PHYSIOPATHOLOGY OF OSTEOCLAST IN BONE

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A thesis submitted to the faculty of the University of North Carolina at Chapel Hill in partial fulfillment of the requirements for the degree of Master of Science in the Curriculum of Oral Biology.

Chapel Hill
2008

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ABSTRACT

XIAOMEI GU: Physiopathology of Osteoclast in Bone
(Under the direction of Dr. Eric T. Everett)

Bone is constantly remodeled by osteoclastic bone resorption and osteoblastic bone formation. Abnormal remodeling can result in bone mass change; bone loss is implicated in a number of bone diseases, representing an increase in bone resorption relative to formation. Therefore, an understanding of osteoclast biology is important to demystify the pathogenesis of bone diseases and to develop treatment strategies. Osteoclasts are formed by fusion of hematopoietic monocyte/macrophage lineage cells, in which osteoblasts/stromal cells play a central role by producing macrophage-colony stimulating factor and receptor activator of nuclear factor κ B ligand. Characterization of osteoclastogenesis has provided new insight into our understanding of bone diseases with excessive bone resorption. Moreover, anti-resorptive drugs, bisphosphonates, have been developed to target osteoclasts and their function. Additionally, a better understanding of the interactions of fluoride between osteoclasts may help harness the desirable effects of fluoride on bone while limiting its undesirable effects.

To my mom who taught me to love.

ACKNOWLEDGEMENTS

This thesis would not have been possible without the support of so many people. Firs of all, I would like to convey my gratitude to my advisor, Dr. Eric T. Everett, for his invaluable support and suggestions. I am also deeply indebted to the members of my committee, Dr. Patrick Flood and Dr. Ching-Chang Ko for their insightful guidance throughout the writing process.

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Besides academic support, I have benefited from the constant moral support and inspiration of a number of persons. Special thanks go to Oral Biology manager, Cindy Blake, for her precious life advice. I am very grateful to my family and friends. I would also like to acknowledge my fellow Oral Biology students.

I am lucky enough to have all these wonderful people in my life; words fail me to express my gratitude to each of them.

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LIST OF ABBREVIATIONS

AP	activator protein
ALP	alkaline phosphatase
ATP or Appp	adenosine triphosphate
aPKC	atypical protein kinase C
BMC	bone mineral content
BMD	bone mineral density
BMP	bone morphogenetic protein
BMU	basic multicellular unit
DXA or DEXA	dual energy x-ray absorptiometry
CA II	carbonic anhydrase II
CaR	calcium receptor
CFU-GEMM	colony forming unit-granulocyte, erythrocyte, monocyte, megakaryocyte
CFU-GM	colony forming unit-granulocyte, monocyte/macrophage
CFU-G	colony forming unit-granulocyte
CFU-M	colony forming unit-monocyte/macrophage
CLC-7	chloride channel 7
CREB	cAMP-response element-binding protein
CTK	cathepsin K
CTR	calcitonin receptor
DAP12	DNAX-activating protein 12

DC-STAMP	dendritic cell-specific transmembrane protein
DMAP	dimethylallyl pyrophosphate
ERα	estrogen receptor alpha
ERβ	estrogen receptor beta
ERK	extracellular-signal-regulated kinase
FasL	Fas ligand
FPP	farnesyl diphosphate
FcRγ	Fc receptor common γ subunit
GDP	guanosine diphosphate
GTP	guanosine triphosphate
GGPP	geranylgeranyl diphosphate
G protein	guanine nucleotide binding protein
GPCR	G protein-coupled protein
GRB2	growth-factor-receptor-bound protein 2
H^+ATPase	H^+-adenosine triphosphatase
HSC	hematopoietic stem cell
IκB	inhibitor of NFκB
IKK	IκB kinase
IL	interleukin
IPP	isopentenyl pyrophosphate
ITAM	immunoreceptor tyrosine-based activation motif
JNK	Jun N-terminal kinase
MAPK	mitogen-activated protein kinase

MCP-1	monocyte chemoattractant protein-1
M-CSF	macrophage-colony stimulating factor
MGF	macrophage growth factor
MGI-IM	macrophage and granulocyte inducer IM
MMP 9	metalloproteinase 9
NFκB	nuclear factor κ B
NFATc1	nuclear factor of activated T cells, cytoplasmic 1
OCIL	osteoclast inhibitory lectin
OCN	osteoclacin
OPG	osteoprotegerin
OSCAR	osteoclast-associated receptor
Osx	osterix
Runx2	runt-related transcription factor-2
PDB	Paget's disease of bone
PIR-A	paired immunoglobulin-like receptor-A
PGE_2	prostaglandin E_2
PI3K	phosphoinositide 3-kinase
PLCγ	phospholipase Cγ
PKB	protein kinase B
PPi	inorganic pyrophosphate
ppm	parts per million
PTH	parathyroid hormone
PTHrP	parathyroid hormone-related protein

RANK	receptor activator of nuclear factor κ B
RANKL	receptor activator of nuclear factor κ B ligand
SCF	stem cell factor
SD	standard deviation
SDF-1	stromal cell-derived factor-1
sFRP1	secreted frizzled-related protein 1
SIRP β1	signal-regulatory protein β1
TACE	metalloprotease disintegrin TNF α convertase
TNF	tumor necrosis factor
TGFβ	transforming growth factor beta
TRAF	TNF receptor-associated factor
TRAP	tartrate resistant alkaline phosphatase
TREM-2	triggering receptor expressed on myeloid cells-2
VCP	valosin-containing protein
Vitamin D_3	1, 25$(OH)_2D_3$ 25-dihydroxy

CHAPTER I. BASIC BONE BIOLOGY

1.1. Thesis Introduction

Regardless of etiologies, bone loss represents an increase in bone resorption by osteoclasts relative to bone formation by osteoblasts. Understanding osteoclast biology and bone resorption are essential in understanding the pathogeneses and therapies of diseases involving excessive bone destruction. This thesis will begin with a very brief introduction of basic bone biology to orient the readers' understanding of osteoclasts in a context of normal bone remodeling. In the subsequent two chapters, origin, differentiation, and function of osteoclasts will be addressed in greater detail. To help understand osteoclast differentiation and its regulation, the role of osteoclast formation in periprosthetic osteolysis, peri-implantitis, and orthodontic tooth movement will also be discussed. In addition to the mechanism of bone resorption, this thesis will also explore two newly-identified functions of osteoclasts: mobilizing hematopoietic stem cells in bone marrow and stimulating osteoblastic bone formation.

Osteoclastic bone resorption has been implicated in a number of diseases; however, this thesis will primarily focus on the cellular and molecular pathogenesis of common metabolic bone disease, such as osteoporosis and Paget's disease of bone. Parathyroid hormone (PTH) plays a central role in calcium homeostasis via its action on targeting tissues

such as bone cells [1, 2]. The anabolic action of PTH will be discussed in a context of osteoclasts being the anabolic signaling sources (Chapter III), whereas the catabolic action of PTH will be discussed in a context of hyperthyroidism pathogenesis. The recent breakthrough in understanding the pathogenesis of a genetic disorder, Cherubism, is a good example of how understanding osteoclast biology can advance our understanding of a disease process.

Osteoclasts are the primary therapeutic targets for conditions with excessive bone resorption. Bisphosphonate is a major class of anti-resorptive drug currently available. In the last chapter, this thesis will discuss the actions of bisphosphonates on bone with a focus on their induction of osteoclast apoptosis. Meanwhile, the actions of fluoride on bone cells will also be addressed. The anabolic action of fluoride on bone has been largely explained by its mitogenic effect on osteoblasts; however, this thesis will mainly focus on the effects of fluoride on osteoclasts. A complete picture of fluoride's effects on the two major cell types in bone—osteoblasts and osteoclasts—is required for the utilization and/or optimization of fluoride's potential in treating diseases with excessive bon resorption. By reviewing the pathophysiology of osteoclasts, this thesis will provide a powerful context to better understand fluoride's effect on osteoclasts.

1.2. Embryonic Development of Bone

The skeletal system develops from three distinct origins: 1) the paraxial mesoderm gives rise to the axial skeleton (vertebrae and ribs); 2) the lateral plate mesoderm gives rise to the appendicular skeleton (limb skeleton); and 3) the cranial neural crest, which is derived from the ectoderm, gives rise to the craniofacial skeleton [3].

Bone development occurs through two distinct developmental processes: intramembranous ossification and endochondral ossification. During intramembranous ossification, mesenchymal cells condense and differentiate directly into osteoblasts to lay down bone matrix [4]. This process occurs in several craniofacial bones and the lateral part of clavicles [5]. During endochondral ossification, mesenchymal cells condense and differentiate directly into chondroblasts to synthesize a cartilage model, which is then replaced by bone and bone marrow [4]. This process occurs in the long bones of the limbs, basal part of the skull, vertebrae, ribs, and medial part of the clavicles [5]. Therefore, the major difference between the two types of ossification is the presence of a cartilaginous precursor template during endochondral ossification.

1.3. Bone Function

Bone tissue has the following functions: 1) it provides structural support for the body; 2) it protects internal organs, such as brain, spinal cord, heart, and lungs; 3) it supports hematopoiesis in bone marrow; 4) it is the basis of posture and locomotion by allowing the attachments of muscles, ligaments, and tendons; 4) it serves as a mineral reservoir and in particular helps regulate calcium homeostasis [6, 7].

1.4. Components of Bone

As in all connective tissues, the fundamental constituents of bone are cells and extracelluar matrix. However, unlike most of other tissues, the extracelluar matrix of bone is mineralized by calcium salts in form of hydroxyapatite, which provides stiffness to the

tissue. The organic matrix is largely composed of type I collagen (95%), and the remaining 5% is composed of proteoglycans and numerous non-collagenous proteins [4, 8]. Three major cell types are present in bone tissue: osteoblasts, osteocytes, and osteoclasts [4].

1.4.1. Osteoblasts

Osteoblasts are the cells responsible for bone formation [7]. They are derived from mesenchymal stem cells in bone marrow [11]. Under appropriate stimuli, these multipotent cells can differentiate along three principal lineages: osteoblastic, adipocytic and chondrocytic lineages [6, 12]. Expression of the transcription factors, runt-related transcription factor-2 (Runx2), is required to shift the progenitors toward the osteoblast lineage and away from adipocytic and chondrocytic lineages. Further differentiation of the preosteoblast into a mature, functional osteoblast phenotype requires the expression of another transcription factor, osterix (Osx). The early stage of osteoblastic differentiation is characterized by markers such as type I collagen and alkaline phosphatase (ALP), while the late stage of osteoblastic differentiation is characterized by markers, such as osteoclacin and mineralization of extracellular matrix [5, 6].

Mature and active osteoblasts synthesize most of the proteins in bone extracellular matrix [6, 7]. Some osteoblasts are buried in bone matrix and become osteocytes, while some osteoblasts become flattened cell on bone surface—bone-lining cells (inactive osteoblasts) [7].

Besides the bone-forming function, osteoblasts provide essential signals for osteoclast differentiation and its regulation (discussed in more detail in Chapter II) [13]. Moreover, recent studies have found that osteoblasts also play a central role in hematopoiesis

by providing many factors essential for the survival, renewal, maturation and lineage commitment of hematopoietic stem cells [14, 15].

1.4.2. Osteocytes

Osteocytes are the most abundant cells in bone (25,000/mm^3 of bone [4]) and yet remain the least characterized [16]. They are terminally differentiated osteoblasts that have become entombed in bone matrix [7, 16]. Osteocytes are strain-sensitive and can transduce mechanical signals. Recent studies have shown that damage-induced osteocyte apoptosis may be involved in the recruitment of osteoclasts and initiation of new bone remodeling [16, 17].

1.4.3. Osteoclasts

Osteoclasts are cells that can resorb bone [18]. They are of hematopoietic origin, and are formed by the maturation and fusion of monocyte/macrophage lineage common precursors [19]. Mature osteoclasts are large, multi-nuclear cells and have morphology highly specialized for its bone-resorbing function [20]. After fulfilling the resorption function, they are likely to be removed by apoptosis [21]. The biology and physiopathology of osteoclasts will be discussed in more detail in the following chapters of this thesis.

1.5. Bone Structure: Cortical Bone and Trabecular Bone

Morphologically, there are two major forms of bone: cortical (compact) and trabecular (cancellous or spongy). In cortical bone, collagen fibrils are densely arranged to form concentric lamellae, with fibrils assembled in perpendicular planes in adjacent lamellae. Trabecular bone is composed of a porous network of thin and mineralized trabeculae with lamellae arranged parallel to each other [4, 8]. The percentage of cortical and cancellous bone varies among different sites in the skeletons system. For example, in the lumbar spine, 66% of the bone is cancellous bone, whereas in the midradius >95% of the bone is cortical bone [4].

1.6. Bone Histology: Lamellar Bone and Woven Bone

Bone can also be classified as lamellar bone and woven bone. Lamellar bone is mature bone, in which the collagen fibers are arranged in a lamellar structure [9, 10]. Lamellar bone is mainly composed of cylindrical units called osteons or Haversian systems, which consist of a central canal surrounded by concentric lamellae. The central canal is called osteonal or Haversian canal and contains blood vessels and nerves [10]. Woven bone is immature bone, which is formed very rapidly during skeletagenesis or fracture healing. The collagen fibers in woven bone are loosely arranged in irregular arrays and woven bone is therefore also called nonlamellar bone. Woven bone contains more cells per unit area than lamellar bone [9].

1.7. Bone Remodeling

Although it appears to be inert and static, bone tissue is metabolically active. Throughout life, bone is constantly broken down and rebuilt; this continuous process of bone resorption and bone formation is called bone remodeling [6].

1.7.1. Purposes of Bone Remodeling

It has been generally accepted that bone remodeling serves two closely linked purposes. First, some remodeling can be directed towards specific sites to repair fatigue damage, continuously replacing fatigued bone with mechanically competent new bone. So bone remodeling is necessary to maintain the mechanical strength and structural integrity of new bone. Secondly, through continuous bone resorption and formation, bone subserves its metabolic functions as a storehouse of calcium and phosphorus. Therefore, bone remodeling plays a very important role in maintaining mineral homeostasis [22, 23].

1.7.2. Cellular Processes of Bone Remodeling

Bone-resorbing osteoclasts and bone-forming osteoblasts work as a team to remodel bone. At the microscopic level, bone remodeling occurs in small areas of the cortical and trabecular surface, known as basic multicellular units (BMUs) [24, 25]. At each BMU, resorption always precedes formation. Osteoclasts remove a certain amount of bone, and osteoblasts subsequently deposit organic matrix and mineral to fill the previously created cavity [25]. Therefore, bone remodeling involves sequential activities of two distinct cell types in bone.

1.7.3. Difference between Bone Remodeling and Bone Modeling

During bone remodeling, bone resorption is always followed by bone formation at a locus, and these two processes are said to be coupled to one another [17]. In fact, it is the coupling of osteoclast activity to osteoblast activity that makes bone remodeling distinctive from bone modeling. Bone modeling refers to a process in which bone are shaped or reshaped by the independent activities of osteoclasts and osteoblasts [17]. Therefore, unlike remodeling, bone modeling at a single site involves either bone resorption or bone formation, but not both. Bone modeling is most pronounced during bone growth and development; it also occurs in adults bone in response to mechanical loads [17, 22].

1.7.4. Regulation of Bone Remodeling

Bone remodeling process is highly regulated by numerous local factors and systemic hormones. These factors include bone morphogenetic protein (BMP), tumor growth factor β (TGFβ), tumor necrosis factor α (TNFα), interleukins, estrogen, prostaglandin E_2 (PGE_2), parathyroid hormone (PTH), calcitonin, and vitamin D [25]. Among them, estrogen is considered the most important hormone in maintaining normal bone turnover [26]. It has a dual effect on bone remodeling: on one hand, it can increase bone formation; on the other hand, it can reduce bone resorption [25].

The delicate balance between bone resorption and bone formation is essential for the maintenance of normal bone functions. Imbalance between the two can result in compromised bone renewal and change in bone mass; skeletal diseases will develop.

Increased bone resorption or a relative decrease in bone formation compared to bone resorption can lead to osteoporosis (osteoporosis will be further discussed in Chapter IV). In contrast, increased bone formation or a relative decrease in bone resorption compared to bone formation can lead to osteopetrosis [6].

1.8. Chapter Summary

Bone is a highly specialized connective tissue. It consists of mineralized extracelluar matrix and three major cell types—the bone-forming osteoblast, the bone-resorbing osteoclast, and the osteocyte. To carry out its functions, bone undergoes constant remodeling throughout life. Bone remodeling is carried out by the coupled activities of osteoclasts and osteoblasts. The balance between bone resorption and bone formation is very important for normal bone function. Disruption of this balance will lead to metabolic bone diseases. Therefore, understanding the normal process of bone remodeling will allow us to better understand the pathogenesis of metabolic bone diseases.

Chapter I. Reference List

[1] Hamann KL and Lane NE. Parathyroid hormone update. Rheum Dis Clin North Am 2006; 32: 703-719.

[2] Cole DE, Webb S and Chan PC. Update on parathyroid hormone: new tests and new challenges for external quality assessment. Clin Biochem 2007; 40: 585-590.

[3] Sadler TW and Langman J. Langman's Medical Embryology. Philadelphia, Lippincott Williams & Wilkins. 2006.

[4] Favus MJ and American Society for Bone and Mineral Research. Primer on the Metabolic Bone Diseases and Disorders of Mineral Metabolism. Philadelphia, Lippincott Williams & Wilkins. 1999.

[5] Nakashima K and de Crombrugghe B. Transcriptional mechanisms in osteoblast differentiation and bone formation. Trends Genet 2003; 19: 458-466.

[6] Cohen MM,Jr. The new bone biology: pathologic, molecular, and clinical correlates. Am J Med Genet A 2006; 140: 2646-2706.

[7] Sommerfeldt DW and Rubin CT. Biology of bone and how it orchestrates the form and function of the skeleton. Eur Spine J 2001; 10 Suppl 2: S86-95.

[8] Bilezikian JP, Raisz LG and Rodan GA. Principles of Bone Biology. San Diego, Academic Press. 2002.

[9] Hernandez CJ, Majeska RJ and Schaffler MB. Osteocyte density in woven bone. Bone 2004; 35: 1095-1099.

[10] Ross MH and Pawlina W. Histology :A Text and Atlas : With Correlated Cell and Molecular Biology. Baltimore, MD, Lippincott Wiliams & Wilkins. 2006.

[11] Ducy P, Schinke T and Karsenty G. The osteoblast: a sophisticated fibroblast under central surveillance. Science 2000; 289: 1501-1504.

[12] Compston JE. Bone marrow and bone: a functional unit. J Endocrinol 2002; 173: 387-394.

[13] Arai F, Miyamoto T, Ohneda O*, et al*. Commitment and differentiation of osteoclast precursor cells by the sequential expression of c-Fms and receptor activator of nuclear factor kappaB (RANK) receptors. J Exp Med 1999; 190: 1741-1754.

[14] Taichman RS. Blood and bone: two tissues whose fates are intertwined to create the hematopoietic stem-cell niche. Blood 2005; 105: 2631-2639.

[15] Haylock DN and Nilsson SK. Stem cell regulation by the hematopoietic stem cell niche. Cell Cycle 2005; 4: 1353-1355.

[16] Kogianni G and Noble BS. The biology of osteocytes. Curr Osteoporos Rep 2007; 5: 81-86.

[17] Robling AG, Castillo AB and Turner CH. Biomechanical and molecular regulation of bone remodeling. Annu Rev Biomed Eng 2006; 8: 455-498.

[18] Teitelbaum SL. Osteoclasts: what do they do and how do they do it? Am J Pathol 2007; 170: 427-435.

[19] Miyamoto T and Suda T. Differentiation and function of osteoclasts. Keio J Med 2003; 52: 1-7.

[20] Takahashi N, Ejiri S, Yanagisawa S and Ozawa H. Regulation of osteoclast polarization. Odontology 2007; 95: 1-9.

[21] Vaananen K. Mechanism of osteoclast mediated bone resorption--rationale for the design of new therapeutics. Adv Drug Deliv Rev 2005; 57: 959-971.

[22] Favus MJ and American Society for Bone and Mineral Research. Primer on the Metabolic Bone Diseases and Disorders of Mineral Metabolism. Washington, DC, American Society for Bone and Mineral Research. 2006.

[23] Heaney RP and Weaver CM. Newer perspectives on calcium nutrition and bone quality. J Am Coll Nutr 2005; 24: 574S-81S.

[24] Parfitt AM. Osteonal and hemi-osteonal remodeling: the spatial and temporal framework for signal traffic in adult human bone. J Cell Biochem 1994; 55: 273-286.

[25] Fernandez-Tresguerres-Hernandez-Gil I, Alobera-Gracia MA, del-Canto-Pingarron M and Blanco-Jerez L. Physiological bases of bone regeneration II. The remodeling process. Med Oral Patol Oral Cir Bucal 2006; 11: E151-7.

[26] Raisz LG. Physiology and pathophysiology of bone remodeling. Clin Chem 1999; 45: 1353-1358.

CHAPTER II. OSTEOCLAST BIOLOGY: ORIGIN AND DIFFERENTIATION

Osteoclasts are the exclusive bone resorptive cells. They are large, multinucleated, and terminally differentiated cells formed via the fusion of their mononuclear precursors [1].

2.1. Osteoclast Origin

Controversy concerning the origin of osteoclasts existed until the late 1970s. The pioneering parabiosis experiments where the circulation of a normal rat was connected to an irradiated rat have demonstrated that cells able to differentiate to osteoclasts were present in the circulation and can be recruited to bone-resorbing sites via the blood stream [2]. This observation was further corroborated by studies using osteopetrotic mouse models. The osteopetrotic phenotype of these mice can be rescued after parabiotic union to normal mice, or after transplantation of bone marrow cells from normal mice [2, 3]. The cure of osteopetrosis by bone marrow transplantation has also been established in humans [4]. It is widely accepted that the osteoclast is of hematopoietic origin [2].

Subsequently, it has been shown that the osteoclast precursor belongs to the monocyte/macrophage family derived from the myeloid progenitors [2, 5-7]. Hematopoietic

stem cells (HSCs) generate all the blood cell types, including the lymphoid and myeloid lineages. The determination of HSCs to the myeloid lineage requires the expression of the transcription factor PU.1 [8, 9]. The myeloid lineage, also known as colony forming unit-granulocyte, erythrocyte, monocyte, megakaryocyte (CFU-GEMM), gives rise to CFU-GM (granulocyte, monocyte/macrophage), and CFU-M (monocyte/macrophage), the latter being the common precursors of osteoclasts and macrophages (Figure 2.1.) [3,10]. Interestingly, compared to the more committed CFU-M-derived cells, CFU-GM-derived cells have greater osteoclast differentiating potential in *in vitro* mouse and human studies [11, 12]. Nevertheless, it has been generally accepted that osteoclasts are derived from CFU-M, the monocyte/macrophage lineage [4].

