Parathyroid Hormone Regulates Osterix Promoter Activity
*In Vitro* and Expression *In Vivo*

by

Richard Barbuto

A thesis submitted in conformity with the requirements for the degree of Master of Science

Graduate Department of Pharmacology and Toxicology
University of Toronto

© Copyright by Richard Barbuto 2011
Parathyroid Hormone Regulates Osterix Promoter Activity \textit{In Vitro}
and Expression \textit{In Vivo}

Richard Barbuto

Master of Science

Department of Pharmacology and Toxicology
University of Toronto

2011

Abstract

Osterix (Osx) is a transcription factor required for osteoblast differentiation and bone formation. We previously demonstrated that continuous parathyroid hormone (PTH) treatment inhibited Osx expression in murine calvaria and osteoblastic UMR106-01 cells through the regulation of two regions on the Osx promoter. Mutational analysis of transcription factor elements within these regions revealed two "Sp-sites" were vital for Osx promoter activity. Blockage of these Sp-sites with Mithramycin A demonstrated their importance for Osx expression. Osx bound to its own promoter at these sites, while PTH treatment inhibited this association. PTH regulation of Osx expression \textit{in vivo} was investigated in mice by: daily injection of PTH for 3 days, continuous infusion of PTH from osmotic pumps for 14 days, or mice fed a calcium-deficient diet for 21 days. Osx expression was decreased by daily injection, while Osx expression was stimulated in mice receiving continuous PTH infusion and mice fed a calcium-deficient diet.
Acknowledgments

First, and foremost, I would like to thank my parents, Enzo and Diana Barbuto, for the years of support in all my endeavors. You have always been there to provide me with the tools I require to succeed in all that I do. I would also like to thank my fiancée, Catarina Lemos, for standing by me through my entire academic career, and inspiring me to be the best I can be.

This thesis would not have been possible without the guidance of my supervisor, Dr. Jane Mitchell. I would like to thank my fellow labmate and friend Colin Claridge, who I was fortunate enough to share my graduate school experience with. I am also indebted to Dr. Kim Sugamori and Ariana dela Cruz for helping me countless times throughout my graduate studies.
# Table of Contents

Abstract ............................................................................................................................................ ii

Acknowledgments .......................................................................................................................... iii

Table of Contents ........................................................................................................................... iv

List of Tables ................................................................................................................................ vii

List of Figures .............................................................................................................................. viii

List of Abbreviations ...................................................................................................................... x

1 Introduction ................................................................................................................................ 1

1.1 Bone and its constituents .................................................................................................... 1

1.2 Skeletal development, growth, and maintenance ................................................................ 2

1.2.1 Skeletogenesis ......................................................................................................... 2

1.2.2 Bone homeostasis .................................................................................................... 5

1.3 Differentiation of Osteoblasts ............................................................................................. 6

1.4 Osx is essential for bone formation and osteoblast differentiation ..................................... 8

1.4.1 The discovery of Osx and its importance to bone ................................................... 8

1.4.2 Characterization of Osx .......................................................................................... 9

1.4.3 Osx Target Genes .................................................................................................. 10

1.4.4 Osx Activity .......................................................................................................... 12

1.4.5 Potential Role of Osterix in Bone Disease ............................................................ 15

1.5 Regulation of Osx Expression .......................................................................................... 16

1.5.1 Bone Morphogenetic Proteins ............................................................................... 16

1.5.2 Insulin-like Growth Factor-1 (IGF-1) ................................................................... 17

1.5.3 IRE1α-XBP1 Pathway .......................................................................................... 17

1.5.4 Tumour Necrosis factor ....................................................................................... 18

1.5.5 Ascorbic Acid ....................................................................................................... 19
1.6 Parathyroid Hormone and Parathyroid Hormone Receptor .............................................. 19

1.6.1 Parathyroid Hormone .................................................................................................. 19

1.6.2 Paradoxical effects of PTH on bone ........................................................................... 20

1.6.3 PTH can regulate Osx expression, but not Runx2 expression, in vitro and ex vivo ........................................................................................................................ 21

1.6.4 The Osx promoter contains two PTH-responsive regions ....................................... 24

1.7 Rationale, Hypotheses, and Goals ................................................................................. 26

2 MATERIALS AND METHODS ............................................................................................. 30

2.1 Reagents and Plasmids .................................................................................................. 30

2.2 Cell culture ..................................................................................................................... 30

2.3 Treatment and RNA Extraction ..................................................................................... 30

2.4 Reverse Transcription and Quantitative Real-time PCR .............................................. 31

2.5 Protein Extraction and Immunoblot Analysis ................................................................ 32

2.6 Site-Directed Mutagenesis ........................................................................................... 34

2.7 Transfection and Luciferase Assays .............................................................................. 40

2.8 Chromatin Immunoprecipitation Assays ..................................................................... 41

2.9 Mice Treatments and Tissue Homogenization .............................................................. 44

2.10 Statistical Analysis ....................................................................................................... 45

3 RESULTS ................................................................................................................................ 46

3.1 PTH inhibits Osx mRNA and protein expression in vitro ............................................. 46

3.2 The -304/+91 region of the Osx promoter retains full PTH responsiveness ................ 49

3.3 Mutational Analysis of Potential Binding Sites on -304/+91 Osx Promoter................... 51

3.4 Mutational Analysis of Potential Binding Sites on -1269/+91 Osx Promoter .............. 53

3.5 Inhibition of transcription factor binding to Sp sites with Mithramycin A ................... 56

3.6 PTH regulation of Sp1, Sp3, and TIEG1 expression ...................................................... 59

3.7 Osx can bind to its own promoter in vivo, and PTH can inhibit this binding.............. 61
3.8 Osx expression decreases as mice increase in age *in vivo* ........................................ 64
3.9 Three days of PTH injections inhibits Osx expression *in vivo* ................................. 66
3.10 The effects of hyperparathyroidism on mice ............................................................... 68
3.11 Changes in mRNA levels using a model for primary hyperparathyroidism ............... 71
3.12 Changes in mRNA levels using a model for secondary hyperparathyroidism .......... 73

4 DISCUSSION ...................................................................................................................... 75
4.1 Prolonged exposure to PTH results in persistent inhibition of Osx expression *in vitro* ... 75
4.2 The two identified Sp elements (Spa/Spb) are required for full Osx promoter activity .... 75
4.3 PTH and Mithramycin A inhibit Osx expression through distinct mechanisms .......... 77
4.4 Osx may autoregulate its own expression, and PTH may be able to inhibit this process. 80
4.5 Osx expression levels mirror developmental stage of the skeleton .............................. 85
4.6 Short-term Intermittent PTH inhibits Osx *in vivo* ........................................................ 86
4.7 Assessing primary and secondary hyperparathyroidism .............................................. 87
4.8 Chronically elevated PTH levels stimulate Osx expression *in vivo* ......................... 89
4.9 SOST may mediate PTH's effects on Osx and osteoblast differentiation in long-term PTH paradigms ................................................................. 91
4.10 Conclusion ................................................................................................................... 93

References............................................................................................................................ 95
List of Tables

Table 1 - Primers used for standard quantitative real-time PCR................................. 33
Table 2 - Primary antibodies used for immunoblot and ChIP experiments..................... 35
Table 3 - Primers used to create Osx promoter mutants........................................... 39
Table 4 - Primers used for ChIP genomic DNA quantification.................................... 43
List of Figures

Figure 1 - Diagram of endochondral bone formation............................................................... 3
Figure 2 - Diagram of osteoblast differentiation...................................................................... 7
Figure 3 - The protein structures of Sp-family proteins........................................................... 11
Figure 4 - A schematic diagram of the Osx protein................................................................. 13
Figure 5 - Mechanism for PTH-mediated inhibition of Osx transcription............................... 23
Figure 6 - Luciferase assay demonstrating PTH's effects on Osx promoter activity.............. 25
Figure 7 - Diagram of potential transcription factor binding sites on the Osx promoter........ 27
Figure 8 - Schematic diagram of mutated -304/+91 Osx promoter constructs....................... 37
Figure 9 - Schematic diagram of mutated full-length Osx promoter constructs.................... 38
Figure 10 - PTH regulation of Osx expression........................................................................ 48
Figure 11 - PTH regulation of the Osx promoter..................................................................... 50
Figure 12 - Mutational analysis of two putative Sp elements.................................................. 52
Figure 13 - Mutational analysis of potential elements using the -1269/+91 Osx promoter.......... 55
Figure 14 - The effects of blocking Sp site binding with Mithramycin A............................... 58
Figure 15 - PTH regulation of Sp1, Sp3, and TIEG1.............................................................. 60
Figure 16 - Investigation of transcription factor association to Osx promoter......................... 63
Figure 17 - Detection of Osx mRNA expression in mice of increasing ages.......................... 65
Figure 18 - The effects of acute PTH treatments on Osx in vivo.......................................... 67
Figure 19 - The effects of hyperparathyroidism in mice......................................................... 70
Figure 20 - The effects of primary hyperparathyroidism on gene expression......................... 72
Figure 21 - The effects of secondary hyperparathyroidism on gene expression.................... 74
Figure 22 - Homology of the two Sp sites............................................................................. 82
Figure 23 - Proposed mechanism of Osx autoregulation.......................................................... 84
List of Abbreviations

aBMD - areal bone mineral density
ALP - Alkaline phosphatase
BMP - Bone morphogenic protein
BSP - Bone sialoprotein
ChlP - Chromatin Immunoprecipitation
Col1a1 - Type I collagen
Cyp27B1 - 25-hydroxyvitamin D₃ 1-alpha-hydroxylase
DEXA - dual-energy X-ray absorptiometry
Dkk1 - Dickkopf-related protein 1
EGFR - Epidermal growth factor receptor
ERK1/2 - Extracellular signal regulated kinases 1 and 2
GAPDH - Glyceraldehyde 3-phosphate dehydrogenase
IHH - Indian hedghog homolog
IGF - insulin-like growth factor
IgG - Immunoglobulin G
IRE1α - inositol-requiring protein-1α
JmjC - Jumonji C
JNK - c-Jun N-terminal kinases
LRP - lipoprotein receptor-related protein family
MSC - Mesenchymal stem cell
NFκB - Nuclear factor-kappa B
NFATc - Nuclear Factor of Activated T Cells
NF-Y - Nuclear transcription factor Y
OC - Osteocalcin
OPG - Osteoprotegrin
Osx- Osterix
PS - Primary spongiosa
PTH - Parathyroid hormone
PTH1R - Parathyroid hormone 1 receptor
PTHrP - Parathyroid hormone related-peptide
RANKL - Receptor activator of nuclear factor kappa-B ligand
Runx2 - Runt-related transcription factor 2
SOC - Secondary Ossification Center
SOST - Sclerostin
SP - Specificity protein
TIEG1 - Transforming growth factor β-inducible early gene 1
TGF-β - Transforming growth factor-β
TNF - Tumour Necrosis Factor
UPR - Unfolded protein response
XBP1 - X-box binding protein 1
1 Introduction

1.1 Bone and its constituents

Bone is an essential organ that serves several important functions in vertebrates. It forms the endoskeleton that supports the body's shape and protects the body's delicate organs, it acts as a depot for calcium and phosphate, and it is involved in the process of blood cell production. Both inorganic and organic components are needed to enable bone to function in these capacities. Hydroxylapatite, $\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$, is the principal inorganic constituent, as it accounts for 40% of human adult bone by weight (1). The organic component of bone consists of specialized cells and organic material. There are three main types of bone-specific cells: osteoblasts, osteoclasts, and osteocytes. Osteoblasts are mesenchymal-derived cells that are responsible for bone formation. They express and secrete extracellular matrix proteins, and are then responsible for their mineralization (2). Osteoclasts, which are derived from monocytes/macrophages, are specialized cells that secrete hydrochloric acid and proteolytic enzymes to digest bone tissue (3). Lastly, osteocytes, which make up over 95% of all bone cells, are mechanosensing cells that detect strain placed on the skeleton. They arise from matured osteoblasts that have been trapped within the bone matrix, and, although they can no longer directly form bone themselves, they are able to initiate bone remodeling processes through the release of cytokines (4). Osteocytes are regularly dispersed throughout the mineralized matrix, and are connected to each other via dendritic processes through tiny canals known as canaliculi (5). The organic material found in bone is mostly comprised of extracellular matrix proteins secreted by osteoblasts, of
which type I collagen (col1a1) makes up 90%, and also includes osteonectin and bone sialoprotein, among others (6).

1.2 Skeletal development, growth, and maintenance

1.2.1 Skeletogenesis

The development of the skeleton can be divided into two phases. Skeletal patterning is the first step in this process, where cells derived from the mesenchyme organize at sites where bone will eventually be formed (7). This step occurs under the regulation of unique genes, such as bone morphogenetic protein-4, and will not be discussed in any greater detail in this thesis (8). Instead, a greater focus will be placed on the next step in which mesenchymal stem cells, having already been aligned into a template of the future skeleton by embryonic day 10, differentiate into the various cell lineages that carry out bone formation (Fig 1).

A population of mesenchymal stem cells differentiate into cartilage-forming cells known as chondrocytes that release type II collagen and aggrecan, and these cells form at the centre of future bone (9). The mesenchymal stem cells that remain undifferentiated surround the chondrocytes and form a structure known as the perichondrium, and these mesenchymal stem cells are responsible for the formation of an extracellular matrix made up of type I collagen (10). At embryonic day 13.5 in mice, the chondrocytes at the core of this structure cease proliferating, and exit the cell cycle, becoming hypertrophic chondrocytes that uniquely express type X collagen (11). At the same time, the mesenchymal stem cells of the perichondrium begin to differentiate into preosteoblasts and form the region known as the bone collar, which will eventually develop into cortical bone.
Figure 1. Diagram of endochondral bone formation. Adapted from (7). (a) Mesenchymal stem cells (blue) form a template of skeleton. (b) Some mesenchymal stem cells differentiate into chondrocytes. (c) Chondrocytes at the centre of future bone hypertrophy. (d) Blood vessels invade the hypertrophic centre. (e) Osteoblasts invade the centre and form the primary spongiosa (PS). (f) Chondrocytes continue to proceed through cycles of proliferation, hypertrophy, and apoptosis, elongating bone. (g) Secondary ossification centers (SOC) form at both ends of the elongating bone.
Through a vascular endothelial growth factor-dependent pathway, capillaries invade the hypertrophic cartilage centre (12). The hypertrophic chondrocytes undergo apoptosis, leaving behind a scaffold of extracellular matrix composed of type X collagen, which is then invaded by osteoblasts through the newly created blood vessels. These osteoblasts then induce the mineralization of type X collagen, and this region, which is referred to as the primary spongiosa, is eventually replaced by trabecular bone (13). On either side of the primary spongiosa, there exists an area called the secondary ossification centre where bone is allowed to grow longitudinally through the continued proliferation, hypertrophy, and apoptosis of chondrocytes, resulting in the eventual replacement of the cartilaginous skeleton with a calcified one (14). This site serves as a growth plate, and is capped by so-called resting chondrocytes that are no longer proliferating rapidly. Eventually, the growth plate disappears, usually at adolescence in humans, and the bones no longer grow in length.

