ABSTRACT

MOUSSA, FOUAD M., Ph.D., December 2016

BIOMEDICAL SCIENCES

THE EPIGENETIC REGULATION OF OSTEOACTIVIN/GPNMB IN BONE CELLS

Dissertation Advisor: Fayez F. Safadi, Ph.D.

Osteoporosis is a devastating disease in elderly patients and postmenopausal women. In present day, there are few treatments available on the market but all have limitations and side effects. Therefore, there is a need for new and more effective therapeutic modalities that stimulate bone formation and inhibit bone resorption. Osteoactivin/GPNMB was first discovered in bone by our lab, where we showed its unique role in stimulating osteoblastogenesis and inhibiting osteoclastogenesis, which may lead to net effect of increased bone mass and volume. Although our lab has published on the role of Osteoactivin/GPNMB in osteoblast and osteoclast, but yet, there are no reports on the epigenetic regulation of Osteoactivin/GPNMB in bone cells.

In this study, we investigated the posttranscriptional regulation of Osteoactivin/GPNMB by miR-150. Our results showed that Osteoactivin/GPNMB is targeted by miR-150 in osteoblast and osteoclast, and lack of miR-150 enhanced the expression of Osteoactivin/GPNMB in both cell types. Our data revealed that deficiency of miR-150 increased osteoblast differentiation and function, whereas this deficiency lead to decreased osteoclast differentiation and function in vitro. Therefore, we propose that miR-150 is a negative regulator for
osteoblast and positive regulator for osteoclast differentiation and function, at least in part, by targeting Osteoactivin/GPNMB.

In our next study, we investigated the epigenetic regulation of Osteoactivin/GPNMB through DNA and histone methylation. Our results showed that Osteoactivin/GPNMB gene is highly methylated at early stage of osteoblast differentiation, and its methylation level decreases as cells progress in differentiation. We also showed that Osteoactivin/GPNMB promoter is occupied by H3K27, which is methylated by Ezh2. Our data showed that inhibition of Ezh2 activity enhanced Osteoactivin/GPNMB expression and osteoblast differentiation and function, represented by increased ALP staining and activity, and mineralization. On the other hand, inhibition of Ezh2 had no effect on Osteoactivin/GPNMB-mutated osteoblast. We also showed that inhibiting Ezh2 activity enhanced bone formation in normal mouse calvarial organ culture, whereas no significant effects were shown in Osteoactivin/GPNMB-mutated calvarial organ culture.

In conclusion, Osteoactivin/GPNMB expression is posttranscriptionally regulated by miR-150 and epigenetically regulated through DNA and histone methylation. Our observations may give new insights or possibilities for potential therapies to treat osteoporosis or stimulate bone formation in cases of fractures. Future studies may focus on investigating the effects of in vivo use of miR-150 and Ezh2 inhibitors in increasing Osteoactivin/GPNMB expression and bone mass.
THE EPIGENETIC REGULATION OF OSTEOACTIVIN/GPNMB IN BONE CELLS

A dissertation submitted
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degree of Doctor of Philosophy

by

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December 2016

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<tr>
<td>GPNMB</td>
<td>Glycoprotein Non-Metastatic B</td>
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<tr>
<td>MSC</td>
<td>Mesenchymal Stem Cell</td>
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<tr>
<td>HSC</td>
<td>Hematopoietic Stem Cells</td>
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<td>RUNX2</td>
<td>Runt Related Transcription Factor</td>
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<td>OSX</td>
<td>Osterix</td>
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<td>ALP</td>
<td>Alkaline Phosphatase</td>
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<td>COL1</td>
<td>Collagen Type I</td>
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<td>Osteocalcin</td>
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<td>Osteoprotegrin</td>
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<tr>
<td>RankL</td>
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<tr>
<td>CTX-1</td>
<td>Carboxy Terminal Collagen Crosslink 1</td>
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<tr>
<td>Ezh2</td>
<td>Enhancer of Zeste Homolog 2</td>
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<tr>
<td>KO</td>
<td>Knockout</td>
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<tr>
<td>WT</td>
<td>Wild Type</td>
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<tr>
<td>BMP</td>
<td>Bone Morphogenetic Protein</td>
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<tr>
<td>PRC</td>
<td>Polycomb Repressive Complex</td>
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<tr>
<td>HMTs</td>
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<td>KDMs</td>
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<td>DNMTs</td>
<td>DNA Methyltransferases</td>
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DEDICATION

I would like to dedicate this work to my family, friends, and those who supported me during the past and present.

To my parents, Moussa and Ghaida for your love, care, help, and support that you gave me through all the years of my life. Although I was away in distance for the past 11 years, you were never far to help me with all that I needed. To my brother and sister, Weam and Ranim, for your love, care, and support.

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Kent, Ohio
Chapter 1

Introduction

Osteoporosis is a common devastating disease in the United States and worldwide, where it leads to bone fractures at multiple sites of the skeletal system, especially at hip, spine and wrist. The estimated number of people with osteoporosis in the United States is to increase from 10 million to more than 14 million people in 2020 [3, 4]. In 2005, more than 2 million fractures were reported in patients with osteoporosis over the age of 50, including: 297,000 hip fractures, 547,000 vertebral fractures, 380,000 wrist fractures, 810,000 fractures at other sites [3]. Based on previous estimation, the annual number of fractures related to osteoporosis is predicted to increase by 50% to more than 3 million by 2025 [3]. In addition to the major health issues that are caused by osteoporosis, there is also a major economic impact, which is represented by $25.3 billion expected annual cost and total of $228 billion for the 10-year period between 2016 to 2025 [3].

The problem of osteoporosis begins with aging from the cellular level, when imbalanced bone remodeling takes place between bone resorbing cells (osteoclasts) and bone forming cells (osteoblasts) [5]. This imbalance is a result
of reduced rate of bone formation due to less osteoblasts proliferation, differentiation, and function, whereas osteoclasts are still functioning well [6].
**Osteoblast**

Osteoblasts are bone forming cells which originate from mesenchymal stem cells (MSCs), the pluripotent cells that differentiate into various type of cells generating different tissues, like bone, cartilage, muscle, and fat tissues [7, 8]. MSCs can differentiate into osteoblasts, fibroblast, and chondrocytes. Differentiation of MSCs into osteoblasts is triggered and regulated by multiple cytokines, transcription factors, and growth factors [9]. Osteoblast differentiation process consists of several stages, they are: cell proliferation, matrix deposition, matrix maturation, and mineralization [10] (Figure 1.1). During proliferation stage, MSCs and pre-osteoblast cells go through mitotic division, and once proliferation ceases, matrix deposition takes place and cells start producing the organic extracellular matrix proteins which mainly consist of type I collagen (COL1). When the unmineralized matrix reaches maturity, Osteoblasts start depositing minerals into the matrix, forming hard mineralized matrix [11, 12]. After full matrix mineralization, osteoblasts may either get trapped within mineralized matrix then become osteocytes, or stay on surface to become bone lining cells, or undergo apoptosis [13-15]. These stages of osteoblast differentiation are regulated by several transcription factors, and the major ones are Runt-related transcription factor 2 (RUNX2) and Osterix (OSX).

Runx2 is a master transcription factor in early stage of osteoblast differentiation, Runx2 deficiency in mice led to osteoblasts deficiency and failure to form mineralized bone in their skeleton which only contain cartilage [16]. By interacting with transcriptional activators and repressors, Runx2 can also positively or negatively regulates osteoblast-specific genes including Collagen
type I (COL1), alkaline phosphatase (ALP), osteopontin (OPN), osteonectin (ON), bone sialoprotein (BSP), and osteocalcin (OCN) [17, 18]. The expression levels of these genes define the stage of osteoblast differentiation, for example, ALP expression is considered as an early marker, while OCN and OPN as late markers of osteoblast differentiation.

In addition to Runx2, OSX is a critical transcription factor for osteoblast differentiation, where OSX is regulated by Runx2 which binds OSX promoter and activates its transcription [19]. As previously reported, OSX expression is directly controlled by Runx2 which acts upstream of OSX in osteoblasts [19]. Runx2 expression is not affected in OSX deficient mice, while OSX expression is lost in Runx2 deficient mice [20].

Osteoblast differentiation is also controlled by bone morphogenetic proteins (BMPs), which are members of transforming growth factor-β (TGF-β) superfamily [21]. BMPs are involved in the induction of osteogenic differentiation and regulation of bone formation. Genetically modified BMP receptors, ligands, and BMP inhibitors have shown the critical role that BMP signaling play in bone formation [22-25]. BMP-2 and BMP-7 recombinant proteins are both approved by the FDA to be used for bone regeneration in spinal fusion surgeries and also frequently used off-label for other orthopedic procedures [26, 27].
Figure 1.1: Schematic diagram representing stages of osteoblast differentiation. Osteoblasts originate from mesenchymal stem cells where they start expressing RUNX2, and OSX. During stages of matrix deposition and maturation, osteoblasts begin forming extracellular matrix (ECM) and expressing markers alkaline phosphatase (ALP) and collagen type I (COL1). In later stage of differentiation, osteoblasts start mineralizing ECM by depositing minerals and expressing gene markers osteocalcin (OCN) and osteopontin (OPN). After mineralization stage, an osteoblast may become osteocyte or bone lining cell, or undergo apoptosis.
Osteoclast

Along with bone formation, there is bone remodeling which is controlled by osteoclasts-multinucleated cells responsible for bone resorption or degradation [28]. Osteoclast originates from hematopoietic stem cells, and is a member of the monocyte-macrophage family [28, 29]. There are two major cytokines that drive osteoclastogenesis, they are: macrophage-colony stimulating factor (MCSF) [30], and receptor activator of nuclear factor-κB ligand (RANKL) [29, 31]. MCSF is responsible for osteoclast precursors proliferation, survival, and differentiation, whereas RANKL is the key cytokine for osteoclast formation [30, 32-35].

Osteoclast differentiation is mainly triggered by RANKL which is mostly produced by osteoblasts and osteocytes. As osteoblasts differentiate and form bone, they release or express RANKL as transmembrane protein which binds to RANK receptor (RANKR) on osteoclast surface to activate its differentiation and trigger bone remodeling. Osteoblasts also release RANKL decoy receptor, osteoprotegerin (OPG), which binds RANKL and inhibits its activity on osteoclast [36]. A previous study reported that OPG deficient mice develop an early osteoporosis condition due to increased level of RANKL to OPG ratio leading to enhanced osteoclasts formation and activity [37].

There are four stages of osteoclast differentiation: proliferation, commitment, fusion, and activation (Figure 1.2). The early stage starts with granulocyte-macrophage colony forming cell, which proliferate and differentiate into committed osteoclast precursor [38]. There are two major transcription factors: PU1 and MITF that control early stage of osteoclast differentiation. These transcription factors stimulate the commitment of these cells into granulocyte-
macrophage colony forming cell lineage which expresses MCSF receptor and c-Fos, which leads to the expression of RANKR [39, 40].

Activation of RANKR by RANKL triggers expression of dendritic cell-specific transmembrane protein (DC-STAMP) and nuclear factor of activated T-cells-1 (NFATc1). DC-STAMP is shown to stimulate the fusion of mononucleated osteoclast precursors leading to formation of multinucleated osteoclasts [41, 42]. NFATc1 on the other hand is shown to stimulate the expression of tartrate-resistant acid phosphatase (TRAP), calcitonin receptor (CTR), and cathepsin K (CTSK) [40, 43-45], which are markers of osteoclast terminal differentiation and activation stage.
Figure 1.2: Schematic diagram representing stages of osteoclast differentiation. Osteoclasts originate from hematopoietic stem cells. At early stage of differentiation, they express PU1 and MITF transcription factors, which stimulate expression of MCSF and RANK receptors, (MCSFR) and (RANKR) during the proliferation and commitment stages. Fusion stage starts in response to RANKL activating RANKR and stimulating expression of DC-STAMP and NFATc1, resulting in formation of multinucleated osteoclasts which express. In later stage of differentiation reaching to active stage, osteoclasts express tartrate-resistant acid phosphatase (TRAP), cathepsin K (CTSK), and calcitonin receptor (CTR).
**microRNAs**

MicroRNAs (miRNAs) are small non-coding RNAs ~23 nucleotides long. These miRNAs with RNA-induced silencing complex (RISC), negatively regulate protein translation by binding to 3'-untranslated regions (3'-UTR) of the target mRNAs, and inhibit translation or trigger mRNAs cleavage [46-48]. The 3'-UTR region has the miRNA antisense complementary, where the protein translation repression is mediated [49]. Studies have shown that a minimum of 6 base pairs match between miRNA and mRNA is sufficient to suppress gene expression [50, 51]. This low number of base pairs match between miRNA and 3'UTR of mRNA, gives one miRNA the ability to target multiple mRNAs of different genes, and one mRNA can also be targeted by multiple miRNAs [52, 53].

miRNAs are generated from long double stranded RNAs, which later are processed into short miRNAs. There are two classes of miRNAs: Intergenic and Intragenic miRNAs which are generated from transcripts of miRNA genes, and transcripts of sequences located within protein coding genes, respectively.

The process of miRNA generation starts within the nucleus with transcript primary miRNA (pri-miRNA), which can be generated from intergenic regions, exonic or intronic sequences [54]. Pri-miRNA then is processed to hairpin shaped premature miRNA (pre-miRNA) by a class 2 ribonuclease III enzyme, called Dorsha. After that, pre-miRNA is exported to the cytoplasm by Exportin-5. Following the export from nucleus to cytoplasm, pre-miRNA is processed into mature miRNA by an enzyme called Dicer. By completion of the maturation process, proteins are recruited to form RISC with one strand of the double
stranded mature miRNA. This one strand of miRNA serves as a guide strand to target complementary sequence at the 3'-UTR of target mRNAs [55] (Figure 1.3).

The biogenesis and function of miRNAs are tightly regulated, and any dysregulation in the process may lead to multiple diseases and disorders, including cancer [56], neurodevelopmental disorders [57], cardiovascular diseases [58, 59], and bone disease [60].
Figure 1.3: Schematic diagram representing miRNA biogenesis and translation inhibition. In the nucleus, miRNA is transcribed by RNA polymerase II in a primary miRNA (pri-miRNA) hairpin which gets cleaved by Drosha. After cleavage, precursor miRNA (pre-miRNA) is formed and translocated to the cytoplasm by exportin 5. In the cytoplasm, pre-miRNA gets digested by Dicer producing miRNA duplex. One strand of the miRNA duplex gets incorporated into RNA-Induced Silencing Complex (RISC) which leads miRNA to the targeted 3’UTR mRNA to repress translation or trigger mRNA degradation.
Inhibition of miRNA Biogenesis and Bone

Multiple studies have reported negative effects on bone homeostasis when disrupting the miRNA biogenesis machinery (Table 1.1). In vivo deletion of Dicer in mouse osteoprogenitors under Col1a1 promoter, resulted in skeletal deformities during development. These deformities were presented by reduced mineralized bone, as a result of defective osteoblasts [61]. When deleting Dicer at later stage of osteoblast differentiation under OCN promoter, mice survived with delayed bone development due to reduced osteoblast numbers [61]. Also Dicer deletion in chondrocytes under collagen type II (Col2a1) promoter, resulted in skeletal defects due to decreased cells proliferation caused by early cell shifting into postmitotic hypertrophic stage [62].