Even though osteoclasts can be generated *in vitro* from mononuclear phagocytes of various tissues, osteoclast precursors primarily reside in bone marrow [4, 5, 13]. The formation of osteoclasts only occurs in the close vicinity of bone, and multinucleated osteoclasts are not present in the circulation [13].

2.2. Osteoclast Differentiation

It was first proposed in 1981 that osteoblasts were involved in osteoclast differentiation [2]. After almost 10 years, a co-culture system of mouse osteoblasts/stromal cells and hematopoietic cells for osteoclast formation (or osteoclastogenesis) established the concept that osteoblast/stromal cells are essentially involved in the formation of osteoclasts. The close contact between cells from the osteoblastic and hematopoietic lineage is necessary for osteoclastogenesis [2]. It is now known that stromal cells/osteoblasts are able to produce two essential factors for osteoclastogenesis: nuclear factor (NF)κB ligand (RANKL) and

macrophage colony-stimulating factor (M-CSF) [7, 10]. Signaling pathways initiated by these two factors lead to the expression of genes typifying osteoclasts [7, 10]. Mature osteoclasts are characterized by markers such as integrin $\alpha_V\beta_3$, calcitonin receptor (CTR), cathepsin K (CTK), metalloproteinase-9 (MMP-9), H^+-ATPase, and tartrate resistant alkaline phosphatase (TRAP) (Figure 2.1.) [2].

2.2.1. Role of M-CSF in Osteoclast Differentiation

M-CSF, also known as colony-stimulating factor-1 (CSF-1), was the first colony-stimulating factor to be purified [14]. It was formerly known as macrophage growth factor (MGF) or macrophage and granulocyte inducer IM (MGI-IM) [14]. M-CSF is a homodimeric glycoprotein synthesized constitutively by bone marrow stromal cells and osteoblasts [14-16]. It induces proliferation and survival of the common precursors of osteoclasts and macrophages. Additionally, M-CSF signaling stimulates RANK expression in osteoclast precursors, rendering them more responsive to RANKL [16-18]. The pivotal role of M-CSF in osteoclastogenesis has been confirmed by studies of osteopetrotic (*op/op*) mice. These mice harbor a null mutation in the coding region of M-CSF gene, which leads to the production of a truncated protein. The biologically inactive M-CSF leads to a severe deficiency in osteoclast formation and hence osteopetrotic phenotypes. Moreover, many of the effects of the *op/op* mutation can be rescued by the administration of soluble M-CSF to neonatal mice [19].

The biological effects of M-CSF are mediated via its sole receptor, c-fms, which is also referred to as CSF-1R or M-CSFR [14-16]. The transcription factor PU.1 binds to the

promoter region of *c-fms* gene and positively regulates its transcription [15]. Mice deficient in PU.1 exhibit an osteopetrotic phenotype similar to the *op/op* mice [9]. c-fms is a member of the class III receptor tyrosine kinase family and is encoded by the proto-oncogene *c-fms* (also known as *csf1r*). It is an integral plasma membrane glycoprotein, expressed primarily on macrophages and osteoclasts [14-16]. The functional linkage between M-CSF and c-fms has been established by the observation that mice lacking *c-fms* gene exhibit the same major osteopetrotic phenotypes as *op/op* mice [16, 18].

The binding of M-CSF to c-fms results in the dimerization and the auto-phosphorylation of c-fms. This leads to the recruitment of c-Src kinase, which then phosphorylates the adaptor protein, c-Cbl. c-Cbl recruits a multiprotein complex containing growth-factor-receptor-bound protein 2 (Grb2) and phosphoinositide 3-kinase (PI3K), with the former activating extracellular-signal-regulated kinase (ERK) and the latter the serine/threonine kinase AKT (or protein kinase B, PKB) (Figure 2.2.) [16, 18, 20]. Collectively, M-CSF induces the proliferation of osteoclast precursor cells and supports their survival through PI3K /AKT and Grb2/ERK axis.

2.2.2. Role of RANKL in Osteoclast Differentiation

RANKL, a type II (carboxy-terminal outside the cell) membrane protein, belongs to the tumor necrosis factor (TNF) superfamily [21-24]. It is also known by the following names: TNF-related activation-induced cytokine (TRANCE), osteoprotegerin ligand (OPGL), tumor necrosis factor (ligand) superfamily member 11 (Tnfsf11), and osteoclast differentiation factor (ODF) [24, 25]. RANKL is extensively expressed on the plasma membrane of osteoblasts/stromal cells [24, 25]. It has been shown to be proteolytically

released from the cell surface by the metalloproteasedisintegrin TNF α convertase (TACE) [26, 27]. In the presence of M-CSF, both membrane-bound and soluble RANKL can support osteoclast differentiation *in vitro* [25]. However, the membrane-bound may be more efficient than the soluble form [26]. The *in vivo* biological and pathological significance of soluble RANKL still remains unclear. The importance of RANKL in osteoclast formation has been collaborated by animal studies. RANKL−/− mice display complete failure of osteoclastogenesis and consequently develop severe osteopetrosis [24, 28].

The receptor for RANKL—RANK (receptor activator of NFκB)—is a type I membrane protein and belongs to the TNF receptor superfamily [25]. RANK is also known as TRANCE-R or TNFRSF11A, and is expressed as a transmembrane heterotrimer on the surface of osteoclasts and their precursors [24, 25]. Mice lacking *rank* have exactly the same osteopetrotic phenotype as *rankl*−/−mice. However, *rank*−/− mice have an intrinsic defect in osteoclasts as indicated by the fact that their phenotype can be reversed by bone marrow transplantation from normal mice [25, 29].

When RANK on osteoclasts is recognized by RANKL, it sends signals into the cells through adapter proteins (Figure 2.2.). Like other TNF receptor superfamily member proteins, the intracellular domain of RANK directly binds to TRAF6 (TNF receptor associated factor 6), which undergoes trimerization and then activates the NF-κB, Akt, and mitogen-activated protein kinases (MAPKs) pathways (Figure 2.2.) [24, 25, 30]. Meanwhile, RANKL induces the expression of c-Fos, which, in turn, activates the transcription factor complex, AP1 (activator protein 1) [31-33]. The common target gene of NF-κB and AP-1 has been recently shown to be NFATc1 (nuclear factor of activated T cells, cytoplasmic 1) [31, 34, 35]. NFATc1, a member of the NFAT family of transcription factor genes, is considered

to be the master transcription factor for osteoclastogenesis since its induction activates the expression of osteoclast-specific genes [32, 33, 36]. Genetics studies have shown that osteopetrosis occurs in mice lacking key genes involved in RANKL signaling, such as TRAP6 and c-Fos [37-39].

2.2.3. Costimulatory Signals for RANK

Since authentic osteoclasts can be generated *in vitro* from bone marrow cells in response to recombinant RANKL and M-CSF, it has been generally accepted that RANKL and M-CSF signaling is not only necessary but also sufficient to support osteoclastogenesis [40, 41]. However, recent studies have revealed a more complex picture: additional costimulatory signals are required to activate the transcription factor, NFATc1 [32, 33, 36, 42].

Through interaction between complementarily charged amino acid residues, transmembrane adaptor proteins (e.g. FcRγ and DAP12) associate with immunoglobulin-like receptors (e.g. OSCAR, PIR-A, TREM-2, and SIRPβ1) [32, 33, 36]. Following the stimulation of immunoglobulin-like receptors likely by endogenous ligands from osteoclast precursors or stromal cells/osteoblasts and RANKL-initiated signaling, the ITAM motifs present in those adaptor proteins are phosphorylated. This leads to the recruitment of the Syk family kinase to the phosphorylated tyrosine residues [32]. Consequently, the calcium signaling pathway is activated through phospholipase Cγ (PLCγ), followed by induction of NFATc1 (Figure 2.3.) [32, 33, 36]. Moreover, DAP12−/−FcRγ−/− mice exhibit severe osteopetrosis due to defective osteoclast differentiation, and the retroviral transfer of normal

DAP12 into DAP12−/−FcRγ−/− cells can rescue osteoclast differentiation deficiency [33]. These data indicate that ITAM signaling is indispensable for RANKL-induced osteoclastogenesis. However, so far it still remains largely unknown how RANKL/RANK signaling synergizes with ITAM signaling.

2.3. Regulation of Osteoclast Differentiation

Osteoclast differentiation is subject to negative and positive regulation by circulating hormones and locally produced cytokines [4, 43]. Major mechanisms underlying the regulation of osteoclast differentiation are discussed in the following sections.

2.3.1. The OPG/RANKL/RANK Regulatory Axis

OPG (osteoprotegerin, i.e. protector of bone), also known as osteoclastogenesis inhibitory factor (OCIF), is a secreted protein expressed by osteoblasts/stromal cells [44-46]. OPG, like RANK, belongs to the TNF receptor superfamily. However, in contrast to all other members, OPG lacks transmembrane and cytoplasmic domains and is secreted as a soluble protein. OPG functions as a soluble decoy receptor to RANKL, competing with RANK for RANKL binding (Figure 2.4.) [41, 46, 47]. OPG is a negatively regulator of RANKL activity. Genetic studies have found that excessive RANKL activity, as in OPG deficient mice, results in early-onset osteoporosis [48, 49]. In contrast, due to a decreased net RANKL level, transgenic mice overexpressing OPG exhibit osteopetrosis [46].

Studies have shown that the balance between RANKL/RANK and OPG levels regulate osteoclast differentiation and bone metabolism [46, 50]. Most hormones and cytokines inhibit or enhance bone resorption via osteoblasts/stromal cells, regulating their

expression of RANKL and OPG (see reference [46] for a thorough list) [4, 43, 46, 47]. For example, TGF-β released from bone matrix during bone resorption has been shown to upregulate OPG expression, serving as a negative feedback mechanism for bone resorption [24, 26]. Interleukins (IL-1, 6, 11, and 17) can increase RANKL expression [24, 25, 46]. Some factors not only decrease OPG expression but also increase RANKL expression, such as parathyroid hormone (PTH), parathyroid hormone-related protein (PTHrP), and prostaglandin E_2 (PGE_2) [25, 46]. Dexamethasone and 25-dihydroxy vitamin D_3 (1, $25(OH)_2D_3$, the active form of vitamin D3) can stimulate RANKL production and are often used in coculture of bone marrow cells and stromal cells to generate osteoclasts *in vitro* [25, 46]. Therefore, the OPG/RANKL/RANK axis is critical for maintaining the balance between bone formation and resorption by providing a means for controlling osteoblast and osteoclast activity (Figure 2.4.).

2.3.2. Other Regulatory Mechanisms

In addition to the OPG/RANKL/RANK regulatory system, recent studies have shown that other local molecules produced by osteoblasts/stromal cells and immune cells can enhance or inhibit osteoclast formation [51, 52]. Secreted frizzled-related protein 1 (sFRP1), an inhibitor of the Wnt signaling pathway, is expressed by osteoblasts/stromal cells [51], and directly inhibits osteoclast formation by binding to RANKL *in vitro* [53]. Osteoclast inhibitory lectin (OCIL) is a membrane-bound lectin expressed by osteoblasts, and its soluble form can inhibit osteoclast formation *in vitro* [54]. Aside from osteoblasts/stromal cells, other cell types are also involved in osteoclastogenesis regulation [50, 55]. *In vitro* studies have suggested that through an unknown mechanism, megakaryocytes can inhibit osteoclast

formation [56]. Taken together, more studies, especially *in vivo* ones, are necessary to further explore other mechanisms that regulate osteoclast differentiation.

2.4. Osteoclast Fusion

During osteoclastogenesis, preosteoclasts fuse to form multinuclear mature osteoclasts. Studies comparing the gene expression of multinuclear osteoclasts and mononuclear macrophages have identified two molecules critically involved in cell–cell fusion of osteoclasts: the dendritic cell-specific transmembrane protein (DC-STAMP) [57, 58] and the d2 isoform of vacuolar H^+-ATPase V_0 domain (Atp6v0d2) [59]. Mice deficient in either DC-STAMP or Atp6v0d2 lack multinuclear osteoclasts and develop osteopetrosis [57-59]. Further studies have demonstrated that the expression of DC-STAMP and Atp6v0d2 are induced by transcription factor, NFATc1 [60, 61]. Interestingly, a recent study has suggested a newly-identified protein induced by RANKL, osteoclast stimulatory transmembrane protein (OC-STAMP), is required in the fusion process during osteoclastogenesis [62]. It appears that multiple factors are involved in osteoclast fusion, and more studies are necessary to further understand this process.

2.5. Osteoclasts in Periprosthetic Osteolysis and Peri-implantitis

Studies have shown that osteoclasts play a key role in periprosthetic osteolysis, one of the major complications of orthopaedic replacement [63]. Particulate wear debris generated by bearing surfaces has been identified as a major contributory component in osteolysis [64-67]. However, the mechanism by which particles induce cellular response and

subsequent osteolysis is largely unclear. The primary cellular response to implants is the phagocytosis of foreign particles, which somehow activates macrophages to release numerous pro-inflammatory cytokines, including TNFα, IL-1α, IL-1β, IL-6, and PGE_2 [64, 68, 69]. These cytokines may explain, at least partially, the increased levels of RANKL and RANK in the interface tissue in loosening orthopaedic replacement, leading to increased osteoclastogenesis and bone resorption [64, 70, 71]. In addition to the RANK/RANKL signaling pathway, some cytokines, especially TNFα, may enhance osteoclast differentiation directly [70].

In addition to the involvement of the macrophage-mediated foreign body immune response to particles, T lymphocytes may also play an important role in periprosthetic osteolysis [72, 73]. Immunohistochemical studies have found a large number of T cells in osteolytic tissues [73]. The interplay of immune system and bone has been well established. Activated T cells are well known to produce RANKL and thus directly support osteoclastogenesis [52, 74]. T cells can also produce other osteoclastogenic factors, further contributing to the process of the bone destruction in inflammatory conditions such as rheumatoid arthritis [75]. Whether T cells in osteolytic tissues can secrete other cytokines to influence the RANKL/RANK/OPG system remained to be defined. Collectively, excessive osteoclastogenesis and bone resorption caused by the imbalance of RANKL/RANK/OPG axis plays an important role in aseptic loosening [64, 70, 71].

Similarly, osteoclasts are also involved in the interface of bone and dental implants [76]. It has been proposed that dental implants can induce production of osteoclastogenic cytokines such as TNFα and IL-1β in peri-implant tissue, resulting in implant failing [76, 77]. Immunohistochemical studies have shown that the expression of TNFα, IL-1α, and IL-6 is

significantly increased in peri-implant tissue from loosened implants [77]. In order to develop diagnostic and treating strategies for peri-implantitis, effort has been made to determine the levels of several proteins associated with bone resorption in the implant crevicular fluid from patients with failing dental implants. These proteins include PGE_2, soluble RANKL, cathepsin K, and OPG [77-80]. Theses data implicate an important role of osteoclasts in the bone loss during peri-implantitis. Additionally, immunohistochemical studies have found that T cells are the most prominent cell in peri-implantitis tissue [81], suggesting a role of T cells in the pathogenesis of peri-implantitis. Although the involvement of T cells in inflammatory conditions such as rheumatoid arthritis has been established [75], little is known how T cells contribute to peri-implantitis. It appears that excessive bone loss caused by the imbalance of RANKL/RANK/OPG axis plays an important role in peri-implantitis; however, more research is needed to better understand the mechanism of osteoclast activation and bone resorption in peri-implantitis

2.6. Osteoclasts in Orthodontic Tooth Movement

Orthodontic treatment induces bone resorption on the pressure side of a tooth and bone formation on the tension side. Interestingly, ankylosed teeth, which lack periodontal ligament (PDL), cannot be moved by orthodontic treatment. PDL cells are considered to play a pivotal role during orthodontic tooth movement [82]. Indeed, it has been shown that PDL cells express both RANKL and OPG, and can support osteoclastogenesis *in vitro* [82]. Under mechanical stress, PDL cells can produce larger amount of RANKL [83, 84]. In addition, during orthodontic tooth movement, RANKL level in gingival crevicular fluid is increased, while OPG level is decreased [85, 86]. Consistently, local OPG gene transfer to periodontal

tissue can decrease orthodontic tooth movement [87], while local RANKL gene transfer to the periodontal tissue can increase orthodontic tooth movement [88]. Meanwhile, local administration of OPG can inhibit orthodontic tooth movement [89]. Collectively, these data indicate the pivotal role of OPG/RANKL/RANK regulatory axis in PDL cells during orthodontic tooth movement.

Attempts have also been made to address the mechanism underlying the increased production of RANKL by PDL cells in response to orthodontic force. Intriguingly, a report has suggested that mechanical stress can induce cyclo-oxygenase 2 (COX-2) mRNA expression and PGE_2 synthesis in PDL cells, which stimulate RANKL expression via an atuocrine mechanism [90]. This piece of evidence is in line with the hypothesis that hypoxia at the pressure side of PDL plays an important role in inducing bone resorption [91]. Early studies have shown that during tooth movement, orthodontic trauma can cause vascular changes and hypoxia on the pressure side of PDL, leading to increased production of PGE_2 [91]. *In vitro* studies have further indicated hypoxia as an important stimulator of osteoclast formation and bone resorption [92-94]. Moreover, recent studies have shown that under hypoxia conditions, PDL cells can produce larger amount of osteoclastogenic cytokines, including IL-6, IL-1β, TNFα, and PGE_2 [95]. This is consistent with *in vivo* findings that these cytokines are elevated in gingival crevicular fluid during orthodontic tooth movement (reviewed in reference [96]). It is likely that these osteoclastogenic cytokines further contribute to the increased osteoclastogenesis by acting on the OPG/RANKL/RANK regulatory axis. The important role of TNFα in orthodontic tooth movement is further confirmed by genetics studies. Mice lacking the two receptors of both of these receptors TNFα (TNFR1 and TNFR1) showed reduced orthodontic tooth movement [97].

Therefore, hypoxia at the pressure side of a tooth undergoing orthodontic tooth movement leads to bone resorption by directly or indirectly disrupting the balance of the OPG/RANKL/RANK axis in PDL.

2.7. Chapter Summary

Osteoclasts are large multinucleated cells present only in bone. They are derived from hematopoietic cells. Their formation is a complex and multi-step process, in which osteoblasts/stromal cells play a central role by providing two essential factors: M-CSF and RANKL. M-CSF induces the proliferation and survival of osteoclast precursors from their hematopoietic progenitors, while RANKL stimulates these precursor cells to commit to the osteoclast phenotype. Interestingly, osteoblasts/stromal cells can produce an inhibitory factor for osteoclastogenesis, OPG, which competes with RANK for RANKL binding. Hormones and local factors can exert their effects on bone resorption by regulating OPG and RANKL expression in osteoblasts/stromal cells; therefore, the OPG/RANKL/RANK axis represents a major means for regulating osteoblast to osteoclast activity and maintaining the balance between bone formation and resorption. Osteoclast differentiation also plays an important role in periprosthetic osteolysis, peri-implantitis, and orthodontic tooth movement.

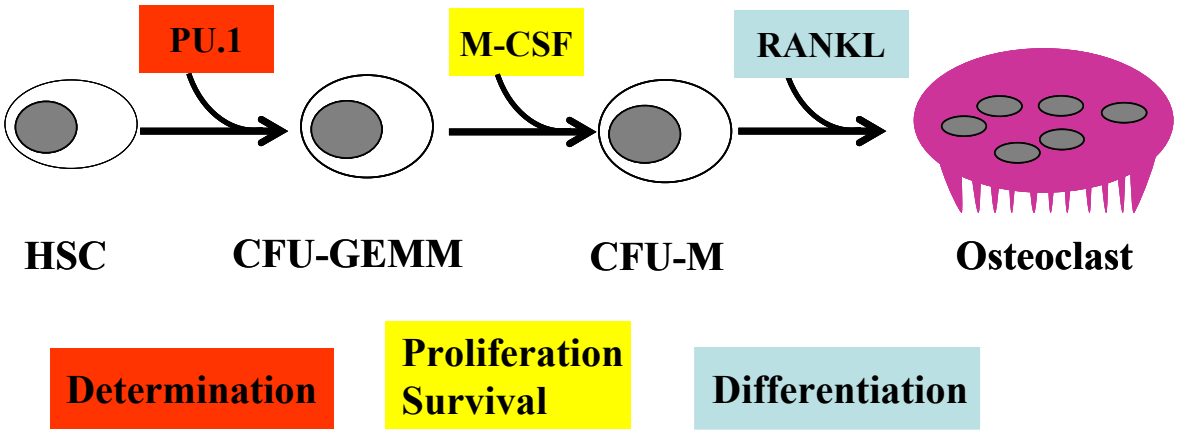

Figure 2.1. Osteoclastogenesis. Osteoclastogenesis is a multi-step process regulated by a sequential series of molecular event. Transcriptional factor PU.1 is critical for the determination of hematopoietic stem cells (HSCs) to the myeloid lineage (CFU-GEMM), and can also upreguate the expression of M-CSF receptor, c-fms. The binding of M-CSF to c-fms ensures the proliferation and survival of monocyte/macrophage lineage precursors (CFU-M) from CFU-GEMM. In addition, M-CSF can increase the expression of RANKL receptor, RANK. RANKL and RANK binding initiates signaling pathways required for CFU-M to assume the osteoclast phenotype.

