OSTEOACTIVIN PLAYS A NOVEL ROLE IN AUTOPHAGY-MEDIATED BONE HOMEOSTASIS (300 PP.)

Dissertation Advisor: Fayez F. Safadi, Ph.D

Osteoporosis is a disease affecting most the population all over the world. There are some medications to treat this disease but with side effects. For example, bisphosphonates therapy has side effects including osteonecrosis. Therefore, it is important to discover new medications that increase bone formation and decrease bone resorption. Osteoactivin was discovered in an osteopetrotic mutant rat model. Our lab found that osteoactivin acts as anabolic factor to stimulate bone formation by osteoblasts. Autophagy is a catabolic process that allows cells to eliminate unwanted material. Autophagy related proteins have shown to be important for bone cells. Loss of autophagy could lead to dysfunction in bone homeostasis.

In this study, we used the autophagy mTOR-independent and dependent pathways to determine its effects on osteoblast differentiation in vitro, ex vivo, and in vivo. First, we treated mesenchymal stem cells (MSCs) and osteoblast with Trehalose (TH) and Rapamycin (R) in culture, and found an increase in osteoblast differentiation and function determined by alkaline phosphatase (ALP) staining, activity, mineralization, and upregulation of osteoblast related gene expression. We found that treatment with TH and R stimulates osteoblast differentiation. Furthermore, we determined that treatments
with TH causes induction of autophagosome formation associated with creating of double membrane of autophagic vacuoles in MC3T3-E1 osteoblast-like cells.

Next, we wanted to determine if autophagy is important for osteoactivin mediated signaling in osteoblast. Our results have shown that osteoactivin co-localizes with microtubule-associated protein light chain 3II (LC3II), the main marker for autophagosome formation. Furthermore, we found that treatment with TH and R caused new bone formation in neonatal calvarial organ cultures from DBA/2J GPNMB\(^+\) and DBA/2J mice. Lastly, we found that osteoactivin enhanced osteoblast differentiation through the autophagy signaling pathway.

In the next study, we wanted to determine the role of osteoactivin on osteoclast differentiation and function by mTOR-independent and dependent pathways. Our results revealed that osteoactivin inhibits osteoclast differentiation and size through mTOR-independent pathway but increases osteoclast function by the mTOR-dependent pathway in vitro study by TRAP staining and activity. While in vivo study showed that both autophagy pathways (mTOR-independent and dependent) causes increased osteoclast differentiation and bone resorption by TRAP staining and µCT.

Taken together, we believe that the osteoactivin and mTOR-dependent pathway rapamycin interact with each other to increase osteoblast differentiation, bone formation and osteoclast mediated resorption. However, mTOR-independent pathway by TH causes increased osteoblast differentiation, bone formation, and osteoclast differentiation.
OSTEOACTIVIN PLAYS A NOVEL ROLE IN AUTOPHAGY-MEDIATED BONE HOMEOSTASIS

A dissertation submitted
To Kent State University in partial fulfillment of the requirements for the degree of Doctor of Philosophy

By
Fatima Jaber

May 2018

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# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>TABLE OF CONTENTS</td>
<td>V</td>
</tr>
<tr>
<td>LIST OF FIGURES</td>
<td>X</td>
</tr>
<tr>
<td>LIST OF TABLES</td>
<td>XVII</td>
</tr>
<tr>
<td>LIST OF ABBREVIATIONS</td>
<td>XIX</td>
</tr>
<tr>
<td>DEDICATION</td>
<td>XXV</td>
</tr>
<tr>
<td>ACKNOWLEDGEMENTS</td>
<td>XXVI</td>
</tr>
<tr>
<td>CHAPTER 1 INTRODUCTION</td>
<td>1-5</td>
</tr>
<tr>
<td>CHAPTER 2 LITERATURE REVIEW</td>
<td>6-75</td>
</tr>
<tr>
<td>2.1 Bone Structure</td>
<td>6</td>
</tr>
<tr>
<td>2.2 Bone Matrix</td>
<td>10</td>
</tr>
<tr>
<td>2.3 Osteoblasts, Osteocytes, and Osteoclasts</td>
<td>14</td>
</tr>
<tr>
<td>2.4 Bone Growth, Modeling and Remodeling</td>
<td>26</td>
</tr>
<tr>
<td>2.5 Interaction between Osteoblasts and Osteoclasts</td>
<td>28</td>
</tr>
<tr>
<td>2.6 Hormonal Influences on Bone</td>
<td>29</td>
</tr>
<tr>
<td>2.7 Bone Fracture</td>
<td>30</td>
</tr>
<tr>
<td>2.8 Osteoactivin</td>
<td>32</td>
</tr>
<tr>
<td>2.9 Autophagy</td>
<td>41</td>
</tr>
<tr>
<td>2.10 Autophagy Regulation</td>
<td>42</td>
</tr>
</tbody>
</table>
3.13 Cells Transfection .................................................................88
3.14 Immunofluorescence ...............................................................88
3.15 Alkaline Phosphatase Staining and Activity .................................89
3.16 Von Kossa Staining ..................................................................89
3.17 New bone formation in Neonatal Calvarial Organ Cultures .............90
3.18 Osteoclast Cultures .................................................................90
3.19 TRAP Staining and Activity .......................................................91
3.20 In Vitro Osteoclast Resorption Assays ........................................91
3.21 In Vivo Calvarial Bone Resorption assays ....................................92
3.22 MicroCT Analysis ...................................................................92
3.23 RT-qPCR ...............................................................................92
3.24 Statistical Analysis .................................................................95

CHAPTER 4 RESULTS ...................................................................97-220
4.1 Osteoblast Differentiation ..........................................................97
4.2 mTOR-Independent Pathway Enhances Viability and Proliferation of MC3T3-E1 Cells .................................................................100
4.3 mTOR-Independent Pathway Induces Autophagy in Osteoblasts ........102
4.4 Trehalose Induces Autophagy Vesicles in Osteoblasts .................105
4.5 Osteoactivin co-Localizes with Autophagosomes .........................107
4.6 Induction of Autophagy Increases Osteoactivin Production ........................................109

4.7 Autophagy is Altered in Mutant Mice for Osteoactivin ..............................................111

4.8 The Effects of mTOR-Independent Autophagy on Primary Osteoblast Differentiation .................................................................................................................................116

4.9 Effects of Chloroquine on mTOR-Independent Pathway in Osteoblasts .................119

4.10 The Effects of the mTOR-Dependent Pathway in Osteoblast Viability and Proliferation .................................................................................................................................122

4.11 The Effect of the mTOR-Dependent Autophagy on Osteoblast Differentiation .................................................................................................................................124

4.12 mTOR-Dependent Pathway Decreases Osteoblast differentiation by Autophagy Inhibitor (Chloroquine) ..........................................................................................................127

4.13 mTOR-Dependent and Independent Pathways Decreases Autophagy in Osteoactivin Mutant Mice .................................................................................................................................130

4.14 Autophagy Stimulates Osteoblast Matrix Deposition ..............................................136

4.15 Autophagy Stimulates Osteoblast Matrix Deposition and Mineralization ..........141

4.16 Inhibition of Autophagy Decreases Osteoblast Differentiation .................................147

4.17 Osteoactivin Plays a Role in Autophagy-Mediated Osteoblast Differentiation and Function .................................................................................................................................152

4.18 Autophagy Signaling During Osteoblast Differentiation ...........................................161

4.19 Osteoactivin Plays a Role in Bone Formation Induced by Autophagy ex-vivo ....163
4.20 Osteoclast Differentiation.................................................................171
4.21 Autophagy is Required for Osteoclast-Podosome Formation...........173
4.22 mTOR-Independent Pathway During Osteoclast Differentiation ........176
4. 23 mTOR-Dependent Pathway During Osteoclast Differentiation ..........179
4.24 Effects of Autophagy During Osteoclast Differentiation ..................182
4.25 Autophagy Pathway Regulates Osteoclast Resorption in vitro ..........185
4.26 Autophagy is Activated During Osteoclast Differentiation ...............187
4.27 Chloroquin Inhibits RANK-Induced Osteoclast Formation................193
4.28 p-mTOR Signaling is Activated During Osteoclast Differentiation .......200
4.29 mTOR-Independent Pathway Alters Osteoclast Podosome Formation ....202
4.30 Autophagy Pathway Increases the Differentiation and Recruitment of TRAP Positive Osteoclasts in Vivo..................................................208

CHAPTER 5 DISCUSSION ...........................................................................221-232

5.1 Aim 1..................................................................................................222

5.2 Aim 2..................................................................................................227

OVERALL SUMMARY, CONCLUSIONS, And Future Studies ...............233-234

REFERENCES .........................................................................................235-273
LIST OF FIGURES

Figure 2.1: Bone histomorphology of the distal femur.................................................................9

Figure 2.2: Schematic diagram illustrating osteoblast differentiation from mesenchymal stem cells (MSCs).........................................................................................................................19

Figure 2.3: Schematic diagram of osteoclast differentiation from hematopoetic stem cells (HSCs) ..................................................................................................................................................25

Figure 2.4: Schematic representation of glycoprotein non-metastatic B (GPNMB)...............38

Figure 2.5: Schematic representation of full length, recombinant and mutant osteoactivin ........................................................................................................................................................................39

Figure 2.6: A schematic representation the role of osteoactivin in bone cells.................40

Figure 2.7: Schematic diagram of the steps of autophagy pathway.........................................46

Figure 2.8: Schematic diagram illustrating protein components of the mTOR complexes ..............................................................................................................................................................................49

Figure 2.9: Schematic diagram illustrating the role of autophagy on osteoblast differentiation and bone formation .................................................................63

Figure 2.10: Schematic diagram illustrating the role of autophagy on osteoclast differentiation and bone resopotion................................................................................................................67

Figure 3.1: Trehalose structure ..................................................................................................81

Figure 3.2: Rapamycin structure............................................................................................82

Figure 3.3: Chloroquine structure..........................................................................................83
Figure 3.4: Bone marrow isolation ................................................................. 84
Figure 3.5: New bone formation in neonatal calvarial organ cultures ................. 93
Figure 3.6: Calvarial bone resorption assays .................................................. 96
Figure 4.1: Schematic diagram illustrates multiple transcription factors important for mesenchymal stem cells to give rise to a variety of cell types ................................. 99
Figure 4.2: Trehalose enhances viability and proliferation of MC3T3-E1 cells ........ 101
Figure 4.3: mTOR-independent pathway induces autophagy in osteoblasts ......... 103
Figure 4.4: Trehalose increases Becline-1 expression in osteoblasts ................. 104
Figure 4.5: Autphagic vesicle formation induced by trehalose in osteoblasts ....... 106
Figure 4.6: Osteoactivin co-localizes with autophagosome in osteoblasts ........... 108
Figure 4.7: Increased osteoactivin protein levels in osteoblasts treated with trehalose .......................................................................................................................................................................................... 110
Figure 4.8: Autophagy is altered in mutant mice for osteoactivin ...................... 113
Figure 4.9: Decreased autophagy in the MSCs of mutant mice for osteoactivin .... 114
Figure 4.10: Decreased autophagy in bones from osteoactivin mutant mice ....... 115
Figure 4.11: The effect of trehalose on MC3T3-E1 differentiation ........................ 117
Figure 4.12: The effect of trehalose on LC3 conversion in osteoblast differentiation .......................................................................................................................................................................................... 118
Figure 4.13: Decreased osteoblast differentiation by chloroquine ...................... 120
Figure 4.14: Increased LC3II protein levels by chloroquine in osteoblasts ..........121

Figure 4.15: mTOR-dependent pathway enhance viability and proliferation of MC3T3-E1 .................................................................123

Figure 4.16: The effects of the mTOR-dependent autophagy pathway by rapamycin on MC3T3-E1 osteoblast differentiation .................................................................125

Figure 4.17: The effects of rapamycin on MC3T3-E1 osteoblast differentiation .......126

Figure 4.18: Chloroquine treatment decreases osteoblast differentiation ..............128

Figure 4.19: Increase LC3II levels by chloroquine in osteoblasts ......................129

Figure 4.20: mTOR-dependent and independent autophagy reduces in osteoactivin mutant mice .................................................................131

Figure 4.21: Trehalose induces autophagy by mTOR-independent pathway in DBA/2J GPNMB+ mice .................................................................132

Figure 4.22: Rapamycin induces autophagy by mTOR-dependent pathway in DBA/2J GPNMB+ mice .................................................................133

Figure 4.23: Trehalose induces autophagy by mTOR-independent pathway in DBA/2J mice .................................................................134

Figure 4.24: Rapamycin induces autophagy by mTOR-independent pathway in DBA/2J mice .................................................................135

Figure 4.25: Autophagy stimulates osteoblast matrix deposition ......................138

Figure 4.26: Autophagy stimulates LC3II conversion in osteoblasts .................139
Figure 4.27: Autophagy stimulates osteoblast gene expression ........................................140

Figure 4.28: Autophagy stimulates osteoblast matrix deposition ....................................143

Figure 4.29: Autophagy stimulates osteoblast matrix mineralization .................................144

Figure 4.30: Autophagy stimulates LC3 conversion during osteoblast differentiation
........................................................................................................................................145

Figure 4.31: Autophagy stimulates osteoblast related markers during osteoblast
differentiation ......................................................................................................................146

Figure 4.32: Inhibition of lysosomal degradation decreases osteoblast differentiation
........................................................................................................................................149

Figure 4.33: Increases levels of LC3II by chloroquine treatment in osteoblasts.............150

Figure 4.34: Chloroquine treatment decreases osteoblast differentiation......................151

Figure 4.35: DBA/2J osteoblast have decrease differentiation induced by autophagy
compared to DBA/2J GPNMB+ cells ..................................................................................154

Figure 4.36: DBA/2J osteoblasts have reduced LC3II conversion induced by autophagy
compared to DBA/2J GPNMB+ cells ..................................................................................155

Figure 4.37: DBA/2J osteoblasts have reduced Beclin-1 expression induced by
autophagy compared to DBA/2J GPNMB+ cells ...............................................................156

Figure 4.38: DBA/2J osteoblasts have reduced OA expression induced by autophagy
compared to DBA/2J GPNMB+ cells ..................................................................................157
Figure 4.39: DBA/2J osteoblasts have reduced OSX expression induced by autophagy compared to DBA/2J GPNMB⁺ cells .................................................................158

Figure 4.40: DBA/2J osteoblasts have reduced ALP expression induced by autophagy compared to DBA/2J GPNMB⁺ cells .................................................................159

Figure 4.41: DBA/2J osteoblasts have reduced OCN expression induced by autophagy compared to DBA/2J GPNMB⁺ cells .................................................................160

Figure 4.42: p-mTOR activation increases during osteoblast differentiation ...........162

Figure 4.43: Photographic of calvaria organ culture.................................................164

Figure 4.44: Calvariae from both DBA/2J GPNMB⁺ and DBA/2J mice ....................165

Figure 4.45: Increased bone formation induced by autophagy in ex- vivo .............166

Figure 4.46: Increased numbers of osteoblasts induces by autophagy ex-vivo ......167

Figure 4.47: Increased new bone area induced by autophagy ex-vivo ..................168

Figure 4.48: Schematic diagram of autophagy signaling during osteoblast differentiation ......................................................................................................................169

Figure 4.49: Schematic diagram illustrate the role of osteoactivin during osteoblast differentiation by mediating autophagy pathways .............................................170

Figure 4.50: Osteoclast differentiation........................................................................172

Figure 4.51: LC3II co-localize with actin in mature osteoclasts in DBA/2J GPNMB⁺ cells ..................................................................................................................174

Figure 4.52: LC3II co-localize with actin in mature osteoclasts in DBA/2J cells ......175
Figure 4.53: Increased osteoclast differentiation through the mTOR-independent pathway .................................................................177

Figure 4.54: Effect of autophagy during osteoclast differentiation through the mTOR-independent pathway .........................................................178

Figure 4.55: Increased osteoclast differentiation through the mTOR-dependent pathway ........................................................................180

Figure 4.56: Effect of autophagy during osteoclast differentiation through the mTOR-dependent pathway ...........................................................181

Figure 4.57: Autophagy pathway is activated in mature osteoclasts .........................183

Figure 4.58: Autophagy pathway regulates osteoclast numbers.........................184

Figure 4.59: Osteoactivin mediates autophagy pathways for osteoclast resorption in vitro ................................................................................186

Figure 4.60: Osteoactivin plays a role in osteoclasts through the autophagy pathways ..................................................................................188

Figure 4.61: DBA/2J mice have reduced OA by mTOR-dependent pathway ..........189

Figure 4.62: DBA/2J mice have reduced Beclin-1 by mTOR-dependent pathway .....190

Figure 4.63: DBA/2J mice have reduced LC3II by mTOR-dependent pathway .......191

Figure 4.64: DBA/2J mice have reduced TRAP by mTOR-dependent pathway ......192

Figure 4.65: Chloroquine inhibits RANKL-induced osteoclast formation ..........195
Figure 4.66: Chloroquine increases LC3II level during osteoclast formation. ..........196
Figure 4.67: Chloroquine decreases Beclin-1 expression during osteoclast formation
..........................................................................................................................197
Figure 4.68: Chloroquine decreases TRAP expression during osteoclast formation
..........................................................................................................................198
Figure 4.69: Chloroquine decreases OA expression during osteoclast formation
..........................................................................................................................199
Figure 4.70: p-mTOR signaling is activated in mature osteoclasts......... ............201
Figure 4.71: Autophagy pathways alters osteoclast podosome formation..........203
Figure 4.72: mTOR-independent pathway alters osteoclast podosome formation in
DBA/2J GPNMB⁺ mice .........................................................................................204
Figure 4.73: mTOR-dependent pathway increases osteoclast podosome formation in
DBA/2J GPNMB⁺ mice .........................................................................................205
Figure 4.74: mTOR-independent pathway alters osteoclast podosome formation in
DBA/2J mice ........................................................................................................206
Figure 4.75: mTOR-dependent pathway increases osteoclast podosome formation in
DBA/2J mice ........................................................................................................207
Figure 4.76: Autophagy pathways increase the differentiation and recuitment of TRAP-positive osteoclast in vivo.................................................................209
Figure 4.77: Calvaria from DBA/2J GPNMB⁺ mice coated with PBS ..............210
Figure 4.78: Calvaria from DBA/2J GPNMB+ mice coated with RANKL .......... 211

Figure 4.79: mTOR-independent pathway increases osteoclast differentiation in
DBA/2J GPNMB+ mice .................................................................................. 212

Figure 4.80: mTOR-dependent pathway increases osteoclast differentiation in DBA/2J
GPNMB+ mice ................................................................................................. 213

Figure 4.81: Calvaria from DBA/2J mice coated with PBS ............................. 214

Figure 4.82: Calvaria from DBA/2J mice coated with RANKL ........................ 215

Figure 4.83: mTOR-independent pathway increases the differentiation of osteoclasts in
DBA/2J mice ....................................................................................................... 216

Figure 4.84: mTOR-dependent pathway increases osteoclast differentiation in DBA/2J
mice .................................................................................................................... 217

Figure 4.85: Autophagy pathways increase the resorption of osteoclasts in vivo
........................................................................................................................... 218

Figure 4.86: Schematic diagram of autophagy signaling during osteoclast differentiation
........................................................................................................................... 219

Figure 4.87: Schematic diagram illustrating the role of autophagy and osteoactivin
during osteoclast differentiation ........................................................................... 220

Figure 5.1: Schematic diagram of mTOR-independent pathway on bone cells....... 231

Figure 5.2: Schematic diagram of mTOR-dependent pathway on bone cells......... 332
LIST OF TABLES

Table 2.1: List of Genes Related to the Autophagy Pathway............................47
Table 2.2. Effects of mTORC1 and mTORC2 on Osteoblast cells...........................58
Table 2.3. Effects of mTORC1 and mTORC2 on Osteoclast cells............................59
Table 2.4: Autophagy Related-Gens Regulated in Bone Cells..............................68
Table 3.1: Types of Animal Models Utilized in the Study..................................76
Table 3.2: List of Primary Antibodies Used in the Study....................................87
Table 3.3: List of Secondary Antibodies Used in the Study..................................87
Table 3.4: List Primers of Osteoblast-Related Markers Used for qPCR Analysis.......94
Table 3.5: List Primers of Osteoclast-Related Markers Used for qPCR Analysis.......94
Table 3.6: List Primers of Autophagy Related Genes Used for qPCR Analysis........94
### LIST OF ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALP</td>
<td>Alkaline Phosphatase</td>
</tr>
<tr>
<td>ATG</td>
<td>Autophagy-Related Gens</td>
</tr>
<tr>
<td>BDGF</td>
<td>Bone Derived Growth Factors</td>
</tr>
<tr>
<td>BMP</td>
<td>Bone Morphogenetic Protein</td>
</tr>
<tr>
<td>BNIP3</td>
<td>Mitogen-Activated Kinases (Pro-Apoptotic gene)</td>
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<tr>
<td>BSP</td>
<td>Bone Sialoprotein</td>
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<tr>
<td>CASMC</td>
<td>Cultured Mouse Coronary Arterial Smooth Muscle Cells</td>
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<td>C/EBPβ</td>
<td>Enhancer-Binding Protein β</td>
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<td>Calcitonin Receptor</td>
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<td>DCHIL</td>
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<td>Family Interaction Protein of 200KD</td>
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<td>FK506</td>
<td>Immunosuppressive Drug</td>
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<tr>
<td>hESCs</td>
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<tr>
<td>MC3T3-E1</td>
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<td>mTOR</td>
<td>Mammalian Target of Rapamycin</td>
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<td>Osteocyte-Like Cells</td>
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DBA/2J Mice Knocked in GPNMB into DBA 2J Mutant

GPNMB

DBA/2J Osteoactivin Mutant Mice

OCN Osteocalcin

OF45 Osteoblast/Osteocyte Factor 45

OP-1 Osteogenic Protein-1

OPG Osteoprotegrin

OPN Osteopontin

PC Prostate Cancer

PDB Paget's Disease of Bone

PI3K Phosphatidylinositol 3-Kinase

P-4E/BP1 Eukaryotic translation initiation factor 4E-binding protein 1

P-S6K1 Ribosomal protein S6 kinase beta-1

PTH Parathyroid Hormon

PLCγ Phosphoinositide Phospholipase C γ

P62 Protein 62

PPARγ Peroxisome Proliferator-Activated Receptor

PKD Polycystic Kidney Disease Domain
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<td>Arginine-Glycine-Aspartic Acid</td>
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<tr>
<td>RANKL</td>
<td>Receptor Activator of NF-kB Ligand</td>
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<tr>
<td>RHEB</td>
<td>Ras Homolog Enriched Brain</td>
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<tr>
<td>Rictor</td>
<td>Rapamycin-Insensitive Companion of mTOR</td>
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<tr>
<td>ROS</td>
<td>Rat Osteoblasts-like Osteosarcoma Cell Line</td>
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<tr>
<td>RUNX2</td>
<td>Runt-Related Transcription Factor 2</td>
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<tr>
<td>TEM</td>
<td>Transmission Electron Microscopy</td>
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<tr>
<td>TGF-β</td>
<td>Transforming Growth Factor</td>
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<td>TH</td>
<td>Trehalose</td>
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<td>TNF-α</td>
<td>Tumor Necrosis Factor-α</td>
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<td>TLR</td>
<td>Toll-Like Receptor</td>
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<tr>
<td>TRAIL</td>
<td>Tumor Necrosis Factor- Related Apoptosis-Inducing Legand</td>
</tr>
<tr>
<td>TRAP</td>
<td>Tartarate Resistant Acid Phosphatase</td>
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</table>
**TSC1/2**  Tuberous Sclerosis Complexes Proteins 1 and 2  
**ULK1**  Unc-51 Like Autophagy Activating Kinase  
**UNC-51**  Serine/Threonine-Protein Kinase  
**UPS**  Ubiquitin-Proteasome System  
**Saos-2**  Osteosarcoma (Cell line)  
**Sin 1**  Mammalian Stress-Activated Protein Kinase Interacting Protein 1  
**SQSTM-1**  Sequestosome-1  
**VEGFR**  Vascular Endothelial Growth Factor Receptor  
**Vps35 and 150**  Vesicular Protein Sorting 34 and-150  
**VSMCs**  Vascular Smooth Muscle Cells
DEDICATION

I would like to dedicate this Dissertation to all of the people who were there when I needed them the most.

To King Abulaziz University, Kent State University, and Northeast Ohio Medical University.

To my parents who had passed away; I wish for them to be around in his presence now so they could share my joy.

To my mother, may God bless her soul. She always prayed and asked for God to be first in my life. Whenever I needed her, she was there with encouragement and support.

To my father, thank you for always being there when I needed you the most.

To my brothers and sisters, thank you for being there and supporting me throughout my long journey. I always felt your encouragement through my journey. Even though you are far away, I always felt you in my presence.

Finally, to my family, my husband and my daughter. Thank you for your moral support and sticking around me during these years. I was constantly busy, going to the lab from morning to evening. I was not able to spend as much time with them as I wanted and would often leave my daughter alone during my long studies.
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To my lab mates and colleagues, Gregory Sandag, Mohammad Yonis, Assad Al-Adlaan, and Nazar Hussein. I cherish all of the experiences that we have shared together. These moments have made the years go by fast and fun; it would not have been the same without you all.

Fatima Jaber

May 2018

Kent, Ohi
Chapter 1

Introduction

The purpose of this research was to assess the role of osteoactivin in osteoblast and osteoclast differentiation and function by mediating autophagy pathways using \textit{in vitro}, ex-vivo and \textit{in vivo} approaches. Moreover, we wanted to determine the effects of autophagy pathways during bone formation. Furthermore, we determined the effects of osteoactivin mediating autophagy \textit{in vivo} using a calvaria osteolysis model. Our hypothesis is that osteoactivin regulates bone cell differentiation and function by mediating the autophagy (mTOR-independent and dependent) pathways. Our results indicate that osteoactivin/autophagy may be used as a therapy to treat diseases related to bone loss.

Bone is a specialized connective tissue consisting of different cell types. Osteoblasts are the cells lie on the bone surface and responsible for bone formation; osteoclasts are multinucleated cells responsible for bone resorption; osteocytes are the responsible for maintaining bone homeostasis. Osteoactivin is a transmembrane protein type I with 572 amino acid that was discovered in osteoporotic mice, osteoactivin is highly expressed in bone cells (Safadi et al., 2001). Our lab has shown that osteoactivin is a positive regulator for osteoblast differentiation and function. In contrast, osteoactivin is a negative regulator for osteoclast differentiation, but positively regulated the function \textit{in vitro} and \textit{vivo} (Abdelmagid, et al., 2008; Frara, et al., 2015; Selim et al., 2003; Sondag,
et al., 2014). In contrast, other groups have reported that osteoactivin is a positive regulator for osteoclast differentiation and bone resorption (Miyazaki et al., 2015; Sheng, et al, 2008; Sheng et al., 2012). Mutation in osteoactivin led to impaired bone formation in vivo and vitro (Abdelmagid et al., 2014).

In the USA, approximately 1.5 million fractures annually are attributable to osteoporosis; these include 700,000 vertebral fractures, 250,000 distal forearm fractures, 250,000 hip fractures, and 300,000 fractures of other limb sites. The lifetime risk of fractures of the spine, hip, and distal radius is 40% for white women and 13% for white men from 50 years of age onwards (Riggs & Melton, 1995). Osteoporosis is a disease leading to bone fractures, marked by bone loss and increased trabecular spacing (Mori, 2004; Suh & Lyles, 2003). In osteoporosis and other bone diseases, imbalances between bone deposition and bone resorption result in bone loss.

Autophagy is a catabolic process that is responsible for removal misfolded protein, damage cell and eliminated intracellular pathogen. Autophagy is the most important and necessary pathway regulates cell growth differentiation and function, and plays an important role in bone homeostasis. There are relationships between autophagy genes, humans, and osteoporosis (Pan, 2010; Zang, 2010). Some studies reported that dysregulation of autophagy may be associated with the process of bone loss and osteoporosis (Onal et al., 2013; Pierrefite-Carle et al., 2015). Autophagy contributes to the maintenance of bone mass by maintaining osteoblast viability. To support this idea, some groups reported that deletion of the FIP200 gene, that is essential for autophagy formation in osteoblasts, causes osteopenia due to decreased bone formation (Liu et al., 2013). In osteocytes, deletion of the ATG7 gene that is essential for autophagy
initiation, led to decreased bone mass in male and female mice (Onal et al., 2013). Other groups reported a relationship between decreased autophagic activity in osteocytes and bone loss during aging in a rat model (Chen et al., 2014). A recent study found that induction of autophagy by rapamycin led to reduced osteoporosis due to activated autophagy in osteocyte cells (Luo et al., 2016). Other groups showed there is an increase in oxidative stress when ATG7 is absent (Onal et al., 2013). Some studies reported that increased oxidative stress in bone is related to bone loss (Almeida & O’Brien, 2013; Manolagas & Parfitt, 2010; Wu et al., 2015). In addition, estrogen deficiency is considered a main factor for bone loss and osteoporosis. There is a correlation between the level of autophagy, oxidative stress, and bone loss caused by a decrease in level of estrogen of overiectomized rats (Yang et al., 2014). In humans, the expression of autophagic pathway regulatory genes was observed in 984 individuals who suffer from osteoporosis (Zhang, 2010).

In this study, we used Trehalose (TH), which acts through the mTOR-independent pathway, and Rapamycin (R), which acts through the mTOR-dependent pathway, to determine the role of osteoactivin on osteoblast and osteoclast differentiation and function. This study consisted of two specific aims and tested our hypotheses that osteoactivin regulated osteoblast and osteoclast differentiation and function by mediating the autophagy pathways. We studied this hypothesis through two specific aims.

Aim 1: To determine the role of osteoactivin on osteoblast differentiation and function by mediating the autophagy pathways in vitro and ex-vivo.
Aim 2: To determine the role of osteoactivin on osteoclast differentiation and function by mediating autophagy pathways \textit{in vitro} and \textit{vivo}.

This study was able to illustrate the role of osteoactivin in both osteoblasts and osteoclasts by mediating autophagy pathways. This study is important for the potential use of osteoactivin-mediated autophagy as treatment for bone diseases such as osteoporosis and fractures of the bone.

Methodology

In this study, we performed approaches to study the role of osteoactivin /autophagy on bone cells \textit{in vitro}, \textit{ex-vivo} and \textit{vivo}.

In Aim 1, we used of cell culture to assess the effect of autophagy on osteoblast differentiation and function. The two different cell types used in this experiment to address the role of osteoactivin/autophagy-mediated osteoblast differentiation were the MC3T3-E1 osteoblast- like cell line and bone marrow derived mesenchymal stem cells (MSCs). I have compared control mice (DBA/2J GPNMB$^+$) and mutant mice for osteoactivin (DBA/2J) with a mutation in osteoactivin resulting in a non-functional protein. Other molecular approaches were used such as: ELISA, qPCR, western blot, immunofluorescence, and transmission electron microscopy (TEM). Moreover, an \textit{ex-vivo} approach we characterized the effect of autophagy on bone formation.

In Aim 2, the use of \textit{in vitro} approaches such as TRAP staining and activity, qPCR, Western blot, immunofluorescence, and transmission electron microscopy (TEM) were used to determine the role of osteoactivin/autophagy on osteoclast differentiation and
function. Moreover, we used an *in vivo* osteolysis model and µ-CT to investigate the role of osteoactivin/autophagy in osteoclast differentiation and bone resorption *in vivo*.

Taken together, our data presented in the body of work are original and this research is the first to show the relationship between osteoactivin and autophagy in mediating bone homeostasis.
Chapter 2

Literature Review

2.1 Bone Structure

Bone is a specialized connective tissue and is the main components of the adult skeleton that provides mechanical support for the body, protects vital organs such as those in the cranium, and regulates mineral homeostasis (Anthony, 2013; Gray et al., 2003), and regulates mineral homeostasis (Taichman, 2005). Bones consist two layers, outer layer and inner layer. The outer surface of the bone called the cortex, corresponding to compact bone, represents 80% of the total bone mass. The inner area called spongy or trabecular bone contains numerous interconnecting cavities representing 20% of the total bone mass (Anthony, 2013). There are four different types of bone: flat bones, short bones, long bones, and irregular bones (Clarke, 2008). Flat bones have two layers of compact bone called plates separated by a thicker layer of spongy bone. Short bones, such as those of the wrist, consist of compact bone covering spongy bone. Long bones, such as the tibia, are divided into three regions: epiphysis, which contains spongy bone, diaphysis which is the cylindrical part that consists mainly of compact bone, and the marrow cavity which is the thin region of spongy bone around the center (Figure 2.1). The outer and inner surfaces of bone are covered by tissue layers called periosteum and endosteum respectively. Both layers contain bone lining cells, osteoblasts, and mesenchymal stem cells called osteoprogenitor cells. The
periosteum contains connective tissue, with small blood vessels, collagen bundles, and fibroblasts. The main functions of the periosteum are bone growth and repair (Anthony, 2013). The endosteum is very thin and covers the trabecular bone matrix (Anthony, 2013). During embryonic development, osteogenesis occurs by one of two processes: intramembranous ossification (osteoblasts differentiate from mesenchymal stem cells to lay down osteoid directly) or endochondral ossification (pre-existing hyaline cartilage is replaced with bone) (Anthony, 2013).