In osteoclast-specific Dicer knockout under CD11b promoter, mice have defective osteoclasogenesis, reduction in multinucleated osteoclast numbers, and increased bone mass [63]. The lack of Dicer prevented the maturity of miR-223, which is normally stimulated by PU1 to suppress nuclear factor I-A (NFI-A) expression. Down regulation of NFI-A is vital for MCSF receptor expression in macrophages in order to differentiate into osteoclasts [63].

miRNAs and Osteoblast

The maintenance of bone homeostasis is within its microenvironment, which depends on cells maintaining precise expression of specific genes [9, 31]. Interestingly, few recent studies have reported about the capacity of individual miRNA to enhance or reduce differentiation and function of cells, like osteoblasts
and osteoclasts. For example, miR-138, miR-204, and miR-143 are shown to inhibit osteogenesis. MiR-138 targets focal adhesion kinase (FAK), a vital factor for osteoblast differentiation. When transfecting human mesenchymal stem cells with anti-miR-138 oligo, increased ectopic bone formation is observed [64]. Runx2 3’-UTR is reported to be a target for miR-204, where retroviral overexpression or transfection of miR-204 oligo in human mesenchymal stem cells, decreases Runx2 protein expression and osteoblast differentiation. On the other hand, the inhibition of miR-204 using anti-miR-204 oligo, results in increased expression of Runx2 and osteoblasts differentiation [65]. The inhibition of miR-143 enhances osteoblast differentiation, leading to increased ALP gene expression and staining. These results are explained by revealing that OSX 3’-UTR is targeted by miR-143 [66].

While the miRNAs mentioned above have negative effects on osteoblast differentiation and function, there are other miRNAs that positively regulate osteogenesis. For example: miR-15b, miR-20a, and miR-181a are shown to promote osteoblast differentiation. miR-15b targets SMAD specific E3 ubiquitin protein ligase 1 (Smurf1), which is a negative regulator for osteoblast differentiation [67, 68]. The Inhibition of miR-15b in human mesenchymal stem cells reduces osteoblast differentiation, which is represented by decreased ALP and Col1a1 gene expression [67].

The positive regulation of BMP2 is very crucial for osteoblast differentiation and function, and this regulation is partially controlled by miR-20a [69]. Overexpression of miR-20a in human mesenchymal stem cells increases
BMP2, BMP4, Runx2, OSX, OCN, and OPN gene expression during osteoblast differentiation; while inhibiting PPARy expression, a negative regulator of BMP2/Runx2 pathway [69].

Transforming growth factor beta (TGF-β) is a member of the BMP superfamily of proteins and negatively regulates osteoblast differentiation [70, 71]. miR-181a is shown to positively regulates osteoblast differentiation by targeting and inhibiting TGF-β-induced and TGF-β receptor [72].

**miRNAs and Osteoclast**

Osteoclast function is also regulated by miRNAs that enhance or reduce its differentiation and function. For example, miR-503 and miR-125a are shown to inhibit osteoclastogenesis [73, 74]. MiR-503 is reported to inhibit osteoclastogenesis by targeting receptor activator of nuclear factor-κB (RANK), where RANK ligand (RANKL) binds and stimulates osteoclast differentiation [73]. The expression of miR-503 is significantly reduced in circulating progenitors of osteoclasts-CD14+ peripheral blood mononuclear cells from postmenopausal osteoporosis patients compared to postmenopausal healthy patients [73]. In vivo silencing of miR-503 using antagonim in ovariectomized (OVX) mice increases RANK expression leading to stimulation in bone resorption, whereas overexpressing miR-503 using agomir inhibits bone resorption by osteoclast [73].

Inhibiting TNF receptor-associated factor 6 (TRAF6), a signaling transduction factor for RANKL/RANK/NFATc1 [75, 76], inhibits osteoclast differentiation [74], where TRAF6 deficient mice develop osteopetrotic bone due
to defected osteoclasts [77]. miR-125a is shown to target TRAF6, which lead to a failure in RANKL/RANK/NFATc1 signal transduction and result in the inhibition of osteoclastogenesis [74].

There are also miRNAs that enhance osteoclastogenesis by targeting specific mRNAs that inhibit osteoclast differentiation. For example, miR-21 and miR-223 are shown to positively regulate osteoclast differentiation. Silencing miR-21 in bone marrow-derived macrophages (BMMs) using antisense miR-21 containing lentivirus particles, inhibits their differentiation into osteoclasts. Inhibition of miR-21 results in up-regulation of programmed cell death 4 (PDCD4) protein levels, down-regulation of RANKL induced c-Fos phosphorylation and NFATc1 and Cathepsin K, which lead to inhibition of osteoclast differentiation and function [78].

The expression of miR-148a increases during differentiation of circulating CD14+ peripheral blood mononuclear cells (PBMCs) into osteoclasts induced by MCSF and RANKL. While overexpression of miR-148a in CD14+ PBMCs promotes osteoclastogenesis, its inhibition reduces osteoclast differentiation. The positive regulation of osteoclastogenesis by miR-148a is due to the inhibition of V-maf musculoaponeurotic fibrosarcoma oncogene homolog B (MAFB) protein expression, a transcription factor that negatively regulates RANKL-induced osteoclastogenesis [79].
### Table 1.1: miRNAs involvement in osteoblast and osteoclast

<table>
<thead>
<tr>
<th>miRNAs</th>
<th>Function</th>
<th>Target</th>
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<td>miR-138</td>
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**miR-150**

It has been reported that miR-150 plays a role in regulating multiple proteins expression in different type of cells and tissues, affecting cells development and controlling diseases [107-112].

miR-150 is highly expressed during the maturation of B cells, where its highest expression is at early stage of B cell development, and decreases as cells progress into maturity. Overexpressing miR-150 in hematopoietic stem cells (HSCs), then transplanting them into bone marrow leads to impaired B cells maturation. Furthermore, early expression of miR-150 during B cells differentiation, inhibits the transition from pro-B to pre-B stage [107]. This inhibition of B cells development by miR-150 is linked to the down-regulation of c-Myb protein [108]. c-Myb is a critical transcription factor for lymphocytes development, where it is highly expressed in lymphocyte progenitors, downregulated upon maturation, then upregulated after activation of mature cells [113, 114]. The specific deletion of c-Myb gene in B cells results in severe inhibition of B cells development from pro-B to pre-B transition, and lack of mature B cells in the animal [114]. On the other hand, in vivo deletion of miR-150 results in 4-fold and 2-fold expansion in splenic and peritoneal cavity B cells numbers, respectively [108].

**miR-150 and Cancer**

Several studies have reported the critical role that miR-150 plays in colorectal cancer [109, 115, 116], considering miR-150 as a potential biomarker
for diagnosis, prognosis, and responding to therapy, where patients with low expression of miR-150 have poor survival outcome compared to patients with higher expression [115]. Using human colorectal cancer cell lines (LoVo), study showed that c-Myb is targeted by miR-150, where c-Myb is known to be a promoting factor for cell proliferation, survival, and metastasis [117, 118]. Overexpressing miR-150 (miR-150 mimic) in colorectal cancer cells down-regulates c-Myb and represses cell proliferation, migration, and invasion compared to cells with miR-150 inhibitor. In vivo experiments using tumor xenograft model with miR-150 mimic or inhibitor transfected colorectal cancer cell lines, shows similar results as in vitro, where animals injected with miR-150 mimic have less tumor growth compared to animals injected with miR-150 inhibitor [109].

In contrast to the role that miR-150 plays in colorectal cancer, it has been reported that miR-150 acts as an oncogene in lung cancer by targeting v-src avian sarcoma (Schmidt-Ruppin A-2) viral oncogene homolog (Src) kinase signaling inhibitor 1 (SRCIN1) [119]. Src is known to be part of multiple signaling pathways that lead to increased proliferation, migration, and invasion of different types of cancer cells [120, 121]. On the other hand, SRCIN1 has recently been identified as a tumor suppressor gene which inactivates Src in tumor cells [122, 123]. Expression of miR-150 in samples from patients with lung cancer is shown to be significantly higher than non-cancerous tissue samples, whereas SRCIN1 expression in cancerous samples is significantly less when compared to non-cancerous cells. By transfecting miR-150 mimic or inhibitor into A549 and H1975
human lung adenocarcinoma epithelial cell lines, SRCIN1 expression showed significant reduction and increase, respectively [119].

**DNA Methylation**

DNA methylation is an essential epigenetic modification to regulate gene expression during embryonic development and cell differentiation [124-126]. This process involves the transfer of a methyl group from methyl group donor, S-adenosyl-L-methionine (SAM), to the carbon-5 position in the cytosine ring by DNA methyltransferase (DNMTs) enzymes [125] (Figure 1.4).

There are two components that structure the DNA methylation machinery, they are: DNMTs, which are responsible to establish and maintain DNA methylation patterns, and methyl-CpG binding proteins (MBDs), which are involved in reading methylation marks [127-129]. The DNMTs family is comprised of: DNMT1, DNMT2, DNMT3A, DNMT3B, and DNMT3L. DNMT1 methylates hemimethylated DNA to copy DNA methylation patterns to the daughter strands during DNA replication [130-132], whereas DNMT2 is a methyltransferase homolog which does not methylate DNA, but specifically methylate cytosine-38 in the anticodon loop of aspartic acid transfer RNA (tRNAAsp) [133]. In contrast to DNMT1, DNMT3A and DNMT3B target unmethylated CpG to perform de novo methylation during development [125]. While DNMT3A and DNMT3B are responsible for de novo methylation, DNMT3L has no catalytic activity, but it assists in the process by enhancing DNMT3A and DNMT3B ability in binding to the methyl group donor, SAM, and stimulates their activities [134, 135]. As each
of these DNMTs has a specific function, they also cooperate with each other, for example, DNMT3A and DNMT3B are involved in maintenance methylation during replication [136], and DNMT1 is involved in de novo methylation of genomic DNA [137].

In mammals, the majority of DNA methylation occurs on cytosine bases located 5’ to guanine bases, a configuration referred to as CpG islands [138]. These islands are associated with about 70% of genes promoters in vertebrates [139], where their methylation can inhibit the binding of transcription factors to DNA promoters resulting in transcriptional repression [129]. Upon methylation of CpG islands, methylated DNA recruit MBDs which bind to methylated CpGs and repress transcription, either by disrupting the formation of RNA polymerase complex, or recruiting chromatin modifiers, which lead to chromatin condensation and gene silencing [140, 141].

These unique modifications of genes, if not regulated correctly, it may result in negative outcomes. For example, disorders such as Angelman syndrome and Prader-Willi syndrome are caused by abnormal DNA methylation patterns during development [142]. Also in cancer, altering DNA methylation patterns of PU.1 promoter can lead to lymphoma and leukemia [143, 144].

**DNA Methylation in Bone Cells**

Several studies have shown the effects of DNA methylation on bone cells-specific genes, where methylation levels of genes vary depending on the stage of differentiation [145, 146]. In osteoblast, CpG island of alkaline phosphatase
(ALP) proximal promoter region is least methylated at early stage of differentiation. This low methylation level (hypomethylated) of ALP promoter is oppositely displayed in osteocyte, where ALP gene is highly methylated (hypermethylated) [145]. The methylation level of ALP promoter increases as osteoblasts differentiate and become bone lining cells. At this point, this methylation level is reached to an intermediate stage between the methylation levels in osteoblasts and osteocytes [145]. Also, the transition of osteoblasts toward osteocytes includes a gradual increase in expression of osteocytes specific genes, like sclerostin (SOST). As transition progress, the methylation level of SOST promoter decreases, allowing SOST to be expressed [147].

The importance of DNA methylation during osteoblasts differentiation is also shown by the regulatory effects it has on Wnt signaling. For example, in the case of receptor tyrosine kinase-like orphan receptor 2 (Ror2), a specific receptor for Wnt5a, the methylation level of Ror2 promoter decreases significantly during the differentiation of human bone marrow stem cells to osteoblasts in vitro [148]. DNA methylation in osteoblast is reported to be influenced by the treatment of BMP-2, where treating C2C12 myoblast cell line with BMP-2 results in hypomethylation the distal-less homeobox 5 (Dlx5) gene promoter and induction of osteogenesis [149].
Figure 1.4: Schematic diagram shows the effects of DNA methylation on gene transcription. During activated gene transcription state, CpG islands are not methylated and transcription factors can reach and bind to promoters (A). In case of Inactivated gene transcription state, CpG islands are methylated by DNA methyltransferase (DNMT) which catalyzes the transfer of -CH$_3$ from the methyl group donor S-Adenosyl-Methionine (SAM) onto cytosine. This methylation of CpG islands prevents transcription factors from binding to promoters and activating gene transcription (B).
**Histone Modifications**

The repeated nucleosomes of chromatin consist of packaged DNA and histones, H2A, H2B, H3, and H4. Modifications of histones usually occur on their extended tails, where specific amino acids go through methylation, acetylation, ubiquitination, or phosphorylation. These modifications can lead to an inhibition or activation of gene transcription depending on the site that was modified. For example, methylation of lysine (K), whether monomethylation (me1), dimethylation (me2), or trimethylation (me3) can enhance or prevent the accessibility for transcription factors to the genes and to activate transcription. Histone 3 for example, can be trimethylated at lysine 4, 36, and 79 (H3K4, H3K36, and H3K79) which lead to transcriptional activation, whereas trimethylation at lysine 9 and 27 (H3K9 and H3K27) causes transcriptional inhibition [150, 151] (Figure 1.5).

There are three families of enzymes that are known to be responsible for catalyzing the transfer of methyl group from methyl donor, S-adenosyl-methionine, into histones [152]. They are: SET-domain-containing proteins [153] and DOT1-like proteins which methylate lysine [154], and protein arginine N-methyltransferase (PRMT) which methylates arginine [155]. Histone methylation can be reversed by members of the two families of histone demethylation enzymes, they are: amine oxidase LSD1 (KDM1), and Jumonji (JmjC) domain protein [156].

Histone acetylation and deacetylation is mainly regulated by two families of enzymes, histone acetyltransferases (HATs) and histone deacetylases
(HDACs) [155]. The activities of HATs and HDACs are regulated through their phosphorylation, and also by other modifiers. For example, in response to DNA damage, HAT gets phosphorylated and regulates the activity of activating transcription factor 2 (ATF2) [157].

