reaction

1 μg of sense strand probe was incubated with 50 μCi γ-32P-ATP and 1 μl of T4 Polynucleotide kinase (Fermentas) in buffer supplied by the enzyme manufacturer at 37ºC for 40 min. The reaction was then heat-inactivated for 5 min at 65ºC followed by addition of 4 μg of anti-sense strand, 1.3 μl of 5M NaCl and 13.7 μl of H2O. Oligonucleotides were denatured at 90ºC for 5 min and annealed by cooling the reaction mix to room temperature.

Purification of oligonucleotides

γ-32P end-labeled double-stranded oligonucleotides were run on 15% polyacrylamide gel in 0.5×TBE at 100 V overnight. The gel was stained with ethidium bromide and the oligonucleotide bands were cut out. Oligonucleotides were transferred to DEAE paper using 1% agarose gel for 20 min at 80 V and recovered from three consecutive elutions using 75 μl TE (10mM Tris-HCL, pH 8.0; 1mM EDTA) containing decreasing concentration of NaCl (5M, 2M, 0.2M respectively). Eluted oligonucleotides were precipitated by adding 200 μl of TE, 2 μl of 10mg/ml tRNA and 1 ml of 100% ethanol at -20ºC overnight and recovered by centrifugation for 15 min and washed with 1ml 70% ethanol, air-dried and resuspended in 100 μl of TE.

2.11 CHROMATIN IMMUNOPRECIPITATION (CHIP) AND RE-CHIP

Osteoclast precursors were plated at a density of 3×10^6 cells per 10cm dish and treated with cytokines for various times as indicated in the Figure legends. Cells were cross-linked with 1% final concentration of formaldehyde
(270 µl for 10 ml culture medium) at 37°C for 10 min. After incubation, 500 µl of 2.5M glycine was added and kept for 5 min at room temperature to inactivate formaldehyde. Cells were then washed three times with ice cold PBS containing protease inhibitors (PI mix). Cells were collected with 1ml PIPES nuclei isolation buffer (20mM PIPES, pH8.0; 85mM KCl; 0.5% NP40) and incubated on ice for 10 min. Samples were centrifuged at 5000 rpm for 5 min and nuclei pellet was resuspended in 1 ml of ChIP lysis buffer (1% SDS, 10mM EDTA, pH 8.0; 50mM Tris-HCl, pH 8.0; protease inhibitors).

Soluble chromatin was prepared following sonication (10 sec pulse with 20 sec pause between each pulse, total 30 pulses at 45% power output) on ice with a Branson-250 digital sonifier (Branson ultrasonics, Danbury, CT) to an average DNA length of 200 to 1000 bp. Samples were then centrifuged at 13,000 rpm for 10 min at 4°C and soluble chromatin was transferred into 15 ml tube and diluted 10 fold in ChIP dilution buffer (0.01% SDS; 1.2mM EDTA, pH 8.0; 16.7mM Tris-HCl, pH 8.0; 167 mM NaCl; 1.1% Triton-X-100; protease inhibitors). The sheared soluble chromatin was pre-cleared with 200 µl t-RNA blocked Protein G agarose slurry for 2 hr at 4°C with gentle rotation. The pre-cleared chromatin was divided into input control, no antibody (no Ab) control, experimental antibody, positive control (Histone H3). Immunoprecipitation was carried out by incubating with 5 µg specific antibodies overnight at 4°C with gentle rotation. Immune-complexes were pulled down using 60 µl Protein G agarose slurry for 2-3 hr at 4°C with gentle rotation. Beads were collected by centrifugation and beads were washed with rotation at room temperature for 15
min using 1 ml of the following buffers: low salt wash buffer (0.1% SDS; 2.0 mM EDTA, pH 8.0; 20 mM Tris-HCl, pH 8.0; 150 mM NaCl; 1.0 % Triton X100), high salt wash buffer (0.1% SDS; 2.0 mM EDTA, pH 8.0; 20 mM Tris-HCl, pH 8.0; 500 mM NaCl; 1.0 % Triton X100), LiCl wash buffer (1 mM EDTA, pH 8.0; 10 mM Tris-HCl, pH 8.0; 250 mM LiCl; 1% NP-40; 1% deoxycholate), twice TE buffer (1 mM EDTA, pH 8.0; 10 mM Tris-HCl, pH 8.0). Immune-complexes were eluted twice with freshly prepared 250 μl of elution buffer (0.1 M NaHCO3, 2% SDS) at room temperature for 15 min. Collected samples were crosslinking reversed in 200mM NaCl with 5 μg/ml RNase A (Sigma-Aldrich) at 65°C overnight. Next day, 10 μl 0.5 M EDTA, 20 μl 1 M Tris-HCl (pH 6.5), and 5 μl proteinase K (10 mg/ml) (Invitrogen Life Technologies, Carlsbad, CA) were added and incubated for 2 h at 55 °C. DNA was recovered by ethanol precipitation and purified with the Qiagen PCR purification kit (Qiagen) using manufacturer's instructions.

For ReChIP assay, pre-cleared soluble chromatin from 6×10^6 cells were immunoprecipitated with Eos antibody. After washing the beads from the first immunoprecipitation, the immune-complex was eluted in 10 mM DTT at 37 °C for 30 min with gentle shaking and diluted 50 fold with ChIP dilution buffer. This eluted immune complex was divided and immunoprecipitated with the second specific antibodies as indicated in Figures. The samples were washed and purified as above.

All ChIP and ReChIP samples were analyzed by Real-Time PCR either by SYBR Green super mix (Bio-Rad) for Ctsk promoter or by Roche universal probe library (Roche) probe using the Faststart TaqMan master kit (Roche) for
TRAP promoter. The primers for Ctsk promoter were: 5'-TGTGCTTCACAGTCCCTCATTT-3' (forward) and 5'-ACTAGGTCCAGTTACAACAGTC-3' (reverse). The following primers were used to amplify TRAP promoter sequence: 5'-CGTTTCAGCCCTAGAACAGC-3' (forward) and 5'-TTTCTCCGAGGATTGTCCAG-3' (reverse). The threshold for the promoter being studied was adjusted by that of input values and represented as relative pull down. All q-PCR reactions were analyzed by melt curve analysis and agarose gels to confirm the presence of a single specific band.

2.12 RNA PURIFICATION AND REVERSE TRANSCRIPTION

RNA was extracted from cultured cells at indicated time points using TRIZOL Kit (Invitrogen) following manufacturer’s direction. At the end of procedure, the RNA pellet was air-dried for 5-10 min and dissolved in 100 μl RNase-free water by passing the solution a few times through a pipette tip and incubating for 10 min at 55 to 60°C. Residual contaminating genomic DNA was removed using 2 units of DNase I (Roche) for 20 min at room temperature. The isolated RNA was subjected to further clean-up using RNeasy Kit (Qiagen) according to the manufacturer’s instruction. The purified RNA was finally eluted in 30-50 μl RNase-free water. RNA concentration was quantified by checking OD$_{260}$, and the purity was determined by the ratio of OD$_{260}$ to OD$_{280}$ (should be greater than 1.8).

1-2 μg of purified total RNA was reverse transcribed by Superscript III reverse transcriptase (Invitrogen) with random hexamer primers (Invitrogen).
Briefly, RNase-free water and 2 μl of 0.2 μg/μl random primers were added to RNA sample to make the volume 13 μl. The RNA-random primer mixture was heated to 65°C for 5 min and incubated on ice for at least 1 min. The mixture was further brought to a final volume of 20 μl by adding other reagents including 4 μl 5×first-strand buffer (Invitrogen), 1 μl of 0.1 M DTT, 1 μl RNaseOUT recombinant RNase inhibitor (Invitrogen), and 1 μl Superscript III reverse transcriptase (Invitrogen). The RT reaction was carried out in a thermocycler at 25°C for 10 min to allow primers to anneal with RNA, then at 50°C for 60 min for reverse transcription, and at 70°C for 15 min to inactivate the reaction. The cDNA sample was diluted to 100 μl using Nuclease-free water. Usually 2 μl of each cDNA sample was used in the Real-Time PCR reaction.

2.13 REAL-TIME PCR AND DATA ANALYSIS

Primers used for Real-Time PCR were picked by Primer 3 software with several additional criteria: the primers are 18-25 bp in length; the Tm are usually between 55°C to 60°C; the G/C contents are about 45-60%; the amplified products are about 100-200 bp; and the forward and reverse primers are usually located in different exons separated by at least by one intron. The Real-Time PCR primers are listed in Table 4. The Real-Time PCR was conducted by an Icycler iQ Real-Time Detection system (Bio-Rad). The typical 25 μl reaction contained 2 μl diluted cDNA sample, 300 nm each primer, 12.5 μl SYBR Green super mix (Bio-Rad), and 7-9 μl Nuclease-free water.
<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acp5 (F)</td>
<td>5'-CGTCTCTGCACAGATTGCA-3'</td>
</tr>
<tr>
<td>Acp5 (R)</td>
<td>5'-GAGTTGCCACACAGCATCAC-3'</td>
</tr>
<tr>
<td>Ctsk (F)</td>
<td>5'-AGGGAAGCAAGCACTGGATA-3'</td>
</tr>
<tr>
<td>Ctsk (R)</td>
<td>5'-GCTGGCTGGAATCACAATTCTCTCTTT-3'</td>
</tr>
<tr>
<td>CTR (F)</td>
<td>5'-TTTCAAGAACCCTAGCTGCCAGAG-3'</td>
</tr>
<tr>
<td>CTF (R)</td>
<td>5'-CAAGGCACGGACATGTGTGAGAG-3'</td>
</tr>
<tr>
<td>Eos (F)</td>
<td>5'-CTCAGTGCCACTCCATCAA-3'</td>
</tr>
<tr>
<td>Eos (R)</td>
<td>5'-GCCTAAGGCGCTGACCTGACATGAATG-3'</td>
</tr>
<tr>
<td>c-fms (F)</td>
<td>5'-CGTCTCCCTAGGACAAAGCA-3'</td>
</tr>
<tr>
<td>c-fms (R)</td>
<td>5'-CACCTGGTACTTGTGACTRESGCTTCT-3'</td>
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<td>RANK (F)</td>
<td>5'-GCTGGCTACCATGGAACTC-3'</td>
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<tr>
<td>RANK (R)</td>
<td>5'-GTGCAGTTGGTCTAAGGTCTTCTTCT-3'</td>
</tr>
<tr>
<td>L4 (F)</td>
<td>5'-AAGATGATGAAACCCGACCTTAGC-3'</td>
</tr>
<tr>
<td>L4 (R)</td>
<td>5'-CCTTCTCTGGAAACCTTCTCAG-3'</td>
</tr>
</tbody>
</table>

Table 4: Real-Time PCR primers for RNA analysis.
The PCR threshold was determined by the iCycler PCR baseline subtracted curve fit method. The threshold for the gene being studied was adjusted by that of a reference gene (ribosomal protein L4). The melting curves were checked for all reactions and agarose gels were examined to confirm the presence of a single specific band in all reactions.