As stated previously, there are two varieties of bone: compact (cortical bone) and trabecular bone (spongy bone). The mature human skeleton consists of 20% trabecular bone and 80% cortical bone; this ratio can change depending on the bone observed (Clarke, 2008). Compact bone consists of two layers: an outer periosteal layer and an inner bone marrow layer. The outer layers contains a fibrous connective tissue with an inner cambium layer (Allen et al., 2004). The inner layers contains fibroblasts, osteoblasts, mesenchymal progenitor cells, and nerves (Squier et al., 1990). The fibrous layer contains collagen and elastin fibers. Both layers play a role in fracture repair and growth.

Most bones in adults are compact bones that are made of osteons and are organized as multiple layers of lamellae that are arranged parallel or concentrically around a central canal, the harvesian canal. The harvesian canal contains blood vessels, nerves, loose connective tissue, and endosteum. Osteons are a long cylindrical hallow and which are important for bearing weight (Marieb, 2007). Lacunae contain one osteocyte, connected by canaliculi (Anthony, 2013; Cowin, 1999). Bone marrow is the soft substance contained in bone cavities and has two types of cells: hematopoietic
stem cells that are the main precursors for all types of blood cells, and mesenchymal stem cells which are common precursors for bone, and fat (Muguruma et al., 2006). All bone marrow cavities contain hematopoietic tissue, macrophages, and immune cells (red bone marrow) that is replaced gradually with age into lipid tissue (yellow bone marrow) (Gurevitch et al., 2009).
Figure 2.1: Bone histomorphology of the distal femur. There are three regions of the long bones (epiphysis, metaphysis, and diaphysis). The epiphysis plate is an area for bones to grow in length. The diaphysis is a shaft area. The metaphysis is bone layers consists of outer layer is cortical bone and the inner layer is trabecular bone.
2.2 Bone Matrix

Bone contains different types of molecules such as water, lipids, minerals, and organic matrix (Seibel et al., 2006). The type of the bone differs based on some factors: age, sex, and health. Bone contains about 50% inorganic material of which the most abundant component is calcium hydroxyapatite $[\text{Ca}_{10} \text{(PO}_4)_6 \text{(OH)}_2]$ (Rosen, 2008a), in addition to other components like bicarbonate, potassium, sodium ions, citrate, and magnesium. These components provide mechanical support to the tissue (Stock, 2015). Bone hydroxyapatite crystals are found in different sizes. During the remodeling, hydroxyapatite crystals undergo chemical reactions to release ions (Rosen, 2008a). Variation in the apatite crystals' size can indicate bone diseases. For example, larger apatite crystals are found within the bone matrix in osteoporosis (Fratzl, 2008).

The organic components are collagen type 1 which represents 85-90% of total bone protein (Knott, 1998) and supplies elasticity and strength for the bone (Seibel, 2006), proteoglycans, and glycoproteins such as osteonectin (Anthony, 2013). Type I collagen contains three polypeptide chains (Viguet-Carrin et al., 2006). In the periosteal bone, collagen fibers are arranged in sheets while in osteonal bone, they occur circumferentially.

Noncollagenous proteins (NCPs) represent 10-15% of bone matrix proteins. These proteins have different functions such as 25% of the NCPs are serum-derived proteins which are found in bone matrix and bind to hydroxyapatites (Rosen, 2008b). These proteins play an important role in matrix mineralization and cell proliferation. The non-collagenous proteins can be divides into a variety of categories, such as proteoglycans.
and glycosylated proteins. The role of these proteins is to regulate both bone mineral deposition and bone cell activity.

Serum osteocalcin, that is produced by osteoblasts and osteocytes (Beresford et al., 1984; Hauschka et al., 1989), serves as a marker of bone formation, and is a promoter of calcium deposition (Akesson et al., 1995; Boiskin et al., 1989). The osteocalcin knockout mouse has a low level of bone mass phenotype (Ducy et al., 1996), which proves that osteocalcin increases bone formation.

The essential glycosylated protein found in bone is alkaline phosphatase (ALP), which has been used as the main marker to evaluate bone formation. ALP is bound to osteoblasts by a phosphoinositol linkage, and is freely present in the mineralized matrix. Alkaline phosphatase plays an essential role in bone mineralization (Whyte, 1994). In humans, lack of ALP leads to a significant reduction in bone mineralization (Orimo, 2010). The role of ALP is hydrolyzing mineral inhibitor pyrophosphates to produce monophosphate ions that are essential for bone mineralization (Anderson et al., 2004).

Another common non-collagenous protein in bone is osteonectin which represents about 2% of the total protein in bone. Osteonectin can affect osteoblast growth, proliferation, and mineralization of the matrix. Osteonectin plays an important role in regulate cell-matrix interactions, increase osteoblast differentiation, and mineralization (Sage & Brekken, 2001). Deficiency in osteonectin leads to osteopenia (Delany et al., 2000). Glycoproteins have several domains such as RGD domains, these domains are essential to the attachment of the cells to other extracellular macromolecules by the matrix through integrin binding (Qin et al., 2004). Some of these glycosylations take
place in the N-terminal protein of the protein which is related to small integrin-binding ligand; the N-glycosylated protein family includes bone sialoprotein, osteopontin, and dentin matrix proteins. Bone sialoprotein (BSP) plays an essential role in matrix mineralization and is 34 kD with an RGD domain (Ganss et al., 1999; Goldberg & Hunter, 1994). BSP plays an important role in both osteoblasts and osteoclasts. Knockout mice for BSP leads to impairs bone growth, mineralization, and reduce bone formation. (Malaval, 2008). In addition, knockout mice also had dysfunctional osteoclast differentiation and function (Boudiffa, 2010; Wade-Gueye, 2012).

Osteoprotegrin (OPG) is a key regulator of bone remodeling. OPG is a glycoprotein expressed in most human tissues including the osteoblast and vasculature tissues (Collin-Osdoby, 2004; Schoppet et al., 2002); it is also a member of the tumor necrosis factor TNF superfamily. OPG is a secreted receptor by NFkB to activate (RANKL) that is an amine cytokine required for osteoclast differentiation (Hsu et al., 1999).

Osteopontin (OPN) is found in different human cells such as macrophages, endothelial cells, osteoblasts, lymphocytes, and vascular smooth muscle cells (Brown et al., 1992). OPN is a cytokine that is capable of activating migration of macrophages and osteoclasts (Giachelli et al., 1998; Suzuki et al., 2002), as well as proliferation of both osteoclasts and vascular smooth muscles (Giachelli et al., 1998; Liaw et al., 1994). OPN overexpression plays an important role in compensatory cardiac fibrosis and hypertrophy (Singh et al., 2010; Xie et al., 2004). OPN regulates different cells on the molecular and cellular levels through several integrins (Bazzichi et al., 2009; Burke et al., 2009). Both OPN and BSP play an essential role in osteoclast resorption by binding to integrin αvβ3 (Ross, 1993).
Proteoglycans are the main protein and acidic polysaccharide side chains (Fisher, 1987). During bone formation, proteoglycans are highly expressed by chondroitin sulfate and hyaluronan (Reddi et al., 1978). Decorin and Biglycan are examples of proteoglycans. Decorin plays a role in collagen synthesis, while Biglycan plays an essential role in bone development (Young et al., 2002). Another proteoglycan is a leucine-rich proteoglycans that may play a role in activating growth factors and cytokines that are found in the extracellular space (Chen & Birk, 2013; Wang et al., 2014).

Fibronectin is an extracellular matrix protein that also plays an important role in osteoblast and osteoclast differentiation (Moursi et al., 1996; Moursi et al., 1997). Fibronectin plays an important role in stimulating osteoclast function by nitric oxide and IL-1β signaling, but inhibiting their differentiation (Gramoun et al., 2010). Another protein that plays role in bone is Vitronectin which stimulates osteoclast function (Salasznyk et al., 2004). In addition, vitronectin plays an essential role in bone resorption through activation of ruffled border formation and stimulate the production of TRAP to resorb bone (Fuller, 2010). Fibrillin-1/2 are extracellular matrix proteins that regulate cytokines and some growth factors in bone. Fibrillin-1 mutation relates to human disease (Marfan Syndrome), an autosomal dominant disorder of connective tissue. Patients suffering from eye, skeletal, have less bone mineral density in the spine and femur (Trifiro et al., 2015). Fibrillin-2 knockout mice have less bone formation compared with controls, while Fibrillin-1 has been shown to be important during neonatal life (Carta et al., 2006).

Another component of bone is lipids which have been shown to play an important role in bone development. For example, caveolins that decreases differentiated of
osteoblast from MSCs and Caveolin-1 Knockout mice have increase bone size. Therefore, Caveolin-1 may use as a target for skeletal homeostasis. (Rubin, 2007), phospholipase D that expressed highly during intramembranous and endochondral ossification and localized to the extracellular matrix result increase developing mouse skeleton (Gregory, 2005), and sphingomyelinase. Some groups reported that deletion in sphingomyelin phosphodiesterase 3 (Smpd3) led to bone disease in the mouse (Aubin et al., 2005; Stoffel et al., 2005).

2.3 Osteoblasts, Osteocytes, and Osteoclasts

Mesenchymal stem cells are (MSCs) found in the bone marrow and have the ability to differentiate into different cell types and tissues such as muscle, cartilage, adipose, ligaments, tendons, and bone (Chamberlain, 2007; Jiang et al., 2002). In ex-vivo, MSCs are able to differentiate into different cells to repair the damaged tissue, and restore function in some tissues including bone and cartilage (Chamberlain et al., 2004; Crevensten et al., 2004).

Bone contains three main cell types: osteoblasts, osteoclast, and osteocytes. These cells represent approximately 15% of the total bone weight (Alberts, 2002), and are responsible for maintaining homeostasis and bone health. There are some factors that regulate bone in both development and function such as, Osteoblast /Osteocyte Factor 45 (OF45) (Petersen et al., 2000), connective tissue growth factor (CTGF) (Xu et al., 2000), the TGF-β superfamily members, lefty-1 (Seth et al., 2000), best-5 (Grewal et al., 2000), PROM-1 (for proliferating cell markers) (Ryoo et al., 1997), murine osteoclast inhibitory lectin (mOCIL) (Zhou, 2001), and RANK-L (Horowitz et al., 2001). Some of
these factors induce osteoblast proliferation and differentiation to increase osteogenesis while others that are released from osteoblasts can regulate osteoclast differentiation and function.

Osteoblasts lie on the surface of the bone matrix and have a cuboidal or columnar shape in addition to a basophilic cytoplasm (Anthony, 2013). These cells differentiate from mesenchymal stem cells in bone marrow (Alberts, 2002; Pittenger et al., 1990). Mesenchymal stem cells proliferate and differentiate into pre-osteoblasts and then into osteoblasts under some factors such as, genetic, local, and systemic factors (Triffitt, 1996; Ducy & Karsenty, 1995). Primary osteoblast cultures are subdivided into three stages. First, cell proliferation is from day 1 to day 7; second, the matrix maturation period is from day 7 to day 14. Finally, matrix mineralization is from day 14 to day 21 (Lian et al., 1991) (Figure 2.2). The proliferation stage expresses high levels of histone 4, collagen type 1, transforming growth factor (TGF-β), and fibronectin. The next stage of matrix maturation is associated with an increased level of alkaline phosphatase (ALP). The ending stage is matrix mineralization in which cells express high levels of osteocalcin, osteopontin, and bone sialoprotein (Owen et al., 1990).

After mineralization, osteoblasts surrounding by material to become osteocytes within the bone matrix, bone lining cells, or day by apoptosis (Dallas & Bonewald, 2010). ALP, osteocalcin, collagen type I, bone sialoprotein, osteopontin, and PTH are markers that are used in different stages of osteoblast differentiation (Aubin, 2001). The most important factor that is responsible for osteoblast differentiation from mesenchymal stem cells is RUNX2, (Abdallah et al., 2015) homologous to the drosophila gene (Runt) (Komori, 2010), which also acts as a transcription factor to
increase the expression of osteoblast in the early promoter region, but as an inhibitor of osteoblastogenesis in the late promoter one. RUNX2 is essential for both intramembranous and endochondral ossification (Bruderer et al., 2014). RUNX2 (osf2/cbfa1) starts to appear in mice to develop the skeleton to osteoblast and is regulated by BMP7 and vitamin D3, it also acts as a regulator of osteoblast differentiation (Andriano & McEnery, 2000; Ducy et al., 1997). Deficiency in RUNX2 leads to abnormalities in the skeleton and lack of osteoblasts (Otto, 1997). In humans, mutation in RUNX2 relates to Cleidocranial Dysplasia disease which is characterized by a lack of collar bones and decreased development of the cranial bones (Mundlos, 1999).

A transcription factor stimulated by p38 and mitogen activated protein kinase (MAPK) is osterix. Osterix is a zinc finger protein which is essential for bone formation (Nakashima et al., 2002). Mice deficient in osterix fail to develop bone (Nakashima et al., 2002). Also, serine threonine kinases bone morphogenetic proteins (BMPs) are important for osteoblastogenesis by smad or transforming growth factor β (TGF-β) (Abdallah et al., 2015).

BMPs are members of the TGF-β superfamily (Lavery et al., 2008; Urist, 1965). In humans, mutations in the BMP receptor results in increased bone formation at ectopic sites (Fibrodysplasia Ossificans Progressiva (FOP)) (Shore & Kaplan, 2008). BMP is able to induce Osterix expression by the MAPK pathway and activated osteoblast differentiation and function (Celil & Campbell, 2005).

Transforming growth factor-β (TGF-β) is cytokine found in the bone matrix; upon activation of the TGF-β type I receptor, it leads to activation of Smad2 and 3 with Smad
4 to regulate osteoblast transcription (Feng & Derynck, 2005; Massague et al., 2005). RUNX2 is inhibited by Smad3 resulting in a decrease of osteoblast mineralization (Alliston et al., 2001). In addition, TGF- β plays an essential role in osteoblast proliferation (Centrella et al., 1994; Linkhart et al. 1996).

Wnts/β-catenin play a critical role in bone development (Logan & Nusse, 2004; Pittenger et al., 1990; Day et al., 2005). Wnts are a family that acts through different signaling pathways. They are expressed by osteoblasts in bone marrow and increases bone mass (Andrade, 2007). Peroxisome proliferator-activated receptor γ (PPARγ) is an inhibitory factor for osteoblast differentiation by decreasing RUNX2 expression (Colaianni et al., 2014), and it has a relationship with β-catenin which inhibits osteoblastogenesis and stimulates the adipogenesis pathway (Fattore et al., 2008).

Parathyroid hormone (PTH) is expressed by the parathyroid glands and it is regulated by different pathway, such as fibroblast growth factor 23 (FGF23), vitamin D, and extracellular calcium (Bergwitz & Juppner, 2010). PTH regulates osteoblastogenesis by its 7-transmembrane receptor PTH1R which stimulates downstream PKA and PKC signaling to produce RUNX2 (Qiu et al., 2015). Mice deficient in PTH have less bone formation (Miao et al., 2002). In humans, abnormalities in PTH signaling lead to premature ossification (Zhang et al., 1998). PTH plays an important role in both osteoblast and osteoclast proliferation and differentiation (Khosla et al., 2008).

Fibroblast growth factor (FGF) plays an essential role in bone formation. FGF binds to tyrosine kinase receptors FGFR and activated signaling pathways (Goetz & Moosa, 2013). FGF KO mice have significantly less trabecular bone and less
mineralization (Montero et al., 2000). FGF/FGFR2 is required for vertebrate limb induction and FGFR2 is important for regulation between FGF8 and FGF10 during limb development (Xu, et al, 1998). Mice deficient in FGFR2 are significantly decreased in bone density, proliferation of osteoprogenitors, and osteoblast function (Yu et al., 2003). FGF2 is considered as therapeutic treatment for patients who have severe osteopenia (Liang et al., 1999). FGF2 has the ability to stimulate the MAPK/RUNX2 pathway to produce osteoblasts (Franceschi & Xiao, 2003). In addition, mice deficient in FGFR3 have osteopenia and decreased bone mineralization (Valverde-Franco et al., 2004). FGF18 is required to regulate bone formation (Ohbayashi et al., 2002).
Figure 2.2: Schematic diagram illustrating osteoblast differentiation from mesenchymal stem cells (MSCs). RUNX2 and osterix are critical transcription factors for osteoblast differentiation. Osteoblasts in culture undergo three distinct stages; proliferation from day 0 to day 7, differentiation from day 7 to day 14, and mineralization from day 14 to day 21. Alkaline phosphatase (ALP) is the early marker, while bone sialoprotein and osteocalcin are late markers for osteoblasts. Osteoblast may undergo apoptosis, become an osteocyte, or lie flat on the bone surface and become a bone lining cell.
Osteocytes are located in cavities called lacunae which lie in between bone matrices and have processes which lie within the canaliculi in mineralize bone (Bonewald, 1999). Osteoblasts are gradually surrounded by the material they secrete and they differentiate to osteoid, they then become osteocytes (Bonewald, 2011; Franz-Odendaal et al., 2006). Osteocytes are flat in shape and maintain bone matrix; they die after matrix resorption (Anthony, 2013). Osteocytes can live many months or years depending on the location and rate of bone remodeling; in contrast, osteoblasts and osteoclasts have a short live span (Manolagas & Parfitt, 2010). The empty lacunae in aging bone suggest that osteocytes undergo apoptosis (Xing & Boyce, 2005). Osteocyte apoptosis relates to a decreased level of estrogen or glucocorticoid treatment and both are risky to bone structure. Estrogen, bisphosphate, and physiologic loading of bones helped avoid osteoblast and osteocyte apoptosis by activating the extracellular signal-regulated kinases (ERKs) (Plotkin et al., 2005). In addition, osteocytes are the main source of the RANKL receptor, activator of NF-κB ligand that regulates osteoclast formation (Nakashima et al., 2011; Xiong et al., 2011). Thus low bone mass contributes to change in osteocyte function (Manolagas & Parfitt, 2010).

Osteoblasts and osteocytes communicate by gap junctions, and both cells are located on the bone surface and within the bone matrix (Rousselle & Heymann, 2002). Osteocytes do not express alkaline phosphatase but they express osteocalcin, galectin 3, and CD44. Osteocytes function as phagocytic cells because they contain lysosomes (Plotkin et al., 2002). The main function of the osteocyte-osteoblast is mechanosensation (Rubin, 1987). E11/gp38/podoplanin is a marker for osteocytes and is found on the cell surface (Wetterwald et al., 1996). Deletion of this molecule leads to
an increase in trabecular bone and a decrease in canaliculi (Bonewald, 2011). The skeletal pathologies are associated with osteocyte cell death. For example, an increase in osteocyte apoptosis associated with osteonecrosis of the hip (Weinstein et al., 2000). Osteocyte cell death produces high levels of RANKL to recruit osteoclast to the damaged site (Kogianni et al., 2008). Glucocorticoids can inhibit bone formation and stimulate bone resorption (Weinstein et al., 2000). Decrease in estrogen production during post-menopausal periods results in increased TNF-α and IL-1 levels can induce osteocyte apoptosis (Emerton et al., 2010). Osteocytes are able to stimulate MSCs proliferation and differentiation to osteoblasts as shown in the MLO-Y4 cell line (Heino et al., 2004).

Osteoclasts are large, multinucleated cells. They play a critical role in bone resorption during bone growth and remolding. Multinucleated osteoclasts are produced from mononuclear precursor cells of the monocyte macrophage lineage (Roodman, 1999). Osteoclasts arise from hematopoietic stem cells that undergo multiple steps to become a multinucleated cell (Suda et al., 1999). Macrophage-colony-stimulating factor (M-CSF) and receptor activator of nuclear factor-kB ligand (RANKL) are required for osteoclast development (Anthony, 2013; Pixley & Stanley, 2004). Both RANKL and M-CSF are produced by marrow stromal cells and osteoblasts in membrane-bound and soluble forms (Sims & Gooi, 2008) (Figure 2.3). OPG that is produce by osteoblasts inhibits osteoclastogenesis (Simonet et al., 1997). Mice deficient in OPG have osteoporosis (decrease in total bone density) (Bucay et al., 1998).

Osteoclastogenesis requires the presence of stromal cells and osteoblasts in bone marrow (Teitelbaum & Ross, 2003). RANKL is essential for osteoclast formation and M-
CSF is important for the proliferation, survival, and differentiation of cells and releases protein which binds to RANKL and inhibits its action at the RANK receptor (Caidahl & Aukrust, 2010; Cohen, 2006). Osteoclasts bind to the bone matrix through integrin receptors; the main integrin receptor for bone resorption is the αvβ3 integrin that binds to osteopontin and bone sialoprotein (Ross & Teitelbaum, 2005; Ross, 1993).

RANKL and M-CSF have the ability to redirect hematopoietic stem cell differentiation into osteoclasts at different stages in their differentiation pathway (Vaananen & Laitala-Leinonen, 2008). They are also crucial for recruiting in sites where there is osteoclast resorption (Alberts, 2002). RANKL binds to the RANKL receptor to stimulate osteoclastogenesis, or bind to ostoprotegrin (OPG) for inhibition. Numerous factors modulate RANKL expression positively such as prostaglandin E2, inflammatory cytokines, parathyroid hormone, and vitamin D, or negatively like estrogen and TGF-β.

RANKL1 and RANKL2 are the transmembrane isoforms of RANKL that are important for osteoclast differentiation. Another isoform is RANKL3 which is a soluble form and it is responsible for inhibiting the other two forms when co-expressed with RANKL1 and RANKL2 (Theoleyre et al., 2004). Other factors play a role in osteoclast differentiation, like ostoprotegerin (OPG) which blocks RANK\ RANKL binding and so inhibits osteolysis. Other factors are phosphoinositide phospholipase Cγ (PLCγ), and ostoprotegrin (OPG) (Marieb, 2007). Another factor that is important for osteoclasogenesis is the Nuclear Factor of Activated T-Cells (NFATc1), leading to the downstream activation of RANK (Wada et al., 2006). In humans, osteoclasts are differentiated to produce approximately eight nuclei fused together; another species of osteoclast contains more than five nuclei to resorb bone (Vaananen & Laitala-Leinonen,
Osteoclast size is controlled by hypoxia and Fra-2 (transcription factors) by LIF/LIF-receptor (Bozec et al., 2008).

The last steps of osteoclast differentiation are cell fusion. This fusion occurs mainly through two different types of cell contacts: phagocytic-cup and broad contact-surfaces. More mature multinucleated osteoclasts fuse with a less mature mono-nucleated pre-osteoclasts. One important factor for osteoclast fusion is dendritic cell-specific transmembrane protein (DC-Stamp) that is found on the surface receptors in pre-osteoclasts (Soe et al., 2015). Afterwards, differentiated osteoclasts attach to the mineralized bone matrices through dot-like, actin-rich structures called podosomes. Furthermore, F-actin is polarized to a circular structure, "actin ring," which is comprised of a dense ring of podosomes. The podosomes rely on the CD44 receptor and αvβ3 integrin which are important for osteoclasts to attachment on the bone matrix (Vaaninen & Laitala-Leinonen, 2008; Holt & Marshall, 1998; Nakamura et al., 1996), this ring is formed by microfilaments and microtubules.

Osteopontin (OPN) is the extracellular matrix protein on integrin plays an important role to connect osteoclasts to bone (Li et al., 2006). RGD peptides inhibit osteoclast attachment and bone resorption and they are able to change osteoclast cytoskeleton (Vaanannen, 2005).

An important factor that plays a role in osteoclast and bone resorption function is tartarte-resistant acid-phosphatase (TRAP) which is a biomarker for osteoclast activity and stimulated by the cathepsin K enzyme (Vaanannen et al., 2000; Raggatt & Partridge 2010). Another factor is reactive oxygen species, generated by TRAP activity, play a
role in the degradation of collagen, and matrix metalloproteinases (Vaaninen & Laitala-Leinonen, 2008). TRAP deficiency in mice leads to an osteoporotic phenotype and a decrease in mineralization of cartilage during bone development (Hayman et al., 1996).

Osteoclasts express a high level of calcitonin receptors (CTR), which inhibit osteoclast resorption, and calcitonin receptors are considered marker of osteoclast differentiation (Wada et al., 1995). Changes in the intracellular level of calcium affects bone resorption (Adebanjo et al., 1998), and it has been shown that increased level of calcium induces apoptosis in osteoclast (Lorget et al., 2000) due to an increase in membrane permeability to protons and other ions (Lehenkari et al., 2003). At the end stage of resorption, osteoclasts die by apoptosis, so apoptosis represents a key step in bone resorption that must be controlled (Kameda et al., 1995). TNF-α, IL-1β, and IL-6 bind to their receptors and regulate downstream signaling that plays an essential role in osteoclast differentiation and function (Lam et al., 2000; Suda et al., 1995).
Figure 2.3: Schematic diagram of osteoclast differentiation from hematopoetic stem cells (HSCs). Myeloid precursor starts at day 0 to proliferate and differentiate to osteoclast. At day three, bone-marrow macrophages (BMM) express high levels of M-CSF and RANK, which stimulates osteoclast precursor cell differentiation. Dendritic cell-specific transmembrane protein (DC-STAMP) is require for osteoclast fusion. At day 7, osteoclasts become mature.
2.4 Bone Growth, Modeling, and Remodeling

During life, bone undergoes longitudinal growth, modeling, and remodeling. Longitudinal growth occurs at the growth plates during childhood and adolescence (Clarke, 2008). Once the bone has reached maturity, there should be a balance between bone formation and bone resorption to ensure there is no loss or gain in this process which is called coupling (Marks, 1996). Modeling is the process in which bones change in shape as a response to mechanical forces or physiologic influences, leading to slow adjustment of the skeleton to the independent action of osteoblasts and osteoclasts (Doblare & Garcia, 2003). During bone modeling, bone formation is not tightly coupled by resorption. Bone modeling is less than remodeling in adults (Kobayashi et al., 2003), and it increases in hypoparathyroidism (Ubara et al., 2005), renal osteodystrophy (Ubara et al., 2003), or treatment with anabolic agents (Lindsay et al., 2006).

Bone remodeling is the process in which bone is renewed. Remodeling includes removal of old bone, replacement with newly synthesized protein matrix and mineralization of that matrix to produce new bone to avoid accumulation of damaged bone. The remodeling cycle consists of four sequential phases: activation, resorption, reversal, and formation respectively. Remodeling sites develop randomly and target the areas which require repair (Burr, 2002; Parfitt, 2002). Activation includes recruitment and stimulation of mononuclear monocyte-macrophage and osteoclast precursors (Roodman, 1999).
The remodeling cycle takes approximately two to four weeks. Osteoclast formation, activation, and resorption are regulated by the proportion of the receptor activator of NF-κB ligand (RANKL) to osteoprotegerin (OPG), IL-1 and IL-6, colony-stimulating factor (CSF), the parathyroid hormone, 1,25-dihydroxyvitamin D, and calcitonin (Boyle et al., 2003; Blair & Athanasou, 2004). Resorbing osteoclasts produce hydrogen ions by H^+ ATPase protein and chloride channels in their cell membranes inside the resorbing compartment to decrease pH in order to help bone mineralization (Silver et al., 1988). In addition, osteoclasts produce tartrate-resistant acid phosphatase, cathepsin K, matrix metalloproteinase 9, and gelatinase from cytoplasmic lysosomes to digest the component of the matrix (Delaisse et al., 2003) leading to formation of the resorption phase by mononuclear cells after multinucleated cells of osteoclast die by apoptosis (Eriksen, 1986; Reddy, 2004). In the reversal phase, bone resorption transitions to bone formation. Resorption cavities consist of a variety of mononuclear cells such as monocytes, osteocytes from bone matrix, and preosteoblasts that accumulate to start formation of new bone. The signals linking the end of bone resorption to the beginning of bone formation are unknown. The coupling signal includes bone matrix derived growth factors (PDGF), transforming growth factor-β (TGF-β), insulin-like growth factor (IGF-1), (IGF-2), bone morphogenetic protein (BMPs), or fibroblast growth factor (bFGF) (Bonedewald & Mundy, 1990; Locklin et al., 1999). TGF-β liberation from bone matrix results in decreased osteoclast resorption by inhibiting production of RANKL. The reversal phase is proposed to be mediated by strain gradient in the lacunae (Smit, 2002a; Smit, 2002b).
Formation of bone takes approximately four to six months to produce osteoblasts which synthesize collagen and regulate mineralization of the matrix by secreting vesicles that contain calcium and phosphate and destroy mineralization inhibitors by enzymes, for example pyrophosphate or proteoglycans (Anderson, 2003). The final result of the bone remodeling cycle is production of new osteon that is important for cortical and trabecular bones; bone remodeling in trabecular bone equals cortical bone remodeling. Bone balance is the difference between bone resorption and formation (Parfitt, 1994).

2.5 Interaction between Osteoblasts and Osteoclasts

Bone is synthesized by osteoblasts and resorbed by osteoclasts (Theill et al., 2002; Karsenty & Wagner, 2002). The communication between these cells occurs through: paracrine factors by cytokines, cell to cell contact, and cell-bone interaction (Matsuo & Irie, 2008); communication between these cells are important for bone remodeling, and this equilibrium is regulated by hormones, cytokines, and mechanical stimulation (Heymann & Rousselle, 2000). Cytokines within the tumor necrosis factor (TNF) superfamily are required to control bone remodeling (Simonet et al., 1997; Yasuda et al., 1998). Osteoclasts function to resorb bone in preparation for the laying down of fresh bone. High levels of tartrate-resistant acid-phosphatase (TRAP) stimulates osteoclast function, mononuclear cells are also important for resorption. The number of osteoblasts controls bone formation independently of their resorption activity (Henriksen, 2009).

Bone usually undergoes a remodeling process known as activation-resorption-formation (ARF) cycle. First, there is activation when osteocyte death leads to
osteoclastogenesis by RANKL. Second, resorption by cytokines, such as OPG and TGF-β that regulate osteoclast numbers results in a decrease in bone resorption. Finally, new matrix formation and mineralization begins through osteoclast-secreted factors to recruit bone lining cells and osteoblasts to the place where bone formation occurs (Roodman, 1999). Osteocytes may work to stop osteoclast resorption by increasing the producing of negative regulators of osteoclasts (Vaaninen, 2005). Two important factors with effects on bone remodeling are calcium controlled by hormones; the other is mechanical and gravitational forces. Parathyroid hormone (PTH) produced by parathyroid glands stimulates bone resorption in response to lower levels of calcium (Marieb, 2007).

