REACTIVE OXYGEN SPECIES ARE ANTICATABOLIC TARGETS FOR OSTEOCLAST-RELATED BONE DISORDERS

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This thesis is presented for the degree of Doctor of Philosophy of The University of Western Australia

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THESIS DECLARATION

I, Kai Chen, certify that:

This thesis has been substantially accomplished during enrolment in this degree.

This thesis does not contain material which has been submitted for the award of any other degree or diploma in my name, in any university or other tertiary institution.

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The research involving animal data reported in this thesis was assessed and approved by The University of Western Australia Animal Ethics Committee (Approval #:RA/3/100/1244) and the Institutional Animal Ethics Committee of Guangzhou University of Chinese Medicine, China (Approval #:20190213001). The research involving animals reported in this thesis followed The University of Western Australia, Guangzhou University of Chinese Medicine and national standards for the care and use of laboratory animals.

The research involving human data reported in this thesis was assessed and approved by the Ethical Committee of the First Affiliated Hospital of Guangzhou University of Chinese Medicine, China Approval #: Y [2019] 141. Written patient consent has been received and archived for the research involving patient data reported in this thesis.

This thesis contains published work and/or work prepared for publication, some of which has been co-authored.

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Signature: 

Date: 24/07/2019
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East Perth, WA
29 June 2019
**AUTHORSHIP DECLARATION: CO-AUTHORED PUBLICATIONS**

The thesis is in agreement with The University of Western Australia Doctor of Philosophy Rules for the content and format of a thesis (39-45) and presented as a series of papers, including two peer-reviewed publications (Chapter 4 and 5) and a manuscript in preparation for submission (chapter 6).

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Bone is a self-renewal connective tissue continually undertaking a process of remodelling, including the removal of bone matrix which is carried out by osteoclasts and replacement of new bone matrix which is deposited by osteoblasts. Osteoclast is considered as the sole type of bone-resorbing cell originated from the monocyte/macrophage lineage. Excessive osteoclast formation and/or activity play a leading role in a variety of bone disorders, including osteoporosis, periprosthetic osteolysis, and subchondral bone destruction in osteonecrosis of femoral head (ONFH). Recently, accumulating evidence demonstrated that reactive oxygen species (ROS) play an essential role in osteoclastogenesis by mediating receptor activator of nuclear factor kappa-B ligand (RANKL)-induced downstream signalling. Thus, approaches targeting aberrant osteoclasts based on antioxidant strategy may remain promising in the treatment of osteoclast-related bone disorders.

In this study, two novel small molecules isolated from secondary metabolites of *Aspergillus fumigatus*, known as helvolic acid (HA) and pseurotin A (Pse) were demonstrated for the first time to inhibit osteoclast formation and resorptive activity by suppressing ROS levels. Pse was further administrated on the ovariectomized (OVX) mice osteoporosis model. Micro-CT and histological data indicated Pse was capable of preventing the OVX-induced bone loss by inhibiting ROS and osteoclasts in vivo. A novel technique using dihydroethidium (DHE) to visualize ROS in bone microenvironment was also developed in this study. Given the inspiring outcome that the treatment of osteoporosis by anti-catabolic interference via ROS suppression, we are also interested the role of ROS and osteoclast in the development of ONFH, which has not been fully elucidated previously. Our results of human patients and rat models demonstrated ROS may play a critical role in the alterations of bone structural integrity during the pathogenesis of ONFH by enhancing osteoclast activity, which provide rationales that antioxidant approaches could be potential for the prevention of the development and/or progression of ONFH.
PUBLICATIONS AND CONFERENCE ABSTRACTS

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CHAPTER ONE - GENERAL BONE BIOLOGY
1.1 Introduction

The skeletal system acts as a rigid framework that provides a structural support for the body, protects internal organs from trauma in all vertebrates, and facilitates locomotion by providing the levers for the muscles (1). Meanwhile, bone also serves as a reservoir of minerals as well as a site for hematopoiesis within the marrow environment (2). Bone is known as a physiologically dynamic tissue with the “paradoxical” properties: stiffness and strength to resist deformation, yet flexibility and lightness to adapt to mechanical loading by changing its size and shape without cracking (3). These unique mechanical properties of bone are achieved by the depositions of its constituent mineral crystals within the extracellular matrix (4, 5).

Bone is a self-renewing connective tissue which continually undertakes a process of remodeling throughout life to adapt to the external circumstances. Bone remodeling includes removal of bone matrix which is carried out by osteoclasts and replacement of new bone matrix which is deposited by osteoblasts. The precise coordination of osteoclastic bone resorption and osteoblastic bone formation is fundamental to maintain bone homeostasis. In this chapter, general bone biology, including bone anatomy and compositions, and bone cell biology will be reviewed.

1.2 Bone Anatomy and Composition

1.2.1 Extracellular bone matrix

Bone matrix (laid down by osteoblasts) is composed of two major components (6): organic matrix, approximately 95% of which is constituted by type I collagen, with the remaining being proteoglycans (such as chondroitin sulfate and hyaluronic acid) and a variety of non-collagenous proteins (osteocalcin, osteonectin, osteopontin, etc.); inorganic minerals, predominantly crystalline salts of calcium and phosphate, primarily deposited in the form of hydroxyapatite crystals \( \text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2 \). Together, collagen and minerals are highly interactive with the mineral distributed throughout the collagen fibrils to construct the bone, in which the minerals are responsible for the stiffness and rigidity whereas the collagen confers the elasticity and flexibility (3). Consequently, the energy derived from mechanical functions can be absorbed or dissipated properly. However, bone fracture can happen if the imposed load exceeds bone’s tolerance of deforming.
Bone matrix does not only physically act as a framework that provides mechanical support, but is also highly involved in regulating cellular activity through several adhesion molecules (7, 8). Integrins, a family of heterodimer transmembrane cell surface receptors, are the most predominant factors that mediate the cell-matrix interaction (9, 10). Osteoblasts interact with the surrounding bone matrix through integrins, which recognize and bind to Arg-Gly-Asp (RGD) motifs contained within several proteins within the bone including osteopontin, collagen, fibronectin, and bone sialoprotein. These interactions transduce extracellular signaling into intracellular responses, and are essential for osteoblasts to achieve their function during osteoid synthesis and bone mass acquisition (8, 11). For osteoclasts, integrins have also been long known to be important for their resorptive function through mediating attachment, cytoskeletal organization, and polarization (12, 13). Bone resorption is only achieved when osteoclastic cells tightly adhere to bone surface which are mineralized (14). Integrins αvβ3 and α2β1 are two major types known to interact with bone matrix when osteoclasts resorb bone by specific binding to proteins containing RGD such as fibronectin and bone sialoproteins, and collagen fibrils respectively (15, 16). The interaction between osteocytes and the bone matrix occurs through the lacuna wall, which is essential for mediating mechanosensitive signaling pathways in osteocytes when responding to dynamic fluid flow (17, 18).

1.2.2 Cortical bone and trabecular bone

Bones are mainly composed of two types of structural tissue termed as cortical (compact) bone and trabecular (cancellous) bone (Figure 1.1). Cortical bone endows the bone with resistance to bending and compressive strength; trabecular bone absorbs loading energy and allows deformation. Both consist of the same matrix components but tremendously differ in structure, porosity, and metabolic activity (19). Cortical bone accounts for 80% of the adult human skeleton by weight (1, 19). It is solid and densely packed, with the porosity ranging from 5% to 20%, featuring a slower metabolic rate than the trabecular bone. Trabecular bone has a porosity of 50 ~ 90%, with a higher surface area/volume ratio and thus leading to a higher remodeling rate.

Cortical bone is principally the diaphysis of long bones and comprises a large number of osteons. The osteon is the major structural unit that constitutes cortical bone. Each osteon appears as a cylindrical structure contained of extracellular matrix and osteocytes within their lacunae. Within each osteon runs the Haversian canal, containing blood vessels, lymphatics, and occasionally nerves (20). Cortical bone is covered by a membrane, called the periosteum on its external surface and
Figure 1.1 Bone structure. Long bone is composed of cortical bone and trabecular bone. Covered by periosteum, cortical bone is principally found in the shaft of long bones and comprises multiple osteons. The osteon is the major structural unit that constitutes cortical bone. Each osteon appears as a cylindrical structure with a central Haversian canal containing blood vessels, lymphatics, and occasionally nerves. Trabecular bone has a porosity of 50 ~ 90%, with a higher surface area/volume ratio. (This Figure was adapted from Structure and Architecture of Bone, R.Bartl and C.Bartl, 2017; DOI: https://doi.org/10.1007/978-3-319-29182-6_2; by permission from Springer Nature, License No. 4624100299450)
endosteum on its internal surface (Figure 1.1). The periosteum provides a pool of precursor cells which facilitates appositional bone growth and expands bone diameters; the endosteum has a higher remodeling activity likely due to a larger exposure to the bone marrow environment. It usually accommodates more osteoclasts to resorb bone from the endosteal surface, activation of bone resorption results in reduced cortical thickness and expansion of the bone marrow space (1). In contrast, trabecular bone is predominantly located in the metaphysis and epiphysis of long bone, with the trabeculae being precisely oriented along the weight-bearing line. It is highly porous and consists of interconnected pore space which is filled by bone marrow.

1.2.3 Bone cells
Bone tissue undergoes dynamic modelling and remodeling through the activity of bone cells. Ten percent of total bone volume is made of bone cells, which arise from two main cell lineages: osteoprogenitor cells (form osteoblasts and osteocytes) and hematopoietic cells (differentiate into osteoclasts) (1, 19). Osteoprogenitor cells are regarded as the stem cells of bone, which usually remain undifferentiated in the periosteum, endosteum, and bone marrow. After receiving signaling, they relocate to a site and “transform” into osteoblasts. Osteoblasts are tightly packed against each other and located at the bone surface, where they synthesize and secrete bone matrix after being activated. Well-known stimulators for osteoblast function are bone morphogenic proteins (BMPs) and the transforming growth factor-β family. Osteoblasts build new bone around themselves, when encased by the bone matrix made by themselves, osteoblasts become osteocytes in their lacunae. Osteocytes have relatively lower bone-forming activity than osteoblasts but account for the majority of bone cells (21, 22). Due to their relative inaccessibility in the bone matrix, the study of osteocytes is challenging. However, an emerging consensus was raised that osteocytes essentially contribute to the transmission of mechanical signals which maintains balanced bone remodeling (18, 22, 23). In the bone microenvironment, osteoclasts act as the only type of “bone eaters”, thus being indispensable for the maintenance of bone homeostasis but also mediating numerous skeletal diseases including osteoporosis (24). The biology of osteoclasts and osteoblasts will be reviewed in more detail in the section 1.3 (osteoclast biology) and section 1.4 (osteoblast biology).

1.2.4 Bone blood supply
Bone is a highly vascularized organ. In general, 10~20 % of resting cardiac output, roughly equivalent to the kidneys, supplies the skeleton (19). The main source of blood supply is from the diaphyseal nutrient arteries, which cross the cortex by penetrating through the nutrient canal. The diaphyseal
arteries supply the inner cortical bone and the medullary area. In the medullary area, these arteries separate into descending and ascending branches. Anastomosed with the diaphyseal capillaries, metaphyseal and epiphyseal arteries supply the two ends of bone and the adjacent joints. Periosteal blood vessels, passing through Volkmann’s canals and connected with diaphyseal capillaries, are another major contributor to the long bone (outer cortex). These vessels are highly interconnected to form a vast network and thus providing the conduits responsible for delivering nutrients and oxygen to bone. Recently, growing evidence also suggests bone-specific blood vessels (specialized type H bone capillaries) positively modulate osteogenesis through the angiocrine production of Noggin (25, 26). Similarly, factors secreted by osteoclasts and osteoblasts can regulate type H vessels in a paracrine manner (27-29).

1.3 Osteoclast Biology
Osteoclasts, originally differentiated from the monocyte/macrophage lineage and featured as large multinucleated giant cells, are considered as the only type of cells programmed to resorb bone (30). They actively participate in the bone remodeling as well as in the pathogenesis of various bone disorders. Macrophage colony stimulating factor (M-CSF) and receptor activator of NF-κB ligand (RANKL) are two essential factors mediating the osteoclast formation and resorption. The osteoclast achieves its resorptive function by adhering to the bone surface with subsequent establishment of a sealing zone and ruffled border, through which it secretes bone degrading substances.

1.3.1 The regulation of osteoclast formation and function
1.3.1.1 The role of osteoblasts in osteoclast formation
A series of experiments using a coculture system of osteoblasts/stromal cells and hematopoietic cells established the concept that osteoblasts support osteoclast differentiation through cell-cell interactions (31, 32). Numerous osteotropic factors including 1α,25-dihydroxyvitamin D3 [1α,25(OH)2D3], parathyroid hormone (PTH), interleukin 11 (IL-11), and prostaglandin E2 (PGE2) were used in this coculture system (32). The resultant osteoclasts were characterized by the presence of tartrate-resistant acid phosphatase (TRACP) activity, the expression of calcitonin receptors and integrin αvβ3, and bone resorptive activity (32). This cell-cell interaction is also found to be critical for osteoclastic bone resorption (33).

1.3.1.2 M-CSF
A major finding in osteoclast biology was established using an osteopetrotic mouse strain (op/op) (34), which demonstrated a decreased number of monocytes and macrophages in peripheral blood (35). It was later found a mutation exists in the coding region of M-CSF (at base pair 262) in op/op mice, which resulted in a lack of functional M-CSF protein (36). Osteoblasts derived from op/op mice are not able to support osteoclast formation when cocultured with normal spleen cells, while the addition of M-CSF to the coculture system could generate osteoclasts (37). Furthermore, the bone phenotype of op/op mice could be reversed by the administration of exogeneous recombinant M-CSF, which restored osteoclast formation and function (38). These findings consolidated that M-CSF is essential for osteoclast differentiation.

In the bone microenvironment, bone marrow stromal cells and osteoblasts are the main sources of M-CSF. By binding to its receptor-c-Fms, M-CSF primarily supports the survival and proliferation of osteoclast precursors (39), which is a prerequisite for terminal osteoclastogenesis. C-Fms-deficient mice present a similar phenotype to the op/op mice, characterized by mononuclear phagocytes and osteopetrosis (39). C-Fms is also a significant marker of early-stage precursor cells which commit to osteoclast differentiation (40).