2.14 **IN VITRO SITE-DIRECTED MUTAGENESIS**

*In vitro* site-directed mutagenesis was carried out by QuickChange Site-directed Mutagenesis Kit (Stratagene, La Jolla, CA) following manufacturer's instruction. Mutagenic primers to introduce desired point mutations in MITF were designed based on manufacturer's guideline and sense strand sequences are listed in Table 5. Mutant strand synthesis was performed in a reaction mixture containing 5 μl 10×reaction buffer, 50 ng of ds-DNA template, 125 ng of each mutagenic primer, 1 μl dNTP mix, 1 μl *Pfu Turbo* DNA polymerase (2.5 U/μl), and double-distilled water to final 50 μl. After amplification using cycling parameters suggested by the manufacturer, each reaction was added with 1 μl of Dpn I restriction enzyme and incubated at 37°C for 1 h to digest nonmutated parental DNA. 1 μl of Dpn I treated DNA from each reaction was transformed into 50 μl of XL1-Blue supercompetent cells. Single colonies from the transformed plates were inoculated into LB medium containing appropriate antibiotics to prepare plasmid DNA. The presence of point mutations was confirmed by DNA sequencing.
<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence</th>
<th>Note</th>
</tr>
</thead>
<tbody>
<tr>
<td>K233-D</td>
<td>5’-GGTACTCTGATCCCCGACTCAAATGATCCA-3’</td>
<td>Mut 1</td>
</tr>
<tr>
<td>N235D236-HA</td>
<td>5’-ATCCCCAAGTCACATGCTCCAGACATGCGG-3’</td>
<td>Mut 2</td>
</tr>
<tr>
<td>P237D238-DN</td>
<td>5’-AAGTCAAATGATGACACATGCGGTGGAAC-3’</td>
<td>Mut 3</td>
</tr>
<tr>
<td>W241-T</td>
<td>5’-GATCCAGACATGCGGACCAACAGGGAACC-3’</td>
<td>Mut 4</td>
</tr>
<tr>
<td>N235-D238</td>
<td>5’-ATCCCCAAGTCACATGCTGACAACATGCGG-3’</td>
<td>Mut 23 after Mut 2</td>
</tr>
<tr>
<td>New W241-T</td>
<td>5’-ACATCGGGACGAAACAGGGAACCATT-3’</td>
<td>Mut 24 after Mut 2</td>
</tr>
</tbody>
</table>

Table 5: Sense strand sequences for primers used in site-directed MITF mutagenesis.
CHAPTER 3

MAPPING AMINO ACIDS IN MITF IMPORTANT FOR INTERACTION WITH PU.1

3.1 INTRODUCTION

Transcription factor MITF has been implicated in differentiation and survival of several developmentally unrelated cell types including osteoclasts, melanocytes and retinal pigmented epithelial cells. Interestingly, MITF transactivates a completely different set of genes in each cell type. MITF regulates Tyrosinase, Tbx-2, TRP-1 in melanocytes and Mouse Mast Cell Proteases (MMCPs) in mast cells (Steingrimsson et al., 2004). In osteoclasts, MITF activates genes including $Acp5/\text{TRAP}$, $Ctsk$, $\text{Oscar}$, and $\text{E-cadherin}$ (Luchin et al., 2000; Motyckova et al., 2001; Mansky et al., 2002a; So et al., 2003). How MITF selectively affects gene expression and differentiation in these unrelated cell types is an interesting biological question.

One possible mechanism to account for MITF action is that MITF acts in concert with a unique combination of transcription factors to affect expression of target genes in each cell type. In mast cells, MITF was shown to interact with
PEBP/CBF transcription factor (Morii et al., 1999), a possible MITF partner necessary for the regulation of mast-cell specific genes. Expression of hematopoietic-restricted Ets family transcription factor PU.1 distinguishes osteoclasts from melanocytes. Cooperation between MITF and PU.1 at least partly accounts for the ability of MITF to selectively regulate target genes during osteoclast differentiation (Chapter 1.4.5.3). *In vitro* DNA-binding assays have demonstrated that PU.1 specifically binds to the GGAA site 10bp upstream of the MITF binding motif M-box and PU.1 forms a ternary complex with MITF on the *Acp5* promoter sequence. Mutation of the binding site for either transcription factor abolished the formation of the complex containing both MITF and PU.1 and abrogated the synergistic activation of the *Acp5* promoter (Luchin et al., 2001). The *Ctsk* promoter also contains a PU.1 site adjacent to the M-box that was shown to be important for the regulation of the promoter by MITF (Motyckova et al., 2001).

MITF and PU.1 form a complex when coexpressed in COS-7 cells. Using *in vitro* GST-pulldown assays, Luchin et al. have mapped the regions of MITF and PU.1 required for the physical interaction: the basic-helix-loop-helix zipper (bHLH-Zip) domain in MITF and the Ets DNA binding domain (DBD), respectively (Luchin et al., 2001). A more precise understanding of the interacting domains of MITF and PU.1, including the identification of the specific residues in MITF essential to mediate MITF/PU.1 interaction, would provide a better understanding of the molecular mechanism of transcriptional control of osteoclast differentiation.
3.2 RESULTS

3.2.1 Construction of mutations in the loop region of MITF

Besides TEF3, TFEB, and TFEC, other bHLH-Zip transcription factors that are most close related to MITF are upstream stimulatory factor (USF) 1 and USF2. These two transcription factors are ubiquitously expressed in eukaryotic cells, and are involved in a broad spectrum of biological activities (Luo and Sawadogo, 1996; Sirito et al., 1998). For instance, both proteins are essential for embryonic development, since an embryonic lethal phenotype was observed with the double-null mouse mutants (Sirito et al., 1998). Similar to MITF, USFs recognize the E-box consensus sequence CANNTG as a homodimer or heterodimer (Sirito et al., 1992). In addition, USF2 can form a heterodimer with MITF (Nechushtan et al., 1997). However, unlike MITF, USF2 can not physically interact with PU.1 and synergistically transactivate promoters with PU.1 (Tian et al., 1999; Gobin et al., 2003). Therefore, protein sequence comparison between MITF and USF2 would facilitate the identification of the specific amino acids in MITF essential for physical and functional interaction with PU.1.

As shown in Figure 17, the bHLH domain of MITF is highly homologous to the bHLH motif in USF2. The only region exhibiting relatively low similarity is the loop region of these two proteins. Based on this observation, in vitro site-directed mutagenesis was conducted to replace the amino acids in MITF with the corresponding amino acids in the loop region of USF2. Four mutations were constructed: K$_{233}$-D, N$_{235}$D$_{236}$-HA, P$_{237}$D$_{238}$-DN, and W$_{241}$-T, which are conveniently denoted as Mut 1, Mut 2, Mut 3, and Mut 4 in this thesis (Figure 17).
Figure 17: Protein sequence comparison of bHLH domain of MITF and USF2. Similar (+) and identical amino acids are labeled in between two sequences. Locations of mutations are indicated.
3.2.2 Identification of mutations in MITF that lack functional interactions with PU.1

To study the effects of these mutations on the functional interaction between MITF and PU.1, we performed transient transfection assays using Acp5/TRAP luciferase reporter in NIH 3T3 cells. MITF or PU.1 alone activated Acp5 promoter activity about 4-6 fold (Figure 18, 1\textsuperscript{st} black bar and 2\textsuperscript{nd} white bar) compared to the basal level of activity (Figure 18, 1\textsuperscript{st} white bar). In agreement with the previous reports that MITF and PU.1 synergistically activate the Acp5 promoter (Luchin et al., 2001), we observed about a 20-fold induction of Acp5 promoter activity by the combination of MITF and PU.1 (Figure 18, 2\textsuperscript{nd} black bar). Interestingly, Mut 1 exhibited much higher activation potential on the Acp5 promoter when present alone or in combination with PU.1 (Figure 18, 3\textsuperscript{rd} white and black bar respectively). This was an unexpected result since we attempted to find mutations that could reduce cooperation between MITF and PU.1. Mut 2 and Mut 4 greatly reduced the ability of MITF to synergistically activate the Acp5 promoter with PU.1 (Figure 18, 4\textsuperscript{th}, 6\textsuperscript{th} black bar respectively), while Mut 3 only slightly reduced coactivation of MITF and PU.1 (Figure 18, 5\textsuperscript{th} black bar). However, MITF proteins with Mut 2, Mut 3, or Mut 4 retained the ability to activate the Acp5 promoter to the same extent as wild type MITF (Figure 18, 4\textsuperscript{th}, 5\textsuperscript{th}, 6\textsuperscript{th} white bar respectively), indicating that these mutations specifically disrupted the synergistic activation mediated by MITF with PU.1. We further constructed double mutations Mut 23 (combined Mut 2 and Mut 3) and Mut 24 (combined Mut 2 and Mut 4) and tested these mutated MITF molecules in the
Figure 18: Identification of mutated MITF proteins that lack functional interaction with PU.1. Transient transfection assays in NIH 3T3 cells. Acp5 luciferase reporter (2µg) was transfected alone or together with expression vectors encoding MITF (2µg) and PU.1 (0.4µg). Total DNA in each transfection was kept constant by adding empty expression vector. Relative luciferase activity was represented as fold induction from basal promoter activity (set as 1). Results from three independent experiments are presented as Mean±SEM.
transient transfection assays. Similar to Mut 2, Mut 23 showed a reduction in cooperation with PU.1. Mut 24 abolished the ability of MITF to synergistically activate Acp5 with PU.1. Neither mutation affected transactivation of the report when tested without PU.1.

3.2.3 DNA-binding affinities of mutated MITF proteins to the Acp5 promoter

Using *in vitro* site-directed mutagenesis, we introduced the MITF loop mutations into the expression construct of GST-MITF (199-298), which contains the bHLH-Zip region of MITF. Recombinant GST-tagged wild type MITF protein, as well as various mutated MITF proteins, were overexpressed in *E. coli* and used in electrophoretic mobility shift assays (EMSAs) to test the effects of these mutations on the DNA-binding abilities to the Acp5 promoter. The Acp5 oligonucleotides used in the present EMSAs represent the mouse Acp5 promoter region and contain the previously identified functional MITF and PU.1 binding sites (Luchin et al., 2000; Luchin et al., 2001), as indicated in Figure 19A.