Polycomb group (PcG) protein complexes are crucial enzymes for maintaining and regulating chromatin structure and histone methylation. Polycomb repressive complex 1 (PRC1) and PRC2 are the two major protein complex families that is formed by the association of PcG proteins. These complexes are known contributors to chromatin compaction, and they are very crucial during development, cell proliferation, and differentiation [158-160]. PRC1 complexes have E3 ligase activity where it catalyzes monoubiquitination of histone 2A at lysine 119 (H2AK119ub1), whereas PRC2 complexes have methyltransferase activity to dimethylates or trimethylates H3K27me2/3 [161]. Each complex contains core components that are essential for the complex function. In PRC1, E3 ubiquitin ligase Ring1B and one polycomb group of ring finger protein are the core components for this complex. On the other hand, PRC2 core components are suppressor of zeste 12 (Suz12), embryonic ectoderm development (Eed), and enhancer of zeste 1 or 2 (Ezh1/2), the methyltransferase subunit of this complex [158].

Ezh1 and Ezh2 are shown to function in complementary manner, where Ezh1 is mostly present in non-dividing cells and adult tissues, whereas Ezh2 is highly expressed in proliferating cells and embryonic tissues [162-166]. PRC2 is known to efficiently methylates H3K27 with Ezh2, whereas the methyltransferase
activity is minor with Ezh1 [164, 165]. The methylation of H3K27 by Ezh2 is recognized and bound by PRC1 complex to enhance transcriptional repression [167], therefore, disruption of PRC2 complex by deletion of any of its subunits leads to decreased levels of H3K27 di-methylation and tri-methylation (H3K27me2 and H3K27me3) [164, 168-172].

**Ezh2 and Osteoblasts**

The importance of Ezh2 for bone development was shown by the study of Ezh2 conditional knock out in mice uncommitted MSCs under Prx1 promoter. This conditional loss of Ezh2 in mice resulted in defects in skeletal development and bone formation, where mice are significantly smaller in size with shortened limbs, craniosynostosis, and clinodactyly [173]. This study also showed that inhibition of Ezh2 using siRNA stimulates osteoblastogenesis in human MSCs, where cells show significant increase in RUNX2, osteopontin (OPN) and osteocalcin (OCN) gene expression. On the other hand, knockdown of Ezh2 inhibits adipogenesis with significant reduction in peroxisome proliferator-activated receptor gamma (PPARγ) and adipsin gene expression [174]. Furthermore, overexpressing Ezh2 using retroviral transduction in human MSCs results in inhibition of osteoblasts differentiation and function represented by downregulation of RUNX2, OPN, and OCN, and less mineral deposition. This overexpression of Ezh2 enhances adipocytes differentiation, where PPARγ and adipsin gene expression are significantly increased and higher oil red uptake [174, 175]. Ezh2 is shown to directly repress Wnt 1, 6, 10a, and 10b genes in
preadipocytes. This repression of Wnt genes downregulates Wnt/β-catenin signaling allowing increased expression of PPARγ, the master regulator of adipogenesis, and resulting in enhanced adipocytes differentiation [175].

Interestingly, exposure to irradiation lead to enhanced expression of Ezh2 which correlated with significant loss in bone mass and volume in rats [176]. After two weeks of exposing four-month-old rats to 20 Gy radiation, it was reported that there was a significant reduction in osteoblasts differentiation and function in vivo represented by decreased expression of BMP2 and RUNX2 at two and twelve weeks after exposure [176].

The regulation of osteoblast differentiation by Ezh2 is also linked to anti-differentiation noncoding RNA (ANCR) which is known to be required to maintain the undifferentiated cell state within the epidermis [177]. The inhibition of ANCR using siRNA in human fetal osteoblastic cell line results in enhanced osteoblasts differentiation with increased RUNX2, ALP and OCN genes expressions. This study also showed that ANCR recruits Ezh2 to catalyze the methylation of H3K27 on RUNX2 promoter leading to inhibition of RUNX2 expression and osteoblast differentiation [177].

It has been shown that methylation of H3K27 by Ezh2 can be reversed by lysine demethylase 6A (KDM6A), which removes methyl groups from H3K27 allowing active chromatin state and genes to be expressed [174]. This group has shown that overexpression of KDM6A gene in human mesenchymal stem cells, and transplanting these cells subcutaneously into the dorsal surface of
immunocompromised mice lead to bone formation, whereas Ezh2 overexpressed cells showed no bone formation [174].
Figure 1.5: Schematic diagram shows the effects of histone methylation and acetylation on chromatin modifications and gene transcription.

Histone deacetylation by histone deacetylases (HDACs) followed by methylation of lysine 9 and 27 on histone 3 (H3K9me3 and H3K27me3) by histone methyltransferases (HMTs), lead to chromatin modifications forming inactive chromatin structure which prevents gene transcription (A). Histone lysine demethylases (KDMs) remove methyl group from lysine allowing histone acetyltransferases (HATs) to add acetyl group to lysine allowing chromatin modifications to form active chromatin structure with active gene transcription (B).
**Osteoactivin/GPNMB**

Osteoactivin/Glycoprotein Non-Metastatic Melanoma Protein B (GPNMB) was first discovered in osteopetrotic rat model, where the animal has high expression level of Osteoactivin/GPNMB correlated excessive bone mass and volume [178]. Osteoactivin/GPNMB is known to have high homology for Dendritic Cell Heparin Sulfate Proteoglycan Integrin Dependent Ligand (DC-HIL), Human Hematopoietic Growth Factor Inducible Neurokinin (HGFIN), and Pmel-17 melanocyte protein [2]. Osteoactivin/GPNMB is 115 kDa transmembrane protein (~572 amino acids) which is heavily glycosylated and contains several functional domains. They are: polycystic kidney disease domain (PKD), proline-rich repeat domain (PRRD), transmembrane domain, dileucine signal, and signal peptide [2, 179] (Figure 1.6).

In cancer, Osteoactivin/GPNMB is shown to be highly expressed in glioma human cancer model, where it triggers MMP-3 and MMP-9 expression and induces metastasis [180]. Furthermore, Osteoactivin/GPNMB is also highly expressed in breast cancer cells which metastasize to bone. In this published study, the group developed breast cancer cells that overexpress Osteoactivin/GPNMB, which resulted in enhanced formation of osteolytic bone metastasis in vivo [181, 182]. Several studies also showed the involvement of Osteoactivin/GPNMB in other types of cancer, including colon [183], brain [184], melanoma [185-187], lung [188], pancreas [189], liver [190, 191], and prostate [192].
It has also been shown that Osteoactivin/GPNMB acts as neuroprotective factor during neurodegenerative disease and injuries. For example, in Amyotrophic Lateral Sclerosis (ALS) disease, Osteoactivin/GPNMB attenuates neurotoxicity of mutated superoxide dismutase and rescue motor neurons [193]. Also in cases of cerebral ischemia reperfusion injury, Osteoactivin/GPNMB has a neuroprotective effect, where after injury, its expression increase and enhance phosphorylation of ERK1/2 and Akt [194].

As anti-inflammatory agent, Osteoactivin/GPNMB expression is shown to be increased after injury in brain [193-195], kidney [196, 197], muscle [198, 199], and liver [200, 201]. The overexpression of Osteoactivin/GPNMB in transgenic rat liver, Osteoactivin/GPNMB attenuated the development of hepatic fibrosis in association with downregulation of genes involved in this pathological state [200]. Also in muscle, Osteoactivin/GPNMB is shown to protect skeletal muscle from degeneration caused by muscle denervation in mice [198]. By increasing the infiltration of fibroblasts and expression of matrix remodeling genes MMP-3, MMP-9, and Col1, Osteoactivin/GPNMB was shown to be involved in the regulation of matrix remodeling [199].

**Osteoactivin/GPNMB and Bone Cells**

The initial work to investigate the role of Osteoactivin/GPNMB in bone was first started in osteoblasts [178, 202]. One of the first studies on Osteoactivin/GPNMB showed the importance of this protein as an essential molecule for osteoblasts maturation, where adding anti-Osteoactivin/GPNMB
antibody in cell culture resulted in the inhibition of osteoblasts differentiation and function [203]. On the other hand, overexpressing Osteoactivin/GPNMB in MC3T3-E1 and C2C12 cell lines results in enhanced osteoblasts differentiation and function [2, 204]. Osteoactivin/GPNMB is also shown to act as matricellular protein to enhance osteoblast adhesion and differentiation. This study demonstrates that coating culture plate surface with recombinant Osteoactivin/GPNMB results in increased osteoblast adhesion through the interaction with αvβ1 integrin and heparin sulfate proteoglycans (HSPG) [205].

In osteoblast, the transcriptional regulations of Osteoactivin/GPNMB has been reported in previous studies [206]. Osteoactivin/GPNMB is shown to act as downstream mediator of BMP-2 through the Smad-1 signaling pathway in rats primary osteoblasts, where the inhibition of Smad-1 using siRNA results in reduced Osteoactivin/GPNMB expression [207]. Another study has shown that Osteoactivin/GPNMB gene transcription is up-regulated by BMP-2-induced homeodomain transcription factors, Dlx3 and Dlx5, which get recruited to Osteoactivin/GPNMB promoter during osteoblast differentiation [206].

**Osteoactivin/GPNMB and Animal Models**

Several animal models are used for studying Osteoactivin/GPNMB in bone. These animal models are: Osteoactivin/GPNMB mutant (D2J), Osteoactivin/GPNMB transgenic (OA-Tg) [208], and Osteoactivin/GPNMB knockout (OA-KO). The mutated mice (D2J) are known to have nonsense mutation in the Osteoactivin/GPNMB gene sequence inducting an early stop
codon, and generating non-functional truncated protein sequence of 150 amino acids (Figure 1.6), instead of full length 562 amino acids [209]. These mice are known to develop hearing loss at time of weaning and glaucoma later when aged [210, 211]. Characterization of the skeletal phenotype of mutated mice showed that these animals have significant reduction in bone mass and volume, and an impairment of osteoblast differentiation and function in vitro and in vivo [209].

Osteoactivin/GPNMB is also shown to be important for osteoclasts differentiation, where the addition of anti-Osteoactivin/GPNMB antibody into osteoclasts culture results in inhibition of cell differentiation through integrin β3 and β1 [212]. Interestingly, ex vivo examination of Osteoactivin/GPNMB mutant osteoclasts showed that these cells differentiate more and have larger size than wild type cells, but they have impaired function [213].

Further evidence that shows the importance of Osteoactivin/GPNMB for bone formation, is the skeletal characterization of Osteoactivin/GPNMB transgenic mouse model. This animal model overexpresses Osteoactivin/GPNMB under the CMV promoter, which results in an increased bone mass and volume in vivo and osteoblasts differentiation ex vivo [208]. On the other hand, deletion of Osteoactivin/GPNMB gene, results in reduction in osteoblasts differentiation and function ex vivo (data not published).

**Osteoactivin/GPNMB and miRNAs**

Further studies have shown that Osteoactivin/GPNMB is targeted and regulated by two miRNAs. They are: miR-150 and miR-508. As mentioned
previously about the role of miR-150 in B cells development and cancer, it is also shown that miR-150 is involved in bronchopulmonary dysplasia disease by targeting Osteoactivin/GPNMB [214, 215]. Bronchopulmonary dysplasia is a chronic lung disease of premature infants caused by ventilation and oxygen toxicity, which results in complications of impaired alveolarization and vascularization of the lung [214]. In vivo experiments in neonatal rats, show that exposure to 95% oxygen in bronchopulmonary dysplasia condition, enhances the expression of Osteoactivin/GPNMB and inhibits miR-150 expression [214]. The expression levels of Osteoactivin/GPNMB in the lungs of normal and miR-150 knockout (KO) mice is the same, but its expression is significantly increased in the lungs of miR-150 KO mice after 6 days of hyperoxia exposure compared to the levels in normal mice. Interestingly, miR-150 KO neonates show more resistance to hyperoxia injury than normal mice. This resistance is possibly linked to the increased expression of Osteoactivin/GPNMB which has been reported to play a role in tissue protection by promoting autophagy, that is involved in cytoprotection during hyperoxia injury [215-217]. On the other hand, there is an increase in vascularity and minimal inflammation in miR-150 KO neonates compared to wild type littermates, due to high level of Osteoactivin/GPNMB expression which stimulates angiogenesis [218, 219].

Beside miR-150, miR-508 is also shown to target Osteoactivin/GPNMB [220]. miR-508 is reported to play a role in inhibiting the progression of glioma by targeting Osteoactivin/GPNMB, where miR-508 expression levels are shown to be lower in glioma cell lines and tissue compared to normal human astrocyte cell
line or tissues [220]. Furthermore, overexpression of miR-508 is shown to decrease glioma cell and tumor growth in vitro and in vivo, while downregulating the expression of Osteoactivin/GPNMB. The progression of pre-miR-508-transfected glioma cell growth is shown to be restored when cells are co-transfected with Osteoactivin/GPNMB overexpression vector that lack the miR-508 binding sequence and allowing more expression of Osteoactivin/GPNMB in these cells [220].

**Osteoactivin/GPNMB and Methylation**

There have been no previous reports on Osteoactivin/GPNMB DNA or histone methylation in bone cells, however, there is one study reported that Osteoactivin/GPNMB DNA methylation is linked to colorectal cancer [221].

This study showed that there is a methylation differences in Osteoactivin/GPNMB gene in patients with colorectal cancer from two populations, Iranians and African Americans. Results from this study revealed that there is a significant increase in Osteoactivin/GPNMB gene methylation in African American patients compared to Iranians, which explain that Osteoactivin/GPNMB might play a role in the higher incidence and aggressiveness of colorectal cancer in African American patients [221].
Figure 1.6: Schematic Diagram of the full length protein and mutant Osteoactivin/GPNMB. The full length Osteoactivin/GPNMB consists of 574 amino acids and has multiple domains. The extracellular domain of Osteoactivin/GPNMB contains: signal peptide (1 – 22), RGD domain, polycystic kidney disease domain (PKD, 256 – 316), proline rich repeat domain (PRRD, 320 – 345). The transmembrane domain (TM, 503 – 523). The intracellular domain contains ITAM motif and Di-leucine motif [1, 2].
Specific Aims

Although the role and signaling mechanism of Osteoactivin/GPNMB has been investigated in both osteoblast and osteoclast in two different animal models, the post-transcriptional and epigenetic regulation of Osteoactivin/GPNMB mRNA and DNA and histone methylation of the Osteoactivin/GPNMB gene have not yet been studied. In this study, we investigated the post-transcriptional regulation of Osteoactivin/GPNMB mRNA mediated by miR-150, and the gene epigenetic regulation through DNA and histone methylation.

Our hypothesis is: Osteoactivin/GPNMB expression is post-transcriptionally and epigenetically regulated by miR-150, DNA and histone methylation during osteoblast differentiation.

Aim I: To determine the effects of miR-150 on bone homeostasis by targeting Osteoactivin/GPNMB

We will test the hypothesis that miR-150 regulates bone mass in vivo, and osteoblast and osteoclast differentiation and function ex vivo, at least in part, by targeting Osteoactivin/GPNMB mRNA

- We will characterize the bone phenotype of miR-150 KO mice compared to C57BL/6 (WT) mice
- We will characterize the differentiation and function ex vivo of osteoblasts and osteoclasts derived from miR-150 KO mice and compared to osteoblasts and osteoclasts derived from WT
Aim II: To determine the epigenetic regulation of Osteoactivin/GPNMB expression during osteoblastogenesis

- We will test the hypothesis that Osteoactivin/GPNMB is methylated in MSC and the state of DNA and histone methylation is reduced during osteoblast differentiation.