1.3.1.2 OPG-RANKL-RANK axis

A major advance in osteoclast research was the discoveries of osteoprotegerin (OPG), receptor activator of NF-κB ligand (RANKL) and its receptor RANK in the 1990s (41), which hugely advanced our understanding of osteoclast formation and activation. Transgenic mice with overexpression of OPG or animals administrated with OPG developed osteopetrosis through the inhibition of osteoclast formation and function (41, 42). In contrast, OPG-deficient mice exhibited a phenotype of low bone mass characterized by high bone porosity and high incidence of bone fracture (43). These results demonstrated OPG functions as a significant negative modulator in osteoclasts in vitro and in vivo. RANKL was then discovered following the initial identification of OPG, a decoy receptor of RANKL. A cDNA encoding OPG ligand (OPGL, also known as RANKL) was cloned from an expression library of mouse stromal cells (41). RANKL belongs to the membrane-associated tumor necrosis factor ligand family and was found to successfully induce osteoclast formation. The soluble extracellular domain of RANKL, together with M-CSF, could induce osteoclastogenesis without the support of osteoblasts/stromal cells (41). RANKL can be secreted by osteoblasts, stromal cells, and osteocytes (44, 45), suggesting osteoblasts support osteoclastogenesis through the expression of RANKL. RANK acts as the receptor for RANKL in osteoclasts and was cloned from a cDNA library of
human dendritic cells (46). RANKL- and RANK- deficient mice result in similar bone phenotypes, which are characterized by osteopetrosis because of an obvious block in osteoclastogenesis (47, 48). RANK is a type I transmembrane protein with the structure of its N-terminal region being similar to OPG. The binding of RANKL to RANK induces the survival, fusion, and resorptive activity of osteoclasts *in vitro* (49). Interestingly, with the addition of RANKL, osteoclasts are able to be induced from spleen cells in the presence of the antibody against the extracellular domains of RANK but fail to form under the interference of anti-RANKL antibody which lacks the Fab fragment (Fc fragment) (50). Moreover, similar to OPG transgenic mice, transgenic mice overexpressing RANK-Fc fusion protein exhibit a bone phenotype of high bone mass which is due to the compromised osteoclast formation and function (51). Collectively, in osteoclast precursor cells, RANK may directly act as the only receptor for RANKL to achieve signaling transduction.

The interaction of RANKL with RANK recruits the TNF receptor-associated factor (TRAF) family proteins, of which TRAF6 was identified to be most involved in the RANKL-induced osteoclast formation and resorptive function (52, 53). Deficiency of TRAF6 leads to the complete absence of osteoclasts or poorly functioning osteoclasts which are detached from the bone surface. Following RANKL stimulation, the trimerization of TRAF6 and RANK causes the stimulation of the nuclear factor-κB (NF-κB) and mitogen-activated kinases (MAPKs) (54), which are essential in both differentiation and maturation of osteoclasts. Signal transduction of TRAF6 is also regulated by other proteins such as FHL2 (four and a half LIM domain 2), which acts as a transcriptional coactivator or corepressor (55). It was found FHL2 inhibits TRAF6-mediated NF-κB activation, FHL2-knockout osteoclasts has an enhanced TRAF6/RANK association whereas FHL2 overexpression delays osteoclastogenesis and cytoskeletal organization.

**1.3.1.3 NF-κB and MAPKs pathway**

The activation of the NF-κB signaling pathway acts as the very early event in RANKL-induced osteoclastogenesis (46) (Figure 1.2). Under resting conditions, the NF-κB complex is bound to the inhibitor of κB (IκB) and remains in the cytoplasm. When stimulated with RANKL, the recruitment of TRAF6 causes IκB kinase (IKK) phosphorylation and leads to IκB degradation, which allows the translocation of NF-κB into the nucleus and subsequent expression of osteoclast-specific genes (56, 57). Mice with a deficiency of either *Nfkbia* (encoding p50 subunit of NF-κB) or *Nfkbiab* (encoding p52 subunit of NF-κB) don’t show developmental defects, while the loss of both *Nfkbia* and *Nfkbiab* genes results in osteopetrosis due to the decreased osteoclast numbers which is accompanied by impaired
hematopoietic components (58). Skeletal abnormalities were also found in mice deficient in IKK, particularly IKK-β rather than IKK-α, because of the defect in osteoclasts (59). These findings together implicate that NF-κB is required for normal osteoclast formation and function. NF-κB is suggested to induce osteoclast formation and function via binding to nuclear factor of activated T cells 1 (NFATc1) promoter, as revealed in chromatin immunoprecipitation (ChiP) experiments (60). Overexpression of NF-κB can lead to NFATc1 promoter activation in a luciferase reporter assay (60). In p50/p52-deficient cells, which have a defect in osteoclastogenesis, the activation of c-Fos is affected while the overexpression of c-Fos is able to rescue osteoclast formation (61). It is likely that NF-κB activates NFATc1 directly by binding to its promoter or indirectly through c-Fos induction.

In addition to the NF-κB pathway, MAPKs, containing three major family members (ERKs, JNKs, and p38), are also suggested to be essentially involved in RANKL-induced osteoclastogenesis in vitro (62) probably by modulating AP-1 activity (63), but in-vivo evidence has not been established. MAPKs are triggered via phosphorylation of tyrosine and threonine by MAPK kinases and deactivated by MAPK phosphatases (MKPs) through dephosphorylation (64). Upon RANKL stimulation, TRAF6 is recruited and MAPKs are shown to be activated to drive osteoclastogenesis (65, 66).

1.3.1.4 Reactive oxygen species (ROS)

Accumulating evidence has demonstrated that intracellular ROS, such as hydrogen peroxide and superoxide, play an essential role in osteoclastogenesis by mediating RANKL-induced downstream signaling (67-69) (Figure 1.2). With the stimulation of RANKL in osteoclast precursors, ROS are endogenously generated via a signaling axis involving TRAF6/Rac1/ Nox1 (nicotinamide adenine dinucleotide phosphate [NADPH] oxidase 1) (67). Oxidant scavengers such as N-acetylcysteine (NAC) or diphenylene iodonium (DPI) are able to suppress osteoclast formation by attenuating intracellular ROS levels (67), suggesting ROS are critical for osteoclastogenesis. The mechanisms underlying ROS-mediated signaling still need to be further elucidated; however, ROS are suggested to promote osteoclast differentiation and bone resorptive function via activating NF-κB and MAPK pathways (70, 71). NF-κB is considered as a crucial downstream target of RANKL-induced ROS signaling (72), ROS may facilitate IkBα’s phosphorylation and degradation, which causes the release of NF-κB dimers and subsequent translocation into the nucleus (73). Additionally, ROS were also clearly suggested to participate in NFATc1 induction via regulating calcium signalling (74, 75). Meanwhile, to protect from the oxidative damage to the cells, a wide range of antioxidant/cytoprotective enzymes, such as heme oxygenase-1 (HO-1), NAD(P)H: quinone
reductase (NQO1), catalase, glutathione-disulfide reductase (GSR), and γ-glutamylcysteine synthetase (GCS) (71, 76) are induced to defend against oxidative stress. Hence, these results deliver a rationale for suppressing the level of ROS, by inhibiting ROS production or enhancing antioxidant enzymes, as a potential strategy for inhibiting osteoclast formation and activity.

1.3.1.5 NFATc1
The NFAT transcription factor family is comprised of 5 members NFATc1, NFATc2, NFATc3, NFATc4, and NFAT5. It was originally discovered in T cells and is important in the immune response (77), but also was shown to be involved in many other physiological processes as well as pathological conditions, such as inflammatory bowel disease and several types of cancer. During RANKL-induced osteoclastogenesis, NFATc1, also referred to as NFAT2, was identified to be the most strongly induced in a genome-wide search (78). NFATc1-deficient embryonic stem cells can’t be induced into osteoclasts under the treatment of RANKL, conversely, the ectopic expression of NFATc1 is able to regulate osteoclast formation and function in the absence of RANKL (78). Calcium signalling is important to activate NFAT family members by a well-known calcium sensor protein-calmodulin (CaM), which subsequently activates a specific phosphatase termed calcineurin. Similarly, the activation of NFATc1 was also found to be mediated by calcineurin, which is further supported by the results that calcineurin inhibitors-such as cyclosporin A and FK50-can effectively suppress osteoclast formation (78, 79).

NFATc2, another one of the NFAT family of transcription factors, is a critical molecule which binds to the promoter of NFATc1 and cooperates with NF-κB to drive the initial stimulation of NFATc1 (60). However, NFATc2 is considered as dispensable for osteoclast formation in vivo (60), this initial induction of NFATc1 is not completely essential for osteoclast differentiation. An interesting observation was obtained by performing ChIP experiments: NFAT-binding site are discovered in both promoters of NFATc1 and NFATc2, but NFATc1 is exclusively recruited to bind NFATc1 promoter in response to RANKL stimulation, which persists through the terminal stage of osteoclastogenesis, suggesting an auto-amplification mechanism exists in RANKL-induced NFATc1 activation (60). Investigation of the transcriptionally active chromatin structure indicated that the rate of histone acetylation and methylation of histone H3 lysine 4 were upregulated in the NFATc1 promoter; while epigenetic modifications such as the methylated DNA-binding proteins lead to the silencing of NFATc2 expression (60).
The AP-1 transcription factor, consisting of proteins including Jun (c-Jun, JunB, JunD), Fos (c-Fos, FosB, Fra-1, Fra-2), and ATF (ATFa, ATF2, ATF3, ATF4, B-ATF) (80), is essential for osteoclastogenesis by activating NFATc1. RANKL-induced signaling activates AP-1 in part through c-Fos, its critical component (Figure 1.2). In c-Fos-deficient cells, the induction of NFATc1 is fully suppressed in response to RANKL stimulation, while overexpression of NFATc1 can rescue osteoclast formation (81). After RANKL stimulation, c-Fos was found to bind to NFATc1 promoter in ChIP experiments, suggesting it acts as a direct transcriptional regulator of NFATc1 (60). However, the mechanisms underlying RANKL-induced activation or modification of c-Fos still remain unclear.

Based on ChIP experiments, a wide range of genes that characterize the osteoclast including Acp5 (encoding tartrate-resistant acid phosphatase [TRAcP]) (78, 81, 82), calcitonin receptor (78, 81, 82), cathepsin K (82), β3 integrin (83), and osteoclast-associated receptor (OSCAR) (82, 84) were found to be regulated by NFATc1. DC-STAMP (Dendrocyte Expressed Seven Transmembrane Protein) and Atp6v0d2 (ATPase H+ Transporting VO Subunit D2), crucial factors for osteoclast fusion, were also revealed to function as downstream targets of NFATc1 (85, 86). It is noteworthy that NFATc1 also interacts with PU.1 and MITF on the promoters of Ctsk and OSCAR. Therefore, NFATc1 represents a master switch for osteoclastogenesis (Figure 1.2) and can be an auspicious target for the management of bone diseases caused by excessive osteoclast formation and function (78).

1.3.2 Osteoclast function

1.3.2.1 Cell-bone attachment and formation of sealing zone

During the development of the skeleton, osteoclasts are required for the resorption of mineralized bone, dentine, and calcified cartilage. Osteoclast bone resorption is initially achieved by attaching tightly to the surface of the bone matrix, which is mediated by integrin complexes that consist of α and β chains. Integrins promote the interactions between the osteoclast and the bone surface by binding to various extracellular matrix proteins, including collagen, vitronectin, bone sialoprotein, osteopontin, disintegrin and metalloproteinase (87, 88). Integrins so far identified in osteoclasts include αvβ3, αvβ5, α2β1, αvβ1, αvβ3, and α6β1. Integrin αvβ3, a classical vitronectin receptor is abundantly expressed in osteoclasts, and recognizes and specifically binds to proteins containing Arg-Gly-Asp (RGD) domain (89). Interestingly, β3 knockout mice showed increased bone mass but with 3.5-fold more osteoclasts characterized by abnormal cytoskeleton and disrupted ruffled membranes which affected cell-bone interaction. Thus, Integrin αvβ3 is required for osteoclast
Figure 1.2 Signaling transduction during osteoclastogenesis. M-CSF, RANKL and its co-stimulatory factors, immunoglobulin-like receptor are required for osteoblast differentiation. M-CSF binds to c-Fms to support survival, proliferation and cytoskeletal organization of osteoclast precursors. The expression is also upregulated by M-CSF. The binding of RANKL to RANK leads to TRAF6 recruitment and subsequent phosphorylation of ITAM. NF-κB is induced by RANK signaling and then initiates the NFATc1 induction. RANK signaling cooperates with ITAM signaling to phosphorylate PLCγ and trigger calcium signaling, which activates NFATc1. AP1, mediated by the activation of c-Fos, is required for the robust expression of NFATc1. ROS is produced by TRAF6/Rac1/Nox1 signaling and partly from mitochondrion, mediating NF-κB and MAPK to regulate osteoclastogenesis. The activation of NFATc1 induces the expression of osteoclast-specific genes such as Acp5, Atp6v0d2, Ctsk, and Mmp9. AP-1, activator protein 1; CREB, cAMP-responsive element-binding protein; DAP12, DNAX-activating protein; FcRγ, Fc receptor common γ subunit; ITAM, immunoreceptor tyrosine-based activation motif; JNK, JUN N-terminal kinase; M-CSF, macrophage colony-stimulating factor; MITF, microphthalmia-associated transcription factor; NFATc1, nuclear factor of activated T-cells cytoplasmic 1; NF-κB, nuclear factor-kB; PLCγ, phospholipase Cγ; RANKL, receptor activator of nuclear factor-kB ligand; TRAF6, tumor necrosis factor receptor-associated factor 6 (This figure was adapted from Nakashima et al., 2012).
resorptive function but has no effect on osteoclastogenesis. The latest integrin to be associated with osteoclast biology is $\alpha_9\beta_1$ which binds to ADAM8 by recognizing RX6DLPEF sequence and plays a fundamental role in osteoclastic resorption (88).

Following the attachment of osteoclasts to the mineralized bone surface, an isolated sealing zone between the ruffled border membrane and its underneath bone matrix is formed, providing a special lacunar space for effective resorption (90) (Figure 1.3). During osteoclastogenesis on bone or mineralized matrix, isolated podosome-type structures on the bone-facing surface of osteoclasts are rapidly accumulated and fuse into a sealing zone for resorption. By expelling $\text{H}^+$ and $\text{Cl}^-$ ions through the ruffled border, a low-PH resorptive microenvironment is created and well maintained due to the tight sealing around the lacunar space, which is needed for the activation of bone-degrading enzymes as well as bone mineral dissolution (91).