2.6 Hormonal Influence on Bone

A variety of hormones influence bone behavior such as androgens, estrogen, and leptin (Marieb, 2007). In vivo, bone resorption is controlled by cellular and hormonal factors; these factors effect osteoclast activity and formation. For example, parathyroid hormone (PTH) increases bone resorption (Kameda et al., 1995). Estrogen inhibits osteoclast differentiation and function (Jilka et al., 1992); some studies demonstrated that estrogen does not directly affect osteoclast formation but mediates its inhibition by osteoblast, OPG, and RANKL (Hofbauer & Schoppet, 2004; Ramalho et al., 2001). Hughes et al 1996 suggested that estrogen could reduce bone resorption by decreasing length of the osteoclast lifespan (Hughes et al., 1996); in contrast, another study has shown that estrogen can prevent osteoclasts from serum starvation, which stimulates apoptosis (Selander et al., 1996).
2.7 Bone Fracture

Bone is a living material that is exposed to a mechanical environment. There are several causes of bone fracture. Fractures occur for reasons such as increased bone fragility, pathology, and skeletal trauma. The most common models proposed for fracture risk are measurements of bone density and cortical thickness by quantitative computed tomography µ-CT (Atkinson, 2012). There are some crucial factors considered for the treatment of bone such as the type of bone, location, morphology, and fracture severity (Marsh et al., 2007). Fracture healing is the most remarkable process in the body. Embryonic and child bone healing can occur in different forms: endochondral or intramembranous ossification. Intramembranous ossification occurs when mesenchymal stem cells (MSCs) directly differentiate into osteoblasts. Endochondral ossification occurs when cartilage is formed, calcified, and replaced by bone (Gittens & Uludag, 2001; Mandracchia & Barp, 2001; Thompson et al., 2002). There are different growth factors expressed during bone healing, such as bone morphogenetic proteins (BMPs), basic fibroblast growth factors (bFGFs), transforming growth factor beta (TGF-β), and platelet derived growth factors (PDGFs) (Sandberg et al., 1993; Schmid et al., 2009; Wong, 2003; Tatsuyama et al., 2000). These factors have been shown to stimulate bone healing (Andriano & McEnery, 2000). Deletion of BMP-2 from limb buds delayed fracture healing (Tsuji, 2006). Also, the presence of vascular endothelial growth factor (VEGF) and its receptor (VEGFR) are increased during healing (Street et al., 2002); increase VEGF at the site of injury in response to BMP suggest a strong link between osteogenic and angiogenic during bone healing.
Inflammatory cytokines are important for skeletal repair. The levels of RANKL, M-CSF, OPG, and TNF-α are increased during cartilage mineralization (Kon et al., 2001). In addition, IL-1 and IL-6 are increased during the remodeling of bone (Yang et al., 2007). Another factor playing an important role in the repair process is Cyclooxygenase-2 COX-2, an enzyme responsible for formation prostaglandin from arachidonic acid (Zhang, 2002).

During skeletal repair after trauma or surgical intervention, bone repair requires two processes, one is intramembranous and the other is endochondral ossification (Gerstenfeld et al., 2003). During intramembranous ossification, MSCs differentiate into osteoblasts (Cooper et al., 2006). In flat bones, bones have no cartilage. During endochondral ossification, a cartilage is developed first followed by vascular invasion. Endochondral bone formation takes place near the fracture site, while intramembranous bone formation takes place distal to the site. The main important source of osteoprogenitor cells during fracture repair is periosteum. The periosteum contains two layers: an outer layer consisting of fibroblasts that is connected to cortical bone and an inner layer containing MSCs and osteoprogenitor cells (Augustin et al., 2007).

During fracture repair, progenitor cells are recruited into the fracture site due to low oxygen tension and vascular disruption; MSCs differentiate to chondrocytes. While, MSCs directly differentiated into osteoblasts to form new bone where oxygen and vascular are normal (Einhorn, 1998).

Fibroblast growth factor receptors (FGFRs) are critical in angiogenesis by their interaction with fibroblast growth receptors (FGFs) (Murakami & Simons, 2008).
Blocking FGFR-1 results in impairment of blood vessel development because it is found in endothelial cells (Presta & Moroni, 2005). FGFR signaling has an important function in chondrogenesis and bone development (Marie et al., 2012). FGFR has a critical mechanism to control bone formation (Jarzabek & Lesniewicz, 2012).

The osteoporotic fracture depends on two factors: one of them is the mechanical strength of bone and the other is forces applied to it (Ralston, 1998).

2.8 Osteoactivin

Osteoactivin was discovered in osteopetrotic rats; these rats were used to examine differential gene expressions in both bone normal and osteopetrotic mutant rats (Marks & Popoff, 1989). A novel cDNA up-regulated in osteopetrotic rats was discovered by our lab by using the mRNA technique (Safadi et al., 2001). Osteoactivin has different names, such as glycoprotein non-metastatic melanoma protein B (GPNMB) (Kuan et al., 2006; Okamoto et al., 2005; Pollack et al., 2007; Tse et al., 2006), melanocytes (Anderson et al., 2006; Anderson et al., 2002), dendritic cell heparin sulfate proteoglycan integrin dependent ligand (DC-HIL) (Chung et al., 2007; Shikano et al., 2001), and human hematopoietic growth factor inducible neurokinin (HGFIN) in tumor cells (Bandari et al., 2003). Osteoactivin is expressed in many cells and tissues including macrophages (Safadi et al., 2001; Schroder, 2002). The osteoactivin gene is found in the human chromosome 7p15.1 and in the mouse chromosome 6 (Wong, 2003). Osteoactivin has high homology with other proteins, such as dendritic cell heparin sulfate proteoglycan integrin dependent ligand (DC-HIL) and human hematopoietic growth factor inducible neurokinin (HGFIN) (Abdelmagid, et al., 2008). Osteoactivin has multiple domains including proline-rich repeat domain (PRRD), and polycystic kidney
disease domain (PKD) which relates to the immunoglobulin (Ig); the function of the PKD domain is unclear. The third is the RGD domain which contains only three amino acids; arginine (R), glycine (G), and aspartic acid (D) and is expressed in many cells; the dileucine motif with its cytoplasmic tail is near the carboxyl group, and hemi TAM motif is an immune receptor tyrosine-based activation motif found in cytoplasmic domains, N-terminal signal peptide, and the C-terminal motif (Selim et al., 2007; Maric, 2013) (Figure 2.4). In addition, osteoactivin has two isoforms: one is a transmembrane, glycosylated form (115kDa), that is heavily glycosylated, the other is the secreted portion (68kDa). The full length of osteoactivin contain 574 amino acids, recombinant osteoactivin contains 477 amino acids and DBA/2J mutant osteoactivin contains 150 amino acids (Maric, 2013) (Figure 2.5).

Osteoactivin plays a role in decreasing inflammatory response. In particular, mice showed a decrease in their peritoneal macrophage and levels of pro inflammatory cytokines in response to lipopolysaccharides (LPS) when osteoactivin is expressed (Ripoll, 2007). Thus, it serves as a negative regulator of inflammation by decreasing the macrophages. Moreover, osteoactivin plays an important role as a phagocytic protein for phagocytosis of cellular debris in a renal injury model (Li, et al, 2010). M2-macrophages display high expression levels of osteoactivin, which showed that it is an anti-inflammatory protein (Pahl et al., 2010). Osteoactivin expression is high in response to injury found in the liver, (Abe et al., 2007; Haralanova-Ilieva et al., 2005), kidney (Nakamura et al., 2007; Patel-Chamberlin, 2011), brain (Murata et al., 2014; Nakano et al., 2014), and muscle (Furochi et al., 2007; Nikawa et al., 2004; Ogawa, et al, 2005). In
addition, osteoactivin acts to control cell proliferation, adhesion, differentiation, and biosynthesis of extracellular matrix proteins in different cell types (Chen et al., 2004; Nakamura et al., 2007). Moreover, osteoactivin has an essential function in the regulation of fracture repair and healing (Abdelmagid et al., 2010). Due to potential mechanisms in cell adhesion, migration, and differentiation, it is thought that osteoactivin is associated with the pathophysiological flow of tissue injury and repair (Haralanova-Ilieva et al., 2005).

Groups of researchers are reported that osteoactivin and osteonectin are expressed in a glioma human cancer and promote metastasis by enhancing the production of matrix metalloproteinase 3 (MMP-3) and 9 (MMP-9); this suggests that osteoactivin plays an important role in cancer metastasis (Rich et al., 2003). Another group showed that osteoactivin is expressed in breast cancer cells (Rose et al., 2007). Osteoactivin is an essential target for several types of cancerous diseases, for example those affecting the prostate (Fiorentini et al., 2014), liver (Tian et al., 2013), melanoma (Tomihari et al., 2010; Turrentine et al., 2014), colon (Ruan et al., 2014), brain (Szulzewsky et al., 2015), pancreas (Torres et al., 2015), and lungs (Li, et al, 2014).

Osteoactivin serves as a neuroprotective factor in neurodegenerative disease; osteoactivin fragments were shown to suppress motor neuron degeneration in amyotrophic lateral sclerosis (Tanaka et al., 2012). In addition, osteoactivin serves as a neuroprotective factor in cerebral ischemia by phosphorylation of ERK and Akt (Nakano et al., 2014).

Angiogenesis plays an important factor in bone development and fracture repair (Gerber & Ferrara, 2000; Schindeler et al., 2008). In addition, osteoactivin has been
shown to play an essential role in angiogenesis through the use of recombinant osteoactivin in vitro which stimulates angiogenesis in vein endothelial cells (Hu et al., 2013). One study reported that osteoactivin plays a role in angiogenesis during hyperoxia injury (Narasaraju et al., 2015). Osteoactivin plays a role in endothelium cells through binding between dendritic cells that express osteoactivin and heparin sulfate proteoglycans on the surface of endothelial cells (Shikano et al., 2001). The osteoactivin extracellular domain stimulates the migration of endothelial cells by ADAM10 in breast cancer (Rose et al., 2010). Osteoactivin plays a role in regulating vascular epithelial growth factor (VEGF) by binding to neuropilin-1 and α5β1 to potentiate VEGF signaling (Maric et al., 2015).

Bone tissue expresses a high level of osteoactivin. The first study of bone and osteoactivin were performed using osteoblasts (Owen et al., 2003; Safadi et al., 2001). The level of osteoactivin is increased during osteoblast differentiation. Stimulating mesenchymal stromal cells, MSCs, from human placenta with recombinant osteoactivin lead to an increase in osteogenic differentiation (Raynaud et al., 2012). Osteoactivin interacts with heparin sulfate proteoglycan (HSPG) to stimulate osteoblast adhesion (Moussa et al., 2014). Moreover, osteoactivin interacts with fibroblast growth factor receptor FGF-R to stimulate downstream signaling (Hu et al., 2013). Osteoactivin antibodies in cell culture inhibit osteoblast differentiation and mineralization by ALP staining and activity (Selim et al., 2003). Moreover, osteoactivin overexpression in cell lining increases osteoblast differentiation (Abdelmagid et al., 2008; Sondag et al., 2014). Bone morphogenetic protein 2 (BMP-2) regulates osteoactivin by enhancing its expression during osteoblast differentiation by homeodomain transcription factors DLX-
There is evidence in mice with increased bone mass in both *in vivo* and *in vitro* as being a result of osteoactivin over expression. These suggest that osteoactivin plays an essential role in bone mineralization (Frara et al., 2015). Osteoactivin mutant mice (DBA/2J) have pigmentary glaucoma and hearing loss (Mckinnon et al., 2009; Anderson et al., 2002; Johnson et al., 2006). Osteoactivin mutant mice have impaired osteoblast differentiation and decreased bone mass *in vivo* and impaired osteoblastogenesis *in vitro* (Abdelmagid et al., 2014).

Osteoactivin also plays an important role in osteoclast differentiation and function. Osteoactivin expression increases during osteoclastogenesis (Ripoll, 2008), and anti-osteoactivin antibodies inhibit osteoclast differentiation (Sheng, et al., 2008). Furthermore, overexpression of osteoactivin decrease osteoclast differentiation and increase bone resorption which leads to decreased bone mass *in vivo* (Sheng et al., 2012). A recent study showed that osteoactivin increases bone resorption by binding to αvβ3 integrin (Miyazaki et al., 2015). Our group showed that there was decreased trabecular bone mass and increased cortical bone mass DBA/2J mice (Abdelmagid et al., 2015). Osteoactivin negatively regulates osteoclast differentiation. In contrast, osteoactivin positively regulates osteoclast resorption (Figure 2.6).

Osteoactivin is highly expressed in osteoblasts, osteocytes, and osteoclasts during bone repair (Abdelmagid et al., 2010); also osteoactivin stimulates bone regeneration in rats (Bateman et al., 2012), and mice (Hu et al., 2013). Osteoactivin expression is increased with time after fracture and reaches its peak at 2 weeks post fracture (Furochi et al., 2007; Hadjiargyrou & Zhao, 2002). Osteoactivin is a positive regulator of fracture
healing that is observed in rat fracture models (Abdelmagid et al., 2010). Fracture repair is associated with spatial and temporal communication between blood vessels and cells (Kanczler & Oreffo, 2008). Angiogenesis has an essential role in bone regeneration. When the vascular supply decreases, the nutrient availability decreases resulting in delay of bone healing (Cinotti et al., 2013).
Figure 2.4: A schematic representation of glycoprotein non-metastatic b (GPNMB). Illustrates the domains and motifs related to GPNMB function. RGD domain comprised only 3 amino acids, arginine (R), glycine (G), and aspartic acid (D), is found near the N-terminus of GPNMB. PKD domain belongs to the immunoglobulin-Ig. hemITAM immunoreceptor tyrosine-based activation motif. Di-leucine sorting motif in cytoplasmic tail near the carboxy-terminus.
Figure 2.5: Schematic representation of full length, recombinant and mutant osteoactivin. The full length of osteoactivin contain 574 amino acids. Recombinant osteoactivin contains 477 amino acids. DBA/2J mutant osteoactivin contains 150 amino acids.
Figure 2.6: A schematic representation the role of osteoactivin in bone cells.

Osteoactivin is a positive regulator of osteoblast differentiation and function. In contrast, osteoactivin is a negative regulator for osteoclast differentiation but is positive for their function.
2.9 Autophagy

The term Autophagy is derived from Greek and means a self-eating process that removes misfolded protein, clears the damaged organelles, and eliminates intracellular pathogens (Reggiori & Klionsky, 2002). Autophagy is important to balance the energy sources during nutrient stress and starvation. Autophagy plays a role in preventing numerous diseases such as autoimmune diseases, diabetes, and infections (Glick et al., 2010). Autophagy is induced in different tissues in response to decreased food in young and adult mice (Mizushima et al., 2004). A defective autophagy process leads to several diseases such as Alzheimer's (Arduino et al., 2013) and cancer (Kundu & Thompson, 2008; Levine & Kroemer, 2008). Autophagy can be protective against prostate cancer (PC) (Nikki, 2012).

Autophagy is the most important and necessary pathway that regulates cell growth, differentiation, and function (Awan & Deng, 2014). Autophagy protects the cells from death (Lamy et al., 2013). Autophagy and apoptosis (programmed cell death) are related to each other; autophagy provides a basis for apoptosis and inhibition of it leads to delayed apoptosis (Wu et al., 2014). Autophagy maintains the cell's balance by recycling the cytosolic components with misfolding proteins and damaged organelles (Cuervo et al., 2004). There are three types of autophagy: microautophagy, chaperone-mediated autophagy, and macroautophagy. Microautophagy in mammalian cells is defined as a non-selective lysosomal degradation which is considered as an autophagy form involved in long-lived protein turnover, while soluble-substrate microautophagy can be stimulated by nitrogen starvation or by rapamycin which leads to high LC3II levels (Kaushik & Cuervo, 2012). Chaperone-mediated autophagy is specialized for soluble
cytosolic protein degradation. Chaperone proteins will translocate the targeted protein across the lysosomal membrane. This type of autophagy is triggered by oxidative stress and toxic material exposure (Kaushik & Cuervo, 2012). Macroautophagy plays a role in eukaryotic cells by regulating arrangement of cellular components that have to be recycled via lysosomes (Kanazawa et al., 2004; Wang & Klionsky, 2003). While in the mammalian cell, it is stimulated by a decreased level of insulin and it delivers the cytoplasmic components to lysosomes through structures called autophagosomes (Mortimore et al., 1989). This mechanism of autophagy starts from isolating the membranes which encounter extra substrates, then attach with the lysosome (Zahm et al., 2011).

2.10 Autophagy Regulation

Autophagy is regulated by several genes related to the formation of the autophagosome a double-membraned vesicle surrounding the damaged cell components (Awan & Deng, 2014). Autophagy is regulated by the autophagy-related gene (ATG); more than 30 that have been identified in yeast and mammals, and they have a role in autophagosome membrane formation (Awan & Deng, 2014; Yang & Klionsky, 2010). ATG1 is the key protein initiating autophagy by forming a complex with ATG13 and ATG17, which both increase the kinase activity of ATG1 to activate the formation of autophagosome (Hara & Mizushima, 2009; Hosokawa et al., 2009). UNC-51-like kinase (ULK1), a major negative regulator of autophagy (mATG13), and family interaction protein of 200 KD (FIP200) are the mammalian homologs of the yeast ATG1, ATG13, and ATG17 respectively. ATG13 stimulates the interaction of ATG1-ATG1, which correlates with the kinase activity of ATG1 and promotes autophagy (Yeh et al.,
Microtubule-associated protein light chain3 (LC3II), the mammalian homolog of ATG8, plays a role in the elongation process during autophagosome formation; ATG8 also binds to ATG1 and increases its activity (Kraft et al., 2012). Beclin-1, the mammalian homolog of ATG6 in yeast, is the molecule associated most directly with autophagy and is required for the initiation step of autophagosome; by being a part of the Beclin-1 class III phosphatidylinositol (PI) 3-kinase (III PI3K) complex, this complex consists of Beclin-1, vesicular protein sorting 34 (Vps34) and p150 (Simonsen & Tooze, 2009) (Table 2.1). Other regulators for autophagy include mitogen-activated kinases, pro-apoptotic gene (BNIP3) (Velde, 2000), AMP-activated protein kinase and calcium (Metcalf et al., 2012). Tumor necrosis factor-related apoptosis-inducing legend (TRAIL) is essential to induce autophagy during formation of lumen in vitro (Mills et al., 2004). Toll-like receptor (TLR) signaling in macrophages links between two pathways: autophagy and phagocytosis (Sanijuan, 2007). All these proteins are participating in other processes, such as extracellular protein secretion. Recently it was reported that deficiency in autophagy proteins leads to impairment of the secretion of lysozyme which breaks down bacterial cell walls (Cadwell et al., 2008).

Autophagy contains various steps to remove unwanted cellular components (Xie & Klionsky, 2007; Meijer & Codogno, 2004). The initiation process of autophagy starts with the formation of a membrane known as phagophore that is derived from the lipid bilayer membrane from the endoplasmic reticulum or trans-Golgi (Axe et al., 2008; Simonsen & Tooze, 2009). Both ends of the phagophore membrane elongate to engulf the targeted components and form the autophagosome structure. The maturity of
autophagosome occurs through the fusion with the lysosome which has lysosomal acid proteases that degrade the engulfed materials (Mizushima, 2007) (Figure 2.7).

There are five stages of signaling pathways regulating autophagy: (1) phagophore formation at the membranes; (2) conjugation of ATG 5-ATG 12, interaction with ATG 16 L and multimerization at the phagophore; (3) LC3II processing insertion into the extending phagophore membrane; (4) capture of arbitrary or selective targets for degradation and (5) autophagosome fusion with lysosome, followed by lysosomal degradation of engulfed molecules (Glick et al., 2010).

During starvation, proteins are broken down by autophagy to release amino acids and increase ATP levels (Singh & Cuervo, 2011). Macroautophagy degrades lipid droplet into free fatty acids (Lipophagy) (Singh et al., 2009) while macroautophagy and microautophagy break down glycogen into oligosaccharides and glucose (Kotoulas et al., 2006). Defective autophagy results in disrupted metabolic homeostasis. For example, defects in lipid degradation lead to toxic accumulation of autophagy related gene TAG (Singh et al., 2009) and defective glycophagy leads to glycogen deposition in the cytosol (Kotoulas et al., 2006). Because it is failure to recycle the degradation products to the next material. Autophagy allows maintenance of energy homeostasis during neonatal starvation (Kuma et al., 2004). Moreover, mice lacking ATG5 display defective engulfment of apoptotic corpse during development (Xueping, 2006). Beclin-1 autophagy gene in mammals can inhibit human malignancies and its level is decreased in breast cancer (Jiang, 2013). Knockout of other autophagy genes in the nervous system leads to neurodegeneration in mice (Hara et al., 2006; Komatsu et al., 2006). Autophagy is needed for preimplantation of embryos for protein recycling.
(Tsukamoto, 2008). In plant cells, nitrogen or carbon starvation induces autophagy (Yoshimoto et al., 2004). In addition to nutrient starvation, autophagy might be responsive to a hypoxia condition (Adhami et al., 2006), such as the ischemic myocardium (Matsui et al., 2007; Yan et al., 2005). This is mediated by HIF-1 which is the main regulator for hypoxic response (Bohensky et al., 2007). Autophagy is important for cell survival for a short period; for example, autophagy stimulates during tumor growth but returns to the normal level after blood supply is established (Degenhardt, 2006). Autophagy is stimulated by rapamycin and causes marked increase of LC3 II levels (Yu et al., 2010). Epigenetic factors also play role in the regulation of autophagy in different pathological conditions (He & Klionsky, 2009). Both autophagic and epigenetic regulation are highly regulated by environmental factors during skeletal remodeling (Zahm et al., 2011).
Figure 2.7: Schematic diagram of the steps of autophagy. (A) Autophagy begins with the isolation of the membrane called the phagophore (1). Collection of cellular components forms autophagosome (2). Autophagosome fuses with the lysosome to form autolysosome (3). The membrane is lysed and autophagic cargo is digested by lysosomal proteases (4). Chemical inhibition of autophagy can be achieved during any step. 3-Methyladenine blocks the early step of autophagy (membrane formation); Bafilomycin A1 is responsible for blocking fusion between the autophagosome and lysosome, and chloroquine blocks the last step autolysosome (B).
### Table 2.1 List of Genes Related to the Autophagy Pathway

<table>
<thead>
<tr>
<th>Name of gene</th>
<th>Role of autophagy</th>
<th>Reference</th>
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<tbody>
<tr>
<td>ATG1</td>
<td>Induction of autophagy</td>
<td>(Reggiori &amp; Klionsky, 2002)</td>
</tr>
<tr>
<td>ATG5</td>
<td>Autophagosome formation</td>
<td>(Reggiori &amp; Klionsky, 2002)</td>
</tr>
<tr>
<td>ATG7</td>
<td>Autophagosome formation</td>
<td>(Reggiori &amp; Klionsky, 2002; Yu et al., 2004)</td>
</tr>
<tr>
<td>ATG8</td>
<td>Marker of autophagosome formation</td>
<td>(Reggiori &amp; Klionsky, 2002)</td>
</tr>
<tr>
<td>ATG12</td>
<td>Autophagosomeosome formation</td>
<td>(Reggiori &amp; Klionsky, 2002)</td>
</tr>
<tr>
<td>ATG13</td>
<td>Induction of autophagy</td>
<td>(Reggiori &amp; Klionsky, 2002)</td>
</tr>
<tr>
<td>Beclin-1</td>
<td>Initiation of autophagosome formation</td>
<td>(Simonsen &amp; Tooze, 2009)</td>
</tr>
</tbody>
</table>
2.11 mTOR Regulation

mTOR or mammalian target of rapamycin is a serine-threonine kinase that shown to play a central role in a number of key cellular processes that have been previously reported in bone formation. A group of studies reported that mTOR does its job through the formation of two multi-protein complexes namely mTORC1 with the raptor responsible for the cell proliferation and protein synthesis, and mTORC2 with the rictor responsible for the cytoskeletal organization (Memmott & Dennis, 2009) (Figure 2.8). The general idea is that the inhibition of mTOR increases the osteoblastic potential of MSCs and increases mineralised matrix production while simultaneously inhibiting adipogenic differentiation (un published); these data suggest that the regulation of mTOR may be helpful in solving diseases regarding low bone mass.

Studies have shown that the regulation or control of mammalian target of rapamycin (mTOR) may result in a lot more discoveries in its roles in the cells, particularly in the bone and muscle. In one particular study mTOR was noted as a central regulator of cell adhesion and proliferation. In addition, mTOR plays an important role in cell homeostasis (Memmott & Dennis, 2009). This study claims that mTOR is also a factor that helps in the growth of tumors, therefore they reported ways to regulate mTOR for it not to contribute to the growth of tumor cells. There are some signaling pathways cause activated of mTOR-independent and dependent pathways including mitogenic signals, energy, and nutrient (Memmott & Dennis, 2009). They also suggested some pharmacologic approaches in order to stop or regulate the mTOR pathway in leading to cancer.
Figure 2.8: Schematic diagram illustrating protein components of the mTOR complexes. mTORC1 contains of the core proteins including raptor (regulatory-associated protein of mTOR), and sec-13 protein 8 (mLST) (mammalian lethal with SEC13 protein 8) are essential for phosphorylation of mTORC1. In addition, mTORC1 binds to other associated proteins such as proline-rich Akt substrate 40 kDa (PRAS40), and DEP domain-containing mTOR-interacting protein (Deptr) both PRAS40 and Deptr is suppress kinase activity. In contrast, mTORC2 contains core proteins including rictor (rapamycin-insensitive companion of mTOR), and mLST8, and other associated proteins such as mammalian stress-activated MAP kinase-interacting protein 1 (Sin1), deptr, and protor 1/2.
Another study reported that the mammalian TOR (mTOR) regulates the autophagy machinery as well as the balance between cell growths. Nutritional status, growth factors, and stress signals causes induction of autophagy (Jung et al., 2010). These data are strengthened by the findings of another study on mTOR which concludes that mTOR regulates cellular homeostasis by stimulating metabolism processes in response to nutrient starvation, lower energy, hypoxia, and reduce growth factor signaling (Sengupta et al., 2010). Moreover, this study also claims that the regulation of mTOR can be attributed to the many and different stresses that affect them.

The cells in the bone and muscle are also affected by mTOR signaling. According to another research study, the differentiation of vascular smooth muscle cells (VSMCs) from the mesenchymal stem cells (MSCs) is controlled by activation of mTOR, lower concentration of rapamycin results in reducing the formation of neointima and lumen loss which is very helpful in the field of medication in solving diseases regarding the vascular smooth muscles (Hegner et al., 2009).

In another study, the regulation of mTOR also helps in the inhibition of osteopenia in mice that has systemic sclerosis (Chen et al., 2015 a; Chen et al., 2015 b). It was noted that Fbn1 regulates BMMSC osteogenic/adipogenic lineage selection via IL4Rα/mTOR (the mammalian target of rapamycin) signaling. Inhibition of the mTOR by rapamycin reduces osteopenia phenotype in Fbn1+/− SS mice (Chen et al., 2015). The findings of this study highly suggest that mTOR regulation is helpful in solving bone diseases and even bone cancers. This claim is also supported by another study which suggests that activation of mTORC1 through phosphorylation of protein kinases leads to increase osteoblast differentiation in vitro and in vivo in response to baicalein (a natural
compound cause induction of autophagy) treatment (Chen et al., 2015). In this study, baicalein helps in the regulation of bone formation, which may help in solving cases of osteoporosis. However, in the first studies mentioned, the inhibition of mTOR reduces risks of cancer and other diseases. Activating mTORC1 in this study helps in reducing risks of osteoporosis; nevertheless, it all boils down to the fact that regulation of mTOR is very much important in solving cases or diseases related to the mechanism of cells in bones.

Other studies have supported the claims that activating the pathway of mTORC1 is really helpful for the bones. In one study, the researchers found out that activation of mTORC1 by rapamycin causes increase expression RANKL that leads to osteoclast resorption (Xu et al., 2016). Moreover, one study has also found out that the activation of the mTORC1 is connected to the WNT7B (a protein that is encoded by the WNT7B) wherein the researchers found out that WNT7B increases osteoblast differentiation, function, and bone formation in vivo via activation mTORC1 signaling pathway (Chen et al., 2014). This conclusion proves that mTORC1 really aids in bone formation.

Lastly, a study was reported in emphasizing the role of mTOR regulation in bone formation as the study proved that shRNA-mediated knockdown of mTOR results in increase osteoblast differentiation and mineralization in vitro (Martin et al., 2010). This data suggests that mTOR helps bone-forming cells like the osteoblasts. But it is important to note that the mTOR is only beneficial when it is being inhibited and regulated because too much mTOR or too little of it may cause damages in the bone.
2.12 mTOR Signaling

Aside from mTOR regulation, mTOR signaling has also been found to be an agent for activating osteoblasts, which are the cells that help in forming bone. This can be proven by a study that concluded that deletion of raptor results in decrease late markers of osteoblast differentiation include osteocalcin, bone sialoprotein, and collagen type I. Conversely, deletion of raptor causes enhancing early osteoblast markers include alkaline phosphatase and runt-related transcription factor 2 (Runx2) expression (Chen & Long, 2015). Raptor, as mentioned earlier, is a component of mTORC1, and deletion of raptor was proven to increase mTORC2 signaling. This study promotes that the increase in mTORC2 signaling notably increased early osteoblast markers that could enhances bone growth.

Another study showed that the mTOR pathway as a key regulator of cellular metabolism must be controlled. mTOR is involved in an enhancing some of pathological conditions, such as cancer, obesity, type 2 diabetes, and neurodegeneration (Laplante & Sabatini, 2012). In another study, it was found that mTORC2 mentioned above could promote skeletal growth formation, particularly in mice. Deletion of rictor (the main component of mTORC2) results in decreased bone mass in the mouse embryo (Chen et al., 2015). Unlike the previous study that reported that deletion of rictor resulted in the reduction of the width of the initial cartilage anlage. Because of this, the researchers found out that mTORC2 signaling is very essential for the optimal skeletal growth and bone anabolism, (Chen et al., 2015) even more than the regulation of mTORC1 discussed earlier.
Recently, some group reported that mTORC2 signaling is more essential to skeletal growth than mTORC1. This regulation is supported by the study that suggest the mTORC1 is essential for osteoblast proliferation; in contrast, inhibition of mTORC1 is important for osteoblast differentiation and mineralization *in vitro* and *in vivo* (Huang et al., 2015). This data indicates that even though mTORC1 is important for the proliferation of the preosteoblast, it can still slow down the maturation of the bones; therefore, it is true that mTORC2 signaling is still the better way for growth and maturation of bones rather than mTORC1. However, the regulation of mTORC1 is still needed for osteoblasts.

Another study on bone formation was also conducted by Shoba and Lee (2003), and suggested that the phosphatidylinositol 3-kinase (PI 3-kinase) contributes with insulin-like growth factor-I (IGF-I) that causes increased alkaline phosphatase (the main marker of osteoblast differentiation) using fetal rat calvaria cells (Shoba & Lee, 2003). In this study, it was noted that the combination of ostogenic protein (OP-1) and IGF-I, stimulate ALP expression for formation of bones using fetal rat calvaria. This means that signaling of mTOR contributes to the growth and formation of bone nodule.

Lastly, the study of Esen and others have also found another way as to how mTOR signaling can help in the formation of bone. They noted in their study that aerobic glycolysis stimulate osteoblast differentiation and bone formation through the WNT signaling (Esen et al., 2013). mTORC2 signaling downstream is one of the contributing factors for WNT-LRP5 signaling; therefore it would be safe to say that the mTORC2 signaling is also another factor involved in bone formation by WNT-LRP5 signaling.
The mTOR regulation and mTOR signaling is not just relevant for bone cells but for other cell mechanisms. In this particular study, it was shown that inhibition of mTOR signaling pathway leads to decrease human embryonic stem cell viability and proliferation. PI3K/AKT kinase, MAPK/ERK are also decreased upon differentiation (Armstrong et al., 2006). Though mTOR was not mentioned here directly, the PI3K/AKT kinase, MAPK/ERK, and NFκβ pathways are components of mTOR. This study has found that mTOR signaling is important in embryonic stem cell proliferation because the decreasing mTOR upon differentiation resulted in a loss of pluripotency or loss of viability which affects the embryonic stem cell differentiation.

Another study has promoted the role of components of mTOR to the embryonic stem cell. Takahashi (2005) and fellow researchers used mouse embryonic stem cells in their study and concluded that activation of PI3K signaling pathway is crucial for proliferation in mouse embryonic stem cells (ESCs), and early embryos (Takahashi et al., 2005). PI3K is a component of mTOR, and can easily activate the mTOR pathway in this scenario. However, there is a reason as to why researchers have been using mouse embryonic stem cells in this experiment. It was noted in the study of Takahashi that the mouse embryonic stem cells share some similarities with cancer cells (Takahashi et al., 2005).

2.13 mTOR-Dependent Pathway and Bone Homeostasis

Rapamycin in bone cells plays a significant role in both osteoblast and osteoclast and many studies have proven this fact. According to one study, lower concentrations of rapamycin cause decrease bone formation by targeting osteoblast proliferation and
differentiation in preosteoblast MC3T3-E1 and primary mouse bone marrow stromal cells (BMSCs) (Singha et al., 2008). Moreover, investigators have also found that rapamycin has also significantly reduced osteoblast markers including osteocalcin, bone sialoprotein and osterix which all contribute to the proliferation and differentiation of osteoblast. Another study noted that this effect was because rapamycin inhibits mTOR that can promote the differentiation of osteoblast using human embryonic stem cells (hESCs). According to the research study, rapamycin is only beneficial for bone cells by activation of bone morphogenetic protein/Smad (BMP/Smad) signaling (Lee et al., 2010).