Endochondral bone formation, described above, is the process whereby osteoblastic ossification first necessitates the creation of a cartilaginous template by chondrocytes. This type of bone development results in the formation of the long bones, such as the femur. Alternatively, intramembranous bone formation occurs without a cartilage template and the osteoblasts are responsible themselves for the secretion of all the proteins that make up the unmineralized extracellular bone matrix (15). Subsequently, osteoblasts induce the mineralization of the matrix, resulting in calcified bone. Intramembranous bone formation is largely responsible for the formation of the bones of the skull.
1.2.2 Bone homeostasis

Bone is a dynamic tissue which requires a mechanism to respond to the stresses and strains put upon the skeleton so that micro-fractures, which may occur as a result of normal daily activities, can be repaired. In addition, there must be a mechanism in place whereby calcium and phosphate can be released to maintain serum concentrations. These requirements are met through the critical process of bone remodeling. This process begins when old or damaged bone tissue is degraded by osteoclasts, which typically takes between 30-40 days in humans, in a process called bone resorption (16). Then, osteoblasts are recruited and new bone is formed over a period of approximately 150 days. Bone remodeling is a highly regulated process, as osteoblastic and osteoclastic activity is tightly coupled to ensure an appropriate response. For example, the presence of osteoblasts is a requirement for the differentiation of mature osteoclasts and initiation of bone resorption (17). This is due to the fact that osteoblasts express receptor activator of nuclear factor-κB ligand (RANKL), both a membrane-bound and soluble protein, which osteoclasts require for their recruitment, differentiation, and survival (3). RANKL interacts with the RANK receptor which is expressed on the surface of osteoclast precursors and mature osteoclasts. Conversely, osteoblasts can abrogate osteoclastic activity through the expression of osteoprotegrin (OPG), a soluble receptor expressed by osteoblasts that can bind and inhibit RANKL (2). Therefore, increased expression of OPG results in decreased RANK binding by RANKL, and decreased osteoclastic activity.

In addition, osteoclasts can modulate osteoblastic activity. During resorption, key factors, such as insulin-like growth factor and transforming growth factor-β, are released from the digested bone matrix by osteoclast activity, which cause an increase in the
number of osteoblasts by increased differentiation from precursors and decreased apoptosis (16).

1.3 Differentiation of Osteoblasts

As noted above, osteoblasts are derived from mesenchymal stem cells through a complex pathway that is still not entirely understood (Fig 2). Mesenchymal stem cells (MSCs) are pluripotent cells that have the ability to differentiate into numerous cell types that form cartilage, fat, tendon, muscle, or bone (18). For osteoblasts to form, the mesenchymal stem cells must first be committed to the osteogenic lineage and become osteochondroprogenitors, which can ultimately differentiate into osteoblasts or chondrocytes (19). Critical for the differentiation to these bipotential progenitors in both endochondral and intramembranous forms of ossification is the expression of Runx2/Cbfa1, a runt-domain containing transcription factor. A crucial role for Runx2 was discovered when Runx2-KO mice displayed a complete absence of osteoblasts and of bone formation, and thus, Runx2 is considered the master regulator of osteoblast differentiation (20). However, the differentiation of mesenchymal stem cells to bipotential cells is complex and varies depending on bone formation type. For example, the expression of the signaling factor Indian hedgehog (IHH) is required for the initial activation of Runx2 expression during endochondral bone formation. In Ihh-KO mice, endochondral bone formation is compromised, while intramembranous bone formation remains unaffected (21).
Figure 2. Diagram of osteoblast differentiation. Osteoblasts are derived from mesenchymal stem cells (MSCs), and require the expression of both Runx2 and Osx to fully mature. Runx2 expression commits the MSCs down the osteogenic lineage, while Osx expression allows for osteoblasts to arise preferentially over chondrocytes. Immature osteoblasts express genes such as type I collagen (Col1a1) and alkaline phosphatase (ALP), while mature osteoblasts express osteocalcin (OC) and the parathyroid hormone receptor (PTHR).
Once the osteochondroprogenitors begin expressing Runx2, they can then differentiate into osteoblasts, and this process requires canonical Wnt/β-catenin signaling (22). Once Wnt/β-catenin signaling is activated, the bipotential precursors begin expressing the transcription factor Osterix (Osx), and thereafter these cells are classified as osteoblasts. Osteoblasts mature through at least two stages, expressing different genes at each stage. Immature osteoblasts characteristically express type I collagen and alkaline phosphatase, and these cells are largely responsible for creating the unmineralized extracellular matrix of bone. Mature osteoblasts, which arise under the influence of Osx expression, express genes such as osteocalcin, bone sialoprotein, and the parathyroid hormone receptor, and they are responsible for the mineralization of bone (2).

1.4 Osx is essential for bone formation and osteoblast differentiation

1.4.1 The discovery of Osx and its importance to bone

The recently discovered transcription factor Osterix (Osx, Sp7) is essential for osteoblast differentiation and embryonic skeletal development, and is considered the second master regulator of osteoblasts. Osx was first discovered through the use of a subtractive hybridization screen in which a muscle progenitor cell-line was forced to differentiate into osteoblasts. To identify its role, Osx was knocked-out in mice. Like Runx2-KO mice, Osx-KO mice displayed no mineralized bone and died at birth, demonstrating the absolute importance of Osx in both endochondral and intramembranous ossification (23). Osx acts downstream of Runx2 in the osteoblast differentiation pathway as Osx-KO mice express Runx2, whereas Runx2-KO mice do not express Osx.
After this initial determination of the importance of Osx in osteoblast differentiation, it was then hypothesized that Osx could also be a key player in healthy bone maintenance. In order to assess the role of Osx on the homeostasis of postnatal bone, conditional Osx knockout mice were created and Osx was inactivated after birth to rescue lethality. These mice displayed an accumulation of bone microfractures, decreased osteoblastic activity, and an impairment of normal osteocyte maturation and function (24,25). Thus, Osx is also vital for the normal maintenance of mature bone.

1.4.2 Characterization of Osx

Osx is expressed primarily in osteoblasts and osteocytes, however, it has been detected transiently in chondrocytes and has recently been found in the olfactory bulb of mouse brain (26). Osx expression can first be detected at low levels at embryonic day 13.5 in the perichondrium, which corresponds to the time in which mesenchymal stem cells are differentiating into osteoblasts.

The Osx gene spans approximately 15 kb, and is located on chromosome 12q13 in both mice and humans, and there is 95% Osx gene homology between these species (23,27). In both, the Osx gene is organized into two exons and one intron, with the second exon containing most of the coding sequence. The Osx gene has a TATA-less promoter, and two alternatively spliced mRNA variants have been identified with 5' RACE experiments (27). The Osx protein can exist as either the long isoform (431 residues), derived from mRNA with both exons, or a short isoform (413 residues), derived from mRNA with only exon 2. Both isoforms can be visualized on an immunoblot as bands at approximately 56 kDa and 46 kDa, respectively (28).
The Osx protein, which is constitutively found in the nucleus, contains 3 Cys2-His2-type (Kruppel-type) zinc fingers near the C-terminus. The 85 amino acid zinc-finger motif has a high degree of homology to the motifs present in the Specificity protein (Sp) family of transcription factors (Fig 3). However, it is unique from the other Sp factors as it has a proline-rich domain near its N-terminus, and it lacks a glutamine-rich transactivation domain. As a member of the Sp family, Osx is predicted to bind to G/C-rich DNA binding sites similar to the other Sp factors (29).

1.4.3 Osx Target Genes
At the cellular level, Osx is able to cause the differentiation of osteoblasts from precursors, although this process is not entirely understood. Normally, marker genes are used to determine whether or not an osteoprogenitor has differentiated into an osteoblast. Immature osteoblasts, which can secrete extracellular matrix proteins but cannot yet induce mineralization, are cells that express type I collagen and alkaline phosphatase (ALP), while mature osteoblasts, which are capable of mineralizing matrix, express osteocalcin (OC). It has been demonstrated that in vitro, forced Osx expression in non-osteoblastic cell-lines can induce the expression of all three of these osteoblast marker genes, in addition to osteopontin and bone sialoprotein (30,31). However, to date, Osx has not been shown to be able to associate with the promoters of these genes, suggesting that Osx may be indirectly stimulating the expression of these osteoblastic markers.

Meanwhile, Osx has been shown to directly associate with, and activate, the promoters of sclerostin, Dickkopf-related protein 1 (Dkk1), and collagen 5a. When Osx was
Figure 3. The protein domain structures of Sp-family proteins. Adapted from (33). Sp box, Buttonhead (Btd) box, serine/threonine-rich (S/T-rich), glutamine rich (Q), highly charged, activation domain (AD), inhibitory domain (ID), and proline-rich (P) regions are all indicated.
inactivated after birth, it was observed that the expression of the osteocyte-specific glycoprotein sclerostin decreased (25). It was then demonstrated that Osx could stimulate sclerostin reporter constructs and directly associate with the sclerostin promoter, providing evidence that Osx increase sclerostin expression (119). Similarly, in the embryos of Osx-KO mice, Dkk1 expression, which is a Wnt signaling antagonist, was also shown to be decreased (120). Further analysis revealed that Osx overexpression increased Dkk1 expression, and that Osx could bind to the Dkk1 promoter (120). Lastly, Osx was shown to be able to directly associate with and stimulate the activity of the collagen 5a1 promoter, which makes up part of the extracellular matrix (32). Thus, Osx has been shown to directly and indirectly regulate a diverse set of genes in order to regulate osteoblast differentiation and skeletogenesis.

1.4.4 Osx Activity

Tissue-specific transcription factors, such as Osx, have the ability to activate a discreet subset of cell-specific genes due to highly regulated processes that include interaction with basal transcriptional machinery, co-activators, and chromatin remodeling complexes, as well as regulation via post-translational modifications (33).

The proline-glycine rich transactivation domain of Osx has been shown to interact with TF-IIIB (Fig 4), a protein required for RNA polymerase II recruitment to the preinitiation complex on target gene promoters, and this interaction may be important for Osx transcriptional activity (34,35). In addition, the transcription factor Nuclear Factor of Activated T Cells (NFATc), which has been shown to be involved in osteogenesis, forms a complex with Osx in order to initiate the transcription of at least one osteoblast-specific gene, type I collagen (36).
Osx/Sp7

Figure 4. A schematic diagram of the Osx protein. The Osx protein has several important regulatory regions, such as binding sites for NO66, TF-IIB, and Brg-1. In addition, the Osx protein can be phosphorylated by p38 via Ser-73 and Ser-77 or dephosphorylated by calcineurin at the LXVP domain.
There is evidence that chromatin remodeling can either positively or negatively regulate Osx activity, depending on the context. The coactivators p300 and Brg-1 have been shown to stimulate Osx-mediated gene transcription (34,37). p300 is an acetyltransferase that causes histone acetylation, while Brg-1 forms part of the SWI-SNF complex that induces transcription through chromatin remodeling, and Brg-1 has been shown to be highly recruited to acetylated histones (38). On the other hand, histone methylation, which is dynamically regulated and can result in inactive and inaccessible chromatin, may play a negative role in Osx functioning.

It has been reported that NO66, a protein that can cause histone demethylation, inhibits Osx-mediated gene transcription, and this process requires the direct interaction between the Jumonji C (JmjC)-domain of NO66 and the Osx protein (39). Interestingly, NO66 has also been shown to inhibit Osx expression itself, and a NO66 knockdown results in accelerated osteoblast differentiation and maturation.

Like the other members of the Sp family, Osx is highly dependent on post-translational modifications for its function. Two residues on the Osx protein, Ser-73 and Ser-77, must be phosphorylated by p38 for Osx to be able to associate with and activate the transcription of the fibromodulin and bone sialoprotein promoters (37). In addition, calcineurin, or protein phosphatase 2B, may be able to regulate Osx activity as it has been shown to cause the dephosphorylation of Osx protein isoforms via interaction with a LXVP domain (40).

It is likely that the regulatory mechanisms controlling Osx activity display a large degree of cross-talk. For example, the phosphorylation of Ser-73 and Ser-77 causes an increase in Brg-1 and p300 recruitment to target promoters, which would allow for more
easily accessible chromatin (37). Therefore, regulation of the activity of Osx is a complex process involving several levels of control to ensure the correct genes are activated at the appropriate time.

1.4.5 Potential Role of Osterix in Bone Disease

The improper functioning and expression of Osx has been implicated in numerous bone diseases and abnormalities. Clinically, a patient's bone strength is correlated to their areal bone mineral density (aBMD), which is commonly measured using dual-energy X-ray absorptiometry (DEXA). aBMD is the single best predictor of fragility fractures (41), and, through the use of twin studies, aBMD has been shown to be a highly heritable trait (42). Interestingly, genome-wide association studies of aBMD in adults have identified several loci corresponding to 20 different genes, including Osx (42,43). This suggests that certain genes can be used to explain the genetic component of aBMD and the risk of bone fracture. However, these studies were performed using adult populations exclusively, where bone mass is also affected by age-related bone loss. Therefore, a genome-wide association study using children was performed, and Osx was the only gene that was found to be associated with differences in aBMD (44). Bone accrual in early life reflects the processes involved in bone development and homeostasis and, as such, is an important determinant of bone structural integrity in adulthood. Thus, Osx may play a role in the development of bone diseases, such as osteoporosis, which are characterized by bone fractures that result in significant hospital stays and a heavy economic burden on our healthcare systems.

In addition to its association with aBMD, Osx has been implicated in a form of osteogenesis imperfecta, a disease characterized by bone deformities. This form of the
disease is caused by a single nucleotide deletion resulting in a truncated Osx protein product that has altered activity (45).

1.5 Regulation of Osx Expression

Osx expression can be regulated by a plethora of signaling proteins and pathways, and, as such, osteoblast differentiation and bone formation can be modulated by a variety of different cellular processes.

1.5.1 Bone Morphogenetic Proteins

Bone Morphogenetic Proteins (BMPs) are a subfamily of cytokines that belong to the Transforming Growth Factor-β superfamily (TGF-β), and are highly conserved across many species. This subfamily received its name because the first identified BMPs were found to induce ectopic bone formation. However, BMPs are now known to play crucial roles in embryonic development, osteogenesis, chondrogenesis, and organogenesis (46). By binding to serine-threonine transmembrane receptors, they cause the phosphorylation and activation of Smad proteins, which then translocate and initiate the transcription of target genes in the nucleus (47). At least 20 BMPs have been discovered to date, but only BMP-2, BMP-4, and BMP-7 have been shown to induce osteoblast differentiation (48,49). In fact, BMP-2 stimulated cells were utilized in the discovery of Osx from the subtractive hybridization screen (23). BMP-2 is the only factor of the BMP family that can directly induce Osx expression upon stimulation, and this induction may be mediated through a number of different pathways (50). BMP-2 can induce the expression of Runx2, which, as mentioned previously, is upstream of Osx in the osteoblast differentiation pathway. Runx2, in turn, stimulates Osx expression, but the exact mechanism remains unclear (51). In addition, there are two proposed Runx2-
independent pathways downstream of BMP-2. Using Runx2-deficient mesenchymal cells, it was discovered that the homeobox transcription factor Msx2 could mediate BMP-2 induction of Osx expression (52). In addition, BMP-2 can also activate the homeodomain-containing transcription factor Dlx5, which can then bind a BMP-responsive site on the Osx promoter and initiate its expression (53). However, in vivo evidence for the importance of Msx2 and Dlx5 is lacking.