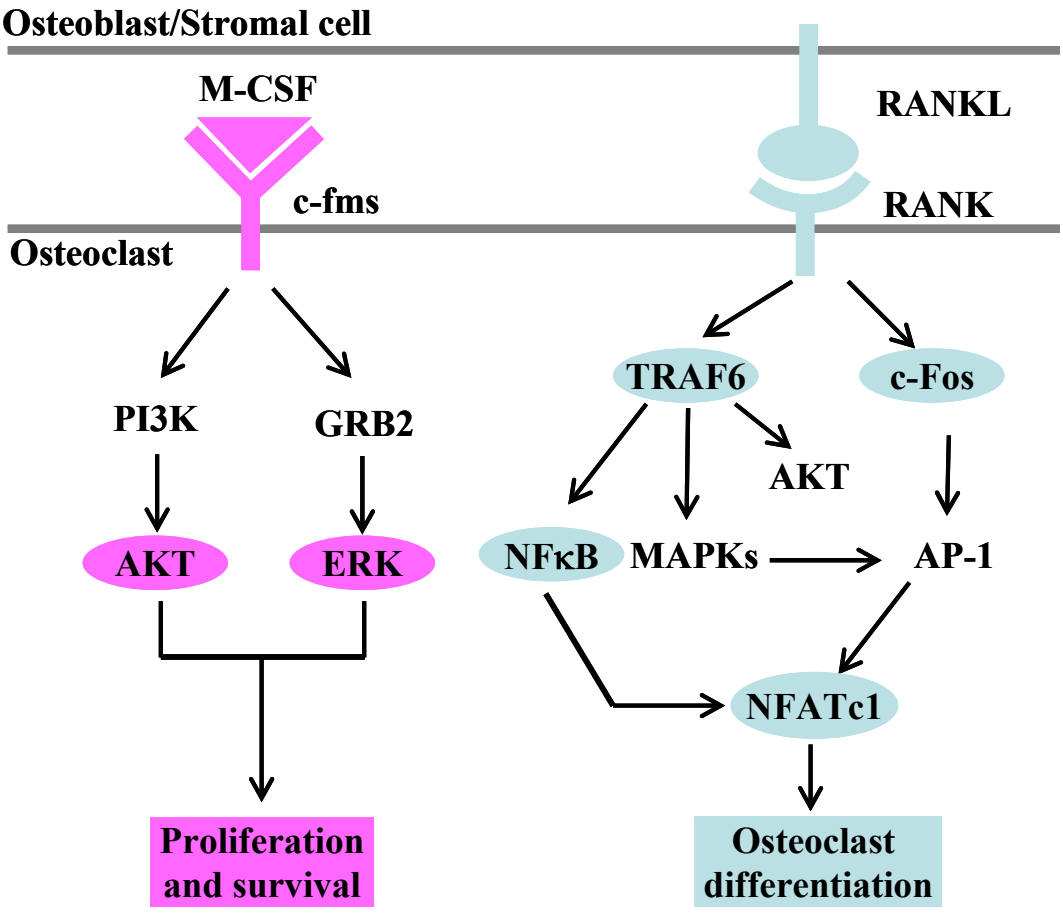

Figure 2.2. M-CSF/RANKL signaling pathways. M-CSF, which is released from osteoblasts/stromal cells, binds to its receptor on osteoclast precursors, c-fms. This leads to the activation of AKT and ERK pathways through PI3K and GRB2, respectively. These signals ensure the survival and proliferation of osteoclast precursors. RANKL on osteoblasts/stromal cells recognizes its receptor on osteoclast precursors, RANK, which then recruits TRAF6. As a result, NF-κB, Akt, and MAPKs pathways are activated. Additionally, RANKL induces the expression of c-Fos, which then activates AP1. The common target gene of both NF-κB and AP-1 is NFATc1, whose induction activates the expression of osteoclast specific genes. Adapted from references 32 and 33.

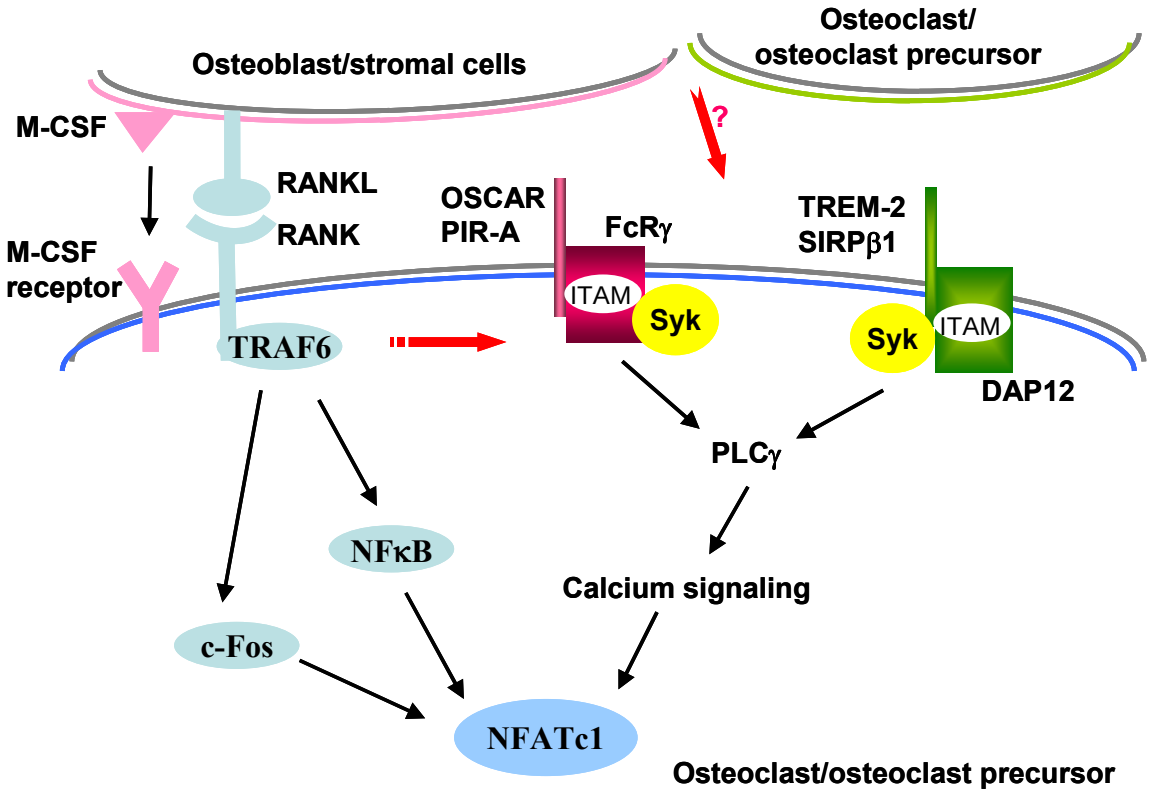

Figure 2.3. Cooperation of RANKL and ITAM induced signals in osteoclastogenesis. Osteoblasts/stromal cells secrete M-CSF, which binds to its receptor on osteoclast precursor cells to ensure proliferation and survival of osteoclast precursor cells. Osteoblasts/stromal cells also express RANKL, which recognizes RANK on osteoclast precursor cells. RANK then binds to TRAF 6, which, in turn, activates NFATc1 through NFκB and c-Fos. In addition, transmembrane adapter proteins, FcRγ and DAP12, associate with immunoglobulin-like receptors, OSCAR, PIR-A, TREM-2 and SIRPβ1. RANKL/RANK signaling (red arrow) and stimulation of the immunoglobulin-like receptors possibly by endogenous signals from osteoblastic cells or osteoclastic cells (red arrow with question mark) collaboratively phosphorylate ITAM motifs present in those adaptor proteins, and then recruit the Syk family kinase to the phosphorylated tyrosine residues. Finally, through phospholipase Cγ (PLCγ), the calcium signaling pathway is activated, leading to NFATc1 induction. Adapted from reference 33.

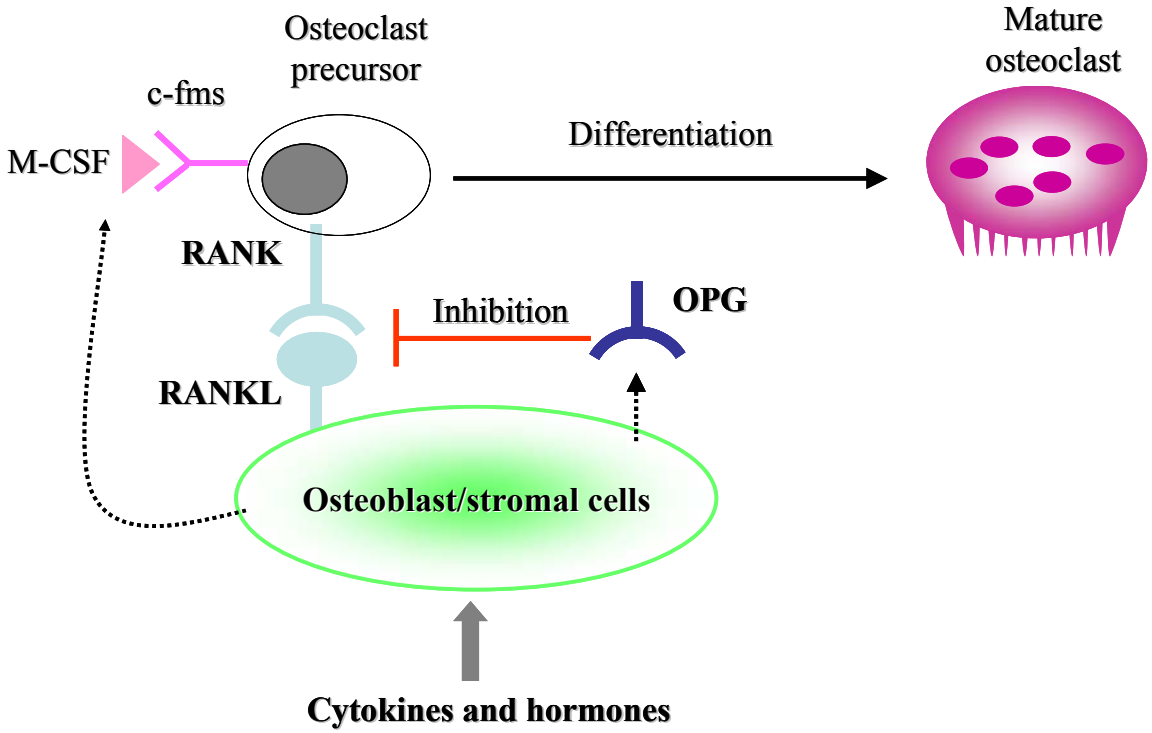

Figure 2.4. RANKL-dependent regulation of osteoclast formation. M-CSF secreted by osteoblasts/stromal cells can drive myeloid progenitors towards osteoclast precursors. RANKL on osteoblasts/stromal cells binds to its receptor RANK on osteoclast precursors and thus initiate a signal transduction cascade, leading to osteoclast differentiation. Intriguingly, osteoblast/stromal cells also produce a decoy receptor for RANKL, OPG, which can block RANK and RANKL binding. This crosstalk mechanism is also an endpoint for the actions of several hormones and cytokines, such as estrogen, interleukins, prostaglandin E_2 (PGE_2), TGF-β and parathyroid hormone (PTH). Adapted from reference 25.

Chapter II. Reference List

[1] Vaananen K. Mechanism of osteoclast mediated bone resorption--rationale for the design of new therapeutics. Adv Drug Deliv Rev 2005; 57: 959-971.

[2] Suda T, Takahashi N and Martin TJ. Modulation of osteoclast differentiation. Endocr Rev 1992; 13: 66-80.

[3] Miyamoto T and Suda T. Differentiation and function of osteoclasts. Keio J Med 2003; 52: 1-7.

[4] Teitelbaum SL. Osteoclasts: what do they do and how do they do it? Am J Pathol 2007; 170: 427-435.

[5] Udagawa N, Takahashi N, Akatsu T*, et al*. Origin of osteoclasts: mature monocytes and macrophages are capable of differentiating into osteoclasts under a suitable microenvironment prepared by bone marrow-derived stromal cells. Proc Natl Acad Sci U S A 1990; 87: 7260-7264.

[6] Miyamoto T, Ohneda O, Arai F*, et al*. Bifurcation of osteoclasts and dendritic cells from common progenitors. Blood 2001; 98: 2544-2554.

[7] Arai F, Miyamoto T, Ohneda O*, et al*. Commitment and differentiation of osteoclast precursor cells by the sequential expression of c-Fms and receptor activator of nuclear factor kappaB (RANK) receptors. J Exp Med 1999; 190: 1741-1754.

[8] Scott EW, Simon MC, Anastasi J and Singh H. Requirement of transcription factor PU.1 in the development of multiple hematopoietic lineages. Science 1994; 265: 1573-1577.

[9] Tondravi MM, McKercher SR, Anderson K*, et al*. Osteopetrosis in mice lacking haematopoietic transcription factor PU.1. Nature 1997; 386: 81-84.

[10] Suda T, Takahashi N, Udagawa N, Jimi E, Gillespie MT and Martin TJ. Modulation of osteoclast differentiation and function by the new members of the tumor necrosis factor receptor and ligand families. Endocr Rev 1999; 20: 345-357.

[11] Menaa C, Kurihara N and Roodman GD. CFU-GM-derived cells form osteoclasts at a very high efficiency. Biochem Biophys Res Commun 2000; 267: 943-946.

[12] Hodge JM, Kirkland MA, Aitken CJ*, et al*. Osteoclastic potential of human CFU-GM: biphasic effect of GM-CSF. J Bone Miner Res 2004; 19: 190-199.

[13] Lerner UH. Osteoclast formation and resorption. Matrix Biol 2000; 19: 107-120.

[14] Barreda DR, Hanington PC and Belosevic M. Regulation of myeloid development and function by colony stimulating factors. Dev Comp Immunol 2004; 28: 509-554.

[15] Hume DA, Yue X, Ross IL, Favot P, Lichanska A and Ostrowski MC. Regulation of CSF-1 receptor expression. Mol Reprod Dev 1997; 46: 46-52; discussion 52-3.

[16] Pixley FJ and Stanley ER. CSF-1 regulation of the wandering macrophage: complexity in action. Trends Cell Biol 2004; 14: 628-638.

[17] Cappellen D, Luong-Nguyen NH, Bongiovanni S, Grenet O, Wanke C and Susa M. Transcriptional program of mouse osteoclast differentiation governed by the macrophage colony-stimulating factor and the ligand for the receptor activator of NFkappa B. J Biol Chem 2002; 277: 21971-21982.

[18] Ross FP and Teitelbaum SL. alphavbeta3 and macrophage colony-stimulating factor: partners in osteoclast biology. Immunol Rev 2005; 208: 88-105.

[19] Yoshida H, Hayashi S, Kunisada T*, et al*. The murine mutation osteopetrosis is in the coding region of the macrophage colony stimulating factor gene. Nature 1990; 345: 442-444.

[20] Ross FP. M-CSF, c-Fms, and signaling in osteoclasts and their precursors. Ann N Y Acad Sci 2006; 1068: 110-116.

[21] Lacey DL, Timms E, Tan HL*, et al*. Osteoprotegerin ligand is a cytokine that regulates osteoclast differentiation and activation. Cell 1998; 93: 165-176.

[22] Yasuda H, Shima N, Nakagawa N*, et al*. Osteoclast differentiation factor is a ligand for osteoprotegerin/osteoclastogenesis-inhibitory factor and is identical to TRANCE/RANKL. Proc Natl Acad Sci U S A 1998; 95: 3597-3602.

[23] Kartsogiannis V, Zhou H, Horwood NJ*, et al*. Localization of RANKL (receptor activator of NF kappa B ligand) mRNA and protein in skeletal and extraskeletal tissues. Bone 1999; 25: 525-534.

[24] Jones DH, Kong YY and Penninger JM. Role of RANKL and RANK in bone loss and arthritis. Ann Rheum Dis 2002; 61 Suppl 2: ii32-9.

[25] Theill LE, Boyle WJ and Penninger JM. RANK-L and RANK: T cells, bone loss, and mammalian evolution. Annu Rev Immunol 2002; 20: 795-823.

[26] Nakashima T, Kobayashi Y, Yamasaki S*, et al*. Protein expression and functional difference of membrane-bound and soluble receptor activator of NF-kappaB ligand: modulation of the expression by osteotropic factors and cytokines. Biochem Biophys Res Commun 2000; 275: 768-775.

[27] Lum L, Wong BR, Josien R*, et al*. Evidence for a role of a tumor necrosis factor-alpha (TNF-alpha)-converting enzyme-like protease in shedding of TRANCE, a TNF family

member involved in osteoclastogenesis and dendritic cell survival. J Biol Chem 1999; 274: 13613-13618.

[28] Kim N, Odgren PR, Kim DK, Marks SC,Jr and Choi Y. Diverse roles of the tumor necrosis factor family member TRANCE in skeletal physiology revealed by TRANCE deficiency and partial rescue by a lymphocyte-expressed TRANCE transgene. Proc Natl Acad Sci U S A 2000; 97: 10905-10910.

[29] Li J, Sarosi I, Yan XQ*, et al*. RANK is the intrinsic hematopoietic cell surface receptor that controls osteoclastogenesis and regulation of bone mass and calcium metabolism. Proc Natl Acad Sci U S A 2000; 97: 1566-1571.

[30] Xing L, Bushnell TP, Carlson L*, et al*. NF-kappaB p50 and p52 expression is not required for RANK-expressing osteoclast progenitor formation but is essential for RANK- and cytokine-mediated osteoclastogenesis. J Bone Miner Res 2002; 17: 1200-1210.

[31] Yamashita T, Yao Z, Li F*, et al*. NF-kB p50 and p52 regulate RANKL and TNF-induced osteoclast precursor differentiation by activating c-Fos and NFATc1. J Biol Chem 2007.

[32] Takayanagi H. Mechanistic insight into osteoclast differentiation in osteoimmunology. J Mol Med 2005; 83: 170-179.

[33] Asagiri M and Takayanagi H. The molecular understanding of osteoclast differentiation. Bone 2007; 40: 251-264.

[34] Winslow MM, Pan M, Starbuck M*, et al*. Calcineurin/NFAT signaling in osteoblasts regulates bone mass. Dev Cell 2006; 10: 771-782.

[35] Gohda J, Akiyama T, Koga T, Takayanagi H, Tanaka S and Inoue J. RANK-mediated amplification of TRAF6 signaling leads to NFATc1 induction during osteoclastogenesis. EMBO J 2005; 24: 790-799.

[36] Asagiri M, Sato K, Usami T*, et al*. Autoamplification of NFATc1 expression determines its essential role in bone homeostasis. J Exp Med 2005; 202: 1261-1269.

[37] Lomaga MA, Yeh WC, Sarosi I*, et al*. TRAF6 deficiency results in osteopetrosis and defective interleukin-1, CD40, and LPS signaling. Genes Dev 1999; 13: 1015-1024.

[38] Johnson RS, Spiegelman BM and Papaioannou V. Pleiotropic effects of a null mutation in the c-fos proto-oncogene. Cell 1992; 71: 577-586.

[39] Wang ZQ, Ovitt C, Grigoriadis AE, Mohle-Steinlein U, Ruther U and Wagner EF. Bone and haematopoietic defects in mice lacking c-fos. Nature 1992; 360: 741-745.

[40] Teitelbaum SL. Bone resorption by osteoclasts. Science 2000; 289: 1504-1508.

[41] Feige U. Osteoprotegerin. Ann Rheum Dis 2001; 60 Suppl 3: iii81-4.

[42] Sato K, Suematsu A, Nakashima T*, et al*. Regulation of osteoclast differentiation and function by the CaMK-CREB pathway. Nat Med 2006; 12: 1410-1416.

[43] Bruzzaniti A and Baron R. Molecular regulation of osteoclast activity. Rev Endocr Metab Disord 2006; 7: 123-139.

[44] Simonet WS, Lacey DL, Dunstan CR*, et al*. Osteoprotegerin: a novel secreted protein involved in the regulation of bone density. Cell 1997; 89: 309-319.

[45] Yasuda H, Shima N, Nakagawa N*, et al*. Identity of osteoclastogenesis inhibitory factor (OCIF) and osteoprotegerin (OPG): a mechanism by which OPG/OCIF inhibits osteoclastogenesis in vitro. Endocrinology 1998; 139: 1329-1337.

[46] Theoleyre S, Wittrant Y, Tat SK, Fortun Y, Redini F and Heymann D. The molecular triad OPG/RANK/RANKL: involvement in the orchestration of pathophysiological bone remodeling. Cytokine Growth Factor Rev 2004; 15: 457-475.

[47] Khosla S. Minireview: the OPG/RANKL/RANK system. Endocrinology 2001; 142: 5050-5055.

[48] Bucay N, Sarosi I, Dunstan CR*, et al*. osteoprotegerin-deficient mice develop early onset osteoporosis and arterial calcification. Genes Dev 1998; 12: 1260-1268.

[49] Mizuno A, Amizuka N, Irie K*, et al*. Severe osteoporosis in mice lacking osteoclastogenesis inhibitory factor/osteoprotegerin. Biochem Biophys Res Commun 1998; 247: 610-615.

[50] Blair JM, Zhou H, Seibel MJ and Dunstan CR. Mechanisms of disease: roles of OPG, RANKL and RANK in the pathophysiology of skeletal metastasis. Nat Clin Pract Oncol 2006; 3: 41-49.

[51] Cohen MM,Jr. The new bone biology: pathologic, molecular, and clinical correlates. Am J Med Genet A 2006; 140: 2646-2706.

[52] Takayanagi H. Osteoimmunology: shared mechanisms and crosstalk between the immune and bone systems. Nat Rev Immunol 2007; 7: 292-304.

[53] Hausler KD, Horwood NJ, Chuman Y*, et al*. Secreted frizzled-related protein-1 inhibits RANKL-dependent osteoclast formation. J Bone Miner Res 2004; 19: 1873-1881.

[54] Hu YS, Zhou H, Myers D*, et al*. Isolation of a human homolog of osteoclast inhibitory lectin that inhibits the formation and function of osteoclasts. J Bone Miner Res 2004; 19: 89-99.

[55] Compston JE. Bone marrow and bone: a functional unit. J Endocrinol 2002; 173: 387-394.

[56] Kacena MA, Nelson T, Clough ME*, et al*. Megakaryocyte-mediated inhibition of osteoclast development. Bone 2006; 39: 991-999.

[57] Kukita T, Wada N, Kukita A*, et al*. RANKL-induced DC-STAMP is essential for osteoclastogenesis. J Exp Med 2004; 200: 941-946.

[58] Yagi M, Miyamoto T, Sawatani Y*, et al*. DC-STAMP is essential for cell-cell fusion in osteoclasts and foreign body giant cells. J Exp Med 2005; 202: 345-351.

[59] Lee SH, Rho J, Jeong D*, et al*. v-ATPase V0 subunit d2-deficient mice exhibit impaired osteoclast fusion and increased bone formation. Nat Med 2006; 12: 1403-1409.

[60] Yagi M, Ninomiya K, Fujita N*, et al*. Induction of DC-STAMP by alternative activation and downstream signaling mechanisms. J Bone Miner Res 2007; 22: 992-1001.

[61] Kim K, Lee SH, Ha Kim J, Choi Y and Kim N. NFATc1 induces osteoclast fusion via up-regulation of Atp6v0d2 and the dendritic cell-specific transmembrane protein (DC-STAMP). Mol Endocrinol 2008; 22: 176-185.

[62] Yang M, Birnbaum MJ, MacKay CA, Mason-Savas A, Thompson B and Odgren PR. Osteoclast stimulatory transmembrane protein (OC-STAMP), a novel protein induced by RANKL that promotes osteoclast differentiation. J Cell Physiol 2008; 215: 497-505.

[63] Abu-Amer Y, Darwech I and Clohisy JC. Aseptic loosening of total joint replacements: mechanisms underlying osteolysis and potential therapies. Arthritis Res Ther 2007; 9 Suppl 1: S6.

[64] Gallo J, Kaminek P, Ticha V, Rihakova P and Ditmar R. Particle disease. A comprehensive theory of periprosthetic osteolysis: a review. Biomed Pap Med Fac Univ Palacky Olomouc Czech Repub 2002; 146: 21-28.