EMSA results demonstrated that Mut 2 and Mut 4 had no apparent effects on DNA binding affinity to the Acp5 promoter sequence, while Mut 1 resulted in increased DNA binding, while Mut 3 exhibited slightly increased DNA binding to the Acp5 probe (Figure 19B, left panel). MITF proteins bearing Mut 23 or Mut 24 demonstrated similar DNA-binding affinities to the wild type protein (Figure 19B, right panel). Cold-competition assays to measure off-time rates of the complexes were also performed for wild type and mutated MITF proteins (Figure 20). The complex containing labeled Acp5 oligonucleotides and wild type
Figure 19: Binding of mutated MITF proteins to the Acp5 promoter sequence. (A) Sequence of Acp5 oligonucleotide used in the present EMSAs. (B) EMSAs using γ-32P end-labeled Acp5 oligonucleotide and recombinant GST, GST-MITF (199-298) wild type (WT), or mutated MITF proteins as indicated. The MITF/Acp5 complex is indicated. The protein loading for each EMSA reaction was indicated by staining with Coomassie Brilliant G-250 (bottom panel).
Figure 20: Cold competition assays for mutated MITF proteins on the Acp5 promoter sequence. Recombinant GST-MITF (199-298) wild type (WT), GST-Mut 1(A), GST-Mut 2(B), and GST-Mut 4 (C) were incubated with γ-32P end-labeled Acp5 oligonucleotides. 100-fold molar excess of the cold Acp5 probe was added to the reaction mix either prior the addition of the labeled probe (+) and incubated for 5 min, or after the addition of the labeled probe and incubated for indicated time period.
MITF protein was mostly competed by unlabeled probe after 10 minutes of incubation. However, the Mut 1/Acp5 complex was very stable, with little competition observed even after 120 min of incubation with cold probe (Figure 20A), indicating a much higher affinity between Mut 1 and Acp5 oligonucleotides. The complex containing either Mut 2 (Figure 20B) or Mut 4 (Figure 20C) proteins behaved very similarly to the complex containing the wild type MITF protein. These observations were consistent with previous EMSA data (Figure 19). Taken together, the results of DNA-binding assays were in close agreement with the transient transfection data (Figure 18). The increased DNA binding of Mut 1 may account for the enhanced coactivation between Mut 1 protein and PU.1. EMSA results also support the notion that Mut 2, Mut 4, Mut23, and Mut24 did not impair the DNA-binding affinity of MITF, but specifically disrupted the functional interaction between MITF and PU.1.

3.2.4 Physical interactions between mutated MITF proteins with PU.1

In vitro GST pulldown assays were performed to determine whether the mutations in the loop region of MITF directly affected its physical association with PU.1 (Figure 21). Bacterially expressed GST protein, GST-MITF (199-298), and various GST-tagged mutated MITF proteins were immobilized on Glutathione-Sepharose beads and used to pulldown His₆-tagged PU.1 protein. Beads were then extensively washed using binding buffer (containing 0.15 M NaCl) and subjected to western blot using antibody against His₆ epitope. The amount of GST-tagged protein bound to the beads was evaluated using GST antibody. As shown in Figure 21A, all four mutated MITF proteins still could bind to PU.1 in
Figure 21: Physical interactions between mutated MITF proteins and PU.1. Recombinant GST, GST-MITF (199-298) wild type or mutated MITF proteins, and His-tagged PU.1 were used for in vitro GST pulldown assays. Bound proteins were analyzed by western blot using anti-His and anti-GST antibodies.
vitro. Similar levels of physical interaction with PU.1 were detected for Mut 1, Mut 2 and Mut 4, whereas Mut 3 showed increased association with PU.1. Mut 23 exhibited a slightly reduced association with PU.1 taking into account the total amount of GST-tagged protein bound to the beads (Figure 21B). Significantly, Mut 24 bound less efficient to PU.1 protein compared to wild type protein (Figure 21B). We further assayed the association of Mut 23 and Mut 24 with PU.1 protein after a more stringent high salt wash (0.4 M NaCl). As shown in Figure 21C, under these conditions both Mut 23 and Mut 24 showed an obvious reduction of association with PU.1 protein, indicating the corresponding amino acids in the loop region of MITF are critical to mediate physical interaction with PU.1.

3.3 CONCLUSION

Using an in vitro site-directed mutagenesis approach, we have introduced mutations into the loop region of MITF protein: K233-D (Mut 1), N235D236-HA (Mut 2), P237D238-DN (Mut 3), W241-T (Mut 4), N235D236-HA&P237D238-DN (Mut 23), and N235D236-HA&W241-T (Mut 24). We demonstrated that Mut 1 has much higher DNA binding affinity to the Acp 5 promoter and enhanced coactivation with PU.1, as shown in the DNA-binding and transient transfection assays. Though MITF proteins bearing the mutation of N235D236-HA (Mut 2), W241-T (Mut 4) or N235D236-HA&W241-T (Mut 24) showed similar DNA binding affinity to wild type protein, mutations of these amino acids disrupted the synergistic activation of MITF with PU.1 on the Acp5 promoter and greatly reduced the physical association with PU.1. This evidence allows us to conclude
that they specifically mediate physical and functional interaction between MITF and PU.1.

Transcription factor PU.1 is an osteoclast-specific partner of MITF in regulating target genes expression. Strong evidence of genetic interaction between MITF and PU.1 has been revealed, indicating the importance of this interaction \textit{in vivo}. Since interaction of these proteins leads to synergistic activation of osteoclast specific genes, by targeting this interaction directly, we can regulate the activity of osteoclasts, thus providing another approach to the treatment of bone-related diseases due to excessive bone resorption. Additional studies are still needed to prove the significance of these identified residues for MITF/PU.1 interaction in the context of osteoclast differentiation.
CHAPTER 4

EOS MODULATES MITF AND PU.1 ACTION ON OSTEOCLAST SPECIFIC GENES IN COMMITTED MYELOID PROGENITORS

4.1 INTRODUCTION

Cooperation between MITF and the hematopoietic-restricted transcription factor PU.1 at least partly accounts for the ability of MITF to selectively regulate target genes during osteoclast differentiation. However, MITF and PU.1 are expressed in macrophages and osteoclasts, and in the common mononuclear precursor for both of these cell types (Scott et al., 1994; Kawaguchi and Noda, 2000). This poses an additional question concerning how gene regulation patterns are maintained in closely related cell lineages. Modulation of MITF and PU.1 activities is likely to play a critical role in maintaining the specific gene expression patterns in these closely related cell lineages. Like many other DNA-binding proteins, the transcriptional activity of MITF and PU.1 are influenced in a complex manner by an array of different intracellular proteins. Recently, we demonstrated that in primary cells deprived of CSF-1, MITF was sequestered to the cell cytoplasm through interactions with 14-3-3 proteins,
providing one potential mechanism that regulates MITF activity in myeloid precursor cells (Bronisz et al., 2006). Work presented in this chapter demonstrates that MITF and PU.1 are regulated by a novel interaction with the Ikaros family protein Eos, which functions to recruit co-repressor complexes and thus represses target genes of MITF and PU.1 in myeloid progenitors in the absence of RANKL signaling.

4.1.1 Ikaros family transcription factors

Ikaros (Molnar and Georgopoulos, 1994; Georgopoulos et al., 1997), a hematopoietic-specific zinc finger protein, is the founding member of a Kruppel zinc finger transcription factor family that also includes Aiolos (Morgan et al., 1997), Helios (Hahm et al., 1998; Kelley et al., 1998), Pegasus (Perdomo et al., 2000) and Eos (Honma et al., 1999). The full-length form of all these proteins is characterized by two functionally distinct domains (Figure 22): four N-terminal zinc fingers crucial for sequence-specific DNA binding and two C-terminal zinc fingers involved in homodimerization with self or heterodimerization with members of the family (Molnar and Georgopoulos, 1994). Alternative mRNA splicing generates at least eight Ikaros isoforms (IK1-IK8) containing subsets of the N-terminal fingers and all sharing the C-terminal zinc finger domain. Ikaros isoforms containing at least three N-terminal fingers are able to bind to the Ikaros consensus recognition sequence (Molnar and Georgopoulos, 1994). Two splice forms of Helios have been reported; the first is similar to full-length Ikaros (IK-1) in that it encodes four N-terminal zinc fingers and two C-terminal zinc fingers, and the second is analogous to IK-2 in that it is missing the first zinc finger in the
N-terminal domain (Hahm et al., 1998). Multiple isoforms of Aiolos have been identified in human, but not in mouse (Liippo et al., 2001). No splicing variants have been reported in Eos so far.

Studies of knockout mutations in mouse models have revealed Ikaros as one of the central regulators of lymphocyte differentiation. Ikaros null mice (Ik^-/-) lack B and natural killer cells but develop T cells postanatally (Wang et al., 1996). Mice overexpressing a dominant negative Ikaros protein that lacks the DNA-binding domain but still retain a dimerization domain exhibit more severe phenotype, with a complete absence of lymphoid cells (T, B, natural killer and dendritic cells) and death from severe infection soon after birth (Georgopoulos et al., 1994), suggesting Ikaros works in concert with other family proteins. Indeed, two other Ikaros family members, Aiolos and Helios, have been shown to have lymphoid-restricted expression patterns and both factors can interact with Ikaros to form a complex regulatory protein network that controls cell fate decisions and regulate homeostasis in the lymphoid system (Morgan et al., 1997; Hahm et al., 1998; Kelley et al., 1998). The C-terminal zinc finger domains are indispensable for these protein-protein interaction (Sun et al., 1996). The physiological effect of Aiolos deficiency is an increase in B cells precursors, spontaneous production of autoantibodies and development of B cell lymphomas, indicating the important role of Aiolos in B cell differentiation, proliferation, and maturation to the effector state (Wang et al., 1998). No knockouts so far have been reported for other Ikaros family members including Eos, Pegasus and Helios, and the biological functions of these proteins remains to be elucidated.
4.1.2 Eos protein

Eos was identified as a novel Ikaros family protein through a two-hybrid screen against a bait protein encompassing a typical C-terminal zinc finger dimerization domain from Aiolos (Perdomo et al., 2000). The Eos protein consists of 532 amino acids and has structural motifs common to the Ikaros family members, namely four N-terminal zinc finger motifs required for DNA recognition and two C-terminal zinc fingers implicated in homo- or hetero-dimerization with the family members (Figure 22). These domains have stronger homologies with corresponding domains of other known Ikaros members than with the remaining regions. The N-terminal DNA binding domain of Eos is capable of recognizing a typical Ikaros binding element (GGGAAAtacc, where GGGAA are the most highly conserved residues) and C-terminal zinc finger domains are able to mediating interactions with all known Ikaros family members (Perdomo et al., 2000). The C-terminal domain of Eos has also been implicated to form a higher order complex in solution (Westman et al., 2003).

In contrast to other Ikaros family members, which are mainly expressed in lymphoid cells, Eos exhibits a distinct expression pattern. Eos is present at highest levels in THP-1 and Mo7e-IL3, human monocytic and early megakaryocytic cell lines respectively. Eos expression is also detected at much lower levels in other selected hematopoietic cell lines: MOLT-4, NALM-6, and K562 (T, B, and erythroid cell lines respectively), while Ikaros and Helios are most abundant in the T and B cell lineages (Perdomo et al., 2000). Expression of Eos in human tissues has also been examined: the Eos signal is highest in
Figure 22: Schematic representation of Eos and Ikaros. The zinc fingers are represented by unfilled semi-ellipses; the positions of the PXDLS-related motif are indicated by filled ellipses.
skeletal muscle, but low level expression can also be detected in heart, thymus, kidney, liver, and spleen (Perdomo et al., 2000). In contrast, the expression of human Ikaros and Helios is essentially confined to lymph nodes, thymus, and spleen (Nietfeld and Meyerhans, 1996; Hosokawa et al., 1999). These observations demonstrate that there are several cell lines and tissues in which Eos is co-expressed with other family members and raise the possibility that Eos may function along with the Ikaros family proteins in these compartments. In addition, Eos appears to be more broadly expressed than other family members like Ikaros and Helios, indicating Eos may play roles that are independent of other Ikaros family members outside of the lymphoid compartment.