1.3.2.2 Bone mineral dissolution

Bone mineral mainly consists of crystalline hydroxyapatite and the localised acid condition within the resorption lacuna has been suggested as the primary process responsible for solubilizing crystals (92). The lacunar acidification results from the presence of electrogenic proton pumps in the ruffled border of the osteoclast, of which the main type is V-type ATPases (93). V-type ATPases contain at least 14 different subunits usually forming different isoforms and a rotary mechanism is used to export protons through the ruffled border (91). For bone biology, a 116-kDa $\alpha_3$ subunit of vacuolar type ATPase (encoded by Tcirg1) was identified to be of significance in human autosomal recessive osteopetrosis (94). This was further highlighted by a knockout mouse model (95). Targeted disruption of Tcirg1 leads to severe osteopetrosis because of a lack of extracellular acidification. However, the balance of acid-base in blood and urine of the knockout mice remain normal, indicating this proton pump is specific in osteoclasts (95). Proto-oncogene Src is known to stimulate the generation of $\text{H}^+$-ATPase and vesicular trafficking to the cell membrane. Osteoclasts lacking Src are unable to form the ruffled border membrane, which consequently affects bone resorptive activity (96). Additionally, the administration of vacuolar proton pump inhibitors like bafilomycin A1 also exhibits similar inhibitory effects on osteoclastic resorption (97). Therefore, the development of specific vacuolar proton pump inhibitors provides a new alternative therapy for osteoclast-related bone disorders like osteoporosis or osteolysis.
Figure 1.3 Schematic illustration of osteoclastic bone resorption. Following the attachment of osteoclasts to the mineralized bone surface, osteoclasts become polarized and an isolated sealing zone between the ruffled border membrane and its underneath bone matrix is formed, providing a special lacunar space for effective resorption. Cathepsin K (CTSK) and protons are secreted through the ruffled border, which is essential for bone resorption. Transcytotic vesicles are responsible for the removal of the degraded bone products. (This figure was adapted from Feng et al., 2013).
Another molecular mechanism essential for osteoclast resorptive activity is the production of H+ ions. Essentially catalyzed by carbonic anhydrase II (CA-II) enzyme, H2O and CO2 convert into H2CO3 which is subsequently dissociated into H+ and HCO3- ions (59). The disruption of the expression of CA-II enzyme by antisense RNA and DNA molecules inhibits bone resorption (98). Consistently, mice lacking CA-II are characterized by smaller tibiae, widened metaphyses and decreased bone formation rate in both cortical and trabecular bone (99). The pumped protons in the resorption lacunar are balanced by other anions, particularly the chloride ions which are secreted through abundant chloride channels in the ruffle border. Chloride channel isoform CIC-7, encoded by CLCN7 gene, is important to regulate the flow of chloride ions across the cell membrane and thus maintain an appropriate acidity. Mice lacking the CIC-7 channel exhibit normal osteoclast formation but impaired osteoclastic resorption, causing severe osteopetrosis characterized by dense and brittle bones (100).

1.3.2.3 Bone organic matrix degradation

Following bone mineral solubilization, the organic matrix is efficiently degraded by various enzymes, including cathepsin K, tartrate-resistant acid phosphatase (TRAcP), and matrix metalloproteinases (MMPs). Cathepsin enzymes include several types, of which cathepsin K is the major cysteine proteinase degrading bone matrix in the resorption lacuna (101, 102). Cathepsin K was discovered to be abundantly expressed and secreted into the resorption lacuna, degrading insoluble collagenous matrix (type I collagen). The cathepsin-K-deficient osteoclasts exhibit poor formation of the resorptive interface with collagen fibrils undigested; consistently, the cathepsin-K-deficient mice manifest a severe osteopetrotic phenotype with a large area of abnormal trabecular bone matrix (103). Histomorphometric and biomechanical analyses further demonstrated that the bone matrix is disorganized, which makes the bone more brittle and more susceptible to fracture due to the impaired osteoclast function (104). Mutations of the gene encoding cathepsin K in humans lead to an autosomal recessive osteochondrodysplasia - pycnodysostosis (105).

MMPs, members of a family of zinc-dependent proteolytic enzymes, were first known as collagenases and are expressed at high levels in bone and cartilage during skeletal modelling and remodeling (106). MMPs function in the skeleton by cleaving extracellular matrix like collagens, growth factors, and cytokines. In osteoclasts, MMPs are secreted through the ruffled border to achieve its resorptive function. MMP9 and MMP13 appear to be of most significance in the
generation of functional osteoclasts. This is supported by the establishment of \textit{Mmp9} and \textit{Mmp13} knockout mice, in which the mice exhibit a higher bone mass and delayed bone fracture healing, indicating the impairment of osteoclast recruitment and function during skeletal development (59).

TRAcP is widely accepted as an osteoclast-specific marker and is also suggested to play a critical role in facilitating the breakdown of bone matrix. TRAcP destroys collagen and other bone matrix by generating ROS which are highly destructive (107). TRAcP contains a redox-active iron center, which actively participates in the generation of hydroxyl radicals by reacting with hydrogen peroxide (108). Following the bone matrix degradation, the resultant products are removed from the resorption site to allow the continuous bone resorption. Transcytosis was discovered as one of the mechanisms to traffic the degradation products, as evidenced by the finding that transcytotic vesicles in resorbing osteoclasts contain various fragments of collagen and other matrix proteins (109). In this process, TRAcP is also found to exist in transcytotic vesicles and contributes to the matrix degradation during transport intracellularly. Mice deficient in TRAcP display an early onset osteopetrotic phenotype because of disrupted ruffled borders and intracellular vesicular transport in osteoclasts in spite of normal osteoclastogenesis (110).

1.4 Osteoblast Biology

Bone is known as a dynamic organ undergoing continuous renewal throughout life, during which the remodeling process is greatly involved partly through osteoblastic activity. Bone formation by osteoblasts essentially support the bone metabolic activity and reparative process, which is achieved by the commitment and differentiation of osteoblast lineage cells. Osteoblasts are bone-forming cells originated from mesenchymal progenitors. The differentiation of osteoblasts is a multi-stage process encompassing a series of signaling cascades: the initial stage is characterized by the expression of transcriptional factors like osterix (also known as transcription factor Sp7) and Runx2 (runt domain-containing transcription factor), followed by the subsequent expression of type I collagen, non-collagenous proteins, and alkaline phosphatase, eventually leading to the deposition and mineralization of bone matrix (111). Next, osteoblasts are trapped in bone as osteocytes or reside on the bone surface as bone lining cells, the rest undergo apoptosis.

1.4.1 Origin, morphology and function of osteoblasts
The origin of osteoblasts is from multipotential mesenchymal stem cells (MSCs), which give rise to another two kinds of cells, chondrocytes and adipocytes, under different conditions. MSCs exist in the bone marrow stroma and periosteal tissue from the skeleton developmental perspective. As MSCs are of increasing interest in various biomedical disciplines, a minimal criteria for identifying human MSCs was proposed in 2006 by the International Society for Cellular Therapy (ISCT) (112), in which the MSCs should meet 3 criteria: 1. MSCs can adhere to plastic under the standard culture conditions; 2. MSCs express CD73, CD90, and CD105 (≥95% positive) but show the absence of CD34, CD45, CD14 or CD11b, CD79a or CD19 and HLA class II; 3. MSCs are able to differentiate into trilineage cells including osteoblasts, chondrocytes, and adipocytes, as examined by specific staining (von Kossa staining or Alizarin Red staining for osteoblasts, Alcian blue staining for chondrocytes, Oil Red O staining for adipocyte).

The commitment of MSCs into specific cell lineages is mediated by a set of transcription factors which initiate the expression of phenotypic genes featured by adipocytes, chondrocytes, or osteoblasts. These transcription factors act as “master switches”. For adipocyte differentiation, peroxisome proliferator-activated receptor λ (PPAR λ) and CCAAT/enhancer-binding protein (C/EBP) family coordinately trigger the robust expression of adipocyte phenotypic genes such as aP2 and CD36 (113, 114). Sox9 is known as a master transcription factor required during chondrogenesis through the induction of a set of chondrocyte-related genes including Aggrecan, Col2a1, Col9a2, and Col11a2 (115). With regard to osteoblast differentiation, Runx2 and osterix are well known to essentially direct MSCs into the osteoblastic lineage.

Osteoblasts usually presented on the bone surface where they are dynamically engaged in laying down organic bone matrix. Morphologically, osteoblasts are compactly lined next to each other and appear to be cuboidal cells encompassing large nuclei (Figure 1.4). On the apical side of the nuclei, Golgi apparatus and extensive endoplasmic reticulum can be observed through transmission electron microscopy. Osteoblasts highly express osteocalcin, alkaline phosphatase (ALP), as well as secreting type I collagen and other essential matrix components, leading to the formation of osteoid. This specialized phase of organic accumulation provides the template for the mineral deposition and subsequent formation of hydroxyapatite.
**Figure 1.4 Osteoblast morphology by histological staining.** Rat bone sections stained with McNeal and von Kossa. (A) Osteoblasts (red arrows) lining up on the bone surface and covered by osteoid. (B) Osteoblasts are embedded in osteoid and become osteocytes (red arrows). (C) Higher magnification images showing the nucleus and Golgi apparatus of osteoblasts. Red arrows indicate the osteocyte transformed from osteoblast; white arrows indicate the osteoid. Scale bars, 40 μm. *(This Figure was adapted from Basic and Applied Bone Biology, Teresita Bellido, Lilian I. Plotkin, Angela Bruzzaniti, 2014; https://doi.org/10.1016/B978-0-12-416015-6.00002-2; by permission from Elsevier, License No. 4623400790633)*
1.4.2 Transcription factors regulating osteoblast differentiation and function

1.4.2.1 Runx2

The discovery of Runx2 (also termed as Pebp2a1, Aml3, or Cbfa1) as the master transcriptional activator of osteoblast formation boosted our understanding of osteoblast biology. Runx2 was firstly revealed to be predominantly expressed in the osteoblast through the analysis of the osteocalcin promoter, which led to the discovery of 2 osteoblast-specific cis-acting elements-OSE1 and OSE2 (116). The sequence analysis of OSE2 subsequently indicated that it’s immunologically related to the Runt family, following which Runx2 was eventually shown to dominate the functional expression of osteoblast-specific osteocalcin gene (117). During mouse development, Runx2 is found to be expressed early at 10.5 days post- coitus (dpc) in the lateral plate mesoderm. After the appearance of osteoblasts, Runx2 expression is well- maintained and becomes highly restricted to osteoblasts as well as perichondrium after birth. In contrast, Runx2 expression negatively correlated with chondrogenesis and is only expressed abundantly in hypertrophic chondrocytes. This spatial and temporal pattern during development revealed that Runx2 is essential for the regulation of osteoblast differentiation (117). In accordance with this, OSE2 elements were also found to be highly functional in the promoter area of other osteoblastic- genes such as COL1A1 (encoding collagen type I alpha 1 chain), OPN (encoding osteopontin), and BSP (encoding bone sialoprotein) (117). Interestingly, the expression of Runx2 in non-osteoblastic cell lineages like fibroblasts is still able to induce the expression of osteoblastic genes. These findings together demonstrated the indispensable role of Runx2 in osteoblast differentiation.

Mice deficient in Runx2 display a bone phenotype characterized by the complete abrogation of osteoblast formation which leads to the absence of ossification (118). Interestingly, Runx2-deficient calvarial cells fail to form mature osteoblasts even under the stimulation of BMP-2, but they can spontaneously gain adipocytic phenotype and switch into chondrocytes with the simulation of BMP-2 (119), suggesting Runx2 may hinder the development of adipogenesis and chondrogenesis. Runx2 has so far been considered as an early osteogenic marker which is highly up-regulated when the MSCs undergoes osteoblast differentiation. Furthermore, cleidocranial dysplasia (CCD), a human autosomal dominant disease characterized by hypoplastic clavicles and abnormal skeletal development, was revealed to have two missense mutations in the Runx2 gene thus altering osteoblast differentiation (120). In addition to promoting osteoblast formation, Runx2 also actively participates in bone matrix deposition. Mice deficient in Stat1 or Shn3, two negative regulators of Runx2, showed the enhancement of Runx2 expression which subsequently increased the deposition
of bone matrix (121, 122). Another well-known Runx2 regulator is the Twist proteins. Twist-1 and -2 are basic helix-loop-helix (bHLH)-containing transcriptional factors which are known as Runx2 inhibitors during skeletogenesis. The inhibitory effects are achieved by the binding of Twist-box (C-terminal 20 amino acids) to Runx2 DNA binding domain (123).

1.4.2.2 Osterix

Osterix is a zinc finger-containing transcription factor which is needed for osteoblast differentiation and widely exists in developing bones (124). In mice deficient of Osterix, cortical and trabecular bone formation through intramembranous or endochondral ossification was abrogated. During endochondral skeletogenesis in Osterix-deficient mice, mesenchymal cells fail to deposit bone matrix and thus no ossification occurs (124). However, the cell organization of cartilage growth plate remains normal in Osterix null mice, which suggests that Osterix is not required for chondrogenesis. Furthermore, unlike Runx2, Osterix has no effect in skeleton patterning as evidenced by the results that Osterix null embryos are similar to wild-type embryos at E12.5, a skeleton patterning decisive time point (124). Evidence regarding human mutations of the Osterix gene still remain to be identified.

In osteoblast differentiation, Runx2 is required for the expression of Osterix. Mice deficient in osterix still express Runx2 while mice deficient in Runx2 show the absence of osterix expression, demonstrating Runx2 is upstream of osterix (119). Runx2 was further determined to bind to activating domains of the Osterix promoter (125). P53 is identified as a negative regulator of Osterix. Mice deficient in p53 display a phenotype of high bone mass, and osteoblasts lacking p53 show enhanced differentiation by increasing the expression of Osterix in a DNA binding-independent manner (126). In addition to Runx2 and Osterix, Schnurri-2 (SHN-2) can also positively regulate the activity of Osterix in bone formation (127). There is still little known how Osterix mediates osteoblast differentiation. It is suggested that a complex forms through the interaction of Osterix and NFAT, which controls osteoblast differentiation by activating the Col1a1 promoter rather than Runx2-dependent Osteocalcin promoter (128).

1.4.2.3 ATF4

Activating transcription factor 4 (ATF4) was first discovered from the genetic data of RSK2 (encoding a kinase) mutated Coffin-Lowry syndrome (CLS), an X-linked disease which is characterized by progressive skeletal abnormalities (129). Consistently, mice deficient in Rsk2 showed a low-bone-
mass phenotype due to the impairment of bone formation (130). ATF4 is identified to be phosphorylated by Rsk2 as examined by in vitro kinase assays, and the phosphorylation of ATF4 becomes undetectable in osteoblasts derived from mice deficient in Rsk2. Further analysis indicated that ATF4 acts as a critical transcriptional factor in bone formation by post-transcriptionally synthesized type I collagen, and ATF4-deficient mouse models also exhibited similar bone phenotype to the Rsk2-deficient mice (130). The synthesis of type I collagen is implicated to be related with the well-organized amino acid introduction into osteoblasts which requires the involvement of ATF4. ATF4 also facilitates the differentiation of MSCs into osteoblastic cell lineage probably by interacting with β-catenin protein (131). Furthermore, ATF4 predominantly secreted from osteoblasts is able to inhibit insulin sensitivity and mice lacking the expression ATF4 specifically in osteoblasts exhibited similar metabolic abnormalities to ATF4 globally deficient mice (132). Thus, by producing ATF4, osteoblasts also function as a type of endocrine cell. ATF4 expressed in osteoblasts can indirectly regulate osteoclast formation and function by binding to the promoter of RANKL gene (133). This is further supported by the finding that osteoclast number was reduced in ATF4-deficient mice.