[65] Sabokbar A, Pandey R and Athanasou NA. The effect of particle size and electrical charge on macrophage-osteoclast differentiation and bone resorption. J Mater Sci Mater Med 2003; 14: 731-738.

[66] Sabokbar A, Pandey R, Diaz J, Quinn JM, Murray DW and Athanasou NA. Hydroxyapatite particles are capable of inducing osteoclast formation. J Mater Sci Mater Med 2001; 12: 659-664.

[67] Sabokbar A, Pandey R, Quinn JM and Athanasou NA. Osteoclastic differentiation by mononuclear phagocytes containing biomaterial particles. Arch Orthop Trauma Surg 1998; 117: 136-140.

[68] Merkel KD, Erdmann JM, McHugh KP, Abu-Amer Y, Ross FP and Teitelbaum SL. Tumor necrosis factor-alpha mediates orthopedic implant osteolysis. Am J Pathol 1999; 154: 203-210.

[69] Neale SD, Sabokbar A, Howie DW, Murray DW and Athanasou NA. Macrophage colony-stimulating factor and interleukin-6 release by periprosthetic cells stimulates osteoclast formation and bone resorption. J Orthop Res 1999; 17: 686-694.

[70] Sabokbar A, Kudo O and Athanasou NA. Two distinct cellular mechanisms of osteoclast formation and bone resorption in periprosthetic osteolysis. J Orthop Res 2003; 21: 73-80.

[71] Mandelin J, Li TF, Liljestrom M*, et al*. Imbalance of RANKL/RANK/OPG system in interface tissue in loosening of total hip replacement. J Bone Joint Surg Br 2003; 85: 1196-1201.

[72] Goodman SB. Wear particles, periprosthetic osteolysis and the immune system. Biomaterials 2007; 28: 5044-5048.

[73] Arora A, Song Y, Chun L*, et al*. The role of the TH1 and TH2 immune responses in loosening and osteolysis of cemented total hip replacements. J Biomed Mater Res A 2003; 64: 693-697.

[74] Sato K and Takayanagi H. Osteoclasts, rheumatoid arthritis, and osteoimmunology. Curr Opin Rheumatol 2006; 18: 419-426.

[75] Sato K, Suematsu A, Okamoto K*, et al*. Th17 functions as an osteoclastogenic helper T cell subset that links T cell activation and bone destruction. J Exp Med 2006; 203: 2673-2682.

[76] Minkin C and Marinho VC. Role of the osteoclast at the bone-implant interface. Adv Dent Res 1999; 13: 49-56.

[77] Konttinen YT, Lappalainen R, Laine P, Kitti U, Santavirta S and Teronen O. Immunohistochemical evaluation of inflammatory mediators in failing implants. Int J Periodontics Restorative Dent 2006; 26: 135-141.

[78] Strbac GD, Monov G, Cei S, Kandler B, Watzek G and Gruber R. Cathepsin K levels in the crevicular fluid of dental implants: a pilot study. J Clin Periodontol 2006; 33: 302-308.

[79] Monov G, Strbac GD, Baron M, Kandler B, Watzek G and Gruber R. Soluble RANKL in crevicular fluid of dental implants: a pilot study. Clin Implant Dent Relat Res 2006; 8: 135-141.

[80] Arikan F, Buduneli N and Kutukculer N. Osteoprotegerin levels in peri-implant crevicular fluid. Clin Oral Implants Res 2008; 19: 283-288.

[81] Bullon P, Fioroni M, Goteri G, Rubini C and Battino M. Immunohistochemical analysis of soft tissues in implants with healthy and peri-implantitis condition, and aggressive periodontitis. Clin Oral Implants Res 2004; 15: 553-559.

[82] Kanzaki H, Chiba M, Shimizu Y and Mitani H. Dual regulation of osteoclast differentiation by periodontal ligament cells through RANKL stimulation and OPG inhibition. J Dent Res 2001; 80: 887-891.

[83] Yamaguchi M, Aihara N, Kojima T and Kasai K. RANKL increase in compressed periodontal ligament cells from root resorption. J Dent Res 2006; 85: 751-756.

[84] Nakajima R, Yamaguchi M, Kojima T, Takano M and Kasai K. Effects of compression force on fibroblast growth factor-2 and receptor activator of nuclear factor kappa B ligand production by periodontal ligament cells in vitro. J Periodontal Res 2008; 43: 168-173.

[85] Kawasaki K, Takahashi T, Yamaguchi M and Kasai K. Effects of aging on RANKL and OPG levels in gingival crevicular fluid during orthodontic tooth movement. Orthod Craniofac Res 2006; 9: 137-142.

[86] Nishijima Y, Yamaguchi M, Kojima T, Aihara N, Nakajima R and Kasai K. Levels of RANKL and OPG in gingival crevicular fluid during orthodontic tooth movement and effect of compression force on releases from periodontal ligament cells in vitro. Orthod Craniofac Res 2006; 9: 63-70.

[87] Chen R, Kanzaki H, Chiba M, Nishimura M, Kanzaki R and Igarashi K. Local osteoprotegerin gene transfer to periodontal tissue inhibits lipopolysaccharide-induced alveolar bone resorption. J Periodontal Res 2008; 43: 237-245.

[88] Kanzaki H, Chiba M, Arai K*, et al*. Local RANKL gene transfer to the periodontal tissue accelerates orthodontic tooth movement. Gene Ther 2006; 13: 678-685.

[89] Dunn MD, Park CH, Kostenuik PJ, Kapila S and Giannobile WV. Local delivery of osteoprotegerin inhibits mechanically mediated bone modeling in orthodontic tooth movement. Bone 2007; 41: 446-455.

[90] Kanzaki H, Chiba M, Shimizu Y and Mitani H. Periodontal ligament cells under mechanical stress induce osteoclastogenesis by receptor activator of nuclear factor kappaB ligand up-regulation via prostaglandin E2 synthesis. J Bone Miner Res 2002; 17: 210-220.

[91] Norton LA and Burstone CJ. The Biology of Tooth Movement. Boca Raton, Fla., CRC Press. 1989.

[92] Arnett TR, Gibbons DC, Utting JC*, et al*. Hypoxia is a major stimulator of osteoclast formation and bone resorption. J Cell Physiol 2003; 196: 2-8.

[93] Srinivasan S and Avadhani NG. Hypoxia-mediated mitochondrial stress in RAW264.7 cells induces osteoclast-like TRAP-positive cells. Ann N Y Acad Sci 2007; 1117: 51-61.

[94] Muzylak M, Price JS and Horton MA. Hypoxia induces giant osteoclast formation and extensive bone resorption in the cat. Calcif Tissue Int 2006; 79: 301-309.

[95] Motohira H, Hayashi J, Tatsumi J, Tajima M, Sakagami H and Shin K. Hypoxia and reoxygenation augment bone-resorbing factor production from human periodontal ligament cells. J Periodontol 2007; 78: 1803-1809.

[96] Ren Y and Vissink A. Cytokines in crevicular fluid and orthodontic tooth movement. Eur J Oral Sci 2008; 116: 89-97.

[97] Yoshimatsu M, Shibata Y, Kitaura H*, et al*. Experimental model of tooth movement by orthodontic force in mice and its application to tumor necrosis factor receptor-deficient mice. J Bone Miner Metab 2006; 24: 20-27.

CHAPTER III. OSTEOCLAST BIOLGOY: FUNCTION

Osteoclasts have a number of unique ultrastructural characteristics, including multiple nuclei surrounded by the full-developed Golgi apparatus, abundant mitochondria, and a large number of vacuoles and lysosomes [1]. Functional osteoclasts can be found both in trabecular and cortical bone; they are located on the surface of trabeculi in trabecular bone, and in the cutting edge of the forming osteons in cortical bone [2].

3.1. Mechanism of Bone Resorption

During bone resorption, osteoclasts undergo a transient morphological change [3]. Absorbing osteoclasts obtain different membrane domains through cytoskeletal reorganization and cellular polarization (Figure 3.1) [2, 3]. The apical membrane domain faces the bone surface, and is called the ruffled border. The ruffled border is the main resorptive organelle of the cell. Away from the bone surface are the basolateral membrane domain and the functional secretory domain. Another important feature of osteoclasts' zone of contact with bone is the sealing zone, an actin-rich area surrounding the ruffled border [3]. In the area of the sealing zone, the cell membrane closely interacts with bone matrix via integrin $\alpha v\beta 3$, thus sealing off an isolated resorptive compartment [1-3].

3.1.1. Dissolving the Mineral Phase

The extracellular compartment between the osteoclast and the bone surface is acidified by protons transported by the ATP (adenosine triphosphate)-dependent proton pumps known as the vacuolar H^+- adenosine triphosphatase (H^+-ATPase) or V-ATPase at the ruffled border. As shown in the reaction below, the low pH value can dissolve the strongly basic hydroxyapatite in solid phase, releasing calcium ions, phosphate ions, and water in solution [4].

$$[Ca_3(PO_4)_2]_3\,Ca(OH)_2 \;+\; 8H^+ \;\longleftrightarrow\; 10Ca^{2+} \;+\; 6HPO_4^{2-} \;+\; 2H_2O$$

Solid hydroxyapatite Solution

Secretion of protons is accompanied by movement of chloride anions via the CLC-7 chloride channel at the basolateral membrane [2, 3]. Ion equilibrium in the cytoplasm is maintained by the following two processes: 1) the production of protons and bicarbonate ions from CO_2 hydration facilitated by carbonic anhydrase II (CA II); and 2) the exchange of bicarbonate to chloride through bicarbonate-chloride (HCO_3/Cl) exchanger at the basolateral membrane (Figure 3.2.) [2, 3]. Key enzymes involved in the acidification process are very important for normal bone resorption. Mutations in H^+ATPase, CLC-7 chloride channel or carbonic anhydrase II can result in osteopetrosis in mice and humans [3, 5, 6].

3.1.2. Degrading the Organic Phase

While the acidic milieu decalcifies bone, lysosomal enzymes are secreted from the ruffled border to degrade the exposed organic matrix in bone [2, 3]. The major enzymes that degrade type I collagen in an acid environment include cathepsin K (CTK), a cysteine

proteinase, matrix metalloproteinase-9 (MMP-9), and tartrate resistant alkaline phosphatase (TRAP) (Figure 3.2.) [2, 7-9]. Genetics studies have demonstrated that mice deficient in lysosomal proteases such as TRAP and cathepsin K are osteopetrotic [8, 9].

3.1.3. Removing Resorption Products

In order to continue resorption, osteoclasts must have a mechanism to dispose the resorption products simultaneously with the ongoing secretion of acid and proteolytic enzymes [2]. Studies have demonstrated that degradation products are endocytosed into the cell from the ruffled border, transcytosed through the cell, and finally secreted into the extracellular fluid through a specific plasma membrane domain. This domain is located in the upper part of a resorbing and is called "functional secretory domain" (Figure 3.1.) [2]. It should be noted that the process of transcytosis in osteoclasts is not just a disposal process. It mobilizes the calcium stored in bone, and it releases growth factors such as transforming growth factor-beta (TGF-β) latent in bone matrix [4].

3.2. Role of Osteoclasts in Hematopoiesis

Besides their anatomical juxtaposition, bone and bone marrow are functionally associated. On one hand, bone marrow cells provide progenitors of bone remodeling cells and regulate their development [10, 11]. On the other hand, osteoblasts have been shown to play a central role in supporting hematopoiesis in bone marrow [11, 12]. Intriguingly, recent data have demonstrated that the bone-resorbing osteoclasts are critically involved in the mobilization of hematopoietic stem cells induced by stress situations such as

lipopolysaccharide (LPS) administration and controlled bleeding [13]. Stimulation of osteoclasts by RANKL, bleeding, or LPS results in elevated production of proteolytic enzymes, MMP-9 and CTK, which, in turn, degrade the hematopoietic stem cell niche components, including stromal cell-derived factor-1(SDF-1), stem cell factor (SCF), and osteopontin [13]. This leads to increased mobilization of HSCs into the circulation. In summary, these data associate osteoclasts with the regulation of hematopoiesis and host defense, further emphasizing the function interdependence between bone and bone marrow/ hematopoiesis (Figure 3.3.).

3.3. Beyond Bone Resorption: Stimulating Bone Formation

Osteoclast activity has been traditionally considered as limited to bone resorption; however, accumulating evidence has suggested an alternative function of osteoclast: stimulating bone formation [14-16].

The very nature of the bone remodeling process, i.e., the coupling between bone resorption and bone formation, has long been noticed. It has been hypothesized that the coupling mechanism is achieved by growth factors released from bone matrix during osteoclastic resorption [14, 15]. However, increasingly more studies have revealed a far more complex coupling mechanism: osteoclasts themselves, not their resorptive activity, can provide the anabolic signals that can couple bone resorption and bone formation [14, 16].

3.3.1. Evidence from the Osteopetrosis Models

In osteopetrosis, bone mass is increased due to reduced bone resorption; however, depending on the etiology, bone formation and resorption is not always coupled. Patients with mutations in the chloride channel CLC-7 or the H^+-ATPase display reduced bone resorption due to impaired acidification of the resorption lacunae. However, rather than decreased bone formation as expected from the decreased bone resorption, these patients exhibit normal or even increased bone formation. Moreover, they have increased number of non-resorbing osteoclasts that are otherwise normal [16]. This observation has also been confirmed by a recent finding that bone formation is increased in mice with deficiency in osteoclast fusion [17]. In contrast to patients with defective acidification process, osteopetrosis patients with defective cathepsin K have no apparent uncoupling of formation and resorption, but they have poorly remodeled bone. [16].

In comparison to osteoclast-rich osteopetrosis mentioned above are osteopetrotic models where the osteoclasts are reduced or absent (osteoclast-poor). Studies from *op/op* mice have shown that in the complete absence of osteoclasts, bone formation is impaired, with disorganized matrix, reduced mineralization, and a lower bone quality [16, 18]. Consistently, *c-fos* deficient mice, which lack osteoclasts, have markedly decreased serum osteocalcin level, suggesting reduced bone formation [16, 19, 20].

Collectively, these data suggest a role of osteoclasts, even those that are not capable of resorbing bone, in coupling bone resorption to bone formation.

3.3.2. Evidence from the Anabolic Action of PTH

Parathyroid hormone (PTH) is a polypeptide hormone secreted by the chief cells of the parathyroid glands [21, 22]. PTH has a dual action on bone remodeling. Sustained elevations of PTH, as in hyperparathyroidism, have a net catabolic effect on bone, favoring bone resorption [21]. The catabolic action of PTH on bone will be discussed in detail in Chapter V. Interestingly, when administered in an intermittent, rather than a continuous mode, PTH has an anabolic action on bone [21].

This has raised the question whether the resorptive action of PTH is necessary for its anabolic action on bone formation. As indicated by both human and animal studies, the anabolic effect of PTH is not solely dependent on osteoblasts; it also requires signals from osteoclasts linking back to osteoblasts [14, 23].

The anabolic effect of PTH is significantly reduced when the subjects are co-administered with agents that promote osteoclast apoptosis, such as bisphosphonate or estrogen [14]. Mice deficient with *c-fos* are osteopetrotic and lack the anabolic response to intermittent PTH treatment [14, 23]. Compared to those from normal mice, osteoblasts from *c-fos* knockout mice showed no different response to PTH treatment *in vitro*. In contrast, the rescue of osteoclast defect in *c-fos* mutant mice can restore the anabolic effect of PTH, suggesting osteoclasts are required for the anabolic response to PTH. Moreover, in normal mice, the anabolic action of PTH is significantly blocked when osteoclast precursors recruitment is inhibited by stromal derived factor-1 (SDF-1) or osteoclast differentiation is inhibited by OPG [23]. Taken together, these data demonstrate that the osteoclast is the intermediate target for the anabolic action of PTH [23].

In addition, the underlying mechanism has been further proposed: PTH can increase production of RANKL, which stimulate the transient activation of osteoclasts. RANKL-activated osteoclasts then provide signals for osteoblast maturation (Figure 3.4.) [14].

In summary, studies from the anabolic action of PTH further emphasize the important role of osteoclasts in providing signals required in coupling bone resorption to bone formation during bone remodeling.

3.3.3. Evidence from *In Vitro* Studies

In order to identify the anabolic factor(s) produced by osteoclasts, a recent study employed an *in vitro* cell culture system, where primary human osteoclasts were induced by recombinant RANKL and M-CSF. The results showed that even conditioned media collected from osteoclasts cultured on plastic can promote bone nodule formation by the murine osteoblastic cell line, MC3T3-E1, in a dose dependent manner [24]. Although the authors claimed that this was the first evidence that osteoclasts can secrete non-bone matrix derived factor(s) to increase bone formation, this is not exactly the case. In fact, studies have been performed to address the question whether osteoclasts control osteoblastic growth and function. Factors expressed by osteoclasts have been found to be able to regulate osteoblast differentiation and proliferation: hepatocyte growth factor (HGF) and myeloid protein-1 precursor (Mim-1) as positive regulators, while platelet-derived growth factor BB (PDGF) as negative regulators [25]. Nevertheless, these preliminary data still remain controversial and more research is required to identify the factor(s) secreted by osteoclasts that can regulate osteoblasts.

3.4. Chapter Summary

Osteoclasts are the sole bone-absorbing cells. They decalcify bone by producing acid and degrade bone matrix by secreting lysosomal proteases. By resorbing bone, osteoclasts also play a critical role in mobilizing hematopoietic stem cells in response to stress situations, such as LPS treatment. Moreover, accumulating data have indicated that osteoclast activity is not limited to bone resorption. Osteoclasts can stimulate bone formation, serving as a source of anabolic signals to couple bone formation to bone resorption. The underlying molecular mechanism, however, still remains to be defined.

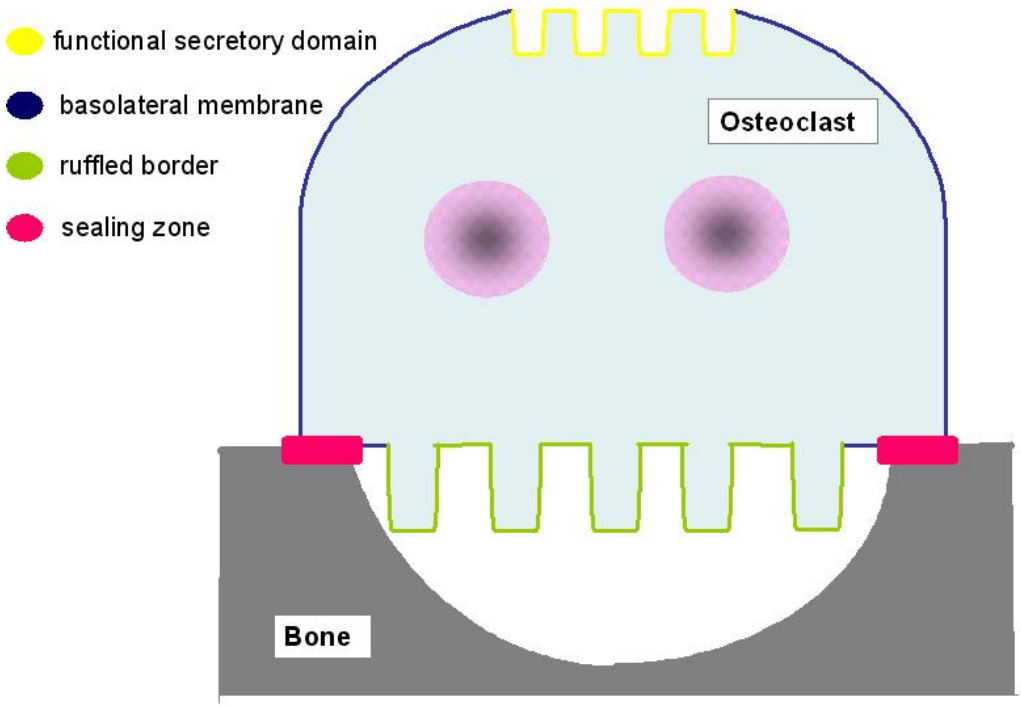

Figure 3.1. Plasma membrane domains present in a resorbing osteoclast. Adapted from reference 2.

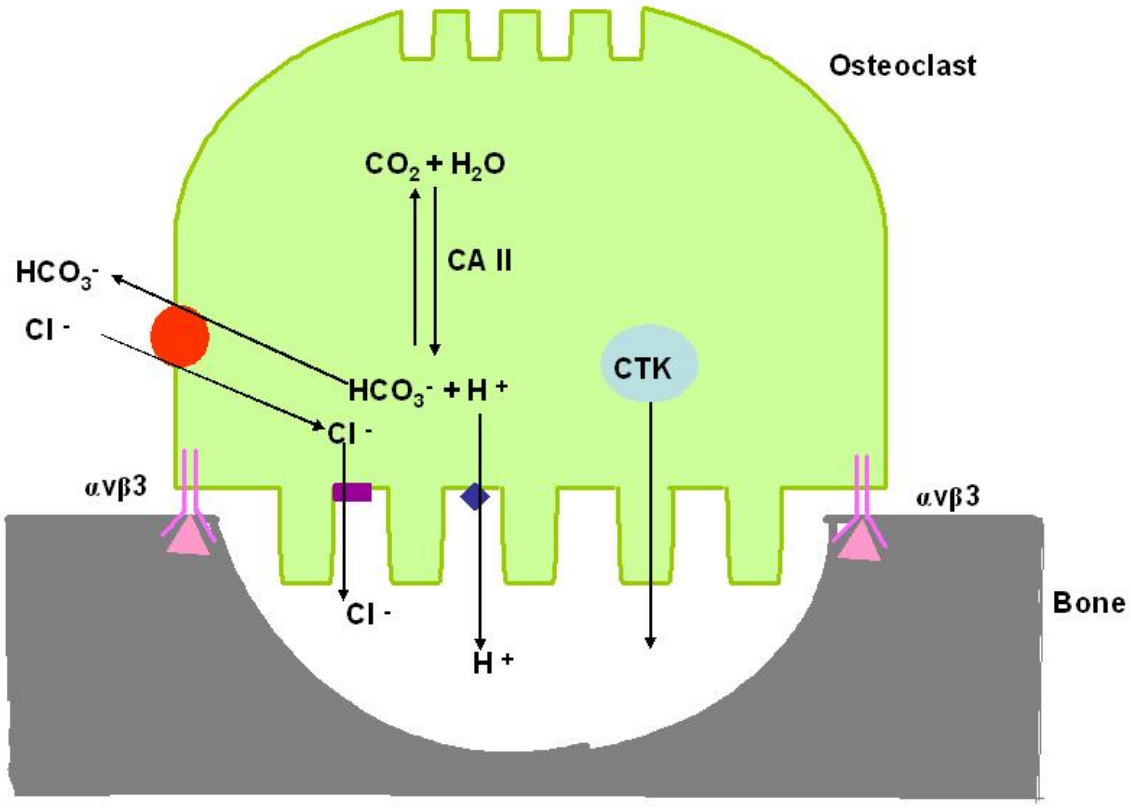

Figure 3.2. Mechanism of osteoclastic bone resorption. The osteoclast adheres to bone surface via integrin αvβ3, and forms a special membrane facing bone: the ruffled border. Hydrochloric acid is secreted into the resorptive lacuna by the combined actions of a vacuolar H^+ ATPase, its coupled Cl^- channel, and a basolateral chloride–bicarbonate exchanger. Carbonic anhydrase II (CA II) converts CO_2 and H_2O into H^+ and HCO_3^-. The acidic milieu mobilizes the mineral phase of bone and thereby exposing the organic phase of bone for degradation by acidic proteases released from lysosomes, such as cathepsin K (CTK). Adapted from reference 2.