Additional studies support the conclusion mentioned above. In another study that was described that rapamycin directly acts on osteosarcoma cell line (ROS) and causes enhancing of alkaline phosphatase activity and increased expression of osteopontin and osteocalcin mRNA by using 25-hydroxyvitamin D (1.25(OH) 2-vitaminD3) (Ogawa et al., 1998), and promotes bone fracture healing (Yang et al., 2015). Though this study has used other means to activate rapamycin, it was still concluded that this drug helped in the differentiation of osteoblast cells in the bones. In contract, another study was emphasized that the liraglutide can also increase osteoblast differentiation by using MC3T3-E1 preosteoblast cell line through activation protein kinase (AMPK) signaling (Hu et al., 2016) (Table 2.2).

In terms of osteoclastic differentiation, rapamycin was also tested to know its implications. In one study, it was found that activation the process of translational control of the gene regulatory transcription factor CCAAT/enhancer binding protein beta (C/EBPβ) isoform expression may use as a therapeutic approach for bone diseases.
The control of C/EBPβ isoform expression is part of the rapamycin, specifically downstream of mTOR in this study; therefore, it would be safe to conclude that the rapamycin can be an alternative in order to produce osteoclast differentiation which is very helpful in solving lytic bone diseases. Moreover, another study showed that rapamycin inhibits osteoclast formation in giant cell tumor of bone through the C/EBPβ-MafB axis (Smink et al., 2012). The study referred to mice wherein when induced with rapamycin, osteoclast differentiation was decreased. Additionally, through the latter study reviewed in this part, it was found that C/EBPβ is a determinant of giant osteoclast formation in giant cell tumor (GCT). The researchers strongly stated that rapamycin treatment cause decrease osteoclast formation and bone resorption in patients with giant cell tumor (GCT) (Smink et al., 2012).

On another note, the rapamycin in mTOR was attributed as well to osteoclast formation. It was noted that protein kinase 1/2 (Akt1/Akt2) causes decrease in osteoclast formation through mTOR inhibition (Sugatani & Hruska, 2005). This study is relevant in knowing more about the effect of rapamycin in mTOR. Rapamycin played a role in the differentiation of osteoclastic cells. Supporting this article is another study that noted that decrease osteoclast formation by using sirolimus or everolimus that have the same effect of rapamycin (Cejka et al., 2010). One way to inhibit mTOR is through the rapamycin. These studies suggest that rapamycin plays significant roles in osteoclast cells.

Rapamycin is also seen, not just to inhibit mTOR but also as a bone sparing immunosuppressant. One study noted that, treatment with rapamycin results in improve bone modeling and remodeling. (Romero et al., 1995). This suggests that rapamycin is
both an immunosuppressant and a bone drug that does not negatively affect the bone structure and growth. A study stated that immunosuppressant causes enhancing receptor activator of NF-kappa B ligand (RANKL) and reduces osteoprotegerin (OPG) by using marrow stromal cells (Hofbauer et al., 2001) which ultimately destroys the bones however, the latter study suggests that even though rapamycin is an immunosuppressant, it does not really harm the bones. However, a study contradicts with the effectiveness of rapamycin, and noted that rapamycin treatment leads to decrease osteoblast differentiation from mesenchymal stem cells through inhibition of dexamethasone effects. In contrast, FK506 is an immunosuppressive drug cause increase osteoblast differentiation and function (Isomoto et al., 2007). These data suggest that FK506 is more ideal than rapamycin in allogeneic transplantation of mesenchymal stem cells. However, rapamycin was also found to inhibit osteolysis and improves survival in bone metastases which is also beneficial for bone formation as authors noted that rapamycin can treat metastases-associated with osteolytic diseases (Hussein et al., 2012) (Table 2.3).
Table 2.2. Effects of mTORC1 and mTORC2 on Osteoblast cells.

<table>
<thead>
<tr>
<th>Osteoblast</th>
<th>Effect</th>
<th>References</th>
</tr>
</thead>
</table>
| **mTORC1** | • Stimulates osteoblast differentiation.  
• Reduces risks of osteoporosis. | (Li et al., 2015) |
|           | • Increase bone formation through WNT7B signaling | (Chen et al., 2015) |
|           | • Deletion of raptor reduces late markers of osteoblast | (Chen et al., 2015) |
|           | • Increases osteoblast proliferation by activation of mTORC1  
• Increases osteoblast differentiation by inhibition of mTORC1 | (Hussein et al., 2012) |
|           | • Improves bone metastases. | (Hussein et al., 2012) |
| **mTORC2** | • Increases osteoblast differentiation | (Chen et al., 2015) |
|           | • Essential for skeletal growth and bone anabolism | (Chen et al., 2015) |
|           | • Increases bone formation | (Soba and Lee 2003) |
|           | • Increases osteoblast proliferation | (Takahashi et al., 2005) |
|           | • Increases osteoblast differentiation in hESCs and osteosarcoma. | (Lee et al., 2010; Ogawa et al., 1998) |
Table 2.3. Effects of mTORC1 and mTORC2 on Osteoclast cells.

<table>
<thead>
<tr>
<th>Osteoclast</th>
<th>Effect</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>mTORC1</td>
<td>• Stimulates RANKL expression.</td>
<td>(Xu et al., 2016; Hofbauer et al., 2001)</td>
</tr>
<tr>
<td></td>
<td>• Induces bone loss and osteoporosis.</td>
<td></td>
</tr>
<tr>
<td>mTORC2</td>
<td>• Increases osteoclast differentiation.</td>
<td>(Smink et al., 2010)</td>
</tr>
<tr>
<td></td>
<td>• Inhibits osteolysis.</td>
<td>(Hussein et al., 2012)</td>
</tr>
<tr>
<td></td>
<td>• Inhibits osteoclast formation.</td>
<td>Smink et al., 2012; Sugatani and Hruska 2005</td>
</tr>
<tr>
<td></td>
<td>• Increases modeling, remodeling, and decreases longitudinal growth rate.</td>
<td>(Romero et al., 1995)</td>
</tr>
</tbody>
</table>
2.14 mTOR-Independent Pathway and Bone Homeostasis

Autophagy related proteins have been shown to be mediators in bone cell differentiation and function, suggesting that autophagy plays a role in bone homeostasis (Hocking et al., 2012), and cartilage repair (Lotz & Carames, 2011). The interaction between osteoblasts, osteoclasts, and osteocyte is very important to maintain bone homeostasis (Crockett et al., 2011). The imbalance between osteoblast and osteoclast function leads to bone disorders such as osteoporosis (Hocking et al., 2012). Loss of autophagy could lead to bone cell dysfunction. In addition, during osteoblast differentiation, cells use autophagic vacuoles as a source of energy (Nuschke et al., 2014).

Osteoblasts are the cells responsible for synthesis of bone matrix and mineralization. Autophagy is a physiological process to recycle protein and acts along the ubiquitin-proteasome system (UPS) to maintain cellular homeostasis. Destroyed organelles, intracellular pathogens, and protein aggregates are enclosed by a newly synthesized membrane (to form autophagosomes), which in turn fuses with lysosomes to form autolysosomes, their enzymes then degrade the contents inside it (Wong et al., 2011). Autophagosome membrane formation needs certain autophagy proteins (Mizushima et al., 2011) and the insertion of lipidated microtubule-associated light chain 3 (LC3II) or gamma-aminobutyric acid A receptor-associated protein (GABARAP) subfamily members. The neighbor of BRCA1 gene (NBR1) is an autophagy receptor that interacts with LC3II and with ubiquitinated, aggregated proteins to degrade the ubiquitinated substrates. Disruption of NBR1 has a tremendous effect on osteoblast differentiation and function. Genetic truncation of murine Nbr1 enhances osteoblast
differentiation and activity which leads to an age-dependent increase in bone mass and density (Whitehouse et al., 2010). Increased levels of autophagy in human mesenchymal stem cells (hMSCs) lead to reduced differentiation to osteoblasts (Oliver et al., 2012). On the contrary, another study showed that a target deletion of FIP200, part of a complex that is important to initiate autophagosome formation, lead to reduction in bone formation (Yang et al., 2012).

Another study, which used a high-throughput small-molecule screening assay, found that rapamycin induced autophagy enhances osteoblast differentiation (Bonewald, 2011). In summary, these studies suggest that autophagy enhances bone formation and this contradicts the NBR1 study, which suggests the opposite. Therefore, it is not clear whether activation or inhibition of autophagy can induce bone formation. By using osteoblast cell lines, a study showed that autophagy is enhanced during osteoblast differentiation and mineralization (Lui, 2013; Nollet et al., 2014). Using primary mouse osteoblast, and osteoblast cell lines they found double-membraned autophagic vesicles containing structures that are released in the extracellular medium (Nollet et al., 2014). The role of autophagy in osteoblast function is confirmed by using 3-methyladenine (3-MA) and chloroquine at day 10 of differentiation to inhibit autophagy which led to a decreased number and size of alkaline phosphatase, and also a reduction in bone mineralization at day 21 (Lui, 2013). Other autophagy inhibitor including bafilomycin, chloroquine, ammonium chloride (NH4CL), and shRNA-mediated knockdown of the autophagy-essential gene LC3-β caused decreased osteogenic differentiation in human dental pulp mesenchymal stem cells (Pantovic et al., 2013). In addition, siATG7 and
siBCN1 were shown to decrease mineralization in the rat osteoblast cell line (Nollet et al., 2014) (Figure 2.9).
**Figure 2.9:** Schematic diagram illustrating the role of autophagy on osteoblast differentiation and bone formation.
Osteocytes are terminally differentiated cells that are formed after osteoblasts transition to osteocytes located in mineralized bone matrix (Bonewald, 2011). During this transition, cells undergo a change in shape and a decrease in organelle contents and size (Bohensky et al., 2007). An increase in autophagy may accompany this transition to adjust the osteocyte to the hypoxia as they live in poor nutrient conditions. In addition, autophagy is induced in osteocytes in response to starvation (Zahm et al., 2011). In addition, autophagy osteocyte increased in rats with an ovariectomy due to increased oxidative stress that is induced by loss of estrogens (Yang et al., 2014). In the same context, another study showed a higher expression of LC3II in rat tibia in osteocytes compared to cell surface osteoblasts (Yoshimoto et al., 2004). Furthermore, in vitro studies showed that the more differentiated osteocytic cells have higher autophagic flux (Zahm et al., 2011). Another study also showed that a deletion of ATG7 from osteocytes results in a significant reduction of bone mass (Zhao et al., 2012). Treatment with Glucocorticoid also led to induced autophagy to save osteocyte viability (Jia et al., 2011; Xia et al., 2010), suppression of autophagy also does not have an effect on glucocorticoid on bone (Piemontese et al., 2015). These data suggest that autophagy plays an important role in osteocyte survival, which in turn protects bone from loss.

Bone resorption occurs with osteoclasts, which are multinucleated cells derived from hematopoietic stem cells in response to stimulation by macrophage colony stimulating factor (M-CSF) and RANK ligand (RANKL). In addition, NFATc1 is very important transcription factor for osteoclasts and osteoblasts differentiation and function (Winslow et al., 2006).
Autophagy has a role in the regulation of osteoclast function. Studies found that Paget’s disease of bone (PDB) which is characterized by increased osteoclast activity, size, multinuclearity, and number can occur as a result of a mutation in the autophagic cargo receptor SQSTM1(or P62) (Helfrich & Hocking, 2008). Deletion of ATG5 and ATG7 reduced the resorptive capacity of osteoclasts without affecting these cells’ differentiation (DeSelm et al., 2011). Furthermore, other studies confirmed that osteoclast increases in size by hypoxia (Arnett, 2010). Another study shows that hypoxia through HIF-α caused an increase in osteoclast differentiation and a significant increase in autophagy (Sun et al., 2015). In addition, osteoclastogenesis is induced by different factors mediated by autophagy such as hypoxia (Zhao et al., 2012) or microgravity (Sambandam et al., 2014).

Induced autophagy by rapamycin leads to a decrease in osteoclast number in rats (Sanchez & He, 2009), a reduction in osteoclast differentiation and bone resorption in mouse models of arthritis (Cejka et al., 2010), a decrease in bone resorption with patients who have renal transplantation (Westenfeld et al., 2011), and limited in vitro osteoclast formation in patients who have giant cell tumors (Smink et al., 2012). Autophagy plays an essential role in the maintenance of hematopoietic stem cells (HSCs). HSCs fail to differentiate to osteoclasts when ATG7 is absent (Mortensen et al., 2011). P62 is an adaptor protein that plays a critical role in RANKL to promote autophagy and osteoclastogenesis (Li et al., 2014) (Figure 2.10).

Recently, it has been reported that in an autophagy-independent manner, in vitro decreased levels of Beclin-1 in osteoclasts lead to a reduction in differentiation and function of these cells through NFATc1 (Chung et al., 2014). Increased expression of
Beclin1 and ATG7 in human rheumatoid arthritis (RA) happens with activated autophagy in osteoclasts by using tumor necrosis factor (TNF-α) \textit{in vivo} and \textit{vitro}, and this lead to regulation of osteoclast differentiation and bone resorption (Lin et al., 2013; Yu et al., 2004). Some groups showed that ATG4B, ATG5, ATG7, and LC3II are essential for generating the osteoclast-ruffled border \textit{in vivo} and \textit{in vitro}. Autophagy mediates the fusion between lysosomal contents and the plasma membrane by participating in polarized secretion between them (DeSelm et al., 2011). ATG7 knockdown inhibits expression of osteoclast markers TRAP and cathepsin K during osteoclast differentiation (Wang et al., 2011). The lysosome fusion with the ruffled border leads to proton and protease secretion into lacuna, (Itzstein et al., 2011) and induced autophagy leads to reduced pathological osteoclast formation (Smink, 2012). Recently a study showed that autophagy is deficient in main organs and tissues from critically ill humans and rabbits, with a decrease in the LC3-II and an increase in P62 accumulation contributing to mitochondrial dysfunction (Derde et al., 2012; Gunst et al., 2013; Vanhorebeek et al., 2011) (Table 2. 4).
**Figure 2.10:** Schematic diagram illustrating the role of autophagy on osteoclast differentiation and bone resorption.
**Table 2.4: Autophagy Relates-Gens Regulated in Bone Cells.**

<table>
<thead>
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<th>Function</th>
<th>References</th>
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<tr>
<td><strong>Osteoblast</strong></td>
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<tr>
<td>NBR1</td>
<td>Increased osteoblast differentiation and activity + increased bone mass and density. (Whitehouse et al., 2010)</td>
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<tr>
<td>Deletion of FIP200</td>
<td>Reduction in bone formation. (Yang &amp; Krebsbach, 2012)</td>
</tr>
<tr>
<td>Rapamycin</td>
<td>Increased osteoblast differentiation (Bonewald, 2011)</td>
</tr>
<tr>
<td>3-MA and chloroquine</td>
<td>Decreased number and size of alkaline phosphatase at day 10 + reduction in bone mineralization at day 21. (Pantovic et al., 2013)</td>
</tr>
<tr>
<td>Bafilomycin, chloroquine, NH₄CL, and shRNA-mediated knockdown of LC3-B</td>
<td>Blocked osteogenesis (Pantovic et al., 2013)</td>
</tr>
<tr>
<td>siATG7 and siBCN1</td>
<td>Decreased osteoblast mineralization (Nollet et al., 2014)</td>
</tr>
<tr>
<td><strong>Osteoclasts</strong></td>
<td></td>
</tr>
<tr>
<td>Mutation in SQSTM1 (P62)</td>
<td>Paget's disease (Helfrich &amp; Hocking, 2008)</td>
</tr>
<tr>
<td>ATG5, ATG7, ATG4B, and LC3II</td>
<td>Generating the osteoclast ruffled border (DeSelm et al., 2011)</td>
</tr>
<tr>
<td>Deletion of ATG5 and ATG7</td>
<td>Reduce the resorptive capacity of osteoclasts (DeSelm et al., 2011)</td>
</tr>
<tr>
<td>Hypoxia</td>
<td>Increased osteoclast (Arnett, 2010; Bozec et al., 2008; Rousselle &amp;</td>
</tr>
<tr>
<td>Treatment</td>
<td>Effect</td>
</tr>
<tr>
<td>-------------------------------</td>
<td>---------------------------------------------</td>
</tr>
<tr>
<td>Rapamycin</td>
<td>Decreased osteoclast number</td>
</tr>
<tr>
<td>Absent of ATG7</td>
<td>HSCs fail to differentiate to osteoclasts</td>
</tr>
<tr>
<td>P62</td>
<td>Increased osteoclastogenesis</td>
</tr>
<tr>
<td>Decrease level of Beclin-1</td>
<td>Reduction in osteoclast differentiation and function</td>
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2.15 Osteoactivin and Autophagy

Osteoactivin is a necessary gene that causes communication between the macroautophagic, degradation pathway, and phagocytosis. Osteoactivin is important for recruitment of autophagy protein LC3II to the phagosome. These proteins increase degradation of the content when the lysosome fuses with the phagosome. These data suggest that osteoactivin plays an essential role in kidney repair after injury by regulation of phagosome degradation (Bing & Thomas, 2010). In addition, osteoactivin may play a role in normal handling of self-antigen and avoidance of aberrant adaptive immune-responses (Anderson et al., 2002; Mo, 2003). Furthermore, retinal pigment epithelial cells express osteoactivin, and accumulation of pigment in macrophages recruited to the eye in these mice points to a possible role for osteoactivin in the normal clearance of photoreceptor outer segment debris and iris pigment debris by retinal pigment epithelial cells (Kuan et al., 2006; Li et al., 2010). Osteoactivin is expressed by macrophages in inflammatory sites of function in clearance and degradation of self-peptides and inflammatory oxidized lipids needed to maintain homeostatic conditions. Therefore, phagocytosis is an essential step to prevent inflammation and autoimmunity (Hanayama et al., 2006). Macroautophagy is a degradation pathway leading to removal of unwanted intracellular organelles by lysosomes (Codogno, 2005). Epithelial cells are up-regulated by osteoactivin and express high levels of phagocytic receptors (Ichimura et al., 2008). The main function of epithelial cells after injury is removal and degradation of the debris cells. Therefore, up-regulation of phagocytic receptors to clear the degradation contents is essential (Bing & Thomas, 2010). Defect in clearance of phagocytosed, apoptotic, and debris cells lead to the death of macrophages (Mo, 2003).
In addition, to the role of epithelial cells in tissue repair and regeneration, another factor plays a role in repairing by clearing debris cells and supporting tissue regeneration which are macrophages (Duffield et al., 2005). Osteoactivin is upregulated after injury (Ichimura et al., 2008). Autophagy has a relationship with innate and adaptive immune response (Deretic, 2005; Levine & Deretic, 2007). Autophagy leads to avoiding intracellular pathogens.

2.16 Bone Diseases and Autophagy

The most important aspect of the bone that results when autophagy components are mutated or knocked out is an age-dependent effect (Hocking et al., 2004) (Daroszewska et al., 2011; Watts et al., 2004; Whitehouse et al., 2010). For example, 8-months-old ATG5 knockout mice, had higher trabecular bone volume compared to, 8-week-old wild-type mice (DeSelm et al., 2011). Autophagy decreases with age and this is clear in the terminally differentiated cells (Hubbard et al., 2012).

Paget’s Disease of Bone

Paget’s disease (PDB) is a pathology related to bone in which there is increased bone resorption and disorganized bone formation. The patients with PDB are elderly; PDB affects both males and females. Patients suffer from pain, deformation of bone, and pathologic fractures (Crisp, 1993). The most important complication of PDB is development of osteosarcoma in pagetic bone (Hansen et al., 1999; Huvos, 1986). PDB is characterized by increased size and nuclei number of osteoclasts (Reddy et al., 1999). In PDB, bone resorption increases in a focal region followed by increased bone formation, resulting in deposition of new bone, but these bones have poor quality and are disorganized. At the end stage of PDB, the bone becomes sclerotic with
replacement of the bone marrow by fibrous tissue, and thickness of the bone increases (Hosking, 1981). In PDB, there is an increase in numbers for both bone remodeling by osteoblasts and bone resorption by osteoclasts. For example, enhanced level of alkaline phosphatase in the serum, suggests increased osteoblast activity; furthermore, increased collagen type I which is released during bone resorption also suggest increased osteoclast activity (Alvarez & Pons, 1997). These data suggest that bone resorption and formation remain coupled in PDB. Osteoclasts increase in number and size and contain 100 nuclei compared to normal osteoclasts which contain 3-20 nuclei (Rebel et al., 1981). This disease is associated with mutation in the ubiquitin associated domain of SQSTM/P62 gene that has been shown with most of PDB (Goode & Layfield, 2010). P62 serves as an autophagy receptor and signaling regulator of osteoclast formation and apoptosis (Goode & Layfield, 2010); also the infection factor has been reported to be essential for this pathology (Roodman & Windle, 2005). Another study reported that mutation in the UBA domain of P62 is the cause of Paget’s disease (Daroszewska et al., 2011). In this study, it is shown that an increased level of LC3-II was shown in the presence of Bafilomycin A1 which increases autophagosome formation. Recently, OPtn gene is shown as a genetic risk for PDB (Albagha et al., 2010). OPtn gene is essential for different physiological processes including NF-kB regulation (Kachaner et al., 2012). Furthermore, OPtn is an autophagy receptor responsible for protein aggregates (Korac et al., 2013).
Osteopetrosis

This disease, also known as malignant infantile osteopetrosis, is characterized by osteoclast dysfunction and increased bone mass (Stark & Savarirayan, 2009). Patients with this disease exhibit increased bone mass with defective ruffled-border formation which results in the inability of osteoclasts to resorb bone (Sobacchi et al., 2013). Osteopetrosis: genetics, treatment and new insights into osteoclast function (Sobacchi et al., 2013). There are two forms of this disease: the first one is autosomal dominant, which has symptoms, and the second one is autosomal recessive infantile, which is fatal (Villa et al., 2009). Some mutations in osteoclast related genes are required for osteoclast differentiation and bone resorption and their loss leads to an osteopetrotic phenotype (Vernejoul & Kornak, 2010; Perdu & Van Hul, 2010; Villa et al., 2009), such as the TCIRG1 and CLCN7 gene (Sobacchi et al., 2013). Both the TCIRG1 and CLCN7 gene are important for formation of the podosome and lysosomal trafficking in bone cell resorption (Frattini et al., 2000; Kornak et al., 2001). The mouse models are shown to be defective in autophagy with a decreased level of LC3-II and an increased level of P62 in mutant osteoclast mice (Ochotny et al., 2013). In addition, mutation in CLCN7 and OSTM1 were associated with neurodegeneration due to increased LC3-II levels (Heraud et al., 2014).

Osteoporosis and Aging Bone

Osteoporosis is bone loss associated with age and increased risk of fracture ("NIH Consensus Development Panel on Osteoporosis Prevention, Diagnosis, and Therapy, March 7-29, 2000: highlights of the conference," 2001). Two million people are reported in United States with fragility fractures associated with low bone mass or osteopenia
Eight percent of patients who have osteoporosis require care (Chrischilles et al., 1991). This disease affects one out of three women and one out of six men over the age of 50 (Sanchez-Riera et al., 2010). This disease is characterized by low bone mass ("Consensus development conference: prophylaxis and treatment of osteoporosis," 1991; Cooper, 2003). There are some mechanisms that can affect the bone homeostasis such as increased bone resorption by osteoclasts and decreased bone formation by osteoblasts (Raisz, 2007). These mechanisms can be effected by genetics and nutrition. Genetics are the most important factor effect on bone, because changes in gene expression leads to some disease. Collagen type I (COL1) is an important marker for low bone mass and fracture (Grant et al., 1996). Polymorphisms in COL1A1 Sp1 “T” allele is associated with decreased osteoblast function to form mineralized bone in vitro and abnormalities of bone mineralization in vivo (Stewart et al., 2005). Lipoprotein receptor-related protein 5 (LRP5) is essential for osteoblast differentiation and function (Ferrari et al., 2004; Meurs et al., 2006). The most important factor that leads to osteoporosis is lack of estrogen. Estrogen leads to decreased activity of osteoclast (Shevde et al., 2000). TCIRG1 and CLCN7 are essential for osteoclast function; defects in these genes result in osteoporosis disease (Balemans et al., 2005; Frattini et al., 2000). Sclerosteosis / Van Buchem disease gene (SOST) have been associated with bone mineral density in elderly whites (Uitterlinden et al., 2004). In addition, deficiencies in Vitamin D and calcium lead to bone loss and fractures (Lips, 2001); smoking, alcohol (Wong et al., 2007) and nutrition (Borer, 2005) can also effect bone. Vitamin D and calcium are important to reduce the risk of hip fracture (Boonen et al., 2007); vitamin K and vitamin B12 are essential for bone health (Baines et al., 2007)

(Raisz, 2007).
The key factors in this disease are loss of sex steroids and increased oxidative stress (Almeida & O'Brien, 2013; Manolagas & Parfitt, 2010), which indicates that autophagy is an essential player in age-related bone loss. Moreover, some studies report that the autophagic pathway is undergoing age related decrease in several organs such as the liver (Roso et al., 2003), kidney (Cui et al., 2012), skeletal muscle (Wohlgemuth et al., 2010) and pancreatic islets (Liu et al., 2013). In bone, decreased autophagic activity was reported in rat osteocytes. A decreased expression of BECN 1, ULK-1, and LC3-II was reported with age while apoptosis and P62 levels increase in osteocytes (Chen et al., 2014). It was recently reported that oxidative stress induced by overiectomy was associated with increased osteocyte autophagic activity (Yang et al., 2014).
Chapter 3

Materials and Methods

3.1 Mice

Osteoactivin rescue (DBA/2J GPNMB\(^+\)), and osteoactivin-mutant mice (DBA/2J) were purchased from Jackson Laboratory. Six or 8-week males were used to avoid hormonal effects on the experiment. All mice were housed and maintained at Northeast Ohio Medical University according to the guidelines set by the Institutional Animal Care and Use Committee (IACUC) (Table 3.1).

Table 3.1: Types of Animal Models Utilized in the Study

<table>
<thead>
<tr>
<th>Mouse model</th>
<th>Phenotype</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>DBA/2J</td>
<td>Pigmentary glaucoma</td>
<td>(Anderson et al., 2002; McKinnon et al., 2009)</td>
</tr>
<tr>
<td></td>
<td>Bone</td>
<td>(Abdelmagid et al., 2014)</td>
</tr>
<tr>
<td>DBA/2J GPNMB(^+)</td>
<td>Wild type with a functional allele of GPNMB</td>
<td>(Abdelmagid et al., 2014)</td>
</tr>
</tbody>
</table>
3.2 Trehalose

In nature, Trehalose (TH) can be found in animals, plants and microorganisms. Trehalose, also known as mycose or tremalose, is a natural alpha-linked disaccharide formed by α, α-1, 1-glucoside bond between two α-glucose units. Giving it the name of α-D-glucopyranosyl-(1→1)-α-D-glucopyranoside (Sapir & Harries, 2011) (Figure 3.1). The bonding makes trehalose very resistant to acid hydrolysis, and therefore is stable in solution at high temperatures. Trehalose directly interacts with nucleic acids, facilitates melting of double stranded DNA and stabilizes single-stranded nucleic acids (Bezrukavnikov et al., 2014). Trehalose is nutritionally equivalent to glucose, because it is rapidly broken down into glucose by the enzyme trehalose, which is present in the brush border of the intestinal mucosa of omnivores. Trehalose induces autophagy by mTOR-independent pathway. A master controller of lysosomal biogenesis and autophagy, (Sardiello et al., 2009), by inhibiting AKT/PKB. It may have use for treatments of Huntington's disease, Parkinson's disease, tauopathies or Batten disease, (Palmieri et al., 2017), as it may correct defects in autophagy seen in these diseases and improve removal of aggregated proteins and other aberrant storage material (Palmieri et al., 2017). In this study, we used D-(+)-T trehalose dihydrate purchased from SIGMA-ALDRICH and diluted in the medium, 100mM concentration was used in this experiment.

3.3 Rapamycin

Rapamycin (R) has immunosuppressant functions in humans and is especially useful in preventing the rejection of kidney transplants (Romero et al., 1995). It inhibits activation of T cells and B cells by reducing the production of interleukin-2 (IL-2). It is
produced by the bacterium *Streptomyces hygroscopicus* for anti-proliferative properties due to its ability to inhibit mTOR. Rapamycin treatment may increase risk of type 2 diabetes (Johnston et al., 2008) due to inhibition of mTOR, which results in diabetes-like symptoms. This includes decreased glucose tolerance and insensitivity to insulin (Lamming et al., 2012). Additionally, rapamycin is used in a rare lung disease called lymphangioleiomyomatosis (Chhajed et al., 2006) (Figure 3.2). In this study, we used rapamycin purchased from Selleckchem and diluted in Dimethyl Sulfoxid, final concentration was 500µM.

**3.4 Chloroquine**

Chloroquine (CQ) is a medication use in health system, used in some autoimmune disorders. Chloroquine use to prevent endosomal acidification (Steinman et al., 1983). Group of research reported that chloroquine accumulates inside the cell especially acidic cells and inhibits fusion between lysosomes and endosomes (Rutz, et al., 2004; Hart et al., 2005). Moreover, chloroquine inhibits autophagy by preventing fusion of autophagosome with lysosome and inhibits lysosomal degradation (Shintani & Klionsky, 2004). In this experiment, chloroquine was purchased from SIGMA-ALDRICH and diluted in water 100µM concentration was used in this experiment (Figure 3.3).

**3.5 Cell culture**

MC3T3-E1 cells were cultured in 100mm dishes with α-MEM medium supplemented with 10% FBS. The medium was changed twice a week until cell confluence. Then cells were counted after trypsinization and plated in a 6-well plate at 2X10^5 densities with α-MEM medium supplemented with 10% FBS. Then cells were
divided into two groups: a control group and a second group treated with 100mM of TH, and cells were then incubated at 37°C and 5% CO₂ for 12 and 24 hour.

### 3.6 Bone Marrow Isolation

Femurs and tibias were isolated from 6-to 8-week-old from DBA/2J GPNMB+ and DBA/2J mice. Bones were dissected from surrounding tissues by removing epiphyseal growth plates and flushing bone marrow with α-MEM medium containing 10% FBS. The medium was changed twice a week. Once MSCs were confluent, they were plated in a density of 2 x10⁵ cells/cm² in a 6-well plate, and then both mouse groups were divided into three groups: one served as a control and the second group treated with 100mM of TH, and the third group treated with 500µM of R all groups treated with differentiation factor β-Glycerophosphate (10mM) and Ascorbic Acid (50µg/mL). Cells were then incubated at 37°C and 5% CO₂ for 7, 14 days (Figure 3. 4).

### 3.7 Bone Tissue Isolation

After flushing bone marrow, we placed clean bones in the mortar with a buffer containing (10mL PBS with 2% FBS and 1m MEDTA). Then we crushed bones gently with a pestle and transferred the bone fragments to a 100mm dish. We covered bone fragments with 2mL of 0.25% collagenase type I in PBS containing 20% FBS. We used a scalpel to cut bone fragment into fine pieces. Next, we transferred the bone fragments and collagenase solution to a 50mL polypropylene tube and added another 0.25% collagenase to the final volume of 2mL per mouse. The tube was covered with parafilm and put in a shaking 37°C water bath at maximum speed for 45 minutes. After 45 minutes, we removed the tube from the shaker and added buffer containing 1mM
EDTA, PBS with 2% FBS to final volume of 30mL. Centrifuge at 300 X g for 10 minutes at room temperature.