4.1.3 Gene repression mediated by Ikaros family proteins

The molecular mechanisms by which Ikaros proteins regulate gene expression are particularly interesting since Ikaros family proteins can act in both transcriptional activation and repression. For example, Ikaros is capable of both activation and repression (Koipally et al., 1999; Koipally et al., 2002); Aiolos and Helios have been reported to function as activators (Morgan et al., 1997). In contrast, Eos and Pegasus have been mainly implicated in transcriptional repression (Perdomo et al., 2000; Perdomo and Crossley, 2002). Recent studies have highlighted the roles of Ikaros family proteins as potent transcriptional repressors. In lymphocytes, Ikaros exists in association with at least two co-repressors Sin3 (Koipally et al., 1999) and Mi-2 (Kim et al., 1999b) respectively, which are integral components of distinct histone deacetylase (HDAC) and
chromatin remodeling complexes. Consistent with a role for HDAC in Ikaros-mediated gene repression, histones are underacetylated in the vicinity of Ikaros recruitment sites and the HDAC inhibitor, trichostatin A (TCA), alleviates repression by Gal4-Ikaros (Koipally et al., 1999). In addition to HDAC-dependent mechanisms of repression, Ikaros also interacts with co-repressor CtBP (C-terminal binding protein), which can repress gene expression in a HDAC activity-independent manner (Koipally and Georgopoulos, 2000). Furthermore, interaction between Ikaros and the CtBP-interacting protein (CtIP) has also been linked to a HDAC-independent strategy of repression (Koipally and Georgopoulos, 2002a).

Interactions between co-repressors and other Ikaros family proteins have also been reported. Using immunoprecipitation assays, Koipally et al. demonstrated that all Ikaros family members could interact with ectopically expressed Sin3A and Sin3B, and endogenous Mi-2 and several HDACs (Koipally and Georgopoulos, 2002b). As for association with CtBP co-repressor, Ikaros and Eos, but not other family members, can interact with CtBP (Koipally and Georgopoulos, 2000; Perdomo and Crossley, 2002). CtBP recognizes a PXDLS motif found in the repression domains of a wide range of transcription factors (Chinnadurai, 2002). CtBP has been shown to associate with Ikaros through a $^{34}$PEDLS$^{38}$ motif in its N-terminus and CtBP contact was required for the repressive activity of this domain (Koipally and Georgopoulos, 2000). The C-terminal region of Eos also contains a sequence $^{372}$PEDLA$^{376}$, that resembles the consensus CtBP binding motif. Eos interacts with CtBP in vitro and in vivo, but
mutation in this putative CtBP binding site does not eliminate the Eos-CtBP interaction (Perdomo and Crossley, 2002), indicating that the interaction does not completely depend on this motif and may take additional contacts through other domains within the Eos protein. The specific mechanisms by which Eos influences gene expression are still ill-defined. Currently available evidence defining Eos as a strong transcriptional repressor are mostly derived from transient reporter assays using constructs containing canonical Ikaros binding sites (Perdomo et al., 2000; Perdomo and Crossley, 2002). In addition, due to the lack of Eos deficient mice, little is known about its target genes in vivo and its overall physiological functions.

4.1.4 Co-repressor complexes and gene repression

Transcriptional repression, like transcriptional activation, is important in regulation of gene expression. DNA sequence-specific repressors mediate their effect by recruiting co-repressors. One of the well-known mechanisms by which several co-repressors function, involves the removal of acetyl groups from histone tails through recruitment of histone deacetylases (HDACs). In general, deacetylated histones correlate with a closed chromatin structure and repression of gene expression. Rapid progress has been made in elucidating the molecular nature and activities of transcriptional co-repressor complexes. In mammals, Sin3 and Mi-2 complexes are two histone deacetylation complexes that both are highly conserved across species (Ahringer, 2000). The Mi-2 complex (also known as NuRD for “nucleosome remodeling and histone deacetylation”) is
approximately 2 MDa in size and consists of at least seven polypeptides (Figure 23), in which Mi-2 protein is the largest component. HDAC1, HDAC2, and two histone-binding proteins (RbAp46 and RbAp48) are also components of the Mi-2 complex. In addition to histone-deacetylase activity, the Mi-2 complex also has an ATP-dependent nucleosome remodeling activity. The Mi-2/CHD protein, which has a chromo domain, a DNA helicase domain, and PHD fingers, is responsible for this remodeling activity. Another distinguishing feature of the Mi-2 complex is the inclusion of the MBD3 protein and MTA1 protein, or the similar protein MTA2, but the biochemical functions of these proteins are not known yet (Ahringer, 2000). The Sin3 complex share four core proteins with the Mi-2 complex (HDAC1, HDAC2, RbAp46, RbAp48) and additionally contains Sin3, SAP18 and SAP30 (Figure 23). The functions of these latter proteins are less clear. Sin3 has no intrinsic DNA-binding activity but is recruited to a target gene promoter through interaction with DNA-binding factors (Silverstein and Ekwall, 2005). A number of proteins have been reported to be involved in gene repression through association with the Sin3 or Mi-2 complexes (Ahringer, 2000; Ng and Bird, 2000). These proteins include transcription factors like Ikaros family proteins, and co-repressors such as N-CoR and SMRT, which facilitate transcriptional repression by physically linking the Sin3 complex with steroid family receptors that are not bound to ligand (Heinzel et al., 1997; Nagy et al., 1997).
Figure 23: Mi-2 and Sin3 complexes (Ahringer, 2000). Yellow boxes indicate core components shared in Mi-2 and Sin3 complexes. The components that are specific for Mi-2 and Sin3 complexes are in dark blue and light blue respectively. Arrows indicate some identified proteins associated with Mi-2 or Sin3 complexes.
CtBP, an unconventional transcriptional co-repressor, can repress transcription in a HDAC-dependent or -independent manner (Chinnadurai, 2002). CtBP is highly conserved among vertebrates as well as invertebrates and was originally identified as a protein that binds to the C-terminal region of the human adenovirus E1A proteins (Boyd et al., 1993). The deletion of the CtBP-binding motif (PXDLS) in E1A increased E1A’s transcriptional activity (Sollerbrant et al., 1996), providing the first indication that CtBP played a role in gene repression. A two hybrid screen identified a number of CtBP-interacting proteins (Vo et al., 2001), and the majority of the identified proteins have previously been known to repress transcription, highlighting the role of CtBP in transcriptional repression. Subsequent work has confirmed that the majority of DNA-binding factors that recruit CtBP family proteins contain related CtBP-binding motifs and some proteins contain multiple motifs (Turner and Crossley, 2001). CtBP has been proposed to be involved in the assembly of a large transcriptional silencing complexe. There is good evidence that CtBP proteins can recruit HDACs. Human CtBP has been reported to associate with HDAC1 in cotransfection experiments (Sundqvist et al., 1998) and with endogenous HDAC2 and Sin3 (Koipally and Georgopoulos, 2000). CtBP may recruit class II HDACs (Chinnadurai, 2002). CtBP also appears to be capable of HDAC-independent transcriptional repression, since CtBP-mediated repression of certain promoters has been reported to be insensitive to HDAC inhibitor trichostatin A (TSA) (Meloni et al., 1999; Koipally and Georgopoulos, 2000). These results suggest CtBP may operate through additional mechanisms. The precise mechanism by which CtBP
mediates transcriptional repression remains to be elucidated and it seems possible that action of CtBP might depend on specific promoter context.

4.2 RESULTS

4.2.1 Eos expression is downregulated during osteoclast differentiation

Gene expression profiles of osteoclast-like cells derived in vitro were determined using DNA microarrays (data not shown). The Ikaros-family member Eos was one gene downregulated during osteoclast differentiation initiated by CSF-1 and RANKL that was selected for further analysis. The kinetics of Eos mRNA expression at various stages of OCL differentiation was validated using qRT-PCR. Bone marrow derived macrophages (BMMs) from wild type mice were stimulated with recombinant CSF-1 and RANKL to induce osteoclast differentiation in vitro (Figure 24A). Consistent with the microarray data, Eos mRNA expression was highest in OCL precursors treated only with CSF-1 (day 0) or during the early stages of differentiation in cells (day 0.5). Eos mRNA expression was reduced more than 5-fold after 3 or 5 days of cytokine stimulation at a time when osteoclast differentiation occurs. In contrast to Eos expression, expression of MITF/PU.1 target genes like Ctsk, and Acp5/TRAP were induced more than 60 fold after 3 or 5 days of combined CSF-1 and RANKL treatment (Figure 24B).
Figure 24: Eos expression is downregulated during osteoclast differentiation. (A) Primary murine bone marrow derived macrophages (BMMs) were stimulated with CSF-1 and RANKL to induce osteoclast differentiation in vitro and cells were stained for TRAP activity. (B) Relative expression of Eos, Acp5, and Ctsk mRNA was measured by qRT-PCR at the indicated time and cytokine treatments. Results from three independent experiments are presented as Mean±SEM. (C) Nuclear extracts from osteoclasts harvested at indicated time were analyzed by western blot using anti-Eos antibody. Histone H3 was used as loading control.
Eos protein expression was evaluated by western blot using an antibody generated and characterized in our lab (see Materials). Histone H3 was used as a loading control for nuclear extracts prepared from cells treated with CSF-1/RANKL for different time. This analysis demonstrated that Eos protein expression was also significantly reduced during CSF-1/RANKL initiated osteoclast differentiation (Figure 24C). These results suggest that Eos is expressed in osteoclast precursors and RANKL signaling downregulates mRNA expression and protein levels of Eos during osteoclast differentiation. The inverse-related expression patterns between Eos and osteoclast specific genes raise the possibility that Eos may play a role in the process of osteoclastogenesis.

4.2.2 Eos inhibits the Acp5 and Ctsk promoter activation by MITF and PU.1

Eos was previously reported to recognize the consensus Ikaros-binding site GGGAA\text{tacc} (where GGGAA are the most highly conserved residues) and repress the activity of the promoters containing such binding sites in transient transfection assays (Molnar and Georgopoulos, 1994; Perdomo et al., 2000). Inspection of the mouse Acp5 and Ctsk promoter region revealed a potential consensus Eos-recognition site that overlaps the functional PU.1 sites (GGAA) present in the Acp5 and Ctsk promoter sequences. As previously demonstrated, this promoter region contains a conserved MITF binding site as well (Figure 25A, (Luchin et al., 2001; Matsumoto et al., 2004)). In addition, these recognition sites
for Eos, PU.1 and MITF are highly conserved across the species including mouse, human and pig.