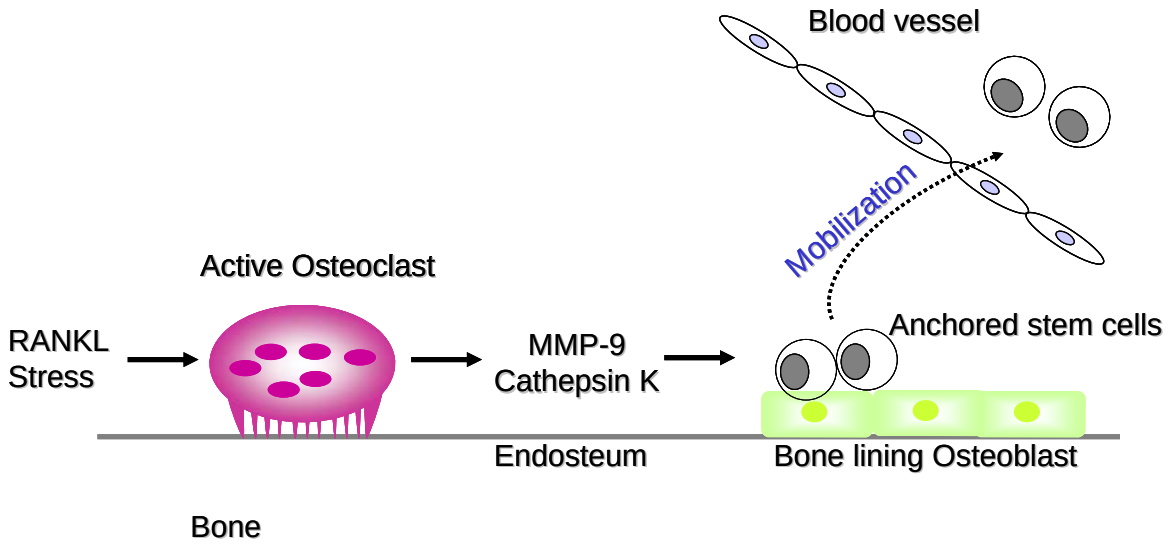

Figure 3.3. Osteoclasts mobilize hematopoietic stem cells into circulation. Stimulation of osteoclasts by RANKL or stress situations such as inflammation and injury results in elevated production of proteolytic enzymes, MMP-9 and CTK, which, in turn, degrade components of the hematopoietic stem cell niche. This leads to increased mobilization of the hematopoietic stem cells from bone marrow into the circulation. Adapted from reference 13.

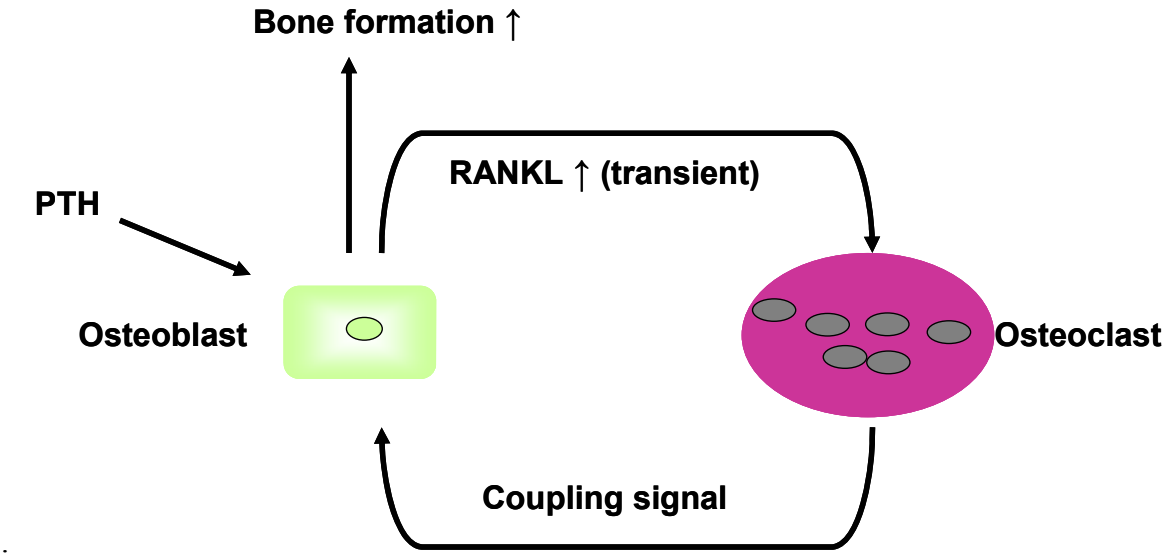

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Figure 3.4. Mechanism of the anabolic action of PTH. An anabolic stimulus, PTH, increase transiently RANKL production by osteoblasts, which results in the activation of osteoclasts. Osteoclasts then send coupling signals to stimulate osteoblast differentiation and bone formation. Adapted from reference 14.

Chapter III. Reference List

[1] Takahashi N, Ejiri S, Yanagisawa S and Ozawa H. Regulation of osteoclast polarization. Odontology 2007; 95: 1-9.

[2] Vaananen K. Mechanism of osteoclast mediated bone resorption--rationale for the design of new therapeutics. Adv Drug Deliv Rev 2005; 57: 959-971.

[3] Bruzzaniti A and Baron R. Molecular regulation of osteoclast activity. Rev Endocr Metab Disord 2006; 7: 123-139.

[4] Cohen MM,Jr. The new bone biology: pathologic, molecular, and clinical correlates. Am J Med Genet A 2006; 140: 2646-2706.

[5] Komarova SV, Dixon SJ and Sims SM. Osteoclast ion channels: potential targets for antiresorptive drugs. Curr Pharm Des 2001; 7: 637-654.

[6] Kornak U, Kasper D, Bosl MR*, et al*. Loss of the ClC-7 chloride channel leads to osteopetrosis in mice and man. Cell 2001; 104: 205-215.

[7] Kusano K, Miyaura C, Inada M*, et al*. Regulation of matrix metalloproteinases (MMP-2, -3, -9, and -13) by interleukin-1 and interleukin-6 in mouse calvaria: association of MMP induction with bone resorption. Endocrinology 1998; 139: 1338-1345.

[8] Gowen M, Lazner F, Dodds R*, et al*. Cathepsin K knockout mice develop osteopetrosis due to a deficit in matrix degradation but not demineralization. J Bone Miner Res 1999; 14: 1654-1663.

[9] Hayman AR and Cox TM. Tartrate-resistant acid phosphatase knockout mice. J Bone Miner Res 2003; 18: 1905-1907.

[10] Compston JE. Bone marrow and bone: a functional unit. J Endocrinol 2002; 173: 387-394.

[11] Taichman RS. Blood and bone: two tissues whose fates are intertwined to create the hematopoietic stem-cell niche. Blood 2005; 105: 2631-2639.

[12] Haylock DN and Nilsson SK. Stem cell regulation by the hematopoietic stem cell niche. Cell Cycle 2005; 4: 1353-1355.

[13] Kollet O, Dar A, Shivtiel S*, et al*. Osteoclasts degrade endosteal components and promote mobilization of hematopoietic progenitor cells. Nat Med 2006; 12: 657-664.

[14] Martin TJ and Sims NA. Osteoclast-derived activity in the coupling of bone formation to resorption. Trends Mol Med 2005; 11: 76-81.

[15] Seibel MJ, Robins SP and Bilezikian JP. Dynamics of Bone and Cartilage Metabolism. San Diego, Academic Press. 2006.

[16] Karsdal MA, Martin TJ, Bollerslev J, Christiansen C and Henriksen K. Are nonresorbing osteoclasts sources of bone anabolic activity? J Bone Miner Res 2007; 22: 487-494.

[17] Lee SH, Rho J, Jeong D*, et al*. v-ATPase V0 subunit d2-deficient mice exhibit impaired osteoclast fusion and increased bone formation. Nat Med 2006; 12: 1403-1409.

[18] Yoshida H, Hayashi S, Kunisada T*, et al*. The murine mutation osteopetrosis is in the coding region of the macrophage colony stimulating factor gene. Nature 1990; 345: 442-444.

[19] Johnson RS, Spiegelman BM and Papaioannou V. Pleiotropic effects of a null mutation in the c-fos proto-oncogene. Cell 1992; 71: 577-586.

[20] Wang ZQ, Ovitt C, Grigoriadis AE, Mohle-Steinlein U, Ruther U and Wagner EF. Bone and haematopoietic defects in mice lacking c-fos. Nature 1992; 360: 741-745.

[21] Hamann KL and Lane NE. Parathyroid hormone update. Rheum Dis Clin North Am 2006; 32: 703-719.

[22] Cole DE, Webb S and Chan PC. Update on parathyroid hormone: new tests and new challenges for external quality assessment. Clin Biochem 2007; 40: 585-590.

[23] Koh AJ, Demiralp B, Neiva KG*, et al*. Cells of the osteoclast lineage as mediators of the anabolic actions of parathyroid hormone in bone. Endocrinology 2005; 146: 4584-4596.

[24] Karsdal MA, Neutzsky-Wulff AV, Dziegiel MH, Christiansen C and Henriksen K. Osteoclasts secrete non-bone derived signals that induce bone formation. Biochem Biophys Res Commun 2008; 366: 483-488.

[25] Phan TC, Xu J and Zheng MH. Interaction between osteoblast and osteoclast: impact in bone disease. Histol Histopathol 2004; 19: 1325-1344.

CHAPTER IV. OSTEOCLAST PATHOLOGY: BONE LOSS

Bone resorption plays an important role not only in bone development and physiology, but also in bone pathology. The discovery of the OPG/RANKL/RANK axis as a major regulatory mechanism of osteoclastogenesis has revolutionized our understanding of normal bone biology and a number of bone diseases as well [1-3].

4.1. Postmenopausal Osteoporosis

4.1.1. Introduction

Osteoporosis is a heterogeneous group of metabolic bone diseases [4] and has been defined as "a skeletal disorder characterized by compromised bone strength predisposing a person to an increased risk of fracture" [5]. Although osteoporosis is characterized by low bone mass, the ratio of bone mineral to the organic matrix in osteoporosis normal, as opposed to a decreased ratio of bone mineral to the organic matrix in osteomalacia [6, 7].

In clinical practice, the measurement of bone mineral density (BMD) is the most commonly used method for osteoporosis diagnosis. BMD is most often assessed by dual energy x-ray absorptiometry (DXA or DEXA) [5, 8]. DXA measures both bone mineral content (BMC, in grams) and area (in cm^2). An "areal" BMD (g/cm^2) is obtained by dividing bone mineral content by area. This value can be converted to a T score or a Z score. A T score

compares a patient to a sex-matched, young, healthy population; it is calculated by subtracting the mean BMD of a young adult healthy population from the patient's BMD and then dividing by the standard deviation (SD) of the reference population. A Z score compares a patient to an age-and sex-matched control population; it is calculated by subtracting the mean BMD of an age- and sex-matched control population from the patient's BMD and then dividing by the SD of the reference population [9]. T scores are often reported in postmenopausal women and men of 50 years age and older, while Z scores are often reported in premenopausal women, men under the age of 50, and children [9]. According to the WHO definition, the diagnosis of osteoporosis is established when BMD is 2.5 SDs below the mean for normal Caucasian women (i.e. the T score is at least −2.5 SDs) [5].

However, it has been argued that the measurement of bone mass does not account for another important determinant of bone strength—bone quality [10, 11]. Bone quality is independently influenced by parameters including bone architecture, bone turnover, microdamage, and mineralization [5]. Although BMD measurement is currently the best diagnostic practice, alternative methods that can detect the deterioration in bone quality will provide significant improvement in osteoporosis prevention, diagnosis, and treatment [11].

Osteoporosis can occur as a primary disorder or as a disorder secondary to a number of systemic diseases (e.g. hyperparathyroidism) or medications (e.g. glucocorticoids) [6]. Primary osteoporosis is the most common metabolic bone disorder in adults, mostly associated with aging. There are two clinical subtypes of age-related osteoporosis: 1) Type I or postmenopausal osteoporosis, which occurs in postmenopausal women; and 2) Type II or senile osteoporosis, which is associated with the normal aging process in both men and women [4, 12]. Menopause refers to the cessation of menstruation, which occurs at ~ 48-50

years of age for healthy women. As a result, the production of ovarian hormones including estrogen is reduced [6]. The pathogenesis of postmenopausal osteoporosis will be discussed below with a focus on the current understanding of estrogen action on osteoclastic bone resorption.

4.1.2. Cellular Pathogenesis

Under normal conditions, bone undergoes constant remodeling, where resorption of the existing bone by osteoclasts is tightly coupled to formation of new bone by osteoblasts [13]. In postmenopausal osteoporosis, the rate of bone remodeling is increased, i.e. increased number of osteoclasts and bone resorption coupled with increased number of osteoblasts and bone formation. However, due to estrogen deficiency, there is an imbalance between bone resorption and bone formation, resulting in a decrease in total bone mass [4, 7].

4.1.3. Indirect Effects of Estrogen Deficiency on Bone Resorption

Since the interplay between the immune system and bone has long been noticed [14], it is no surprise that the pathogenesis of bone loss in postmenopausal osteoporosis is mainly mediated by immune cells [15, 16]. Indeed, many studies have suggested that stimulation of bone resorption in response to estrogen deficiency is largely mediated by inflammatory and osteoclastogenic cytokines, such as IL-1, IL-6, TNFα, and IL-7 [7, 15, 17, 18]. These cytokines are able to increase the production of M-CSF by osteoblasts/stromal cells and/or the ratio of RANKL to OPG, thereby upregulating osteoclastogenesis [7, 18].

Among these cytokines, TNFα and IL-7 have been shown to be the major ones. The source of TNFα in postmenopausal osteoporosis has been identified to be T cells [16]. TNFα can stimulate osteoclastogenesis by enhancing osteoblasts/stromal cells to produce more RANKL and M-CSF, and by priming osteoclast precursors to the stimulation of RANKL. Meanwhile, TNFα can inhibit osteoblast differentiation by repressing transcription factor, Runx2 [15]. A lack of estrogen results in elevated levels of circulating cytokines, such as IL-1 and TNFα, which, in turn, stimulate osteoblasts/stromal cells to release more IL-7 [17]. On one hand, IL-7 decreases Runx2 activity, and hence inhibits osteoblast function [17, 19]. On the other hand, IL-7 stimulates the expression of M-CSF and RANKL and simultaneously decreases OPG expression by osteoblasts/stromal cells. Moreover, IL-7 targets T cells to induce RANKL production [15, 17].

In summary, TNFα and IL-7 are the key mediators involved in bone lose induced by estrogen withdrawal.

4.1.4. Direct Effects of Estrogen Deficiency on Bone Resorption

The physiological effects of estrogen are mediated by two nuclear hormone receptors: estrogen receptor alpha (ERα) and estrogen receptor beta (ERβ), with the former being the major one in most target tissues [7, 18]. Estrogen receptors are also expressed in bone cells, including bone-absorbing osteoclasts and their precursors [7, 18], which indicates that the effects of estrogen on bone resorption may be mediated, at least in part, directly. However, until recently little is known regarding the direct action of estrogen on osteoclasts [18].

Using an osteoporosis mouse model, a recent publication has elegantly demonstrated that estrogen can directly induce osteoclast apoptosis [20]. To specifically disrupt ERα gene in mature osteoclasts, the authors inserted the Cre recombinase into the cathepsin K gene locus. In another word, ERα gene is selectively deleted during osteoclastogenesis. Adult female, but not male, $ER^{\Delta Oc/\Delta Oc}$ mice displayed high bone turnover characterized by increased osteoclast numbers, increased bone formation, but decreased trabecular bone mass [20]. So these mice mimic, to a certain degree, human postmenopausal osteoporosis. However, in $ER^{\Delta Oc/\Delta Oc}$ mice, ovariectomy did not result in trabecular bone loss or increased osteoclast numbers. In addition, estrogen administration could not rescue the osteoporotic phenotypes of these mice. These results suggest that estrogen may directly target osteoclasts to exert its osteoprotective action.

This report further demonstrated that estrogen can upregulate Fas ligand (FasL) expression in osteoclasts and thus induce apoptosis of osteoclasts having wildtype ERα , but not those lacking ERα [20]. Since Fas is also expressed by osteoclasts, it appears that estrogen can upregulate FasL expression and affect osteoclast survival through an autocrine mechanism [21]. Collectively, these data suggested that estrogen can directly induce osteoclast apoptosis via its receptor ERα in osteoclasts [21]. In contrast, a more recent article has described a paracrine mechanism in which estrogen control osteoclast life span by upregulating FasL in osteoblasts, not osteoclasts [22]. Therefore, to generate a complete picture of the extremely complex process of postmenopausal osteoporosis, more research is necessary to identify the central cellular target(s) of estrogen.

4.2. Paget's Disease of Bone

Paget's disease of bone (PDB), also known as osteitis deformans, is a nonmalignant, localized, metabolic bone disorder [23]. It is characterized by enlarged and deformed bone, occurring mainly in the axial skeleton [23].

4.2.1. Cellular Pathogenesis

Histological studies have shown that the primary cellular abnormality in Paget's disease of bone is the increased activity of osteoclasts [6, 23]. Compared to normal osteoclasts, Pagetic osteoclasts are larger and contain more nuclei, causing excessive bone resorption. Subsequently, more osteoblasts are recruited to the site, and bone formation is increased. However, new bone is formed at such a rapid rate that bone is formed in a sporadic and haphazard way, and woven bone rather than lamellar bone develop. Pagetic bone is expanded in size, structurally disorganized, and mechanically weak, leading to bone deformity, pathological fracture, and various other complications [23, 24]. In Paget's disease of bone, increased osteoclastic bone resorption coupled to increased bone formation leads to accelerated and abnormal bone remodeling.

4.2.2. Molecular Pathogenesis

Paget's disease of bone is the second most common bone disease after osteoporosis, but its etiology remains largely unknown [23]. Two main theories have been proposed: viral and genetic. Infection of virus, such as the measles virus, the canine distemper virus, and the respiratory syncytial virus, may contribute to the osteoclast hyperactivity in genetically

susceptible individuals [23]. Although the virus theory is still controversial, genetic factors are certainly involved in Paget's disease of bone. This disorder shows autodominant inheritance, and is called familial Paget's disease. Ethnic difference in the prevalence also suggests the importance of genetic factors [25]. Several rare familial bone conditions have been described to share clinical features phenotypes with Paget's disease of bone (classical PDB) [25]. These diseases are summarized in Table 4.1.

Several susceptibility loci for Paget's disease of bone and related syndromes have been identified by genome-wide scans. Mutations that predispose individuals to PDB and related disorders have been subsequently identified in four genes, all of which are involved in one of the pathways essential for osteoclastogenesis — the RANKL–RANK–NFκB signaling pathway [25].

As discussed in Chapter II, the binding of RANKL produced by osteoblasts/stromal cells to its receptor, RANK on osteoclasts and their precursors initiates signaling pathways (e.g. NFκB pathway) to induce osteoclast differentiation (Figure 2.2.)[26-28]. Meanwhile, osteoblasts/stromal cells also produce a decoy receptor to RANKL— OPG— to negatively regulate RANKL induced signals (Figure 2.2.) [3, 29, 30]. The familial expansile osteolysis, early-onset PDB, and ESH are caused by insertion mutations in *TNFRSF11A*, which encodes RANK. Inactivating mutations in TNFRSF11B, which encodes osteoprotegerin (OPG) cause JPD, and polymorphisms in this gene are associated with an increased risk for PDB (Figure 4.1.) [25].

The most important cause of classical PDB is the mutations of *SQSTM1* gene, which encodes ubiquitin-binding protein p62 (sequestosome 1) [31]. The ubiquitin-binding protein p62 (SQSTM1) functions as a scaffold in a range of signaling pathways including the NFκB

pathway [32, 33]. Studies have suggested that RANKL stimulation leads to the formation of a complex involving TRAF6, p62, and atypical protein kinase C (aPKC), the latter activating IKK (IκB kinase) and NF-kB (Figure 4.1.) [32, 34].

IBMPFD is caused by mutations in the gene encoding valosin-containing protein (VCP) [25]. VCP is a protein involved in targeting the inhibitor of NFκB (IκB) for proteasomal degradation. The degradation of IκB results in the release of NFκB, which then translocates to the nucleus to activate responsive genes. Together with other signaling pathways described in Chapter II, NFκB signaling eventually leads to osteoclast differentiation (Figure 4.3.) [35].

In summary, mutations or polymorphisms in genes implicated in PDB and related disorders are all involved in the osteoclastogenesis signaling pathway, RANKL–RANK–NFκB pathway.

4.3. Hyperparathyroidism

Primary hyperparathyroidism is a hypercalcemia state resulting from excessive secretion of parathyroid hormone (PTH) by parathyroid glands [36].This can be caused by adenoma, hyperplasia, or carcinoma of the parathyroid glands [6]. Secondary hyperparathyroidism is an acquired disorder in which parathyroid glands secrete excessive PTH in response to a low extracellular calcium concentration [6].

4.3.1. Cellular and Molecular Pathogenesis

PTH is a polypeptide hormone secreted by the chief cells of the parathyroid. PTH has a dual action on bone remodeling. When administered in an intermittent, rather than a continuous mode, PTH has an anabolic action on bone [37]; the underlying mechanism has been discussed in detail in Chapter III. As discussed in the following sections, sustained elevations of PTH have a net catabolic effect on bone [37].

Small fluctuations in plasma Ca^{2+} level is sensed by a cell surface Ca^{2+} receptor (CaR) on parathyroid gland cells. CaR belongs to the superfamily of G protein-coupled receptors (GPCR) [38]. Under hypocalcemic conditions, the parathyroid CaR senses the decreased Ca^{2+} level and enhances PTH secretion. PTH can mobilize Ca^{2+} into the extracellular fluid through the following mechanisms: 1) stimulation of bone resorption; 2) increasing renal calcium reabsorption; and 3) stimulation of renal 1α, 25-dihydroxyvitamin D_3 [$1,25(OH)_2D_3$] (Figure 4.2.) [38]. Under hypercalcemic conditions, the parathyroid CaR senses the elevated Ca^{2+} level and inhibits PTH secretion, leading to suppressed calcium mobilization (Figure 4.2.) [38].