3.8 Osteoblast Cultures

Mouse bone marrow-derived stem cells were isolated from 6-8 week male DBA/2J GPNMB+ and DBA/2J mice as previously described (Abdelmagid et al., 2015). In order to generate osteoblasts, bone marrow cells were plated at a cell density of 1 X10⁵ cells in a 24-well plate with αMEM with 10% FBS (VWR, GA, USA) and 1% penicillin-streptomycin (ThermoFisher, NY, USA). In parallel cultures, we add differentiation factors β-glycerophosphate (10mM) (Sigma, MO, USA), and ascorbic acid (50µg/mL) (Sigma, MO, USA) with TH (100mM), or R (500µM), every medium change 7, 14 days. Cells were then fixed, and ALP activity and staining was assessed. ALP positive osteoblast images were taken using a Nikon Ti Eclipse inverted microscopy.
Figure 3.1: Trehalose Structure. Molecular Formula: $C_{12}H_{22}O_{11}$. Trehalose occurs in mushrooms. Trehalose is a source of energy, containing two glucose molecules (taken from SIGMA-ALDRICH Company).
Figure 3.2: Rapamycin Structure. Molecular Formula: C_{51}H_{79}NO_{13}. Rapamycin is a macrolide compound obtained from \textit{(Streptomyces hygroscopicus)} that acts by selectively blocking the transcriptional activation of cytokines thereby inhibiting cytokine production (taken from Selleckchem Company).
Figure 3.2: Chloroquine Structure. Molecular Formula: $C_{18}H_{26}ClN_3$. Chloroquine is the phosphate salt, work as anti-malarial and anti-inflammatory properties. Chloroquine is shown to inhibit conversion the toxic material into non-toxic material molecules (taken from SIGMA-ALDRICH Company).
**Figure 3.4: Bone marrow isolation.** Flushing bone marrow from bone DBA/2J GPNMB^+^ and DBA/2J mice. MSCs were cultured with α-MEM medium. Detecting cells (MSCs) use for osteoblast were culture with α-MEM medium supplement with β-glycerophosphate (10mM) and ascorbic acid (50ng/mL) for day 7, 14, and 21. Floating cells are (HSCs) and used to generate osteoclast. Cells were cultured with α-MEM medium supplement with RANKL (40ng/mL) and M-CSF (20ng/mL) for 7 days.
3.9 Cell Viability and Proliferation Assays

Cell viability was evaluated using the MTT assay. MC3T3-E1 osteoblast like cell line treated with different doses of TH, or R for 72 hours was determined by the colorimetric MTT assay. MC3T3-E1 cells were cultured into 96-well plates for 24 hours prior treatment with TH (25, 50, 100, and 200mM) or R (125, 250, 500 µM). Cells were treated following 72 hours with 20µl of MTT solution (3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyl tetrazolium bromide Thermofisher) was added into wells and incubated for 4 hours at 37°C and 5% CO₂. After 4 hours, dimethyl sulphoxide was added for 15 min. Absorbance was measured on an ELISA plate reader at 570nm using Bio Tek plate reader. For cell proliferation assay, DNA was determined by using CyQuant NF cell proliferation assay (Life Technologies). Briefly, MC3T3-E1 cells were plated at 1.2x10⁵ cells/well in 96-well plates using 10% FBS in DMEM supplemented with Penicillin/Streptomycin (Penicillin: 50 U/ml; Streptomycin: 50µg/mL) after 24 hours; cells were starved serum and treated with different concentrations of TH (25, 50, 100, and 200mM) or R (125, 250, 500, and 600 µM) for 72 hours. We replaced medium with 100µl of 1X dye binding solution per well and incubated for 1 hour at 37 °C. A BioTek plate reader was used to measure the fluorescence intensity (excitation at 485 and emission at 530 nm). Proliferation and viability in experimental data were compared to control cells.

3.10 ELISA

Serum samples were obtained from the MC3T3-E1 preosteoblast cell line. Osteoactivin protein was measured by enzyme-linked immunosorbent assay (ELISA)
according to the manufacturer’s guidelines. The Osteoactivin ELISA (R&D) was used to determine the concentration of the protein in cell cultures.

### 3.11 Western Blot Analysis

Protein extracts were prepared from the preosteoblast cell line (MC3T3-E1), osteoblasts and osteoclasts were differentiated from 6-8 week-old male DBA/2J GPNMB⁺ and DBA/2J mice. Cells were lysed by using radio immunoprecipitation (RIPA) assay buffer (Millipore, Billerica, MA) with phosphatase and proteinase inhibitor cocktail. Protein concentration was determined by using a pierce bicinchoninic acid protein assay kit (BCA) protein assay kit. Protein samples were mixed with loading buffer, heated at 100° C for 5 minutes, and then were loaded in 10% SDS-PAGE gel in 1X Tris-glycine-SDS running buffer. The gel was transferred by using Trans-Blot Turbo system to separate SDS-PAGE and transfer to polyvinylidene fluoride (PVDF) membranes. The blots were blocked with 5% BSA 1X TBS, 0.1% Tween-20 for an hour, and then membranes were incubated overnight on a shaker at 4° C with LC3B rabbit antibody (1:1000). The next day, we washed the blots 3 times in 1X Tris-buffered saline/Tween 20 (TBS-T) followed by one hour in secondary anti-rabbit antibody incubation and washed again with TBS-T. Finally, membranes were covered in 3mL of Luminata substrate (1:1) for 5 mins and images were taken using the GENE SYS program. The membranes were stripped, washed in TBS-T, and reprobed for GAPDH (Table 3.2) and (Table 3.3).
Table 3.2: List of Primary Antibodies Used in the Study

<table>
<thead>
<tr>
<th>Primary Antibody</th>
<th>Source</th>
<th>Company</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAPDH</td>
<td>Rabbit</td>
<td>Cell Signaling</td>
</tr>
<tr>
<td>LC3 II</td>
<td>Rabbit</td>
<td>Signaling</td>
</tr>
<tr>
<td>β-Actin</td>
<td>Rabbit</td>
<td>Signaling</td>
</tr>
<tr>
<td>p-mTOR</td>
<td>Rabbit</td>
<td>Cell Signaling</td>
</tr>
<tr>
<td>mTOR</td>
<td>Rabbit</td>
<td>Cell Signaling</td>
</tr>
</tbody>
</table>

Table 3.3 List of Secondary Antibodies Used in the Study

<table>
<thead>
<tr>
<th>Secondary Antibody</th>
<th>Conjugate</th>
<th>Company</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-Rabbit</td>
<td>HRP</td>
<td>Cell Signaling</td>
</tr>
</tbody>
</table>

3.12 Transmission Electron Microscopy

Samples were prepared as described previously. Briefly, MC3T3-E1, osteoblasts from bones, and osteoclasts differentiated from DBA/2J GPNMB+ and DBA/2J mice were cultured in α-MEM medium supplemented with 10% FBS in 33mm petri dishes until cells were confluent, then cells were divided into groups: one group served as a control and the other group was treated with 100mM TH or 500µM R for 24 hours. Cells then were pelleted down, and fixed in 2.5% glutaraldehyde in 0.1M Sorensen’s buffer for 1 hour. Cells were then fixed in 1% osmium tetroxide and rinsed in double distilled water. The samples were dehydrated in ethanol, rinsed two times in propylene oxide 2 times each for 30 minutes at room temperature, and then embedded 3 times in resin with propylene oxide for 30 minutes followed by twice embedding with resin alone for 1 hour. Samples were removed from the resin and placed in a tip of silicone mold with a label. Blocks were trimmed and cut on a Leica EM UC6 Ultramicrotome. Thin sections were obtained with a diamond knife and were picked up on uncoated 200 mesh copper grids. The grids were stained with 2% aqueous uranyl acetate for 45 minutes. Then they were rinsed in double distilled water and allowed to dry before additional staining in
Reynold’s lead citrate for 2 minutes. The grids were again rinsed in double distilled water and allowed to dry before storing in grid boxes.

3.13 Cell Transfection

MC3T3-E1 were cultured in 12-well chamber slides with α-MEM medium supplemented with 10% FBS for 24 hours until cell confluency; cells were then divided into two groups: one group served as a control and the other group was treated with 100mM TH for 24 hours. Cells were plated cells in 1mL complete growth medium per well in a 12-well plate 18-24 hours before transfection. Cells were 90% confluent prior to transfection. We prepared X-treme GENE HP Reagent:DNA complex (Clontech, USA) according to manufacture) immediately before transfection by mixing 200ul of serum-free medium in a sterile tube, and then added 2mg of plasmid DNA pipet gently to mix completely before adding 6ul of X-treme GENE HP DNA transfection reagent to the diluted DNA mixture (3:1 reagent to DNA ratio). A pipet was gently mix the solution. It was incubated at room temperature for 30 minutes for complex formation. We added 200ul of X-treme GENE HP Reagent:DNA complex drop-wise to different areas of one well. We gently rocked the culture vessel back and forth and side to side to distribute the X-treme GENE HP Reagent:DNA complex. It was incubated for 24 hours, and then cells were treated with 100mM of TH for 24 hours.

3.14 Immunofluorescence

MC3T3-E1 cells were cultured in 12-well plates at 0.1x10⁵ chamber slides with α-MEM medium supplemented with 10% serum FBS for 24 hours. Cells were then fixed with 4% paraformaldehyde (PFA) 20 minutes and permeabilized using 0.5% Triton x-100 in PBS (Tween-20;0.1%). The cells were then blocked with 5% BSA in PBS-T 30
minutes followed by incubation with the anti-rabbit primary antibody GPNMB (R&D Systems), (1: 1000) for 1 hour then washed with PBS three times, 5 minutes per wash. Next, the cells were incubated with secondary antibodies of anti-rabbit IgG for 1 hour followed by washing with PBS three times, then counterstaining by diamidino-2-phenylindole (DAPI) mounting media. Images were taken using the Olympus Fluoview program images were taken using a Nikon Ti Eclipse inverted microscope.

3.15 Alkaline Phosphatase Staining and Activity

Osteoblast differentiation was determined by ALP staining and activity at day 7 and 14. By using ALP staining kit (Sigma-Aldrich) and according to the manufacturer’s instructions, medium was removed, and cells were washed with PBS and fixed in 10% formaldehyde (ThermoFisher, NY, USA) for 1 hour. Next, cells were rinsed with PBS, stained with buffer containing 0.1mg/mL naphthol AS-MX phosphate disodium salt (Sigma, MO, USA) and 0.6 mg/mL Fast Bleu TR salt (Sigma, MO, USA) at room temperature in the dark for 30 seconds. Cells were then washed with dH2O for 2 minutes, and imaged by using Nikon microscopy. ALP activity was determined by using an ALP activity kit (BioAssay Systems). In brief, 50μL of cell lysate was added to 50μL of working solution, and then absorbance was measured at 405 nm after 30 minutes of incubation at room temperature. ALP activity was calculated in enzyme units (U = μmol/L) per μg protein.

3.16 Von Kossa Staining

Osteoblast mineralization was determined by Von Kossa staining at day 21. Briefly, osteoblast cultures were fixed with 10% formalin in water for 30 minutes and stained with fresh 5% AgNO₃ silver nitrate solution under UV light for 30 minutes, cells
were then washed 3 times with water. Next, fresh 5% Na$_2$CO$_3$ sodium carbonate in 10% formalin was added for up to 5 minutes for minerals and matrix staining in bone nodule, 5% Na$_2$SO$_3$ sodium thiosulfate for 2 minutes was added then washed 3 times with water. Hydroxyapatite crystals of mineralized nodules were stained by using Nikon microscopy.

3.17 New Bone Formation in Neonatal Calvarial Organ Cultures

Calvariae were harvested from 3-5 day-old DBA/2J GPNMB$^+$ and DBA/2J mouse pups. In a 12-well tissue culture plate, calvariae were plated on the grid in each well with BGJb medium supplemented with 0.1% bovine serum albumin and 100u/ml each of penicillin and streptomycin incubation at 37$^\circ$C and 5% CO$_2$. The following day, we removed the old medium and replaced it with medium containing 100mM/mL of TH or 500µM/mL of R and changed it after 72 hours. At the end of the culture period, calvariae were harvested by removal from the culture medium and directly placed in fixative, decalcified, embedded in paraffin wax, and underwent tissue sectioning and staining (Figure 3.5).

3.18 Osteoclast Cultures

Bone marrow-derived stem cells were isolated from 6-8 week male DBA/2J GPNMB$^+$ and DBA/2J mice as previously described (Abdelmagid et al., 2015). Briefly, primary bone marrow cells were flushed out of long bones, cells were then cultured in minimum essential medium α- (α-MEM) supplemented with 10% fetal bovine serum (FBS). After 24 hours, the non-adherent cells which contained osteoclast precursors plated at a cell density of 1.2X10$^5$ cells in a 96-well plate and primed with M-CSF (20ng/mL) for the 3 days. After 3 days, the first dose of RANKL (40ng/mL) and M-CSF
(20ng/mL) was added. After 48 days, a second dose of RANKL and M-CSF to the cells was added. In parallel cultures, osteoclasts were treated with TH (100mM), or R (500µM) with RANKL and M-CSF. At day 7, cells were fixed and TRAP activity and staining was assessed. Cells were then imaged by using a Nikon microscope.

3.19 TRAP Staining and Activity

Osteoclasts were fixed by adding 10% formalin to each well of the plate for 20 minutes, cells where then washed twice with dH20. For TRAP activity assays, we added 1:1 (Methanol:Acetone) 100ul was added for 3 minutes, then Methanol:Acetone was removed from the wells and allowed dry. Next, incubation with TRAP buffer (52mM of Na-tartrate in 0.1 M Na-acetate buffer pH 5.2) containing 0.1 mg/mL of p-nitrophenyl phosphate (p-NPP) for 1 hours at 37C. The reaction was stopped by adding 1 N NaOH and read at an optical density of 405nm using a BioTek Synergy microplate reader. For TRAP staining, mature osteoclasts were incubated with 100ul of TRAP staining solution containing 1.5mM naphthol AX-MX phosphate and 0.5mM Fast Red Violet LB Salt. Into each well and incubated for 20-30 minutes, plates were checking every 10-20 minutes. When adequate staining is achieved, remove TRAP and add 50ul of 60% glycerol with PBS to protect cells from drying and shrinking.

3.20 In Vitro Osteoclast Resorption Assays

BMM obtained from DBA/2J GPNMB+ and DBA/2J mice were plated on Corning OsteoAssay surfaces (Corning) for 7 days with M-CSF (20ng/mL) and RANKL (40ng/mL). In parallel cultures, we added TH (100mM), or R (500µM) every medium change. After 24 hours, osteoclast cultures were terminated using 10% bleach. Resorption from osteoclasts was analyzed by using NIS-Elements software.
3.21 *In Vivo* Calvarial Bone Resorption Assay

A collagen sheet (100mm2) (Medline) was coated with PBS (control), RANKL (40μg/mL), RANKL and TH (100μg/mL) and RANKL and R (500μM) in 30μL volume and placed on the calvaria in 6-8 week old mice from DBA/2J GPNMB⁺ and DBA/2J mice (n=3). One week later, the calvaria were isolated and fixed in 4% paraformaldehyde and stained for TRAP. Images were taken using a Nikon microscopy (Figure 3.6).

3.22 Micro CT Analysis

Male mice calvaria derived from 6-8 weeks old DBA/2J GPNMB+ and DBA/2J mice (n =3 per group) were scanned using the SkyScan 1172 high-resolution microtomography (micro-CT) system. All scanned images were reconstructed into 3-dimensional images and were regenerated using SkyScan NRecon software.

3.23 RT-qPCR

Total RNA was isolated from MC3T3-E1, Bone cells, and differentiated osteoblasts, and osteoclast cultured in 6-well plate by adding 1mL Qiazol (QIAGEN, Hilden, Germany). Total RNA was extract using an RNA extraction kit (QIAGEN). Next, the concentrations of RNA was determined by using a NanoDrop 2000 Spectrophotometer (Thermo Scientific). Then, cDNA was prepared using a high capacity cDNA reverse transcription kit (Thermo Fisher). Gene expression for Beclin-1, ATG5, ATG7, LC3II, OA, ALP, OSX, OCN, and GAPDH were determined by using the ▲▲CT. For MC3T3-E1, bones, osteoblasts and osteoclasts are lasted (Table 3.4), (Table 3.5), and (Table 3.6).
Figure 3.5: New bone formation in neonatal calvarial organ cultures. Calvariae were harvested from 3-5 day-old DBA/2J GPNMB⁺ and DBA/2J mouse pups. Calvariae were cut in two halves and then placed on the grid in 12-well tissue culture plates, with BGJb medium supplemented with 0.1% bovine serum albumin and 100u/mL each of penicillin and streptomycin. Calvariae were treated twice with either TH or R. One week later calvaria were stained with H&E.
### Table 3.4: List Primers of Osteoblast-Related Markers used for qPCR Analysis

<table>
<thead>
<tr>
<th>Primers</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>GAPDH</strong></td>
<td>5'-CGACTTCAACAGCAACTCCACTCTTTCC-3'</td>
</tr>
<tr>
<td></td>
<td>5'-TGGGTGGTGTCAGGGTTCTACTCT-3'</td>
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<tr>
<td><strong>Runx2</strong></td>
<td>5'-GACGGAGAGGTCCACC-3'</td>
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<td>5'-GGACCGTCCACTG-3'</td>
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<tr>
<td><strong>Osterix</strong></td>
<td>5'-GCAACTGCCAGTGGTGGGTGTC-3'</td>
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<td></td>
<td>5'-GCAAGTGCAGGTGGTAGTG-3'</td>
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<tr>
<td><strong>Alkaline Phosphatase (ALP)</strong></td>
<td>5'-CCGATGCCACACCTCT-3'</td>
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<td>5'-GGAGGCATACGCACACATC-3'</td>
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<td><strong>Osteocalcin (OCN)</strong></td>
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<td>5'-CCAGTACTCTCCGCCTTTCCA-3'</td>
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<tr>
<td><strong>Type I Collagen (Col1α1)</strong></td>
<td>5'-CTCGAGTCAGGACTGATACTGGAGAACA-3'</td>
</tr>
<tr>
<td></td>
<td>5'-CCAGTACTCTCCGCCTTTCCA-3'</td>
</tr>
<tr>
<td><strong>Osteoactivin/GPNMB</strong></td>
<td>5'-AATGGGTCTGGACACCTACTG-3'</td>
</tr>
<tr>
<td></td>
<td>5'-GCTCTTGTACGCCTTGTGTT-3'</td>
</tr>
</tbody>
</table>

### Table 3.5: List Primers of Osteoclast-Related Markers used for qPCR Analysis

<table>
<thead>
<tr>
<th>Primers</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>RANK</strong></td>
<td>5'-AGTTAAGCCAGTGGTCTACG-3'</td>
</tr>
<tr>
<td></td>
<td>5'-ACGTAGACCCAGTGTG-3'</td>
</tr>
<tr>
<td><strong>TRAP</strong></td>
<td>5'-GCAGTATCTCAGCAGGAAAC-3'</td>
</tr>
<tr>
<td></td>
<td>5'-TCCATAGTGAAAGACAG-3'</td>
</tr>
<tr>
<td><strong>NFATc1</strong></td>
<td>5'-CTCGAAGACAGCAGTGGGACAT-3'</td>
</tr>
<tr>
<td></td>
<td>5'-CCGGCTGCCAGTGGTCTTANGT-3'</td>
</tr>
<tr>
<td><strong>Calcitonin Receptor</strong></td>
<td>5'-AGTTGCCCCTCTTATGAAAGGAGAAG-3'</td>
</tr>
<tr>
<td></td>
<td>5'-GGAGTGTCGCCACACAT-3'</td>
</tr>
</tbody>
</table>

### Table 3.6 List Primers for Autophagy-Related Genes used for qPCR Analysis

<table>
<thead>
<tr>
<th>Primers</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Biclin-1</strong></td>
<td>5'-AGCTGCCCTTACTGTTCT-3'</td>
</tr>
<tr>
<td></td>
<td>5'-ACTGCGCTCCTGTGTCCTAATCT-3'</td>
</tr>
<tr>
<td><strong>ATG5</strong></td>
<td>5'-TGCGGATCCTAAGGAAACACT-3'</td>
</tr>
<tr>
<td></td>
<td>5'-TGCGGGCTCAGGAGAAGCAG-3'</td>
</tr>
<tr>
<td><strong>ATG7</strong></td>
<td>5'-CTTGAGTGGATGAGTGGCCT-3'</td>
</tr>
<tr>
<td></td>
<td>5'-TGGATTGAGGAGTGAGTGGCCT-3'</td>
</tr>
<tr>
<td><strong>ATG12</strong></td>
<td>5'-CTCTGAGGCGCCGGGCCTA-3'</td>
</tr>
<tr>
<td></td>
<td>5'-AAGGAGGCCGGAGGAGG-3'</td>
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<tr>
<td><strong>LC3II</strong></td>
<td>5'-CGGAGCTTTGAAACAAAAAGATG-3'</td>
</tr>
<tr>
<td></td>
<td>5'-TCTCTCAGCTTGCACCTC-3'</td>
</tr>
</tbody>
</table>
3.24 Statistical Analysis

For all generated data, Prism 5 software, version 5.04 (GraphPad, La Jolla, CA) was used. For multiple group comparison, analysis of variance (1-way ANOVA) was used, followed by a Tukey’s multiple comparison post hoc test. For two groups, an unpaired t-test was used. All differences where p<0.05 were considered as statistically significant. Group means or means + standard error of the mean (±SEM) were graphed.
**Figure 3.6 Calvarial bone resorption assays.** Collagen sponge coated with 30µL of either PBS, RANKL (5µg) alone, RANKL with TH (100mM), or RANKL with R (500µM) was placed on DBA/2J GPNMB+ and DBA/2J mice calvaria (n=3) for 7 days. A week later calvaria were removed and TRAP staining was performed.
Chapter 4

Results

4.1 Osteoblast Differentiation

Osteoblasts originate from mesenchymal stem cells (MSCs) that have the ability to differentiate into several cell types such as chondrocytes, adipocytes, myoblast, and osteoblasts (Jiang et al., 2002). Differentiation of osteoblasts from MSCs requires certain transcription factors. RUNX2 and Osterix are the most crucial transcription factors for MSCs to become osteoblasts (Figure 4.1).

In addition to transcription factors, MSCs also require some growth factors such as transforming growth factors, including insulin-like growth factors I and II (IGF-I, IGF-II) transforming growth factor (TGF-beta 1, TGF-beta 2), platelet-derived growth factor (PDGF), basic and acidic fibroblast growth factor (FGF), and bone morphogenetic proteins (BMPs) that play a role in osteoblast differentiation and function. Another factor that plays a role in osteoblast differentiation and bone formation is glycoprotein nmb (GPNMB) that is known as osteoactivin. Osteoactivin is a type I transmembrane glycoprotein expressed in both osteoblasts and osteoclasts. The role of osteoactivin in osteoblast differentiation and function has shown to increase bone formation in vivo and vitro (Abdelmagid et al., 2008; Abdelmagid et al., 2014; Frara et al., 2016; Sondag et al.,
2014); however, the mechanism involved in osteoactivin mediated bone formation is still not fully understood.

Osteoblasts and osteoclasts are bone cells with opposing roles; osteoblasts build bone while osteoclasts resorb bone. Imbalances between bone formation and resorption results in skeletal disorders including osteoporosis. Autophagy-related proteins have been shown to be important for bone cell differentiation and function (Lu et al., 2011).

In this study, we used trehalose (TH) which acts through the mTOR-independent pathway and rapamycin (R) which acts through the mTOR-dependent pathway to study the role of osteoactivin in autophagy mediated signaling in both osteoblasts and osteoclasts. Our data shows that osteoactivin stimulates osteoblast differentiation, function, and bone formation through autophagy (mTOR-independent and dependent) pathways. However, osteoactivin increased osteoclast differentiation through the mTOR-independent pathway, and increase osteoclast function through mTOR-dependent pathway.
Figure 4.1: Schematic diagram illustrates multiple transcription factors important for mesenchymal stem cells to give rise to a variety of cell types. RUNX2 indicates Runt-related transcription factor 2 (transcription factor for osteoblast differentiation), SCX indicates scleraxis, PPARγ indicates to peroxisome proliferator-activated receptor, C/EBPα indicates CCAAT/enhancer binding protein α, MRF indicates myogenic regulatory factor, and MEF2 indicates myocyte enhancer binding factor-2, SOX9 is a transcription factor for development of the skeleton and plays a key role in the determination of sex before birth.
4.2 mTOR-Independent Pathway Enhances Viability and Proliferation of MC3T3-E1 Cells

To investigate the role of TH on MC3T3-E1 viability, we used the MTT colorimetric assay for assessing MC3T3-E1 metabolic activity and CyQuant assay for evaluating cell proliferation through measuring total DNA content. MC3T3-E1 osteoblast-like cells were cultured with different concentrations of TH (25, 50, 100, and 200 mM) for 72 hours. Cell viability and proliferation were assessed using the MTT and CyQuant assays, respectively. Data show that there is a significant increase in MC3T3-E1 viability and proliferation in a dose-dependent manner reaching maximum effects at 100 mM of TH. These results suggest that TH stimulates MC3T3-E1 cell viability and proliferation (Figure 4.2 A) and (Figure 4.2 B). It is important to note that presence or absent of serum in our cellular system did not alter the effects of TH (data not shown). For all experiments in the study we used 10% serum in culture media in all of our assay.
Figure 4.2: Trehalose enhances viability and proliferation of MC3T3-E1 cells.

MC3T3-E1 osteoblast-like cells were plated at a seeding density of 5,000 cells per well in 96-well plate and serum starved overnight prior the addition of different concentrations (25, 50, 100, and 200 mM) of TH for 72 hours. Cell viability was measured using the MTT assay (A). For the proliferation assays, MC3T3-E1 cells were plated as above for 72 hours. Proliferation was measured by quantitating the fluorescently labeled DNA with the CyQuant assay (B). Results show increased cell viability and proliferation reaching maxima with treatment of 100 mM TH. Experiment was repeated 3 times with 2-3 replicates per experiment. Data represent Mean + SEM. **P < 0.01, ***P < 0.001 compared to control.
4.3 mTOR-Independent Pathway Induces Autophagy in Osteoblasts

In order to investigate the role of TH in mediating mTOR-independent autophagy in osteoblasts, we induced autophagy using the MC3T3-E1 osteoblast-like cell line treated with TH (100mM) for 12 or 24 hours. We evaluated autophagy in osteoblasts by western blot analysis for microtubule-associated protein light chain 3 (LC3II), a marker for autophagosome formation. Western blot shows the conversion of the cytosolic form of microtubule-associated protein light chain 3I (LC3-I) to LC3-II. Our data revealed that there were significant increases in LC3-II level (Figure 4.3 A and B). This indicates that TH plays a role in inducing autophagy in osteoblasts.

Next, we examined the role of TH by the mTOR-independent pathway using MC3T3-E1 osteoblast-like cell line for 12 and 24 hours using qPCR for the main marker of autophagy, Beclin-1. The expression of Beclin-1 was significantly increased in both time points, (Figure 4.4). These data indicate that TH induces autophagy in osteoblasts.
Figure 4.3: mTOR-independent pathway induces autophagy in osteoblasts.

Western blot analysis of MC3T3-E1 cells plated at a seeding density of $2 \times 10^5$ cells per well in a 6-well plate and serum starved overnight prior to the addition of 100mM of TH for 12 or 24 hours at $37^\circ$C. Western blot analysis (A). Quantitation of multiple blots (B). The experiment was repeated 3 times with similar results. Data represent Mean + SEM. *P <0.05, **P<0.01 compared to control.
Figure 4.4: Trehalose increases Beclin-1 expression in osteoblasts. qPCR analysis of Beclin-1 in MC3T3-E1 treated with 100mM of TH for 12 or 24 hours as in (Figure 4.3). TH upregulated Beclin-1 expression in a time-dependent manner. The experiment was repeated 3 times with similar results. Data represent Mean + SEM. *P <0.05, **P<0.01 compared to untreated control.
4.4 Trehalose Induces Autophagic Vesicles in Osteoblasts

In order to investigate the role of TH treatment on autophagy in osteoblasts, we wanted to determine its effect on autophagosome formation using the MC3T3-E1 osteoblast-like cell line. We examined the role of TH treatment in autophagosome formation using transmission electron microscopy (TEM). Briefly, MC3T3-E1 cells were plated in 6-well plates at a seeding density of $2 \times 10^5$ cells per well. Cells were then treated with 100mM of TH for 24 hours. Cells were then prepared for TEM as in material and methods.

TH treatment causes induction of autophagosome formation associated with the formation of double membrane autophagic vesicles compared to untreated control cells (Figure 4.5). This indicates that the mTOR-independent pathway induced by TH plays a role in autophagosome formation in osteoblasts.
Figure 4.5: Autophagic vesicle formation induced by trehalose in osteoblasts.

MC3T3-E1 cells were plated into 6-well plate at a seeding density of $2 \times 10^5$ cells per well. Cells were then treated with 100mM TH for 24 hours. Cells were then prepared for TEM. Control untreated cells (A), (B-D) TH treated cells; the white arrow indicates phagophore (B), the red arrow indicates autophagosome double membrane vesicles (C), and black indicates autolysosome (D). The experiment was repeated 3 times with similar results.
4.5 Osteoactivin co-Localizes with Autophagosomes

Several studies have shown that osteoactivin is essential for the recruitment of autophagy proteins to the phagosome to create the autophagosome and then fusion with the lysosome to degrade their contents (Bing & Thomas, 2010). Other studies reported that osteoactivin co-localizes with lysosomes by delivering the contents of membrane proteins to lysosomes (Furochi et al., 2007; Hoashi & Yamaguchi, 2010). Therefore, in order to understand the role of osteoactivin in autophagy in osteoblasts, we first accessed whether osteoactivin localizes within the autophagy pathway. Hence we transfected the MC3T3-E1 osteoblast-like cell line with either GFP-LC3II or control empty vector (GFP-EV) followed by TH treatment. Immunofluorescent analysis revealed that osteoblasts display a punctate distribution of LC3II that co-localized with osteoactivin compared to untreated control cells. This result indicates that osteoactivin may interact and bind to the autophagosome. These data suggest a role of osteoactivin in autophagy (Figure 4.6).
Figure 4.6: Osteoactivin co-localizes with autophagosomes in osteoblasts.

Immunofluorescent analysis of the co-localization of autophagosome and osteoactivin. MC3T3-E1 cells were seeded at a density of $1 \times 10^5$ cells per well and cultured in chamber slides. Cells were then transfected with a vector containing cDNA for LC3II Target GFP. Cells were then treated with 100mM TH for 24 hours. Cells were then fixed, stained with anti-osteoactivin antibody, and counterstained with DAPI in order to visualize the nucleus. LC3II (green), osteoactivin (red), and DAPI (blue) were imaged in order to visualize co-localization of autophagosome with osteoactivin. Scale bars: 10μm. The experiment was repeated 3 times with similar results.
4.6 Induction of Autophagy Increases of Osteoactivin Production

Previous literature has shown that osteoactivin plays a role in autophagy (Li et al., 2010). In this study, we determined whether osteoactivin expression is increased in response to TH treatment. We induced autophagy using the MC3T3-E1 osteoblast-like cell line treated with TH (100 mM) for 12 or 24 hours. We then measured the levels of osteoactivin protein by enzyme-linked immunosorbent assay (ELISA). ELISA shows that there was a significant increase in osteoactivin protein levels in cells treated with TH compared to untreated control (Figure 4.7). These data indicate that osteoactivin may play a role in the mTOR-independent pathway in osteoblasts.
Figure 4.7: Increased osteoactivin protein levels in osteoblasts treated with trehalose. Enzyme-linked immunosorbent assay (ELISA) of MC3T3-E1 cells treated with TH. Briefly, MC3T3-E1 cells were plated in 6-well plate at a seeding density of 2X10^5 cells per well and serum starved overnight. Cells were then treated with 100mM of TH for 12 or 24 hours and conditioned medium samples were obtained and analyzed by ELISA. Data show a significant increase in levels of secreted osteoactivin in response to TH treatment. The experiment was repeated 3 times with similar results. Data represent Mean + SEM. *P <0.05, **P<0.01 compared to untreated control.
4.7 Autophagy is Altered in Mutant Mice for Osteoactivin

Several studies reported that osteoactivin is a major protein essential for bone formation. Osteoactivin is highly expressed by osteoblasts and plays an important role in bone growth and maintenance (Abdelmagid et al., 2008; Ornitz & Marie, 2002; Sheng et al., 2012). Previous studies have also shown that mutant mice for osteoactivin (DBA/2J) have impaired osteoblast differentiation both in vitro and in vivo (Abdelmagid et al., 2014). Therefore, in order to understand the role of osteoactivin in autophagy in bone homeostasis, we tested the ability of osteoblasts derived from bones of osteoactivin mutant mice (DBA/2J) and DBA/2J GPNMB+ to induce autophagy using western blot analysis. Osteoactivin mutant osteoblasts were impaired to induce autophagy compared to control cells (DBA/2J GPNMB+) (Figure 4.8). These data indicate that osteoactivin plays a role in autophagy in osteoblasts.

Next, we wanted to determine the role of osteoactivin mutant protein on autophagy related gene expression. We cultured MSCs that were obtained from either DBA/2J GPNMB+ or DBA/2J mice and assessed for different autophagy markers including osteoactivin (OA) (Figure 4.9 A), Beclin-1(Figure 4.9 B), ATG5 (Figure 4.9 C), and ATG7 (Figure 4.9 D). The mutant osteoactivin mice has significant reduction in autophagy related genes. These results suggest that mutant osteoactivin has impaired osteoblast differentiation by downregulating autophagy-related markers.