The function of ATF4 is mostly modified post-translationally. ATF4 is abundant in cells and tissues. The application of MG115, a proteasome inhibitor, in non-osteoblastic cells promotes ATF4 accumulation, which induces the expression of osteoblast-specific gene (134). The elimination of the expression of a ubiquitin-protein isopeptide ligase (β-TrCP1) by RNA interference leads to the accumulation of ATF4 and thus enhanced Osteocalcin expression in non-osteoblastic fibroblasts (134). These results demonstrated a post-translational mechanism exists in the osteoblast-specific function of ATF4.

1.4.2.4 Other transcription factors

In addition to Runx2, Osterix, and ATF4, there is a couple of other transcriptional regulators which are essential for bone formation. Activator protein 1 (AP1) is a heterodimeric complex consisting of the Fos and Jun family, of which Fra-2 and JunD are the major components in differentiated osteoblasts (135). Osteocalcin and col1a2 are identified as the targets of Fra-2, and mice overexpressing Fra-2 display an osteosclerotic phenotype due to increased osteoblasts (136). Transcriptional factors of Tcf/Lef family, downstream factors of wnt signaling which are activated in response to the nuclear translocation of β-catenin, also participate in the regulation of osteoblast differentiation. In particular, Tcf1 was identified to be essential for the expression of osteoblastic
genes such as \textit{Opg}, the promoter of which was revealed to contain a Tcf-binding site (137). Other transcription factors such as \textit{Msx2}, \textit{Bpx}, and \textit{Hoxa2} are known to regulate osteoblast differentiation through affecting the master transcriptional regulator-\textit{Runx2}.

\subsection*{1.4.3 Extracellular signaling regulating osteoblast differentiation and function}

\subsection*{1.4.3.1 Wnt signaling}

Wnt proteins are highly post-translationally modified (glycosylation and palmitoylation) and secreted proteins which have crucial effects on osteoblast differentiation and function (138). Wnt signaling is transduced into a cell through its receptors on the cell surface: the frizzled (Fzd) family and a coreceptor of Lrp5/Lrp6 or transmembrane tyrosine kinase - Ryk or Ror. Upon the binding of a Wnt protein to its receptor, several intracellular signaling cascades, including canonical and noncanonical pathways, are activated (139). The difference between Wnt canonical pathway and noncanonical pathway is the involvement of \(\beta\)-catenin. The best depicted is the canonical pathway. \(\beta\)-catenin is encoded by \textit{Catnb1} and acts as a critical transcriptional co-activator in response to Wnt signaling. Without Wnt, the intracellular \(\beta\)-catenin is normally maintained at a low level because of a destruction complex which consists mainly of Axin, adenomatosis polyposis coli (APC), glycogen synthase kinase 3\(\beta\) (GSK-3\(\beta\)), and some other proteins (140). Among them, GSK-3\(\beta\) is specifically responsible for the ubiquitination and degradation of \(\beta\)-catenin through phosphorylation. Following Wnt proteins’ binding to the Lrp5/6-Fzd coreceptors, the intracellular protein Dishevelled (Dsh) is activated and thus leads to the phosphorylation and deactivation of GSK-3\(\beta\). Meanwhile, the destruction complex is entrapped due to the binding of Axin to the cytoplasmic Lrp5/6 tail. Therefore, \(\beta\)-catenin is released and accumulated in the cytoplasm, followed by translocation into the nucleus where it interacts with the Lymphoid-enhancing factor/T cell factor (Lef/Tcf) family of transcription factors to stimulate the targeted genes (138).

Furthermore, a wide range of signaling cascades including the calcium calmodulin dependent kinase 2 (CaMK2), JNK, p38, protein kinase C (PKC), PI3K/AKT, and mTOR are also mediated by Fzd which may be determined by the specific ligand-receptor interaction (138). In addition to Wnt proteins, there are also a few extracellular antagonists such as Sclerostin (Sost) and Dickkopf (Dkk1/2) which can compete for the Lrp5/6 extracellular domains and impede their interaction with Wnt proteins (141, 142).

The significance of Wnt/ \(\beta\)-catenin signaling in skeletal patterning and development has been well established (143). Some fundamental component alterations can result in various bone disorders.
In patients with osteoporosis pseudoglioma syndrome (OPPG), a rare bone disorder characterized by skeletal abnormalities such as short stature, juvenile osteoporosis, and a higher risk of bone fracture, loss-of-function Lrp5 mutation was identified as the leading cause (144, 145). In contrast, gain-of-function Lrp5 mutation leads to a high bone mass phenotype (146). This is further evidenced by the studies that indicated common Lrp5 variants are responsible for fracture risk to a genome-wide significance level (147). In addition, Wnt proteins are also considered to be associated with bone mineral density (BMD) according to several genome-wide association studies (GWAS) in human populations (148, 149). For instance, Wnt1 mutation was found to be responsible for the development of osteogenesis imperfecta (OI) based on the whole exome sequencing (150). Wnt16 was identified to be associated with cortical bone thickness, bone strength, and risk of bone fracture (149). The establishment of mice gene defect models consistently implies the importance of Wnt signaling in bone formation. Mice with an Lrp5 knock-in mutation which contains a missense mutation result in a bone phenotype which is comparable to human high bone mass (151). Wnt16-deficient mice developed a bone phenotype characterized by altered cortical parameters and poor mechanical properties which increase fracture susceptibility by affecting Wnt signaling (152). The mechanisms underlying the regulation of Wnt signaling in osteoblast differentiation are multiple: Wnt5a promotes osteoblast differentiation by indirectly inhibiting adipogenesis due to suppressive effects on the promoter of PPARα; Wnt10b enhances osteoblastogenesis through the induction of Runx2, Dlx5 and Osterix (153); Wnt7b increases bone formation partly through the activation of mTORC1 (154); β-catenin, together with Tcf1, directs osteoblast differentiation by targeting the promoter of Runx2 (155).

1.4.3.2 Notch signaling

Notch signaling is an evolutionarily conserved pathway involved in the regulation of skeletal development. The notch receptors (Notch1-4) are single transmembrane proteins which are cleaved by γ-secretases (presenilin-1/2) to generate the active Notch intracellular domain (NICD) in response to ligand (JAG 1/2 and DLL 1-4) binding (156). After release from the cytoplasm, NICD relocates to the nucleus and subsequently interacts with immunoglobulin kappa J region (Rbpj) and Mastermind-like (MAML), followed by the induction of downstream genes including Hes family (-1, -3, -5, -7) and Hey family (-1, -2, -L) (157). Notch signaling was first found to play a critical role in somitogenesis and patterning, mice embryos deficient in Notch1, Dll1, and Rbpj all displayed significant skeletal developmental defects (156). Furthermore, Notch signaling gene mutations in humans can also lead to bone abnormalities including Alagille syndrome (AGS), Adams–Oliver
syndrome (AOS), and spondylcostal dysostosis (SCDO) (158). In a GWAS, a higher expression of JAG1 gene was associated with a higher BMD, which suggests that Notch signaling regulates bone metabolism (159).

Genetic mice studies provide the evidence of Notch function in osteoblast differentiation and activity. The disruption of Notch signaling (deletion of presenilin 2) specifically in mesenchymal progenitor cells reduced the number of mesenchymal progenitors which leads to increased trabecular bone mass in adolescent mice who eventually developed osteopenia when they aged, suggesting Notch signaling is important to maintain the osteoblast precursors in bone marrow (160). Moreover, mice with conditional deletion of Rbpj in skeletal progenitors show a phenotype of fracture nonunion, whereas the fracture repair process is normal when Rbpj is deleted in mature osteoblasts or chondrocytes (161). Notch signaling in the osteoblastic cell lineage was investigated in osteoblast-specific gain- and loss-of-function mouse models (162). Transgenic mice expressing N1ICD under the control of the 2.3kb collagen type 1 (Colla1) promoter demonstrate severe osteosclerosis, which is characterized by disorganized woven bone probably due to osteoblast immaturities. Further analysis revealed that N1ICD physically inhibits the interaction of Runx2 with Osteocalcin. In contrast, mice lacking Notch signaling in osteoblasts due to the deletion of presenilin-1 and presenilin-2 display an osteoporotic phenotype through changes in Opg expression. Interestingly, transgenic mice overexpressing NICD under the control of the 3.6kb Colla1 promoter had decreased bone mass secondary to the inhibition of osteoblast progenitor differentiation (163). Taken together, Notch signaling regulates skeletal development in a stage-dependent manner.

1.4.3.3 BMP signaling

BMP signaling exhibits fundamental importance in the development of the skeleton as well as the maintenance of bone homeostasis (164). Following the binding of BMPs to BMP receptors, homomeric type I/II receptors, either Smad-dependent or non-Smad-dependent signaling is activated. In the Smad-dependent (canonical signaling) pathway, receptor-activated Smads (R-Smads) which are composed of Smad1/5/8-Smad4, translocate into the nucleus and interact with Runx2 to initiate osteoblast differentiation. In the non-Smad-dependent (noncanonical signaling) pathway, TAK1-MKK3/6-p38 MAPK axis is highly involved in mediating the activation of Runx2 and Osterix upon the binding of BMPs (165). Disruption to components of these pathways result in multiple developmental problems in the skeleton.
BMPs have been well elucidated in directing osteoblast differentiation through genetic studies and some recombinant proteins are in pre-clinical trials for fracture healing and spine surgery. BMP2 can hugely enhance the expression of the osteoblastic gene marker Osteocalcin in mesenchymal stem cells (166). Limb specific BMP2-deficient mice developed spontaneous bone fracture and compromised bone repair which can’t be compensated by other osteogenic stimuli (167). BMP7 is able to facilitate the osteoblastic differentiation of MSCs in vitro and may have therapeutic effects on tissue repair (168). However, mice lacking BMP7 in limbs show normal development of the skeleton and bone healing after fracture, suggesting other BMPs may compensate for the absence of BMP7 (169). Likewise, limb-specific removal of Bmp4 also showed normal limb skeletogenesis and bone fracture-healing (170). Interestingly, among BMP family members, BMP3 perhaps counteracts BMP2/4 and is known to negatively regulate bone volume as evidenced by the establishment of mouse models lacking or overexpressing BMP3 (171, 172).

1.4.3.4 Other Signaling
There are also some other signaling pathways known to regulate osteoblast differentiation (173). Indian hedgehog (IHH) signaling is essential for osteoblast differentiation by inhibiting GLI3 and promoting GLI2A, which eventually initiates Runx2 expression as well as further enhancing Osterix expression. Fibroblast growth factor (FGF) signaling functions through FGF receptors and results in the stimulation of numerous signaling events including MAPK, phosphoinositide 3-kinase (PI3K), signal transducer and activator of transcription 1 (STAT1) to regulate osteoblast differentiation.

1.5 References


Yamashita T, Yao Z, Li F, Zhang Q, Badell IR, Schwarz EM, et al. NF-kappaB p50 and p52 regulate receptor activator of NF-kappaB ligand (RANKL) and tumor necrosis factor-induced


CHAPTER TWO - OSTEOCLAST AND BONE DISORDERS
2.1 Introduction

To adapt to the influences of circumstances, bone tissue continuously renews itself by performing bone remodelling which is carried out by a specific functional structure known as the basic multicellular unit (BMUs) (1). BMUs locate within a closed system named as the bone-remodelling compartment (BRC) where they conduct a complex process which starts with trenches created by osteoclasts, followed by the deposition of new bone matrix by osteoblasts. A well-maintained bone homeostasis depends on the proper balance between osteoclastic bone destruction and osteoblastic bone formation. Under physiological conditions, no net change exists in bone mass due to bone remodelling. However, a couple of bone disorders may occur when the balance derails because of various causes, including menopausal estrogen deficiency, aging, physical activities, drugs, and secondary diseases (2). When bone resorption outweighs bone formation, the bone becomes fragile which leads to fracture; in contrast, when bone formation exceeds bone resorption, a pathological condition known as osteopetrosis can occur as a result.

As described in the previous chapter, osteoclasts are considered as the sole type of bone-resorbing cell and originate from the monocyte/macrophage lineage. Excessive formation and activity of osteoclasts leads to a variety of bone and joint disorders (3), including osteoporosis, periprosthetic osteolysis after joint arthroplasty, Paget’s disease of bone, rheumatoid arthritis, and subchondral bone destruction in osteonecrosis of the femoral head (ONFH). Moreover, the tumour metastases-induced bone destruction is found to be related with the secretion of RANKL by cancer cells (4). Given the leading role osteoclasts play in the pathogenesis of the above bone disorders, strategies targeting aberrant osteoclast formation and function remain as the first mode of action in the expansion of bone-protective treatments. Indeed, most therapeutic interventions were developed for either reducing osteoclast formation or inhibiting its resorptive activity and thus slowing bone loss (5). Anticatabolic agents currently available include selective estrogen receptor modulators (SERMs), bisphosphonates (BPs), calcitonin, and Denosumab (monoclonal antibody against RANKL). Despite the remarkable advance they have achieved, these approved treatments are still facing huge challenges posed by various unexpected side effects. For instances, the usage of BPs increase the risks of gastrointestinal irritation, atypical fractures, and mandible necrosis (6); treatment with SERMs may lead to the occurrence of stroke and cardiovascular events (7). As such, there remains urgent need for continued identification and development of novel anticatabolic drugs with less adverse effects. In-depth studies are also required for elucidating the underlying mechanisms.
The aim of this chapter is to briefly review some common osteoclast-related bone disorders and the role of osteoclasts played in these pathological conditions, with particular highlights on osteoporosis and osteonecrosis of the femoral head (ONFH). We then focus on the tremendous progress that the anticatabolic agents have currently made as well as some ongoing challenges presented. Finally, we review our novel discoveries of drugs which are promising for the treatment of osteoporosis and ONFH by targeting osteoclasts. Anabolic therapies may also be therapeutic in treating some of these bone disorders. For instance, teriparatide, a truncated form of parathyroid hormone, has been approved for the treatment of osteoporosis by enhancing osteoblast activity. These anabolic approaches are beyond the scope of this thesis and will not be reviewed in this chapter.

