Phosvitin Extraction from Egg Yolk and Its Potential as a Functional Food Ingredient for Improving Bone Health

by

Jiandong Ren

A thesis submitted in partial fulfillment of the requirements for the degree of

Doctor of Philosophy

in

Food Science and Technology

Department of Agricultural, Food and Nutritional Science

University of Alberta

ABSTRACT

Osteoporosis is a common disease afflicting a quarter of the population of 50 years old and above worldwide. An osteoporotic fracture brings great pain and inconvenience to the patients, and sometimes causes immobility or even mortality. Drugs are available for treating osteoporosis but are associated with serious side-effects. There is an interest in discovering food components for improving bone health.

Egg yolk phosvitin is one of the most phosphorylated proteins in nature with a potential for increasing bone health. The overall objectives of this thesis were to develop a scalable method of phosvitin extraction and to study the effects of phosvitin and phosvitin hydrolysate on bone cells as well as osteoporosis rat model. Since inflammation stimulates bone cells and causes bone loss, the effects of phosvitin and phosvitin hydrolysate on bone cells under inflammatory stimulation were investigated.

To develop a scale-up method of phosvitin extraction for animal study and potential industrial application, the first study focused on the effects of pH, low centrifugal force, ultra filtration desalting, use of industrial separators and different water dilution factors on the extraction. The results suggested that decreasing pH from neutral to acidic could increase phosvitin purity and recovery. The highest recovery of 67.3% was obtained at pH 3.0 with a purity of 86.3%. Desalting achieved by ultrafiltration was comparable to that of dialysis. Increasing water dilution ratio from 1/1 to 1/4 (yolk/water, weight/weight) increased phosvitin recovery in the pellets, as well as phosvitin purity and recovery in the final extracts at pH 3.0. Low centrifugal force resulted in low phosvitin recovery (11.4% at 2,000 g vs 67.3% at 10,000 g, at pH 3.0), although the purity remained at a high level. None of the industrial separators (decanter and disc stack separators) were sufficient to separate the pellet from egg yolk and thus

resulted in significantly lower yield (~20-30%), while the purity from both separators was comparable (~70%).

Both phosvitin and its hydrolysate promoted osteoblast differentiation by upregulating expression of runt-related transcription factor 2 (RUNX2), alkaline phosphatase, osteocalcin production and collagen synthesis. Tumor necrosis factor alpha (TNF-α) induced production of inflammatory proteins including regulated on activation, normal T cell expressed and secreted (RANTES), monocyte chemoattractant protein-1 (MCP-1) and cyclooxygenase-2 (COX-2). Both phosvitin and phosvitin hydrolysate inhibited the production of these proteins in osteoblast cells. The expression of osteoprotegerin (OPG) and alkaline phosphatase (ALP) was not affected. Considering the activation of both extracellular signal–regulated kinases (ERK) and protein kinase B (AKT) signaling pathways, phosvitin and its hydrolysate may promote osteoblast differentiation and exert anti-inflammation effects via ERK and AKT pathways.

Both phosvitin and phosvitin hydrolysate inhibited receptor activator of nuclear factor kappa-B ligand (RANKL) induced osteoclastogenesis in RAW264.7 cells. The number of tartrate-resistant acid phosphatase (TRAP) positive cells and TRAP activity were both reduced upon treatment of phosvitin/phosvitin hydrolysate. The expression of osteoclastogenesis-associated transcription factors, c-Fos and nuclear factor of activated T-cells 1 (NFATc1) was downregulated. Inflammatory biomarkers, such as TNF-α, MCP-1, RANTES, and inducible nitric oxide synthases (iNOS), were significantly suppressed by phosvitin/phosvitin hydrolysate. The results suggested that the inhibitory effects of phosvitin/phosvitin hydrolysate on RAW264.7 cells differentiation might be mediated through c-Jun N-terminal kinase (JNK), p38 and nuclear factor kappa beta (NF-κB) pathways.

It was also found that phosvitin hydrolysate could improve bone micro structure and prevent bone loss in ovariectomized (OVX) rats. To be specific, 2% phosvitin hydrolysate in animal diet resulted in significantly lower bone separation, structure model index (SMI), and higher bone thickness, trabecular bone number, volumetric bone mineral density (vBMD) and bone volume fraction compared to the control animals at certain time points. Correspondingly, some changes in bone turnover markers were also detected as higher concentrations of serum osteocalcin (OCN) and N-terminal propeptide of type 1 collagen (P1NP) and lower serum tartrate-resistant acid phosphatase isoform 5b (TRACP 5b) were found in animals fed with 2% phosvitin hydrolysate. The rise of serum OCN and P1NP levels are usually associated with an increase in bone formation activities whereas a drop in TRACP-5b levels could be a sign of suppressed osteoclast number/activities. These results suggested the prevention activity of phosvitin hydrolysate on bone loss in OVX rats might be due to a combination of both anabolic and anti-resorptive effects.