1.5.2 Insulin-like Growth Factor-1 (IGF-1)
Insulin-like growth factors (IGFs) are structurally related to insulin, but promote growth on a more general scale in various tissues. IGF-I is important for bone formation, as osteoblast-specific KO models have abnormal bones and show impaired mineralization (54). IGF-I can positively regulate Osx expression, and this stimulation requires the MAPK pathways and all of its downstream effectors: ERK, p38, and JNK (50,55).

1.5.3 IRE1α-XBP1 Pathway
Cells that synthesize a large quantity of extracellular proteins, such as osteoblasts, require an efficient means to produce and secrete their products without interfering with critical cell processes. Thus, in response to the stress of accumulating proteins in the endoplasmic reticulum (ER), these cells trigger the unfolded protein response (UPR), which is a system that is highly conserved in eukaryotic cells. This response results in the suppression of apoptosis and expression of proteins, such as chaperones, that increase the folding and secretory abilities of the cells (56). One such mediator of this pathway is inositol-requiring protein-1α (IRE1α), which senses ER stress and activates its effector, X-box binding protein-1 (XBP1). XBP1 is then responsible for initiating the transcription of target genes. Interestingly, XBP1 can bind to the proximal region of the
Osx promoter and initiate its transcription in response to ER stress, and this pathway may be the initial trigger for Osx expression and osteoblast differentiation (57). Before osteoblasts differentiate from Runx2-expressing pre-osteoblasts, these pre-osteoblasts express high levels of col1a1, which causes significant ER stress. This has been proposed to necessitate the expression of genes such as BSP and ALP, which may facilitate the production and deposition of ECM components in order to relieve ER stress. But before they can be expressed, Osx must be present (58). However, further investigation is required to confirm that these are the conditions that enable initial Osx expression.

1.5.4 Tumour Necrosis factor

Tumour necrosis factor (TNF) is an inflammatory cytokine that is able to both recruit osteoclast precursors and inhibit osteoblast differentiation, and is elevated in inflammatory disease states such as rheumatoid arthritis (59). TNF is a negative regulator of Osx expression, as it can inhibit the Osx promoter (60). The epidermal growth factor receptor (EGFR) may be the receptor that mediates the inhibitory effects of TNF, as EGFR stimulation has been demonstrated to cause an inhibition of Osx expression and TNF is considered to be an epidermal growth factor-like ligand (61). In any case, it was recently discovered that the transcription factor paired related homeobox protein (Prx1) is the downstream effector mediating TNF inhibition of Osx expression (62). It has been suggested that an unbalanced activation of this pathway, and the resulting inhibition of Osx expression, may predispose some individuals to certain inflammatory diseases (62).
1.5.5 Ascorbic Acid

Ascorbic acid, or vitamin C, is a key antioxidant vitamin that has been found to be important for the differentiation of osteoblasts. Disrupting gulonolactone oxidase, which is involved in the synthesis of ascorbic acid, causes increased bone fractures in mouse models (63). Stimulation of differentiating cells with ascorbic acid causes an induction of Osx and increased osteoblast differentiation (64). Ascorbic acid is a co-factor of prolyhydroxylases, enzymes that can regulate certain transcription factors, such as hypoxia-inducible factor-α, and it may be through this pathway that ascorbic acid may mediate its effects although investigations are still ongoing (65).

1.6 Parathyroid Hormone and Parathyroid Hormone Receptor

Lastly, parathyroid hormone is able to regulate Osx expression, and this regulation will be the focus of this thesis.

1.6.1 Parathyroid Hormone

Parathyroid hormone (PTH) is a major regulator of serum calcium levels and bone remodeling. It is produced by the chief cells of the parathyroid glands as a prepropeptide, and its cleavage and release is triggered when calcium-sensing receptors detect decreases in extracellular calcium concentrations (66). PTH is released as an 84 amino acid polypeptide, and directly exerts its actions on kidney and bone. In kidney, PTH increases calcium reabsorption through the activation of specific ion channels in the distal tubule, and increases phosphate excretion in the proximal tubule (67). PTH also stimulates the expression of 25-hydroxyvitamin D-1α-hydroxylase (CYP27B1), which converts 25-hydroxyvitamin D₃ to 1,25-dihydroxyvitamin D₃, the most potent form of vitamin D (68). 1,25-dihydroxyvitamin D₃, in turn, acts in the intestine to
stimulate the absorption of dietary calcium and phosphate (68). In bone, PTH stimulates bone remodeling with a net increase in bone resorption, culminating in the release of calcium and phosphate from bone. The PTH-1 receptor (PTH1R) mediates PTH's effects, and, in bone tissue, osteoblasts and osteocytes are the only cells that express this receptor (69,70). Therefore, PTH must regulate osteoclastic activity indirectly, which, as mentioned previously, is responsible for bone resorption. It accomplishes this by stimulating osteoblasts to express key genes that promote osteoclast differentiation and activity, such as RANKL (71).

The PTH1R is a G-protein coupled receptor that exerts its effects in osteoblasts by coupling to both the $G_s\alpha$ and $G_q\alpha$ G-proteins. Coupling to $G_s\alpha$ causes the activation of adenylyl cyclase and an increase in cAMP, whereas $G_q\alpha$ signaling stimulates phospholipase C activity and the formation of diacylglycerol and inositol trisphosphate. Another PTH1R agonist is PTH-related peptide (PTHrP), which is synthesized and released by osteoblasts upon PTH exposure (72). In addition, a synthetic N-terminal fragment of PTH, PTH (1-34), retains all of the signaling activity of the original peptide, and it is often used clinically and experimentally instead of the full peptide (66).

1.6.2 Paradoxical effects of PTH on bone

Depending on the duration of exposure, PTH exerts clinically significant effects on the bones of both developing and mature individuals. As one might expect, excessive PTH release, which occurs in certain disease states such as parathyroid hyperplasia, results in a significant reduction in aBMD and increase in fracture risk (73). Paradoxically, it was serendipitously discovered that when PTH is administered intermittently, as once-daily injections, aBMD is actually increased (74). Presently, the PTH analogue teriparatide, or
PTH(1-34), is the only approved treatment for osteoporosis that results in bone anabolism, and has been shown to significantly increase aBMD in patients as early as one month after the start of treatment.

It has been concluded that the biphasic effects of PTH on bone are dependent on the duration of exposure to this hormone. Continuous elevation of PTH in the bloodstream, defined as longer than 2 hours/day, is catabolic to bone, while intermittent doses of PTH, where exposure is less than 2 hours/day, is anabolic (75). The exact mechanisms that account for these different effects remain unclear. As osteoblast and osteoclast activities are normally coupled, it may be that transient activation of the PTH1R on osteoblasts results only in the proximal effects, which are its effects on the osteoblasts themselves. The more distal effects exerted on osteoclasts may require prolonged receptor activation to be triggered. Intermittent treatment with PTH has been shown to enhance the activity of existing osteoblasts, increase the differentiation of osteoblast precursors, and reduce osteoblast apoptosis (76). A likely candidate that may play a significant role in mediating these effects is a transcription factor that can both activate osteoblastic genes and promote osteoblast differentiation. Therefore, understanding the regulation of Runx2 and Osx, both considered master regulators of osteoblast differentiation, by PTH could be beneficial in uncovering the mechanisms of these effects.

1.6.3 PTH can regulate Osx expression, but not Runx2 expression, in vitro and ex vivo

Our lab has previously shown that continuous PTH treatment causes a decrease in Osx mRNA and protein levels ex vivo in calvaria isolated from embryonic and postnatal mice and in vitro in the osteoblastic rat cell line UMR106-01 (77). Osx mRNA levels were
decreased as early as 3h after treatment, while Osx protein was significantly decreased after 6h. This inhibition does not require new protein synthesis and is not mediated by alteration of Osx mRNA stability. It is likely that this inhibition occurs at the transcriptional level, as luciferase activity from reporter constructs driven by the full-length Osx promoter was inhibited in a time-dependent manner that mirrored Osx mRNA and protein declines. It was then discovered that this inhibition is mediated through the activation of $G_s\alpha$ by the PTH1R, and subsequent stimulation of cAMP production (Fig 4). Precisely how cAMP is able to suppress Osx promoter activity is still under investigation. Interestingly, PTH had no effect on Runx2 expression in either calvaria or UMR106-01 cells.
Figure 5. Mechanism for PTH-mediated inhibition of Osx transcription. The PTH1R activates the G_sα signaling pathway, resulting in an inhibition of Osx transcription and expression.
1.6.4 The Osx promoter contains two PTH-responsive regions

Using luciferase reporter constructs with progressive truncation of the murine Osx promoter, two PTH-regulated regions were identified (Fig 6).

The first region, located between base pairs -304/-119, was determined to be important as its removal partially abrogated PTH-inhibition of luciferase activity. *In silico* analysis of this region revealed putative transcription factor binding sites for nuclear factor-kappa B (NF-κB), Ets-1, and nuclear factor-Y (NF-Y) (51,60) (Fig 7). The putative site for NF-κB, which is involved in cellular responses to stimuli such as cytokines and UV radiation, was previously mutated and deleted within the Osx promoter construct by our lab. These changes had no effect on the ability of PTH to inhibit promoter construct activity, suggesting that NF-κB is not involved in the regulation of Osx by PTH (77). The two remaining elements have yet to be investigated. Ets-1 is a transcription factor that is expressed by numerous cell types, and has been shown to modulate differentiation of many different cell types, although it is uncertain if it can be regulated by PTH. NF-Y is a ubiquitous transcription factor, and it is again unknown if PTH is able to regulate its expression or activity.

The second region on the Osx promoter, located between base pairs -71/+91, was considered PTH responsive as PTH was still able to inhibit the luciferase activity of this construct by approximately 40%. In this region, two identical Sp-binding sites, situated together, were identified (51). For the purposes of distinguishing them, they will be referred to as Sp_a and Sp_b (Fig 7).
Figure 6. Luciferase assay demonstrating PTH's effects on Osx promoter activity. Adapted from (78). Two PTH-responsive regions were identified on the Osx promoter: between base-pairs -304/-119 and -71/+91.
These contiguous sites can potentially be bound by several different transcription factors that belong to the Sp-family, however, likely candidates must be expressed in osteoblasts. The transcription factors that can bind these sites, and have previously been shown to be expressed in osteoblasts, include: TGF-beta-inducible early-response gene-1 (TIEG1), Sp1, Sp3, and Osx itself. TIEG1 is a downstream effector that mediates Smad signaling, and may play a role in bone formation as TIEG1 knockout mice displayed decreased mineralized bone and decreased Osx expression levels (78). Lastly, Sp1, Sp3, and Osx belong to the same Sp family, and have been shown to regulate the transcription of a number of osteoblastic genes. Sp1 and Osx are primarily transcriptional activators, while Sp3 can act as either an activator or repressor, depending on the context.

1.7 Rationale, Hypotheses, and Goals

The differentiation of osteoblasts from mesenchymal stem cells is vital for bone formation and bone homeostasis. However, the regulation of this process remains inadequately understood, especially from the standpoint of how bone remodeling is directed. It still remains to be elucidated how PTH, a key regulator of bone remodeling, is able to alter bone morphology towards both ends of the spectrum. Osx has been identified as a potential mediator of PTH's effects, as it is critical to osteoblast differentiation and can be regulated by PTH. Following on from previous work in our lab demonstrating inhibition of Osx expression and promoter activity by PTH, I hypothesize that:
Figure 7. Diagram of potential transcription factor binding sites on the Osx promoter. NFκB, Ets-1, NF-Y, and Sp elements have been previously identified.
1. The -304/+91 region of the Osx promoter is bound, and transcriptional activity is activated, by a transcription factor that is important for Osx expression.

To date, XBP1 is the only transcription factor that has been shown to associate with and activate the Osx promoter. Previous luciferase assays have revealed that the -304/+91 region of the Osx promoter contains most of the activity of the Osx promoter. My goal is to determine which transcription factor(s) play a role in stimulating Osx expression. Using the identified putative binding sites found within the -304/+91 proximal promoter region to direct my studies, I wished to test which, if any, of these sites are important for Osx promoter activity using mutational studies. Once the key site(s) have been defined, I will test which transcription factor(s) bind to these sites under normal cellular conditions using Chromatin Immunoprecipitation (ChIP) assays.

2. PTH can regulate the transcriptional activity of the Osx promoter by regulating one or more transcriptional factors that can associate with the Osx promoter.

My goal is to determine the mechanism by which PTH represses Osx transcription. Since two PTH-responsive regions have been identified on the proximal Osx promoter (-304/-119 and -71/+91), I will test which, if any, of the potential transcription factor sites mediate PTH's inhibitory effects on Osx promoter activity using mutational studies. Then, using ChIP assays, I will determine which transcription factors bind to these sites, and whether or not PTH is able to regulate their binding.

3. PTH can regulate the expression of Osx in vivo

Currently, little is known about the regulation of Osx expression by PTH in vivo. Since PTH is able to regulate Osx expression in vitro, I hypothesize that PTH will be able to
regulate Osx expression in vivo, and I will test this using both continuous and intermittent PTH paradigms. Since excessive PTH is catabolic to bone, which implies a disproportionate increase in the amount of osteoclastic activity and bone resorption compared to bone formation, I hypothesize that continuous PTH administration will result in an inhibition of Osx expression. Conversely, intermittent PTH treatment is anabolic to bone, and, as such, implies a relative increase in osteoblastic activity in comparison to osteoclastic activity. I hypothesize that PTH given in this manner will result in an increase in Osx expression.

The cell-line UMR106-01, which is derived from rat osteosarcoma cells, was utilized as a model for osteoblasts in all subsequent in vitro experiments, as previous work has shown that PTH-mediated inhibition of Osx expression in these cells is comparable to what is observed ex vivo using mouse calvaria (77). These cells express all of the osteoblastic marker genes except osteocalcin, and also express abundant PTH1R that is well coupled to the Gsα and adenylyl cyclase pathway (79).
2 MATERIALS AND METHODS

2.1 Reagents and Plasmids
Rat PTH (1-34) was purchased from Bachem Bioscience Inc. (King of Prussia, PA, USA). Mithramycin A was purchased from Tocris Bioscience (Ellisville, MO, USA). Primers were synthesized by Integrated DNA Technologies (IDT). Automated sequencing of PCR products was performed by the The Centre for Applied Genomics (Hospital for Sick Children, MaRS Centre, Toronto, ON, Canada). The luciferase Osx promoter plasmids -1269/+91 and -304/+91 were obtained from Dr. Mark Nanes (Emory University, Atlanta, GA, USA).

2.2 Cell culture
The cell line used in this study was UMR 106-01 osteosarcoma cells, a gift from Dr. Nicola Partridge (UMDNJ-Robert Wood Johnson Medical School, Piscataway, NJ, USA). Cells were cultured at 37°C in a humidified 5% CO₂ atmosphere. UMR cells were cultured in 1:1 DMEM/F12 medium (Gibco) supplemented with 5% FBS (Gibco) and an antibiotic-antimycotic cocktail (100 U/ml penicillin, 100 µg/mL streptomycin, 0.25 µg/mL amphotericin B; Gibco).