To further confirm our results, we isolated RNA from bones (femur and tibia) of DBA/2J and DBA/2J GPNMB+ mice and assessed different autophagy markers including OA (Figure 4.10 A), Beclin-1(Figure 4.10 B), ATG5 (Figure 4.10 C), and ATG7 (Figure 4.10 D). We found that autophagy markers are decreased in DBA/2J bones.
compared to the control groups DBA/2J GPNMB+. Taken together, these data indicate that osteoactivin plays an important role in autophagy in bone.
Figure 4.8: Autophagy is altered in mutant mice for osteoactivin. Primary osteoblasts derived from DBA/2J and DBA/2J GPNMB+ were plated at a density of 2X10^5 cells per well in 6-well plate. LC3II was detected using Western blot analysis, GAPDH was used as loading control (A), quantitation of multiple blots (B) using the GENE SYS software. The experiment was repeated 3 times with similar results. Data in B represent Mean ± SEM. **P<0.01 compared to DBA/2J GPNMB+.
Figure 4.9: Decreased autophagy in the MSCs of mutant mice for osteoactivin.

qPCR analysis of autophagy-related genes. MSCs derived from 8-week old DBA/2J and DBA/2J GPNMB+ mice were plated in a 6-well plate. MSCs derived from osteoactivin mutant mice showed downregulation of several autophagy genes that include osteoactivin (OA) (A), Beclin-1 (B), ATG5 (C), and ATG7 (D). The experiment was repeated 3 times with similar results. Data presented in all graphs represent Mean + SEM. **P <0.01, ***P <0.001 compared to DBA/2J GPNMB+.
Figure 4.10: Decreased autophagy in bones from osteoactivin mutant mice. qPCR analysis of RNA from bones isolated from DBA/2J and DBA/2J GPNMB+ mice. Osteoactivin (OA) (A), Beclin-1 (B), ATG5 (C) and ATG7 (D) mRNA expression. The experiment was repeated 3 times with similar results. Data presented in all graphs represent Mean + SEM. **P <0.01, ***P<0.001 compared to DBA/2J GPNMB+. 
4.8 The Effects mTOR-Independent Autophagy on Primary Osteoblast Differentiation

In order to investigate the role of the mTOR-independent pathway on osteoblast differentiation, we used the MC3T3-E1 osteoblast-like cell line. MC3T3-E1 cells were cultured with different concentrations of TH (25, 50, 100, and 200mM) for 7 days. We then evaluated osteoblast differentiation by alkaline phosphatase (ALP) staining (Figure 4.11 A) and activity (Figure 4.11 B). Our data showed that TH treatment caused increased ALP staining and activity with 100mM of TH compared to control.

Next, we examined the role of the mTOR-independent pathway on osteoblast differentiation using western blot analysis for LC3II. MC3T3-E1 cells were treated with different concentrations of TH. Western blot analysis showed that 100mM of TH causes maximum conversion of microtubule-associated protein light chain3 (LC3-I) to LC3-II (Figure 4.12 A) and (Figure 4.12 B). These data suggest that induction of mTOR-independent pathway stimulates osteoblast differentiation.
Figure 4.11: The effects of trehalose on MC3T3-E1 differentiation. Briefly, MC3T3-E1 cells were plated in 24-well plate at a density of 1X10^5 cells per well, and serum starved overnight prior the addition of different concentrations of TH (25, 50, 100, and 200mM) for 24 hours. ALP staining (A), and ALP activity (B). Experiment was repeated 3 times with 2-3 replicates per experiment. Data represent Mean + SEM. *P<0.05, **P <0.01, ***P<0.001 compared to control. Cells in (A) were imaged at 10X magnification.
Figure 4.1: The effects of trehalose on LC3 conversion in osteoblasts

differentiation. Briefly, MC3T3-E1 cells were plated at $2 \times 10^5$ cells per well in 6-well plate and serum starved overnight prior to the addition of different concentrations of TH (25, 50, 100, and 200 mM) for 24 hours. Untreated cells served as control. Total protein was isolated and analyzed by western blot with anti-LC3 and GAPDH antibodies. 100 mM of TH stimulates the conversion of LC3I into LC3II in a dose-dependent manner. Western blot analysis (A) and quantification of multiple blots (B). Experiment was repeated 3 times. Data represent Mean + SEM. *P<0.05, **P <0.01, ***P<0.001 compared to control.
4.9 Effects of Chloroquine on mTOR-Independent Pathway in Osteoblasts

Previous studies showed that under starvation or in the presence of chloroquine (CQ) in osteoblast leads to higher LC3II levels in deficient mice for FIP200 (Liu et al., 2013). In order to determine the effects of inhibiting autophagy by CQ on osteoblast differentiation induced by TH. We divided MC3T3-E1 cells into three groups: the first group served as control, the second group was treated with 100mM of TH, and the third group was treated with 100µM of CQ for two hours followed by treatment with 100mM of TH for 24 hours. There was a significant reduction in ALP staining and activity in cells treated with CQ compared to cells treated with TH alone (Figure 4.13 A) and (Figure 4.13 B).

Next, we wanted to determine if CQ has an effect on LC3II by western blot. We found that LC3-II protein levels were significantly higher under CQ treatment (Figure 4.14 A) and (Figure 4.14 B). These data indicates that an increase in autophagy in MC3T3-E1 cell results in decreased osteoblast differentiation by accumulation of LC3II.
Figure 4.13: Decreased osteoblast differentiation by chloroquine. MC3T3-E1 cells treated with osteogenic differentiation factors β-glycerophosphate (10mM) and ascorbic acid (50µg/mL) or osteogenic differentiation factors alone (control). Briefly, 1X10⁵ cells per well were plated in 24-well plate and cultured for 7 days. MC3T3-E1 cells were divided into three groups, the first group served as control, the second group was treated with 100mM of TH, and the third group was treated with 100µM of CQ for two hours followed by 100mM of TH for 24 hours. ALP staining (A), ALP activity (B) were evaluated. Experiment was repeated 3 times. Data represent Mean + SEM. *P<0.05 compared to control. Cells were imaged at 10X magnification.
Figure 4.14: Increased LC3II protein levels by chloroquine in osteoblasts. MC3T3-E1 cells were treated with osteogenic differentiation factors β-glycerophosphate (10mM) and ascorbic acid (50µg/mL) or osteogenic differentiation factors alone. Briefly, 2X10^5 cells per well were plated in 6-well plate and cultured for 7 days. MC3T3-E1 osteoblast-like cells were divided into three groups: the first group served as control, the second group was treated with 100mM of TH, and the third group was treated with CQ for two hours followed by 100mM of TH. LC3 and GAPDH proteins were analyzed using western blotting with anti-LC3 and GAPDH antibodies, respectively. Western blot (A) and quantification of multiple blots (B). Experiment was repeated 3 times. Data represent Mean + SEM. **P<0.01, ***P<0.001 compared to control.
4.10 The Effects of the mTOR-Dependent pathway in Osteoblast Viability and Proliferation

To investigate the effects of Rapamycin (R) on MC3T3-E1 viability, we used the MTT colorimetric assay for assessing MC3T3-E1 metabolic activity and CyQuant assay for evaluating cell proliferation through the measuring the total DNA content. MC3T3-E1 osteoblast like cells were cultured with different concentrations of R (125, 250, 500, and 600µM) for 72 hours. Cell viability and proliferation were assessed using the MTT and CyQuant assays, respectively. Data revealed that there were significant increase in MC3T3-E1 viability and proliferation with reaching maximum effect at 500µM concentrations of R. These results suggest that induction of the mTOR-dependent pathway increases MC3T3-E1 cell viability and proliferation (Figure 4.15 A) and (Figure 4.15 B).
Figure 4.15: mTOR-dependent pathway enhances viability and proliferation of MC3T3-E1. MC3T3-E1 osteoblast-like cells were plated at a density of 5,000 cells per well in 96-well plate and serum starved overnight prior to addition of different concentrations (125, 250, 500, and 600 µM) of R for 72 hours. Cell viability was measured using the MTT assay (A). For cell proliferation, MC3T3-E1 cells were plated for 72 hours. Proliferation was measured by quantitating the fluorescently labeled DNA with the CyQuant assay (B). Results showed increase cell viability and proliferation with maximum response at 500 µM of R. Experiment was repeated 3 times with 2-3 replicates per experiment. Data represent Mean + SEM. *P<0.05, **P <0.01, ***P<0.001 compared to control.
4.11 The Effects of the mTOR-Dependent Autophagy on Osteoblast Differentiation

In order to investigate the role of mTOR-dependent pathway on osteoblast differentiation, we used the MC3T3-E1 osteoblast-like cell line, and cultured them with different concentrations of R (125, 250, 500, and 600µM of R) for 7 days. Alkaline phosphatase (ALP) staining (Figure 4.16 A) and activity (Figure 4.16 B), data revealed that an increase of ALP positive osteoblasts with maximum response at 500 µM of R.

Next, we examined the role of mTOR-dependent pathway on osteoblast using western blot analysis for LC3II. MC3T3-E1 cells were treated with different concentrations of R. Western blot analysis showed that 500µM of R caused maximum conversion of microtubule-associated protein light chain3, LC3-I to LC3-II (Figure 4.17 A) and (Figure 4.17 B). These data suggest that induction of the mTOR-dependent pathway induces osteoblast differentiation.
Figure 4.16: The effects of the mTOR-dependent autophagy pathway by rapamycin on MC3T3-E1 osteoblast differentiation. MC3T3-E1 cells were treated with different concentrations of R. Briefly, MC3T3-E1 cells were plated in 24-well plate and serum starved overnight prior to the addition of different concentrations of R (125, 250, 500, and 600µM) for 24 hours. ALP staining (A) and ALP activity (B). Experiment was repeated 3 times with 2-3 replicates per experiment. Data represent Mean + SEM. **P <0.01, ***P<0.001 compared to control. Cells were imaged at 10X magnification.
Figure 4.17: The effects of rapamycin on MC3T3-E1 osteoblast differentiation.

MC3T3-E1 osteoblast-like cells were treated with different concentrations of R. Untreated cells served as control. Briefly, MC3T3-E1 cells were plated in 6-well plate and serum starved overnight prior to the addition of different concentrations of R (125, 250, 500, and 600µM) for 24 hours. LC3 and GAPDH proteins were analyzed using western blotting with anti-LC3 and GAPDH antibodies, respectively. 500µM of R stimulates the conversion of LC3I into LC3II in a dose-dependent manner. Western blot (A) and quantification of multiple blots (B). Experiment was repeated 3 times. Data represent Mean + SEM. ***P<0.001 compared to control.
4.12 mTOR-Dependent Pathway and Decreases Osteoblast by Autophagy Inhibitor (Chloroquine)

Previous studies showed that under starvation or treatment with chloroquine (CQ) in osteoblast leads to higher LC3II levels in mice (Liu et al., 2013). In order to determine the effects of inhibiting autophagy by CQ on osteoblast differentiation induced by rapamycin, we divided MC3T3-E1 cells into three groups: first group served as control, the second group was treated with 500 µM of R, and the third group was treated with 100µM of CQ for two hours followed by treatment with 500µM of R for 24 hours. ALP staining and activity show a significant reduction in cells treated with CQ compared to cells treated with rapamycin alone (Figure 4.18 A) and (Figure 4.18 B).

Next, we wanted to determine if CQ has an effect on autophagy using western blot. We found that LC3-II protein levels were significantly higher under CQ treatment (Figure 4.19 A) and (Figure 4.19 B). These data indicate that inhibition of late stage autophagy decreases osteoblast differentiation compared to rapamycin alone untreated cells.
Figure 4.18: Chloroquine treatment decreases osteoblast differentiation. Briefly, MC3T3-E1 cells were plated in 1X10^5 cells per well in 24-well plate and cultured for 7 days. Cells were divided into three groups, the first group served as control, the second group was treated with 500µM of R alone, and the last group was treated with 100µM of CQ for two hours followed by 500µM of rapamycin for 24 hours. ALP staining (A), ALP activity (B). Experiment was repeated 3 times. Data represent Mean + SEM. *P<0.05 compared to control. Cells were imaged at 10X magnification.
Figure 4.19: Increases LC3II levels by chloroquine in osteoblasts. Briefly, 2X10^5 MC3T3-E1 cells per well were plated in 6-well plate and cultured for 7 days. Cells were divided into three groups: the first group was untreated control, the second group was treated with 500µM of R, and the third group was treated with 100µM of CQ for two hours followed by 500µM of R. LC3 and GAPDH proteins were analyzed using western blotting with anti-LC3 and GAPDH antibodies, respectively. Western blot (A) and quantification of multiple blots (B). Experiment was repeated 3 times. Data represent Mean ± SEM. *P<0.05, **P<0.01 compared to control.
4.13 mTOR-Dependent and Independent Pathways Decrease Autophagy in Osteoactivin Mutant Mice

Our laboratory previously showed that osteoblasts derived from mutant mice for osteoactivin (DBA/2J) had a reduced level of osteoactivin protein and this expression level is associated with decreased osteoblast differentiation and function. Our earliest data showed that autophagy induces osteoactivin expression (Figure 4.7), which suggests that osteoactivin plays a role in autophagy in osteoblast. Previous literature has also shown that deletion of focal adhesion kinase family interacting protein of 200 kD (FIP200) is an essential component of mammalian autophagy leading to multiple autophagic defects in osteoblasts including absence of autophagosome-like structures (Liu et al., 2013). In this study, we were interested to determine whether autophagy plays a role on osteoblast formation. Transmission electron microscopy (TEM) analysis revealed a dramatic reduction in autophagic vacuole formation in mutant DBA/2J mice (Figure 4.20). These data indicate that autophagy induce autophagy formation in osteoblasts.
Figure 4.20: mTOR-dependent and independent pathways reduces autophagy in osteoactivin mutant mice. Osteoblasts were isolated from the bone of DBA/2J GPNMB+ and DBA/2J mice, treated with TH (100mM) or R (500µM) for 24h. The red arrow indicates different autophagic vacuoles observed after treatment with TH or R. Note incomplete autophagosome formation in DBA/2J compared to GPNMB+ DBA/2J.
Figure 4.21: Trehalose induces autophagy by the mTOR-independent pathway in DBA/2J GPNMB⁺ mice. Osteoblast cells were isolated from bones of DBA/2J GPNMB⁺ mice and treated with TH (100mM) for 24 hours. Cells were then prepared for TEM. The nucleus (N) and autophagic vacuole are shown by the red arrow.
Figure 4.22: Rapamycin induces autophagy by mTOR-dependent pathway in DBA/2J GPNMB<sup>+</sup> mice. Osteoblast cells were isolated from the bone of DBA/2J GPNMB<sup>+</sup> mice and treated with R (500µM) for 24 hours. Cells were then prepared for TEM. TEM shows the nucleus (<b>N</b>) and autophagosome formation with the red arrow.
Figure 4.23: Trehalose induces autophagy by mTOR-independent pathway in DBA/2J mice. Osteoblast cells were isolated from the bone of DBA/2J mice and treated with TH (100mM) for 24 hours. Cells were then prepared for TEM. The nucleus (N) and the autophagic vacuole are shown by the red arrow.
Figure 4.24: Rapamycin induces autophagy by mTOR-dependent pathway in DBA/2J mice. Osteoblast cell were isolated from the bone of DBA/2J mice and treated with R (500µM) for 24 hours. Cells were then prepared for TEM. TEM shows the nucleus (N) and the autophagosome formation with the red arrow.
4.14 Autophagy Stimulates Osteoblast Matrix Deposition

Previous studies reported that osteoactivin enhances osteoblast differentiation and function (Selim et al., 2003). Other studies reported that autophagy is activated during osteoblast differentiation (Liu et al., 2013). In the present study, we were interested to examine the effect of the mTOR-independent and mTOR-dependent pathways on osteoblast differentiation mediated by osteoactivin. We used the MC3T3-E1 cells to examine the effects of TH (mTOR-independent) and R (mTOR-dependent) pathways on osteoblast differentiation. Cells were treated with a dose of (100mM and 500µM, respectively) with every medium change. Osteoblast differentiation was determined by ALP staining and activity. Alkaline phosphatase (ALP) staining and activity revealed that an increase in osteoblast differentiation at day 7 in both groups treated with TH or R (Figure 4.25 A) and (Figure 4.25 B). This indicates that both the mTOR-independent and dependent pathways stimulates early matrix deposition of osteoblasts in vitro.

Next, in order to test whether autophagy plays a role in osteoblast differentiation, we assessed the dynamic autophagy activation during osteoblast differentiation. We determined this by measuring the conversion of the cytosolic form of microtubule associated protein LC3-I to LC3-II. MC3T3-E1 osteoblast like cells were treated with TH or R in the present of differentiation factors. Autophagy (mTOR-independent and dependent) treatments in osteoblasts displayed an enhancement in the conversion of LC3-I to LC3-II determined by western blot analysis (Figure 4.26 A) and (Figure 4.26 B).

To further elaborate the role of autophagy in osteoblast differentiation, qPCR analysis revealed a significant increase in the levels of Beclin-1 (Figure 4.27 A), ATG5
(Figure 4.27 B), OA (Figure 4.27 C), OSX (Figure 4.27 D), ALP (Figure 4.27 E), and OCN (Figure 4.27 F) in MC3T3-E1 treated with either TH or R compared to control. These data indicate that the autophagy increases osteoblast differentiation.
Figure 4.25: Autophagy stimulates osteoblast matrix deposition. MC3T3-E1 cells were treated with either TH (100mM) or R (500µM) with osteogenic differentiation factors; β-glycerophosphate (10mM) and ascorbic acid (50µg/mL) or osteogenic differentiation factor alone (control). Briefly, 1X10⁵ cells were plated in 24-well plate and cultured for 7 days before termination for ALP staining (A), ALP activity (B). The experiment was repeated 3 times with similar results. Data presented in the graph represent Mean + SEM. *p<0.05, **p<0.01 compared to control. Plates were imaged using a Nikon microscopy with 10X magnification.
Figure 4.26: Autophagy stimulates LC3II conversion in osteoblasts. Western blot analysis of MC3T3-E1 cells treated with either TH (100mM) or R (500µM). Briefly, cells were plated in 6-well plate at a seeding density of 2x10^5 cells per well and treated with TH (100mM) or R (500µM) with osteogenic factors; β-glycerophosphat (10mM) and ascorbic acid (50 µg/mL) or osteogenic differentiation factors alone (control). Total protein was subjected to western blot analysis and probed with antibodies against LC3 and GAPDH (A). Quantitation of multiple blots (B). The experiment was repeated 3 times with similar results. Data presented in the graph represent Mean ± SEM. *p<0.05, **p<0.01 compared.
Figure 4.27: Autophagy stimulates osteoblast gene expression. qPCR analysis of mRNA isolated from MC3T3-E1 cells during osteoblast differentiation. MC3T3-E1 cells were plated in 6-well plate at a seeding density of 2x10^5 cells per well and treated with either TH (100mM) or R (500µM) with osteogenic factors; β-glycerophosphate (10mM) and ascorbic acid (50 µg/mL) or osteogenic differentiation factors alone (control). Total RNA was isolated at day 7. Relative expression of autophagy and osteoblast related genes were evaluated; Beclin-1 (A), ATG5 (B), OA (C), OSX (D), ALP (E), and OCN (F) was determined by qPCR analysis. Data presented in all graphs represent Mean + SEM. *p<0.05, **p<0.01, ***p<0.001 compared to control.
4.15 Autophagy Stimulates Osteoblast Matrix Deposition and Mineralization

Previous literature has shown that autophagy plays an important role in osteoblast mineralization and bone homeostasis (Liu et al., 2013; Nollet et al., 2014). Since osteoactivin has a role in osteoblast differentiation and function, we hypothesized that autophagy may increase osteoblast mineralization. First, we evaluated the role of autophagy during osteoblast differentiation using MC3T3-E1 cells for ALP staining (Figure 4.28 A) and activity (Figure 4.28 B). We found induction of autophagy increases osteoblast differentiation in both groups treated with TH or R.

To investigate the potential direct relationship between autophagy and osteoblast mineralization, we treated MC3T3-E1 osteoblast like cells with either TH or R for 21 days with every medium change. Von Kossa staining was used to evaluate matrix mineralization and showed a dramatic increase in osteoblast mineralization with both treatments compared to untreated controls (Figure 4.29).

Next, we analyzed the conversion of LC3 during osteoblast matrix mineralization using the MC3T3-E1 osteoblast like cell line. MC3T3-E1 cells were treated with either TH (100mM) or R (500µM) with differentiation factors; β-glycerophosphate (10mM) and ascorbic acid (50 µg/mL) with every medium change for 14 days. The essential autophagy protein LC3-I becomes lipidated to LC3-II determined by western blot analysis (Figure 4.30 A) and (Figure 4.30 B).

To confirm the role of autophagy on osteoblast terminal differentiation by qPCR, we found increased gene expression of Beclin-1 (Figure 4.31 A), ATG5 (Figure 4.31 B), OA (Figure 4.31 C), OSX (Figure 4.31 D), ALP (Figure 4.31 E), and OCN (Figure 4.31 F) in
MC3T3-E1 treated with either TH or R compared to control. These data indicate that autophagy treatment increases osteoblast terminal differentiation and function.
**Figure 4.28: Autophagy stimulates osteoblast matrix deposition.** MC3T3-E1 cells were treated with either TH (100mM) or R (500µM) with osteogenic differentiation factors; β-glycerophosphate (10mM) and ascorbic acid (50 µg/mL) or osteogenic differentiation factors alone (control). Briefly, 1X10⁵ cells per well were plated in 24-well plate and cultured for 14 days before termination for ALP staining (A), ALP activity (B). The experiment was repeated 3 times with similar results. Data presented in the graph represent Mean + SEM. **p<0.01, ***p<0.001 compared to control. Plates were imaged using a Nikon microscopy with 10X magnification (Bottom panel).
Figure 4.29: Autophagy stimulates osteoblast matrix mineralization. MC3T3-E1 cells were treated with either TH (100mM) or R (500µM) and osteogenic differentiation factors; β-glycerophosphate (10mM) and ascorbic acid (50µg/mL) or osteogenic differentiation factors alone (control). Briefly, 1X10^5 cells per well were plated in 24-well plate and cultured for 21 days prior to termination for Von Koss staining. Plates were imaged using a Nikon microscopy with 10X magnification.
Figure 4.30: Autophagy stimulates LC3 conversion during osteoblast differentiation. Western blot analysis of MC3T3-E1 cells treated with either TH or R. Briefly, cells were plated in 6-well plate at a seeding density of 2X10^5 cells per well and treated with either TH (100mM) or R (500µM) with osteogenic factors; β-glycerophosphate (10mM) and ascorbic acid (50µg/mL) or osteogenic differentiation factor alone (control). Total protein was isolated at day 14 and run using 12% SDS gel. Gel was then transformed to PVDF membrane, and membrane was probed with antibodies against LC3 and GAPDH (A). Quantitation of multiple blots (B). The experiment was repeated 3 times with similar results. Data presented in the graph represent Mean + SEM. *p<0.05, **p<0.01 compared to control.
Figure 4.31: Autophagy stimulates osteoblast related markers during osteoblast differentiation. qPCR analysis of mRNA from MC3T3-E1 cells during osteoblast mineralization. MC3T3-E1 cells were plated in 6-well plate at a seeding density of 2×10⁵ cells per well and treated with either TH (100mM) or R (500µM) with osteogenic factors; β-glycerophosphate (10mM) and ascorbic acid (50µg/mL) or osteogenic differentiation factors alone (control). Total RNA was isolated at day 14. Relative expression of autophagy and osteoblast related genes; Beclin-1 (A), ATG5 (B), OA (C), OSX (D), ALP (E), and OCN (F) was determined by qPCR analysis. Data presented in all graphs represent Mean + SEM. *p<0.05, **p<0.01, ***p<0.001 compared to untreated control.
4.16 Inhibition of Autophagy Decreases Osteoblast Differentiation

Studies reported that deletion of FIP200 an essential component of mammalian autophagy, led to osteopenia (Liu et al., 2013). Other studies reported that 3-methyladenine which blocks the early stages of autophagosome formation (Salvioli et al., 2006), bafilomycin A which blocks fusion of the autophagosome with the lysosome (Lewinski et al., 2008; McLean & Le Couteur, 2004), and chloroquine (CQ), which blocks the last step of lysosomal degradation (Li et al., 2007). To confirm the role of lysosomal degradation on osteoblast differentiation and function, we used CQ as autophagy inhibitor, to determine whether lysosomal degradation indeed plays a role in osteoblast differentiation. MC3T3-E1 cells were treated with CQ for two hours followed by treatment with either TH (100mM) or R (500µM). ALP staining (Figure 4.32 A) and activity (Figure 4.32 B), revealed that CQ treatment decreased matrix deposition determined by ALP staining and activity at day 14. This indicates that CQ inhibits osteoblast deposition and mineralization.

To confirm previous results, we measured the LC3 conversion by western blot analysis. MC3T3-E1 cells were differentiated in the presence of differentiation factors, and pre-treated for two hours with CQ (100µM) followed by the addition of TH (100mM) or R (500µM) for 24 hours. Total protein was isolated and analyzed by western blotting. Results revealed that treatment with CQ increases autophagosome formation. Surprisingly, there is an increase of LC3II levels led to inhibition of osteoblast differentiation and function (Figure 4.33 A) and (Figure 4.33 B). These results suggest that autophagy inhibitor led to inhibit the osteoblast differentiation.
Next, we wanted to determine if autophagy inhibitor was responsible for decreased osteoblast differentiation determined by gene expression. CQ (100µM) was added to MC3T3-E1 cells two hours prior to treatment with TH (100mM) or R (500µM). qPCR analysis revealed a significant decrease of Beclin-1 (Figure 4.34 A), ATG5 (Figure 4.34 B), OA (Figure 4.34 C), and ALP (Figure 4.34 D). These indicate that autophagy inhibition decreases osteoblast gene expression.
Figure 4.32: Inhibition of lysosomal degradation decreases osteoblast differentiation. MC3T3-E1 cells were treated with CQ (100μM) two hours prior treatment with either TH (100mM) or R (500μM) Briefly, 1X10^5 cells per well were plated in 24-well plate and cultured for 14 days with osteogenic differentiation factors; β-glycerophosphat (10mM) and ascorbic acid (50 μg/mL) or osteogenic differentiation factors alone (control), before termination for ALP staining (A) and ALP activity (B) at day 14. The experiment was repeated 3 times with similar results. Data presented in graph represent Mean ± SEM. *p<0.05 compared to control. Plates were imaged using Nikon microscopy with10X magnification (Bottom panel in A).
Figure 4.33: Increases levels of LC3II by Chloroquine treatment in osteoblasts.

MC3T3-E1 cells were treated with CQ (100µM) two hours prior treatment with either TH (100mM) or R (500µM). Briefly, MC3T3-E1 cells were plated in 2X10^5 cells per well in 6-well plate with osteogenic factors; β-glycerophosphate (10mM) and ascorbic acid (50µg/mL) or osteogenic differentiation factors alone (control) and cultured for 14 days before termination. Total protein was isolated at day 14 and run using 12% SDS gel. Gel was then transferred to PVDF membrane, and the membrane was probed with antibodies against LC3 and GAPDH (A) and densitometry analysis (B). The experiment was repeated 3 times with similar results. Data presented in the graph represent Mean + SEM. **p<0.01 compared to control.
Figure 4.34: Chloroquine treatment decreases osteoblast differentiation. qPCR analysis of mRNA from MC3T3-E1 cells during osteoblast mineralization. MC3T3-E1 were plated in 6-well plate and treated with CQ (100µM) two hours prior treatment with either TH (100mM) or R (500µM). Total RNA was isolated at day 14. Relative expression of autophagy and osteoblast related genes; Beclin-1 (A), ATG5 (B), OA (C), and ALP (D) was determined by qPCR analysis. Data presented in all graphs represent Mean ± SEM. *p<0.05, ***p<0.001 compared to control.
4.17 Osteoactivin Plays a Role in Autophagy-Mediated Osteoblast Differentiation and Function

Previous literature has indicated that two signaling pathways control autophagy; one is the mTOR-independent pathway and the other is mTOR-dependent pathway (Memmott & Dennis, 2009). Our data indicate that mice with natural mutation of osteoactivin have less bone mass and lower levels of LC3-II by western blot analysis (Figure 4.8) and gene expression analysis (Figure 4.9). In order to understand the role of osteoactivin in autophagy mediating osteoblast differentiation and function, we used TH that acts as the mTOR-independent pathway and R that acts as the mTOR-dependent pathway to determine which pathways is involved in osteoactivin plays a role in autophagy. We isolated bone marrow-derived mesenchymal stem cells from both DBA/2J GPNMB+ (control) and DBA/2J (osteoactivin mutant mice) and cultured them ex vivo. Osteoblast differentiation and function in both DBA/2J GPNMB+ and DBA/2J mice was evaluated by ALP staining and activity. We plated DBA/2J GPNMB+ and DBA/2J MSCs and treated them with osteogenic factors; β-glycerophosphate (10mM) and ascorbic acid (50µg/mL) with or TH (100mM) or R (500µM) for 14 days with every medium change or osteogenic differentiation factors alone (control). In order to determine the role of autophagy in osteoblast matrix deposition. ALP staining (Figure 4.35 A and C) and activity (Figure 4.35 B, D and E) revealed an increase in osteoblast differentiation in both TH and R treatments in both control and osteoactivin mutant mice. These indicate that osteoactivin plays a role in both mTOR-independent and dependent pathways. However, the increase in osteoblast differentiation was significantly reduced in osteoactivin mutant DBA/2J cells compared to control, DBA/2J GPNMB+ cells.
To further elaborate the role of autophagy during osteoblast differentiation, we analyzed the autophagy process during differentiation by western blot analysis. We observed increased levels of LC3-II protein (Figure 4.36 A) and (Figure 4.36 B-D), suggesting an increase in the number of autophagosome formation during osteoblast differentiation. It is interesting to mention that LC3II levels were significantly reduced in osteoactivin mutant DBA/2J mice compared to control DBA/2J GPNMB+ mice.