2.2 Osteoclast-related Bone Disorders

2.2.1 Osteoporosis

Osteoporosis is defined as a metabolic skeletal disorder characterized by decreased bone mass and bone strength (8), which ultimately predisposes a person to fragility fracture commonly seen in vertebrae, wrist, and hip sites. A more quantitative technique to diagnose osteoporosis is to measure bone mineral density (BMD), which is conducted by dual-energy X-ray absorptiometry (DXA). A widely accepted criterion established by the World Health Organization (WHO) to define osteoporosis is a patient whose BMD lies 2.5 standard deviations (SD) or more below the young adult reference mean. Osteoporosis poses a significant health and social burden to Australia (9): In 2012, it was estimated that 4.74 million (66%) Australians older than 50 years of age had poor bone health, the number of which suffered osteoporosis is more than one million; moreover, 6.2 million Australians older than 50 years of age are predicted to develop osteoporosis in 2022; a total of $2.75 billion was put into the management of osteoporosis and osteopenia in 2012, and the total cost is predicted to reach $3.84 billion. Osteoporotic fractures or fragility fractures, which are catastrophic results of osteoporosis and are of high morbidity and mortality, are usually simply caused by just a fall from a standing height or even less.

The development of primary osteoporosis is considered to result from multiple reasons, including endocrine disorders, nutritional deficiency, and genetic mutations. In contrast, secondary osteoporosis was defined as bone loss secondary to other pathological conditions, such as multiple
myeloma, hyperparathyroidism, and hyperthyroidism (2). Below, we focus on estrogen-deficiency induced osteoporosis and the role of osteoclasts on this process.

Estrogen-deficiency osteoporosis is the most common type of osteoporosis which primarily occurs in postmenopausal women. The negative relationship between estrogen and osteoporosis was first proposed by Albright et al., which was further supported by the evidence that estrogen treatment could prevent osteoporosis (10). Subsequently, a few studies demonstrated that estrogen deficiency mainly causes hyperactive osteoclast formation and resorption which exceeds the extent of bone formation (11-13). Hence, the central role of osteoclasts on the pathogenesis of postmenopausal osteoporosis was established and large amounts of studies were then conducted to investigate the underlying cellular mechanisms. Discoveries were first made on the relationships between the cytokines and estrogen. It was found that human peripheral-blood monocytes from postmenopausal women or ovariectomized premenopausal women secreted larger amounts of IL-1 and TNF (14, 15), which were positive regulators of osteoclastogenesis. Followed by the unravelling of the essential effect of the RANKL/RANK/OPG axis on osteoclast biology, the effects of estrogen on osteoporosis were better understood. Estrogen specifically enhances OPG expression in osteoblasts and stromal cells (16), which leads to the inhibition of RANKL signalling. Consistently, bone marrow cells of postmenopausal women also showed the upregulation of RANKL (17). Recently, growing evidence also indicated that reactive oxygen species (ROS) play a critical role in the development of osteoporosis by activating osteoclasts (18-20). Therefore, estrogen deficiency promotes osteoclast formation and activity by upregulating IL-1, TNF, as well as RANKL signalling. These findings suggest that anticatabolic agents are promising therapeutic approaches for osteoporosis by targeting osteoclasts.

2.2.2 Osteonecrosis of femoral head
Non-traumatic or spontaneous osteonecrosis of femoral head (ONFH) is a devastating bone disorder in which the femoral head blood supply is disrupted and subsequently leads to cellular death, subchondral microfracture, and eventually the collapse of the femoral head (21, 22). It frequently occurs in relatively active, middle-aged patients. An epidemiologic survey conducted in Japan revealed that the peak age of definitive diagnosis were the 30s in female patients and 40s in males (23). Unfortunately, ONFH is often progressive, with a rate of 67% in asymptomatic patients who proceed to collapse of the femoral head and receive total hip arthroplasties (THA) within a short time period (24). In the United States, it was estimated over 10% of more than 500,000 THA are
performed in patents due to ONFH (25, 26). Several factors including head collapse, lesion size, presence of head depression, and acetabular involvement are believed to determine the prognosis and treatment options (22, 27). Given the high incidence and catastrophic consequences, development of joint-preserving approaches are fundamental to prevent the collapse of femoral head and thus slow or even avoid the progression to THA, particularly in the young population.

A couple of risk factors have been suggested to be associated with ONFH, but there is still a lack of consensus regarding a specific or common pathophysiologic mechanism (22). The administration of high-dose steroids is considered to be the most commonly known risk factor, which accounts for 51% of cases in Japan (23). Alcohol abuse is also a major aetiology which represents 31% of the affected population. Other assumed risk factors include tobacco use, systemic lupus erythematosus (SLE), coagulation abnormalities, radiation, human immunodeficiency virus (HIV) infection, chemotherapy, and genetic factors (22, 23). Below we focus on the major causative factor, steroid-induced ONFH.

It still remains obscure how ONFH develops following the use of steroids, which is regarded as an independent risk factor for ONFH. The occurrence of ONFH is highly related with the steroid dose used in patients. It was reported that for SLE patients receiving corticosteroid treatment, an increase of 10 mg/d resulted in an increase of 3.6% in the rate of the development of osteonecrosis, and a daily dose higher than 40 mg predisposed a person to develop ONFH (28). A time sequential MRI investigation was conducted in a cohort of renal allograft recipients who were administrated with steroid post-operatively for immune-suppression purposes. All necrotic lesions occurred within 16 weeks after renal transplantation, and the onset of ischemic events was believed to arise within 12 weeks considering the time lag due to reparative processes (29). The hip symptoms caused by femoral head collapse usually presented 6 months to 2 years after the start of use of steroids (29-31). The initial site and size of the necrotic lesion are thought to be fundamental to the prognosis of ONFH and the continuation of steroid administration is rarely associated with the expansion of necrotic area or additional recurrence of bone necrosis (29, 32, 33).

In the mouse ONFH model, the early stage of steroid-induced ONFH is characterized by the decreased expression of vascular markers such as hypoxia-inducible factor (Hif)-α and vascular endothelial growth factor (VEGF), reduced osteoblast activity, but increased osteoclast formation (34). As the osteonecrosis progressed, the femoral head cancellous density decreased and bone
architecture was damaged (35), which ultimately resulted in end-stage of ONFH showing the collapse of the femoral head. It was implicated that the resultant bone loss and subchondral fracture was predominantly due to the hyperactive osteoclastic resorption rather than the necrosis itself (35). Thus, an early diagnosis of ONFH and the implementation of anticatabolic treatments to prevent the collapse of femoral head may remain as one promising strategy. Moreover, both in animal models and humans, the well-known anti-resorptive agents bisphosphonates were demonstrated to have therapeutic effects on ONFH to slow its progression by targeting osteoclasts (36-42). However, there are also some studies challenging the efficacy regarding the use of bisphosphonates on ONFH (43). More anticatabolic therapies are still needed at this stage.

2.2.3 Periprosthetic osteolysis
Joint arthroplasty, a reliable approach for end-stage arthritis, severe trauma as well as periarticular tumours, is considered to be successful up to 10- to 15-year follow-up intervals. However, the long durability of the joint prostheses after surgery is limited because of the generation of wear particles which cause periprosthetic osteolysis and subsequent aseptic loosening (44). Although the mechanisms of periprosthetic osteolysis remain unclear, it is widely accepted the wear-particle induced hyperactivation of osteoclasts plays a leading role in the pathogenesis (45). Implant-derived wear particles stimulate osteoclastogenesis and bone resorption by enhancing RANKL expression in osteoblasts as well as several cytokines including TNFα, interleukin 1 (IL-1), IL6 and MCSF which are induced from monocytes, fibroblasts, and T lymphocytes. (46-48). Other factors that promote the bone resorption include mechanical stimuli within the joint space, devitalized bone produced by the surgical procedure, disuse osteopenia, endosteal bone remodeling, and age-related bone marrow expansion (45, 48). In addition to surgical replacement of implants, pharmacological approaches based on anti-resorptive agents are considered to be promising both in animal models (49) and patients after joint arthroplasty (50). Bisphosphonate significantly reduced the periprosthetic bone loss after total hip arthroplasty in a 6-year follow-up study, for example (50). However, further evidence to establish the safety and efficacy of using these agents is still required.

2.2.4 Paget disease of bone
Paget disease of bone (PDB) is a metabolic bone disorder featured by a high rate of bone turnover due to an abnormal bone remodelling cycle (51). The incidence of PDB remains the second highest bone remodelling disorder in the United States (2). The primary abnormality observed in the pathogenesis of PDB is excessive osteoclast bone resorption, which is then compensated by high,
but abnormal, bone formation, thus leading to the development of woven bone prone to deformity and fracture (51). PDB primarily affects, and is restricted to, focal areas of one or more skeletal sites. The aetiology of PDB is still not fully understood. Peripheral mononuclear cells derived from the affected bone were shown to be of higher sensitivity to RANKL than those from normal bone (52). Meanwhile, several genetic loci including TNFRSF11A [encoding RANK], CSF1 (colony stimulating factor 1), and OPTN (optineurin) were identified to be associated with PDB in a genome-wide association study (GWAS) (53). Through the analyses of familial PDB, SQSTM1 gene was revealed to be the most common mutation leading to PDB (51). Current treatments are mainly directed to inhibit osteoclast activity.

2.2.5 Other bone disorders related to hyperactive osteoclasts

Other common osteoclast-related bone diseases include rheumatoid arthritis (RA) and bone tumours. RA is a chronic autoimmune disorder which causes swelling, pain, and tenderness to touch in joints. The development of RA is usually irreversibly progressive, which leads to the bone and cartilage destruction. Excessive mature osteoclasts are observed in the localized lesions and the imbalanced ratio of RANKL and OPG is believed to be the responsible factor (54). Similarly, RANKL/RANK/OPG system also plays a critical role on the invasion, metastasis, and recurrence of primary or secondary bone tumours by mediating osteoclast activity (55, 56).

2.3 Overview of Anticatabolic Agents

As the excessive activation of osteoclasts dominantly mediates the development of a wide spectrum of bone diseases, a variety of anticatabolic agents have been emerging to inhibit the osteoclasts over the past decades. For some of them, the efficacy and safety were examined by clinical trials and follow-up investigations, from which people have better understanding of the indications to achieve better outcomes. An overview of current approved anticatabolic agents and their limitations are shown in Table 2.1. Also, there are some ongoing developments of novel drugs based on strategies to inhibit osteoclast formation and function, which may need further clinical trials to assess their safety and feasibility.
Table 2.1. Currently approved anticatabolic agents for osteoclast-related diseases.

<table>
<thead>
<tr>
<th>Anticatabolic agents</th>
<th>Potential mechanisms</th>
<th>Limitations</th>
</tr>
</thead>
<tbody>
<tr>
<td>Estrogens:</td>
<td>Acting on estrogen receptor to enhance osteoclast apoptosis; Increase the ratio of OPG/RANKL expression in osteoblasts</td>
<td>• Cardiovascular events</td>
</tr>
<tr>
<td>• Estrogen</td>
<td></td>
<td>• Breast cancer</td>
</tr>
<tr>
<td>• Estrogen plus progestin</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Selective estrogen-receptor modulators (SERMs):</td>
<td>Estrogen agonist effects by interacting with estrogen receptor</td>
<td>• A modest effect on BMD;</td>
</tr>
<tr>
<td>• Raloxifene,</td>
<td></td>
<td>• Reduce vertebral fracture but not non-vertebral fracture;</td>
</tr>
<tr>
<td>• Bazedoxifene plus oestrogen</td>
<td></td>
<td>• Increased risks of venous thromboembolism</td>
</tr>
<tr>
<td>Calcitonin:</td>
<td>Reduce osteoclastic resorption by interacting with specific G protein-coupled receptors</td>
<td>• Inconsistent effect on fracture prevention;</td>
</tr>
<tr>
<td>Miacalcin (calcitonin-salmon)</td>
<td></td>
<td>• Increased risk of cancer with long-term use</td>
</tr>
<tr>
<td>Bisphosphonates:</td>
<td>High affinity to bone mineral and inhibitory effects on osteoclasts</td>
<td>• Inconvenient administration: nasal spray or injection</td>
</tr>
<tr>
<td>• Alendronate,</td>
<td></td>
<td></td>
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<tr>
<td>• Iseдрonate,</td>
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<td>• Ibandronate,</td>
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<tr>
<td>• Zoledronic acid</td>
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<tr>
<td>RANKL antibody Denosumab</td>
<td>Specifically deactivate RANKL signalling</td>
<td>• Osteonecrosis of the jaw</td>
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<tr>
<td></td>
<td></td>
<td>• Atypical fractures</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Serious infections</td>
</tr>
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<td></td>
<td></td>
<td>• Malignancy</td>
</tr>
</tbody>
</table>

2.3.1 Estrogens

Seventy-nine years ago Fuller Albright recognized that osteoporosis developed before 65 years old mostly in women and established for the first time a relationship between estrogen deficiency and osteoporosis (10). It was later found long-term estrogen treatment can effectively prevent the reduction in bone mineral content of patients after oophorectomy, particularly in the first 3 years of treatment (57). In postmenopausal women with diagnosed osteoporosis, estrogen also showed therapeutic effects (58). The direct and indirect effects of estrogen on bone remain yet to be fully elucidated, but partly include the enhancement of osteoclast apoptosis, induction of OPG.
expression and suppression of RANKL expression in osteoblasts (59, 60). Estrogens are always administrated simultaneously with progestin which can reduce the risk of endometrial cancer. In 2003, a definitive study of the Women’s Health Initiative (WHI) was conducted, in which 16,608 postmenopausal women were randomly given estrogen plus progestin or placebo and followed up for an average of 5.6 years. They found estrogen could increase BMD and result in a lower risk of bone fracture, especially in women whose calcium intake was above 1200 mg/day (61). However, the WHI study also revealed estrogen treatment significantly increase the risk of cardiovascular events and breast cancer (62). The protective effects of ultra-low dose estrogen (0.014 mg/day) on the prevention of BMD reduction in postmenopausal women have also been investigated, but whether this dose is related with other adverse events remains unknown (63). Hence, guidelines for the use of estrogen have been altered, estrogen is now primarily used for short-term relief of menopausal hot flushes (6).