To examine whether Eos could regulate Acp5 and Ctsk genes through these recognition motifs, transient transfection assays with luciferase reporters were performed in NIH 3T3 cells (Figure 25B). Consistent with previous reports (Luchin et al., 2001; Matsumoto et al., 2004), MITF or PU.1 alone could induce Acp5 and Ctsk promoter activity. The combination of MITF and PU.1 synergistically activated the Acp5 and Ctsk promoters in the transient assays. However, co-transfection of Eos repressed both Acp5 and Ctsk reporter activation by MITF and PU.1, either singly or in combination. These results demonstrated Eos could regulate Acp5 and Ctsk gene expression and inhibit MITF and PU.1 action on their target genes.

4.2.3 Eos binds to the Acp5 promoter sequence in vitro

Eos shares two domains in common with other Ikaros family members: an N-terminal domain containing four zinc fingers crucial for sequence-specific DNA binding, and a C-terminal domain with two zinc fingers, involved in homo- or heterodimerization (Molnar and Georgopoulos, 1994; Perdomo et al., 2000). EMSA assays were performed with oligonucleotides representing mouse Acp5 promoter region and recombinant Eos protein containing the N-terminal DNA binding domain, which has successfully been used in gel-shift assays by other laboratories (Perdomo et al., 2000; Kwan et al., 2003). As shown in Figure 26A, the Acp5 promoter sequence formed a complex with GST-Eos (1-230), but not with control protein GST. Additionally, the formation of this complex was
efficiently competed by the addition of increasing amounts of unlabeled probe to the EMSA reaction. These results demonstrated that Eos could directly bind to the Acp5 target sequence in vitro.

PU.1 has been previously reported to bind the Acp5 promoter in vitro (Luchin et al., 2001) and we confirmed this by using recombinant PU.1 protein in the EMSA reaction (Figure 26B, lane 2). Compared to recombinant PU.1, weaker binding of Eos to the Acp5 probe was observed (Figure 26B, lane 3 vs. lane 2). Since the Eos recognition site overlaps with PU.1 binding site, one hypothesis we considered was that Eos abrogates PU.1 activation on the Acp5 promoter through competitive binding to the same region of Acp5 promoter. Unexpectedly, incubation of both recombinant PU.1 and Eos resulted in formation of a larger complex that contained both factors (Figure 26B, lane 4).

We next examined the effect of mutating the putative Eos binding site on complex formation. Mutation of either half of the Eos consensus sequence (M1: GGAA mutated to TTTT; M2: GTCC mutated to CAAA; Figure 26A) resulted in loss of Eos binding (Figure 26B, lanes 6 and 9), suggesting both regions of the Eos consensus sequence are indispensable for Eos binding to the Acp5 probe. Acp5 mutation M1 also significantly reduced PU.1 binding, as well as the larger complex formation (Figure 26B, lane 5 and 7). However, PU.1 binding were largely unaffected by Acp5 mutation M2 and the larger PU.1/Eos complex could still form with Acp5 mutation M2 (Figure 26B, lane 8 and 10). This result indicated that formation of the larger complex might not require Eos to bind directly to DNA.
Figure 25: Eos represses both Acp5 and Ctsk promoter activity. (A) Acp5 and Ctsk promoter sequences from mouse and human with conserved MITF and PU.1 binding sites (shown with brackets), and the putative Eos binding site (underlined). M1 and M2 show the sequence replacements within the Eos DNA consensus, as indicated. (B) Transient transfection assays in NIH 3T3 cells. Either Acp5 luciferase reporter (Acp5-luc) or the Ctsk luciferase reporter (Ctsk-luc) was transfected alone or together with indicated combinations of expression vectors encoding MITF, PU.1, and Eos. Total DNA in each transfection was kept constant by adding empty expression vector. Relative luciferase activity was represented as fold difference from basal promoter activity (set as 1). Results from three independent experiments are presented as Mean±SEM.
Figure 26: Eos binds to Acp5 promoter sequence in vitro and forms complex with PU.1 on the Acp5 promoter. (A) EMSAs using γ-32P end-labeled Acp5 oligonucleotide and recombinant GST or GST-Eos (101-230) protein. The formation of the DNA-Eos complex (arrow) was competed with increasing amount of cold Acp5 probe. (B) EMSAs using γ-32P end-labeled wild-type or mutated Acp5 oligonucleotides in the presence of recombinant His_{6}-PU.1 and GST-Eos (101-230) protein. The Eos-DNA complex (thick arrow), PU.1-DNA complex (thin arrow) and the supershifted band containing both Eos and PU.1 (broken arrow) are indicated.
4.2.4 Physical interactions between Eos and PU.1

The EMSA results suggested that Eos and PU.1 might directly interact. This possibility was directly tested by *in vitro* GST-pulldown assays using bacterially expressed recombinant Eos and PU.1 proteins. The various PU.1 and Eos constructs used for these experiments are graphically depicted in Figure 27A. PU.1 is a 272-amino acid protein that consists of the N-terminal activation domain, PEST domain and C-terminal DNA binding domain (DBD). GST-Eos (101-230) protein, which has been shown to form a complex with PU.1 and Acp5 probe in above EMSAs, was used to pulldown His-tagged PU.1. As shown in Figure 27B, GST-Eos (101-230), but not control GST protein, was able to pulldown full length PU.1 protein. To map the Eos interaction domain of PU.1, the series of deletions in PU.1 were also tested in pulldown binding assays. Deletion of the PU.1 DBD domain (Δ201-272) abolished association between PU.1 and Eos, indicating the DBD domain of PU.1 is necessary to mediate interaction with Eos. Additionally, recombinant PU.1 protein containing the DBD domain alone (161-272) was sufficient for complex formation with Eos. These experiments mapped the Eos interaction region to the C-terminal DBD/ETS domain of PU.1 (Figure 27B). We also conducted *in vitro* GST pulldown using purified recombinant PU.1 and C-terminal Eos (231-532) proteins. No physical association was observed between Eos (231-532) with either full length PU.1 or DBD of PU.1 (Figure 27C). Therefore, these results demonstrated that the regions to mediate the Eos/PU.1 physical interaction are the N-terminal zinc fingers in Eos (101-230) and DBD domain in PU.1.
Figure 27: Physical interaction of Eos with PU.1 in vitro. (A) Schematic representation of Eos and PU.1 domains and the respective deletion mutations used in the present study. AD-activation domain; DBD- DNA binding domain. (B) Recombinant GST-Eos (101-230) and His-tagged full length (f.l), as well as different deletions of PU.1 as indicated, were used for in vitro GST pull down assays and analyzed by western blot with anti-His antibody. (C) GST pull down assays between recombinant GST-Eos (231-532) with full length (f.l) His-PU.1 or DBD domain of PU.1. Input controls for both (B) and (C) were analyzed by western blot using His and GST antibodies respectively, as indicated.
**Figure 28: Association of Eos with PU.1 in vivo.** Co-IP assays using extracts from COS-7 cells co-transfected with expression vector encoding Flag-Eos (50-230) and HA-PU.1, as indicated. Arrows indicate heavy chain (H.C) and light chain (L.C). Input controls were analyzed by western blot using Flag and HA antibodies respectively, as indicated.
To confirm the *in vitro* results, co-immunoprecipitation (Co-IP) assays were performed (Figure 28). Expression vectors encoding Flag-tagged Eos (aa 50-230) and HA-tagged PU.1 expression constructs were cotransfected into COS-7 cells. Flag-Eos (50-230) was immunoprecipitated from cells by Flag antibody and the immunoprecipitates were examined by western blot using HA antibody. Consistent with the *in vitro* data, PU.1 proteins lacking either the activation domain (Δ33-100) or the PEST domain (ΔPEST) were able to form a complex with Eos, but the protein without the DBD domain (ΔDBD) lost the ability to interact with Eos (Figure 28). PU.1 DBD was again sufficient for this interaction with Eos (Figure 28). Identical results were obtained when Flag-tagged full length Eos protein was used in the Co-IP assays (data not shown). These results demonstrated the physical interaction of Eos and PU.1 both in *vitro* and *in vivo* and identified the minimal domains in PU.1 necessary to mediate this interaction.

### 4.2.5 Physical interactions between Eos and MITF

The results demonstrating that Eos attenuated the activation of *Acp5* and *Ctsk* promoter by MITF in transient transfection assays prompted us to speculate Eos can also interact with MITF. To test this possibility, we performed GST pull down assays using COS-7 cells co-transfected with pEBG-GST-Eos and Flag-MITF expression constructs. pEBG is an expression vector that expresses GST-tagged protein in mammalian cells. MITF was pulled down together with full length GST-Eos (Figure 29B, lane 3), but not with GST protein (Figure 29B, lane 1). To identify the region of MITF responsible for this association, N-terminal (aa 1-218)
and C-terminal (aa 219-418) truncations of MITF (Figure 29A) were created in the Flag-tagged expression vector and used in GST pulldown assays. The N-terminal region of MITF (aa 1-218) (Figure 29A, lane 4), but not the C terminal of MITF (Figure 29B, lane 5), was able to interact with Eos. To further map the region of Eos required for association with MITF, a series of Eos deletion mutations (Figure 29A) were constructed in the pEBG vector and co-transfected with Flag-MITF (aa 1-218) into COS-7 cells. MITF (aa 1-218) bound to C-terminal Eos (aa 231-532) (Figure 29C, lane 7) but not to the N terminal Eos fragments (Figure 29C, lane 4-6) or GST alone (Figure 29C, lane 1).

To test whether this is a direct physical interaction between Eos and MITF, we performed in vitro GST pull down assays using E.Coli expressed recombinant GST-tagged full length MITF and His-tagged Eos (231-532), which contained the C-terminal dimerization zinc fingers of Eos protein. As shown in Figure 30A, His-Eos (231-532) was pulled down along with GST-MITF (lane 2), but not with GST alone (lane 1) by glutathione-sepharose beads. We also confirmed that MITF and Eos form a complex in the presence of Acp5 DNA target sequence as determined by EMSA. MITF itself could bind to the Acp5 promoter sequence (Figure 30B, lane 2) and a super-shifted band was detected with the addition of GST-Eos (231-532) (Figure 30B, lane 3), while Eos (231-532) itself didn’t bind to the DNA probe (Figure 30B, lane 1). These results demonstrated that Eos can associate with PU.1 and MITF via its N-terminal zinc fingers (aa 101-230) and C-terminal zinc fingers (aa 231-532) respectively.
Figure 29: Identification of Eos and MITF interacting domain. (A) Schematic representation of MITF and Eos constructs used in the present study. Zinc fingers in Eos are represented as a black vertical eclipse. (B) GST pull down assays using COS-7 cells expressing full length (f.l) GST-Eos and Flag-tagged full length, N-terminal (1-218) and C-terminal (219-419) fragments of MITF. (C) GST pull down assays using COS-7 cells co-transfected with Flag-tagged N-terminal MITF (1-218) and full length (f.l) GST-Eos, or Eos deletion mutations as indicated.
Figure 30: Eos and MITF physically interact and form a ternary complex on the *Acp5* promoter. (A) Recombinant GST-MITF and His-Eos (231-532) expressed in *E. coli* were used for *in vitro* GST pull down assays and probed with anti-His antibody in western blot. Input controls were analyzed by western blot using His and GSI antibodies, as indicated. (B) EMSAs using γ-32P end-labeled *Acp5* oligonucleotides in the presence of recombinant His-Eos (231-532) and GST-MITF proteins. The MITF-DNA complex (thick arrow) and the supershifted band due to MITF and Eos binding with *Acp5* DNA (broken arrow) are indicated.
4.2.6 Association of Eos with *Acp5* and *Ctsk* promoters decreases during osteoclast differentiation

The preceding EMSA results suggested that recombinant Eos protein can bind to the *Acp5* promoter *in vitro*. To determine whether endogenous Eos actually binds to the target promoter sequences *in vivo*, we used chromatin immunoprecipitation (ChIP) to study the association of Eos with the *Acp5* and *Ctsk* promoters during osteoclast differentiation. The regions of *Ctsk* and *Acp5* genes that were analyzed by qPCR following the ChIP procedures are depicted in Figure 31A. PCR primers were designed to amplify *Ctsk* (-1548 to -1396) and *Acp5* (-210 to -1) promoter regions which have been previously shown to contain functional MITF and PU.1 binding sites (Luchin et al., 2000; Motyckova et al., 2001). In addition to the 5' region of the *Ctsk* and *Acp5* genes, respective internal exon/intron regions were used as negative controls (Figure 31A). An additional negative control was a reaction in which no primary antibody was added to ChIP reactions. A representative control experiment is shown for the *Ctsk* gene with Eos antibody over the time course studied (Figure 31B). Following 40 cycles of PCR, no product could be detected in the negative controls. Histone H3 was routinely used as a positive control. The same set of controls was used for all subsequent ChIP experiments presented for both *Ctsk* and *Acp5* genes (data not shown).