Next, we confirmed our previous data using qPCR analysis; we found a significant increase in gene expression of osteoblast and autophagy markers derived from both DBA/2J and DBA/2J GPNMB+ mice, Beclin-1 (Figure 4.37), OA (Figure 4.38), OSX (Figure 4.39), ALP (Figure 4.40), and OCN (Figure 4.41). This indicates that the autophagy treatment increases osteoblast differentiation and function. Taken together, these data clearly suggest that osteoactivin regulated osteoblast differentiation and function, at least in part by mediating the autophagy pathways.
Figure 4.35: DBA/2J osteoblasts have decreased differentiation induced by autophagy compared to DBA/2J GPNMB+ cells. Bone marrow-derived MSCs from DBA/2J GPNMB+ and DBA/2J mice were plated at a density of 1X10^5 cells per well in 24-well plate. Osteoblasts were differentiated with osteogenic factors; β-glycerophosphate (10mM) and ascorbic acid (50µg/mL), and treated with TH (100mM) or R (500µM) with every medium change or osteogenic differentiation factors alone (control) for 14 days and terminated for ALP staining for DBA/2J GPNMB+ (A), and DBA/2J (C). ALP activity for DBA/2J GPNMB+ (B), and DBA/2J (D). Comparison between DBA/2J GPNMB+ and DBA/2J mice (E). The experiment was repeated 3 times with similar results. **p<0.01, ***p<0.001 compared to control. Plates were imaged using Nikon microscopy with 10X magnification.
Figure 4.36: DBA/2J osteoblasts have reduced LC3II conversion induced by autophagy compared to DBA/2J GPNMB+ cells. Bone marrow-derived MSCs from DBA/2J GPNMB+ and DBA/2J mice were plated at a density of 2X10^5 cells per well in 6-well plate. Osteoblasts were differentiated with osteogenic factors; β-glycerophosphate (10mM) and ascorbic acid (50µg/mL), and treated with TH (100mM) or R (500µM) with every medium change or osteogenic differentiation factors alone (control) for 14 days. Total protein was isolated at day 14 and run using a 12% SDS gel. The gel was then transferred to PVDF membrane, and the membrane was probed with antibodies against LC3 and GAPDH. Western blot analysis (A) and quantification of multiple blots from DBA/2J GPNMB+ (B), and DBA/2J mice (C). Comparison between DBA/2J GPNMB+ and DBA/2J mice (D). The experiment was repeated 3 times with similar results. Data presented in all graphs represent Mean± SEM. *p<0.05 **p<0.01, ***p<0.001 compared to control.
Figure 4.37: DBA/2J osteoblasts have reduced Beclin-1 expression induced by autophagy compared to DBA/2J GPNMB+ cells. We conducted qPCR analysis of mRNA isolated from DBA/2J GPNMB+ and DBA/2J MSCs during osteoblast differentiation at day 14. MSCs derived from 6-8 week old DBA/2J GPNMB+ and DBA/2J male mice were plated in 6-well plate at a density of 2X10^5 cells per well and differentiated with osteogenic factors; β-glycerophosphate (10mM) and ascorbic acid (50μg/mL) and treated with TH (100mM) or R (500μM) with every medium change or osteogenic differentiation factors alone (control) for 14 days. RNA was isolated at day 14 from both DBA/2J GPNMB+ and DBA/2J mice and the relative expression of Beclin-1 was determined by qPCR analysis. DBA/2J GPNMB+ (A), DBA/2J (B), and comparison between DBA/2J GPNMB+ and DBA/2J mice (C). Data presented in all graphs represent Mean + SEM. *P<0.05, **P <0.01, ***P<0.001 compared to control.
Figure 4.38: DBA/2J osteoblasts have reduced OA expression induced by autophagy compared to DBA/2J GPNMB+ cells. We conducted qPCR analysis of mRNA isolated from DBA/2J GPNMB+ and DBA/2J MSCs during osteoblast differentiation at day 14. MSCs derived from 6-8 week old DBA/2J GPNMB+ and DBA/2J male mice were plated in 6-well plate at a density of 2X10^5 cells per well and differentiated with osteogenic factors; β-glycerophosphate (10mM) and ascorbic acid (50µg/mL) and treated with TH (100mM) or R (500µM) with every time change medium or osteogenic differentiation factors alone (control) for 14 days. RNA was isolated at day 14 from both DBA/2J GPNMB+ and DBA/2J mice and the relative expression of OA was determined by qPCR analysis. DBA/2J GPNMB+ (A), DBA/2J (B), and comparison between DBA/2J GPNMB+ and DBA/2J mice (C). Data presented in all graphs represent Mean + SEM. *P<0.05, **P <0.01, ***P<0.001 compared to control.
Figure 4.39: DBA/2J osteoblasts have reduced OSX expression induced by autophagy compared to DBA/2J GPNMB+ cells. We conducted qPCR analysis of mRNA isolated from DBA/2J GPNMB+ and DBA/2J MSCs during osteoblast differentiation at day 14. MSCs derived from 6-8 week old DBA/2J GPNMB+ and DBA/2J male mice were plated in 6-well plate at a density of 2X10^5 cells per well and differentiated with osteogenic factors; β-glycerophosphate (10mM) and ascorbic acid (50µg/mL) and treated with TH (100mM) or R (500µM) with every medium change or osteogenic differentiation factors alone (control) for 14 days. RNA was isolated at day 14 from both DBA/2J GPNMB+ and DBA/2J mice and the relative expression of OSX was determined by qPCR analysis. DBA/2J GPNMB+ (A), DBA/2J (B), and comparison between DBA/2J GPNMB+ and DBA/2J mice (C). Data presented in all graphs represent Mean + SEM. *P<0.05, **P <0.01, ***P<0.001 compared to control.
Figure 4.40: DBA/2J osteoblasts have reduced ALP expression induced by autophagy compared to DBA/2J GPNMB+ cells. We conducted qPCR analysis of mRNA isolated from DBA/2J GPNMB+ and DBA/2J MSCs during osteoblast differentiation at day 14. MSCs derived from 6-8 week old DBA/2J GPNMB+ and DBA/2J male mice were plated in 6-well plate at a density of 2X10^5 cells per well and differentiated with osteogenic factors; β-glycerophosphate (10mM) and ascorbic acid (50µg/mL) and treated with TH (100mM) or R (500µM) with every medium change or osteogenic differentiation factors alone (control) for 14 days. RNA was isolated at day 14 from both DBA/2J GPNMB+ and DBA/2J mice and the relative expression of ALP was determined by qPCR analysis. DBA/2J GPNMB+ (A), DBA/2J (B), and comparison between DBA/2J GPNMB+ and DBA/2J mice (C). Data presented in all graphs represent Mean + SEM. *P<0.05, **P <0.01, ***P<0.001 compared to control.
Figure 4.41: DBA/2J osteoblasts have reduced OCN expression induced by autophagy compared to DBA/2J GPNMB⁺ cells. We conducted qPCR analysis of mRNA isolated from DBA/2J GPNMB⁺ and DBA/2J MSCs during osteoblast differentiation at day 14. MSCs derived from 6-8 week old DBA/2J GPNMB⁺ and DBA/2J male mice were plated in 6-well plate at a density of 2X10⁵ cells per well and differentiated with osteogenic factors; β-glycerophosphate (10mM) and ascorbic acid (50µg/mL) and treated with TH (100mM) or R (500µM) with every medium change or osteogenic differentiation factor alone (control) for 14 days. RNA was isolated at day 14 from both DBA/2J GPNMB⁺ and DBA/2J mice and the relative expression of OCN was determined by qPCR analysis. DBA/2J GPNMB⁺ (A), DBA/2J (B), and comparison between DBA/2J GPNMB⁺ and DBA/2J mice (C). Data presented in all graphs represent Mean + SEM. *P<0.05, **P <0.01, ***P<0.001 compared to control.
**4.18 Autophagy Signaling During Osteoblast Differentiation**

There is very little literature describing the signaling pathway of mTOR in bone cells. Some groups has shown that (phospho) p-mTOR gradually increased over time during osteoblast differentiation (Hu et al., 2016). In order to determine the role of osteoactivin in autophagy signaling pathway during osteoblast differentiation, total protein was isolated from osteoblasts differentiated from MSCs obtained from DBA/2J GPNMB+ and DBA/2J mice treated with osteogenic differentiation factors in presence or absent of R during osteoblasts differentiation for (0, 7, 14,) days. Western blot analysis of p-mTOR showed that the levels of activated mTOR significantly increased over time reaching maximum activation levels at day 14 in culture in both DBA/2J GPNMB+ cells and DBA/2J cells. However, in DBA/2J the activated of mTOR was significantly reduced compared to control, DBA/2J GPNMB+ (Figure 4.4 A-D).
Figure 4.42: p-mTOR activation increases during osteoblast differentiation. Bone marrow derived MSCs was isolated from DBA/2J GPNMB+ and DBA/2J. Briefly, cells were plated in 6-well plates at a seeding density of $2 \times 10^5$ cells per well and treated with R (500µM) with osteogenic factors; β-glycerophosphate (10mM) and ascorbic acid (50µg/mL) or osteogenic differentiation factors alone (control) and terminated at day 0, 7, 14. Total protein was isolated and run using 12% SDS gel. Gel was then transferred to PVDF membrane, and membrane was probed with antibodies against p-mTOR, mTOR, β-actin, and GAPDH control. The experiment was repeated 3 times with similar results. Data presented in all graphs represent Mean ± SEM. *P<0.05, **P <0.01, ***P<0.001 compared to control.
4.19 Osteoactivin Plays a Role in Bone Formation Induced by Autophagy ex-Vivo

Studies reported an increase in the number of autophagic vesicles during post-natal bone development (Cinque et al., 2015). Other studies also found that deletion of rictor, the main component of m-TORC2 led to decrease bone mass in mice (Chen et al., 2015). In order to investigate the role of osteoactivin in autophagy mediated bone formation *in ex-vivo*, we isolated calvaria from 3-5 day old DBA/2J GPNMB+ and DBA/2J pups and cultured with either TH (100mM) or R (500µM). One week later, calvarae were processed, embedded and sectioned at 5 µm thickness and stained with hematoxylin and eosin. Samples were analyzed for new bone formation using ImageJ software. Our data indicates that autophagy treatment led to increased bone formation in DBA/2J mice compared to DBA/2J GPNMB+ (Figure 4.45), increased osteoblast numbers (Figure 4.46 A-C) and new bone area (Figure 4.47 A-C), whereas this increase was significantly reduced in DBA/2J compared to DBA/2J GPNMB+. 
Figure 4.43: Photographic of calvaria organ culture. Calvariae from DBA/2J GPNMB⁺ and DBA/2J (n=4) mice were plated on stainless steel mesh in a 12-well plate (A). Calvariae were incubated in medium for 24 hours prior to treatment with TH (100mM) or R (500µM) (B).
Figure 4.44: Calvariae from both DBA/2J GPNMB+ and DBA/2J mice. Images were taken at 4X magnification a using Nikon microscopy.
Figure 4.5: Increased bone formation induced by autophagy in ex-vivo. Calvariae from both DBA/2J GPNMB+ and DBA/2J mice (n=4) were treated with either TH (100mM) or R (500µM) untreated culture served as control. Note increased bone formation in DBA/2J GPNMB+ by TH and R while this increase was reduced in DBA/2J mice.
Figure 4.46: Increased numbers of osteoblasts induced by autophagy ex-vivo.

Calvariae from both DBA/2J GPNMB+ and DBA/2J mice were treated with either TH (100mM) or R (500µM). Number of osteoblasts from DBA/2J GPNMB+ (A) and DBA/2J mice (B), and comparison between DBA/2J GPNMB+ and DBA/2J mice (C). Data presented in all graphs represent Mean + SEM. **P <0.01, ***P<0.001 compared to control.
Figure 4.47: Increased new bone area induced by autophagy ex-vivo. Calvariae from both DBA/2J GPNMB+ and DBA/2J mice were treated with either TH (100µM) or R (500µM). New bone area from DBA/2J GPNMB+ (A), and DBA/2J mice (B), and comparison between DBA/2J GPNMB+ and DBA/2J mice (C). Data presented in all graphs represent Mean ± SEM. **P <0.01, ***P<0.001 compared to control.
**Figure 4.48: Schematic diagram of autophagy signaling during osteoblast differentiation.** Autophagy signaling molecule binds to receptor threonine kinase leads to phosphorylation of the receptor and produces phosphatidylinositol 3-kinases (PI3K) and protein kinase (Akt) which results in inhibition of 4 eukaryotic binding protein 1 (4E/BP1) and tuberous sclerosis complex 1 and 2 (TSC1/2) and activated mTOR pathways. The basic idea is an inhibition of mTOR by rapamycin leads to activate of autophagy pathways. mTOR-independent and dependent pathways regulate osteoblast differentiation, function, and bone formation. Chloroquine (CQ) inhibit osteoblasts differentiation and function.
Figure 4.49: Schematic diagram illustrating the role of osteoactivin during osteoblast differentiation by mediating the autophagy pathways. mTOR-independent and dependent pathway are a positive regulator of osteoblast differentiation and function.
4.20 Osteoclast Differentiation

Osteoclasts differentiate from bone marrow macrophages (BMM). Differentiation of osteoclasts requires certain transcription factors. M-CSF and RANKL are the most important factors for BMM to become osteoclasts. In order to see all stages of osteoclast differentiation, we isolated BMM from DBA/2J GPNMB\(^+\) cells and differentiated them with M-CSF for 2 days, M-CSF plus RANKL on day 4 and 6 and stained them for TRAP on day 0, 2, 5, and 7. We terminated osteoclast precursor cells (OCPs) at day 2, pre osteoclasts (pre-OCs) at day 5, and mature osteoclasts (OCs) at day 7 by TRAP staining (Figure 4.50 A) and TRAP activity (Figure 4.50 B).

Next, we wanted to determine the effects of gene expression during osteoclast differentiation by qPCR, we found increased osteoactivin (OA) expression (Figure 4.50 C), cathepsin K, (Figure 4.50 D), and Beclin-1 (Figure 4.50 E) compared to untreated control.
Figure 4.50: Osteoclast differentiation. Bone marrow derived from DBA/2J GPNMB+ mice was flushed and non-adherent cells were cultured and treated with (20ng/mL) of M-CSF for 72 hours. Followed by first dose of RANKL (40ng/mL) and M-CSF to differentiate into pre-osteoclast (day 5). After two days, pre-osteoclasts were given a second dose of RANKL and M-CSF for additional 2 days to give rise to mature osteoclasts on day 7, TRAP staining (A), and TRAP activity (B). qPCR analysis of osteoactivin (OA) mRNA expression during osteoclast differentiation (C), cathepsin K (D), and Beclin-1 (E). Data presented in all graphs represent Mean ± SEM. *P<0.05, **P<0.01, ***P<0.001. Cells were imaged at 10X magnification by using Nikon microscopy.
4.21 Autophagy is Required for Osteoclast-Podosome Formation

Previous literature reported that ATG5 and ATG7 are required for lysosomal secretion at the ruffled border (Maeda et al., 1999; Palokangas et al., 1997). Another groups found that mTOR/S6K signaling is important for osteoclasts survival and function (Glantschnig et al., 2003). In this study, we wanted to determine if LC3II co-localizes with the actin ring on the osteoclast cell membrane. BMM isolated from DBA/2J GPNMB+ and DBA/2J mice were plated in chamber slides and differentiated into mature osteoclasts with M-CSF (20ng/mL) and RANK (50ng/mL) and treated with TH (100mM) or R (500µM) for 24 hours. Cells were terminated at day 7. Immunofluorescent analysis shows that LC3II co-localizes and interacts on the plasma membrane of mature osteoclasts DBA/2J GPNMB+ (Figure 4.51) and DBA/2J mice (Figure 4.52).
Figure 4.51: LC3II co-localizes with actin in mature osteoclasts in DBA/2J GPNMB\(^+\) cells. BMM derived from DBA/2J GPNMB\(^+\) mice were differentiated into mature osteoclasts at seeding density 5x 10\(^4\) cell/cm\(^2\) onto coverslips with M-CSF (20ng/mL) and RANKL (50ng/mL) treated with 100mM of TH or 500µM of R for 24 hours and terminated at day 7. Osteoclasts were seeded at a density of 1X10\(^3\) cells per well and cultured in chamber slides. Cells were then fixed, stained with anti-LC3II antibody, and stain for actin and counterstained with DAPI in order to visualize the localization of these molecules in multinucleated osteoclasts. Merged images of LC3II (green), actin (red), and DAPI (blue) were generated in order to visualize co-localization of LC3II and actin. Scale bars: 10µm. Experiment was repeated three times with similar results.
**Figure 4.52: LC3II co-localizes with actin in mature osteoclasts in DBA/2J cells.**

BMM derived from DBA/2J were differentiated into mature osteoclasts at seeding density $5 \times 10^4$ cell/cm$^2$ onto coverslips with M-CSF (20ng/mL) and RANK (50ng/mL) treated with TH (100mM) or R (500µM) for 24 hours and terminated at day 7. Osteoclasts were seeded at a density of $1 \times 10^3$ cells per well and cultured in chamber slides. Cells were then fixed, stained with anti-LC3II antibody, and stain for actin and counterstained with DAPI in order to visualize the localization of these molecules in multinucleated osteoclast. Merged images of LC3II (green), actin (red), and DAPI (blue) were generated in order to visualize co-localization of LC3II and actin. Scale bars: 10µm. Experiment was repeated three times with similar results.
4.22 mTOR-Independent Pathway During Osteoclast Differentiation

In order to determine the role of osteoactivin on osteoclast differentiation by mediating mTOR-independent pathway, we added different concentrations of TH (25, 50, 100, and 200 mM) to BMM isolated from DBA/2J GPNMB+ and DBA/2J mice during osteoclast differentiation. We evaluated osteoclast differentiation by TRAP staining (Figure 4.53 A and B) and TRAP activity (Figure 4.53 C-E). We found that a 100 mM concentration of TH was the optimal dose to induce osteoclast differentiation.

In order to examine the role of autophagy during osteoclast differentiation by the mTOR-independent pathway. We derived BMM from DBA/2J GPNMB+ and DBA/2J mice and divided them into five groups: the first group served as a control, the second group was treated with M-CSF plus TH, the third group was treated with one dose of RANKL plus TH, and the fourth group was treated with two doses of RANKL plus TH, and the last group of mature osteoclast (M-OC) was treated with M-CSF, two doses of RANKL, and TH (during osteoclast differentiation). TRAP staining (Figure 4.54 A and B) and TRAP activity (Figure 4.54 C-E) show increased osteoclast differentiation by the m-TOR independent pathway only when mature osteoclast (M-OC) was treated with M-CSF, two doses of RANKL and TH compared to other treatment groups when osteoclast differentiation was decreased compared to control.
Figure 4.3: Increased osteoclast differentiation through the mTOR-independent pathway. BMM-HSC derived from DBA/2J GPNMB+ and DBA/2J mice were differentiated with M-CSF (20ng/mL) and RANKL (40ng/mL) along with increasing doses of TH (25, 50, 100, and 200mM) TRAP staining from DBA/2J GPNMB+ (A), and DBA/2J (B), TRAP activity from DBA/2J GPNMB+ (C) and DBA/2J (D), and comparison between DBA/2J GPNMB+ and DBA/2J (E). Data presented in all graphs represent Mean + SEM. *P<0.05, **P <0.01, ***P<0.001. Osteoclasts were imaged at 10X magnification using Nikon microscopy.
Figure 4.5: Effects of autophagy during osteoclast differentiation through the mTOR-independent pathway. Bone marrow derived from DBA/2J GPNMB+ and DBA/2J mice was flushed, and non-adherent cells were divided into five groups. The first group served as a control, the second group was cultured with (20ng/mL) of M-CSF and (100mM) of TH, the third group was cultured with one dose of RANKL (40ng/mL) and TH, the fourth group was cultured with first and second dose of RANKL plus TH, and the last group of mature osteoclast (M-OC) was treated with M-CSF, two doses of RANKL, and TH. TRAP staining from DBA/2J GPNMB+ (A) and DBA/2J (B). TRAP activity from DBA/2J GPNMB+ (C) and DBA/2J (D), and comparison between DBA/2J GPNMB+ and DBA/2J (E). Data presented in all graphs represent Mean + SEM. *P<0.05, **P <0.01, ***P<0.001. Cells were imaged at 10X magnification using Nikon microscopy.
4.23 mTOR-Dependent Pathway During Osteoclast Differentiation

In order to determine the role of the mTOR-dependent pathway in osteoclast differentiation, we added different concentrations of R (125, 250, 500, and 600 µM) to BMM isolated from DBA/2J GPNMB⁺ and DBA/2J during osteoclast differentiation. We evaluated osteoclast differentiation by TRAP staining (Figure 4.55 A and B) and TRAP activity (Figure 4.55 C-E). We found that a 500 µM concentration of R is the optimal dose to induce autophagy during osteoclast differentiation.

In order to examine the role of autophagy during osteoclast differentiation by the mTOR-dependent pathway, we treated BMM isolated from DBA/2J GPNMB⁺ and DBA/2J mice and divided them into five groups: the first group served as a control, the second group was treated with M-CSF plus R, the third group was treated with one dose of RANKL plus R, the fourth group was treated with two doses of RANKL plus R, and the last group of mature osteoclast (M-OC) was treated with M-CSF, two doses of RANKL and R. TRAP staining (Figure 4. 56 A and B) and TRAP activity (Figure 4.56 C-E) show increased osteoclast differentiation by mTOR-dependent pathway.
Figure 4.55: Increased osteoclast differentiation through the mTOR-dependent pathway. BMM derived from DBA/2J GPNMB+ and DBA/2J mice were differentiated with M-CSF (20ng/mL) and RANKL (40ng/mL) along with increasing doses of R (125, 250, 500, and 600µM). Then cells were subjected to TRAP staining from DBA/2J GPNMB+ (A) and DBA/2J (B), TRAP activity from DBA/2J GPNMB+ (C) and DBA/2J (D), and comparison between DBA/2J GPNMB+ and DBA/2J (E). Data presented in all graphs represent Mean + SEM. *P<0.05, **P <0.01, ***P<0.001. Cells were imaged at 10X magnification using Nikon microscopy.
Figure 4.56: Effects of autophagy during osteoclast differentiation through the mTOR-dependent pathway. Bone marrow derived from DBA/2J GPNMB+ and DBA/2J mice was flushed, and non-adherent cells were divided in five groups. The first group served as a control, the second group was cultured with (20ng/mL) of M-CSF and (500uM) of R, the third group was cultured with one dose of RANKL (40ng/mL) and (500µM) of R, the fourth group cultured with first and second dose of RANKL plus R, and the last group of mature osteoclast (M-OC) was treated with M-CSF, two doses of RANKL, and R. TRAP staining from DBA/2J GPNMB+ (A) and DBA/2J (B), TRAP activity DBA/2J GPNMB+ (C) and DBA/2J (D), and comparison between DBA/2J GPNMB+ and DBA/2J (E). Data presented in all graphs represent Mean + SEM. *P <0.05, **P <0.01, ***P <0.001. Cells were imaged at 10X magnification using Nikon microscopy.
4.24 Effects of Autophagy During Osteoclast Differentiation

Previous studies have shown that osteoactivin is expressed in osteoclasts (Abdelmagid et al., 2010; Abdelmagid et al., 2015; Sheng et al., 2008). The role of osteoactivin in autophagy mediated osteoclastogenesis is still not fully understood. Some group found that mutation in osteoactivin in DBA/2J mice led to increased osteoclast differentiation (Sheng et al., 2008). Studies found that Paget’s disease of bone (PDB) which is characterized by increased osteoclast activity, size, multinuclearity, and number can occur as a result of a mutation in the autophagic cargo receptor SQSTM1 (or P62) (Helfrich & Hocking, 2008). Furthermore, ATG7 knockdown inhibits expression of osteoclast markers TRAP and cathepsin K during osteoclast differentiation (Wang et al., 2011). In order to investigate the role of osteoactivin in osteoclast differentiation by mediating the autophagy pathway, we added TH and R with RANKL and M-CSF to BMM derived from DBA/2J GPNMB+ and DBA/2J in order to differentiate them into mature osteoclasts. TH treatment enhances osteoclast differentiation assessed by TRAP staining (Figure 4.57 A and C), activity (Figure 4. 57 B, D and E), and osteoclast count (Figure 4.58 A-C). While R treatment decreased osteoclast differentiation. These results indicate that osteoactivin regulates osteoclast differentiation in part by the mTOR-independent pathway.
Figure 4.57: Autophagy pathway is activated in mature osteoclasts. Bone marrow-derived osteoclasts (BMM) from DBA/2J GPNMB+ and DBA/2J mice. Cells were differentiated with M-CSF (20ng/mL) and RANKL (40ng/mL) alone (control), or treated with M-CSF (20ng/mL) and RANKL (40ng/mL) plus TH (100mM), or R (500µM). Osteoclast terminated at day 7. Microscopic images of TRAP staining show an increase in osteoclast upon TH treatment in both DBA/2J GPNMB+ (A) and DBA/2J (C). TRAP activity from DBA/2J GPNMB+ (B) and DBA/2J (D). The experiment was repeated 3 times with similar results, and comparison between DBA/2J GPNMB+ and DBA/2J mice (E). Data presented in all graphs represent Mean + SEM. *P <0.05, **P <0.01, ***P <0.001. Cells were imaged at 10X magnification using Nikon microscopy.
Figure 4.58: Autophagy pathways regulate osteoclast numbers. BMM from DBA/2J GPNMB+ and DBA/2J mice were differentiated with M-CSF (20ng/mL) and RANKL (40ng/mL) alone (control), or treated with M-CSF (20ng/mL) and RANKL (40ng/ml) plus TH (100mM), or R (500µM). Mature osteoclasts terminated at day 7. TH treatment shows an increase numbers of osteoclast in both DBA/2J GPNMB+ (A), DBA/2J (B), while R treatment also decreased osteoclast numbers. Comparison between DBA/2J GPNMB+ and DBA/2J (C). The experiment was repeated 3 times with similar results. Data presented in all graphs represent Mean + SEM. **P <0.01, ***P <0.001.
4.25 Autophagy Pathway Regulates Osteoclast Resorption in vitro

Previous studies have shown that osteoactivin has a positive role on osteoclast function both in vitro and in vivo (Abdelmagid et al., 2015; Sheng et al., 2012; Sheng et al., 2008). We wanted to determine if osteoactivin mediated autophagy stimulated osteoclast resorption. The addition of R to DBA/2J GPNMB+ and DBA/2J significantly increased osteoclast resorption when cultured on corning discs. Interestingly, when R was added to DBA/2J deficient osteoclasts, a similar response as the DBA/2J GPNMB+ was observed. However, TH treatment decreased osteoclast mediated bone resorption in control DBA/2J GPNMB+ and DBA/2J osteoclast. These data indicate that osteoactivin regulates osteoclast resorption by R (mTOR-dependent pathway) (Figure 4.59 A-E).
Figure 4.59: Osteoactivin mediates autophagy pathways for osteoclast resorption *in vitro*. BMM from DBA/2J GPNMB+ and DBA/2J mice were cultured on corning calcium phosphate treated with M-CSF (20ng/mL) and RANKL (40ng/mL) alone (control), or treated with M-CSF (20ng/mL) and RANKL (40ng/mL) with TH (100mM), or R (500µM). Microscopic images of corning discs reveal the resorption pits from the osteoclasts from DBA/2J GPNMB+ (A) and DBA/2J (C), quantification from the corning discs of DBA/2J GPNMB+ (B) and DBA/2J (D), and comparison between DBA/2J GPNMB+ and DBA/2J (E). The experiment was repeated 3 times with similar results. Data presented in all graphs represent Mean ± SEM. *P <0.05, **P <0.01, ***P <0.001 compared to control. Cells were imaged at 10X magnification using Nikon microscopy.
4.26 Autophagy is Activated During Osteoclast Differentiation

Previous literature has shown that osteoactivin is expressed in osteoclasts and is important for both differentiation and function (Abdelmagid et al., 2015; Sheng et al., 2008). Recently, our lab has found that mutation in osteoactivin in DBA/2J mice led to increased osteoclast differentiation and an inhibition in their function both in vivo and ex-vivo. These data suggest that osteoactivin plays a crucial role in osteoclast differentiation and function. Recently, it has been reported that in an autophagy-independent manner, in vitro, a decreased level of Beclin-1 in osteoclasts leads to a reduction in differentiation and function of these cells through NFATc1 (Chung, 2014). In this study, we examined the effects of TH and R on DBA/2J GPNMB⁺ and DBA/2J osteoclast in vitro to determine the role of osteoactivin in autophagy mediated osteoclast differentiation by western blot analysis. We added TH or R during osteoclast differentiation with RANKL and M-CSF and the cells were terminated at day 7. We found that the control DBA/2J treated group has higher levels of LC3II which were still high when treated with TH (mTOR-independent pathway) while R (mTOR-dependent pathway) seemed to decrease LC3II levels compared to the control group (Figure 4.60 A-D).

In order to confirm that osteoclasts were able to be successfully differentiate by qPCR analysis osteoclast transcription factor and the autophagy markers OA (Figure 4.61), Beclin-1 (Figure 4.62), LC3II (Figure 4.63), and TRAP (Figure 4.64). Interestingly, autophagy levels with TH (mTOR-independent pathway) treatment were higher compared to R (mTOR-dependent pathway) treatment.
Figure 4.60: Osteoactivin plays a role in osteoclast through autophagy pathways.

BMM from DBA/2J GPNMB+ and DBA/2J mice were isolated and differentiated into osteoclasts with M-CSF (20ng/mL) and RANKL (40ng/mL) alone (control), or treated with M-CSF (20ng/mL) and RANKL (40ng/mL) with TH (100mM), or R (500µM). Total protein was isolated at day 7 and run using 12% SDS gel. Gel was then transferred to PVDF membrane, and membrane was probed with antibodies against LC3, and GAPDH. Western blot image (A), quantification of multiple blots from DBA/2J GPNMB+ (B) and DBA/2J (C), and comparison between DBA/2J GPNMB+ and DBA/2J (D). The experiment was repeated 3 times with similar results. Data presented in all graphs represent Mean + SEM. *P <0.05, **P <0.01, ***P <0.001 compared to control.
Figure 4.61: DBA/2J mice have reduced OA by mTOR-dependent Pathway. BMM derived from DBA/2J GPNMB+ and DBA/2J mice were isolated and differentiated into osteoclasts with M-CSF (20ng/mL) and RANKL (40ng/mL) control, or treated with M-CSF (20ng/mL) and RANKL (40ng/mL) with TH (100mM) or R (500µM). Total RNA was isolated at day 7, relative expression of OA was determined by qPCR. DBA/2J GPNMB+ (A), DBA/2J (B), and comparison between DBA/2J GPNMB+ and DBA/2J (C). The experiment was repeated 3 times with similar results. Data presented in all graphs represent Mean + SEM. **P <0.01, ***P<0.001 compared to control.
Figure 4.62: DBA/2J mice have reduced Beclin-1 by mTOR-dependent Pathway.

BMM derived from DBA/2J GPNMB+ and DBA/2J mice were isolated and differentiated into osteoclasts with M-CSF (20ng/mL) and RANKL (40ng/mL) control, or treated with M-CSF (20ng/mL) and RANKL (40ng/mL) plus TH (100mM) or R (500µM). Total RNA was isolated at day 7, relative expression of Beclin-1 was determined by qPCR. DBA/2J GPNMB+ (A), DBA/2J (B), and comparison between DBA/2J GPNMB+ and DBA/2J (C). The experiment was repeated 3 times with similar results. Data presented in all graphs represent Mean + SEM. *P <0.05, ***P<0.001 compared to control.
Figure 4.63: DBA/2J mice have reduced LC3II by mTOR-dependent Pathway. BMM derived from DBA/2J GPNMB+ and DBA/2J mice were isolated and differentiated into osteoclasts with M-CSF (20ng/mL) and RANKL (40ng/mL) control, or treated with M-CSF (20ng/mL) and RANKL (40ng/mL) plus TH (100mM) or R (500µM). Total RNA was isolated at day 7, relative expression of LC3II was determined by qPCR. DBA/2J GPNMB+ (A), DBA/2J (B), and comparison between DBA/2J GPNMB+ and DBA/2J (C). The experiment was repeated 3 times with similar results. Data presented in all graphs represent Mean + SEM. *P<0.05, **P <0.01, ***P<0.001 compared to control.
Figure 4.64: DBA/2J mice have reduced TRAP by mTOR-dependent Pathway.

BMM derived from DBA/2J GPNMB+ and DBA/2J mice were isolated and differentiated into osteoclasts with M-CSF (20ng/mL) and RANKL (40ng/mL) control, or treated with M-CSF (20ng/mL) and RANKL (40ng/mL) plus TH (100mM) or R (500µM). Total RNA was isolated at day 7, relative expression of TRAP was determined by qPCR. DBA/2J GPNMB+ (A), DBA/2J (B), and comparison between DBA/2J GPNMB+ and DBA/2J (C). The experiment was repeated 3 times with similar results. Data presented in all graphs represent Mean + SEM. *P<0.05, ***P<0.001 compared to control.
4.27 Chloroquine Inhibits RANKL-Induced Osteoclast Formation

Previous studies reported that chloroquine (CQ) inhibits osteoclast differentiation by preventing lysosomal degradation through TRAF3 (Xiu et al., 2014). Another study found that 3-MA (autophagy inhibitor) causes decreased osteoclast differentiation in mouse bone marrow cultures (Sambandam et al., 2014). Other groups also found that CQ treatment reduced numbers and size of TRAP-positive multinucleated osteoclasts (Weinstein et al., 2000). To determine whether CQ affects osteoclast formation, we cultured BMM DBA/2J GPNMB+ and DBA/2J cells treated with M-CSF and RANKL for 6 days. Cultures were then treated with CQ for 2 hours followed by TH or R for 24 hours and stained for TRAP at day 7. The total number of osteoclasts and osteoclast size were significantly reduced in response to CQ in combination with TH or R in both DBA/2J GPNMB+ and DBA/2J (Figure 4.65 A and C) and there was a reduction in TRAP activity (Figure 4.65 B, D and E). These data indicate that CQ inhibits the effect on TH or R on osteoclast differentiation in vitro.

The effects of autophagy inhibitor on LC3II levels during osteoclast differentiation were examined by western blot. We used BMM from DBA/2J GPNMB+ and DBA/2J mice treated with M-CSF and RANKL 6 days then treated with CQ for 2 hours followed by TH or R. Protein was collected at day 7 for western blot analysis, as we expected, CQ increased the level of LC3-II induces by TH or R, would favor accumulation of LC3-II (autophagosom formation) as shown in (Figure 4. 66 A-D). These data indicate that CQ accumulates in the autophagosome and inhibits of the late stage autophagy.