2.3.2 SERMs
Compared with estrogen, SERMs were developed to provide a more acceptable overall balance of benefits and risks. Mechanistically, this agent class exerts estrogen agonist effects by interacting with estrogen receptor (ER) with a high affinity but causes different conformational changes to estrogen, thus decreasing some potential side effects (3). Modified unique ligand structure provides the rationale for tissue-specific targeting. The clinical utility of Raloxifene, a SERM, has been determined in a multicentre, randomized, placebo-controlled trial which is known as the Multiple Outcomes of Raloxifene Evaluation (MORE) study (64). In this study, a total of 7705 women diagnosed with osteoporosis received raloxifene or a placebo for up to 3 years and the BMD as well as fracture outcomes were examined. The outcome showed that the administration of Raloxifene modestly improved the BMD and reduced the risks of vertebral fracture but had no effect on non-vertebral fracture. Moreover, Raloxifene also reduced the risk of breast cancer, and did not increase the risks of endometrial hyperplasia and coronary events. However, there were more reported events including venous thromboembolism and menopausal hot flushes in patients receiving Raloxifene. The combination of Bazedoxifene, another SERM, with estrogen was found to prevent the bone loss in postmenopausal women and approved for osteoporotic patients with menopausal symptoms (65). However, the adverse effects of long-term use still remain unclear.

2.3.3 Calcitonin
Calcitonin is a 32-amino-acid hormone produced by the thyroid gland. The secretion of calcitonin is triggered by the increased level of serum calcium and thus it is viewed as a calcium-regulating factor (66). Calcitonin inhibits osteoclastic resorptive function by interacting with its specific G protein-coupled receptors (Gs and Gq). It was first used to treat Paget’s disease of bone and later was found to be effective in the prevention of osteoporosis by modestly improving BMD. However, considering its limited efficacy on fracture prevention compared to other available agents (67), uncertainty for long-term use, and concerns on increased risks of cancer (68), it is now rarely used in the clinic (6).

### 2.3.4 Bisphosphonates

Bisphosphonates currently are the most widely used anticatabolic agents and implicated to be potentially therapeutic for a wide range of bone diseases in which osteoclast formation and activity become excessive. The management of Pagets disease of bone is exclusively reliant on the use of bisphosphonates (51). Several studies demonstrated the efficacy of bisphosphonates on preventing the collapse of femoral head in patients with early stage of ONFH but with some inconsistent outcomes (26, 39, 69-71). Bisphosphonates are commonly prescribed to patients with osteoporosis and are shown to reduce fracture risk by maintaining BMD (72). Additionally, bisphosphonates are also indicated to be effective for periprosthetic osteolysis (73, 74), osteosarcoma-mediated osteolysis (75), myeloma bone disease (76), and rheumatoid arthritis (77). Bisphosphonates are stable analogs of pyrophosphates, which are naturally occurring compounds. They inhibit bone resorption by binding to hydroxyapatite with high affinity, which suppresses the expression of farnesyl pyrophosphate synthase and subsequently leads to the blocking of protein prenylation (65). Without prenylated proteins, the formation and function of osteoclasts is affected. Due to the high binding affinity to bone minerals as well as the possibility of re-attachment when released, bisphosphonates have a long retention and thus provide a long interval between administrations. The use of bisphosphonates has been reported to have some adverse effects including gastrointestinal irritation, atypical femur fractures (78), and osteonecrosis of the jaw (79).

### 2.3.5 Denosumab

The development of denosumab was driven by an improved understanding of osteoclast biology. As outlined in the Chapter 1, RANKL is a major factor that determines osteoclast differentiation and function. Denosumab is a synthetic IgG2 monoclonal antibody targeting RANKL, which can specifically prevent the binding of RANKL to RANK, leading to the inhibition of osteoclast formation and activation. In a phase III clinical trial, osteoporotic women receiving 60 mg of denosumab
subcutaneously every 6 months for 36 months showed a significantly lower risk of both vertebral and nonvertebral fracture compared to the patients receiving placebo (80). Further study was conducted to examine the long-term safety and efficacy of denosumab for osteoporosis, the outcome showed 10 years of denosumab treatment could maintain the fracture risk at low level but was also related with a low incidence of adverse effects including atypical femur fracture, osteonecrosis of the jaw, malignancy as well as infections (81). Denosumab also exhibits therapeutic effects on rheumatoid arthritis. In a multicentre, randomised, phase II clinical trial, 350 Japanese patients with rheumatoid arthritis were randomly assigned to injections of placebo or denosumab and the outcome indicated denosumab considerably repressed the progression of bone erosion, suggesting denosumab may serve as an alternative therapy for rheumatoid arthritis (81). Furthermore, by improving the overall survival and disease progression rate, denosumab is also emerging as a promising therapy for bone tumours which feature excessive osteoclastic activity (82, 83).

2.3.6 Other anticatabolic agents

Cathepsin K, secreted by osteoclasts, serves as a key enzyme to degrade bone matrix. A strategy to develop cathepsin K inhibitors remains promising for the treatment of osteoclast-related bone disorders. Indeed, odanacatib was develop as a specific inhibitor for cathepsin K and exhibited therapeutic effects on postmenopausal osteoporosis, with reductions in the incidence of fragility fracture (84, 85). The use of odanacatib may lead to a low incidence of adverse events including subtrochanteric atypical fracture and morphea-like skin lesions (85). The FDA approval for odanacatib is currently pending. Another specific cathepsin K inhibitor, balicatib, was reported to also cause morphea-like skin changes in patients during phase II clinical trials (86), which were then terminated. V-ATPase contributes to the acidic microenvironment by pumping protons, which is necessary for osteoclastic bone resorption. Thus, V-ATPase remain as a promising target in treating osteoclast-hyperactive bone disorders as outlined above. A few specific V-ATPase inhibitors, including bafilomycin (87), concanamycin A (88), and diphyllin (89) were discovered to potentially inhibit osteoclasts. However, none of these has proceeded to clinical trial so far and further studies regarding the effects in vivo remain to be conducted.

2.4 Closing Remarks
Osteoclasts have a leading role in many bone disorders characterized by a bone destructive process, which makes them an essential target in the treatment of these diseases. The evolution of anticatabolic agents has been advancing over the past decades, from clinical observations, opportunistic inventions to the more specific osteoclast biology investigations which established a large number of novel therapeutic approaches for the osteoclast-related bone disorders. With currently available anticatabolic therapies, the burden of bone disorders such as osteoporosis has been remarkably reduced. However, concerns with regard to the side-effects and long-term efficacy were raised across this expanding list of drugs. Some bone disorders such as ONFH and periprosthetic osteolysis are still waiting for more solid solutions. This problem may be well addressed by more novel discoveries of anticatabolic agents with less adverse effects but more convincing results on reversing bone loss. In the following chapter novel anticatabolic approaches will be presented, including several small molecules, for osteoclast-related bone disorders. In particular, the role of ROS in osteoclasts in the development of ONFH will be investigated and the efficacy of preventing the femoral subchondral bone loss by inhibiting osteoclast formation and function via suppressing ROS level is investigated.

2.5 Reference


CHAPTER THREE - HYPOTHESIS AND AIMS
3.1 General background
To adapt to the influences of circumstances, bone tissue continuously renews itself by performing bone remodelling, which is a complex process maintained by the proper balance between osteoclastic bone destruction and osteoblastic bone formation. Osteoclasts are considered as the sole type of bone-resorbing cells, and excessive formation and activity of osteoclasts lead to various bone and joint disorders, including osteoporosis, periprosthetic osteolysis after joint arthroplasty, Paget disease of bone, rheumatoid arthritis, subchondral bone destruction in osteonecrosis of femoral head (ONFH). Accumulating evidence also indicated that reactive oxygen species (ROS) are critical for the osteoclast-related signalling events. Given the leading role that osteoclasts play in the pathogenesis of the above bone disorders, we hypothesized that anti-catabolic strategies targeting aberrant osteoclast formation and function remain promising in developing bone-protective treatments, particularly the antioxidant agents.

3.2 Hypothesis and aims 1
Two novel secondary metabolites isolated from *Aspergillus fumigatus*, known as helvolic acid (HA) and pseurotin A (Pse), which were previously identified to have antioxidant activity. Herein, we hypothesized HA and Pse may have anti-catabolic effects on bone disorders through the antioxidant properties. We aim to:

1. Examine the inhibitory effects of HA and Pse on osteoclast formation *in vitro*;
2. Determine the cytotoxicity of HA and Pse on osteoclast precursors using cell proliferation assay *in vitro*;
3. Determine the intracellular ROS production of osteoclast precursors in the presence of HA and Pse;
4. Explore the mechanisms underlying the inhibitory effects of HA and Pse on osteoclast using qPCR and Western Blot (WB) assay;
5. Validate the oxidative stress in the ovariectomised (OVX)-induced osteoporosis mice model and investigate the therapeutic effect of Pse *in vivo*.

3.3 Hypothesis and aims 2
In the pathogenesis of ONFH, it is hyperactive osteoclasts, rather than the necrosis itself, that cause the loss of bone structural integrity and femoral head collapse. However, the pathophysiologic mechanisms underlying the activation of osteoclasts in ONFH have not been fully elucidated. It is
further hypothesized that ROS may enhance the activation of osteoclast during the progression of ONFH. We aim to:

1. Determine the expression of osteoclast-related markers and antioxidant enzymes at mRNA and protein levels in the healthy bone tissues and osteonecrotic bone tissues of steroid-induced ONFH patients;
2. Establish the steroid-induced rat ONFH model and examine the ex-vivo ROS signaling using fluorescence probe;
3. Evaluate the femoral head bone volume in the rat ONFH model;
4. Investigate the correlation between the osteoclast and antioxidant enzymes.
CHAPTER FOUR - HELVOLIC ACID ATTENUATES OSTEOCLAST FORMATION AND FUNCTION VIA SUPPRESSING RANKL-INDUCED NFATC1 ACTIVATION

Publication arising from this chapter:
4.1 Abstract
Excessive osteoclast formation and function are considered as the main causes of bone lytic disorders such as osteoporosis and osteolysis. Therefore, the osteoclast is a potential therapeutic target for the treatment of osteoporosis or other osteoclast-related diseases. Helvolic acid (HA), a mycotoxin originally isolated from Aspergillus fumigatus, has been discovered as an effective broad-spectrum antibacterial agent and has a wide range of pharmacological properties. Herein, for the first time, HA was demonstrated to be capable of significantly inhibiting RANKL-induced osteoclastogenesis and bone resorption in vitro by suppressing NFATc1 activation. This inhibition was followed by the dramatically decreased expression of NFATc1-targeted genes including calcitonin receptor (Ctr), TRAcP (Acp5), cathepsin K (Ctsk), V-ATPase-d2 (Atp6v0d2), and matrix metallopeptidase 9 (Mmp9) which are osteoclastic-specific genes required for osteoclast formation and function. Mechanistically, HA was shown to greatly attenuate multiple upstream pathways including ERK phosphorylation, c-Fos signalling, and intracellular Ca\(^{2+}\) oscillation, but had little effect on NF-κB activation. In addition, HA also diminished the RANKL-induced generation of intracellular ROS. Taken together, our study indicated HA effectively suppressed RANKL-induced osteoclast formation and function. Thus, we propose that HA can be potentially used in the development of a novel drug for osteoclast-related bone diseases.

4.2 Introduction
Bone undergoes continuous remodelling that consists of a tight coupling of bone resorption and formation throughout life. Changes in either resorption or formation can lead to gross perturbations in the skeletal system, and potentially to life-threatening morbidity and mortality (1, 2). Under normal age-related physiological conditions and certain pathological changes, osteoclasts act as key participants regulating bone loss. For instance, excessive osteoclastic resorption is commonly present in estrogen deficiency-induced osteoporosis (3, 4) and osteolysis such as periprosthetic loosening after total joint arthroplasty (5, 6).

Osteoclasts are large, multinucleated cells derived from hematopoietic cells of the monocyte/macrophage lineage (7). Osteoclasts form through fusion of mononuclear precursor cells and are unique in the ability to resorb mineralised bone matrix. Macrophage-colony stimulating factor (M-CSF) and receptor activator of nuclear factor-κB ligand (RANKL) are two critical factors
responsible for osteoclastogenesis and bone resorption. M-CSF is required for the survival, proliferation and differentiation of osteoclasts via binding to its receptor c-fms (8). It is also involved in the upregulation of RANK, a transmembrane receptor which efficiently interacts with RANKL (8). Interaction between RANK and RANKL is indispensable for osteoclast development and function. Both RANK- (9) and RANKL-deficient (10) mice were similarly characterized by severe osteopetrosis resulting from compromised osteoclast formation in bone.

The binding of RANKL to RANK induces the trimerization of an adaptor molecule, TNF receptor-associate factor 6 (TRAF6), leading to activation of NF-κB, extracellular signal-regulated kinase (ERK) phosphorylation (11), and Ca\(^{2+}\) signalling (12), which is then followed by the initial induction and activation of c-Fos and nuclear factor of activated T cells, cytoplasmic 1 (NFATc1). A number of osteoclast-specific genes such as Acp5 (encoding Tartrate resistant acid phosphatase; TRAcP), Ctr (encoding calcitonin receptor), Ctsk (encoding cathepsin K), Atp6v0d2 (encoding V-ATPase-d2), and Mmp9 (encoding matrix metallopeptidase 9) are regulated by NFATc1 (11, 13). In addition, accumulating studies also demonstrated that RANKL-induced intracellular reactive oxygen species (ROS) generation plays a crucial role in osteoclast formation and activity (14-16).

Helvolic acid (HA), a mycotoxin originally isolated from Aspergillus fumigatus, was discovered as an effective broad-spectrum antibacterial agent (17-19) and exerted enhanced antitumor efficacy in vivo when co-administrated with a well-known chemotherapeutic drug, cyclophosphamide (CTX) (20). HA was also shown to inhibit oxidized LDL metabolism in macrophages (21, 22). However, HA’s effects on RANKL-induced osteoclast formation and function still remain unknown. Given HA’s wide range of pharmacological properties, we investigated its in vitro effects on osteoclast development and its underlying molecular mechanisms. Our results demonstrated that HA effectively inhibited osteoclast formation and function through attenuation of NFATc1 activation via suppressing Ca\(^{2+}\) oscillation, intracellular ROS production, and c-Fos signalling. Taken together, HA is a potential candidate for the treatment of osteoclast-related bone diseases.