2.3 Treatment and RNA Extraction
Cells were plated at a density of 5.4x10⁵ cells/well in six-well plates. Cells were treated with either rPTH (1-34) or Mithramycin A the following day. Treatments were stopped after the indicated treatment times by aspirating medium and washing cells twice with sterile PBS. Cells were lysed and RNA was extracted using TRIzol (Invitrogen, Life Technologies) by adding 1 mL/well of reagent and pipetting for 5 minutes. Solubilized RNA samples were then transferred to 1.5 mL eppendorf tubes, 200 µL of chloroform
was added, and tubes were shaken. Tubes were then centrifuged for 15 minutes at 12,000 RPM at 4°C. Supernatants were transferred to fresh centrifuge tubes, and 0.75 mL of isopropanol was added per tube and shaken vigorously to precipitate RNA. After a 10 minute room temperature incubation period, samples were centrifuged for 10 minutes at 12,000 RPM at 4°C to pellet the RNA. The RNA pellets were washed with 75% ethanol, and the pellets were dried for 10 minutes at room temperature. Depending on size, pellets were resuspended in 20-100 µL of DEPC-ddH₂O. The RNA concentration was then measured using the UV absorbance at wavelength 260 nm in a spectrophotometer.

2.4 Reverse Transcription and Quantitative Real-time PCR

Reactions were performed in 0.2 mL thin-walled PCR tubes. Any genomic DNA contamination was removed from extracted RNA samples by treatment with 1 U of DNase for each 1 µg of sample RNA. DNase activity was quenched with addition of EDTA at final concentration of 2.7 mM and incubating at 65°C for 10 minutes. Then, random hexamer primers (Fermentas) and dNTPs (Invitrogen) were added to each sample to a final concentration of 7.5 µM and 750 µM, respectively, and incubated for 5 minutes at 65°C. Subsequently, a master mix of First Strand buffer (Invitrogen), 10mM DTT, 28 U RiboLock Ribonuclease Inhibitor (Fermentas), were added to a final volume of 30 µL and incubated at 37°C for 2 minutes. Lastly, 300 U of M-MLV reverse transcriptase (Invitrogen) was added to the samples. Reverse transcription was carried out using the following cycle: 25°C for 10 minutes, 37°C for 50 minutes, and 70°C for 15 minutes.
Real-time PCR was then performed using RT product derived from 25 ng of RNA added to a master mix containing SYBR Green Reagent (Applied Biosystems), 300 nM each of forward and reverse primers for target gene of interest (Table 1), in a total volume of 20 µL. Reactions were performed in triplicate using the 7500 Real-time PCR system (Applied Biosystems, Foster City, CA, USA) which utilized the following cycle: 50°C for 2 minutes, 95°C for 10 minutes, 40 cycles of 95°C for 15 seconds, and 60°C for 1 minute. Ct values were measured and averaged across the three triplicates, where Ct is the cycle number required to reach the threshold fluorescence. Relative quantification (RQ) was calculated as:

\[ RQ = 2^{-\Delta\Delta Ct} \]

where \( \Delta\Delta Ct = \Delta Ct (\text{treatment}) - \Delta Ct (\text{control}) \)

and \( \Delta Ct = Ct (\text{gene of interest}) - Ct (\text{housekeeping gene}) \)

In all cases, glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as the internal control gene.

### 2.5 Protein Extraction and Immunoblot Analysis

Cells were treated and plated in the same manner as for RNA extraction. Treatments were stopped by aspirating medium and washing wells twice with 1 mL of ice-cold PBS. Then, 100 µL of whole cell lysis buffer, which consisted of 50 mM Tris pH 7.5, 16 mM EDTA, 1 mM DTT, 1X protease inhibitor cocktail, 0.1% NP40, and 0.2% DOC, was added, and cells were scraped into solution. The lysis buffer containing cells was then transferred to 1.5 mL eppendorf tubes and incubated on ice for 30 minutes. Then, cell debris was pelleted by centrifugation for 15 minutes at 13,000 RPM at 4°C, and supernatants containing extracted protein were transferred to fresh 1.5 mL eppendorf tubes. Protein concentrations were determined using amido black protein assays.
**Table 1. Primers used for standard quantitative real-time PCR.** Primers were designed for use in mouse (m) or rat (r) samples. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH), Osx (Osterix), Sclerostin (SOST), Bone sialoprotein (BSP), Runt-related transcription factor 2 (Runx2), Receptor activator of nuclear factor kappa-B ligand (RANKL), Osteoprotegrin (OPG)

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>mGAPDH Fwd</td>
<td>5’ – CAG CCT CCC GTA GAC A – 3’</td>
</tr>
<tr>
<td></td>
<td>Rvr : 5’ – CGC CCA ATA CGG CCA AA– 3’</td>
</tr>
<tr>
<td>rGAPDH Fwd</td>
<td>5’ – CAT GGC CTT CCG TGT TCC TA</td>
</tr>
<tr>
<td></td>
<td>Rvr : 5’ – GCG GCA CGT CAG ATC CA– 3’</td>
</tr>
<tr>
<td>mOsx Fwd</td>
<td>5’ – GGT CCC CAG CTC GAG GAT– 3’</td>
</tr>
<tr>
<td></td>
<td>Rvr : 5’ – CTA GAG CCG CCA AAT TTG CT– 3’</td>
</tr>
<tr>
<td>rOsx Fwd</td>
<td>5’ – CAG CCT GCA GCA AGT TTG G– 3’</td>
</tr>
<tr>
<td></td>
<td>Rvr : 5’ – TTT TCC CAG GGC TGT TGA GT– 3’</td>
</tr>
<tr>
<td>mSOST Fwd</td>
<td>5’ – CGC CAA AGA TGT GTC CGA GTA– 3’</td>
</tr>
<tr>
<td></td>
<td>Rvr : 5’ – TGT CAG GAA CGC GGT GTA GTG– 3’</td>
</tr>
<tr>
<td>mOsteocalcin Fwd</td>
<td>5’ – CTG ACA AAG CCT TCA TGT CCA A– 3’</td>
</tr>
<tr>
<td></td>
<td>Rvr : 5’ – GGT AGC GCC GGA GTC TGT T– 3’</td>
</tr>
<tr>
<td>mBSP Fwd</td>
<td>5’ – CCA AGA AGG CTG GAG ATG CA– 3’</td>
</tr>
<tr>
<td></td>
<td>Rvr : 5’ – TTC CTC GTC GCT TTC CTT CA– 3’</td>
</tr>
<tr>
<td>mRunx2 Fwd</td>
<td>5’ – ACT GGC GGT GCA ACA AGA C– 3’</td>
</tr>
<tr>
<td></td>
<td>Rvr : 5’ – ACC ACA GTC CCA TCT GGT ACC T– 3’</td>
</tr>
<tr>
<td>mRANKL Fwd</td>
<td>5’ – GGC CAC AGC GCT TCT CA– 3’</td>
</tr>
<tr>
<td></td>
<td>Rvr : 5’ – CCT CGC TGG GCC ACA TC– 3’</td>
</tr>
<tr>
<td>mOPG Fwd</td>
<td>5’ – CAC TCC TGG TGT TCT TGG ACA TC– 3’</td>
</tr>
<tr>
<td></td>
<td>Rvr : 5’ – GGG TCA TAA TGC AAG TAT TTT GGA– 3’</td>
</tr>
</tbody>
</table>
Designated amounts of protein were loaded onto SDS-PAGE gels, consisting of a 5% acrylamide stacking gel and an 11% acrylamide running gel. Protein samples were separated by electrophoresis, and transferred to nitrocellulose membranes overnight at 4°C. Membranes were then blocked in Tris-based buffer with 0.1% Tween (TBST) and 5% milk for 5-7 h, washed two times with TBST and incubated overnight with primary antibodies (Table 2) with gentle mixing on a Nutator at 4°C. The following day, the nitrocellulose was washed 2 times with TBST, and a secondary HRP anti-rabbit IgG antibody (GE Healthcare) was added at a 1:2000 dilution in TBST and 5% milk, and incubated for 1h at RT. The membrane was then washed 3 times with TBST, and 2 times with TBS, before adding Amersham ECL Western Blotting Detecting reagents (GE Healthcare). The blots were then exposed to Bioflex photographic film (Clonex). Protein expression of the samples was quantified by densitometry imaging using ImageQuant software (Molecular Dynamics). All target protein expression levels were normalized to the amount of α−actin detected in the same sample.

2.6 Site-Directed Mutagenesis

The QuikChange II XL Site-Directed Mutagenesis Kit (Agilent Technologies) was utilized to mutate the NF-Y, Ets-1, and Sp sites, as well as a control mutation (ΔCon) on the -304/+91 and -1269/+91 luciferase Osx promoter plasmids (Fig 6-7). Mutations were introduced as highlighted in bold: ΔSp_a: CAC CCC CAC CCC > CAA AAA CAA AAC C; ΔSp_b: CAC CCC CAC CCC > CAA AAA CCA CCC C; ΔSp_ab: CAC CCC CAC CCC > CAC CCC AAA AAC C; ΔCon: CGG AGT CTT > AGC AGC CA; ΔEts-1: AGG AAA > TAA CTA; ΔNF-Y: TCA TTG GAT > TCT CGC TAT. Double mutants were created using the ΔSp_a mutated construct as the template.
Table 2. Primary antibodies used for immunoblot and ChIP experiments.

<table>
<thead>
<tr>
<th>Name</th>
<th>Company</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Actin</td>
<td>Boehringer Mannheim Biochemica</td>
<td>1:1000 in TBST + 1% BSA</td>
</tr>
<tr>
<td>Sp1</td>
<td>Millipore (07-645)</td>
<td>1:500 in TBST + 1% BSA</td>
</tr>
<tr>
<td>Sp3</td>
<td>Santa Cruz (sc-644)</td>
<td>1:500 in TBST + 1% BSA</td>
</tr>
<tr>
<td>Osterix</td>
<td>Abcam (22552)</td>
<td>1:500 in TBST + 1% BSA</td>
</tr>
<tr>
<td>TIEG1</td>
<td>Santa Cruz (sc-67062)</td>
<td>1:500 in TBST + 3% BSA</td>
</tr>
<tr>
<td>IgG</td>
<td>Santa Cruz (sc-2027)</td>
<td>ChIP only</td>
</tr>
</tbody>
</table>
First, complementary oligonucleotides containing the desired mutations were designed using the QuikChange Primer Design Program and synthesized and HPLC purified by IDT (Table 3). 125 ng of each pair of primers was added to a mix containing 10 ng of the Osx promoter plasmid template, 1X reaction buffer, 1 µL of dNTP mix, 3 µL dilution of QuikSolution, and 2.5 U of *Pfu Ultra* High Fidelity DNA polymerase in a 50 µL reaction volume. Each sample was then amplified in a Thermocycler using the following cycle: 1 minute at 95°C, followed by 18 cycles of 95°C for 50 seconds, 60°C for 50 seconds, and 68°C for 1 minute/kb plasmid length, and lastly 68°C for 7 minutes. Following amplification, unmutated templates were degraded with *Dpn I* at 37°C for 1 hour. All sequences were confirmed with automated sequencing.

The mutated plasmids were then transformed into XL 10-Gold ultracompetent cells. Cells were first pre-chilled followed by addition of β-mercaptoethanol and further incubation on ice for 10 minutes, with periodic swirling. 2 µL of the mutated plasmids were then added, incubated on ice for 30 minutes with periodic swirling and then heat-shocked at 42°C for 30 seconds followed by cooling for 2 minutes on ice. 0.5 mL of pre-warmed NZY+ broth was then added, and cells were shaken for 1 hour at 225 RPM at 37°C. The transformed cells were then plated on ampicillin-LB agar plates and incubated overnight at 37°C. Individual bacterial colonies were chosen at random from the plates, and grown in LB medium containing ampicillin overnight at 37°C. The plasmids were then harvested using an EndoFree MaxiPrep Kit (Qiagen). The base pair numbering is in reference to the predicted transcription start site (+1) designated by the first investigators to sequence the Osx promoter (61).
Figure 8. Schematic diagram of mutated -304/+91 Osx promoter constructs. Mutations, shown in black, were introduced to putative Sp transcription factor elements (Sp_a and Sp_b) or to a control (Con) area of the promoter to assess their importance on promoter activity.
Figure 9. Schematic diagram of mutated full-length Osx promoter constructs. Mutations, shown in black, were introduced to putative transcription factor elements to assess their importance on promoter activity.
Table 3. Primers used to create Osx promoter mutants.

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>∆Sp_a</td>
<td>Fwd: 5' – CAG AGA GCA GCC CCC CAA AAA CCA C – 3'</td>
</tr>
<tr>
<td></td>
<td>Rvr: 5' – AGG GAC TGG GGG GTG TTT GGG G – 3'</td>
</tr>
<tr>
<td>∆Sp_b</td>
<td>Fwd: 5' – AGC AGC CCC CCC ACC CCA AAA ACC C – 3'</td>
</tr>
<tr>
<td></td>
<td>Rvr: 5' – TCG TCG GGG GGG TGG GTT TGG G – 3'</td>
</tr>
<tr>
<td>∆Sp_a/b</td>
<td>Fwd: 5' – GCA GCC CCC CAA AAA CAA AAC CCC A – 3'</td>
</tr>
<tr>
<td></td>
<td>Rvr: 5' – CGA CGG GGG GTT TTT GTT TGG GGG T – 3'</td>
</tr>
<tr>
<td>∆Con</td>
<td>5' – CGC CTT CCT CAT TGG ATC AGC AGC CA – 3'</td>
</tr>
<tr>
<td></td>
<td>5' – GCG GAA GGA GTA ACC TAG TCG TGG G – 3'</td>
</tr>
<tr>
<td>∆Ets-1</td>
<td>Fwd: 5' – GGG GAG CTC AGC AAA TGG AGC TAA CTA TTT GGA CCC TCT GCC TCC CT – 3'</td>
</tr>
<tr>
<td></td>
<td>Rvr: 5' – AGG GAG GCA GAG GGT CCA AAT AGT TAG CTC CAT TTG CTG AGC TCC CC – 3'</td>
</tr>
<tr>
<td>∆NF-Y</td>
<td>Fwd: 5' – CTC CCT CTC TCG CCT TCC TCT CGC TAT CCG GAG TCT TCT CGG CTG – 3'</td>
</tr>
<tr>
<td></td>
<td>Rvr: 5' – CAG CGG AGA AGA CTC CGG ATA GCG AGA GGA AGG CGA GAG AGG GAG – 3'</td>
</tr>
</tbody>
</table>
2.7 Transfection and Luciferase Assays

Cells were plated at a density of 2x10^5 cells/well in 12-well plates and allowed to grow in DMEM:F12 medium with 5% FBS for two days. Two days after plating transfections were performed using 6 µL of lipofectamine reagent (Invitrogen), 0.4 µg luciferase reporter plasmid and 0.8 µg of β-gal reporter plasmid were mixed to a final volume of 800 µL/well in serum-free DMEM:F12 medium for 6 hours, after which the medium was aspirated and fresh DMEM:F12 medium with 5% FBS and 1% AA was added. pSV-β-gal is a galactosidase expression plasmid under the control of an SV40 promoter (Promega), and was used to correct for variable transfection efficiencies. On the day after transfection cells were treated with PTH or Mithramycin A at various concentrations in serum-containing medium for 16 hours. Reactions were stopped by removal of medium and rinsing cells with ice-cold PBS. 200 µl/well of reporter lysis buffer (Promega) was added and cells were scraped into the solution and transferred to a 1.5 mL eppendorf tube. Cell lysis was further aided by freezing and thawing the samples at -80°C for 2 hours. Luciferase activity was measured in a luminometer (Turner Instruments) by addition of a 1:3 ratio of sample: luciferin (Promega) in luminometer tubes for 15 seconds.