In order to determine the role of osteoactivin on osteoclast differentiation mediated by autophagy inhibiton, we used the same models of mice and examined the gene
expression of autophagy markers and the osteoclast marker (TRAP). Our data show a decrease in Beclin-1 (Figure 4.67), TRAP (Figure 4.68), and OA (Figure 4.69) expression. This indicates that autophagy inhibition negatively regulates osteoclast differentiation.
Figure 4.65: Chloroquine inhibits RANKL-induced osteoclast formation. BMM derived from DBA/2J GPNMB+ and DBA/2J treated with RANKL (40ng/mL) and M-CSF (20ng/mL), at day 6 osteoclast treated with CQ (100µM) for two hours followed by TH (100mM) or R (500µM). TRAP staining from DBA/2J GPNMB+ (A) and DBA/2J (C). Quantitative analysis from TRAP activity from DBA/2J GPNMB+ (B) and DBA/2J (D). The experiment was repeated 3 times with similar results. Data presented in all graphs represent Mean + SEM. *P <0.05, **P <0.01, ***P <0.001 compared to control. Cells were imaged at 10X magnification by using Nikon microscopy.
Figure 4.66: Chloroquine increases LC3II level during osteoclast formation. BMM derived from DBA/2J GPNMB$^+$ and DBA/2J treated with RANKL (40ng/mL) and M-CSF (20ng/mL), at day 6 osteoclast treated with CQ (100µM) for two hours followed by TH (100mM) or R (500µM). Total protein was isolated at day 7 and run using 12% SDS gel. Gel was then transferred to PVDF membrane, and membrane was probed with antibodies against LC3, and GAPDH. Western blot (A), quantification of multiple blots from DBA/2J GPNMB$^+$ (B) and DBA/2J (C), and comparison between DBA/2J GPNMB$^+$ and DBA/2J (D). The experiment was repeated 3 times with similar results. Data presented in all graphs represent Mean + SEM. **P <0.01, ***P <0.001 compared to control.
Figure 4.67: Chloroquine decreases Beclin-1 expression during osteoclast formation. BMM derived from DBA/2J GPNMB+ and DBA/2 treated with RANKL (40ng/mL), M-CSF (20ng/mL), at day 6 we added CQ (100µM) for two hours followed by TH (100mM) or R (500µM). Total RNA was isolated at day 7, relative expression of Beclin-1 was determined by qPCR. DBA/2J GPNMB+ (A), DBA/2J (B), and comparison between DBA/2J GPNMB+ and DBA/2J (C). The experiment was repeated 3 times with similar results. Data presented in all graphs represent Mean + SEM. *P <0.05, **P <0.01, ***P<0.001 compared to control.
Figure 4.68: Chloroquine deceases TRAP expression during osteoclast formation.

BMM derived from DBA/2J GPNMB+ and DBA/2 treated with RANKL (40ng/mL), M-CSF (20ng/mL), at day 6 we added CQ (100µM) for two hours followed by TH (100mM) or R (500µM). RNA was isolated at day 7, relative expression of TRAP was determined by qPCR. DBA/2J GPNMB+ (A), DBA/2J (B), and comparison between DBA/2J GPNMB+ and DBA/2J (C). The experiment was repeated 3 times with similar results. Data presented in all graphs represent Mean + SEM. **P <0.01, ***P<0.001.
Figure 4.69: Chloroquine decreases OA expression during osteoclast formation.

BMM derived from DBA/2J GPNMB+ and DBA/2 treated with RANKL (40ng/mL), M-CSF (20ng/mL), at day 6 we added CQ (100µM) for two hours followed by TH (100mM) or R (500µM). Total RNA was isolated at day 7, relative expression of OA was determined by qPCR. DBA/2J GPNMB+ (A), DBA/2J (B), and comparison between DBA/2J GPNMB+ and DBA/2J (C). The experiment was repeated 3 times with similar results. Data presented in all graphs represent Mean + SEM. **P <0.01, ***P<0.001.
4.28 p-mTOR Signaling is Activated During Osteoclast Differentiation

Previous studies reported that RANKL stimulation was associated with an increased phosphorylation of S6 ribosomal protein and 4E-BP1 protein and rapamycin effectively blocked activation of the mTOR pathway in RANKL stimulated osteoclast precursors (Hussein et al., 2012). In order to determine the signaling pathway involved in autophagy mediated osteoclast differentiation, we used BMM from DBA/2J GPNMB$^+$ and DBA/2 mice treated with M-CSF (20ng/mL) and RANKL (40ng/mL) control, or treated with M-CSF (20ng/mL) and RANKL (40ng/mL) plus R (500µM). Protein was then collected at day 7 for (0, 30, and 60 minutes). Western blot analysis revealed that treatment with R increases phosphorylated mTOR in a time dependent manner (Figure 4.70 A-D). These data indicate that mTOR signaling plays a role in stimulating osteoclast differentiation.
Figure 4.70: p-mTOR signaling is activated in mature osteoclasts. BMM derived from DBA/2J GPNMB + and DBA/2 cells were treated with M-CSF (20ng/mL) and RANKL (40ng/mL) with 500µM of R, protein collected at day 7 for (0, 10, 30, and 60 minutes). Total protein cell lysates were collected and analyzed by western blot. Blots were probed for p-mTOR, mTOR, β-actin, and GAPDH antibodies. Notice there are slight increases in p-mTOR compared to control mTOR. The experiment was repeated 3 times with similar results. Data presented in all graphs represent Mean + SEM. *P <0.05, **P <0.01, ***P<0.001 compared to control.
4.29 mTOR-Independent Pathway Alters Osteoclast Podosome Formation

Previous literature has shown that ATG4B, ATG5, ATG7, and LC3 are essential for generating the osteoclast-ruffled border in vivo and in vitro. Autophagy mediates the fusion between lysosomal contents and the plasma membrane by participating in the polarized secretion between them (Deselm et al., 2011). The lysosome fusion with the ruffled border leads to proton and protease secretion into the lacuna (Itzstein et al., 2011) and induced autophagy leads to reduced pathological osteoclast formation (Smink et al., 2012). In order to determine if mTOR-independent or dependent pathway will cause formation of the osteoclast-podosome. BMM isolated from DBA/2J GPNMB\(^+\) and DBA/2J were plated in chamber slides and differentiated into mature osteoclasts, then treated with TH or R for 24 hours. Transmission electron microscopy (TEM) analysis revealed that treatment with R causes formation of osteoclast-podosome, while treatment with TH causes disrupts of osteoclast-podosome formation (Figure 4.71).
Figure 4.71: Autophagy pathways alters osteoclast podosome formation. BMM derived from DBA/2J GPNMB+ and DBA/2J mice were plated in chamber slides with RANKL (40ng/mL) and M-CSF (20ng/mL) with TH (100mM) or R (500µM). N indicates nucleus; P indicates podosomes; the yellow arrow indicates autophagosome; * indicates autolysosome.
Figure 4.72: mTOR-independent pathway alters osteoclast podosome formation in DBA/2J GPNMB\(^+\) mice. BMM derived from DBA/2J GPNMB\(^+\) were plated in chamber slides with RANKL (40ng/mL) and M-CSF (20ng/mL) with TH (100mM) for 24 hours. Transmission electron microscopy shows the numerous of autophagosome formation with the white arrow, N indicates nucleus; P indicates podosomes.
Figure 4.73: mTOR-dependent pathway increases osteoclast podosome formation in DBA/2J GPNMB+ mice. BMM derived from DBA/2J GPNMB+ were plated in chamber slides with RANKL (40ng/mL) and M-CSF (20ng/mL) with R (500µM) for 24 hours. Transmission electron microscopy shows the numerous of autophagosome formation with the white arrow; N indicates nucleus; P indicates podosomes.
Figure 4.74: mTOR-independent pathway alters osteoclast podosome formation in DBA/2J mice. BMM derived from DBA/2J were plated in chamber slides with RANKL (40ng/mL) and M-CSF (20ng/mL) with TH (100mM) for 24 hours. Transmission electron microscopy shows the disruption of podosome formation, N indicates nucleus; P indicates podosomes.
Figure 4.75: mTOR-dependent pathway increases osteoclast podosome formation in DBA/2J mice. BMM derived from DBA/2J were plated in chamber slides with RANKL (40ng/mL) and M-CSF (20ng/mL) with R (500 µM) for 24 hours. Transmission electron microscopy shows increased podosome formation, N indicates nucleus; P indicates podosomes.
**4.30 Autophagy Pathway Increases the Differentiation and Recruitment of TRAP-Positive Osteoclasts in Vivo**

Previous studies have shown that addition of anti-inflammatory cytokines led to decreased osteoclast differentiation and migration in a calvaria osteolysis model (Lee et al., 2009). Therefore, we wanted to examine the effects of TH and R on osteoclast differentiation and recruitment *in vivo*. We preformed small surgery on calvaria DBA/2J and GPNMB+ DBA/2J mice by adding a collagen sheet coated with either PBS, RANKL, RANKL plus TH or RANKL plus R for one week. After 7 days, we isolated calvaria and stained them with TRAP. We found that in DBA/2J GPNMB+ and DBA/2J mice, addition of RANKL alone increases the number of TRAP positive osteoclasts as expected; however, addition of RANKL in combination with TH or R results in a significant increase of osteoclast differentiation and recruitment of TRAP-Positive Osteoclasts in Vivo (Figure 4.76 A-E).

To further elucidate the role of osteoactivin on osteoclast function mediated by autophagy pathways by using µCT. We tested the effects of TH and R using the same model of mice. Interestingly, R (mTOR-dependent) and TH (m-TOR independent) pathways causes increased osteoclast resorption compared to control untreated (Figure 4.85). These data indicate that osteoactivin increased osteoclast resorption by mediating autophagy (mTOR-independent and dependent) pathways *in vivo*. 
Figure 4.76: Autophagy pathways increases the differentiation and recruitment of TRAP-positive osteoclasts in vivo. A collagen sponge coated with 30μL of either PBS, RANKL (5μg) alone, RANKL and TH (100mM), or RANKL and R (5000μM) was placed on DBA/2J GPNMB+ and DBA/2J calvaria (n=3) for seven days. After a week, calvaria were removed and TRAP staining was performed. By TRAP staining, we found an increased number of osteoclasts in RANKL alone and RANKL with TH in both DBA/2J GPNMB+ and DBA/2J mice. Note that R and TH treatments in both DBA/2J GPNMB+ and DBA/2J calvaria led to a increased in the TRAP staining from DBA/2J GPNMB+ (A), DBA/2J (C). Staining area analysis reveals an increase in the overall TRAP stained area in RANKL and TH treated DBA/2J GPNMB+ (B), DBA/2J mice (D), and comparison between DBA/2J GPNMB+ and DBA/2J mice (E). The experiment was repeated 3 times with similar results. Data presented in all graphs represent Mean + SEM. *P <0.05, **P <0.01, ***P <0.001.
Figure 4.77: Calvaria from DBA/2J GPNMB⁺ mice coated with PBS. A collagen sponge coated with 30 μL of PBS was plated on the calvaria of DBA/2J GPNMB⁺ mice for a week. Calvaria were then stained with TRAP. Arrows indicate a small osteoclast.
Figure 4.78: Calvaria from DBA/2J GPNMB⁺ mice coated with RANKL. A collagen sponge coated with RANKL (5μg) alone, was placed on the calvaria of DBA/2J GPNMB⁺ mice for a week. Calvaria were then stained with TRAP. Arrows indicates a large osteoclast.
Figure 4.79: mTOR-independent pathway increases osteoclast differentiation

**DBA/2J GPNMB+ mice.** A collagen sponge coated with RANKL (5μg) with combination with (100mM) of TH, was placed on the calvaria DBA/2J GPNMB+ mice for a week. Calvaria were then stained with TRAP. Arrows indicates to huge osteoclast.
Figure 4.80: mTOR-dependent pathway increases osteoclast differentiation

**DBA/2J GPNMB+ mice.** A collagen sponge coated with RANKL (5µg) with combination
with (500µM) of R was placed on the calvaria of DBA/2J GPNMB+ mice for a week.
Calvaria were then stained with TRAP. Arrows indicates to nice and big osteoclast.
Figure 4.81: Calvaria from DBA/2J mice coated with PBS. A collagen sponge coated with 30μL of PBS was plated on calvaria of DBA/2J mice for a week. Calvaria were then stained with TRAP. Arrows indicates an osteoclast, as expected there was an increase in osteoclasts in control DBA/2J mice.
Figure 4.82: Calvaria from DBA/2J mice coated with RANKL. A collagen sponge coated with RANKL (5μg) alone was placed on the calvaria of DBA/2J mice for a week. Calvaria were then stained with TRAP. Arrows indicates to increase osteoclast differentiation with RANKL.
**Figure 4.83: mTOR-independent pathway increases the differentiation of osteoclasts in DBA/2J mice.** A collagen sponge coated with RANKL (5µg) in combination with (500µM) of R was placed on the calvaria of DBA/2J mice for a week. Calvaria were then stained with TRAP. Arrows indicate an increase of osteoclast.
Figure 4.84: mTOR-dependent pathway increases osteoclast differentiation in DBA/2J mice. A collagen sponge coated with RANKL (5µg) in combination with (500µM) of R was placed on calvaria of DBA/2J mice for a week. Calvaria were then stained with TRAP. Arrows indicates to osteoclast.
Figure 4.85: Autophagy pathways increases the resorption of osteoclasts *in vivo*.

A collagen sponge coated with 30μL of either PBS, RANKL (5μg) alone, RANKL plus TH (100mM), or RANKL plus R (500μM) was placed on the calvaria of DBA/2J GPNMB\(^+\) and DBA/2J mice (n=3) for one week. Calvaria were removed and scanned by μCT. We found an increase in the number of pores (red arrows) in RANKL plus R compared with the group treated with RANKL plus TH.
Figure 4.86: Schematic diagram of autophagy signaling during osteoclast differentiation. Autophagy signaling binds to receptor threonine kinase leads to phosphorylation of the receptor and produces phosphatidylinositol 3-kinases (PI3K) and protein kinase (Akt) which results in inhibition of 4 eukaryotic binding protein 1(4E/BP1) and tuberous sclerosis complex 1 and 2 (TSC1/2) and activated mTOR pathways. The basic idea is an inhibition of mTOR by rapamycin leads to activate autophagy pathways. mTOR-independent pathway plays a positive regulatory role of osteoclast differentiation, while mTOR-dependent pathway plays a positive regulatory for osteoclast function in vitro. In contrast, mTOR-independent and dependent pathway stimulates osteoclasts differentiation and function in vivo.
Figure 4.87: Schematic diagram illustrating the role of autophagy and osteoactivin during osteoclast differentiation. The mTOR-independent pathway increases osteoclast differentiation but inhibits their function, while osteoactivin and the mTOR-dependent pathway are a positive regulator of osteoclast function.
Chapter 5

Discussion

Summary

The aim of this study was to determine the role of osteoactivin in both osteoblast and osteoclasts by mediating the autophagy pathways. We determined that osteoactivin regulated both osteoblast and osteoclast differentiation and function by the autophagy pathways. For osteoblasts, osteoactivin stimulated osteoblast differentiation and function by mTOR-independent and dependent pathways and increased bone formation in mutant mice DBA/2J less compared to control DBA/2J GPNMB+ mice. Interestingly in osteoblasts, osteoactivin binds to the LC3II, main marker for autophagosome formation, and displays punctate distribution in osteoblasts. For osteoclasts, osteoactivin inhibited osteoclast differentiation by rapamycin treatment in a dose dependent manner in vitro. Furthermore, in vivo results from a mouse osteolysis model revealed that rapamycin might stimulate osteoclast differentiation and increase its function in vivo. These results support our hypothesis that osteoactivin stimulates osteoblast differentiation and function by mediating the autophagy pathways while inhibiting osteoclast differentiation and increasing their function by mTOR-dependent pathway.
5.1 Aim 1: The Role of Osteoactivin on Osteoblast Differentiation and Function by Mediating the Autophagy Pathways in Vitro and ex-Vivo

In this study, we determined that osteoactivin stimulates osteoblast differentiation and function through the autophagy pathways. Previous literature has shown that osteoactivin is essential for osteoblast differentiation (Abdelmagid et al., 2014; Frara et al., 2016; Sondag et al., 2014). A previous report from our lab showed that overexpression of osteoactivin enhances osteoblast differentiation and function in vitro and ex-vivo (Abdelmagid et al., 2008; Frara et al., 2016). Several other groups found that autophagy stimulates osteoblast differentiation and function (Whitehouse et al., 2010; Zahm et al., 2011). Our data supports this finding that osteoactivin increases osteoblast differentiation and function by mediating the autophagy pathways.

Our data indicates that autophagy is activated during osteoblast differentiation in MC3T3-E1 cells. Previous studies have shown that autophagy increases during osteoblast differentiation and mineralization using osteoblast cell lines (Liu et al., 2013; Nollet et al., 2014). Other studies emphasize that induction of autophagy by liraglutide increases osteoblast differentiation in MC3T3-E1 cells (Hu et al., 2016). Other groups confirm that autophagy is a developmentally regulated process necessary for bone growth, and that FGF is a crucial regulator of autophagy in chondrocytes (Cinque et al., 2015). This evidence supports our study that osteoactivin increases osteoblast differentiation by autophagy pathways.

Transmission electronic microscopy and confocal imaging indicate that treatment with TH causes autophagosome formation associated with autophagic vacuoles that act
as vehicles to secreteapatite crystals into the matrix in MC3T3-1 osteoblast cells. In addition, increased autophagosome formation was found in treatments with both TH and R in bone cells taken from DBA/2J GPNMB+ and DBA/2J. Previous studies using transmission electron microscopy have shown double-membranated autophagic vesicles contain crystal-like structures that are released in the extracellular medium and through X-ray microanalysis they found that calcium and phosphorus in primary mouse osteoblast and osteoblast cell line is present after induction of autophagy (Nollet et al., 2014). Another study using transmission electron microscopy detected autophagic vacuoles in control osteoblasts, while autophagosome-like structures were absent in knockout mice for FIP200 (Liu et al., 2013). This evidence supports our study that autophagy treatment causes formation of autophagosome structures in osteoblasts.

Our data also indicates that osteoblasts recruit to LC3II and display punctate distribution of LC3II and co-localizes with osteoactivin. Osteoactivin has been shown to be highly expressed in epithelial cells and it is essential for recruitment of autophagy proteins to the phagosome to create the autophagosome followed by fusion with the lysosome to degrade their contents (Bing & Thomas, 2010). Furthermore, retinal pigment epithelial cells express osteoactivin and accumulation of pigment in macrophages requisite to the eye; this data confirms the role of osteoactivin in the normal clearance of photoreceptors (Anderson, 2002; Li et al., 2010). Osteoactivin is also expressed by macrophages at the inflammatory site, and helps to clean and degrade self-peptides and inflammatory oxidized lipids to maintain homeostatic conditions (Hanayama et al., 2006). Epithelial cells have up-regulated osteoactivin and
express high levels of the phagocytic receptor (Ichimura et al., 2008). This evidence supports our study that osteoactivin is located phagocytic cells.

Furthermore, our data show that DBA/2J mutation in osteoactivin mice have significantly less bone in both \textit{vitro} and \textit{ex-vivo} indicating that osteoactivin acts as a positive regulator of osteoblast differentiation and function. Furthermore, we showed that autophagy (mTOR-independent by TH or mTOR-dependent by R) treatment in DBA/2J mutant mice stimulated bone formation \textit{in vitro} and \textit{ex-vivo}. Furthermore, the TH and R treatment led to increased osteoblastic colony size and increased bone formation in calvaria explant culture of mutant osteoactivin mice was less than control mice. Previous studies have shown that disruption of neighbor of BRCA1 gene 1 protein (NBR1) has a negative effect on osteoblast differentiation and function; genetic truncation of murine Nbr1 enhances osteoblast differentiation and activity which leads to an increase in bone mass and density (Whitehouse et al., 2010). Another study showed that deletion of FIP200, part of a complex essential to initiation of autophagosome formation, leads to a reduction in bone formation (Yang et al., 2012). Another group reported that deletion of FIP200 led to osteopenia in mice through the inhibition of osteoblast terminal differentiation (Liu et al., 2013). Further, terminal differentiation of osteoblasts is controlled by autophagy (Guignandon, 2013). We previously showed that mTORC1 contributed to the hyperactive osteoblast activity in response to Wnt overexpression (Chen et al., 2014). Others also reported that osteoblast specific deletion of mTORC2 led to decreased trabecular bone mass, whereas hyperactivation of mTORC1 through tuberous sclerosis complex 2 (TSC2) deletion results in excessive production of immature osteoblasts (Riddle et al., 2014). Additionally, another study
found that mTORC2 is necessary for skeletal growth and bone anabolism because the rictor main component of mTORC2 not only enhances osteoblast activity directly but also promotes osteoclastogenesis indirectly (Chen et al., 2015). Treatment with rapamycin led to reduced bone loss in critically ill rabbits in vivo (Owen et al., 2015), and promotes bone fracture healing in rats (Yang et al., 2015). Rapamycin also caused a marked increase in LC3-II levels in rat kidney (Yu et al., 2010). This data supports our data that osteoactivin regulated bone formation through autophagy.

In the current study, we have shown that increase levels of LC3-II protein during osteoblast matrix deposition and mineralization, suggesting an increase in the number of autophagosome formation. Increases in LC3II levels could be due to an increase conversion of LC3I to LC3II or inhibition of lysosomal function. The role of autophagy in osteoblast function is confirmed by using the autophagy inhibitor chloroquine (CQ) at day 14 which results in decreased mineralization capacity in osteoblast cells. Our data suggest an inability of osteoblasts to switch from proliferation to mineralization upon CQ treatment due to accumulate toxic materials inside the cells. Some groups uses 3-methyladenine (3-MA) autophagy inhibitor and CQ at day 10 of differentiation to inhibit autophagy which led to a decreased number and size of alkaline phosphatase as well as a reduction in bone mineralization at day 21 (Liu et al., 2013). Similarly, bafilomycin A1, CQ, ammonium chloride (NH4CL), and shRNA-mediated knockdown of the autophagy essential gene LC3-β caused blocked osteogenic differentiation in human dental pulp mesenchymal stem cells (Pantovic et al., 2013). In addition, siATG7 and siBCN1 were shown to decrease mineralization in the rat osteoblast cell line (Nollet et al., 2014). This evidence supports our study that treatment with TH results in increased
osteoblast differentiation and bone formation. There is little literature describing the role of rapamycin on osteoblast differentiation and function. One group found that rapamycin stimulated osteoblastic differentiation of hESCs (human embryonic stem cells) (Lee et al., 2010). Another study noted that, rapamycin directly acts on rat osteoblasts-like osteosarcoma cell line (ROS) and increases osteoblast differentiation (Ogawa et al., 1998). This evidence supports our findings that treatment with rapamycin results in increased bone formation. On the contrary, another group found that increased levels of autophagy in the mesenchymal stem cell leads to reduced differentiation of osteoblasts (Oliver et al., 2012).

In our study, we have shown upregulation of OSX, ALP, OA, and OCN mRNA in DBA/2J GBNMB+ osteoblasts. Interestingly, when DBA/2J mutant mice for osteoactivin are treated with mTOR-independent TH or mTOR-dependent R, we found an increase in osteoblast markers by qPCR in both groups. However, DBA/2J mice show less compared to DBA/2J GPNMB+ groups. Previously it was found that the expression levels of alkaline phosphates (ALP), bone sialoprotein (BPS), and osteocalcin (OCN), (osteoblast differentiation markers), as well as the osteoblast transcription factor osterix (OSX) were significantly decreased in mice with deletion of FIP200 (essential component of mammalian autophagy) (Liu et al., 2013). Other groups reported that an increase in osteocalcin (OSN) specific gene of osteoblast mineralization with autophagy during osteoblast differentiation (Ha et al., 2014).

Taken together, our data demonstrate that osteoactivin regulates osteoblast differentiation, function, and bone formation by mediating the autophagy pathway.
5.2 Aim 2: The Role of Osteoactivin on Osteoclast Differentiation and Function by Mediating the Autophagy Pathways *in vitro* and *in vivo*

In this aim, we study the role of osteoactivin in osteoclasts by mediating the autophagy pathways. Our lab also discovered that a mutation in osteoactivin results in enhanced osteoclast differentiation but inhibits their function (Abdelmagid et al., 2015). On the contrary, other groups have reported that osteoactivin stimulates osteoclast differentiation and function (Sheng et al., 2012; Sheng et al., 2008). We showed that rapamycin inhibits osteoclast differentiation and size by downregulating osteoclast-specific markers. We also demonstrated that osteoactivin increases osteoclast differentiation *in vitro* through the mTOR-independent pathway. Previous literature has shown that rapamycin inhibits osteoclast formation in giant cell tumor of the bone through the C/EBPβ-MAfB; they found that treatment of giant cell tumor (GCT) treated with rapamycin cell cultures derived from seven different patients strongly reduced formation of giant osteoclasts and bone resorption (Smink & Leutz, 2010; Owen et al., 2015). Another study found that stimulation of AKt1/AKT2, the key elements for osteoclast differentiation, by rapamycin inhibits osteoclast precursors cell (Sugatani & Hruska, 2005). Furthermore, inhibition of mTOR by sirolimus or everolimus (same group with rapamycin) results in reduced osteoclast formation and protection against local bone erosion and cartilage loss (Cejka et al., 2010). Several other groups found that rapamycin inhibits osteolysis and improves survival in bone metastases which is also good for bone formation (Hussein et al., 2012). Additionally, induced autophagy by rapamycin leads to a decreased in osteoclast number in rats (Sanchez & He, 2009). Decrease bone resorption occurs in patients who have renal transplantation when
treated with rapamycin (Westenfeld et al., 2011). This data supports our hypotheses that osteoactivin inhibits osteoclast differentiation through the mTOR-dependent pathway (Rapamycin).

In this study, we demonstrated increased osteoclast differentiation by the mTOR-independent pathway with TH treatment. Autophagy plays an essential role in the maintenance of hematopoietic stem cells (HSCs); HSCs fail to differentiate into osteoclasts when ATG7 is absent (Mortensen et al., 2011). ATG7 knockdown inhibits expression of osteoclast markers TRAP and cathapsin K during osteoclast differentiation (Wang et al., 2011). P62 is an adaptor protein that plays a critical role in RANKL to promote autophagy and osteoclastogenesis (Li et al., 2014). A previous study reported that hypoxia caused induction of autophagy and led to an increase in osteoclast number and size (Arnett, 2010). Furthermore, other studies confirmed that hypoxia HIF-α caused an increase in osteoclast differentiation and a significant increase in autophagy markers (Sun et al., 2015). In addition, other groups used microgravity to induce autophagy and they found enhanced osteoclastogenesis (Sambandam et al., 2014; Zhao et al., 2012). In addition, P62 plays important roles in RANKL-induced autophagy and osteoclastogenesis (Li et al., 2014). Ovariectomy induced oxidative stress, was also appeared to induce autophagy and bone loss (Yang et al., 2014). This evidence supports our study that there is an increased in osteoclast differentiation by the mTOR-independent pathway.

In this study, we demonstrated that the mTOR-dependent pathway stimulation of osteoclast resorption in vitro. Previous literature reported that autophagy has a role in the regulation of osteoclasts’ function; they found that Paget's disease of bone, which is
characterized by an increased number and size of osteoclasts, can occur as a result of mutation in the autophagic cargo receptor SQSTM1 or (P62) (Helfrich & Hocking, 2008). Furthermore, other studies confirmed that deletion of ATG5 and ATG7 reduced the resorptive capacity of osteoclasts without affecting differentiation (DeSelm et al., 2011). Recently, other groups, reported that decreased levels of Beclin-1 in osteoclast by the autophagy-independent pathway lead to a reduction in osteoclast function through NFATc1 (Chung et al., 2014). Other groups found increased expression of Beclin-1 and ATG7 in human rheumatoid arthritis with activated autophagy in osteoclasts by using the tumor necrosis factor \textit{in vivo} and \textit{in vitro} and regulated osteoclast function (Lin et al., 2013). Also other, showed that ATG4B, ATG5, ATG7, and LC3II are essential for generating the osteoclast-ruffled border \textit{in vivo} and \textit{in vitro} (DeSelm et al., 2011). This data supports our hypothesis that osteoactivin increases osteoclast function by mTOR-dependent pathway.

Our data suggests that the mTOR-dependent pathway inhibits osteoclast differentiation \textit{in vitro}, but stimulates resorption \textit{vivo}. Other group have shown that fibronectin inhibits osteoclast differentiation but stimulates function through IL-1β and nitric oxide (Gramoun et al., 2010). Previous literature has shown several different anti-inflammatory molecules suppress osteoclast formation \textit{in vivo} (Hu et al., 2003; Lee et al., 2009). It is interesting to note that rapamycin has a negative effect on osteoclast differentiation and a positive response on osteoclast resorption \textit{in vitro}. There may be the explanation for these phenomena, that rapamycin may be utilized to osteoclast in a same manner as osteoactivin do. Our lab also found that addition of recombinant osteoactivin in osteoblasts led to a decreased level of RANKL. This supported the idea
that mice with a mutation mice of osteoactivin have higher levels of RANKL (Abdelmagid et al., 2015).

Taken together, our data show that mTOR-independent pathway stimulates osteoclast differentiation but inhibits their function, while mTOR-dependent pathway inhibits osteoclast differentiation but increases their function \textit{in vitro}. However, mTOR-independent and dependent pathway stimulate osteoclast differentiation and increase their function \textit{in vivo}.
Figure 5.1: Schematic diagram of mTOR-independent pathway on bone cells. TH and osteoactivin proteins are positive regulators of osteoblast differentiation and function. TH is a positive regulator of osteoclast differentiation but inhibits their function, however, osteoactivin is a negative regulator for osteoclast differentiation but a positive regulator of their function.
Figure 5.2: Schematic diagram of mTOR-dependent pathway on bone cells. R and osteoactivin protein are positive regulators of osteoblast differentiation and function, while R and osteoactivin are a negative regulator factor for osteoclast differentiation and a positive regulator for their function.
Overall Summary, Conclusions, And Future Studies

In this study, we have shown that autophagy stimulates osteoblast differentiation \textit{in vitro}. This was shown by the increased ALP staining, activity, and mineralization. Furthermore, autophagy was shown to upregulate several osteoblast specific genes. We identified LC3II, Beclin-1, ATG5, and ATG7 as markers responsible for autophagy to be upregulated during osteoblast differentiation and function.

In order to understand the role of osteoactivin in osteoblast differentiation mediated by the autophagy pathways, we measured the level of LC3II protein by western blot and serum analysis. We detected autophagosome associated with autophagic vesicles by TEM. We observed punctate distribution of LC3II and co-localized with osteoactivin by immunofluorescent. We concluded that osteoactivin contributes with autophagy to control osteoblast differentiation.

Next, we determined the role of osteoactivin on osteoblast differentiation by the autophagy pathways. We demonstrated that addition of TH or R resulted in an increase in osteoblast differentiation and function. We treated MC3T3-E1, DBA/2J GPNMB\textsuperscript{+} and DBA/2J mice with TH or R. Both TH and R treatment enhance ALP staining and activity in MC3T3-E1 and both mice. TH and R upregulated osteoblast related genes (ALP, OCN, OSX, and OA).

We determined the role of osteoactivin on osteoclast differentiation and function by mediating the autophagy pathways. Our data suggest that osteoactivin inhibits osteoclast differentiation but increases its function by mTOR-dependent pathway shown by TRAP staining, activity, and genes expression analysis. R treatment resulted in the
inhibition of RANKL mediated osteoclast differentiation in vitro. Furthermore, R and TH increased RANK-induced osteoclast differentiation and recruitment in vivo using an osteolysis model.

In conclusion, we have shown that osteoactivin act as a positive regulators for osteoblast differentiation, function and bone formation by mediated autophagy (mTOR-independent and dependent) pathways. In addition, osteoactivin is a positive regulator for osteoclast differentiation by mTOR-independent pathway, and positive regulator for osteoclast function by mTOR-dependent pathway.

Future studies will look into the effect of autophagy (mTOR-independent and dependent pathways) on the CD44 knockout mice model. Also we will evaluate the function of autophagy in the CD44 knockout mouse model compared to the transgenic mouse model. Moreover, our future studies will be focused on the treatment of autophagy with osteoactivin as a therapeutic approach for people who suffer from osteoporosis to stimulate bone formation.
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