- We will test the hypothesis that Osteoactivin/GPNMB expression level is regulated by methylation of lysine 27 in histone 3 (H3K27me3) by EZH2 during osteoblast differentiation.
Chapter 2

A Novel Role of miR-150 in Bone Homeostasis

Introduction

Osteoporosis is a debilitating bone disease, due to an imbalance of bone remodeling and bone formation [222]. Patients with osteoporosis have higher rates of bone resorption resulting in lower bone mass, thus, increasing the risk of fracture [223]. Furthermore, patients with osteoporosis have a reduced rate of bone formation. The decreased bone formation by osteoblast is due to the reduction in osteoblast cell proliferation, differentiation or function [6].

Osteoblasts (OBs) originate from mesenchymal stem cells (MSCs), which have the tendency to differentiate into osteoblasts, adipocytes or chondrocytes [224]. Osteoblasts are responsible for the bone matrix maturation and minerals deposition. As an early marker of differentiation, osteoblasts express Runt-Related Transcription Factor 2 (Runx2) and alkaline phosphatase (ALP), then deposit calcium phosphate minerals into matrix at a later stage of differentiation [11, 12]. On the other hand, Osteoclasts (OCs) are multinucleated cells that originate from hematopoietic stem cells (HSCs). These cells are responsible for bone resorption and remodeling by forming a sealing zone at the area that need
to be resorbed, and then secreting acidic enzymes within the sealing zone and degrade bone matrix [28, 29].
Osteoactivin/glycoprotein nonmetastatic melanoma protein B (Osteoactivin/GPNMB) is a type I transmembrane protein expressed in bone. This protein was first identified by our group in natural mutant osteopetrotic rats [178]. Osteoactivin/GPNMB has also been identified in other tissues and cell types, referred to as mouse dendritic cell associated heparan sulfate proteoglycan dependent integrin ligand (DC-HIL) in dendritic cells [225, 226]. Previously, our lab has shown that Osteoactivin/GPNMB plays a crucial role in osteoblast differentiation and function. Overexpression of Osteoactivin/GPNMB was shown to enhance osteoblast differentiation and function, while inhibition of Osteoactivin/GPNMB reduced alkaline phosphatase staining and activity and mineral deposition [208, 209, 225, 226]. Osteoactivin/GPNMB mRNA and protein expression were also shown to increase during osteoblast differentiation [209]. On the other hand, Osteoactivin/GPNMB was also shown to play a role in osteoclast differentiation and function. Previously our group reported that ex vivo differentiated osteoclasts from D2J mice that have non-functional truncated Osteoactivin/GPNMB protein were more in number and larger in size compared to normal osteoclasts [213]. Interestingly, these larger osteoclasts showed impaired resorption compared to normal osteoclasts [213], indicating that Osteoactivin/GPNMB negatively regulates osteoclast differentiation but positively regulates its function.

MicroRNAs (miRNAs) are small noncoding 22 nucleotide bp long single stranded RNA that bind to the 3’ untranslated region (3’-UTR) of the target mRNA, resulting in inhibition of mRNA translation into the protein by degradation of the
target mRNA [46, 47, 49, 50]. This inhibition of mRNA translation by miRNAs can have a major effect on cell proliferation, differentiation, and function. Multiple miRNAs have been reported to play a role in bone cell differentiation and function. Several miRNAs have previously been shown to inhibit osteoblast differentiation such as miR-34a, miR-204, and miR-214 [65, 227, 228]. MiRNAs have also been shown to play a role in osteoclast differentiation and function. Certain miRNAs such as miR-26a and miR-124 have been shown to attenuate osteoclast differentiation [102, 229]. Previous studies have shown that miR-150 targets Osteoactivin/GPNMB in a model of angiogenesis during hyperoxia-induced neonatal lung injury [214, 215]. Since miRNA150 has been shown to regulate Osteoactivin, we believe that this miRNA may play a role in bone homeostasis.

In this study, we investigate the role of miR-150 in osteoblasts and osteoclasts differentiation and function in vivo and ex vivo by targeting Osteoactivin/GPNMB. Our results show that miR-150 KO mice have decreased bone mass compared to WT mice, for ages 4, 8, and 16 weeks old as shown by micro-CT analysis. Interestingly, our findings demonstrate that miR-150 deficiency in MSCs and osteoclast progenitors in ex vivo cultures enhanced osteoblastogenesis and decreased osteoclastogenesis, respectively. Based on our results, we show that miR-150 is a negative regulator for osteoblastogenesis and positive regulator for osteoclastogenesis.

Taken together, miR-150 can be utilized for therapeutic strategies to either enhance bone formation or reduce bone resorption in osteopathology conditions.
Materials and Methods

Mice

Wild type (WT) (C57BL/6J) and miR-150 KO (Mir150tm1Rsky, stock no: 007750) mice were purchased from the Jackson Laboratory. Mice colonies were housed and maintained in cages at Northeast Ohio Medical University in a facility accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC). Animals were kept in 21°C environment with 12-hour light-dark cycle. All studies associated with mice are approved by the institution IACUC at NEOMED.

Micro-CT

Male mice femurs derived from ages 4, 8, and 16 weeks old from WT and miR-150 KO mice (n ≥ 4 per group) were scanned using SkyScan 1172 high-resolution microtomography (micro-CT) system (Bruker). All scanned images were reconstructed into 3-dimensional images were regenerated using SkyScan NRecon software. Analysis was performed on trabecular bone starting at 400 µm below the distal growth plate of femur at a resolution of 7.6 µm. All analyses were measured and calculated using SkyScan CT analyzer software (Bruker).

Biochemical Analysis

Serum samples were collected from WT and miR-150 KO male mice at 8 and 16 weeks of age (n ≥ 4 per group). Serum RANKL, OPG (R&D Systems), and CTX-1 (Bioassay Systems) were measured using ELISA based on the
manufacturer’s instructions. All ELISA data were normalized to the total µg protein concentration per sample using a Pierce bicinchoninic acid protein assay kit (Thermo Fisher Scientific).

**Tissue Preparation and Bone Histomorphometry**

Distal femurs from WT and miR-150 KO male mice at age 4 weeks, were collected, fixed at 4% paraformaldehyde, dehydrated, and embedded undecalcified in plastic methylmethacrylate (MMA) resin. Femurs were sectioned at 5 µm thick sections. Quantitative histomorphometric analysis was performed in an area located at 100 µm to 600 µm proximal to the growth plate, using OsteoMeasure software version 3.2.1 (Osteometrics) (n ≥ 3 per group). Brightfield images were taken using microscope Nikon Eclipse Ti inverted microscope (Nikon).

For kinetic histomorphometry, we used 7 weeks old WT and miR-150 KO male mice were injected twice with 10mg/kg of calcein AM (Santa Cruz Biotechnology), five days apart between the two injections, then animals were sacrificed after seven days of the first injection. Femurs were collected, fixed at 4% paraformaldehyde, dehydrated, and embedded undecalcified in plastic methylmethacrylate resin. Femurs were sectioned at 5 µm thick, then samples were analyzed with transmitted and epifluorescence microscopy using a Nikon Eclipse Ti inverted microscope (Nikon). Mineral apposition rate (MAR) was calculated using average double labeled surface per day, and bone formation rate (BFR) was calculated as MAR × mineralizing surface (MS).
**Proliferation, Survival and Apoptosis**

MSCs and Osteoclast progenitors were collected from femurs and tibias of WT and miR-150 KO male mice at ages between 4 and 8 weeks. Cells were cultured in 96-well plate at density of $10 \times 10^3$ cells per well and cultured in minimum essential medium $\alpha$ (α MEM) supplemented with 10% FBS with 20 ng/ml MCSF for Osteoclast progenitors for 48 and 72 hours, then measured proliferation using CyQUANT NF cell proliferation assay (Invitrogen). For measuring survival, cells were plated similar to above but with 2% serum medium then measured using Cell Counting Kit-SK (Dojindo Molecular Technologies) for cell survival were performed.

For assessing cell apoptosis, MSCs were cultured in 96-well plate at density of $10 \times 10^3$ and serum starved for 72 hours, then cell apoptosis was measured using SensoLyte Homogeneous AFC Caspase - 3/7 Assay Kit (Ana Spec, Fremont).

**Alkaline Phosphatase Staining and Activity**

Osteoblast cultures maturation were determined by ALP staining and activity. ALP staining was performed for osteoblasts differentiated from mesenchymal stem cells at day 7. By using ALP staining kit (Sigma-Aldrich) and according to the manufacturer’s instructions, ALP-positive cells were stained in purple color. On the other hand, ALP activity was determined in total cell lysates using an ALP activity kit (BioAssay Systems) according to the manufacturer’s protocol. In brief, 50 μL of cell lysate was added to 50 μL of working solution, then
absorbance was measured at 405 nm after 30 minutes’ incubation at room temperature. ALP activity was calculated in enzyme units (U = μmol/L) per μg protein.

**Von Kossa Staining**

Osteoblast cultures mineralization were determined by Von Kossa staining at day 14, where cells were differentiated from mesenchymal stem cells. Osteoblast cultures were stained with 5% silver nitrate solution under UV light for 30 minutes, then washed with water. Hydroxyapatite crystals of mineralized nodules were stained black and fixed with 5% sodium thiosulfate.

**Osteoblast Differentiation and Function**

Bone marrow cells were flushed and collected from femurs and tibias of WT and miR-150 KO male mice of age between 4 and 8 weeks. Adherent cells were incubated to proliferate in 10 cm dish. When cells reached confluency, they were trypsinized, counted, and re-plated at a density of 5 x 10⁵ cells and at 1 x 10⁵ cells in 6-well and 24-well plates, respectively. Cells were cultured differentiated in minimum essential medium α (α MEM) supplemented with 10% FBS, 50 μg/ml ascorbic acid, 10 mM β-glycerophosphate, and 10 nM dexamethasone. Cell cultures were terminated on day 7 and 14 for RNA isolation, and stained for alkaline phosphatase and minerals on day 7 and 14, respectively.
**Osteoclast Differentiation and Function**

Femurs and tibias were flushed with minimum essential medium α supplemented with 10% FBS, 1% penicillin streptomycin, and 0.1% Amphotericin. Cells were centrifuged in 4°C at 1200 rpm for 12 minutes then medium was replaced and cells plated in 10 cm cell culture dish overnight. After 24 hours, non-adherent cells (osteoclast progenitors) were collected, counted and plated at 10 x 10^4 and 2 x 10^6 seeding density in 96-well plate and 6-well plate, respectively, with 20 ng/ml of MCSF (R&D systems) to be primed. After 72 hours, cells were treated twice on day 1 and 3 with 20 ng/ml MCSF and 30 ng/ml of RankL (R&D systems). Cultures were terminated on day 4 for staining and measuring TRAP activity. For RNA isolation, cultures were terminated after 72 hours of priming with MCSF and on days 2 and 4 after RankL treatments.

For Osteoclasts function, Osteoclast progenitors were plated at 10 x 10^4 in 96-well Corning osteoassay (Corning) plate. Cells were differentiated to osteoclasts as above and terminated on day 4.

**TRAP Activity and Staining**

Differentiated osteoclasts were fixed with 10% formalin, then washed with water. For TRAP activity assay, 1:1 of methanol and acetone mixture solution was added to the fixed cells, then removed and replaced with TRAP buffer containing 0.1 mg/ml p-nitrophenyl phosphate and incubated at 37 °C for one hour. After that, 1 N NaOH was added and absorbance was read at 405 nm using BioTek
Synergy microplate reader. TRAP activity was calculated in enzyme units (U = μmol/L) per μg protein.

For TRAP staining, osteoclasts were incubated at 37 °C with TRAP buffer containing 1.5 mM naphthol (Sigma) and 0.5 mM Fast Red Violet LB salt (Sigma).

**RNA Isolation and RT-qPCR**

For RNA isolation from femurs and other organs, Qiazol reagent (miRNeasy Mini Kit, Qiagen) and a tissue homogenizer were used.

RNA isolation from cell cultures was also done using Qiazol reagent (miRNeasy Mini Kit, Qiagen) on day 7 and 14 for osteoblasts, and primed Osteoclast progenitors, then on day 2 and 4 for osteoclasts. RNA quantities and quality were measured using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific). Complementary DNA was generated using High-capacity cDNA reverse transcription kit (Applied Biosystems). For genes of GAPDH, RUNX2, OSX, ALP, OCN, COL1, TRAP, CTR, CTSK, NFATc1, DC-STAMP and Osteoactivin/GPNMB, qPCR was performed with an ABI 7500 Fast real-time PCR system (Life Technologies) in triplicate 20-μL volume per reaction, each reaction contained 10 ng cDNA, 100 nmol/L primer, and 10 μL 2× SYBR Green PCR master mix (Life Technologies). Primers used in these measurements are reported in Table 2.1. For measuring miR-150 gene expression, cDNA was generated using miScript II RT Kit (Qiagen), and qPCR was performed using the mmu-miR-150-1 miScript Primer Assay and miScript SYBR Green PCR Kit (Qiagen), where U6 was used as internal control.
### Table 2.1. qPCR Primers

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
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<tr>
<td>GAPDH</td>
<td>5' - CGACTTCAACAGCAACTCCCACTCTCC-3'</td>
</tr>
<tr>
<td></td>
<td>5' - TGGGTGGTGCCAGGTTTCTTACTCTTT-3'</td>
</tr>
<tr>
<td>RUNX2</td>
<td>5' - GACGAGGAAGAGATTTCCACC-3'</td>
</tr>
<tr>
<td></td>
<td>5' - GCAACGCTGACTGTCACACTTT-3'</td>
</tr>
<tr>
<td>OSX</td>
<td>5' - GCAACTGCTGCTGTGGGTC-3'</td>
</tr>
<tr>
<td></td>
<td>5' - GCAAAATCATGGAGTGAGGAC-3'</td>
</tr>
<tr>
<td>ALP</td>
<td>5' - CCGATGGCACACCTGCTT-3'</td>
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<tr>
<td></td>
<td>5' - GGAGGCATACGCATCACAT-3'</td>
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<tr>
<td>OCN</td>
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</tr>
<tr>
<td></td>
<td>5' - GCGCCGGAGCTGCTGACTA-3'</td>
</tr>
<tr>
<td>COL1</td>
<td>5' - CCTGAGTCAGCAGATTTGAAACA-3'</td>
</tr>
<tr>
<td></td>
<td>5' - CCAGTACTCTCCGCCTTGCA-3'</td>
</tr>
<tr>
<td>TRAP</td>
<td>5' - GCAGTATCTTCCAGGAGGAGA-3'</td>
</tr>
<tr>
<td></td>
<td>5' - TCCATAGTGAACCCGCAAGTA-3'</td>
</tr>
<tr>
<td>CTR</td>
<td>5' - AGTTGCCCTCTTATGAAGGAGAAG-3'</td>
</tr>
<tr>
<td></td>
<td>5' - GGAGTGTCGCTCAGACAT-3'</td>
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<tr>
<td>CTSK</td>
<td>5' - CTTAGCTTCCGCTCAGTAG-3'</td>
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<td></td>
<td>5' - ACTTGACACCCATCCTTG-3'</td>
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<td>NFATc1</td>
<td>5' - CTC GAA AGA CAG CAC TGGAGCT AT-3'</td>
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<td></td>
<td>5' - CGG CTG CCT TCC GTTCATAG-3'</td>
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<tr>
<td>DC-Stamp</td>
<td>5' - TGATCGCTACATCTCCAT-3'</td>
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<tr>
<td></td>
<td>5' - GACTCCTTGCTTCTGCTT-3'</td>
</tr>
<tr>
<td>Osteoactivin/GPNMB</td>
<td>5' - AATGGGTCTGCGACCTACTG-3'</td>
</tr>
<tr>
<td></td>
<td>5' - GGCTTGTACGCTGTGGTT-3'</td>
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**3´-Untranslated Region Luciferase Assay**

MSCs of WT and miR-150 KO mice femurs and tibia were collected and plated in 24-well plate at cell density of 10 x 10^4 cells per well. Cells were then transfected with 3´-Untranslated region (3´-UTR) reporter vector of Osteoactivin/GPNMB (GeneCopoeia) using Lipofectamine 2000. The 3´-UTR Osteoactivin/GPNMB vector contains Renilla luciferase as control, then luciferase activities of Firefly and Renilla were measured using Luc-Pair Duo-Luciferase assay kit (GeneCopoeia).