To measure β-gal activity, 30 µL of each sample was incubated with 10 mM MgCl₂, 3 mM o-nitrophenyl-β-D-galactoside (ONPG), diluted in 100 mM NaPO₄ in a final volume of 300 µL and incubated at 37°C. After the solution became a mustard-yellow (approximately 2 hours) the reaction was stopped with addition of 1M Na₂CO₃ and absorbance was measured with a spectrophotometer at a wavelength of 420 nm.
2.8 Chromatin Immunoprecipitation Assays

For chromatin immunoprecipitation (ChIP) assays cells were plated at a density of \(2 \times 10^6\) cells in 100mm tissue culture dishes in DMEM:F12 with 5% FBS. Cells were treated with 1 nM rPTH (1-34) the following day. After incubation with PTH for 2-24 hours, cells were cross-linked by adding formaldehyde directly to tissue culture medium to a final concentration of 1% while rocking for 10 minutes at room-temperature (RT). Cross-linking was then stopped by addition of glycine to a final concentration of 0.125 M with rocking for an additional 5 minutes at RT. Medium was then removed and the cells were rinsed with sterile PBS three times. 750 µL of PBS with 0.1% Tween was added to each dish and cells were scraped from the culture dish into this solution and transferred to 1.5 mL eppendorf tubes, and centrifuged for 3 minutes at 10,000 RPM at 4°C. The supernatants were aspirated, and the pellets were resuspended in a solution of 400 µL TSE I buffer (20 mM Tris pH 8.0, 1% Triton, 150 mM NaCl, 0.1% SDS, 2 mM EDTA) and 1% Protease Inhibitor Cocktail (BioShop). DNA was sheared to 500-800 bp fragments by eighty 10-second pulses of sonication while samples were kept on ice. The samples were then centrifuged for 10 minutes at 13,000 RPM at 4°C, and the supernatant was transferred to fresh 1.5 mL eppendorf tubes and stored at -80°C.

Chromatin samples were precleared by addition of 30 µL of a 50% protein A slurry (GE Healthcare, pre-equilibrated in TSE I buffer) with mixing for 2 hours at 4°C on a rotating wheel. Samples were subsequently centrifuged for 1 minute at 5,000 RPM, and 110 µL of each sample was aliquoted to a new low DNA-binding 1.5 mL eppendorf tube (SafeSeal) for immunoprecipitation. For each antibody used a master mix consisting of 0.1 mg/mL sheared sperm DNA and 2.5 mg/mL BSA in 100 µL of TSE I buffer and 3 µg
of antibody was made. The antibodies used were IgG (Millipore), Osx, Sp1, Sp3, and TIEG1 (Table 2). Each antibody-containing master mix was then added to 110 µL of sample and incubated on a rotating wheel at 4°C overnight.

The following day immunoprecipitated DNA was isolated by addition of pre-equilibrated protein A slurry and incubation for 1 hr at 4 C on a rotating wheel. Samples were pelleted by centrifugation and extensively washed 3 times using TSE I buffer, once using TSE II buffer (20 mM Tris pH 8.0, 1% Triton, 500 mM NaCl, 0.1% SDS, 2 mM EDTA), once using LiCl buffer (20 mM Tris pH 8.0, 1 mM EDTA, 250 mM LiCl, 1% NP-40, 1% Na-deoxycholate), and twice using TE buffer (10 mM Tris pH 8.0, 1mM EDTA). Samples were then resuspended in 110 µL of TE buffer containing 1% SDS and incubated for 1 hour on the rotating wheel at RT. To measure total input of DNA, 5 µL of each sample that was not immunoprecipitated was added to 105 µL of 1% SDS/TE and incubated for 1 hour on rotating wheel at room temperature. All samples were incubated overnight at 66°C to uncrosslink DNA.

DNA in the samples was purified using a PCR purification kit (Invitrogen). Purified DNA was eluted into 50 µL of elution buffer and Osx promoter fragments were quantified by real-time PCR using SYBR Green, 0.3 µM forward and reverse ChIP primers (Table 4), and 2 µL DNA sample, in a final volume of 15 µL. Input DNA and immunoprecipitated samples were assayed in triplicate. Total amounts of binding by a specific antibody were determined by expressing the amount of DNA obtained for each immunoprecipitated sample as a percentage of total DNA using the following formula:

\[
\text{% of Input} = 2^{\Delta \text{Ct Antibody}} \times 100 = \text{% of Input}
\]

where \( \Delta \text{Ct Antibody} = C_t \text{ Total DNA} - C_t \text{ Ab} \)
Table 4. Primers used for ChIP genomic DNA quantification.

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Osx Promoter Sp Sites</td>
<td>Fwd : 5’ – CTC ATT GGA TCC GGA GTC TTC T – 3’</td>
</tr>
<tr>
<td></td>
<td>Rvr : 5’ – TGT CTG TAG GGA TCC ACC CTC TA – 3’</td>
</tr>
</tbody>
</table>
2.9 Mice Treatments and Tissue Homogenization

Animals were treated according to the guidelines of the University of Toronto animal care committee. FEV mice were all sacrificed at 8 weeks of age, however, treatment began at varying ages depending on three different treatment regimens. In the first regimen, osmotic pumps purchased from Alzet (Cupertino, CA, USA) preloaded with either PBS or 26.28 µg rat PTH (1-34), based on previous studies, were implanted subcutaneously on the back of 6 week old mice. These pumps released their contents continuously for 2 weeks, mimicking primary hyperparathyroidism, and were used as a model for continuous PTH exposure. In the second paradigm, 5 week old mice were fed normal or calcium deficient chow for 3 weeks, which induced secondary hyperparathyroidism, and this was used as a second model for continuous PTH exposure. Hyperparathyroidism was assessed by measurement of serum phosphate levels, serum calcium levels, changes in Cyp27b1 mRNA levels, and changes in aBMD as assessed by DEXA by Ariana dela Cruz. The last treatment required that 7.5 week old mice received single, daily injections of either PBS or rat PTH (1-34) for 3 days at a dose of 40 µg/kg as a model for acute intermittent PTH exposure.

Mice were sacrificed 24 hours after the end of each treatment regimen, and their calvaria, tibia, and femurea were dissected and snap frozen using liquid nitrogen. One pair of tibia and femur from each mouse were pulverized with a mortar and pestle kept cold with dry ice. The remaining femur was used to assess areal bone mineral density (aBMD), which was performed by Ariana dela Cruz using dual-energy X-ray absorptiometry. For RNA extraction 1 mL of TRlzlol was added, and RNA was extracted as previously outlined for UMR cells. The calvaria were snap frozen, placed in 1.5 mL
eppendorf tubes, and 1 mL of TRIzol was added. The tissue was homogenized with a Polytron Homogenizer while in TRIzol, and RNA was extracted as previously described.

2.10 Statistical Analysis

All experiments performed were repeated at least twice with most repeated three times. Statistical analyses were performed using the GraphPad Prism software (San Diego, CA, USA). All data are shown with error bars indicating the standard error of the mean. One-way ANOVA, where P < 0.05 was considered statistically significant, followed by Bonferroni post hoc analysis was used to determine: the effect of PTH on Osx expression, the effect of mutating the Osx promoter luciferase constructs, the effect of Mithramycin A on reporter constructs in luciferase assays, and transcription factor binding in ChIP assays. Unpaired, two-tailed T-tests were used to assess the in vitro effect of PTH on Osx expression and the in vivo effects of the three different PTH regimens on Osx in mice. Statistical significance was represented by * P < 0.05.
3 RESULTS

3.1 PTH inhibits Osx mRNA and protein expression in vitro

As previously reported by our lab, PTH inhibits Osx expression in UMR106-01 cells with a time course (3-24h) and dose-response similar to that seen in mouse calvaria (77). To extend these previous findings, the ability of PTH to inhibit Osx expression was assessed in the UMR106-01 cell-line at time-points after 24 hours in culture. Cells were treated with 10 nM PTH, and mRNA was extracted from the cells at various times following PTH addition (3-56h). PTH significantly decreased Osx mRNA levels at all time points (Fig 10A). As shown previously by our lab, the earliest time-point at which PTH is able to decrease Osx mRNA is after 3h of treatment.

A dose-response curve was generated to identify suitable PTH concentrations for use in subsequent studies. To this end, UMR106-01 cells were treated with varying doses of PTH ($10^{-7}$-$10^{-11}$M), and protein was extracted after 16h. As expected, immunoblot analysis using primary antibodies against Osx revealed multiple Osx bands of different mobility. There were two major Osx bands at 56 kDa and 46 kDa, which corresponded to the long and short isoforms of the Osx protein, respectively (Fig 10B). In addition, minor bands were visualized that run slightly above each of those 56 and 46 kDa bands. Both long and short isoforms of the Osx protein can undergo post-translational modification through phosphorylation by p38. Previous work has reported that these phosphorylated forms of the Osx protein isoforms can be visualized on immunoblots as minor bands corresponding to proteins of greater molecular weight, and treatment with phosphatases results in the disappearance of these bands (37). Therefore, the major bands at each size likely corresponded to unphosphorylated Osx proteins, while the
minor bands corresponded to phosphorylated forms. Interestingly, quantification of Osx protein levels of all bands revealed a dose-dependent inhibition by PTH. However, the 56 kDa phosphorylated forms of Osx decreased to almost negligible levels. All future studies therefore used PTH concentrations between $10^{-8}$ M and $10^{-9}$ M, as following exposure to these concentrations, Osx protein was significantly inhibited.
Figure 10. PTH regulation of Osx expression. (A) UMR106-01 cells were treated with 10 nM PTH for 3-56h and Osx mRNA was significantly decreased at all time-points. (B) Osx protein levels were decreased by PTH in a dose-dependent manner after 16h of treatment. Average Osx mRNA and protein levels were plotted with bars representing mean ± S.E.M. Statistical significance was represented by * P < 0.05 by t-test and # P < 0.05 by ANOVA compared to cells treated with PTH-free medium.
3.2 The -304/+91 region of the Osx promoter retains full PTH responsiveness

As previously reported by our lab, PTH inhibits Osx expression by inhibiting Osx promoter activity. To corroborate those findings, luciferase expression vectors under the control of Osx promoter fragments of various sizes (-1269/+91, -304/+91, and -119/+91) were transfected into UMR106-01 cells. In addition, the PGL3-Basic vector, which has no promoter driving luciferase expression, was transfected and used as a control as its activity should not be affected by PTH. Transfected cells were then treated with 1 nM PTH or PTH-free medium, and protein was extracted after 16h. Luciferase activity was then measured, and normalized with β-gal to correct for different transfection efficiencies. The responsiveness of each construct to PTH was expressed by averaging luciferase activity in the presence of PTH over luciferase activity in the absence of PTH (Fig 11A). Thus, PTH caused luciferase expression of the full-length Osx promoter (-1269/+91) construct to be inhibited by 70.8%. Similarly, the luciferase expression of the -304/+91 construct was inhibited by PTH by 71.1%. Interestingly, the luciferase expression of the -119/+91 construct displayed a reduced inhibition by PTH, as PTH was only able to inhibit the luciferase expression by 29.1%. When compared to the PTH inhibition of the luciferase expression of the -119/+91 constructs, both -1269/+91 and -304/+91 constructs showed significantly greater inhibition of luciferase expression after PTH stimulation.

Within the -304/+91 region of the Osx promoter, several potential transcription factor binding sites have been identified in silico (51). As mentioned previously, the Osx promoter lacks a TATA box in the vicinity of its transcription start site (Fig 11B).
Figure 11. PTH regulation of the Osx promoter. (A) Cells transfected with luciferase constructs driven by Osx promoter fragments were inhibited by PTH after 16h. Average luciferase activity obtained from at least 2 experiments with 2 duplicates were plotted with bars representing mean ± S.E.M. Statistical significance was represented by * P < 0.05. (B) The sequence of the PTH-responsive fragment of the Osx promoter contained several putative transcription factor binding elements. TSS; Transcription start site
3.3 Mutational Analysis of Potential Binding Sites on -304/+91 Osx Promoter

To examine the importance of the potential transcription factor binding sites on Osx promoter activity, the luciferase Osx promoter construct containing only the PTH-responsive regions (-304/+91) was mutated to specifically disrupt these sites. At this juncture, the smaller Osx promoter fragment was utilized because the -304/+91 region retained full PTH-responsiveness, and it would allow for a more precise assessment of this area. All mutations were introduced using site-directed mutagenesis, after which UMR106-01 cells were transfected with each mutant construct and luciferase activity was measured 48h after transfection (Fig 12). Surprisingly, when both Spa and Spb sites were mutated simultaneously (ΔSpa/b), Osx promoter activity significantly decreased by 97.5% compared to the activity of the wild-type construct (-304/+91). Mutation of the Spa site (ΔSpa) or Spb (ΔSpb) individually resulted in a 96.9% or 76.2% decrease in activity, respectively. The activity of all three mutants (ΔSpa/b, ΔSpa, ΔSpb) was significantly higher than PGL3-Basic transfected cells, which were used to measure background activity of the luciferase expression vector. Lastly, a control mutant was created (ΔCon), where mutations were introduced that did not disrupt any identified transcription factor binding sites. As expected, ΔCon did not have significantly altered activity in comparison to the unmutated wild-type construct.
Figure 12. Mutational analysis of two putative Sp elements. Mutational analysis was employed to assess the importance of potential transcription factor binding sites on luciferase activity in unstimulated UMR-01 cells using the luciferase construct driven by the -304/+91 Osx promoter. Average luciferase activity obtained from at least 3 experiments was plotted with bars representing mean ± S.E.M. Statistical significance was represented by * P < 0.05 compared to cells transfected with the unmutated -304/+91 construct.
3.4 Mutational Analysis of Potential Binding Sites on -1269/+91 Osx Promoter

To ensure that the altered activity displayed by the -304/+91 Osx promoter mutant constructs were not specific to the smaller Osx promoter fragment, the same mutations were introduced in the context of the entire Osx promoter (-1269/+91). In addition, the remainder of the potential binding sites, Ets-1 and NF-Y, were mutated. Similar to what was observed from the -304/+91 constructs, the ΔSpa/b mutant resulted in a decrease of activity by 94.6%, while the Sp_a and Sp_b mutants displayed a 91.1% and 89.7% decrease, respectively (Fig 13A). Conversely, when the NF-Y site was mutated (ΔNF-Y), activity significantly increased by 60%, while mutation of the potential Ets-1 site (ΔEts-1) showed no significant effect. In addition, two double-mutant constructs were created which either had both Sp_a and NF-Y sites mutated simultaneously (ΔSp_a/NF-Y), or both Sp_a and Ets-1 sites mutated simultaneously (ΔSp_a/Ets-1). While both double-mutants displayed a significant loss of activity, ΔSp_a/NF-Y displayed significantly more activity than the ΔSp_a mutant.