The purified immunoprecipitated DNA fragments were analyzed by Real-Time PCR, in which relative pull down was determined by normalizing to the DNA input control. As shown in Figure 31C, the association of Eos with *Ctsk* and
Acp5 promoter was highest in bone marrow-derived macrophages (BMMs) grown in the presence of CSF-1 only (day 0) and in cells after only 12 h of CSF1 and RANKL treatment (day 0.5). The recruitment of Eos decreased by 7-10 fold following treatment of cells with both CSF-1 and RANKL for 3-5 days, when osteoclast differentiation occurs.

In the same ChIP experiment, we also investigated the recruitment of MITF and PU.1 to the Acp5 and Ctsk promoters during osteoclast differentiation. To our surprise, we detected relatively abundant MITF and PU.1 occupancy at both promoters before the addition of RANKL (day 0) and only a small increase of 2 fold in occupancy of these two promoters by MITF or PU.1 after addition of both cytokines for 3-5 days, when osteoclast differentiation occurs (Figure 31C). This data indicates that there are only small increases in the recruitment of MITF and PU.1 to these target promoters over the time course of OCL differentiation, which doesn’t sufficiently explain the dramatic induction in gene expression observed after three days of CSF-1/RANKL treatment.

4.2.7 Dynamic recruitment of co-repressors and co-activators to the Acp5 and Ctsk promoters during osteoclast differentiation

Since MITF and PU.1 occupancy at target promoters did not correlate with induction of gene expression, we hypothesized that these two factors were also associated with repressor complexes on the Acp5 and Ctsk promoter in the presence of CSF-1 alone. Eos could be one such repressor that could tightly control osteoclast specific genes expression before the initiation of the
Figure 31: Recruitment of Eos, MITF and PU.1 to the Acp5 and Ctsk promoter during osteoclast differentiation. (A) Graphical representation of regions analyzed for Ctsk and Acp5 genes in ChIP assays (left panel). Representative gel pictures for Eos ChIPs on Ctsk gene, following 40 cycles of PCR (right panel). Input indicates the total DNA in each assay before antibody was added. Negative controls included no antibody (no Ab) and 3' exon/intron region (exon). Anti-Histone H3 was used as a positive control. (B) ChIP assays to study the association of Eos, MITF and PU.1 with Ctsk and Acp5 promoters in cells treated with CSF-1 alone (day 0) or CSF-1 and RANKL for 0.5, 3, 5 days. The relative enrichment was measured by qPCR and normalized to input level. Results from three independent experiments are represented as Mean±SEM.
differentiation program by RANKL. Eos has been reported to interact with several co-repressors, including Sin3, Mi-2, and CtBP (Kim et al., 1999b; Koipally et al., 1999; Koipally and Georgopoulos, 2000; Koipally and Georgopoulos, 2002b; Perdomo and Crossley, 2002). ChIP assays were used to determine if these co-repressors were enriched at Ctsk and Acp5 promoters (Figure 32A, top panels). For this analysis, cells grown in CSF-1 alone were compared to cells grown for three days with CSF-1 and RANKL, a time when visible differentiation of OCLs is first apparent and robust expression of target genes occurs. These experiments demonstrated that co-repressors CtBP, HDAC1 and Sin3A were all enriched at Ctsk and Acp5 promoters in cells grown with CSF-1 only, but levels of their association with these target promoters were significantly reduced following three days of CSF-1/RANKL treatment. The Mi-2 protein was also detected at Ctsk and Acp5 promoters, but its association with these promoters upon CSF-1/RANKL stimulation was not significantly different (Figure 32A).

The recruitment of the co-activators CBP/p300 and BRG1 was also studied, since these co-activators were previously reported to interact with both MITF and PU.1 (Sato et al., 1997; Price et al., 1998; Yamamoto et al., 1999; de la Serna et al., 2006). This analysis indicated that CBP and BRG1 were not detected at Ctsk and Acp5 promoters in cells grown with CSF-1 alone. However, both of the co-factors were enriched at these target promoters in cells grown for three days with CSF-1 and RANKL (Figure 32B), concurrent with the robust expression of these target genes at day 3. Collectively, these data indicate the
dynamic assembly of distinct co-repressor and co-activator complexes at the 
*Acp5* and *Ctsk* promoter during OCL differentiation.

**4.2.8 Eos associates with MITF, PU.1, and co-repressors in osteoclast precursors**

To determine whether MITF, PU.1, Eos and co-repressors complexes were assembled at the target promoters simultaneously within the same cells, we performed ReChIP (also called Sequential ChIP) assays using cells treated with CSF-1 alone, in which gene expression of *Acp5* and *Ctsk* is not induced. The first round of immunoprecipitation was carried out with an Eos antibody and the immunoprecipitated cross-linked DNA-protein complexes were then isolated and disassociated from the beads. Aliquots of this sample were subjected to re-immunoprecipitation using antibodies against Eos, MITF, PU.1, CtBP, HDAC1, Sin3A and Mi-2. As expected, the cross-linked DNA-protein complexes isolated by the first round of immunoprecipitation were re-immunoprecipitated by anti-Eos antibody. The amount of DNA present in the ReChIP samples obtained using these second antibodies was determined by Real-Time qPCR and compared to the amount of DNA present in the Eos ReChIP sample (Figure 33A). This analysis indicated that more than 80% of the recovered Eos complex also contained MITF and PU.1 at both *Ctsk* and *Acp5* promoters. In addition, more than 60% of the Eos complex contained CtBP, HDAC1, and Sin3A, at the *Ctsk* promoter. Similar levels of CtBP were seen at the *Acp5* promoter, but the levels of both HDAC1 and Sin3A measured at the *Acp5* promoter were slightly lower,
Figure 32: Dynamic recruitment of co-repressors and co-activators to the Acp5 and Ctsk promoters during osteoclast differentiation. Analysis of association of co-repressors (A) and co-activators (B) with Ctsk and Acp5 promoters in osteoclast precursors grown in CSF-1 alone (day 0) and osteoclast like cells after 3 days of CSF-1 and RANKL treatment (day 3) by ChIP assays. Results from three independent experiments are represented as Mean±SEM.
Figure 33: Association of Eos, MITF and PU.1 with co-repressors on Ctsk and Acp5 promoters in osteoclast precursors. ReChIP to analyze the simultaneous presence of Eos, MITF, PU.1 and co-repressors in osteoclast precursors. Results from three independent experiments are represented as Mean±SEM.
approximately 40% (Figure 33B). The level of Mi-2 complex was much lower than the other co-repressor complexes, indicating that Mi-2 may not be strongly associated with the Eos/MITF/PU.1 complex.

4.2.9 Overexpression of Eos disrupts osteoclast differentiation

4.2.9.1 Retroviral transduction of Eos in BMMs

To confirm the role of Eos as a negative regulator of osteoclast differentiation, Eos was overexpressed in BMMs via retroviral transduction. The bi-cistronic retroviral construct (MSCV-FlagEos-IRES-GFP) used to express Flag-tagged Eos also expressed the green fluorescent protein (GFP) under control of an internal ribosome entry site (IRES), allowing individual infected cells to be identified. Viral stocks produced from packaging cells mock-transfected (mock control), transfected with MSCV-GFP alone (vector control), or transfected with Flag-tagged Eos/GFP vector were used to transduce BMMs derived from wild type mice. The efficiency of transduction as determined by expression of GFP was 70-80% for both vector control and MSCV-Flag-Eos (Figure 34A). Western blotting was performed to verify expression of exogenous Flag-Eos. As seen in Figure 34B, Flag antibody recognized a band only in BMMs transduced with MSCV-FlagEos-IRES-GFP virus but not in mock or vector control. Total Eos protein level was evaluated by anti-Eos antibody. Analysis by densitometer (using the ImageJ program) indicated that retroviral transduction resulted in a modest approximately 2-fold increase in Eos protein production, using Histone H3 as the loading control.
Figure 34: Retroviral transduction of Eos in BMMs. Primary osteoclast precursors were either mock infected or retroviral transduced with empty MSCV-IRES-GFP (mscv) and MSCV-Flag-Eos-IRES-GFP (mscv-Flag-Eos). (A) Individual infected cells were identified by GFP signal under a fluorescence microscope and nuclei of cells were stained for DAPI. (B) Nuclear extracts from infected BMMs were analyzed one day post-infection by Western Blot using antibody against Flag, Eos and Histone H3 respectively. Eos protein expression is about 2 fold increases in BMMs infected with MSCV-FlagEos virus compared to control virus, based on Image J software when using Histone H3 as loading control.
4.2.9.2 Overexpression of Eos inhibits multinuclear osteoclast formation

After transduction, cells were treated with both CSF-1 and RANKL for 3 days. Control virus-infected BMMs matured into large multinuclear (MNC, ≥ 3 nuclei) osteoclasts following this treatment. In contrast, we rarely detected GFP positive multinuclear osteoclasts formation in cells infected with retrovirus encoding Eos (Figure 35A). Osteoclast precursors infected with vector control virus formed approximately the same number of OCLs as mock infected cells (318.5±23.2 Acp5⁺ MNCs per well in vector control; 333±19.5 Acp5⁺ MNCs per well in mock infected control). Infection of osteoclast precursors with the virus encoding Eos led to about 3-fold fewer OCLs formation (133.5±26.9 Acp5⁺MNCs per well), a reduction that was statistically significant (p value <0.001, compared with mock and vector controls) (Figure 35B, bottom left panel). We observed a more dramatic reduction when we counted only GFP-positive OCLs, cells which were successfully transduced. In the vector control, about 19% of the cells positive for GFP underwent differentiation into Acp5⁺ MNCs after 3 days. On the other hand, only 3% of cells expressing Eos and GFP could differentiate into Acp5⁺ MNCs (p value <0.001) (Figure 35B, bottom right panel). These results demonstrated that overexpression of Eos in BMMs inhibited multinuclear osteoclast formation.
Figure 35: Overexpression of Eos in BMMs inhibits multinuclear osteoclast formation. Transduced BMMs were treated with CSF-1 and RANKL for 3 days. (A) Individual infected cells were identified by GFP signal under a fluorescence microscope and nuclei of cells were stained for DAPI. (B) Representative TRAP staining for infected BMMs after 3 days CSF-1 and RANKL treatment. Formation of TRAP⁺ multinuclear cells (MNCs, nuclei ≥3 per osteoclast) was counted for each well (left bar graph) and among infected BMMs (GFP⁺ cells) (right bar graph). Results from three independent experiments were represented as Mean+SEM. Based on two-sample t-test, there is no significant difference between mock and control virus infected BMMs, while mscv-FlagEos virus differ significantly from control virus (p<0.01, labeled with *).