**Statistical Analysis**

Data were analyzed using the Prism 5 software (GraphPad). Individual experiments were repeated a minimum of three times. One way Anova (1-way-Anova) analysis followed by Tukey’s post hoc test were performed when comparing multiple groups. Unpaired t-test was performed for two groups comparison. Differences were considered statistically significant when p value is less than 0.05. Group means or means ± standard error of the mean (±SEM) were graphed.
Results

**miR-150 is expressed in different tissues including long and flat bones**

miR-150 has been reported in lungs [215], thymus [230], brain [231], heart [232], kidney [233], muscle [234], spleen [107], and liver [235]. By RT-qPCR, we examined the expression of miR-150 in lungs, thymus, brain, heart, kidney, muscle, testis, tibia, calvaria, spleen, and liver (Figure 2.1), and found that miR-150 is highly expressed in the lungs, brain, heart, skeletal muscle, tibia, calvaria, and spleen. This expression of miR-150 in different tissues and specifically in long and flat bones, suggests that this molecule may play a role in bone cells differentiation and function.
Figure 2.1: miR-150 gene expression in different tissues of mice. miR-150 has different expression levels in different organs of C57BL/6 male mice at age of 8 weeks. miR-150 is also expressed in long and flat bones. This experiment was repeated twice. Data presented in this graph represent Mean ± SEM.
**miR-150 expression increases with age in mice long bone**

To study how miR-150 expression is affected during aging in bone, we collected femurs from male C57BL/6 mice at different ages: 3 days, 4, 12 weeks, 6 months, and 1 year old. RT-qPCR was performed for miR-150 from these 5 different bones, and our findings showed that miR-150 expression starts low at early age and increases as the animal gets older (Figure 2.2). These results suggest that miR-150 has a high impact on bone development.
Figure 2.2: miR-150 gene expression in long bone during aging. miR-150 has low expression in male mouse long bone at age of 3 days, and its expression increases as animal gets older. This experiment was repeated twice. Data presented in this graph represent Mean ± SEM.
miR-150 KO mice have significant reduction in bone mass and volume

Several miRNAs have been reported to play a role in bone homeostasis in vivo [64, 228, 236], some have been shown to act as positive regulators to increase bone volume [64] and other miRNAs act as a negative regulators and decrease bone volume [228, 236]. Here we show that deletion of miR-150 in mice leads to significant reduction in bone volume as shown by BV/TV, Tb.N, Tb.Th, and Tb.Sp measurements (Figure 2.3, 2.4, 2.5 B – E). Micro-CT scanning and analyses of distal femur trabecular bones of 4, 8, and 16 weeks old mice, showed reduced bone mass in miR-150 KO compared to WT mice (Figure 2.3, 2.4, and 2.5). This reduction in bone mass is also shown in three-dimensional micro-CT images (Figure 2.3A, 2.4, 2.5 A) at ages 4, 8, and 16 weeks. Significant decrease in the trabecular number was also shown in miR-150 KO distal femurs at ages of 4 and 8 weeks (Figure 2.3, 2.4 C), while at 16 weeks they showed a similar trend but the difference was not significant (Figure 2.5 C). Trabecular separation at all three ages showed a significant increase in miR-150 KO mice (Figure 2.3, 2.4, 2.5 E), while trabecular thickness was significantly less in 4 and 16 weeks old miR-150 KO mice (Figure 2.3, 2.5 D), but there was no difference trabecular thickness in the bones of 8 weeks old miR-150 KO mice (Figure 2.4 D). These results indicate that absence of miR-150 profoundly affects bone homeostasis in vivo.
Figure 2.3: miR-150 KO mice has significant reduction in bone mass compared to WT. Micro-CT 3-dimensional images of distal femur in 4 weeks old mouse (A) shows less trabecular bone in miR-150 KO than WT (n=6). Other parameters also show significant differences between miR-150 KO and WT, BV/TV (B), Tb.N (C), Tb.Th (D), and Tb.Sp (E). Data presented in all graphs represent Mean ± SEM. * = p<0.05, ** = p<0.01.
Figure 2.4: miR-150 KO mice has significant reduction in bone mass compared to WT animals. Micro-CT 3-dimensional images of distal femur in 8 weeks old mouse (A) shows less trabecular bone in miR-150 KO than WT (n=6). Other parameters also show significant differences between miR-150 KO and WT, BV/TV (B), Tb.N (C), Tb.Th (D), and Tb.Sp (E). Data presented in all graphs represent Mean ± SEM. *=p<0.05, ***=p<0.001.
Figure 2.5: miR-150 KO has significant reduction in bone mass compared to WT mice. Micro-CT 3-dimensional images of distal femur in 16 weeks old mouse (A) shows less trabecular bone in miR-150 KO than WT (n=6). Other parameters also show significant differences between miR-150 KO and WT, BV/TV (B), Tb.N (C), Tb.Th (D), and Tb.Sp (E). Data presented in all graphs represent Mean ± SEM. *=p<0.05, **=p<0.01.
**miR-150 KO mice have decreased bone mass and osteoblast number in vivo**

In order to further characterize the bone phenotype of miR-150 KO mice, we performed histomorphometric analysis on femoral metaphysis in 4 and 8 weeks old WT and miR-150 KO mice. Analysis of von kossa stained histology sections showed a similar trend to the micro-CT data shown in Figures 2.3, 2.4, and 2.5. miR-150 KO have significantly less trabecular number and thickness compared to WT mice (Figure 2.7, 2.8 A and D), while trabecular separation was significantly increased in miR-150 KO compared to WT mice (Figure 2.7, 2.8 C). Moreover, osteoblast numbers in miR-150 KO animals were less when counted and compared to numbers in WT animals (Figure 2.7, 2.8 B). Furthermore, we calculated the ratio of trabecular number and bone perimeter to tissue area where results showed the same trend with less value in miR-150 KO compared to WT (Figure 2.7, 2.8 E and F). Microscopic images of 4 weeks old miR-150 KO and WT distal femurs that are stained for mineralized matrix with von kossa staining, show less mineralized matrix in miR-150 KO compared to WT mice (Figure 2.6).
Figure 2.6: miR-150 KO mice have decreased mineralized bone matrix in vivo. Sagittal sections from 4 weeks old WT and miR-150 KO mice femurs (n=4) are stained with von kossa to visualize minerals (black) and counterstained with toluidine blue.
Figure 2.7: Histomorphometric analysis showed significant decrease in osteoblast number and bone mass in miR-150 KO mice. Sagittal sections from 4 weeks old mice femurs (n=4) were stained for von kossa. Histomorphometric parameters including Tb.N (A), N.Ob (B), Tb.Sp (C), Tb.Th (D), Tb.N/T.Ar (E), and B.Pm/T.Ar (F) were all significantly different in miR-150 KO compared to WT mice. Data presented in all graphs represent Mean ± SEM. *=p<0.05.
**Figure 2.8: Histomorphometric analysis of 8 weeks old mice femurs.**

Sagittal sections from 8 weeks old mice femurs (n=4) were stained for von kossa. Histomorphometric parameters including Tb.N (A), N.Ob (B), Tb.Sp (C), Tb.Th (D), Tb.N/T.Ar (E), and B.Pm/T.Ar (F) were all significantly different in miR-150 KO compared to WT mice. Data presented in all graphs represent Mean ± SEM. *=p<0.05.
miR-150 KO mice have slower bone formation rate compared to WT mice as revealed by kinetic histomorphometry

To investigate if the reduction in bone volume is related to osteoblast function, we performed bone dynamic analyses by measuring the level of mineralized matrix formation in WT and miR-150 KO femurs. Bone kinetic images and analyses showed less bone mineralization with decreased width of interlabeled surfaces in miR-150 KO (Figure 2.9 A, B, and C). From these data, we found a significant reduction in mineral apposition and bone formation rates in miR-150 KO mice compared to WT mice (Figure 2.9 B and C). These results indicate that miR-150 regulates osteoblasts differentiation and function in vivo.
Figure 2.9: miR-150 KO mice have significant reduction mineral apposition and bone formation rates in vivo. WT and miR-150 KO mice of age 8 weeks (n=3) were injected with calcein AM which label mineralized matrix in bone (green) (A). Analyses of injected mice femurs sagittal sections show miR-150 KO MAR (B) and BFR/B.S. (C) rate are significantly less than WT. Data presented in all graphs represent Mean ± SEM. **=p<0.01, ***=p<0.001.
**miR-150 KO mice have higher bone resorption and lower bone formation serum biomarkers**

Recently, a study reported that OPG levels were significantly less in miR-150 KO compared to WT mouse serum at 13 weeks old [237]. Here we show that OPG and RANKL levels in miR-150 KO mice serum is less than WT at age of 8 weeks (Figure 2.10 A and B); however, there was no difference in the RANKL to OPG ratio (Figure 2.10 C). Interestingly, levels of C-terminal telopeptides of type I collagen (CTX-1) in 8 weeks old miR-150 KO mouse serum was slightly higher than WT but not significant (Figure 2.10 D). At age of 16 weeks, we show a significant increase in CTX-1 levels in miR-150 KO serum as result of age over active osteoclasts compared to WT (Figure 2.10 E). On the other hand, osteoblasts serum markers like Procollagen I Intact N-terminal (PINP) and osteocalcin (OCN) in miR-150 KO mice were significantly reduced compared to WT at age of 16 weeks (Figure 2.11 A and B), which may explain the reduction in osteoblasts function.
Figure 2.10: miR-150 KO mice have significant increase in bone resorption serum biomarkers compared to WT mice. Biochemical analysis of osteoclast serum markers RANKL (A) and OPG (B), ratio of RANKL/OPG (C), CTX-1 (D) at age of 8 weeks (n=5), CTX-1 (E) at age of 16 weeks (n=5). Data presented in all graphs represent Mean ± SEM. *=p<0.05, ***=p<0.001, ****=p<0.0001.
Figure 2.11: miR-150 KO mice have significant reduction in bone formation serum biomarkers compared to WT mice. Biochemical analysis of osteoblast serum markers PINP (A) and OCN (B) at age of 16 weeks (n=5).

Data presented in all graphs represent Mean ± SEM. *=p<0.05, **=p<0.01.
miR-150 deficiency in MSCs decreases cells apoptotic rate

To investigate the effects of miR-150 deficiency on MSC proliferation and survival, we performed ex vivo proliferation and viability assays on mouse MSCs from WT and miR-150 KO. There was no significant difference in proliferation and viability between the WT and miR-150 KO mice MSCs in ex vivo (Figure 2.12 A and B). Next, we examined whether miR-150 deficiency has any effect on MSCs apoptosis level. Our results showed that miR-150 KO mouse MSCs have a lower apoptotic rate compared to WT. These findings were further supported by low expression of pro-apoptotic markers caspase-3/7 and Bax (Figure 2.13 A and B) in miR-150 KO MSCs compared to WT.
Figure 2.12: miR-150 KO MSCs proliferation and viability have no difference compared to WT. Proliferation of MSCs measured by quantitation of fluorescence-labeled DNA in MSCs culture at 48 and 72 hours’ time points (A). Mitochondrial activity and cell viability of MSCs at 48 and 72 hours’ time points (B). This experiment was repeated three times.
Figure 2.13: miR-150 deficient MSCs have lower levels of apoptotic markers compared to WT. MSCs apoptosis assay measured by quantitation of caspase-3/7 after 72 hours of cell starvation (A). qPCR analysis of the pro-apoptotic Bax level in MSCs after 72 hours’ cell starvation (B). Data presented represent Mean ± SEM. **=p<0.01.
**Ex vivo miR-150 KO osteoblasts have significant increase in differentiation compared to WT**

To test the effect of miR-150 deficiency in *ex vivo* osteoblast differentiation and function, we stained for alkaline phosphatase (ALP) (Figure 2.14 A), an early marker of osteoblast differentiation, and analyzed ALP protein activity levels (Figure 2.14 B). We also stained for mineralization (Figure 2.15 A), and measured mineralized nodules area (Figure 2.15 B). Our results show a significant increase in osteoblast differentiation from miR-150 KO compared to WT, reflected by the increase of ALP staining and activity on day 7, and mineralization and nodules area on day 14 (Figures 2.14 and 2.15). Furthermore, osteoblast gene expression markers also were all significantly increased in miR-150 KO compared to WT, including RUNX2, Osterix (OSX), ALP, Collagen type I (COL1), and Osteocalcin (OCN) (Figure 2.17 A – E). Also by analyzing gene expression of miR-150 in WT cells during osteoblasts differentiation, we notice a significant down-regulation of miR-150 expression between undifferentiated cells on day 0 and differentiated cells on days 7 and 14 (Figure 2.16). With these findings we believe that miR-150 has an inhibitory effect ex-vivo on osteoblast differentiation and function.
Figure 2.14: miR-150 deficient osteoblasts have significant increase in ex vivo differentiation compared to WT. Images of ALP stained osteoblasts on day 7 in culture (A). ALP activity in differentiated osteoblasts on day 7 (B). This experiment was repeated three times. Data presented represent Mean ± SEM. **=p<0.01.
Figure 2.15: miR-150 deficient osteoblasts have increased mineralization ex vivo compared to WT. Images of von kossa stained osteoblasts on day 14 in culture (A). Average size or area of total mineralized nodules activity in differentiated osteoblasts on day 14 (B). This experiment was repeated three times. Data presented represent Mean ± SEM. **=p<0.01.
**Figure 2.16: miR-150 expression decreases during osteoblasts differentiation in WT cells.** Expression of miR-150 is at highest in undifferentiated MSCs, and this expression decreases as cells progress into later stage of differentiation. This experiment was repeated twice.
Figure 2.17: miR-150 KO osteoblasts have increased expression of osteoblast specific genes ex vivo compared to WT. qPCR analysis of osteoblast related genes are significantly enhanced in miR-150 KO cells compared to WT, RUNX2 (A), OSX (B), ALP (C), COL1 (D), and OCN (E). This experiment was repeated three times. Data presented in all graphs represent Mean ± SEM. ***=p<0.001, ****=p<0.0001.
**miR-150 deficiency decreases ex-vivo osteoclastogenesis**