In order to determine which, if any, of these mutations altered the PTH-responsiveness of the Osx promoter, cells were treated with 1 nM PTH for 16h. PTH responsiveness was expressed as the average luciferase activity in the presence of PTH over luciferase activity of that same construct in the absence of PTH (Fig 13B). The wild-type full-length Osx promoter (-1269/+91) was inhibited by PTH by 70.8%, which represented the entire inhibitory effect of PTH on Osx promoter activity. The double Sp mutant (ΔSp_a/b) displayed a significant decrease in PTH-responsiveness, as it was inhibited by 50.5% by PTH. Similarly, mutation of both Sp sites individually resulted in a significant decrease in PTH-responsiveness, as the ΔSp_a and ΔSp_b constructs were inhibited by
53.4% and 57.3%, respectively. In addition, the ΔEts-1 mutant displayed a significant decrease in PTH responsiveness, while the ΔNF-Y mutant displayed similar PTH-mediated inhibition as that seen for the -1269/+91 construct. The PTH-responsiveness of the double-mutants was then measured to determine if there were any additive effects seen by mutating two different sites at once. When both Spα and Ets-1 were mutated simultaneously, PTH-responsiveness was significantly decreased to 58%, while simultaneous mutation of Spα and NF-Y elicited no change.
Figure 13. Mutational analysis of potential elements using the -1269/+91 Osx promoter. The importance of potential transcription factor binding sites was assessed with (A) luciferase activity in unstimulated cells (B) PTH-responsiveness as cells were treated with 1 nM PTH for 16h. Average luciferase activity obtained from at least 3 experiments was plotted with bars representing mean ± S.E.M. Statistical significance was represented by * and # P < 0.05. * compared to cells transfected with the unmutated -1269/+91 construct. # compared to cells transfected with the ΔSpα construct.
3.5 Inhibition of transcription factor binding to Sp sites with Mithramycin A

Mutation of the two Sp sites found on the Osx promoter revealed that these sites were vital for Osx promoter activity as it related to driving luciferase expression under normal cellular conditions. To further assess the importance of these Sp sites on Osx expression, the effect of blocking these sites was investigated. To this end, the compound Mithramycin A, which is used experimentally as an Sp-site blocker, was utilized. Mithramycin A has been demonstrated to specifically inhibit the binding of transcription factors to Sp sites, and it has been postulated to be able to do so by binding to the DNA directly itself (80,81).

If Mithramycin A could inhibit transcription factor binding to these potentially vital Sp sites, it was postulated that Mithramycin A would inhibit Osx promoter activity, and this was tested using luciferase assays. Therefore, UMR106-01 cells were transfected with the full-length luciferase Osx promoter construct (-1269/+91), and cells were treated with increasing concentrations of Mithramycin A (0.5-2 µM) for 16h (Fig 14), after which luciferase activity was measured. Luciferase activity was normalized to β-gal. Mithramycin A treatment resulted in a concentration-dependent decrease in luciferase activity up to 84% (Figure 14A).

To determine if this decrease in promoter activity translated into a decrease in Osx expression, untransfected UMR106-01 cells were treated with 1 µM Mithramycin A for 16h and Osx mRNA levels were measured using real-time PCR. In comparison to untreated cells, Mithramycin A treatment resulted in a significant 86% decrease in Osx mRNA levels (Fig 14B). In addition, the effect of Mithramycin A on Osx protein levels
was tested. UMR106-01 cells were treated with either 1 µM Mithramycin A or 10 nM PTH, and after 16h, cells were harvested and Osx protein levels were measured using immunoblot analysis (Fig 14C). Actin levels were also measured, and used as an internal control. As mentioned previously, Osx protein can exist as either of two isoforms, and each isoform can be either phosphorylated or unphosphorylated. Both Mithramycin A and PTH significantly decreased levels of both phosphorylated and unphosphorylated Osx protein isoforms compared to control. Interestingly, PTH significantly decreased levels of the 46 kDa phosphorylated Osx protein to a greater degree than Mithramycin A, while both PTH and Mithramycin A almost completely eliminated expression of the 56 kDa phosphorylated protein.
Figure 14. The effects of blocking Sp site binding with Mithramycin A. (A) UMR106-01 cells transfected with the luciferase -1269/+91 Osx promoter construct were treated with increasing doses of Mithramycin A (0.5-2 µM) for 16h. In addition, untransfected UMR106-01 cells were treated with 1 µM Mithramycin A for 16h, and levels of Osx (B) mRNA and (C) protein were measured. Average values obtained from at least 3 experiments were plotted with bars representing mean ± S.E.M. Statistical significance was represented by * P < 0.05 compared to control.
3.6 PTH regulation of Sp1, Sp3, and TIEG1 expression

As shown previously using luciferase assays, the two Sp sites that are responsible for a large amount of basal Osx expression are also responsible for partially mediating PTH's inhibitory effects on Osx expression, as mutating these sites resulted in a partial abrogation of PTH inhibition of Osx promoter activity. Thus, the mechanism by which PTH can accomplish this was investigated. Since these Sp sites were shown to be vital for the expression of Osx under normal cellular conditions, it was postulated that PTH may be able to inhibit the expression of the transcription factors that bind these sites, which would disrupt normal promoter function. To date, 9 distinct transcription factors belonging to the Sp family have been identified (82). However, only a subset of these Sp factors would be expected to be expressed in osteoblasts. For example, Sp1 and Sp3 are ubiquitously expressed across many different cell-types, and we have previously identified these Sp proteins in UMR106-01 cells (77), while Osx/Sp7 is an osteoblast-specific transcription factor. In addition, the osteoblast-specific transcription factor TIEG1 has been previously shown to be able to bind Sp sites. Therefore, PTH's ability to regulate the expression of Sp1, Sp3, and TIEG1 was investigated as a preliminary step in determining if any of these factors mediated PTH's inhibitory effects on Osx expression. UMR106-01 cells were treated with 1 nM PTH for either 8h or 24h, and protein expression levels were measured using immunoblot analysis. Actin was used as an internal control. As expected, all three transcription factors were highly expressed in the UMR106-01 cell-line. However, PTH did not alter the expression of Sp1, Sp3, or TIEG1 (Fig 15A-C). Thus, if these transcription factors played a role in mediating PTH's effects, it would not be as result of changes in their expression levels.
Figure 15. The PTH regulation of (A) Sp1, (B) Sp3, and (C) TIEG1 was assessed using immunoblot analysis in UMR106-01 cells treated with 1 nM PTH. Average values obtained were plotted with bars representing mean ± S.E.M.
3.7 Osx can bind to its own promoter in vivo, and PTH can inhibit this binding

Since PTH could not alter the expression of Sp1, Sp3, and TIEG1, all factors that could potentially bind the Sp sites on the Osx promoter, the inhibitory effects of PTH on Osx expression must be carried out through another mechanism. Alternatively, PTH downstream effects may induce a post-translational modification that either 1) inhibits the activity of a transcription factor needed for Osx expression or 2) stimulates the activity of a transcriptional repressor. Of the potential factors identified, only Sp3 has been shown to act as a repressor (83). But first, it was important to determine which, if any, of the identified transcription factors could bind to the Osx promoter via the Sp sites. Therefore, ChIP assays, which permit the investigation of protein-DNA interactions under normal cellular conditions, were utilized. Using antibodies against Sp1, Sp3, Osx, and TIEG1, the ability of these transcription factors to bind the Osx promoter at these Sp sites was assessed. Immunoglobulin G (IgG) binding was used as a negative control to account for non-specific protein-DNA interactions. Of all the transcription factors assessed, Osx was the only factor that displayed significant Osx promoter binding, while Sp1, Sp3, and TIEG binding were not significantly different from the background binding with IgG (Fig 16A).

Next, it was investigated whether or not PTH could alter the binding of Osx to its own promoter. UMR106-01 cells were treated with 10 nM PTH for various times (2h, 4h, 16h, and 24h), and Osx occupancy on the Osx promoter was assessed using ChIP assays (Fig 16B). Treatment with PTH for 2h and 4h resulted in a comparable decrease in Osx binding by 46.8% and 43.8% compared to untreated controls, respectively. After 16h, Osx binding decreased by 68.1% compared to untreated controls, and by 24h, Osx
displayed negligible binding to the Osx promoter. As a control, IgG binding was not significantly changed by PTH. Thus, in the presence of PTH, Osx occupancy on its own promoter underwent a time-dependent decrease.
Figure 16. Investigation of transcription factor association to Osx promoter. ChIP assays were employed to assess: (A) the ability of Osx, TIEG1, Sp1, and Sp3 to bind the Osx promoter via the Sp sites (B) If 10 nM PTH treatments of various durations could regulate Osx binding to its own promoter. Average values obtained from at least 3 experiments were plotted with bars representing mean ± S.E.M. Statistical significance was represented by * P < 0.05 by T-test and # P < 0.05 by ANOVA compared to cells treated with PTH-free medium.
3.8 Osx expression decreases as mice increase in age in vivo

Since it was demonstrated that PTH regulated Osx expression in vitro, the next stage of assessment was to determine the effects of PTH on Osx in vivo. Before this could be investigated, it was important to determine the relative changes in Osx expression that may occur as an animal matures. Thus, 4 male mice at ages of 5, 28, 52 and 95 days old were sacrificed, and their calvaria, tibia, and femur were dissected. These ages were chosen as they gave a good approximation of mice during adolescence up to early adulthood. Calvaria were used to measure the changes in Osx expression in intramembranous bone, while the tibia and femur were combined and used to assess Osx levels in endochondral bone. Osx mRNA levels were quantified by real-time PCR, and levels were expressed as a percentage of the value found for 5 day old mouse calvaria, which displayed the highest expression levels.

Osx mRNA expression showed a progressive decline in both calvaria and tibia/femur samples with age, and expression reached as low as 4% in 95 day old mice compared to 5 day mouse calvaria (Fig 17). In order to avoid the rapid growth phase of pubescent mice, future experiments were performed at the oldest possible age of mice where Osx expression levels could be detected with enough accuracy to ensure quantitative comparisons. Thus, mice at the approximate age of 56 days (8 weeks) were deemed sufficiently mature, and have Osx expression levels that could be accurately compared. At around 8 weeks, Osx mRNA levels were 14% in calvaria and 11% in tibia and femur of the expression levels found in 5 day old mouse calvaria. At ages past 8 weeks, comparisons might not be quantitative due to inadequate amounts of detectable mRNA.
Figure 17. Detection of Osx mRNA expression in mice of increasing ages. Calvaria were used to measure levels in intramembranous bone, while the tibia and femur were used to assess levels in endochondral bone. Values plotted are from bones of single mice at each age.
3.9 Three days of PTH injections inhibits Osx expression in vivo

As postulated above, PTH was expected to inhibit Osx expression in mice. Thus, mice were given i.p. injections of either PBS or PTH at a dose of 40 µg/kg body weight once a day for three consecutive days. This dosage of PTH is commonly utilized in mice to stimulate bone growth, however, detectable changes in bone mineral density require a minimum of 28 days (84,85). Single injections were previously shown to have no effect on Osx levels (unpublished observations in our lab). This could be attributed to the possibility that sufficient concentrations of PTH did not reach all of the osteoblasts and osteocytes found in bone tissue, as blood flow to bone differs significantly among the different areas of bone tissue (86). Thus, three days of PTH injections were used as an acute exposure paradigm that would allow PTH to stimulate more cells. To eliminate confounding effects of sex hormones, as estrogen is a potent regulator of bone, the injections were carried out using only male mice (87,88). After three injection days, PTH was able to significantly inhibit Osx mRNA levels in calvaria, while a trend of inhibition was observed in the tibia and femur (Fig 18).
Figure 18. The effects of acute PTH treatments on Osx \textit{in vivo}. 8 week old mice were given one injection of either PBS (n=13) or PTH (n=11) for three consecutive days, and Osx mRNA levels were measured in calvaria and tibia and femur. Average Osx mRNA levels were plotted with bars representing mean ± S.E.M. Statistical significance was represented by * P < 0.05 compared to mice injected with PBS.
3.10 The effects of hyperparathyroidism on mice

As PTH inhibited Osx expression in vivo in the short-term paradigm, the effects of PTH on Osx expression were explored in paradigms of continuous PTH exposure for two weeks or longer. As mentioned previously, continuously elevated circulating PTH levels results in decreased bone mineral density and frailer bones (75). Clinically, hyperparathyroidism, caused by overactive parathyroid glands, may be either primary or secondary in nature.

Primary hyperparathyroidism occurs when the parathyroid glands secrete large amounts of PTH in the absence of appropriate stimuli, and this is observed when the glands become enlarged, or develop adenoma or carcinoma (89,90). As a model for primary hyperparathyroidism, osmotic pumps preloaded with PTH were implanted onto the backs of 6 week old mice; their contents were released continuously for 2 weeks. As a control, a second group of mice received pumps preloaded with PBS.

Distinct from primary hyperparathyroidism, secondary hyperparathyroidism occurs in response to hypocalcemia, hyperphosphatemia, or impaired 1,25-dihydroxyvitamin D$_3$, and results in chronic elevation of circulating PTH (91,92). Clinically, common causes of secondary hyperparathyroidism are insufficient dietary calcium intake, renal failure, or vitamin D deficiency. For the purposes of this study, secondary hyperparathyroidism was induced by placing 5-week old mice on a calcium deficient diet (0% Ca, 0.4% phosphate) for three weeks and comparing them to mice maintained on normal mouse chow (0.6% calcium, 0.4% phosphate) (93,94).

All mice were sacrificed when they reached 8 weeks of age. Analysis of serum chemistry revealed that both models of hyperparathyroidism did not alter serum
phosphate levels in the mice, whereas the model for primary hyperparathyroidism (PTH pumps) resulted in significantly elevated serum calcium levels (Fig 19A-B). To assess the effect of hyperparathyroidism on bone density, the areal bone mineral density (aBMD) of mouse femurs were measured by dual-energy X-ray absorptiometry (95).