Such a feedback mechanism is disrupted in both primary hyperparathyroidism and secondary hyperparathyroidism. In primary hyperparathyroidism, the pathology of parathyroid glands themselves causes sustained elevation of plasma PTH level, which, in turn causes excessive bone resorption and diminished bone mineral density [36, 38, 39]. Primary hyperparathyroidism is the most common cause of hypercalcemia [6]. In secondary hyperparathyroidism, in response to a low extracellular calcium concentration, parathyroid glands synthesize more PTH to restore the normal plasma calcium level. However, due to a disorder within organs responsible for calcium or reduced availability of calcium,

hypocalcemia can occur. Secondary hyperparathyroidism is the most common complication of end stage renal diseases, and is also found in vitamin D-deficiency and Vitamin-D-resistant conditions [6].

4.3.2. Mechanism of PTH Catabolic Effect on Bone

The physiological activities of PTH are mediated by its receptor, PTH1R, a G protein-coupled protein (GPCR) [40]. As in primary hyperparathyroidism, continuous increase of PTH level leads to the activation of PTH1R expressed on the plasma membrane of osteoblasts. The binding of PTH and its receptor initiates downstream signaling to stimulate RANKL expression and inhibit OPG expression by osteoblasts. The increased RANKL to OPG ratio results in increased osteoclastogenesis [1, 2, 41]. The responsible signal transduction pathway is the classic cAMP-dependent protein kinase A (PKA) pathway, in which Gαs subunit of PTH1R stimulates cAMP production and PKA activation; PKA subsequently phosphorylates transcription factors, such as Cbfa1 and cAMP-response element-binding protein (CREB), regulating target gene expression [2, 42]. Additionally, recent studies have demonstrated that PTH can increase osteoblastic production of monocyte chemoattractant protein-1(MCP-1), which, in turn, increases osteoclastogenesis [43].

Osteoblasts have been widely shown to express PTH receptors [1, 44], while it has been controversial whether osteoclasts express high-affinity PTH receptors [45-47]. It is generally accepted that even though osteoclast may express PTH receptors, these receptors do not respond directly to PTH; the functional significance of the osteoclastic PTH receptors, if any, need to be further confirmed and elucidated [46, 47]. Therefore, PTH exerts its catabolic action on bone indirectly via its action on osteoblasts [1, 44].

4.4. Cherubism

Cherubism (OMIM reference number #118400) is an autosomal-dominant benign disorder characterized by painless symmetrical swelling of cheeks and jaws [48-50]. Dental abnormalities such as hypodontia are also a feature [49, 51]. With an early onset at ~2-4 years of age, this condition continues through childhood before regressing in adulthood [52]. Histologically, cherubism is characterized by fibrous connective tissue with abundant osteoclastic cells and inflammation cells [52].

4.4.1. Molecular Pathogenesis

The cherubism locus has been mapped to chromosome 4p [53, 54], and genetic studies have subsequently detected several point mutations in *SH3BP2,* which encodes the widely expressed scaffold protein, the SH3-domain binding protein 2 (SH3BP2) [48]. However, the exact pathogenesis of this disease is largely unclear.

A recent publication has shed new light on this condition by using a mouse model of cherubism. This mouse model is created by introducing the most common SH3BP2 mutation found in cherubism patients, P418R (a proline to arginine substitution affecting amino acid 418), into the mouse SH3BP2 gene (P416R) [55]. Mice homozygous for this mutation exhibited bone resorption in the jaws associated with increased osteoclast numbers, a global decrease in bone mass, and systemic macrophage-infiltrated inflammation [55].

The following two major findings were generated from this knockin mouse model. First, several pieces of evidence have suggested that the primary cell type involved in

cherubism pathogenesis is the myeloid cell: 1) Myeloid osteoclast precursors from SH3BP2 knockin mice showed increased osteoclastic potential in culture in response to macrophage colony-stimulating factor (M-CSF) and receptor activator of nuclear factor-κB ligand (RANKL); 2) The phenotypes can be transferred into wildtype mouse by transplantation of fetal liver cells from SH3BP2 knockin mice; 3) When SH3BP2 knockin mice were crossed with *rag1−/−*mice, which lack functional lymphocytes, the phenotypes were not changed; however, when SH3BP2 knockin mice were crossed with *op/op* mice, which lack myeloid cells, the phenotypes were eliminated [55]. Secondly, the pathogenesis of cherubism is dependent on TNFα [55], a potent stimulator of osteoclastogenesis [56]. SH3BP2 knockin mice had elevated level of serum TNFα, and myeloid cells isolated from the knockin mice produced an increased amount of TNF-α in response to M-CSF treatment in an ERK dependent manner. Moreover, when SH3BP2 knockin mice were crossed with TNFα-deficient mice, they did not exhibit bone loss or inflammation [55].

The authors have further demonstrated that P416R mutation of SH3BP2 in the osteoclast lineage cells causes gain of function, leading to hyperphosphorylation of Syk and increased Syk activity in response to RANKL [55]. Syk is a tyrosine kinase involved in the costimulatory signals for RANK in osteoclastogenesis (see Figure 2.3. for detail) [57]. Since SH3BP2 can mediate ITAM-dependent activation of calcium signaling and NFATc1 induction in lymphocytes, it has been proposed that it may also act as an ITAM-dependent scaffold protein in osteoclast precursors [58]. Based on their findings, the authors have proposed a mechanism of Cherubism pathogenesis shown in Figure 4.3.

4.5. Chapter Summary

Despite of distinct etiologies, metabolic bone diseases, such as osteoporosis, Paget's disease of bone, and hyperparathyroidism are all characterized by excessive osteoclast activities and bone loss [2]. Estrogen deficiency leads to increased levels of osteoclastogenic cytokines produced by immune cells, which stimulate osteoclastogenesis through the RANKL/RANK/OPG system. Moreover, recent research has shown that estrogen can also directly target osteoclasts, inhibiting their apoptosis. Increased osteoclastogenesis in Paget's disease of bone and related syndromes is caused by mutations in genes involved in the RANKL–RANK–NFκB signaling pathway. In hyperparathyroidism, sustained increased of PTH levels results in increased bone resorption to mobilize calcium from bone. PTH binds to its receptor, PTH1R on osteoblasts, leading to increased production of RANKL and decreased production of OPG. In addition, excessive bone resorption in the jaws is also an important characteristic of Cherubism, an autosomal-dominant disorder. [48]. Animal studies have recently shown that mutation in SH3BP2 gene, which encode a scaffold protein, results in increased responsiveness of osteoclast precursors to M-CSF/RANKL and increased production of TNFα by macrophages. TNFα further stimulate osteoclastogenesis by acting on osteoblasts/stromal cells. Further investigation is needed to define the extent to which the cherubism mouse model can recapitulate what happens in humans with cherubism.

Table 1. Familial bone conditions with similar clinical features as Paget's disease of bone (PDB)

Juvenile Paget's Disease (JPD)
Familial Expansile Osteolysis (FEO) and Expansile Skeletal Hyperphosphatasia (ESH)
Early-Onset Paget's Disease of Bone
Inclusion-Body Myopathy, PDB and Frontotemporal Dementia (IBMPFD)

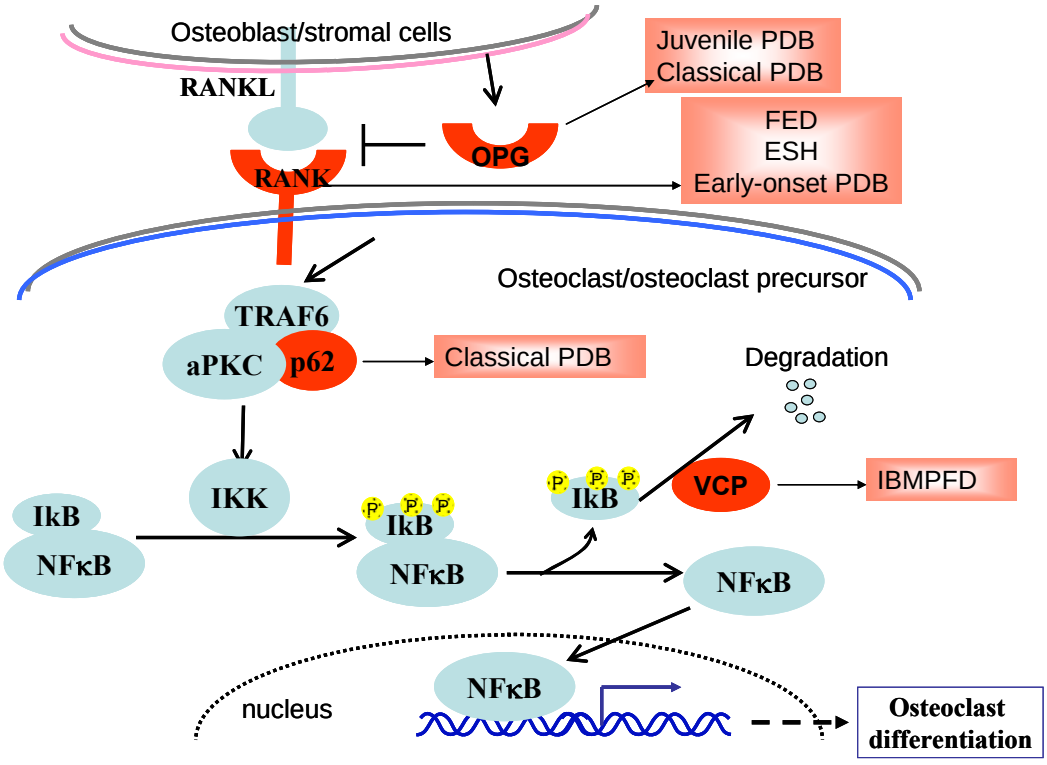

Figure 4.1. Mutations in components of RANK–NFκB signaling pathway cause Paget's disease of bone and related disorders. The binding of RANKL produced by osteoblasts/stromal cells to its receptor, RANK on osteoclasts and their precursors leads to the formation of a complex involving TRAF6, ubiquitin-binding protein p62, and aPKC, the latter activating IKK (IκB kinase) to phosphorylate IκB. IκB is then targeted for proteasomal degradation, a process that involves valosin-containing protein (VCP). The degradation of IκB results in the release of NFκB that translocates to the nucleus to activate the responsive genes. Together with other signaling pathways described in Chapter II, NFκB signaling eventually leads to osteoclast differentiation. Stromal cells/osteoblasts also produce a decoy receptor for RANKL, osteoprotegerin (OPG), which negatively regulate RANK signaling. Components of the RANK−NFκB pathway that are mutated or polymorphic in Paget's disease of bone and related disorders are highlighted in red: 1) RANK mutations can cause the familial expansile osteolysis (FED), ESH and early-onset PDB; 2) OPG mutations can cause juvenile PDB, and polymorphisms in this gene increases the risk for PDB; 3) Mutations of genes encoding p62 can cause classical PDB; 4) Mutations of genes encoding VCP can cause IBMPFD. Adapted from reference 25.

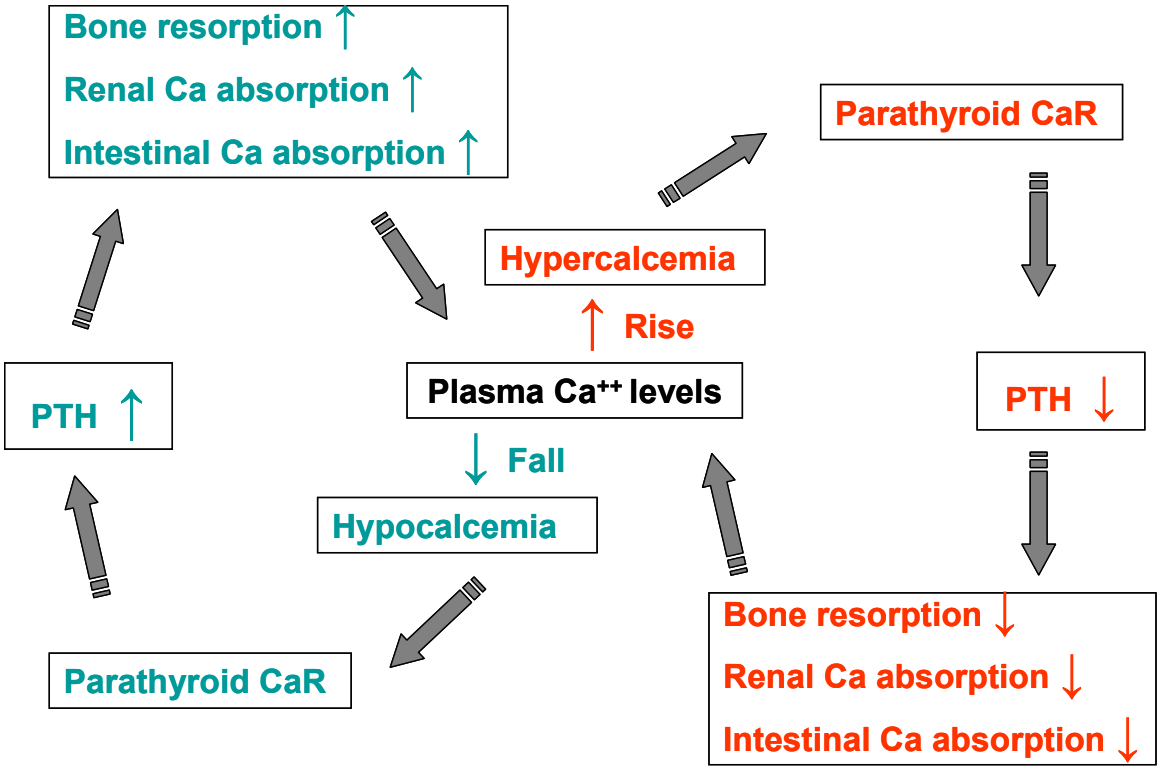

Figure 4.2. Reciprocal relationship between PTH and extracellular calcium level. PTH secretion is triggered by hypocalcemia, and suppressed by hypercalcemia. Hypocalcemic conditions: the parathyroid CaR senses the decreased Ca^{2+} level and enhances PTH secretion. PTH can mobilize Ca^{2+} into the extracellular fluid through the following mechanisms: (1) stimulation of bone resorption; (2) increasing renal calcium reabsorption; (3) stimulation of renal 1α, 25-dihydroxyvitamin D_3 [$1,25(OH)_2D_3$]. Consequently, hypocalcemia returns to normocalcemia. In contrast, under hypercalcemic conditions, the parathyroid CaR senses the elevated Ca^{2+} level and inhibits PTH secretion, leading to suppressed calcium mobilization. Consequently, hypercalcemia returns to normocalcemia. Adapted from reference 38.

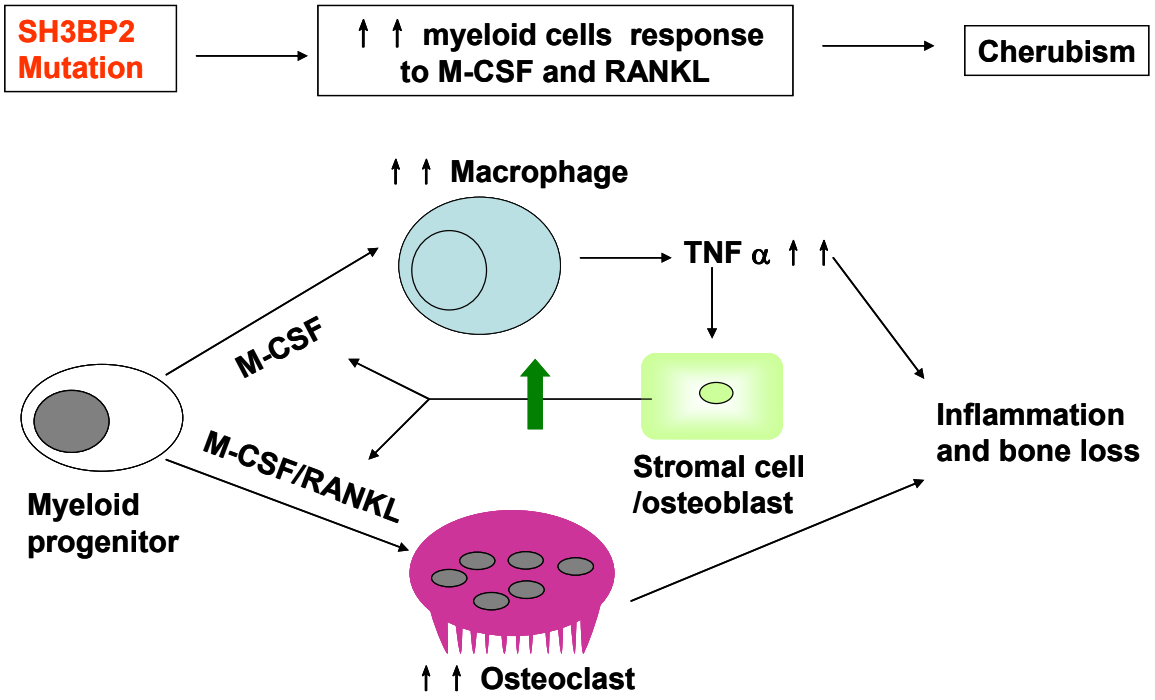

Figure 4.3. Proposed mechanism of cherubism pathogenesis. Mutation in SH3BP2 gene results in an increased response of myeloid cells to M-CSF and RANKL. In the presence of M-CSF, the cells differentiate into macrophages with increased production of TNFα. When exposed to both M-CSF and RANKL, they differentiate into osteoclasts with increased bone-resorbing capability. Moreover, TNFα can increase the production of M-CSF and RANKL by stromal cells/osteoblasts, further promoting macrophage and osteoclast differentiation. Therefore, macrophage/TNFα mediated inflammation and osteoclastic bone loss becomes the pathological characteristic of cherubism. Adapted from references 55 and 58.

Chapter IV. Reference List

[1] Suda T, Takahashi N, Udagawa N, Jimi E, Gillespie MT and Martin TJ. Modulation of osteoclast differentiation and function by the new members of the tumor necrosis factor receptor and ligand families. Endocr Rev 1999; 20: 345-357.

[2] Hofbauer LC, Kuhne CA and Viereck V. The OPG/RANKL/RANK system in metabolic bone diseases. J Musculoskelet Neuronal Interact 2004; 4: 268-275.

[3] Theoleyre S, Wittrant Y, Tat SK, Fortun Y, Redini F and Heymann D. The molecular triad OPG/RANK/RANKL: involvement in the orchestration of pathophysiological bone remodeling. Cytokine Growth Factor Rev 2004; 15: 457-475.

[4] Simon LS. Osteoporosis. Rheum Dis Clin North Am 2007; 33: 149-176.

[5] NIH Consensus Development Panel on Osteoporosis Prevention, Diagnosis, and Therapy. Osteoporosis prevention, diagnosis, and therapy. JAMA 2001; 285: 785-795.

[6] Favus MJ and American Society for Bone and Mineral Research. Primer on the Metabolic Bone Diseases and Disorders of Mineral Metabolism. Washington, DC, American Society for Bone and Mineral Research. 2006.

[7] Lerner UH. Bone remodeling in post-menopausal osteoporosis. J Dent Res 2006; 85: 584-595.

[8] Becker C. Pathophysiology and clinical manifestations of osteoporosis. Clin Cornerstone 2006; 8: 19-27.

[9] Marcus R. Osteoporosis. Amsterdam; Boston, Elsevier Academic Press. 2008.

[10] Briggs AM, Greig AM and Wark JD. The vertebral fracture cascade in osteoporosis: a review of aetiopathogenesis. Osteoporos Int 2007; 18: 575-584.

[11] McDonnell P, McHugh PE and O'Mahoney D. Vertebral osteoporosis and trabecular bone quality. Ann Biomed Eng 2007; 35: 170-189.

[12] Raisz LG. Physiology and pathophysiology of bone remodeling. Clin Chem 1999; 45: 1353-1358.

[13] Hadjidakis DJ and Androulakis II. Bone remodeling. Ann N Y Acad Sci 2006; 1092: 385-396.

[14] Takayanagi H. Osteoimmunology: shared mechanisms and crosstalk between the immune and bone systems. Nat Rev Immunol 2007; 7: 292-304.

[15] Weitzmann MN and Pacifici R. Estrogen regulation of immune cell bone interactions. Ann N Y Acad Sci 2006; 1068: 256-274.

[16] Weitzmann MN and Pacifici R. T cells: unexpected players in the bone loss induced by estrogen deficiency and in basal bone homeostasis. Ann N Y Acad Sci 2007; 1116: 360-375.

[17] Ross FP. Interleukin 7 and estrogen-induced bone loss. Trends Endocrinol Metab 2003; 14: 147-149.

[18] Zallone A. Direct and indirect estrogen actions on osteoblasts and osteoclasts. Ann N Y Acad Sci 2006; 1068: 173-179.

[19] Weitzmann MN, Roggia C, Toraldo G, Weitzmann L and Pacifici R. Increased production of IL-7 uncouples bone formation from bone resorption during estrogen deficiency. J Clin Invest 2002; 110: 1643-1650.

[20] Nakamura T, Imai Y, Matsumoto T*, et al*. Estrogen prevents bone loss via estrogen receptor alpha and induction of Fas ligand in osteoclasts. Cell 2007; 130: 811-823.

[21] Novack DV. Estrogen and bone: osteoclasts take center stage. Cell Metab 2007; 6: 254-256.

[22] Krum SA, Miranda-Carboni GA, Hauschka PV*, et al*. Estrogen protects bone by inducing Fas ligand in osteoblasts to regulate osteoclast survival. EMBO J 2008; 27: 535-545.

[23] Chaffins JA. Paget disease of bone. Radiol Technol 2007; 79: 27-40; quiz 41-3.

[24] Roodman GD and Windle JJ. Paget disease of bone. J Clin Invest 2005; 115: 200-208.

[25] Daroszewska A and Ralston SH. Mechanisms of disease: genetics of Paget's disease of bone and related disorders. Nat Clin Pract Rheumatol 2006; 2: 270-277.

[26] Xing L, Bushnell TP, Carlson L*, et al*. NF-kappaB p50 and p52 expression is not required for RANK-expressing osteoclast progenitor formation but is essential for RANK- and cytokine-mediated osteoclastogenesis. J Bone Miner Res 2002; 17: 1200-1210.

[27] Jones DH, Kong YY and Penninger JM. Role of RANKL and RANK in bone loss and arthritis. Ann Rheum Dis 2002; 61 Suppl 2: ii32-9.

[28] Theill LE, Boyle WJ and Penninger JM. RANK-L and RANK: T cells, bone loss, and mammalian evolution. Annu Rev Immunol 2002; 20: 795-823.

[29] Khosla S. Minireview: the OPG/RANKL/RANK system. Endocrinology 2001; 142: 5050-5055.