160
4.2.9.3 Overexpression of Eos suppresses the expression of subset of osteoclast specific genes

Expression of Acp5 and Ctsk mRNA were greatly induced after 3 days of CSF-1 and RANKL stimulation (Figure 24B). In contrast, qRT-PCR demonstrated that the levels of Ctsk and Acp5 mRNA were reduced 6-8 fold in cells expressing Flag-Eos versus controls (Figure 36, top panels). In contrast, Eos overexpression did not affect the expression of two known PU.1 targets, c-fms and RANK, the receptors for CSF-1 and RANKL, respectively (Hume et al., 1997; Kwon et al., 2005). In addition, expression of the calcitonin receptor (CTR) was greatly induced after 3 days of CSF-1 and RANKL stimulation, but no differences were detected in cells overexpressing Eos compared with controls (Figure 36, bottom panel). These results indicated that Eos repressed subsets of osteoclast marker genes, such as Acp5 and Ctsk, which are also known MITF and PU.1 targets.

4.3 CONCLUSION

We demonstrated that expression of the Ikaros family protein Eos is temporally regulated during osteoclast differentiation. Eos expression is inversely related with the expression of osteoclast marker genes such as Acp5 and Ctsk during osteoclastogenesis. Overexpression of Eos via retroviral transduction in bone marrow-derived precursors disrupted osteoclast differentiation and suppressed transcription of Ctsk and Acp5. Both in vitro and in vivo analyses demonstrated that Eos interacts with both MITF and PU.1. We have also
Figure 36: Overexpression of Eos inhibits expression of subset of osteoclast specific genes. Relative mRNA expression of Acp5, Ctsk, c-fms, RANK and CTR genes measured by qRT-PCR at indicated time point. Results were from three independent experiments and presented as Mean±SEM. Statistical analysis were conducted using two-sample t-test (*, p<0.01).
provided evidence that Eos represses *Ctsk* and *Acp5* transcription activation by MITF and PU.1 in transient reporter assays. ChIP and ReChIP analyses suggested that Eos form a complex with MITF and PU.1 to repress transcription fromed specific promoters through recruitment of co-repressors Sin3A and CtBP in osteoclast precursors. This work provides a mechanism by which MITF and PU.1 activity is regulated in myeloid precursors and macrophages to prevent inappropriate expression of osteoclast specific target genes (Figure 37).
Figure 37: A schematic model of Acp5 and Ctsk gene regulation by transcription factors and their co-factors during osteoclast differentiation. (A) In the presence of CSF-1 only, MITF, PU.1 and Eos complexes recruit with co-repressors, thus inhibiting Acp5 or Ctsk expression. (B) Combined CSF-1 and RANKL stimulation triggers dissociation of Eos and co-repressor complexes and recruitment of co-activators. (C) Continued CSF-1 and RANKL treatment subsequently leading to robust induction of Acp5 and Ctsk expression.
CHAPTER 5

DISCUSSION

5.1 COLLABORATIVE ACTION OF MITF AND PU.1 IN OSTEOCLAST

When considering transcriptional regulation in osteoclasts, one interesting fact that emerges from the available data is that osteoclast-specific transcription factors that can account for differentiation of this cell type have not been identified. Instead, a group of transcription factors, that individually are expressed in many different cell types and cell lineages, collectively regulate osteoclast differentiation (Sharma et al., 2006a). Several transcription factors have been identified to be important for osteoclast differentiation and activation and these factors include PU.1, NF-κB, AP-1, NFATc1, and MITF (Chapter 1). Some of these factors may act independently at specific stages of osteoclast differentiation. For example, PU.1 plays a critical role in the differentiation of the monocyte/macrophage lineage cells. However, especially during the later stage of osteoclast differentiation, these factors appear to act in concert to insure that the gene products necessary for osteoclast function are appropriately expressed. Thus, the specificity of osteoclast gene expression is shaped largely by
cooperative interactions between a set of fairly ubiquitous transcription factors. In contrast, for some cell types it has become clear that the commitment to a specific lineage may be determined by the action of relatively few transcription factors expressed in a cell-type specific or restricted fashion, for example, the myo D family in muscle differentiation (Weintraub, 1993; Molkentin and Olson, 1996).

Work from our laboratory and others have established that PU.1 is an osteoclast-specific partner of MITF (Chapter 1.4.5.3). MITF and PU.1 have been reported to synergistically activate three definitive osteoclast specific genes, Acp5/TRAP (Luchin et al., 2001), Ctsk (Matsumoto et al., 2004), and Oscar (So et al., 2003). We have also mapped the regions of MITF and PU.1 required for the physical interactions: the basic-helix-loop-helix zipper (bHLH-Zip) domain in MITF and the Ets DNA binding domain (DBD), respectively (Luchin et al., 2001). In order to further characterize the interaction between MITF and PU.1, we attempted to map the amino acids in MITF essential to mediate the physical and functional interaction with PU.1 (Chapter 3). Using in vitro site-directed mutagenesis, we constructed point mutations in the loop region of MITF protein based on sequence comparisons between MITF and USF2, which is also a bHLH-Zip protein, but can not physically and functionally interact with PU.1 (Gobin et al., 2003). The properties of the mutated MITF proteins were studied by transient transfection assays, EMSAs, and in vitro GST pulldown assays. We found several amino acids including N_{235}, D_{236}, and W_{241} in the loop region of MITF were involved in this interaction. Replacing these residues with
corresponding amino acids in USF2 disrupted the synergistic action of MITF and PU.1 and reduced physical association between MITF and PU.1. These mutations did not affect DNA binding affinity of MITF, or affect its ability to activate the Acp5 promoter by MITF. These results indicate that these residues are important to specifically mediate physical and functional interaction between MITF and PU.1. Unexpectedly, our results also revealed a mutation of K<sub>233</sub> to D resulting greater DNA binding affinity and enhanced coactivation with PU.1. However MITF protein bearing K<sub>233</sub>D mutation exhibited similar physical association with PU.1 as to the wild type protein, therefore it is likely that the increased DNA binding may facilitate or stabilize MITF/PU.1/DNA complex to enhance the transcriptional potential of MITF/PU.1 complex.

Identification of amino acids essential to mediate MITF/PU.1 interaction would also allow us to better understand the molecular mechanism of this interaction. In our study, we have demonstrated reduced physical association of mutated MITF protein with PU.1 using in vitro GST pulldown assays. In order to more accurately quantitate the interaction strength between mutated MITF proteins and PU.1, other techniques can be additionally used, such as Biacore’s SPR technology, which allows the real-time detection and monitoring of biomolecular binding events. To further prove the significance of these residues in MITF/PU.1 interaction, it is important to study the behavior of these mutated MITF proteins during osteoclast differentiation. The rescue of mi/mi osteoclasts with retroviral-mediated overexpression of the mutated MITF proteins would be one approach that could provide us with this evidence.
5.2 NEGATIVE REGULATION OF MITF AND PU.1 ACTIVITY BY EOS

The transcriptional activity of MITF is regulated through its interactions with other nuclear proteins. Besides PU.1, other proteins known to interact with MITF include the related bHLH-zip TFE proteins (Hemesath et al., 1994); the HLH Id proteins (Lee et al., 2006); the retinoblastoma (Rb) proteins (Yavuzer et al., 1995); the co-activator CBP/p300 and BRG1 (Sato et al., 1997; Price et al., 1998; de la Serna et al., 2006; Sharma et al., 2006b); the ubiquitin-conjugating enzyme hUBC9 (Xu et al., 2000); the transcription factors PEBP2/CBF (Morii et al., 1999); Fos (Sato et al., 1999), Pax6 (Planque et al., 2001), PIAS3 (Levy et al., 2002), and PIP (PU.1 interacting proteins)/IRF4 (Matsumoto et al., 2001).

Some of these interactions appear to be important for enhancing transactivation activity of MITF, whereas others appear to inhibit MITF action on the target genes. For instance, PIAS3, initially identified as an inhibitor of STAT3, was found to suppress the transcriptional activity of MITF through inhibiting its DNA binding activity to the promoter of mouse mast cell proteases-6 (mMCP-6) (Levy et al., 2002). Id (Inhibitors of differentiation/DNA binding) proteins are HLH proteins, which can bind to bHLH transcription factors via their HLH domain and the resulting heterodimers are unable to bind to DNA because Id proteins lack the necessary basic motif (Benezra et al., 1990). Thus, Id proteins have been proposed to act as dominant negative regulators of bHLH transcription factors. Recently, Ids have been reported to inhibit MITF transactivation of OSCAR.
Additionally, overexpression of Ids in BMMs inhibited the formation of ACP5\(^+\) multinuclear osteoclasts (Lee et al., 2006).

For lineage-determining factors like PU.1, mechanisms for repressing potential target genes in progenitors is critical for maintaining the appropriate control of gene expression patterns. Repression of PU.1 function is a critical step for erythroid cell differentiation, because overexpression of PU.1 in erythroblast blocks erythroid cell differentiation (Moreau-Gachelin et al., 1996). PU.1 and GATA-1 are expressed in both early hematopoietic progenitor cells and are essential for the development of myeloid and erythroid lineages, respectively. PU.1 interacts with GATA-1 and represses GATA-1 mediated transcriptional activation (Rekhtman et al., 1999). Conversely, GATA-1 is a repressor of PU.1 function in myeloid cells and a possible mechanism for this repression is competition between c-Jun and GATA-1 for interacting with PU.1 (Zhang et al., 1999). At an early stage of myeloid commitment, one mechanism by which PU.1 accomplishes this is to directly regulate expression of transcription factors like Erg1 and Erg2 that can in turn repress neutrophil specific genes while activating macrophage specific genes (Laslo et al., 2006). At the same time, the zinc-finger factor Gfi-1 antagonizes macrophage differentiation by inhibiting expression of these PU.1 target genes (Laslo et al., 2006).