4.3 Methods

4.3.1 Materials and reagents

HA was obtained from SHANGHAI ZZBIO CO., LTD (Shanghai, China) and dissolved in dimethyl sulfoxide (DMSO) at a stock concentration of 100 mM. It was further diluted to working
concentrations with culture medium. DMSO of the same dilution was used as vehicle control in assays. Alpha modified Minimal Essential Medium (a-MEM) and fetal bovine serum (FBS) were purchased from Thermo Fisher Scientific (Scoresby, Australia). MTS assay kit and luciferase analysis system were purchased from Promega (Sydney, Australia). Primary antibodies for IκB-α, β-actin, phospho-ERK, ERK, NFATc1, and cathepsin K were purchased from Santa Cruz Biotechnology (Dallas, CA, USA). Primary antibody for c-Fos was purchased from Cell Signaling Technology (Danvers, MA, USA). Recombinant macrophage colony stimulating factor (M-CSF) was purchased from R&D Systems (Minneapolis, MN). Recombinant Glutathione S-transferase (GST)-rRANKL protein was expressed and purified as previously described (23). Rhodamine Phalloidin, Fluo-4 and 2',7'-dichlorodihydrofluorescein diacetate (H₂DCFDA) were obtained from Thermo Fisher Scientific (Scoresby, Australia) and DAPI was purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

4.3.2 In vitro osteoclast differentiation
Primary bone marrow macrophages (BMMs) were isolated from the long bones of euthanized C57BL/6 mice using methods approved by the University of Western Australia Animal Ethics Committee (RA/3/100/1244) and then cultured in α-MEM supplemented with 10% (v/v) fetal bovine serum, 1% (v/v) penicillin/streptomycin and 50ng/ml M-CSF. To induce osteoclast differentiation BMMs were seeded into a 96-well plate at a density of 6 × 10³ cells/well. After adhering to the bottom of the plate, BMMs were stimulated with M-CSF (50 ng/mL) and RANKL (50 ng/mL) in the presence of HA at a range of concentrations (0, 2.5, 5, 7.5, 10µM). The culture medium was changed every 2 days for 5 days until osteoclasts formed. Cells were then fixed with 2.5% glutaraldehyde solution and stained for tartrate resistant acid phosphatase (TRAcP) activity. Multinucleated cells with more than 3 nuclei were identified as osteoclasts.

4.3.3 Staining for actin ring formation
To observe actin rings, osteoclasts were induced as above and were fixed with 4% paraformaldehyde for 10 minutes, permeabilized with 0.1% (v/v) Triton X-100 for 10 minutes, blocked with 3% bovine serum albumin (BSA), and subsequently stained with Rhodamine Phalloidin (1:300 dilution) in the dark for 2 h. Cells were then washed with PBS twice, followed by incubation with DAPI for 10 minutes to visualize nuclei. After staining, cells were mounted in ProLong Gold Antifade Mountant and observed under NIKON A1Si confocal microscopy (Nikon Corporation, Tokyo, Japan). Three images were randomly captured for each group and the number of nuclei and the area of the actin rings were analysed using Image J software.
4.3.4 MTS cell viability assay
To determine whether HA has cytotoxic effects on osteoclast precursors, MTS assay was performed following the manufacturer’s instructions. In brief, BMMs were plated in a 96-well plate at a density of \(6 \times 10^3\) cells/well in triplicate. The following day cells were treated with a range of concentrations of HA (0, 2.5, 5, 7.5, 10 \(\mu\)M) for 48 h. Next, 20 \(\mu\)L MTS solution was added in each well and cells were then incubated at 37 °C for 2 h. The optical density (OD) was measured by spectrophotometric absorbance using a microplate reader (BMG LABTECH, Ortenberg, Germany) at a wavelength of 490nm.

4.3.5 Hydroxyapatite resorption assay
The function of osteoclasts was evaluated by hydroxyapatite resorption assay. BMMs were seeded into 6-well collagen-coated plates (Corning, Inc., Corning, NY) at a density of 1 \(\times\) 10^5 cells/well and stimulated with M-CSF and RANKL to induce osteoclast differentiation. When osteoclasts started to form, cells were harvested gently by cell dissociation solution (Sigma-Aldrich, Sydney, Australia) and seeded onto hydroxyapatite-coated plates (Corning, Inc., NY, USA). Cells were cultured in complete medium containing 50 ng/mL RANKL and M-CSF with or without HA (0, 5, 10\(\mu\)M). After mature osteoclasts formed, half of the wells in each group were stained for TRAcP to count the number of osteoclasts and the rest of wells were bleached to capture the images of hydroxyapatite resorption areas using a Nikon microscope (Nikon Corporation, Tokyo, Japan). The resorption area per osteoclast was analysed to determine the osteoclastic resorption activity.

4.3.6 Intracellular Ca^{2+} oscillation
Calcium oscillations were measured using the calcium fluorophore Fluo4 as described previously (24). Briefly, a total of \(2 \times 10^4\) BMMs were seeded onto a 48-well plate and cultured with RANKL (50 ng/ml) and M-CSF (50 ng/ml) in the presence or absence of 10\(\mu\)M HA for 48 hrs. Cells were then rinsed with assay buffer (HANKS balanced salt solution, 1mM probenecid, and 1% FBS), followed by incubation with 4 \(\mu\)M Fluo4 staining solution (Fluo4-AM dissolved in 20% pluronic-F127 (w/v) in DMSO diluted in Assay buffer) for 45 min. The dye-loaded cells were washed twice with assay buffer and viewed on an inverted fluorescent microscope (Nikon, Tokyo, Japan) at an excitation wavelength of 488 nm, images were captured at 2s intervals for 3 min and the results were analysed using Nikon Basic Research Software. Cells showing 2 intensity peaks within the observed time
frame were identified as oscillating cells and the oscillation intensity change was calculated by the maximum peak intensity minus the minimum intensity.

4.3.7 Determination of intracellular ROS generation

Intracellular generation of ROS was measured as described (14). In brief, after treatment with RANKL (50 ng/ml) and HA at different concentrations (0, 5, 10μM) for 60 minutes, BMMs were then washed and incubated with Hank's balanced salt solution containing 5 mM H_2DCFDA in the dark for 5 minutes. The DCF fluorescence was detected at an excitation wavelength of 488 nm and an emission wavelength of 515–540 nm using a NIKON A1Si confocal microscopy. The average fluorescence intensity was analysed using Image J software.

4.3.8 NFATc1 luciferase reporter gene activity assay

NFATc1 transcriptional activation was evaluated using luciferase reporter gene assay as described previously (25). Briefly, RAW264.7 cells transfected with an NFATc1 luciferase reporter (26, 27) were seeded in 48-well plates and maintained overnight. Next, cells were pre-treated with or without different concentrations of HA (0, 2.5, 5, 10 μM) for 1 h, followed by the addition of RANKL (50 ng/mL) for 24 h. In the end, cells were then lysed, and luciferase activity was measured using a luciferase reporter assay kit (Promega, Sydney, Australia). The results were normalized to that of vehicle control.

4.3.9 Western blot analysis

Signalling pathways were analysed using Western blot. For activation of early signalling pathways BMMs were seeded into 6-well plates at a density of 8 × 10^5 cells/well with complete α-MEM containing M-CSF. Cells were pre-treated with or without HA (10 μM) for 2h prior to RANKL stimulation for the stated times (0, 10, 20, 30 or 60 min). For analysis of long term effects on osteoclast formation BMMs (1 × 10^5 cells/well) were seeded into 6-well plates and treated with RANKL with or without HA (10 μM) for 0, 1, 3 or 5 days. Protein was harvested by lysing cells using radioimmunoprecipitation (RIPA) lysis buffer, followed by centrifugation at 12,000 g for 20 min. The supernatants were collected and total protein was separated on SDS-polyacrylamide gels and transferred to a nitrocellulose membrane (GE Healthcare, Silverwater, Australia). Non-specific binding was blocked with 5% skim milk, and membranes were then incubated with primary antibodies (1:1000) at 4 °C overnight. The next day, membranes were incubated with secondary antibodies conjugated to horseradish peroxidase (HRP) for 2h at room temperature. Antibodies
were detected with enhanced chemiluminesence substrate (PerkinElmer, MA, USA). The antibody reactivity was detected on an Image-quant LAS 4000 (GE Healthcare, Silverwater, Australia) and analysed by ImageJ software.

4.3.10 Quantitative PCR analysis
For real-time PCR, BMMs (1 × 10^5 cells/well) were cultured in 6-well plates and induced to form osteoclasts as above with or without HA (0, 5, 10 μM). Total RNA was prepared using an RNeasy Mini Kit (Qiagen, Chadstone VIC, Australia) and single stranded cDNA was synthesized from 1 μg of total RNA template using Moloney murine leukemia virus (MMLV) reverse transcriptase with oligo-dT primer (Promega). SYBR Green PCR MasterMix was used to perform relative quantitative real-time PCR (qPCR). Cycling conditions were as follows: 94°C for 5 min, followed by 30 cycles of 94°C for 40 s, 60°C for 40 s, and 72°C for 40 s, followed by an elongation step of 5 min at 72°C. The qPCR procedure was performed on a ViiA7 Real-time PCR machine (Applied Biosystems, Warrington, UK). Hprt1 was utilized as the housekeeping gene and all reactions were performed in triplicate. The specific primers used are as follows: Atp6v0d2 (Forward: 5′-GTGACCTTGGGAAGACCTGAA-3′; Reverse: 5′-GAGAAATGTGCTCAGGGCT-3′), Acp5 (forward: 5′-TGTTGCCCATTTATGCT-3′; reverse: 5′-GTGATTCTTGGGGCTT-3′), Ctr (forward: 5′-TGTTGAGGTTGTGCCC-3′; reverse: 5′-CTCGTGGGTGGCTCATC-3′), Nfatc1 (Forward: 5′-CAACGCCCTGACCACCGATAG-3′; Reverse: 5′-GGCTGCTTCGCTCATG-3′), c-Fos (Forward: 5′-GCGAGCAACTGAGAAGAC-3′; Reverse: 5′-TTGAAAACCAGAACATC-3′), Ctsk (forward: 5′-GGAGAAAACCTGAGAGC-3′; reverse: 5′-ATTCTGGGACTCAGAGC-3′), Hprt1 (Forward: 5′-TCAGTCAACGGGGACATAAA-3′; Reverse: 5′-GGGGCTGTACTGCTTAACCAG-3′).

4.3.11 Data analysis
All quantitative data are presented as mean ± standard deviation (SD) obtained from at least three experiments. Statistical analyses were determined by Student’s t test and One-way ANOVA. A p value of less than 0.05 was considered to be significant.

4.4 Results

4.4.1 HA inhibits RANKL-induced osteoclastogenesis in vitro
The chemical structure of HA is demonstrated in Figure 4.1A. To determine the effects of HA on osteoclastogenesis, freshly isolated BMMs were induced to form osteoclasts under the stimulation
of RANKL and M-CSF in the presence of HA at different concentrations. The results showed that HA was able to interfere with osteoclastogenesis in a dose-dependent manner as demonstrated by the significant reduction in the number of TRAcP-positive multinucleated osteoclasts (cells with more than three nuclei) at concentrations of 2.5 µM HA and higher (Figure 4.1B and C). To examine whether HA has cytotoxic effects on BMMs, an MTS assay was performed to assess cell viability. It was found that HA had no cytotoxicity on BMMs within the range of concentrations as indicated in our study (Figure 4.1D). Mature osteoclasts were also stained with rhodamine-phalloidin to visualize any morphological changes in formation of F-actin structures (Figure 4.2A). F-actin ring formation is critical for osteoclast function and well-defined actin rings were observed in the control group. However, the area of actin rings per field and the number of nuclei per osteoclast in the HA-treated group were reduced (Figure 4.2B and C). To further evaluate which stage of osteoclast formation was most sensitive to HA treatment, cells were exposed to 10 µM HA at different time points (Figure 4.3A). We found that osteoclast formation was interrupted by treatment with HA at all three stages; however, treatment with HA at early stages (Day 1-3) inhibited osteoclastogenesis most significantly (Figure 4.3B and C). Taken together, these results indicate that HA inhibits RANKL-induced osteoclast differentiation without any obvious cytotoxicity in osteoclast precursors.
Figure 4.1 Helvolic acid (HA) inhibited RANKL-induced osteoclastogenesis in a dose-dependent manner, without cytotoxicity. (A) The chemical structure of HA. (B) Representative images of TRAcP staining. BMMs were cultured in the presence of M-CSF (50 ng/mL) and RANKL (50 ng/mL) with indicated concentrations of HA for 5 days. Cells were fixed and stained for TRAcP. (C) Quantification of TRAcP-positive osteoclasts. Multinucleated cells with more than 3 nuclei were identified as osteoclasts (n=3). (D) BMMs were treated with a serial of concentrations of HA (0, 2.5, 5, 7.5, 10 µM) for 48 h. Cell viability was then measured using MTT assay (n=3). **P<0.01 relative to RANKL-induced control group. Scale bar = 200 µm. BMMs, bone marrow macrophages; RANKL, receptor activator of nuclear factor-κB ligand; TRAcP, tartrate-resistant acid phosphatase
Figure 4.2 HA affects F-actin ring formation. (A) Representative confocal images of mature osteoclasts stained for actin ring and nuclei using Rhodamine Phalloidin and DAPI respectively; (B) The average number of nuclei per osteoclast (C) and the average of actin ring area per field were analysed quantitatively (n=3). *P<0.05, **P<0.01 relative to RANKL-induced control group. Scale bar = 200 µm. BMMs, bone marrow macrophages; RANKL, receptor activator of nuclear factor-κB ligand; TRAcP, tartrate-resistant acid phosphatase
Figure 4.3 HA majorly inhibited RANKL-induced osteoclastogenesis at early stage. (A) The time periods of the treatment of HA. (B) Representative images of the effect of 10 μM HA’s treatment on the different time periods. Osteoclast formation was induced by RANKL (50 ng/ml) and M-CSF (50 ng/mL), and the cells were treated by HA in the indicated time period. (C) TRAcP-positive multinucleated cells (nuclei >3) with the treatment of HA were counted (n=3). *P<0.05, **P<0.01 relative to RANKL-induced control group. Scale bar = 200 μm. RANKL, receptor activator of nuclear factor-κB ligand; TRAcP, tartrate-resistant acid phosphatase.
4.4.2 HA suppresses osteoclast hydroxyapatite resorption and osteoclast-specific gene expression

As HA inhibited osteoclastogenesis and F-actin ring formation, we hypothesized that HA might also affect osteoclastic resorption. Hydroxyapatite-coated plates were used to examine the function of osteoclasts. Relative to the control group, the area of resorption per osteoclast was reduced substantially after treatment with 5 and 10 µM HA (Figure 4.4). In addition, osteoclast-specific genes Atp6v0d2, Acp5, Ctr and Mmp9 were assessed using quantitative real-time PCR. As demonstrated in Figure 4.5, RANKL up-regulated these genes expression of mature osteoclast while HA dramatically suppressed this upregulation. Therefore, these data showed that HA attenuated osteoclast hydroxyapatite resorption and mRNA expression of osteoclast-specific genes.