[30] Feige U. Osteoprotegerin. Ann Rheum Dis 2001; 60 Suppl 3: iii81-4.

[31] Ciani B, Layfield R, Cavey JR, Sheppard PW and Searle MS. Structure of the ubiquitin-associated domain of p62 (SQSTM1) and implications for mutations that cause Paget's disease of bone. J Biol Chem 2003; 278: 37409-37412.

[32] Duran A, Serrano M, Leitges M*, et al*. The atypical PKC-interacting protein p62 is an important mediator of RANK-activated osteoclastogenesis. Dev Cell 2004; 6: 303-309.

[33] Moscat J and Diaz-Meco MT. The atypical PKC scaffold protein P62 is a novel target for anti-inflammatory and anti-cancer therapies. Adv Enzyme Regul 2002; 42: 173-179.

[34] Zhang P, Chan J, Dragoi AM*, et al*. Activation of IKK by thymosin alpha1 requires the TRAF6 signalling pathway. EMBO Rep 2005; 6: 531-537.

[35] Dai RM and Li CC. Valosin-containing protein is a multi-ubiquitin chain-targeting factor required in ubiquitin-proteasome degradation. Nat Cell Biol 2001; 3: 740-744.

[36] Rodgers SE, Lew JI and Solorzano CC. Primary hyperparathyroidism. Curr Opin Oncol 2008; 20: 52-58.

[37] Hamann KL and Lane NE. Parathyroid hormone update. Rheum Dis Clin North Am 2006; 32: 703-719.

[38] Nagano N. Pharmacological and clinical properties of calcimimetics: calcium receptor activators that afford an innovative approach to controlling hyperparathyroidism. Pharmacol Ther 2006; 109: 339-365.

[39] Shoback D. Update in osteoporosis and metabolic bone disorders. J Clin Endocrinol Metab 2007; 92: 747-753.

[40] Murray TM, Rao LG, Divieti P and Bringhurst FR. Parathyroid hormone secretion and action: evidence for discrete receptors for the carboxyl-terminal region and related biological actions of carboxyl- terminal ligands. Endocr Rev 2005; 26: 78-113.

[41] Huang JC, Sakata T, Pfleger LL*, et al*. PTH differentially regulates expression of RANKL and OPG. J Bone Miner Res 2004; 19: 235-244.

[42] Qin L, Raggatt LJ and Partridge NC. Parathyroid hormone: a double-edged sword for bone metabolism. Trends Endocrinol Metab 2004; 15: 60-65.

[43] Li X, Qin L, Bergenstock M, Bevelock LM, Novack DV and Partridge NC. Parathyroid hormone stimulates osteoblastic expression of MCP-1 to recruit and increase the fusion of pre/osteoclasts. J Biol Chem 2007; 282: 33098-33106.

[44] Teitelbaum SL. Bone resorption by osteoclasts. Science 2000; 289: 1504-1508.

[45] Malluche HH, Koszewski N, Monier-Faugere MC, Williams JP and Mawad H. Influence of the parathyroid glands on bone metabolism. Eur J Clin Invest 2006; 36 Suppl 2: 23-33.

[46] Roodman GD. Advances in bone biology: the osteoclast. Endocr Rev 1996; 17: 308-332.

[47] Blair HC, Robinson LJ and Zaidi M. Osteoclast signalling pathways. Biochem Biophys Res Commun 2005; 328: 728-738.

[48] Ferguson PJ and El-Shanti HI. Autoinflammatory bone disorders. Curr Opin Rheumatol 2007; 19: 492-498.

[49] Yamaguchi T, Dorfman HD and Eisig S. Cherubism: clinicopathologic features. Skeletal Radiol 1999; 28: 350-353.

[50] FRANKLIN AW. Three cases of cherubism. Proc R Soc Med 1958; 51: 286-288.

[51] Schultze-Mosgau S, Holbach LM and Wiltfang J. Cherubism: clinical evidence and therapy. J Craniofac Surg 2003; 14: 201-6; discussion 207-8.

[52] JONES WA, GERRIE J and PRITCHARD J. Cherubism--a familial fibrous dysplasia of the jaws. Oral Surg Oral Med Oral Pathol 1952; 5: 292-305.

[53] Tiziani V, Reichenberger E, Buzzo CL*, et al*. The gene for cherubism maps to chromosome 4p16. Am J Hum Genet 1999; 65: 158-166.

[54] Mangion J, Rahman N, Edkins S*, et al*. The gene for cherubism maps to chromosome 4p16.3. Am J Hum Genet 1999; 65: 151-157.

[55] Ueki Y, Lin CY, Senoo M*, et al*. Increased myeloid cell responses to M-CSF and RANKL cause bone loss and inflammation in SH3BP2 "cherubism" mice. Cell 2007; 128: 71-83.

[56] Kitaura H, Zhou P, Kim HJ, Novack DV, Ross FP and Teitelbaum SL. M-CSF mediates TNF-induced inflammatory osteolysis. J Clin Invest 2005; 115: 3418-3427.

[57] Asagiri M and Takayanagi H. The molecular understanding of osteoclast differentiation. Bone 2007; 40: 251-264.

[58] Novack DV and Faccio R. Jawing about TNF: new hope for cherubism. Cell 2007; 128: 15-17.

CHAPTER V. BONE PHARMACOLOGY: TARGETING THE OSTEOCLAST

For diseases associated with excessive bone resorption, osteoclasts are the primary therapeutic targets [1]. The bisphosphonate is currently considered a major class of antiresorptive drug because of its ability to induce osteoclast apoptosis [2]. Meanwhile, stimulators of bone formation can also be a desirable addition to antiresorptive therapy. However, clinical application of the most potent anabolic agent available, fluoride, still remains controversial [3].

5.1. Bisphosphonates

Bisphosphonates are the primary drugs prescribed for the treatment of osteoporosis and other bone diseases associated with excessive bone resorption [2]. Bisphosphonates, formerly called diphosphonates, are stable analogues of naturally-occurring inorganic pyrophosphate (PPi) [2]. In bisphosphonates, the two phosphate groups are connected by a carbon atom, rather than an oxygen atom as in pyrophosphate (Figure 5.1.), which makes the compounds resistant to chemical and enzymatic hydrolysis [4].

Like pyrophosphate, bisphosphonates have high binding affinity for bone mineral. Exploration of bisphosphonates as potential inhibitors of bone absorption was initiated by its property of inhibiting hydroxyapatite dissolution [4]. However, later studies have shown that

the anti-resorptive action of these compounds is mediated by cellular mechanisms rather than simply physicochemical mechanisms [2].

5.1.1. Bone Uptake of Bisphosphonates

After administration, approximately 1/3~2/3 of bisphosphonates are localized in bone, and the remaining are removed by urinary excretion within the first few hours. Intestinal absorption of bisphosphonates is very low [4]. Bisphosphonates initially localize to bone-forming sites and bone-resorbing sites, where a large amount of exposed mineral is available [2]. Much of the bisphosphonates binding to bone are rapidly buried in bone and become pharmacologically inactive. These bisphosphonates can be retained for a long period of time within bone and can also be released from bone surfaces [2, 4]. Two mechanisms of the release of bisphosphonates from bone surfaces have been proposed: 1) chemical desorption occurring when bisphosphonate concentration in the extracellular fluid is dropped; 2) osteoclastic bone resorption [2].

5.1.2. Cellular Uptake of Bisphosphonates

Due to their marked binding affinity for bone rather than other tissues, bisphosphonates are in close contact with bone cells [2]. Using fluorescent-labeled bisphosphonates, researchers have localized bisphosphonates in intracellular endocytic vesicles [5, 6]; acidification of these vesicles appears to be necessary for the movement of bisphosphonates from vesicles into the cytosol [6]. Compared to non-resorbing cells such as osteoblasts and macrophages, osteoclasts are able to internalize a larger amount of

bisphosphonates because of their ability to release these compounds from hydroxyapatite during bone resorption [5].

5.1.3. Biochemical Effects of Bisphosphonates on Osteoclasts

According to their modes of action, bisphosphonates can be classified into two major groups (Figure 5.2.). The first group is the simpler, non–nitrogen-containing bisphosphonates, including clodronate and etidronate; while the second group is the more potent, nitrogen-containing bisphosphonates, including alendronate, risedronate, and zoledronate [4].

5.1.3.1. Non–nitrogen-containing Bisphosphonates

The simple, non–nitrogen-containing bisphosphonate can be incorporated into a non-hydrolyzable analogue of adenosine triphosphate (ATP or Appp), AppCp, which contains the P-C-P moiety in place of the β, γ-phosphate groups present in ATP [4, 7]. This incorporation is brought about by the cyoplasmic enzyme, aminoacyl-tRNA synthetase, which plays an essential role in protein synthesis. This enzyme can catalyse a two-step reaction to form an aminoacyl-tRNA (Figure 5.3.) [4, 8]. First, in a reversible reaction, an amino acid condenses with ATP (Appp) to form an aminoacyl-adenylate (amino acid-AMP), releasing pyrophosphate (PPi) (reaction I). The amino acid-AMP then condenses with a molecule of tRNA to form aminoacyl-tRNA (reaction II). Since bisphosphonates (pCp) with short side chains resemble PPi structure, the reverse reaction of reaction I can occur with pCp in place of PPi, forming an analogue of ATP, AppCp [7]. The ATP analogues are nonhydrolysable and are accumulated intracellularly in osteoclasts, causing osteoclast

apoptosis, possibly by the inhibition of intracellular ATP-dependent enzymes [4]. In summary, non–nitrogen-containing bisphosphonates can be metabolically incorporated into non-hydrolysable analogues of ATP and thereby cause osteoclast apoptosis.

5.1.3.2. Nitrogen-containing Bisphosphonates

Nitrogen-containing bisphosphonates induce osteoclast apoptosis predominantly by the inhibition of the mevalonate pathway. The mevalonate pathway is an important biosynthetic pathway responsible for the production of cholesterol and isoprenoid lipids, such as isopentenyl pyrophosphate (IPP), farnesyl pyrophosphate (FPP), and geranylgeranyl pyrophosphate (GGPP) (Figure5.4.) [8, 9]. The sequential condensation of isopentenyl pyrophosphate (IPP) with dimethylallyl pyrophosphate (DMAP) and geranyl pyrophosphate (GPP) is catalyzed by an enzyme named farnesyl pyrophosphate synthase (FPPS) (Figure 5.4.). Nitrogen-containing bisphosphonates act as substrate analogues for GPP and compete with GPP for the binding of FPPS, leading to competitive inhibition of FPPS [4, 7].

FPP and GGPP are substrates for the prenylation (post-translational modification) of small GTPases, which are important for osteoclast functions and survival [4, 10]. Therefore, by inhibiting FPPS, nitrogen-containing bisphosphonates can block the synthesis of FPP and GGPP and subsequent prenylation of small GTPase proteins, leading to osteoclast apoptosis [2].

In summary, by inhibiting farnesyl pyrophosphate synthase (FPPS) in the mevolonate pathway, nitrogen-containing bisphosphonates can inhibit the synthesis of isoprenoid compounds, which are substrates for the prenylation of GTPases. This leads to the disruption of GTPases functions and eventually the apoptosis of osteoclasts.

5.1.4. Biochemical Effects of Bisphosphonates on Osteoblasts and Osteocytes

Recent studies have suggested that bisphosphonates can protect osteocytes and osteoblasts from apoptosis [2, 11]. Bisphosphonate treatment leads to the opening of a gap junction protein present in osteoblasts and osteocytes—connexin 43 hemichannels [11-14]. Connexin 43 hemichannel opening results in the activation extracellular signal-regulated kinases (ERKs), promoting cell survival [11, 12]. In contrast, induction of osteoblast apoptosis by bisphosphonates has also been reported [15]. Moreover, investigation regarding the direct action of bisphosphonates on osteoblast differentiation and proliferation has yielded mixed results [15-18]. Therefore, to fully understand the effects of bisphosphonate on bone, the potential interaction of bisphosphonates with osteoblasts/osteocytes needs to be further determined.

5.2. Fluoride

Fluorine is the 17th most abundant element in the earth's crust [19]. Fluoride can be found from a variety of sources and is present in the food chain consumed by humans. The normal food chain supports plasma levels in adults ranging from 0.5 to 2.3 μM [19]. Fluoride is rapidly and extensively absorbed from the gastrointestinal tract. It is removed from plasma by urinary excretion and bone uptake [19, 20].

5.2.1. *In Vivo* Effects on Bone

It has long been noticed that fluoride exerts a biphasic action on bone [21, 22]. There is a correlation of the severity of skeletal changes and the magnitude and duration of fluoride exposure. At low levels of chronic exposure, fluoride can increase bone density with little effect on the overall health of bone, while prolonged and heavy exposure results in marked skeletal changes, including periosteal bone formation, ligament calcification, and joint stiffness [19]. The latter is known as skeletal fluorosis, a bone and joint condition that can be categorized into occupational and endemic skeletal fluorosis [23].

This dual action of fluoride on bone has led to exploration of the therapeutic potential of fluoride in osteoporosis and other bone disease associated with bone loss. Clinical trials have consistently shown that fluoride can increase spinal bone mass in a dose dependent manner; however, there is disagreement as to its ability to reduce vertebral fracture risk [3, 19]. Morphology studies in humans and animal models have found that new bone formed with fluoride treatment is often histologically abnormal. Woven bone rather lamellar bone is formed. Generalized or focal osteomalacia is also observed, indicating a deficiency in mineralization [24]. It is therefore proposed that the failure of fluoride to reduce vertebral fracture risk may be partially caused by the abnormal structure and compromised strength of fluoride-treated bone [3, 24].

Due to fluoride's similar size and charge to the hydroxyl ion— the normal component of hydroxyapatite, fluoride can substitute the hydroxyl group to form fluorapatite [25]. Compared to hydroxyapatite, fluoroapatite has a greater crystallinity, larger crystal size, and lower solubility. Moreover, fluoride can inhibit the dissolution of pre-formed hydroxyapatite, likely by forming a surface layer of less soluble fluoroapatite [26]. However,

the biological significance of these findings is not clear. No evidence has suggested that the formation of fluoroapatite *per se* alters the mechanical property of bone [21]. Therefore, the defective structure of fluoride-treated bone is probably caused by the cellular action of fluoride, rather than to its physicochemical action.

5.2.2. *In Vitro* Effects on Osteoblasts

The first *in vitro* evidence demonstrated that fluoride (~10μM) can increase the proliferation and differentiation of bone-forming cells derived from chick embryonic calvaria [27]. Despite some negative results reported [28-31], the direct action of fluoride on osteoblastic proliferation and differentiation has been largely confirmed in similar or other osteoblast-like cell culture system [32-35]. Studies have also showed that *in vitro* fluoride treatment at similar concentrations can stimulate the proliferation and differentiation of osteoprogenitors (pre-osteoblasts) [34, 36], which are more sensitive to fluoride than mature osteoblasts [35]. It is therefore largely accepted that fluoride can enhance osteoblastic proliferation and activity, but the underlying mechanism has not been completely defined yet.

It appears that fluoride can enhance the Ras-activated MAP Kinase (MAPK) pathway and thereby increase osteoblast proliferation. Two competing models regarding the underlying mechanism have been proposed [3, 37]. 1) The tyrosine phosphatase hypothesis: fluoride directly inhibits a tyrosine phosphatase, resulting in increased tyrosine phosphorylation levels of signaling molecules involved in the Ras-Raf-MEK-ERK pathway [37-40]. This unique fluoride-sensitive tyrosine phosphatase has been recently suggested to be the tartrate-resistant acid phosphatase (TRAP), which is specifically expressed by

osteoblasts [41, 42]. 2) The G protein (guanine nucleotide binding protein) hypothesis: fluoride first forms a fluoroalumino complex with aluminum—fluoroaluminate. This complex interacts with GDP (guanosine diphosphate) to form a GTP (guanosine triphosphate)-like molecule, thereby activating a G protein. This leads to the stimulation of an unknown tyrosine kinase, which then increases the phosphorylation levels of signaling molecules in Ras-Raf-MEK-ERK pathway [43-48].

The development of osteoclast biology in the late 1990s has provided us a great opportunity to study fluoride's effects on bone cells in a context of osteoblasts and osteoclasts interaction. Interestingly, it has been reported that fluoride treatment of osteoporosis patients with Crohn's disease can cause increased serum level of osteoprotegerin (OPG), an osteoblast-derived negative regulator of osteoclastogenesis [49]. This result indicates the effects of fluoride on bone cells may not be limited to osteoblasts and bone formation.

5.2.3. Effects on Osteoclasts and Bone Marrow Cells

Based on both *in vivo* and *in vitro* data, researchers have hypothesized that fluoride can promote osteoblastic bone formation independent of any coupling between bone resorption and formation [22, 50]. Effects of fluoride on bone formation have been explained solely by its stimulation of osteoblasts, so fewer attempts have been made to address fluoride's effects on bone resorption or cells of the osteoclastic lineage.

Using isolated chick or rabbit osteoclast culture system, two groups have found that fluoride can inhibit osteoclastic resorption at 500-1000μM and 15 mg/l /30mg/l (i.e. ~360μM/720μM), respectively [51, 52]. Although it is tempting to interpret this as a direct

action of fluoride on osteoclasts, it should be noted that in these studies cells from animal long bones represented a mixed cell population. In fact, it was shown that after 48 hours in such isolated osteoclast cultures stromal cells became the major cell population [53]. Therefore, inhibition of fluoride on osteoclastic resorption could be indirectly mediated by factors released by stimulated osteoblast or other cell types [53]. Another point that needs to consider is the relatively high fluoride dose used in these experiments. Millimolar (mM) of fluoride has long been used as a potent inhibitor of enzyme function; however, the optimal fluoride dose to stimulate osteoblasts *in vitro* is between 10-100μM [38]. Although the therapeutic window for serum fluoride levels has not been firmly established, the widely quoted range is 5 -10 micromolar (μM) [19], while other ranges were also used, such as 5-15 μM [54], ~8μM [55], and ~12μM [56]. Therefore, it is not likely that fluoride concentrations used in these studies and those described below is applicable to fluoride exposure seen in patients who may subject to fluoride therapy.

Studies of fluoride's effects on bone have also been extended to bone marrow, where the progenitors of bone cells reside. Studies have shown that fluoride at 100-500μM can upregulate the expression of granulocyte-specific markers by the HL-60 (human promyelocytic leukemia cells) cell line or primary mouse bone marrow cells [57, 58]. The authors concluded that fluoride can shift the differentiation of myeloid cell line, HL-60 or primary bone marrow cells along the granulocytic but not the monocytic pathway, suggesting that fluoride may suppress the development of osteoclasts from bone marrow hematopoietic progenitor cells [57, 58]. However, stimulation of one differentiation pathway does not necessarily lead to suppression of another. Collectively, so far there is no

direct evidence for an inhibitory action of fluoride on osteoclasts or their precursors in bone marrow.

However, a recent publication has described a novel stimulatory action of fluoride on osteoclast differentiation [59]. C57BL/6J and C3H/HeJ mice—two inbred strains of mice widely used in the study of bone biology—were treated with fluoride in drinking water for three weeks (0 ppm, 50 ppm, and 100 ppm). Anabolic effects of fluoride in C57BL/6J mice were observed. Surprisingly, stimulation of osteoclastic lineages cells was observed in C3H/HeJ mice [59]. Bone marrow cells from C3H/HeJ mice showed increased osteoclast potential in culture in response to soluble RANKL and M-CSF with increasing *in vivo* fluoride exposure [59]. Consistently, *in situ* trabecular osteoclast number, osteoclast hematopoietic colony forming units, and serum makers for osteoclastic bone resorption (PTH, RANKL, and TRAP5b) were all increased dose-dependently. Moreover, serum OPG was decreased [59]. To date, this is the first report of a stimulatory effect of fluoride on osteoclasts. The underlying mechanism needs to be further determined as it will provide us a new perspective on fluoride effect on bone remodeling process.

5.3. Chapter Summary

The bisphosphonate is currently considered a major class of drug for osteoporosis and other diseases associated with increased bone resorption. These compounds can induce osteoclast apoptosis. Based on their modes of action, bisphosphonates can be classified into two major groups. Non–nitrogen-containing bisphosphonates induce osteoclast apoptosis by forming toxic non-hydrolysable analogues of ATP, while nitrogen-containing bisphosphonates do so by inhibiting production of substrates for the prenylation of proteins

that are essential for osteoclast survival. Interestingly, the effects of bisphosphonates on osteoblast cell lineages have also been explored but with inconsistent results.

Fluoride is a potent anabolic agent, but its clinical application in treatment of osteoporosis and other bone diseases has been controversial. Fluoride's stimulation on osteoblast proliferation has been well established, but the underlying mechanism is unclear. Moreover, little is known about its action on another important cell type involved in bone remodeling—osteoclasts. A recent study has revealed a stimulatory effect of fluoride on osteoclastogenesis. To harness the desirable action of fluoride while limiting its undesirable effects, we need to further understand fluoride's effects on bone at both cellular and molecular levels.

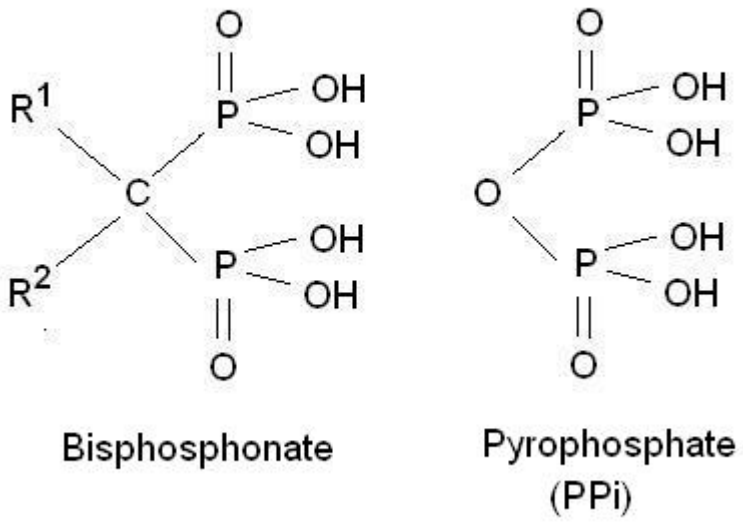

Figure 5.1. The structure of bisphosphonate and pyrophosphate.