In the mice receiving calcium deficient diets, aBMD decreased by 28% whereas mice with preloaded PTH-pumps showed only a 13% decrease in aBMD when compared to control animals (Fig 19C). As a further measure of the effect of elevated PTH levels in the mice, expression of renal CYP27B1, which is known to be stimulated by PTH, was measured by real-time PCR. For both models of hyperparathyroidism, Cyp27b1 levels were significantly increased compared to controls, however, the increase in mice receiving the calcium deficient was far greater than that seen in the mice with PTH pumps (Fig 19D).
Figure 19. The effects of hyperparathyroidism in mice. Mice were either placed on calcium deficient diets (2o; n=5) or implanted with pumps that release PTH (1o; n=4) for 3 or 2 weeks, respectively. In order to assess the effects of each model, (A) serum phosphate (P), (B) serum calcium (Ca), (C) areal bone mineral density (aBMD) (D) renal Cyp27b1 mRNA levels. Bars represented mean ± S.E.M. Statistical significance was represented by * P < 0.05 compared to mice fed normal chow (n=4) or implanted with PBS pumps (n=5), as indicated. Areal bone mineral density and Cyp27b1 mRNA levels were measured by Ariana dela Cruz.
3.11 Changes in mRNA levels using a model for primary hyperparathyroidism

After sacrifice, the calvaria, tibia, and femur of mice with implants were also dissected so that the effect of primary hyperparathyroidism on the expression of key genes related to bone remodeling processes could be investigated (Fig 20). Surprisingly, continuous elevation of serum PTH resulted in a significant stimulation of Osx in both calvaria and tibia and femur. Although it did not reach significance, RANKL expression in PTH-pump mice displayed a trend of increase in both calvaria and tibia and femur, and osteoprotegrin (OPG) remained unchanged, resulting in a trend of increasing RANKL:OPG. To gain greater insight and to explain the mechanism by which PTH could stimulate Osx expression, the expression of key osteoblastic genes were measured. Runx2, which functions upstream of Osx in the osteoblast differentiation pathway, showed no significant changes. In addition, bone sialoprotein (BSP), a marker of immature osteoblasts, remained unchanged, while osteocalcin (OC), a marker of mature osteoblasts, was significantly stimulated in both calvaria and tibia and femur. Lastly, the osteocytic gene sclerostin (SOST) was measured, and its expression was significantly inhibited in both calvaria and tibia and femur.
Figure 20. The effects of primary hyperparathyroidism on gene expression. Osmotic pumps continuously released PTH (n=4) or PBS (n=5) for 2 weeks, and expression of key marker genes was assessed by real-time PCR. Average mRNA levels obtained were plotted with bars representing mean ± S.E.M. Statistical significance was represented by * P < 0.05 compared to mice implanted with PBS pumps.
3.12 Changes in mRNA levels using a model for secondary hyperparathyroidism

In order to compare the effect of PTH in the two models of hyperparathyroidism, the expression of the same marker genes were also measured in the mice receiving calcium-deficient diets (Fig 21). In concordance with what was observed using the PTH pumps, inducing secondary hyperparathyroidism resulted in stimulation of Osx expression, although these effects reached significance only in calvaria, while there was a trend of stimulation observed in the tibia and femur. In addition, RANKL expression was significantly stimulated in the tibia and femur, while only a trend of increase was observed in the calvaria. OPG expression remained unchanged in mouse calvaria, while it was significantly stimulated in the tibia and femur, which resulted in a RANKL:OPG ratio that displayed a trend of increase in calvaria, but a trend of decrease in the tibia and femur. As opposed to what was observed in the PTH pump mice, Runx2 levels in the bones of mice fed the calcium-deficient diet displayed a trend of increased expression in both the calvaria and tibia and femur, although no significance was reached. In addition, BSP and OC levels remained unchanged. Lastly, SOST levels were significantly inhibited in calvaria, while in the tibia and femur only a trend of inhibition could be observed, although statistical significance was not reached.
Figure 21. The effects of secondary hyperparathyroidism on gene expression. Mice were fed calcium deficient (n=5) or normal chow (n=4) diets for 3 wks to induce hyperparathyroidism, and expression of key marker genes was assessed with real-time PCR. Average mRNA levels obtained were plotted with bars representing mean ± S.E.M. Statistical significance was represented by * P < 0.05 compared to normal chow.
4 DISCUSSION

4.1 Prolonged exposure to PTH results in persistent inhibition of Osx expression in vitro

Osx mRNA was previously demonstrated to be significantly decreased by PTH after 3h of treatment, and at all times up to 24h (77). Prolonged stimulation of the PTH1R results in desensitization and downregulation of the receptors (79), thus we wished to explore if longer stimulation by PTH would eventually recover Osx levels. However, PTH-mediated inhibition of Osx expression did not diminish after continuous treatment up to 56h, although a slight recovery of Osx mRNA was seen between 24-32h. Previous studies in the UMR106-01 cell-line have shown that approximately 80% of PTH1Rs are downregulated after 14h of continuous PTH treatment; however, only 50% of PTH-stimulated adenylyl cyclase activity was lost over this same timeframe (80). These results suggested that approximately 20% of PTH1R are resistant to downregulation in these cells and, furthermore, these remaining receptors continue to stimulate significant amounts of cAMP. This may account for the prolonged decrease of Osx mRNA levels seen in our experiments here.

4.2 The two identified Sp elements (Sp\textsubscript{a}/Sp\textsubscript{b}) are required for full Osx promoter activity

In agreement with previous results from our lab, PTH inhibited luciferase expression vectors driven by the Osx promoter, indicating that PTH inhibited Osx through transcriptional repression. In addition, the presence of two distinct PTH-responsive regions was affirmed. These two regions were found between the base-pairs -304/-119, which contained NF-Y and Ets-1 elements, and -71/+91, which contained two Sp elements (Sp\textsubscript{a} and Sp\textsubscript{b}). Initially, mutations were introduced into the luciferase
construct driven by the smaller Osx promoter fragment -304/+91 to evaluate the importance of the identified transcription factor elements. This smaller fragment was used because it contained both PTH-responsive regions, and mutating potential PTH-responsive sites was expected to solely alter the degree to which PTH could inhibit Osx promoter activity. Instead, mutation of the Sp sites resulted in a loss of basal Osx promoter activity. However, the effects of mutations within small promoter fragments can be misleading. Therefore, mutations were introduced using the entire Osx promoter, and again, a loss of much of the basal promoter activity was observed. In addition, mutation of the Sp sites also resulted in a partial abrogation of PTH's ability to inhibit the Osx promoter. However, because the majority of the luciferase activity was lost in the Sp site mutants, it was difficult to determine how much of PTH's effects were mediated through these two sites. As such, it remained unclear whether or not these Sp elements were involved in PTH-mediated inhibition of Osx promoter activity. Therefore, further characterization of these Sp elements became a focal point for subsequent experiments.

In addition to these Sp sites, the importance of the Ets-1 and NF-Y elements were explored. Mutation of the Ets-1 site, which was found in the -304/-119 PTH-responsive region, did not alter basal promoter activity, but it decreased PTH responsiveness. As stated previously, PTH can inhibit the Osx promoter by acting on two regions: -304/-119 and -71/+91. Since the Sp and Ets-1 sites were identified as PTH-responsive elements, and each site is located within a different PTH-responsive region, then mutation of both sites simultaneously was expected to cause an additive effect. However, PTH was able to inhibit the double mutation construct (ΔSp/Ets-1) to the same extent it could inhibit the constructs carrying a mutation to only a single element.
Conversely, mutation of the NF-Y site resulted in a significant increase in luciferase activity, and its mutation was even able to increase the loss of activity caused by mutating Sp$_a$ (ΔSp$_a$/NF-Y mutant). Thus, the NF-Y binding site was demonstrated to be a site of transcriptional repression under basal conditions. NF-Y is a transcription factor that has been shown to be highly expressed in a number of osteoblastic cell-lines (96). However, NF-Y has only previously been shown to activate, and not repress, the transcription of target genes (96). Thus, further studies are required to determine which transcription factor is able to bind to this NF-Y element on the Osx promoter. This could be explored with an electrophoretic mobility shift assay using the NF-Y element found on the Osx promoter as a DNA probe. To confirm the identity of the protein of interest, this technique can be coupled to a supershift assay by using primary antibodies for NF-Y to ensure the identity of the protein bound to the DNA probe. Further tests to determine if NF-Y is binding to the Osx promoter itself could then be pursued using ChIP assays.

4.3 PTH and Mithramycin A inhibit Osx expression through distinct mechanisms

Since mutation of the Sp sites resulted in decreased promoter activity, it was expected that blockade of the Sp sites would elicit a similar response. Treatment with Mithramycin A, a Sp element blocker, confirmed that Osx promoter activity required fully functional Sp elements. However, Osx promoter activity may function differently in the context of chromatin as opposed to the relatively artificial microenvironment of a luciferase reporter construct. It was found that blocking access to the Sp sites with Mithramycin A also resulted in decreased Osx mRNA and protein levels, providing further evidence that these Sp sites are vital for the expression of Osx in osteoblastic cells. Mithramycin
A may potentially have non-specific effects on cells, as it is used clinically as an antineoplastic agent and may decrease the expression of many genes by being toxic to cells. However, upon visual inspection, Mithramycin A treated cells showed no differences in appearance compared to untreated cells. In addition, GAPDH mRNA levels and actin protein levels did not significantly change in response to Mithramycin A treatment. This indicated that Mithramycin A was likely acting specifically to block Sp elements at the concentrations used. Nevertheless, it will be important to investigate whether or not Mithramycin A can inhibit the expression of genes that are known to be regulated by Sp factors, such as protein kinase Cδ as a positive control.

The changes in Osx protein levels achieved by Mithramycin A were also compared to those achieved by PTH exposure in order to investigate whether or not both compounds elicited a similar response. As stated previously, Osx proteins can exist in two isoforms. Each isoform can be visualized on an immunoblot as a major band that corresponds approximately to a 56 or 46 kDa band, with the larger protein having an additional 18 amino acids at the N-terminus. Relative to each band, a minor band that is slightly larger in size can also be detected, which has been shown to correspond to the phosphorylated forms of the Osx protein (38, 41). Previously, it was demonstrated that the Osx protein requires phosphorylation at Ser-73 and Ser-77 by p38 to be fully active (37). Thus, it is not surprising that the relative amount of phosphorylated Osx is less in comparison to the unphosphorylated forms, so that Osx activity can be precisely regulated.

Both Mithramycin A and PTH caused a comparable decrease of Osx unphosphorylated protein levels of both isoforms, indicating that blockade of the Sp sites was as effective
as PTH stimulation. However, PTH caused a significantly larger decrease in phosphorylated Osx protein levels, which is suggestive that the two compounds may function through unique mechanisms. The significant decrease in the phosphorylated isoforms of Osx by Mithramycin A in comparison to control is likely attributable to competition for binding to the Sp sites on the Osx promoter. The decrease in Osx transcription would eventually result in less Osx protein available for post-translational modification, decreasing the likelihood of phosphorylation events.

On the other hand, PTH may actively facilitate the dephosphorylation of Osx protein. PTH has been shown to inhibit the MAPK ERK1/2 and JNK in UMR106-01 cells (97), but not p38 (unpublished observations in our lab). However, the phosphorylation consensus sequence for p38 and JNK are the same (S/TP) and, therefore, it is possible that PTH inhibition of JNK, which is both rapid and prolonged, could result in decreased phosphorylation of Osx at Ser-73 and Ser-77. This possible mechanism of PTH inhibition of Osx could be explored by utilizing a number of techniques. First, UMR106-01 cells can be treated with the specific JNK inhibitor SP600125, which would be expected to decrease levels of phosphorylated Osx protein if JNK is needed for Osx phosphorylation. The changes in amount of phosphorylated Osx protein could be quantified using immunoblot analysis. In addition, having UMR106-01 cells express a constitutively active form of JNK, which is a fusion protein of JNK1 and its upstream activator JNKK2 (98), could be employed, which would be expected to increase phosphorylated Osx levels on its own. Lastly, the constitutively active JNK fusion protein can be combined with either PTH or SP600125 treatments to determine if the inhibition of phosphorylated Osx could be abrogated by maintaining JNK activity.
4.4 Osx may autoregulate its own expression, and PTH may be able to inhibit this process

Since intact and unblocked Sp sites are needed for Osx to be expressed in UMR106-01 cells, it was likely that a transcriptional activator was driving Osx transcription through these sites. If PTH stimulation could inhibit the expression or activity of the transcriptional activator(s) needed for Osx expression, it could indirectly decrease Osx promoter activity. However, it was evident that PTH did not regulate the expression of TIEG1, Sp1, or Sp3, all of which were identified as potential factors that could bind these Sp elements. However, the possibility remained that PTH could modulate the activity of one or more of these transcription factors by regulating their post-translational modifications.

Before this could be investigated, it was crucial to determine which transcription factors were actually binding the Osx promoter, and this was assessed using chromatin immunoprecipitation (ChIP) assays. One of the advantages of using a ChIP assay over other techniques is that it is able to capture DNA-protein interactions as they occur in intact, living cells, as opposed to more artificial environments as in the case of electrophoretic mobility shift assays. Surprisingly, it was found that Osx was the only factor that associated with the Osx promoter. An inherent disadvantage of a ChIP assay is that the primary antibodies may not co-immunoprecipitate the target proteins when bound to DNA. However, all antibodies used were ChIP-grade, minimizing the risk that the negligible binding of TIEG1, Sp1, and Sp3 resulted from inefficient co-immunoprecipitation. As a further test to ensure their efficiency, after the immunoprecipitation step in the ChIP assay, samples can be diluted and electrophoresed on an SDS-PAGE, and immunoblot analysis can be performed. With
increasing amounts of samples, increasing amounts of each specific target protein would be detected (99). In addition, as a positive control, ChIP assays can be performed to measure the binding of TIEG1, Sp1, and Sp3 on genes that have previously been demonstrated to be regulated by these transcription factors.

Since Osx is a stimulatory transcription factor, and it binds its own promoter to a high degree under basal conditions, it is plausible that Osx may autoregulate its own expression. In fact, it has previously been suggested that Osx stimulated its own expression as forced expression of Osx protein increased the Osx promoter activity of a luciferase construct (100). In addition, the sequence identified as potential Sp elements (CACCC) on the Osx promoter do not conform to the traditional Sp consensus sequence (GGGCGGG). However, Osx has recently been shown to specifically bind to these unorthodox Sp sites (Spa/Spb sites) (32). A comparison of these Sp sites between rat, mouse, and human revealed that the Spa site was highly conserved across all three species, indicating the requirement of at least one of these Sp elements within the Osx promoter (Fig 22). These observations suggest Osx stimulates its own expression through unique Sp sites, and this process may occur across species.

Another advantage of utilizing ChIP assays is that they can be quantitative when paired to real-time PCR. Thus, changes in protein binding to the Osx promoter in the presence of PTH were analyzed after various treatment durations. This lead to the observation that PTH decreased Osx binding on its own promoter. Given that PTH inhibits Osx expression, it was anticipated that a decrease in absolute Osx protein would result in less protein being present to occupy promoters. However, the timeline of the decrease in Osx binding was important, and it appeared to occur in two distinct phases.
Figure 22. Homology of the two Sp sites. The Sp elements on the Osx promoter were compared across mouse, rat, and human.
First, a significant decrease in Osx binding was observed after 2h of PTH treatment. However, at this time, Osx mRNA levels would not have even been significantly changed by PTH, which, as shown previously, requires at least 3h of continuous PTH exposure. Also, even after 4h of continuous PTH treatment, Osx protein levels would remain unaffected. Therefore, the initial decrease in Osx binding between 2-4h cannot be attributed to changes in the amount of Osx available to occupy promoters. In addition, it is important to note that the amount of Osx promoter binding is comparable at 2h and 4h, indicating a constant change. This is suggestive that PTH stimulation induces a post-translational modification of the Osx protein, which impedes it from associating with its target sites. As noted above, a possible mechanism that could explain this process is that PTH downstream effects may result in the dephosphorylation of Osx at Ser-73 and Ser-77 through the inhibition of JNK activity, and a dephosphorylated Osx protein is less likely to associate with target promoters.

Then, a second phase was observed in which there was a progressive decrease in Osx binding after 16h of PTH treatment, which culminated in almost negligible Osx promoter binding by 24h. During this timeframe, Osx protein levels would have been significantly decreased due to PTH's effects. One mechanism that could explain this second phase stems from the possibility that Osx is needed, at least partially, for its own expression. The initial decrease in Osx binding during the first phase, as a result of Osx dephosphorylation, would then initiate a cascade of decreasing Osx promoter activity, lower levels of active Osx, and decreasing Osx expression. As a result, even less Osx would be available to drive its own expression (Fig 23).
Figure 23. Proposed mechanism of Osx autoregulation. PTH may be able to not only interfere with this pathway, but have it work against itself in the opposite direction.
However, since PTH cannot completely abolish Osx expression, and Osx protein levels recover after PTH is removed, other transcription factors must be important in driving Osx expression. In order to elucidate which other factors fulfill this role, a DNase footprinting assay could be employed comparing the DNase protected regions in the absence of PTH with DNase protected regions in the presence of PTH. This would reveal other areas of the Osx promoter that are bound by proteins, and either electrophoretic shift assays or ChIP assays could be carried out to confirm the identity of these proteins.