The findings of this thesis research suggest that phosvitin and phosvitin hydrolysate have good potential to be incorporated into functional foods to reduce bone loss and/or promote bone growth.

PREFACE

This thesis is an original work by Jiandong Ren and has been written according to the guidelines provided by the Faculty of Graduate Studies and Research at the University of Alberta. The concept of the thesis originated from my supervisor Dr. Jianping Wu and the research was funded by the grants from Michael Foods Inc., Alberta Livestock Meat Agency Inc. (ALMA), Alberta Innovates and Natural Sciences and Engineering Research Council (NSERC) of Canada to Dr. Wu. The experimental protocol for the animal study was approved by the Animal Care and Use Committee at the University of Alberta (Protocol # 00001960) in accordance with the guidelines issued by the Canada Council on Animal Care.

This thesis consists of seven chapters: Chapter 1 provides a general introduction and thesis objectives; Chapter 2 is a literature review of relevant topics including bone remodeling, osteoporosis, prevention of osteoporosis and an introduction of phosvitin; Chapter 3 presents a simple protocol to extract phosvitin from egg yolk and investigates the possibility for scale up production; Chapter 4 discusses the effect of phosvitin and phosvitin hydrolysate on osteoblast cells function and response to inflammatory stimulation; Chapter 5 discusses the effect of phosvitin and phosvitin hydrolysate on osteoclast cells function and response to inflammatory stimulation; Chapter 6 investigates the effect of phosvitin hydrolysate on osteoporosis animal model (ovariectomized rats); Chapter 7 is the final remarks and recommendations for future work.

Dr. Jianping Wu greatly contributed to the experimental design, data interpretation, and thesis preparation. I was responsible for searching relevant literature for the above studies, designing and performing laboratory experiments, data collection and analysis, and drafting the thesis. Dr. Subhadeep Chakrabarti has provided substantial guidance and assistance for the cell

culture study. Mrs. Nicole Coursen, Miss Wang Liao and Miss Nan Shang have provided technical assistance in animal studies, including animal husbandry and tissue sampling upon termination. Mr. Jiancheng Qi provided technical assistance by operating the pilot equipment in the scale up production of phosvitin in Chapter 3. Dr. Michael Doschak and Dr. Wenlin Yu helped draft and review the animal protocol in Chapter 6. Dr. Michael Doschak also provided professional training on Micro CT imaging, animal handling and discussion on the results in Chapter 6. Nan Shang helped to run the flow cytometer and analyze corresponding data in Chapter 6.

DEDICATION

This thesis is dedicated to my beloved parents, Shumin Yang and Fengqun Ren.

ACKNOWLDEGMENTS

This is a great university with great professors and students. Here I met lots of nice people and made friends with many of them. Without their support and company, I could not finish such a long journey.

First of all, I would like to express my sincere gratitude to my supervisor Dr. Jianping Wu for his continuous support and encouragement. The experience that I gained while working with him is valuable not only for scientific research but also for how to deal with the difficulties in my life. It was the best of my luck to have Dr. Temelli and Dr. Korver in my supervisory committee. They provide professional advice and endless patience for me through the whole program.

I am very grateful to Dr. Bejjani and Dr. Chakrabarti for the cell culture experiment training, and Dr. Doschak for the animal handling and Micro CT training. My gratitude also goes to our department technician Nicole Coursen and my lab mate Nan Shang and Wang Liao, who helped me greatly with the animal experiment.

I would like to thank all the people working in Dr. Wu's lab during these years. It feels like a big family and people are always ready to help each other. I feel very lucky to have many friends in this department. I will remember all of you and it is my great pleasure to talk and work with you. These enjoyable friendships enable me with energy to face the challenges in research and life.

I also want to express my appreciation to Michael Foods Inc., Alberta Livestock Meat Agency Inc. and Alberta-Innovates and Natural Sciences and Engineering Research Council (NSERC) of Canada who provided financial support for my thesis research.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