To investigate if ex-vivo deficiency of miR-150 can have an effect on osteoclast progenitors proliferation and viability, and osteoclasts differentiation and function, we conducted an ex-vivo experiment to differentiate osteoclasts from WT and miR-150 KO mouse osteoclast progenitors. Our data show a reduction in miR-150 KO osteoclast progenitors proliferation after 72 hours (Figure 2.18 A), while there was no difference in cell viability compared to WT (Figure 2.18 B). On the other hand, ex-vivo osteoclasts differentiation and function was significantly decreased in miR-150 KO compared to WT. Tartrate-resistant acid phosphatase (TRAP) activity (Figure 2.19 B) and staining (Figure 2.19 A) was shown to be significantly reduced in miR-150 KO osteoclasts. Also, total and deferential counting of osteoclasts show significant decrease in miR-150 KO compared to WT (Figure 2.19 C). Osteoclasts gene expression markers TRAP, NFATc1, DC-STAMP, CTR, and CTSK were all down regulated in miR-150 KO cells compared to WT (Figure 2.22 A – E). By analyzing miR-150 gene expression during WT osteoclastogenesis, data show an increase in miR-150 expression until day 2 and then decreases as differentiation progress to day 4 (Figure 2.21). To study the effects of miR-150 deficiency on osteoclasts function, we conducted osteoassay experiment where osteoclasts were differentiated on artificial mineralized matrix. Our images and results show significant reduction in resorbed area by miR-150 KO osteoclasts compared to WT (Figure 2.20 A and B).
Figure 2.18: overall miR-150 KO osteoclast progenitors proliferation and survival have no difference, except proliferation at 72 hours compared to WT. Proliferation of osteoclast progenitors measured by quantitation of fluorescence-labeled DNA in osteoclast progenitors culture at 48 and 72 hours’ time points (A). Mitochondrial activity and cell viability of osteoclast progenitors at 48 and 72 hours’ time points (B). This experiment was repeated three times. Data presented represent Mean ± SEM. *=p<0.05.
Figure 2.19: miR-150 KO has significant reduction in osteoclasts differentiation compared to WT. TRAP staining of mature osteoclasts of WT and miR-150 KO mice (A). miR-150 KO osteoclasts have significantly less TRAP activity (B). Deferential counting of osteoclasts show that miR-150 KO mice have less number of total and large osteoclasts compared to WT (C). This experiment was repeated three times. Data presented represent Mean ± SEM. **=p<0.01, ***=p<0.001.
Figure 2.20: miR-150 KO osteoclasts have reduced function compared to WT. Images resorbed patches (white) of minerals-coated wells by osteoclasts (A). Total area of resorbed surface by miR-150 KO osteoclasts is significantly less than WT cells (B). This experiment was repeated three times. Data presented represent Mean ± SEM. *=p<0.05.
Figure 2.21: miR-150 expression during osteoclast differentiation in WT cells. miR-150 gene expression is low in primed osteoclast progenitors but increases during osteoclast differentiation then decreases at terminal stage of differentiation. This experiment was repeated twice.
Figure 2.22: miR-150 KO osteoclasts have decreased expression of osteoclast specific genes ex vivo compared to WT. qPCR analysis of osteoclast related genes are significantly reduced in miR-150 KO cells compared to WT, TRAP (A), NFATc1 (B), DC-STAMP (C), CTR (D), and CTSK (E). This experiment was repeated three times. Data presented represent Mean ± SEM. *=p<0.05, ***=p<0.001, ****=p<0.0001.
**Osteoactivin/GPNMB is targeted by miR-150**

To test if Osteoactivin/GPNMB is a target for miR-150 in bone cell, we analyzed Osteoactivin/GPNMB gene expression in ex-vivo osteoblast and osteoclast cultures, and conducted a luciferase activity assay in WT and miR-150 KO MSCs. Our results show significant increase in Osteoactivin/GPNMB gene expression in miR-150 KO osteoblasts (Figure 2.23 A) and osteoclasts (Figure 2.23 B) compared to WT. When conducting a luciferase activity assay in WT and miR-150 KO MSCs by transfecting Osteoactivin/GPNMB 3'UTR into these cells, data showed an increased luciferase activity in miR-150 KO MSCs compared to WT (Figure 2.24).
Figure 2.23: Osteoactivin/GPNMB gene expression is higher in differentiated miR-150 KO osteoblasts and osteoclasts ex vivo compared to WT. qPCR analysis of Osteoactivin/GPNMB gene expression is significantly increased in miR-150 KO osteoblasts (A), and osteoclasts (B) compared to WT. This experiment was repeated twice. Data presented represent Mean ± SEM. *=p<0.05, **=p<0.01.
Figure 2.24: Osteoactivin/GPNMB 3’UTR is targeted by miR-150. Schematic diagram represents Osteoactivin/GPNMB 3’UTR bases targeted by miR-150 (A). Luciferase activity of Osteoactivin/GPNMB in WT and miR-150 KO MSCs shows an increased luciferase activity in the absence of miR-150 (B). Data presented represent Mean ± SEM. *p<0.05.
Discussion

Our interest in studying how miR-150 regulates bone homeostasis arose from the role that miR-150 plays in B-cell differentiation and function [107], and the link between B-cells and bone homeostasis in vivo [238]. Studies have shown that B-cells are critical for maintenance of bone mass where B-cells deficient mice had significant reduction in bone mass and volume [239]. MiR-150 was shown to target c-Myb, a very important transcription factor for B-cells development [108, 240]. By linking the importance of B-cells to bone homeostasis, and miR-150 to B-cells, we find that miR-150 can also regulate bone homeostasis. In this study, we show that miR-150 is expressed in different tissues, and when measuring its expression in mouse long bone, we find that miR-150 expression increases as animal ages. Our data demonstrate that miR-150 plays a significant role in bone homeostasis in vivo and osteoblast and osteoclast differentiation and function ex vivo.

Our micro-CT analyses of trabecular bone in mice distal femurs showed a decrease in trabecular bone mass and volume in miR-150 KO compared to WT. This reduction in bone mass and volume were significant and similar in trend in all three ages of 4, 8, and 16 weeks old mice. To further confirm the in vivo data of micro-CT, we performed a histomorphometric study, and analyses showed a significant reduction in trabecular bone mass and volume. Osteoblasts number in the miR-150 KO was significantly less compared to WT mice at age of 8 weeks. In addition to these findings, dynamic bone analyses indicated decreased bone formation and minerals opposition rate in miR-150 KO mice compared to WT, thus
these data suggest a reduction in osteoblasts function, and the global loss of miR150 in vivo has negative effects on bone formation.

We further measured bone markers in serum: OPG, RANKL, OCN and CTX-1. At age of 8 weeks, OPG and RANKL levels in serum were significantly reduced in miR-150 KO compared to WT. Interestingly, RANKL to OPG ratio showed no difference, and CTX-1 levels were also same between both WT and miR-150 KO mice. These findings indicate that there is no in vivo osteoclasts phenotype at age of 8 weeks. The reduction in bone mass and volume at age of 8 weeks in miR-150 KO mice is mainly a result from decreased osteoblast function which might be linked to low OPG levels in serum, as previously reported that OPG is important for osteoblast maturation, and overexpression of OPG enhanced osteoblast differentiation and function [241].

According to recent study, miR-150 KO mice femurs showed to have more osteoclasts number when compared to WT at age of 13 weeks [237]. The group reported a reduction in OPG and no change in RANKL levels at age of 13 weeks in miR-150 KO compared to WT. Also, it was suggested that the expansion of B-cells caused by the loss of miR-150, and the low level of OPG is triggering B-cells fusion by RANKL into forming osteoclasts [237]. Here our data support their findings where we show an increase in CTX-1 serum levels at age of 16 weeks in miR-150 KO compared to WT mice. These findings suggest that miR-150 KO mice have very active osteoclasts at this specific age. Also our data show significant reduction in OCN levels in serum in miR-150 KO, reflected by decreased osteoblasts function at age of 16 weeks. Taken together, miR-150 KO
mice have decreased osteoblasts function and increased in osteoclasts function leading to in vivo reduced bone mass and volume phenotype.

Surprisingly, in contrast to our observations on reduced function of osteoblast in vivo in miR-150 KO, we observed enhanced differentiation and function of miR-150 KO osteoblasts ex vivo compared to WT. Our results demonstrated that ex vivo miR-150 KO osteoblasts have increased matrix production which was shown by a significant increase in ALP staining, activity, and von kossa staining for minerals deposition, on day 7 and 14, respectively. Osteoblast marker genes and master transcription factors expression were significantly increased in miR-150 KO cells compared to WT. These findings suggest that miR-150 has an inhibitory effect on osteoblast differentiation and function.

To further understand if miR-150 plays a role in MSCs proliferation and survival in vitro, we conducted a proliferation and survival assays to understand how miR-150 is regulating osteoblast. Our results show no difference in proliferation level between miR-150 KO and WT MSCs in vitro, and same trend was shown also in survival assay. Interestingly, pro-apoptotic markers, caspase 3/7 and Bax, expression levels were significantly less in miR-150 KO MSCs compared to WT. Caspase 3/7 has been reported to negatively regulate osteoblastogenesis [242, 243], which could be one of reasons that caused enhanced differentiation and function of ex-vivo miR-150 KO osteoblasts, due to low level of caspase 3/7.
As ex vivo miR-150 KO osteoblasts showed significant increase in differentiation and function, ex vivo osteoclasts showed opposite phenotype. The deficiency of miR-150 in osteoclasts lead to decrease in miR-150 KO osteoclasts differentiation and function, which was reflected by decreased TRAP staining and activity, osteoclasts number, and function. Also, osteoclasts marker genes were significantly down regulated in miR-150 KO cells. The role of miR-150 in targeting β-catenin might have a significant effect on osteoclastogenesis in vitro, where NF-κB activation upregulates miR-150 which targets β-catenin and inhibits osteogenesis in MSCs [244]. Recent published article reported that activation of Wnt signaling inhibits osteoclastogenesis in vitro, and β-catenin negatively regulates osteoclast differentiation [245]. Based on these reports and our data, we think that the lack of miR-150 in osteoclast ex vivo leads to decreased osteoclastogenesis by active β-catenin.

Previous studies have reported that miR-150 is targeting Osteoactivin/GPNMB mRNA 3'UTR in a rat lung model, where rat pups were exposed to hyperoxia condition to induce the Bronchopulmonary Dysplasia [214, 215]. Gene expression from hyperoxia-exposed lung tissue showed a significant decrease of miR-150 and increase of Osteoactivin/GPNMB levels [215]. Osteoactivin/GPNMB is shown to play an important role during osteoblast and osteoclast differentiation and function [2, 178, 203-205, 207-209, 213, 225], where Osteoactivin/GPNMB gene expression increases during osteoblastogenesis, and overexpression of Osteoactivin/GPNMB increases osteoblast differentiation and function [209]. Osteoactivin/GPNMB was also shown to inhibit osteoclastogenesis.
[213], and therefore Osteoactivin/GPNMB is a positive regulator for osteoblast and negative regulator for osteoclast.

In our observations we find that miR-150 is targeting Osteoactivin/GPNMB in osteoblast and osteoclast. Our data demonstrate that Osteoactivin/GPNMB gene expression is significantly higher in miR-150 KO osteoblasts and osteoclasts compared to WT in ex vivo experiments. Also, by conducting luciferase assay in WT and miR-150 KO MSCs we confirmed that Osteoactivin/GPNMB mRNA 3'UTR is directly targeted by miR-150.

Taken together, we report that miR-150 is a major regulator molecule for bone homeostasis by regulating osteoblast and osteoclast differentiation and function. Our data suggest that miR-150 is a negative regulator for osteoblast, and positive regulator for osteoclast.
Chapter 3

Osteoactivin/GPNMB Gene is Regulated by DNA and Histone Methylation

Introduction

Osteoporosis is a debilitating disease that leads to significant reduction in bone mass in elderly patients and post-menopausal in women. The development of this disease begins with a reduction in osteoblast differentiation and function, followed by an increase in osteoclast bone resorption resulting in low bone mass. Osteoblasts originate from mesenchymal stem cells (MSCs), and are responsible for bone formation by laying down primarily collagen type I and other proteins as part of the matrix, followed by mineralizing the matrix by depositing calcium-phosphate (hydroxyapatite). Osteoclasts are multinucleated cells that originate from hematopoietic stem cells (HSCs). Osteoclasts function as bone degrading and remodeling cells by forming sealing zones around bone and releasing acidic enzymes to resorb the bone. Osteoblasts and osteoclasts differentiation and function can be altered as a result of epigenetic modifications in their genes and histones.

Epigenetic is the study of the change in gene expression without altering the DNA sequence. The mechanisms where the gene is epigenetically affected
are: DNA methylation and histone modifications [246, 247]. DNA methylation is a mechanism by which DNA methyltransferase (DNMT) enzymes methylate the cytosine base of DNA and convert it to 5-methylcytosine [125]. This methylation process in mammals is restricted to cytosine bases located 5’ to guanine bases which are referred to as CpGs islands [127-129]. The methylation of these islands can inhibit the binding of transcription factors to DNA promoters and repress transcription [129].

There are several types of histone modifications that include covalent modifications to specific amino acids in histones by methylation on lysine or arginine, phosphorylation on serine or threonine, acetylation and deacetylation of lysine residues [150, 151]. These modifications lead to a changes in the chromatin structure and affect transcription activity.

Osteoactivin/GPNMB (glycoprotein non-metastatic melanoma B) is type I transmembrane protein that is important for osteoblast differentiation and function [2, 178, 203-205, 208, 209]. Osteoactivin/GPNMB has been shown to be a positive regulator of osteoblast differentiation, and its expression increases during osteoblast differentiation [2, 204, 205]. Studies have shown that inhibition of Osteoactivin/GPNMB using an anti-osteoactivin antibody inhibited osteoblast differentiation and function [203].

Several genetically engineered animal models have been established to study the role of Osteoactivin/GPNMB in bone and related signaling pathways [2, 203-205, 208, 209, 213]; however, to date there has been no reports about the epigenetic regulation of Osteoactivin/GPNMB in bone cells. Here we report that Osteoactivin/GPNMB gene is highly methylated early during mesenchymal stem
cell and MC3T3-E1 proliferation, followed by decreasing methylation levels during osteoblast differentiation. Furthermore, we show that Osteoactivin/GPNMB expression is regulated by methylation of lysine 27 in histone 3 (H3K27me3) by EZH2 during osteoblast differentiation.
Materials and Methods

Mice

C57BL/6J normal (WT) and Osteoactivin/GPNMB mutant (D2J) mice were purchased from the Jackson Laboratory (Bar Harbor, ME, USA). Mice colonies were housed and maintained in cages at Northeast Ohio Medical University in a facility accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC). Mice were kept in a stable environment, at 21°C with a 12-hour light-dark cycle. All studies associated with mice are approved by the institution IACUC at NEOMED.