Our work presented in Chapter 4 identified Ikaros family protein Eos as a novel interaction partner for both MITF and PU.1. We showed that Eos inhibits activation of Acp5 and Ctsk by MITF and PU.1 through a previously unknown mechanism. Unlike PIAS3, which itself does not bind to DNA and exerts its
inhibitory effects by preventing MITF from binding DNA, Eos is capable of direct binding to the Acp5 promoter and does not appear to interfere with binding of MITF or PU.1 to the target genes. Instead Eos forms a ternary complex with MITF and PU.1 on the Acp5 promoter sequence. Eos also functions in a manner different to Id proteins, which inhibit other bHLH factors in general by forming biologically inactive heterodimers.

The ability to interact with PU.1 might provide additional specificity for Eos to regulate MITF target genes. The Eos binding site overlaps with PU.1 recognition site on the Acp5 promoter, raising the possibility that Eos might inhibit PU.1 action through competitively binding to the Acp5 promoter sequence. Surprisingly, EMSA results showed that Eos forms a complex with PU.1 in the presence of Acp5 promoter. Further mutation analysis indicated that this complex formation does not require Eos to directly bind to the Acp5 promoter.

We also demonstrated that Eos interacts with MITF and PU.1 in mammalian cells and in vitro using purified recombinant proteins. Interestingly, Eos associates with PU.1 and MITF via its distinct domains: the N-terminal DNA-binding zinc fingers and C-terminal dimerization zinc fingers, for PU.1 and MITF respectively. We can not currently rule out the possibility that Eos might compete with MITF to associate with PU.1 since both MITF and Eos interact with the DBD domain of PU.1 protein. However, our Re-ChIP assays demonstrated that more than 80% of the recovered Eos-DNA complex also contained MITF and PU.1 at both Ctsk and Acp5 promoters, indicating Eos likely exists in a complex containing both MITF and PU.1 in osteoclast progenitors. This notion can be
further strengthened by Co-IP assays using COS-7 cotransfected with expression vectors for Eos, MITF, and PU.1.

MITF and PU.1 are expressed in macrophages and osteoclasts, and in the common mononuclear precursor for both of these cell types (Scott et al., 1994; Kawaguchi and Noda, 2000). Our work provides a novel mechanism by which MITF and PU.1 activity is regulated to achieve the specific gene expression patterns in these closely related cell lineages. In this case the Ikaros family protein Eos inhibits MITF and PU.1 activity at the Acp5 and Ctsk promoters in committed myeloid precursors prior to the initiation of osteoclast differentiation. To our knowledge, Eos is the first repressor in osteoclasts that can directly interact with both MITF and PU.1 to regulate their transcriptional activities. An important feature of this modulation is that the effect of Eos appears to be specific for the MITF/PU.1 complex, as other PU.1 target genes in both macrophages and osteoclasts, for example \textit{c-fms} and RANK, were not affected when Eos are overexpressed. Since Eos itself binds only weakly to the Acp5 target sequences \textit{in vitro}, interactions through distinct zinc-finger domains with both MITF and PU.1 likely provides the specificity for target gene repression. Our results reveal an unexpected role of Ikaros protein Eos during osteoclast differentiation and therefore expand our knowledge of Ikaros family proteins, which have been previously mainly implicated in lymphocyte differentiation.
5.3 DYNAMIC ASSOCIATION OF CO-REPRESSORS AND CO-ACTIVATORS WITH TARGET GENES DURING OSTEOCLAST DIFFERENTIATION

Both MITF and PU.1 are known transcriptional activators in osteoclasts, but results reported in Chapter 4 demonstrate that they can also act as components of repressor complexes that suppress target gene expression in the absence of the appropriate extracellular signals necessary for osteoclast differentiation. The mechanisms that control transcription factor switching between activator and repressor remain to be determined. In our study, interaction of MITF and PU.1 with Eos appears to be critical for recruiting co-repressors to the target genes, as the ability of Eos to interact directly with these co-repressors is well documented (Koipally and Georgopoulos, 2002b; Perdomo and Crossley, 2002). Using Chromatin immunoprecipitation (ChIP) approach, we demonstrated that recruitment of Eos to both Acp5 and Ctsk promoters was highest in precursor cells grown in CSF-1 only and significantly reduced in differentiating osteoclasts treated with CSF-1 and RANKL. To our surprise, we observed relative constant association of MITF and PU.1 to the Acp5 and Ctsk promoter during osteoclastogenesis, which apparently does not explain the dramatic induction of mRNA expression of Acp5 and Ctsk. This unexpected result also prompted us to speculate that in the absence of RANKL signaling, MITF and PU.1 may exist in a transcriptional inactive complex to prevent Acp5 and Ctsk expression and this may be achieved through interacting with Eos protein and co-repressor complexes. ChIP assays showed that the promoter
association of several known Eos-interacting co-repressors CtBP, HDAC1, and Sin3A, was greatly reduced following three days of CSF-1/RANKL treatment, whereas the recruitment of the co-activators CBP and BRG1 exhibited the opposite pattern. The physical and functional interactions between MITF and PU.1 with co-activator CBP/p300 and BRG1 have been well documented (Price et al., 1998; Yamamoto et al., 1999; Weilbaecher et al., 2001; de la Serna et al., 2006). Our results indicated dynamic association of co-repressors and co-activators to the Acp5 and Ctsk promoter, concurrent with the target gene expression during osteoclastogenesis.

Our results demonstrate a molecular sequence for target gene expression during osteoclast differentiation (Figure 37). In this model, MITF and PU.1 are bifunctional transcription factors capable of repressing or activating transcription during different stages of osteoclast differentiation. Their dynamic association with either co-repressors or co-activators might play a critical role for switching their roles as transcriptional repressors or activators. Zinc-finger protein Eos provides a link between MITF/PU.1 and co-repressors to tightly control osteoclast-specific gene expression in the absence of appropriate signaling. At the same time, the relatively constant presence of MITF and PU.1 at the target promoters allows committed precursors the flexibility to respond rapidly to the bone microenvironment to reprogram gene expression in response to CSF-1/RANKL signaling. This tightly regulated production of Acp5 and Ctsk, important enzymes involved in bone resorption, is likely to play a critical role in maintaining
bone homeostasis, as most of bone-related disorders result from excessive bone resorption by osteoclast.

5.4 POTENTIAL INVOLVEMENT OF CSF-1/RANKL SIGNALING IN EOS ACTION

The program of gene expression necessary for the development of a specific cell lineage is largely controlled by signal transduction pathways acting to coordinate the transcriptional response to environmental cues. CSF-1 and RANKL signaling are essential for osteoclast differentiation (Chapter 1.3). CSF-1 stimulation in OCLs leads to MAPK/Erk1/2 mediated phosphorylation of MITF at Ser73 and RANKL signaling triggers phosphorylation of MITF at Ser307 via p38 MAPK pathway. These post-translational modifications have been shown to be important for MITF transcriptional activity in osteoclast differentiation (Chapter 1.4.5.3). For instance, CBP/p300, a potent co-activator of MITF, is preferentially recruited to Ser73-phosphorylated MITF (Price et al., 1998; Weilbaecher et al., 2001). Whether RANKL-induced phosphorylation of Ser307 in MITF also directly affects MITF association with co-activators is presently unknown. Very recently, we demonstrated the increased recruitment of both pSer73-MITF and pSer307-MITF, as well as active p38 MAPK, to the Acp5 and Ctsk promoter in cells simulated with CSF-1/RANKL for 3 days (Sharma et al., 2006b), concurrent with our results of increased association of CBP and BRG1 with target promoter. Though phosphorylation of PU.1 in response to CSF-1 has been reported in macrophages (Chapter 1.4.3), the contribution of PU.1 phosphorylation in
osteoclast differentiation remains to be determined. It is likely that distinct phosphorylation of PU.1 events may dictate the function of PU.1, possibly by changing its transcriptional potential and affecting the affinity for interaction partners such as MITF and Eos during different stages of osteoclastogenesis.

Our results demonstrated that Eos expression is downregulated at both mRNA and protein level during osteoclast differentiation initiated by combined CSF-1 and RANKL stimulation. Whether CSF-1/RANKL signaling directly targets the post-translational modification of Eos, affecting either its stability or activity, remains to be determined. Though post-translational modification of Eos has not been reported yet, work on the related Ikaros protein is likely to shed light on the function of Eos modification. For example, Ikaros phosphorylation reduces its DNA-binding ability and plays a role in cell cycle control (Gomez-del Arco et al., 2004). SUMO-modification of Ikaros has been reported to disrupt interaction with co-repressors during lymphoid development (Gomez-del Arco et al., 2005). Potential sumoylation sites in Eos were found to be highly conserved to reported sumoylation sites in Ikaros and our preliminary data showed that Eos can also be modified by SUMO (RH and MCO, unpublished observation). In the future, it will be interesting to investigate whether sumoylation of Eos is regulated by CSF-1/RANKL signal and affects Eos interaction with co-repressors, and/or other nuclear regulators such as MITF and PU.1.

CSF-1/RANKL signaling can activate expression of osteoclast target genes by two mechanisms. First is through the down-regulation of Eos expression, both at the level of mRNA and protein, leading to dissociation of the
co-repressors from target genes (Figure 37). Second is direct phosphorylation
and activation of MITF by both Erk and p38 MAPK pathways, leading to
recruitment of co-activators like CBP/p300 and BRG1 (Weilbaecher et al., 2001;
Mansky et al., 2002b; Hershey and Fisher, 2004). Release of the negative
regulation is necessary before target genes can be fully activated as
demonstrated by the Eos overexpression studies. Retroviral-mediated
overexpression of Eos in BMMs significantly reduced multinuclear osteoclast
formation, as well as mRNA expression of Acp5 and Ctsk, indicating the
importance of balanced Eos expression for osteoclast differentiation. To further
confirm the role of Eos as a negative regulator of osteoclastogenesis, it is
worthwhile to test whether knock-down of Eos, either by siRNA approach in
BMMs or targeted gene deletion in mice, would lead to increased multinuclear
osteoclast formation.

Changes in gene regulation are critical for proper cellular differentiation.
Many transcription factors play important roles in controlling the processes for
lineage development. Whether Eos actually functions as a lineage-decision factor
in myeloid cells in a manner analogous to Gfi-1, or to Ikaros in lymphoid cells,
seems unlikely based on current evidence. For example, the CTR gene, another
definitive marker of osteoclast differentiation, is still upregulated in cells that
overexpress Eos. Thus, Eos does not regulate the entire osteoclast gene
expression program, but seems to selectively modulate the activity of the
MITF/PU.1 complex and a subset of genes necessary for full activity of the
differentiated osteoclast. This model to explain how MITF/PU.1 activity is
modulated in osteoclast progenitors may be generally applicable to other cell types in which committed progenitors can give rise to closely related cell types.

How the actions of MITF/PU.1 and other factors are coordinated, especially at distinct stages of osteoclast differentiation still remains largely unknown. A more detailed understanding of how these factors act during osteoclast differentiation will likely not only provide insights into how osteoclast dysfunction leads to common human bone disorders, but also help to define molecular targets that might have translational potential in diagnosis and treatment of bone diseases.
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