4.5 Osx expression levels mirror developmental stage of the skeleton

As noted in the introduction, skeletal development begins during early embryogenesis and continues long after birth, and this process necessitates the continuous differentiation of large numbers of osteoblasts from precursors. As such, expression levels of Osx are expected to be at their highest during this stage in development to accommodate the immense rate of bone formation. In fact, it has been reported that Osx expression remains strong after birth in bone trabeculae and secondary ossification centres (23). This was confirmed in our preliminary study in which 5 day old mice displayed the highest levels of Osx expression in both calvaria and tibia and femur relative to the older mice that were investigated.

An important stage in the skeletal development of an animal is adolescence, as skeletal mass doubles between puberty and the onset of adulthood (101). This period is triggered by the increased release of gonadotropin-releasing hormone from the hypothalamus, which stimulates the gonadal secretion of sex steroids. Mice reach
sexual maturity between 28 and 48 days, and, as such, by this time, bone formation and osteoblastic activity would be expected to remain high to accommodate the rapid acceleration of bone mineral accrual. In our study, Osx expression in the tibia and femur was similar at 28 days to that observed 5 days after birth. However, Osx expression in the calvaria was observed to have decreased by 50% at 28 days, which can be attributed to the fact that most of the growth of the skull bones occurs prenatally and in early postnatal life.

As skeletal development approaches completion, bone formation steadily declines and plateaus to meet only the requirement of replacing old or damaged bone. This timeframe coincides with mice in early adulthood, which would include mice at 56 days and older. By this age, mouse body weight begins to plateau, signaling the end of the adolescent growth spurt. As anticipated, Osx levels showed a progressive decline from this age onwards. In order for us to use the Alzet pumps in mice, they had to be at least 20 g in weight. Therefore, we carried out all of our experimental manipulations of diet and implants to finish with mice of 56 days of age.

4.6 Short-term Intermittent PTH inhibits Osx in vivo

PTH administration in vivo inhibited Osx expression by 60% in intramembranous bone when given in the form of daily injections for 3 days, which was similar to the effects that we have previously reported for PTH on calvaria ex vivo (77). The effect of PTH on endochondral bone, however, was not significant. This may reflect differences in response of the two types of bones to PTH, or it may reflect differences in bone remodeling rates between the two types of bone. In addition, the cortical bone of the tibia and femur may not have been exposed to PTH as much as the capillary-rich
trabecular bone. It was anticipated that PTH would inhibit Osx expression, as all previous *in vitro* experiments indicated that PTH stimulation directly inhibited Osx expression in osteoblasts. Indeed, that was the case in our short term *in vivo* study, at least in calvaria.

From our studies in the UMR 106-01 cell line, PTH inhibition of Osx was shown to be mediated by PTH1R stimulation of cAMP production. To determine if this pathway is also responsible for mediating PTH inhibition of Osx *in vivo*, the expression of other genes known to be inhibited by this mechanism, such as SOST and OPG, could also be measured in future studies (2,102).

### 4.7 Assessing primary and secondary hyperparathyroidism

The effects of chronic elevation of PTH levels on bone were investigated using two different methods to induce hyperparathyroidism. As noted above, primary and secondary hyperparathyroidism both result in excess PTH circulating in the bloodstream and have the same catabolic effects on bones. The main difference between each type of hyperparathyroidism is the underlying mechanisms that result in overactivity of the parathyroid glands, and, as such, their effects on the body are expected to be similar (90,91). However, additional factors, such as hypercalcemia and hypophosphatemia, can exert effects in addition to PTH itself, and must be considered when interpreting the effects of hyperparathyroidism on bone.

While circulating PTH levels were not measured in our mice, analyses of the mice were undertaken to determine evidence for hyperparathyroidism in the two mouse models. Analysis of blood chemistry revealed that serum phosphate levels were unchanged in all mice, suggesting that excess phosphate liberated from bone tissue during resorption
was successfully cleared by the kidney. Renal excretion of phosphate is stimulated by PTH (103), and this seems to have been the case in both our animal models. Serum calcium levels, on the other hand, were different in the two sets of mice, as they were elevated only in mice with primary hyperparathyroidism. In these mice, PTH stimulation of bone resorption, along with increases in calcium absorption from the diet by elevated vitamin D₃ and renal reabsorption, would result in hypercalcemia. In the face of continuous hypercalcemia, the kidneys of these mice were unable to maintain calcium homeostasis. On the other hand, despite receiving no calcium in their diets, the calcium-deficient diet mice were able to maintain normal serum calcium levels. This was likely a result of PTH-stimulated bone resorption, as bone mineral density decreased by 28% over three weeks in these mice.

The effect of PTH on expression of renal Cyp27b1 was also different in the two mouse models of hyperparathyroidism. Cyp27b1 encodes 25-hydroxyvitamin D-1α-hydroxylase, the enzyme that is responsible for the final activation step of 1,25-dihydroxyvitamin D₃ (104). PTH increases calcium absorption in the intestine by increasing 1,25-dihydroxyvitamin D₃, and this is accomplished, in part, by stimulating Cyp27b1 expression in the kidney (105,106). Therefore, increased Cyp27b1 expression suggested that 1,25-dihydroxyvitamin D₃ levels were increased as a result of elevated serum PTH. In the mice receiving calcium-deficient diet, the increase in Cyp27b1 was far greater than that seen in the mice with PTH pumps. This likely reflected the differences in serum calcium in the two sets of mice, as hypercalcemia in the PTH pump mice would suppress Cyp27b1 transcription (118). However, in the calcium-deficient mice, serum calcium levels were normal. It is also possible that the total PTH in the two sets of animals were not the same. In future studies, it will be important to measure the
precise concentrations of serum PTH in both types of hyperparathyroidism. This will enable us to gain greater insight into the degree by which PTH was elevated, and to determine if differences in PTH elevation resulted in more profound effects on the animals. In addition, it will also be useful to determine the concentrations of 1,25-dihydroxyvitamin D₃ directly, instead of using Cyp27b1 expression as a proxy. These measurements will definitively reveal the extent to which these mice had hyperparathyroidism.

4.8 Chronically elevated PTH levels stimulate Osx expression *in vivo*

If we can assume that both of our mouse models achieved chronically elevated PTH levels, which is reasonable based on the observations of decreased aBMD and stimulation of Cyp27b1 expression in these mice, the effects on Osx could then be explored. Paradoxically, continuously elevated serum PTH caused an increase in Osx expression in mice using the two distinct models of hyperparathyroidism. This increase occurred despite having demonstrated that PTH inhibits Osx expression *in vitro* and after 3 days of intermittent exposure *in vivo*. It was originally hypothesized that chronically elevated PTH would result in increased osteoclast activity at the expense of osteoblast activity, and, as such, genes responsible for osteoblast activity, such as Osx, would be inhibited.

In order to assess the differences between osteoblast and osteoclast activity, key genes involved in both processes were analyzed. However, the majority of the genes that were assessed only displayed trends of stimulation or inhibition. In most cases, it was likely that statistical significance was not achieved because of the small sample sizes that
were utilized, as there were 4 mice with PTH-pump implants and 5 mice receiving calcium-deficient diets. Thus, it will be important to increase the sample sizes of all groups so that more reliable conclusions can be drawn from these studies. That being said, osteocalcin, which is a marker for late osteoblasts, was significantly stimulated in the model of primary hyperparathyroidism. This observation, coupled with the finding of increased Osx expression, suggest that chronically elevated PTH acts to increase osteoblast activity.

It is important to note that, while continuous PTH exposure is catabolic to bone, bone remodeling is carried out through the coupling of bone formation and bone resorption, and any changes in bone mineral density reflect a relative increase in one process over the other (16). Thus, PTH does increase both osteoblastic and osteoclastic activity, but whether or not the end result is net bone formation or net bone loss is dependent on intermittent or chronic PTH exposure. Thus, the increases in osteoblastic genes in the present studies may be reflective of this stimulation of bone remodeling processes by PTH. Since both chronically elevated PTH models resulted in decreases in aBMD, it is likely that bone formation was being outweighed by osteoclastic activity and bone resorption.

In fact, it is well established that chronic PTH treatment results in a stimulation of RANKL, inhibition of OPG, and increase in osteoclastic activity (16,75,76,107). However, the present studies were not able to detect significant changes in the RANKL:OPG ratio at the mRNA level, which may be attributable to small sample sizes. Thus, it will be critical to measure key biochemical indices of bone resorption in the future to more accurately assess changes in osteoclastic activity. For example,
measuring urinary hydroxyproline excretion is an indicator of collagen breakdown, and is used in the assessment of bone tissue degradation in patients at risk of osteoporosis (108). In addition, measuring plasma tartrate-resistant acid phosphatase activity would also reveal the extent of osteoclast activity, as osteoclasts express and utilize these phosphatases to aid bone degradation (109). On the other hand, biochemical markers for bone formation that can be assessed are serum bone alkaline phosphatase and serum osteocalcin (110).

Conversely, for PTH to be anabolic to bone, it would be expected that osteoblastic activity is increased relative to osteoclastic activity. In fact, it has been reported that intermittent PTH injections increase Osx expression (85). Therefore, the mechanism of PTH action may revolve around distinguishing how PTH alters relative levels of osteoblast and osteoclast activity. However, an explanation is lacking for how PTH can stimulate osteoblast activity when it is responsible for the inhibition of many osteoblastic genes in vitro.

4.9 SOST may mediate PTH's effects on Osx and osteoblast differentiation in long-term PTH paradigms

Perhaps most important to the explanation of increased osteoblastic activity, and more specifically Osx expression, is the observation that SOST was inhibited in both models of chronic PTH exposure. SOST is a glycoprotein that is secreted primarily by osteocytes to suppress bone formation. Its disruption, as is seen in the bone disorders sclerosteosis and Van Buchem’s disease, causes generalized bone overgrowth (111). SOST migrates through the many osteocyte canaliculi that exist throughout bone tissue, and it is able to reach the surface of bone by traveling through this network (4). SOST
regulates bone formation by inhibiting canonical Wnt/β-catenin signaling (112). Wnts are secreted glycoproteins that can act through canonical or non-canonical pathways by binding to Frizzled receptors. Canonical Wnt/β-catenin signaling occurs when Wnt proteins bind to a Frizzled receptor and a member of the lipoprotein receptor-related protein family (Lrp), either Lrp5 or Lrp6, and cause them to associate and form a receptor complex (113). This receptor complex then initiates a cascade of downstream signaling that results in β-catenin mediated transcriptional activation of various genes (4). SOST is able to inhibit the formation of the receptor complex by binding to either Lrp5 or Lrp6, and preventing their association with Frizzled (114). As mentioned previously, canonical Wnt/β-catenin signaling is critical for the differentiation of osteoblasts from precursors, and, through the regulation of this pathway, SOST is able to regulate bone formation.

Interestingly, continuous PTH exposure has been shown to inhibit SOST expression in osteocytes in vitro via a cAMP pathway similar to PTH inhibition of Osx (102). In addition, primary hyperparathyroidism in humans has recently been shown to result in significantly inhibited SOST expression levels (115). This inhibition of SOST expression was also observed in our studies using the two different models of hyperparathyroidism in mice. By inhibiting SOST expression, PTH indirectly stimulates Wnt/β-catenin signaling, which increases osteoblast differentiation and expression of osteoblastic genes. Therefore, the effects observed on Osx, and osteoblasts, from exposure to chronically elevated PTH may be a secondary effect of inhibition of SOST expression. It appears that this effect on SOST occurs independent of PTH treatment regimen, as long-term intermittent PTH injections have also been demonstrated to inhibit SOST expression (70,116).
Meanwhile, acute exposure to PTH may not activate this process, and thus PTH's direct effects on Osx, which is inhibitory, are the only effects that can be observed. This secondary process of SOST inhibition resulting in Osx stimulation may not occur in short-term exposures to PTH for two reasons. First, it requires more time for PTH to reach osteocytes than it does to reach the more accessible osteoblasts, as osteocytes are embedded within the bone matrix. Secondly, there may be a lag in time before PTH inhibition of SOST expression results in increased Wnt/β-catenin signaling and subsequent Osx expression increases. In sum, PTH directly inhibits Osx, but long-term exposure indirectly stimulates Osx expression by increasing Wnt/β-catenin signaling and osteoblast differentiation through the inhibition of SOST.

It is important to note that, as mentioned above, Osx has been shown to increase SOST expression in osteocytes by directly stimulating SOST promoter activity. Therefore, it is counterintuitive that SOST expression should decrease despite increases in Osx expression levels brought on by long-term PTH stimulation. However, in osteocytes, the direct effects of PTH signaling on Osx, which is inhibitory, may be keeping Osx levels low specifically in these cells. As such, Osx would not be able to increase SOST expression in osteocytes.

4.10 Conclusion
Osx is essential to bone formation and maintenance, and yet details about its regulation are lacking. In this thesis, it was demonstrated that Osx directly binds to its own promoter via unorthodox Sp binding sites (CACCCC). Given that it was previously demonstrated that Osx can stimulate the activity of a luciferase expression vector driven by the Osx promoter, it is possible that Osx may be responsible, at least partially, for
promoting its own expression in osteoblasts. The mechanism by which PTH inhibits Osx expression may occur through the inhibition of this autoregulatory pathway. PTH was shown to be able to decrease Osx binding to its own promoter before any detectable changes in absolute Osx protein levels were observed. Phosphorylation status of the Osx protein regulates its ability to be recruited to target promoters and promote transcription (37). Interestingly, PTH was found to cause a decrease in the phosphorylated forms of Osx in comparison to Mithramycin A, despite both having equally decreased levels of the unphosphorylated forms. Thus, PTH inhibition of protein kinases, such as JNK, that are needed to phosphorylate Osx proteins would decrease the ability of Osx to act as a transcriptional activator. With fewer active Osx proteins available to drive its own expression, Osx protein levels would then drop, and Osx autoregulation would be disrupted.

In addition, PTH has been demonstrated to inhibit Osx expression in vivo in acute, intermittent regimens. Conversely, long-term elevation of serum PTH, by primary or secondary hyperparathyroidism, resulted in the stimulation of Osx expression. This result was surprising as all previous in vitro and ex vivo work by our lab demonstrated a consistent inhibition of Osx expression by PTH. It is possible that PTH's inhibitory effects on SOST, and its indirect stimulatory effects on canonical Wnt signaling, may override PTH's direct effects on Osx. As such, PTH may have been able to stimulate osteoblast differentiation and activity. However, given that aBMD was shown to have been decreased in both studies of hyperparathyroidism, it is possible that PTH was able to stimulate osteoclast differentiation and activity to a greater extent. Therefore, it will be important to follow these studies with an investigation into the effects of long-term intermittent PTH on Osx expression and other key marker genes in vivo.
References


55. Celil AB, Campbell PG. BMP-2 and insulin-like growth factor-1 mediate Osterix (Osx) expression in human mesenchymal stem cells via the MAPK and protein kinase D signaling pathways. The Journal of biological chemistry. 2005 Sep 9;280(36):31353-31359.


64. Xing W, Singgih A, Kapoor A, Alarcon CM, Baylink DJ, Mohan S. Nuclear factor-E2-related factor-1 mediates ascorbic acid induction of osterix expression via interaction with


72. Walsh C a, Bowler WB, Bilbe G, Fraser WD, Gallagher J a. Effects of PTH on PTHrP gene expression in human osteoblasts: up-regulation with the kinetics of an immediate