Genomic DNA Isolation from Cells

Bone marrow was flushed from femurs and tibias of 4 to 8 weeks old C57BL/6 male mice, and mesenchymal stem cells were collected and allowed to proliferate in 10 cm dishes. After reaching confluency, cells were trypsinized, counted, and re-plated at a seeding density of $5 \times 10^5$ cells per well. Cells were differentiated in minimum essential medium α supplemented with 10% FBS, 50 µg/ml ascorbic acid, 10 mM β-glycerophosphate, and 10 nM dexamethasone. Cultures were terminated on day 0, 7, and 14 for genomic DNA isolation using Quick-gDNA MiniPrep kit (Zymo Research) according to the manufacturer protocol.
Isolation of Genomic DNA from Long Bone

Femurs were collected from C57BL/6 male mice at 3 days, 4, 12 weeks, 6 months, and 1-year-old. In order to collect genomic DNA, tissues were frozen in liquid nitrogen and grounded with mortar and pestle. Samples were collected in 15 ml tubes that contained 3 mL of white cell lysis buffer and collagenase B (1mg/ml). After that, samples were incubated at 37°C for 1 Hour with shacking, then added 25 ul of proteinase K (20mg/ml) and 50 ul of 10% SDS solution and incubate at 42°C overnight with shaking. One ml of saturate sodium acetate was added to each tube and mixed manually for 30 seconds, then centrifuged at 4000 x G for 10 minutes. Supernatant was collected and transferred to new 15 ml tube contains 4 ml of 100% isopropanol, which then was mixed for 10 minutes on a rotator to precipitate genomic DNA. After that, samples were centrifuged at 4000 x G for 10 minutes, where supernatant was discarded and DNA pellet was washed in 500 µl of 70% ethanol to be transferred into 1.5 ml eppendorf tube. Samples were centrifuged at 13000 x G for 10 minutes, and supernatant was discarded and pellet was let to dry at 37°C to be dissolved later in 500 µl of TE buffer.

RNA Isolation and RT-qPCR

Cell cultures were terminated and RNA was isolated using (miRNeasy Mini Kit, Qiagen, Valencia, CA, USA) on day 7 and 14. For RNA isolation from femurs, Qiazol reagent (miRNeasy Mini Kit, Qiagen, Valencia, CA, USA) and a tissue homogenizer were used. RNA quantities and quality were measured using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific). Complementary
DNA was generated using High-capacity cDNA reverse transcription kit (Applied Biosystems). For genes of GAPDH, RUNX2, COL1, and Osteoactivin/GPNMB, qPCR was performed with an ABI 7500 Fast real-time PCR system (Life Technologies) in triplicate 20-µL volume per reaction, each reaction contained 10 ng cDNA, 100 nmol/L primer, and 10 µL 2× SYBR Green PCR master mix (Life Technologies). Primers used in these measurements are reported in Table 3.

**Osteoblast Differentiation and Function**

Mesenchymal stem cells were flushed and collected from femurs and tibias of C57BL/6 and D2J male mice of age between 4 and 8 weeks. Cells were left to proliferate in 10 cm dish. When cells reached confluency, they were trypsinized, counted, and re-plated at a density of 5 x 10^5 cells and at 1 x 10^5 cells in 6-well and 24-well plates, respectively. Cells were cultured and differentiated in minimum essential medium α supplemented with 10% FBS, 50 µg/ml ascorbic acid, 10 mM β-glycerophosphate, and 10 nM dexamethasone with or without 2 µM of GSK126 (Selleck Chemicals). Cell cultures were terminated on day 0, 7, and 14 for RNA isolation, and stained for alkaline phosphatase and minerals on day 7 and 14, respectively.

**Cell Survival Assay**

C57BL/6 mouse mesenchymal stem cells cell line (GIBCO) were cultured in 96-well plate at seeding density of 1.5 x 10^3 cells per well and kept in Serum free minimum essential α medium with or without 2, 4, and 8 µM of GSK126 for 48
and 72 hours, then cell survival rate was analyzed using AlamarBlue (Life Technologies).

**Tissue Preparation and Bone Histomorphometry**

Three or four days old C57BL/6 and D2J mouse pups were sacrificed and calvarias were collected. Each calvaria was split into two halves, left and right. Calvarias organ cultures were kept on stainless steel grids (Amazon) in 24-well plates loaded with BGJb medium containing 0.1% bovine serum albumin. Left calvarial halves were used as control, and right ones were treated twice with 10 µM GSK126 for 7 days culture period. On day 7, calvarias were fixed in 10% buffered formalin for 24 hours then decalcified in 10% EDTA for 48 hours. After that, tissues were processed and embedded in paraffin. Calvarias were sectioned at 5 µm thick and stained with hematoxylin and eosin. Samples were analyzed using ImageJ software.

**Quantitative Methylation Specific PCR**

Genomic DNA was isolated from C57BL/6 male mouse femurs at ages of 3 days, 4, 12 weeks, 6 months, and 1 year old, or cultures of differentiated osteoblasts which originated from C57BL/6 mouse mesenchymal stem cells on day 0, 3, and 7. DNA quantities and quality were measured using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific). One µg of genomic DNA was bisulfite converted using EZ DNA Methylation-Gold kit (Qiagen) according to the manufacturer protocol. After bisulfite conversion, 1 µl of converted DNA was divided into three reactions. QPCR was performed with an ABI 7500 Fast real-
time PCR system (Life Technologies) in triplicate 20-μL volume per reaction, each reaction contained 1/3 μl of converted genomic DNA, 100 nmol/L primer, and 10 μL 2x SYBR Green PCR master mix (Life Technologies). Primers used in this study are ACTB forward: 5’ TTAATGGTTTTTGAGTTTTTTTTTTA 3’, ACTB reverse: 5’ AAAAAAAACTCCCTCCCTAACC 3’, Osteoactivin/GPNMB M forward: 5’ TTCGTATTTTATAAACATAGACGGGATC 3’, Osteoactivin/GPNMB M reverse: 5’ AACTTTCCATACTACTCCGAACG 3’.

**Combined Bisulfite Restriction Analysis (COBRA)**

One μg of genomic DNA was subjected to bisulfite modification using the EpiTect kit (Qiagen). Modified DNA was then subjected to 35 cycles of PCR amplification with primers forward: 5’ TGGAATTTGGGAGATTAAGTTATATTG 3’, and reverse: 5’ AAAAAAACTCCCTACAACCAAC 3’, which are specific to modified Osteoactivin/GPNMB sequences. One hundred ng of a 700 bp custom amplimer was added to the 226 bp amplification product as a digestion control. Samples were digested with Hpy188I. To control for completeness of DNA modification, duplicate reactions were digested with SspI. Digested samples were separated in polyacrylamide gels and visualized by GelRed staining. The proportion of methylated Osteoactivin/GPNMB alleles was calculated as 65bp + 161bp bands/65bp + 161bp + 226bp bands.
**RNA Sequencing and Bioinformatic Analysis**

mRNA sequencing was performed on RNA from MC3T3-E1 cells and 3 days old WT and Ezh2 cKO mice calvarias. MC3T3-E1 cells were treated with 5 µM GSK126 in osteogenic medium for 3 days or GSK126-untreated cells. High throughput mRNA sequencing and bioinformatic analyses (mRNASeq) were performed as previously reported by our collaborators [248, 249]. Gene expression is expressed in reads per kilobasepair per million mapped reads (RPKM).

**ChIP Sequencing and Bioinformatic Analysis**

MC3T3-E1 cells were plated in 10 cm plate and treated with 5 µM GSK126 or untreated. Both GSK126-treated and untreated cells were maintained in osteogenic medium for 24 hours. Cells were then trypsinized and analyzed using a chromatin immunoprecipitation assay (ChIP) as previously reported [249, 250] using H3K27me3 (17-622, Lot 2213948, Millipore) and control IgG (PP64B, Lot 2056666A, Millipore) antibodies.

**Statistical analysis**

Data were analyzed using the Prism 5 software (GraphPad, La Jolla, CA). Individual experiments were repeated a minimum of three times. One way Anova (1-way-Anova) analysis followed by Tukey’s post hoc test were performed when comparing multiple groups. Unpaired t-test was performed for two groups comparison. Differences were considered statistically significant when p value is
less than 0.05. Group means or means ± standard error of the mean (±SEM) were graphed.
Results

**Osteoactivin/GPNMB gene methylation decreases during osteoblast differentiation**

Previous literature has shown that osteoblast gene markers change their methylation patterns as cell progress toward differentiation and maturity. For example, osterix (OSX) gene methylation levels were shown to be high in undifferentiated human mesenchymal stem cells, but decreases during osteoblasts differentiation [251]. Here we report that Osteoactivin/GPNMB gene is highly methylated in proliferating pre-osteoblast MC3T3-E1 cell line and mouse mesenchymal stem cells. Furthermore, methylation level of Osteoactivin/GPNMB decreases as cells differentiate into mature osteoblasts in both cell types, shown by combined bisulfite restriction analysis (COBRA) and methylation specific PCR (MSP) (Figure 3.1).
Figure 3.1: Osteoactivin/GPNMB gene is highly methylated in undifferentiated osteoblasts. Analysis of Osteoactivin/GPNMB gene methylation level in MC3T3-E1 cells, using COBRA technique (A), and mouse mesenchymal stem cells using MSP (B). In both cell types Osteoactivin/GPNMB methylation patterns show similar trend, where it is high in proliferative cells and decreases during differentiation. This experiment was repeated three times.
Osteoactivin/GPNMB expression increases during early age in mouse long bone and decreases during aging

Osteoactivin/GPNMB expression has been shown to be low at early stage of osteoblasts differentiation, and increases as cells progress to become fully differentiated osteoblasts [209]. Analysis of Osteoactivin/GPNMB expression in mouse long bone reveals an increase from 3 days to 12 week old mice. Furthermore, expression decreases in older animals between 12 week and 6 month old mice. Osteoactivin/GPNMB expression is then surprisingly upregulated at age of 1-year-old, which could be an indication that cells are compensating to stimulate bone formation (Figure 3.2).
Figure 3.2: Osteoactivin/GPNMB is highly expressed in young mouse long bone. Osteoactivin/GPNMB expression in mouse long bone increases from 3 days until between 4 and 8 weeks old mouse. Expression decreases as animal ages at 6 months old, then increases at 1-year-old mouse. This experiment was repeated twice.
Osteoactivin/GPNMB gene methylation increases with age in mouse long bone

Gene methylation has a direct negative impact on gene expression, by preventing transcription factors binding and repressing transcription activity [129]. Analysis of Osteoactivin/GPNMB gene methylation level during age in mouse long bone revealed low methylation level in young age mouse. Our data show that Osteoactivin/GPNMB gene is hypo-methylated at ages of 3 days, 4, and 12 weeks old. The gene becomes hyper-methylated later at age of 6 months, and surprisingly methylation level decreases at age of 1-year-old mouse (Figure 3.3).
Figure 3.3: Osteoactivin/GPNMB gene has low methylation level in young mouse long bone. Using methylation specific PCR technique, Osteoactivin/GPNMB gene is shown to be hypo-methylated between ages 3 days and 12 weeks old. The gene becomes hyper-methylated in 6 months old mice, but then hypo-methylated in 1-year-old mice.
**Ezh2 conditional knock out calvaria has increased expression of Osteoactivin/GPNMB**

Ezh2 is a known methyltransferase that methylates lysine 27 in histone 3 (H3K27) tail \[164, 165, 167\]. The methylation process carried by Ezh2 leads to silencing genes that are located near or occupied by the methylated H3K27, as a result of chromatin compaction \[150, 151\]. Our data indicates that absence of Ezh2 leads to an increase in Osteoactivin/GPNMB expression. Furthermore, RNA-Seq data from Ezh2 conditional knock out (Ezh2 cKO) under the Prx1 promoter in mouse mesenchymal stem cells show higher expression of Osteoactivin/GPNMB compared to WT when analyzed from calvarial RNA (Figure 3.4).
Figure 3.4: Ezh2 cKO mouse calvaria has high Osteoactivin/GPNMB expression. RNA-Seq data indicate an increase in Osteoactivin/GPNMB expression from 3 week old Ezh2 cKO mouse calvaria compared to WT.
Inhibition of Ezh2 activity increases Osteoactivin/GPNMB expression

Previous literature has shown that inhibition of Ezh2 activity in human mesenchymal stem cells using GSK126, an Ezh2 specific inhibitor, leads to enhanced osteoblast differentiation and function [249]. Here our data revealed that inhibition of Ezh2 activity in MC3T3-E1 cells increases Osteoactivin/GPNMB expression during osteoblasts differentiation as shown by RNA-Seq. Osteoactivin/GPNMB expression was shown to be higher on day 6 and 10 compared to untreated cells (Figure 3.5).
Figure 3.5: Ezh2 inhibitor-treated Osteoblasts have higher Osteoactivin/GPNMB expression. RNA-Seq data from differentiated MC3T3-E1 treated with GSK126 show an increased Osteoactivin/GPNMB expression on day 6 and 10 compared to untreated cells.
**Osteoactivin/GPNMB promoter is occupied by H3K27**

It has been shown that Ezh2 is the methyltransferase that mono-, di-, and tri-methylates lysine 27 in histone 3 (H3K27) [167, 175, 249]. To investigate if Ezh2 has direct effect on Osteoactivin/GPNMB gene, chromatin immunoprecipitation sequencing (ChIP-Seq) analysis was performed from Ezh2 inhibitor treated or untreated MC3T3-E1 cells. Our data show that Osteoactivin/GPNMB promoter is occupied by tri-methylated H3K27 (H3K27me3) using ChIP-Seq. Osteoactivin/GPNMB promoter sequence is shown to be pulled by H3K27me3 antibody in undifferentiated MC3T3-E1 cells. Inhibition of Ezh2 activity using GSK126 in MC3T3-E1 cells, indicate that Osteoactivin/GPNMB promoter sequence is not pulled with H3K27me3 antibody (Figure 3.6).
Figure 3.6: Tri-methylated H3K27 occupies Osteoactivin/GPNMB promoter. ChIP-Seq technique was performed in undifferentiated MC3T3-E1 cells treated or untreated with GSK126 using H3K27me3 antibody. Untreated cells have high levels of H3K27me3 (blue peaks) occupying Osteoactivin/GPNMB promoter compared to treated cells (dark green peaks).
Inhibition Ezh2 activity enhances osteoblast differentiation and increases Osteoactivin/GPNMB expression

Previous literature has shown that inhibiting Ezh2 activity increases osteoblast differentiation in human mesenchymal stem cells [249]. In our studies, we tested the effects of GSK126 on osteoblast differentiation from mouse mesenchymal stem cells. Three different doses were tested for mesenchymal stem cell viability, 2, 4, and 8 µM of GSK126 for 48 and 72 hours. Our data show that cells treated with 4 and 8 µM have less viability (Figure 3.7 A and B), therefore differentiated cells were treated with 2 µM of GSK126. Our results show that osteoblasts treated with 2 µM of GSK126 have enhanced differentiation and function represented by increased alkaline phosphatase (ALP) staining and activity, and increased mineral deposition (Figure 3.8 and 3.9). Furthermore, Osteoactivin/GPNMB mRNA expression and other osteoblast markers are also increased in GSK126-treated cells compared to untreated cells (Figure 3.10).
Figure 3.7: Cell viability affected by GSK126 higher doses. Mouse mesenchymal stem cells cell line were plated in 96 well plates and treated with 2, 4, and 8 µM of GSK126 or left untreated. Doses of 4 and 8 µM significantly and reduce cell viability after 48 (A) and 72 (B) hours incubation. This experiment was repeated three times. Data presented in all graphs represent Mean ± SEM. *=p<0.05, **=p<0.01, ***=p<0.001.
Figure 3.8: Inhibition of Ezh2 enhances osteoblast differentiation.