4.3.3 HA abrogates RANKL-induced Ca^{2+} oscillation

Next, to explore the molecular mechanism by which HA inhibited osteoclast formation and activity, we investigated the effects of HA on RANKL-induced intracellular calcium oscillation. Activation of calcium oscillation is a well-defined process that is critical for the development of osteoclast via NFATc1 activation (12, 28). As is shown in Figure 4.6A and B, compared with the control group, Ca^{2+} oscillation was enhanced by stimulation with RANKL. In contrast, after treatment with HA, RANKL-induced Ca^{2+} oscillation was significantly attenuated (Figure 4.6C and D).

4.4.4 HA suppresses RANKL-induced ROS generation

ROS generation has been shown to increase in response to RANKL stimulation and they act as an intracellular mediator for osteoclast formation (14, 16). Therefore, we tested whether HA would affect ROS generation in BMMs. Intracellular ROS generation was detected by the cell permeant, oxidation-sensitive dye H$_2$DCFHDA (DCF) using confocal microscopy. We found that the addition of HA abolished the rise in DCF fluorescence by RANKL stimulation in a dose-dependent manner (Figure 4.7A). The intensity of DCF fluorescence per positive cell (Figure 4.7B) decreased significantly following HA treatment.
Figure 4.4 HA suppressed osteoclast hydroxyapatite resorption. Representative images of the osteoclastogenesis and hydroxyapatite resorption in each group. Osteoclasts were seeded in hydroxyapatite-coated plates and treated by RANKL with or without HA. Half of the wells for each group were stained by (A) TRAcP or (B) washed with 10% bleach solution. (C) Quantification of the number of osteoclasts per well in each group (n=3). (D) Quantification of resorbed hydroxyapatite surface area per osteoclast (n=3). *P<0.05, **P<0.01 relative to RANKL-induced control group. Scale bar = 200 µm.
Figure 4.5 HA suppressed osteoclast-specific genes expression. Real-time PCR results of osteoclast-specific genes (A) Atp6v0d2, (B) Acp5, (C) Ctr, and (D) Mmp9. The expression levels were normalized to the expression of Hprt1. **P<0.01 relative to RANKL-induced control group. Scale bar = 200 µm. Acp5, acid phosphatase 5, tartrate resistant; Atp6v0d2, ATPase H+ Transporting V0 Subunit D2; BMMs, bone marrow macrophages; Ctr, calcitonin receptor; Mmp9, matrix metallopeptidase 9;
Figure 4.6 HA abrogated RANKL-induced Ca\textsuperscript{2+} oscillation. Representative images of fluorescence intensity waves of Ca\textsuperscript{2+} oscillation in (A) negative group, (B) positive group, and (C) HA (10μM) treated group. Each color indicates a different cell. (D) Quantification of intensity change of Ca\textsuperscript{2+} oscillation in each group (n=6). The values of intensity change were calculated by the maximum peak intensity minus the minimum intensity. **P<0.01 relative to RANKL-induced control group.
Figure 4.7 HA suppressed RANKL-induced ROS generation. (A) BMMs were stimulated with RANKL and with or without the addition of HA. Intracellular ROS generation was detected by the cell permeant, oxidation-sensitive dye H$_2$DCFHDA using confocal microscopy. (B) Quantification of DCF fluorescence intensity averaged on cells. Results are representative of 3 independent experiments. *P<0.05 relative to RANKL-induced control group. Scale bar = 200 μm. BMMs, bone marrow macrophages; DCF, 2',7'-dichlorofluorescein; H$_2$DCFDA, 2',7'-dichlorodihydrofluorescein diacetate; RANKL, receptor activator of nuclear factor-κB ligand; ROS, reactive oxygen species.
4.4.5 HA inhibits RANKL-induced NFATc1 activation

NFATc1 is well known as a master transcriptional regulator of osteoclast differentiation (29), and thus the activation of NFATc1 may be a target of HA treatment. To determine NFATc1 activation, we first utilized a NFATc1 luciferase reporter gene construct in RAW264.7 cells to investigate the NFATc1 transcriptional activity. Cells pre-treated with HA showed a dose-dependent impairment of NFATc1 activity (Figure 4.8A). Consistently, in western blot assay, the protein level of NFATc1 was dramatically upregulated on day 3 and day 5 in RANKL stimulated cultures, but it was suppressed significantly when treated with HA (Figure 4.8B and C). This suppression was also found in mRNA expression of Nfatc1 (Figure 4.8D). Cathepsin K is a crucial downstream protein of functional mature osteoclasts. Following HA treatment, the protein expression of cathepsin K exhibited a substantially decreased trend (Figure 4.8B and E). This result was consistently supported by real-time PCR, which demonstrated the attenuation of Ctsk mRNA expression after HA treatment (Figure 4.8F). These data strongly suggested HA inhibited RANKL-induced NFATc1 activation during osteoclast formation.

4.4.6 HA attenuates c-Fos signaling and ERK phosphorylation

To further elucidate the mechanisms through which HA mediated the suppression of NFATc1 activation, we investigated upstream signaling pathways of NFATc1 such as c-Fos, ERK, and NF-κB. We studied the expression of c-Fos, an upstream component which binds to the promoter of NFATc1 to promote its transcriptional activities (30). C-Fos protein expression in BMMs increased on day 3 after the stimulation with RANKL (Figure 4.8B). However, treatment with HA significantly inhibited the expression of c-Fos in both mRNA and protein level (Figure 4.8G and H). Due to the reduced expression of c-Fos by the interference of HA, we next determined whether its upstream of ERK phosphorylation had been affected. As shown in Figure 4.9A and B, following the stimulation of RANKL, the phosphorylation of ERK increased particularly after 10 and 20 min while the HA treatment could significantly block this upregulation. The effect of HA on RANKL-stimulated NF-κB pathway was also studied, the protein expression of IκBα, an inhibitor of NF-κB, was tested using western blot. After RANKL stimulation, IκBα degraded to activate NF-κB (Figure 4.9A). Treatment with HA had no effect on the degradation of IκBα (Figure 4.9C), indicating it does not impact the NF-κB pathway. Collectively, these data indicated that HA suppressed c-Fos signalling via the inhibition of ERK phosphorylation, but without effects on NF-κB activation.
Figure 4.8 HA attenuated NFATc1 activation and c-Fos signaling. (A) HA affected luciferase activity of RAW 264.7 cells transfected with NFATc1 luciferase construct. Cells were pre-treated with HA of different concentrations as indicated for 1h, followed by the stimulation of RANKL (50 ng/ml) for 24 hours. The luciferase activity was measured using luciferase reported reporter assay (n=3). (B) Representative Western Blot images of the effects of HA on the protein expression of NFATc1, cathepsin K, and c-Fos during osteoclastogenesis. (C-H) Quantification of the ratios of band intensity and real-time PCR results of genes of NFATc1 (C and D), cathepsin K (E and F), and c-Fos (G and H). The band intensity was relative to β-actin (n=3) and the mRNA expression levels were normalized to the expression of Hprt1 (n=3). (I) Representative Western Blot images of the effects of HA on ERK phosphorylation and IκBα degradation and induced by RANKL. BMMs were treated with RANKL for the indicated time points with or without the addition of 10 μM HA. (J and K) Quantification of the ratios of band intensity of phosphorylated ERK (J) and IκBα (K) relative to total ERK and β-actin respectively (n=3). *P<0.05, **P<0.01 relative to RANKL-induced control group.
Figure 4.9 HA attenuated ERK phosphorylation but had no effect on NF-κB pathway. (A)
Representative Western Blot images of the effects of HA on ERK phosphorylation and IkBα degradation and induced by RANKL. BMMs were treated with RANKL for the indicated time points with or without the addition of 10 μM HA. (B and C) Quantification of the ratios of band intensity of phosphorylated ERK and IkBα relative to total ERK and β-actin respectively (n=3). *P<0.05, **P<0.01 relative to RANKL-induced control group.
4.5 Discussion

Osteoporosis is a highly prevalent disease characterized by low bone mass and increased susceptibility to bone fracture, which results in massive costs to both the individual and society (2). Excessive osteoclast formation and function are considered as the main causes of bone lytic disorders such as osteoporosis and osteolysis (5, 31). Therefore, osteoclasts remain one of the potential therapeutic targets for the treatment of osteoporosis and osteoclast-related diseases. In this study, for the first time, HA was demonstrated to be capable of inhibiting osteoclast formation and function by suppressing RANKL-induced NFATc1 activation.

Our results indicated that HA effectively inhibited RANKL-induced osteoclast formation, particularly at early stages, in dose-dependent manner but without exerting any obvious cytotoxicity. HA was also effective at inhibiting resorption suggesting that it might be a drug prototype for an alternative treatment of osteoclast-related bone disease.

We further studied the mechanisms behind HA’s inhibitory effects on osteoclasts. NFATc1 represents a master regulator of terminal RANKL-induced differentiation of osteoclasts (29). The physiological significance of NFATc1 has been well explored. Embryonic stem (ES) cells were able to differentiate into osteoclasts in response to stimulation with RANKL and M-CSF while NFATc1-deficient ES cells failed (29). In addition, osteoclast-specific conditional NFATc1-deficient mice developed osteopetrosis (32). Growing evidence shows that a number of osteoclast-specific genes, including Acp5 (29, 30), Ctr (29, 33), Itgb3 (encoding integrin beta 3) (34), Atp6v0d2(13), and Mmp9(35) are regulated by NFATc1. Cathepsin K is one of the targets regulated by NFATc1 (36). It is found at the ruffled border of active mature osteoclasts and acts as an essential mediator of bone resorption (37). This evidence collectively demonstrates that NFATc1 has an indispensable function in osteoclast differentiation in vitro and in vivo. Therefore, we evaluated the expression level and transcriptional activity of NFATc1 during HA-treated osteoclast formation and demonstrated that NFATc1 activation was dramatically suppressed by HA treatment, followed by suppressing expression of a number of NFATc1-targeted genes including Atp6v0d2, Acp5, Ctr, Ctsk, and Mmp9 leading to the compromised osteoclastogenesis and osteoclastic resorption.

The molecular mechanisms underlying activation of NFATc1 have also been extensively studied previously. NFATc1 is a master transcriptional switch for osteoclast differentiation (29). NFATc1
activation is mediated by calcineurin, which is a specific phosphatase activated by calcium/calmodulin signalling (12). The evident and long-lasting Ca\(^{2+}\) oscillations observed during osteoclastogenesis are required to maintain the induction of NFATc1 (12). Chromatin immunoprecipitation (CHIP) experiments showed that NFATc1 was selectively recruited to the NFATc1 promoter in response to RANKL stimulation and this binding persisted during the terminal differentiation of osteoclasts, suggesting the mechanism of NFATc1 autoamplification (38). In this study, Ca\(^{2+}\) oscillation was induced by RANKL while diminished by the addition of HA, suggesting HA affected NFATc1 partly at least via suppressing Ca\(^{2+}\) oscillation.

C-Fos, a critical component of the transcription factor complex AP-1, is also known as a prerequisite for the robust induction of NFATc1 by functioning as a direct transcriptional regulator of NFATc1 (29, 39). C-Fos-deficient cells failed to induce NFATc1 activation following RANKL stimulation (29) and overexpression of NFATc1 rescued osteoclast differentiation in C-Fos-deficient cells (30). Consistent with HA’s inhibition of NFATc1 activation, we also found that HA strongly reduced the mRNA expression and protein level of c-Fos. Because the expression and stabilization of c-Fos were shown to be linked to the ERK signalling pathway (40, 41), along with inhibition of c-Fos by HA, we attempted to examine its upstream ERK phosphorylation. As expected, RANKL greatly stimulated the phosphorylation of ERK in BMMs, but BMMs pre-treated with HA saw a decreased expression of phosphorylated ERK. In addition, Ca\(^{2+}\) signalling is also responsible for the initial induction of c-Fos in the early stage of osteoclast formation (12). We thus reasoned HA suppressed c-Fos via the suppression of ERK signalling and Ca\(^{2+}\) oscillation.

Recently, growing studies have shown that ROS are generated via nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (Nox) in activated osteoclasts, which are thought to be highly associated with the process of osteoclastogenesis and bone resorption (14, 42, 43). Furthermore, a variety of antioxidants were also found to be capable of inhibiting the formation and bone resorption of osteoclasts by reducing the production of intracellular ROS (14, 44, 45). As Ca\(^{2+}\) oscillations mediated the production of mitochondrial ROS (46) and ROS was clearly demonstrated to be involved in NFATc1 induction (28, 47), we were interested to investigate whether HA’s inhibitory effects on NFATc1 were linked to its attenuation on ROS generation during RANKL-induced osteoclastogenesis. Interestingly, HA had a negative effect on RANKL-induced ROS production. These findings suggest that HA can also suppress NFATc1 via acting as a ROS scavenger or inhibitor. However, HA was previously reported to be ineffective in inhibiting phorbol myristate
acetate (PMA)-stimulated ROS generation in human polymorphonuclear leukocytes (PMN) (48). This contradiction may lie in the different stimulation conditions and different type of cells used in the experiments.

NF-κB activation is a very early event in response to RANKL stimulation (49) and it contributes to the initial induction of NFATc1 through binding to its promoter directly (38). In our study, we observed that the RANKL-induced degradation of IκBα was not affected by the treatment with HA, suggesting HA failed to inhibit the activation of NF-κB. However, whether HA can affect the downstream NF-κB complex, including p65/Rel A, to translocate to the nucleus and achieve its transcription of targeted genes still remains to be further investigated.

In summary (Figure 4.10), our studies provide evidence that HA exerted inhibitory effects on osteoclastogenesis via strongly suppressing RANKL-induced NFATc1 activation, the master regulator involved in osteoclast formation. Furthermore, we also clarified that the attenuated NFATc1 expression was related to the suppression of multiple pathways, including Ca2+ oscillation, intracellular ROS production, and c-Fos signalling. Considering its strong inhibitory effects, we propose that HA, or related molecules, could be potentially used in the development of an alternative drug for osteoclast-related bone disease.
**Figure 4.10** A proposed scheme depicting the inhibition of HA on RANKL-induced NFATc1 activation during osteoclast differentiation. The binding of RANKL to RANK activates NF-κB, ERK phosphorylation, Ca^{2+} oscillation, and ROS production, followed by the activation of c-Fos and NFATc1. A number of osteoclast-specific genes such as *Atp6v0d2*, *Acp5*, *Ctr*, *Ctsk* and *Mmp9* are then regulated by NFATc1 and result in the formation of mature osteoclasts. Our results indicated that the presence of HA exerted multiple inhibitions including attenuating ERK phosphorylation, diminishing Ca^{2+} oscillation, reducing the production of ROS, but without effects on NF-κB pathway, thus eventually suppressing RANKL-induced osteoclast formation and function in vitro.
4.6 References