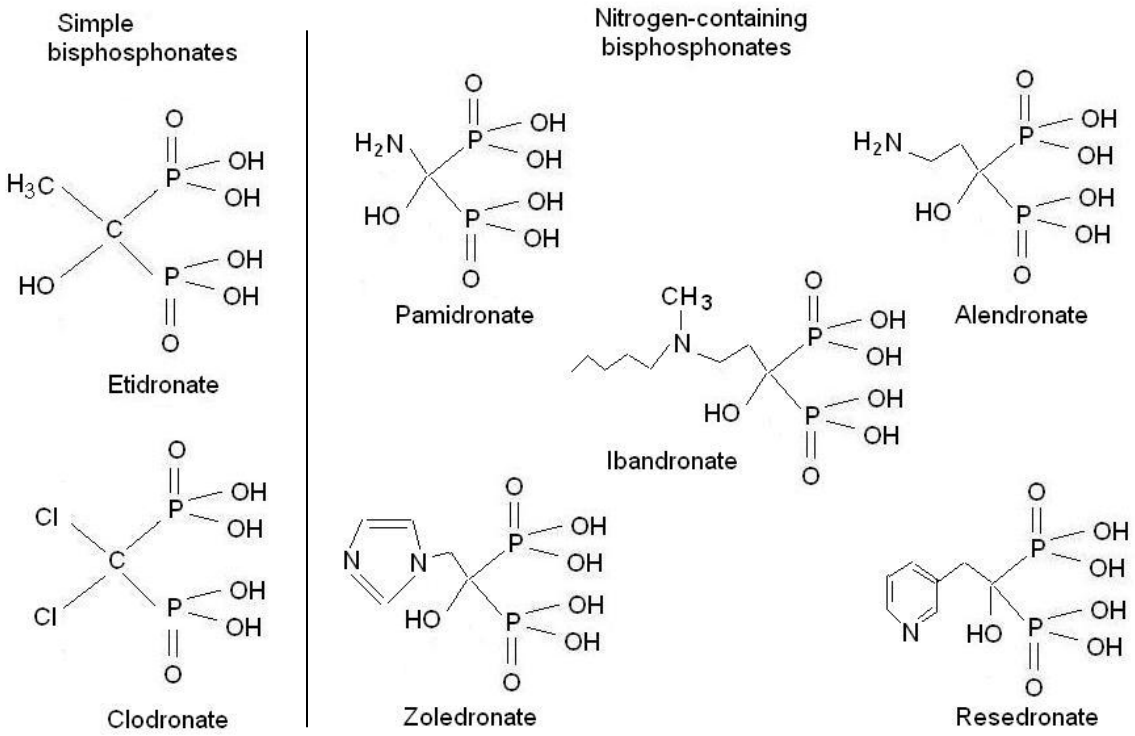

Figure 5.2. Structures of bisphosphonates classified according to their biochemical modes of action. Adapted from reference 4.

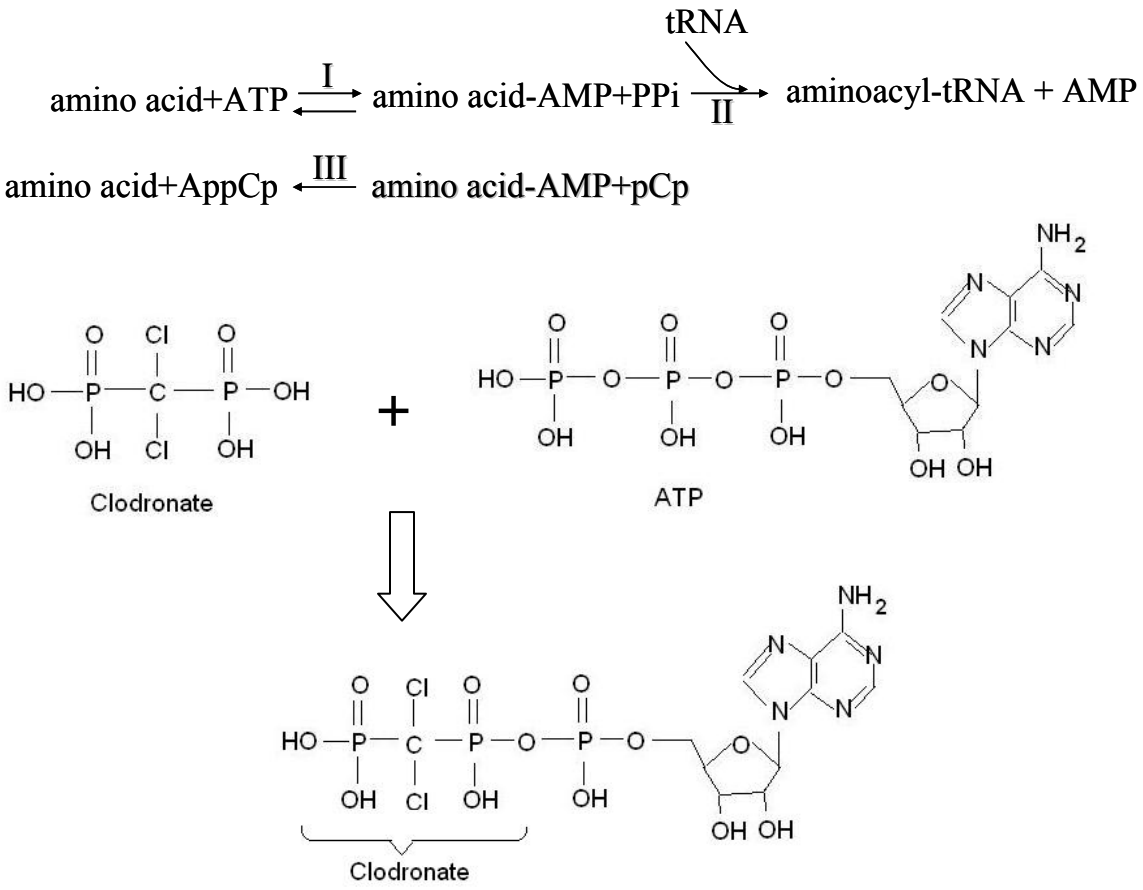

Figure 5.3. Formation of AppCp-type metabolites of simple bisphosphonates. Upper panel: Mechanism by which aminoacyl-tRNA synthetases catalyze formation of AppCp-type metabolites of bisphosphonates. In a reversible reaction, an amino acid condenses with ATP (Appp) to form an aminoacyl-adenylate (amino acid-AMP), releasing pyrophosphate (PPi) (reaction I). The aminoacyl-adenylate then condenses with a molecule of tRNA to form aminoacyl-tRNA (reaction II). Since non–nitrogen-containing bisphosphonates (pCp) resemble PPi in structure, the reverse reaction of (I) can occur with pCp in place of PPi, forming an analogue of ATP (AppCp). Lower panel: Non–nitrogen-containing bisphosphonate, clodronate, substitutes the pyrophosphate group in ATP to form an ATP analogue. Adapted from references 4 and 7.

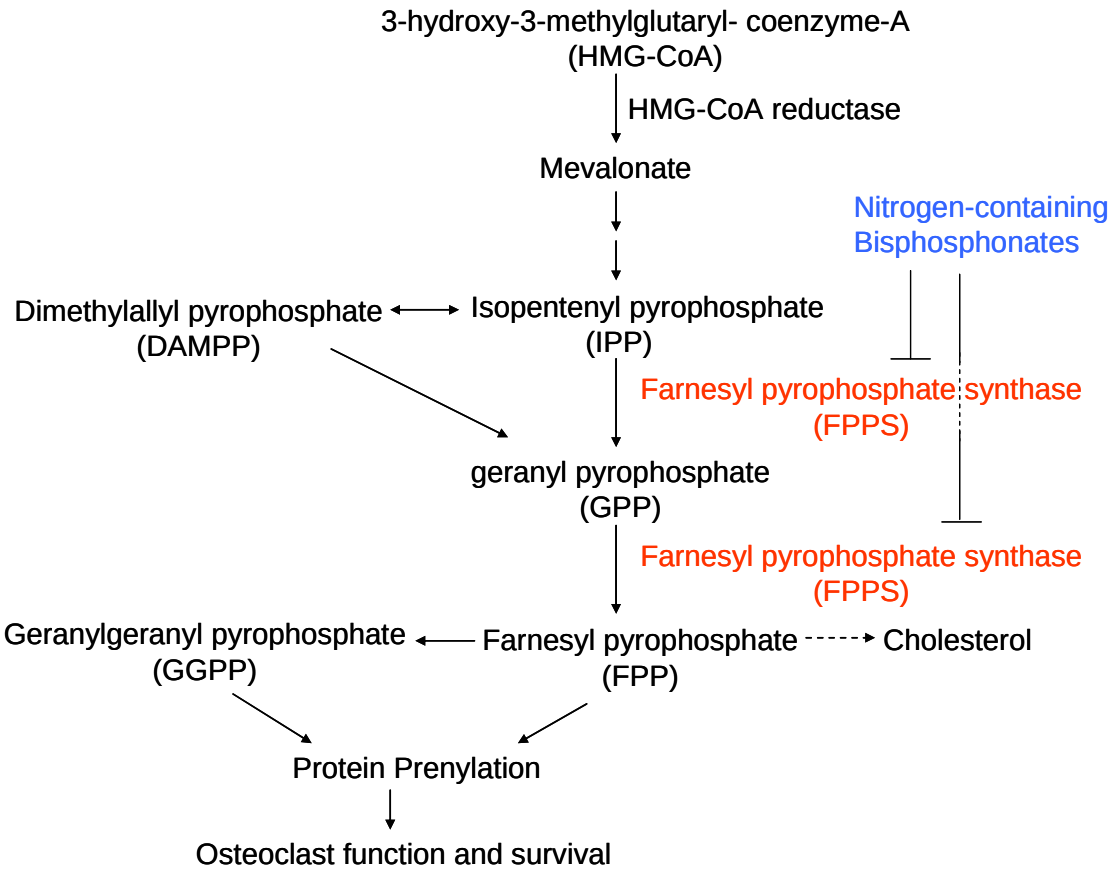

Figure 5.4. Mechanism of action of nitrogen-containing bisphosphonates on osteoclasts. The mevalonate pathway is a biosynthetic pathway responsible for generating cholesterol and isoprenoid lipids. HMG-CoA reductase catalyzes conversion of HMG-CoA to mevolonate. Successive phosphorylation of mevalonate with ATP leads to formation of isopentenyl pyrophosphate (IPP). IPP is then isomerized to dimethylallyl pyrophosphate (DAMPP). Condensation of IPP and DAMPP results in formation of geranyl pyrophosphate (GPP). GPP then condenses with IPP to form farnesyl pyrophosphate (FPP). FPP can be further converted to cholesterol. Meanwhile, condensation of FPP and IPP leads to formation of geranylgeranyl pyrophosphate (GGPP). FPP and GGPP are substrates for prenylation of small GTPases, which are important for osteoclast functions and survival. Farnesyl pyrophosphate synthase (FPPS) catalyzes sequential condensation of IPP with DAMAP and GPP. Nitrogen-containing bisphosphonates act as substrate analogues for GPP and compete with GPP for FPPS binding, leading to competitive inhibition of FPPS. Therefore, by inhibiting FPPS, nitrogen-containing bisphosphonates block synthesis of FPP and GGPP and subsequent prenylation of small GTPase proteins, leading to osteoclast apoptosis. Adapted from references 4 and 7.

Chapter V. Reference List

[1] Rodan GA and Martin TJ. Therapeutic approaches to bone diseases. Science 2000; 289: 1508-1514.

[2] Russell RG, Watts NB, Ebetino FH and Rogers MJ. Mechanisms of action of bisphosphonates: similarities and differences and their potential influence on clinical efficacy. Osteoporos Int 2008.

[3] Caverzasio J, Palmer G and Bonjour JP. Fluoride: mode of action. Bone 1998; 22: 585-589.

[4] Russell RG. Bisphosphonates: mode of action and pharmacology. Pediatrics 2007; 119 Suppl 2: S150-62.

[5] Coxon FP, Thompson K, Roelofs AJ, Ebetino FH and Rogers MJ. Visualizing mineral binding and uptake of bisphosphonate by osteoclasts and non-resorbing cells. Bone 2008.

[6] Thompson K, Rogers MJ, Coxon FP and Crockett JC. Cytosolic entry of bisphosphonate drugs requires acidification of vesicles after fluid-phase endocytosis. Mol Pharmacol 2006; 69: 1624-1632.

[7] Russell RG. Bisphosphonates: from bench to bedside. Ann N Y Acad Sci 2006; 1068: 367-401.

[8] Rogers MJ, Gordon S, Benford HL*, et al*. Cellular and molecular mechanisms of action of bisphosphonates. Cancer 2000; 88: 2961-2978.

[9] Bhagavan NV. Medical Biochemistry. San Diego, Harcourt/Academic Press. 2002.

[10] Coxon FP and Rogers MJ. The role of prenylated small GTP-binding proteins in the regulation of osteoclast function. Calcif Tissue Int 2003; 72: 80-84.

[11] Plotkin LI, Manolagas SC and Bellido T. Dissociation of the pro-apoptotic effects of bisphosphonates on osteoclasts from their anti-apoptotic effects on osteoblasts/osteocytes with novel analogs. Bone 2006; 39: 443-452.

[12] Plotkin LI, Aguirre JI, Kousteni S, Manolagas SC and Bellido T. Bisphosphonates and estrogens inhibit osteocyte apoptosis via distinct molecular mechanisms downstream of extracellular signal-regulated kinase activation. J Biol Chem 2005; 280: 7317-7325.

[13] Plotkin LI and Bellido T. Bisphosphonate-induced, hemichannel-mediated, anti-apoptosis through the Src/ERK pathway: a gap junction-independent action of connexin43. Cell Commun Adhes 2001; 8: 377-382.

[14] Stains JP and Civitelli R. Gap junctions in skeletal development and function. Biochim Biophys Acta 2005; 1719: 69-81.

[15] Idris AI, Rojas J, Greig IR, Van't Hof RJ and Ralston SH. Aminobisphosphonates Cause Osteoblast Apoptosis and Inhibit Bone Nodule Formation In Vitro. Calcif Tissue Int 2008.

[16] Reinholz GG, Getz B, Pederson L*, et al*. Bisphosphonates directly regulate cell proliferation, differentiation, and gene expression in human osteoblasts. Cancer Res 2000; 60: 6001-6007.

[17] Giuliani N, Pedrazzoni M, Negri G, Passeri G, Impicciatore M and Girasole G. Bisphosphonates stimulate formation of osteoblast precursors and mineralized nodules in murine and human bone marrow cultures in vitro and promote early osteoblastogenesis in young and aged mice in vivo. Bone 1998; 22: 455-461.

[18] Sarin J, Derossi SS and Akintoye SO. Updates on bisphosphonates and potential pathobiology of bisphosphonate-induced jaw osteonecrosis. Oral Dis 2008; 14: 277-285.

[19] Kleerekoper M. The role of fluoride in the prevention of osteoporosis. Endocrinol Metab Clin North Am 1998; 27: 441-452.

[20] Whitford GM. Intake and metabolism of fluoride. Adv Dent Res 1994; 8: 5-14.

[21] Pak CY, Zerwekh JE and Antich P. Anabolic effects of fluoride on bone. Trends Endocrinol Metab 1995; 6: 229-234.

[22] Kleerekoper M. Fluoride and the skeleton. Crit Rev Clin Lab Sci 1996; 33: 139-161.

[23] Cundy T. Recovery from skeletal fluorosis. J Bone Miner Res 2007; 22: 1475; author reply 1476.

[24] Kleerekoper M and Mendlovic DB. Sodium fluoride therapy of postmenopausal osteoporosis. Endocr Rev 1993; 14: 312-323.

[25] NEUMAN WF, NEUMAN MW, MAIN ER, O'LEARY J and SMITH FA. The surface chemistry of bone. II. Fluoride deposition. J Biol Chem 1950; 187: 655-661.

[26] Grynpas MD and Cheng PT. Fluoride reduces the rate of dissolution of bone. Bone Miner 1988; 5: 1-9.

[27] Farley JR, Wergedal JE and Baylink DJ. Fluoride directly stimulates proliferation and alkaline phosphatase activity of bone-forming cells. Science 1983; 222: 330-332.

[28] Kopp JB and Robey PG. Sodium fluoride does not increase human bone cell proliferation or protein synthesis in vitro. Calcif Tissue Int 1990; 47: 221-229.

[29] Kopp JB and Robey PG. Sodium fluoride lacks mitogenic activity for fetal human bone cells in vitro. J Bone Miner Res 1990; 5 Suppl 1: S137-41.

[30] Chavassieux P, Boivin G, Serre CM and Meunier PJ. Fluoride increases rat osteoblast function and population after in vivo administration but not after in vitro exposure. Bone 1993; 14: 721-725.

[31] Chavassieux P, Chenu C, Valentin-Opran A*, et al*. In vitro exposure to sodium fluoride does not modify activity or proliferation of human osteoblastic cells in primary cultures. J Bone Miner Res 1993; 8: 37-44.

[32] Reed BY, Zerwekh JE, Antich PP and Pak CY. Fluoride-stimulated [3H]thymidine uptake in a human osteoblastic osteosarcoma cell line is dependent on transforming growth factor beta. J Bone Miner Res 1993; 8: 19-25.

[33] Khokher MA and Dandona P. Fluoride stimulates [3H]thymidine incorporation and alkaline phosphatase production by human osteoblasts. Metabolism 1990; 39: 1118-1121.

[34] Hall BK. Sodium fluoride as an initiator of osteogenesis from embryonic mesenchyme in vitro. Bone 1987; 8: 111-116.

[35] Kassem M, Mosekilde L and Eriksen EF. Effects of fluoride on human bone cells in vitro: differences in responsiveness between stromal osteoblast precursors and mature osteoblasts. Eur J Endocrinol 1994; 130: 381-386.

[36] Bellows CG, Heersche JN and Aubin JE. The effects of fluoride on osteoblast progenitors in vitro. J Bone Miner Res 1990; 5 Suppl 1: S101-5.

[37] Lau KH and Baylink DJ. Molecular mechanism of action of fluoride on bone cells. J Bone Miner Res 1998; 13: 1660-1667.

[38] Wu LW, Yoon HK, Baylink DJ, Graves LM and Lau KH. Fluoride at mitogenic doses induces a sustained activation of p44mapk, but not p42mapk, in human TE85 osteosarcoma cells. J Clin Endocrinol Metab 1997; 82: 1126-1135.

[39] Thomas AB, Hashimoto H, Baylink DJ and Lau KH. Fluoride at mitogenic concentrations increases the steady state phosphotyrosyl phosphorylation level of cellular proteins in human bone cells. J Clin Endocrinol Metab 1996; 81: 2570-2578.

[40] Hashimoto H and Lau KH. Differential effects of bacterial toxins on mitogenic actions of sodium fluoride and those of aluminum fluoride in human TE85 osteosarcoma cells. Mol Cell Biochem 2001; 228: 91-98.

[41] Lau KH and Baylink DJ. Osteoblastic tartrate-resistant acid phosphatase: its potential role in the molecular mechanism of osteogenic action of fluoride. J Bone Miner Res 2003; 18: 1897-1900.

[42] Perez-Amodio S, Vogels IM, Schoenmaker T*, et al*. Endogenous expression and endocytosis of tartrate-resistant acid phosphatase (TRACP) by osteoblast-like cells. Bone 2005; 36: 1065-1077.

[43] Caverzasio J, Palmer G, Suzuki A and Bonjour JP. Mechanism of the mitogenic effect of fluoride on osteoblast-like cells: evidences for a G protein-dependent tyrosine phosphorylation process. J Bone Miner Res 1997; 12: 1975-1983.

[44] Chae HJ, Chae SW, Kang JS, Kim DE and Kim HR. Mechanism of mitogenic effect of fluoride on fetal rat osteoblastic cells: evidence for Shc, Grb2 and P-CREB-dependent pathways. Res Commun Mol Pathol Pharmacol 1999; 105: 185-199.

[45] Freitas F, Jeschke M, Majstorovic I*, et al*. Fluoroaluminate stimulates phosphorylation of p130 Cas and Fak and increases attachment and spreading of preosteoblastic MC3T3-E1 cells. Bone 2002; 30: 99-108.

[46] Jeschke M, Standke GJ and Scaronuscarona M. Fluoroaluminate induces activation and association of Src and Pyk2 tyrosine kinases in osteoblastic MC3T3-E1 cells. J Biol Chem 1998; 273: 11354-11361.

[47] Susa M. Heterotrimeric G proteins as fluoride targets in bone (review). Int J Mol Med 1999; 3: 115-126.

[48] Susa M, Standke GJ, Jeschke M and Rohner D. Fluoroaluminate induces pertussis toxin-sensitive protein phosphorylation: differences in MC3T3-E1 osteoblastic and NIH3T3 fibroblastic cells. Biochem Biophys Res Commun 1997; 235: 680-684.

[49] von Tirpitz C, Klaus J, Steinkamp M*, et al*. Therapy of osteoporosis in patients with Crohn's disease: a randomized study comparing sodium fluoride and ibandronate. Aliment Pharmacol Ther 2003; 17: 807-816.

[50] Chavassieux P, Pastoureau P, Boivin G, Chapuy MC, Delmas PD and Meunier PJ. Dose effects on ewe bone remodeling of short-term sodium fluoride administration--a histomorphometric and biochemical study. Bone 1991; 12: 421-427.

[51] Okuda A, Kanehisa J and Heersche JN. The effects of sodium fluoride on the resorptive activity of isolated osteoclasts. J Bone Miner Res 1990; 5 Suppl 1: S115-20.

[52] Taylor ML, Maconnachie E, Frank K, Boyde A and Jones SJ. The effect of fluoride on the resorption of dentine by osteoclasts in vitro. J Bone Miner Res 1990; 5 Suppl 1: S121-30.

[53] Okuda A, Taylor LM and Heersche JN. Prostaglandin E2 initially inhibits and then stimulates bone resorption in isolated rabbit osteoclast cultures. Bone Miner 1989; 7: 255-266.

[54] Budden FH, Bayley TA, Harrison JE*, et al*. The effect of fluoride on bone histology in postmenopausal osteoporosis depends on adequate fluoride absorption and retention. J Bone Miner Res 1988; 3: 127-132.

[55] Kraenzlin ME, Kraenzlin C, Farley SM, Fitzsimmons RJ and Baylink DJ. Fluoride pharmacokinetics in good and poor responders to fluoride therapy. J Bone Miner Res 1990; 5 Suppl 1: S49-52.

[56] Wiers BH, Francis MD, Hovancik K, Ritchie CK and Baylink DJ. Theoretical physical chemical studies of the cause of fluoride-induced osteomalacia. J Bone Miner Res 1990; 5 Suppl 1: S63-70.

[57] Kawase T, Oguro A, Orikasa M and Burns DM. Characteristics of NaF-induced differentiation of HL-60 cells. J Bone Miner Res 1996; 11: 1676-1687.

[58] Oguro A, Kawase T and Orikasa M. NaF induces early differentiation of murine bone marrow cells along the granulocytic pathway but not the monocytic or preosteoclastic pathway in vitro. In Vitro Cell Dev Biol Anim 2003; 39: 243-248.

[59] Yan D, Gurumurthy A, Wright M, Pfeiler TW, Loboa EG and Everett ET. Genetic background influences fluoride's effects on osteoclastogenesis. Bone 2007; 41: 1036-1044.