Images of alkaline phosphatase stained osteoblasts matrix on day 7 in culture (A). ALP activity in differentiated osteoblasts on day 7 (B). GSK126 treatment enhanced ALP protein expression in matrix compared to untreated cells. This experiment was repeated three times. Data presented in all graphs represent Mean ± SEM. **=p<0.01.
**Figure 3.9: Inhibition of Ezh2 increases osteoblast function.** Images of von kossa stained osteoblasts mineralized matrix on day 14 in culture (A). Average area of mineralized nodules in untreated and treated differentiated osteoblasts on day 14 (B). GSK126 treatment enhanced osteoblasts mineral deposition compared to untreated cells. This experiment was repeated three times. Data presented in all graphs represent Mean ± SEM. *=p<0.05.
Figure 3.10: Inhibition of Ezh2 activity increases Osteoactivin/GPNMB, RUNX2, and COL1 expression in osteoblast. qPCR analysis of Osteoactivin/GPNMB and osteoblast related genes are significantly enhanced in GSK126-treated cells compared to untreated. Osteoactivin/GPNMB (A), RUNX2 (B), and COL1 (C). Data presented in all graphs represent Mean ± SEM. *=p<0.05, **=p<0.01, ***=p<0.001.
**Ezh2 inhibitor has no effect on Osteoactivin/GPNMB mutated calvarial organ culture or osteoblast differentiation and function**

In order to investigate if Ezh2 inhibitor has an effect on bone formation in cells that lack functional Osteoactivin/GPNMB protein, we treated C57BL/6 and D2J calvarial organ culture with GSK126 (Figure 3.11). Our data indicates that GSK126 treatment to D2J calvarial organ culture has no effect on bone formation compared to C57BL/6 calvaria (Figure 3.12 and 3.13). Similar results are seen when D2J osteoblasts are differentiated and treated with GSK126. GSK126-treated D2J osteoblasts show only 3% increase in mineralization compared to untreated D2J osteoblasts; whereas GSK126-treated C57BL/6 osteoblasts have about 22% increase in mineralization compared to untreated C57BL/6 cells (Figure 3.14).
Figure 3.11: Photographic images of plated calvaria organ culture.

Calvarias of C57BL/6 and D2J (n=4) were plated on stainless steel mesh in 12-well plate. Calvarias were incubated in media for 24 hours before GSK126 treatment.
Figure 3.12: GSK126-treated C57BL/6 calvarias have increased bone formation. Calvarias from both C57BL/6 and D2J mice (n=4) were treated with or without GSK126. GSK126-treated calvarias have new bone growth only in C57BL/6 (A) but not in D2J (B) compared to controls of each genotype. Images were taken at 20X magnification.
Figure 3.13: Analysis of GSK126-treated calvarias thickness. C57BL/6 calvarias that were treated with GSK126 have 33% increase in thickness compared to untreated C57BL/6 controls; whereas D2J calvarias that were treated with GSK126 have only 7% increase in thickness compared to untreated D2J controls. Calvarias were treated with 10 µM GSK126. Data presented in all graphs represent Mean ± SEM. **=p<0.01.
Figure 3.14: Analysis of mineralized matrix by GSK126-treated osteoblasts from mouse MSCs. C57BL/6 GSK126-treated osteoblasts have 22% increase in mineralized matrix compared to untreated C57BL/6 controls; whereas D2J GSK126-treated osteoblasts have only 2% increase in mineralized matrix compared to untreated D2J controls. Cells were treated with 2 µM GSK126. This experiment was repeated twice. Data presented in all graphs represent Mean ± SEM. **=p<0.01.
Discussion

Our interest in investigating the epigenetic regulation of Osteoactivin/GPNMB arose from work has published in our lab on molecular regulation of Osteoactivin/GPNMB in bone cells. Previous studies have reported on epigenetic regulation of multiple osteoblast related genes [145, 147-149]; however, there are no reports on the epigenetic regulation of Osteoactivin/GPNMB in bone cells. In this study, we show that Osteoactivin/GPNMB gene is highly methylated in mesenchymal stem cells and MC3T3-E1 osteoblasts during their proliferative stage, and the state of methylation decreases during osteoblast differentiation. Also, we show that Osteoactivin/GPNMB promoter is occupied by H3K27 which is directly methylated by Ezh2.

To understand how Osteoactivin/GPNMB gene is regulated by methylation, we analyzed the changes in its methylation state at different stages during osteoblasts differentiation. As previously published, expression of Osteoactivin/GPNMB at early stage of osteoblast differentiation is low, and increases as osteoblasts progress into maturation [209]. Therefore, when Osteoactivin/GPNMB expression is low, we found methylation levels were high and vice versa during the rest of time points in osteoblast differentiation. In order for Osteoactivin/GPNMB gene to be highly expressed, it needs to be hypo-methylated, especially during osteoblast differentiation.

Our analysis of Osteoactivin/GPNMB expression in mouse femurs from different ages, shows that Osteoactivin/GPNMB expression is high at young age.
This might indicate that Osteoactivin/GPNMB is needed during the period of active bone formation. Furthermore, Osteoactivin/GPNMB expression starts decreasing during aging up until one-year-old in which expression begins to increase again. In our methylation studies, our results show Osteoactivin/GPNMB gene methylation in mouse femurs is low at younger ages but increases at later ages until one-year-old where it decreases. These analyses indicate that Osteoactivin/GPNMB gene expression and methylation in mouse long bone shows that when Osteoactivin/GPNMB is highly expressed, its gene is hypomethylated, and when it has low expression, the gene is hypermethylated. Therefore, Osteoactivin/GPNMB gene is always hypomethylated when its expression is needed for osteoblasts differentiation and during active bone formation age in mouse long bone.

Histone methylation has been shown to play a major role in skeletal development and osteoblast differentiation and function [173]. Enhancer of zeste homolog 2 (Ezh2), the methyltransferase for H3K27, is shown to be a negative regulator for osteoblasts and positive regulator for adipocytes by repressing Wnt genes and facilitating adipogenesis [175]. Recently, a study has reported that Ezh2 conditional knock out in mouse mesenchymal stem cells under the Prx1 promoter resulted in mice with major skeletal deformities and shorter limbs [173]. Interestingly, analysis of RNA-Seq data from Ezh2 conditional knock out mouse calvaria show higher expression of Osteoactivin/GPNMB compared to normal mice. To further understand if there is a direct relation between Ezh2 and Osteoactivin/GPNMB gene, ChIP-Seq data analysis from MC3T3-E1 cells reveals
that Osteoactivin/GPNMB gene promoter is associated with H3K27 and directly regulated by Ezh2. These findings explain the increased expression of Osteoactivin/GPNMB in these cells when treated with Ezh2 inhibitor (GSK126). Similar trend of increased expression of Osteoactivin/GPNMB in GSK126-treated MC3T3-E1 is also shown in mouse mesenchymal stem cells during osteoblast differentiation. Our results indicate, GSK126-treated osteoblasts show enhanced alkaline phosphatase staining and activity, and enhanced mineralization. These results further support the direct connection between Ezh2, Osteoactivin/GPNMB, and osteoblast differentiation and function.

To further look at the direct correlation between Ezh2 and Osteoactivin/GPNMB and its influence on osteoblast differentiation and bone formation, we conducted calvarial organ and osteoblast cultures from C57BL/6 and D2J mice. As shown in previous reports, D2J mice have a natural mutation in Osteoactivin/GPNMB gene which results in a truncated non-functional Osteoactivin/GPNMB protein. These mice were shown to have significant reduction in bone mass, and reduced ex-vivo osteoblast differentiation and function compared to normal mice. Osteoblasts from D2J mice only express the truncated non-functional form of osteoactivin/GPNMB, and therefore these cells have reduced differentiation and function. Using calvarias and mesenchymal stem cells from D2J mice helped us to better look at effects of Ezh2 inhibitor on bone formation and osteoblasts differentiation when Osteoactivin/GPNMB function is absent.
Our data from C57BL/6 and D2J mice calvarial organ culture indicates that using Ezh2 inhibitor has little to no effect on bone formation in D2J compared to C57BL/6 calvaria. Treatment of Ezh2 inhibitor increased C57BL/6 calvaria thickness by 33% compared to untreated control calvaria from C57BL/6 mice, whereas D2J clavaria thickness increased by only 7% compared to untreated control calvaria from D2J mice. Similar trends were shown in GSK126-treated osteoblasts from D2J and C57BL/6, where treatment of GSK126 enhanced C57BL/6 osteoblasts minerals deposition by 22% compared to untreated, whereas D2J treated cells show only 2% increase in mineralization compared to untreated. These data indicate that the absence of functional Osteoactivin/GPNMB protein results in diminished effects of Ezh2 inhibitor on osteoblast differentiation and function. This also supports the existence of a direct link between Ezh2 and Osteoactivin/GPNMB, in which inhibition of Ezh2 activity stimulates bone formation, at least in part, through Osteoactivin/GPNMB expression.

Taken together, we report that Osteoactivin/GPNMB gene is hypermethylated during proliferation and early stage of differentiation in Osteoblast, and gene becomes hypomethylated during later stages of differentiation. Furthermore, our data show that Osteoactivin/GPNMB promoter is occupied by H3K27 which is methylated by Ezh2, and therefore, Osteoactivin/GPNMB expression is directly regulated by Ezh2.
Chapter 4

Summary and Conclusion

The purpose of this study was to investigate the regulation of Osteoactivin/GPNMB by microRNA, gene and histone methylation. In this study, we have shown that Osteoactivin/GPNMB mRNA is targeted by miR-150, and its gene expression is regulated by DNA and histone methylation (Figure 4.1, 4.2, and 4.3).

In order to investigate the effects of miR-150 targeting Osteoactivin/GPNMB mRNA on bone cell differentiation and function, we used miR-150 KO mice. Deficiency of miR-150 in mice lead to a reduction in bone mass and volume, despite the increased expression of Osteoactivin/GPNMB in their bone cells. These results require further investigation through the use of miR-150 specific knock out in osteoblast or osteoclast in order to clearly understand the reason behind this reduction in bone volume, despite the increased expression of Osteoactivin/GPNMB.

Next, we aimed to investigate the effects of miR-150 loss on osteoblast differentiation. Our data shows enhanced ex vivo osteoblast differentiation and function in miR-150 KO cells compared to WT. Osteoactivin/GPNMB expression
is shown to be significantly increased in miR-150 deficient cells compared to WT. Furthermore, our data show a reduced ex vivo osteoclast differentiation and function in miR-150 KO cells, where Osteoactivin/GPNMB expression is significantly increased compared to WT. These findings suggest that miR-150 is a negative regulator for osteoblast and positive regulator for osteoclast, at least in part, due to the increased expression of Osteoactivin/GPNMB which has been shown to stimulate osteoblast and inhibit osteoclast differentiation.

The purpose of our second aim was to investigate if Osteoactivin/GPNMB is epigenetically regulated by DNA and histone methylation. Our data suggest that Osteoactivin/GPNMB gene is hypermethylated at early stage of osteoblast differentiation, then becomes hypomethylated at later stages of differentiation. Osteoactivin/GPNMB is also shown to be hypomethylated in young mice long bone, whereas older mice have hypermethylated Osteoactivin/GPNMB gene in long bone.

We further investigated if Osteoactivin/GPNMB gene is affected by histone methylation. Our data suggest that Osteoactivin/GPNMB is occupied by H3K27 which is methylated by Ezh2, a methyltransferase that mono-, di, and tri-methylates H3K27. Our results show that inhibition of Ezh2 activity yields less methylation of H3K27, higher expression of Osteoactivin/GPNMB, and enhanced osteoblast differentiation and function. Furthermore, inhibition of Ezh2 activity in C57BL/6 mouse calvarial organ culture increases bone formation and calvaria thickness, whereas Ezh2 inhibition in D2J calvaria has no effect on bone formation.
Overall, these findings support our hypotheses that Osteoactivin/GPNMB is post-transcriptionally and epigenetically regulated by miR-150, and DNA and histone methylation, respectively (Figure 4.4). Future studies should focus on the use of miR-150 and Ezh2 inhibitors for therapeutic treatment of osteoporosis and stimulating bone formation and healing in cases of bone fractures.
Figure 4.1: Schematic diagram showing Osteoactivin/GPNMB mRNA targeted by miR-150. (A) Normal translation of Osteoactivin/GPNMB mRNA into protein by ribosome and tRNA. (B) Osteoactivin/GPNMB mRNA translation is inhibited by miR-150, therefore Osteoactivin/GPNMB protein is not expressed.
Figure 4.2: Schematic diagram showing DNA methylation and Osteoactivin/GPNMB expression. (A) CpGs in Osteoactivin/GPNMB gene are not bound to methyl groups, therefore Osteoactivin/GPNMB gene is active and available for transcription. (B) Osteoactivin/GPNMB gene CpGs are occupied by methyl groups and therefore the gene is repressed.
Figure 4.3: Schematic diagram showing H3K27 methylation and chromatin structure in respect to Osteoactivin/GPNMB. (A) Active chromatin with acetylated H3K27 allowing Osteoactivin/GPNMB promoter to be accessible for transcription factors. (B) Inactive, compacted chromatin with de-acetylated and methylated H3K27 by HDACs and Ezh2, respectively, blocking Osteoactivin/GPNMB promoter from being accessible to transcription factors.
**Figure 4.4: Diagram showing Osteoactivin/GPNMB regulation by miR-150 and Ezh2.**

Aim I conclusion suggests that silencing Osteoactivin/GPNMB by miR-150 inhibits osteoblast and enhances osteoclast differentiation. Aim II conclusion suggests that Osteoactivin/GPNMB expression is inhibited by Ezh2, therefore, inhibiting Ezh2 activity enhances Osteoactivin/GPNMB expression and osteoblast differentiation. Future studies should focus on investigating if there is connection between Ezh2 and miR-150.
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