AA: Arachidonic acid

AKT: Protein kinase B

ALP: Alkaline phosphatase

ANOVA: Analysis of variance

AP-1: Activator protein 1

BMD: Bone mineral density

BMP-2: Bone morphogenetic protein-2

BMU: Basic multicellular unit

BSP: Bone sialoprotein

COX-2: Cyclooxygenase-2

CPP: Casein phosphopeptides

CTX: Type 1 collagen cross-linked C-telopeptide

DD: Deionized distilled

DHA: Docosahexaenoic acid

DMEM: Dulbecco's modified eagle's medium

DPPH: 2,2-diphenyl-1-picrylhydrazyl

DTT: Dithiothreitol

DV: Diavolumes

EDTA: Ethylenediaminetetraacetic acid

ELISA: Enzyme-linked immunosorbent assay

EPA: Eicosapentaenoic acid

ERK: Extracellular signal–regulated kinases

ERs: Estrogen receptors

FBS: Fetal bovine serum

FOS: Fructooligosaccharides

GAPDH: Glyceraldehyde 3-phosphate dehydrogenase

GOS: Galactooligosaccharides

GRO-α: Growth-regulated oncogene-alpha

HDL: High-density lipoprotein

HPLC: High-performance liquid chromatography

IBD: Inflammatory bowel disease

IFNγ: Interferon gamma

IGF: Insulin-like growth factor

IL: Interleukin

iNOS: Inducible nitric oxide synthases

JNK: c-Jun N-terminal kinase

LDL: Low-density lipoproteins

LPS: Lipopolysaccharide

MAPK: Mitogen-activated protein kinases

MCP-1: Monocyte chemoattractant protein-1

M-CSFR, or c-FMS: Macrophage colony-stimulating factor receptor

M-CSF: Macrophage-colony stimulating factor

MEM-α: Minimum essential medium alpha

MGP: Matrix Gla-protein

MIP-1: Macrophage inflammatory proteins-1

MMPs: Matrix metalloproteinases

MWCO: Molecular weight cut-off

NDOs: Non-digestible oligosaccharides

NFATc1: Nuclear factor of activated T-cells 1

NF-κB: Nuclear factor kappa beta

NTX: Cross-linked N-telopeptide

OCN: Osteocalcin

OPG: Osteoprotegerin

OPN: Osteopontin

OVX: Ovariectomized

PBM: Peak bone mass

PBS: Phosphate buffered saline

PDGF: Platelet-derived growth factor

PGE2: Prostaglandin E2

pI: Isoelectric point

PI3K/AKT: Phosphoinositide-3-kinase/protein kinase B

PICP: C-propeptide of type 1 collagen

P1NP: N-terminal propeptide of type I collagen

PPARγ: Peroxisome proliferator activator receptor gamma

PTH: Parathyroid hormone

PUFA: Polyunsaturated fatty acids

PV: Phosvitin

PVH: Phosvitin hydrolysate

RANK: Receptor activator of nuclear factor kappa-B

RANKL: Receptor activator of nuclear factor kappa-B ligand

RANTES: Regulated on activation, normal T cell expressed and secreted

RhoA: Ras homolog gene family, member A

RUNX2: Runt-related transcription factor 2

SD rats: Sprague Dawley rats

SD: Standard deviation

SDF-1: Stromal-derived factor-1

Sema4D: Semaphorin 4D

SMI: Structure model index

TGF-β: Transforming growth factor beta

TNF-α: Tumor necrosis factor alpha

TRACP 5b: Tartrate-resistant acid phosphatase isoform 5b

TRAF6: Tumor necrosis factor receptor-associated factor 6

TRAP: Tartrate-resistant acid phosphatase

UF: Ultrafiltration unit

vBMD: Volumetric bone mineral density

VCR: Volume concentration ratio

CHAPTER 1 - General Introduction and Thesis Objectives

1.1 General introduction

Osteoporosis is a very common disease afflicting a quarter of the population of 50 years old and above worldwide (Berger et al., 2010). The loss of bone mass and density, and degraded bone microarchitecture contribute to the elevated bone fragility and risk of fracture (Kanis, Melton, Christiansen, Johnston, & Khaltaev, 1994). In 2010, 10.2 million of US population over 50 years old were diagnosed with osteoporosis and another 43.4 million had low bone mass (Wright et al., 2014). In 2009, 1.4 million Canadians were reported to have osteoporosis (Statistics Canada, 2009). In Europe, 22.0 million women and 5.6 million men were diagnosed with osteoporosis (Hernlund et al., 2013). This prevalence tends to increase due to the aging of world population. An osteoporotic fracture brings great pain/inconvenience to the patients, and sometimes causes immobility or even mortality. Although the risk for osteoporotic fracture for women is twice that for men, the consequence of the fracture is often more serious for men than women (Laster et al., 2011; Hernlund et al., 2013). The cost to treat osteoporosis and related complications is growing rapidly. The annual cost for treating osteoporosis and the fractures was over $2.3 billion as of 2010 in Canada (Tarride et al., 2012). In the USA, this cost was $16.9 billion as of 2006 and is expected to reach $25.3 billion by 2025 (Burge et al., 2007). Europe has the highest incidence of osteoporosis in the world, probably due to its high percentage of elderly population. The cost for osteoporotic fractures was estimated to reach € 76.8 billion by the year of 2050 (Kanis & Johnell, 2005).

Osteoporosis is a result of imbalance of bone remodeling in which bone resorption excels bone formation. In the process of bone remodeling, old bone tissue is resorbed by osteoclast cells

and new bone tissue is formed by osteoblast cells (Seeman, 2009). Currently, most of the treatments, including chemical drugs and hormone therapies, have good performance to reduce bone loss. Anabolic drugs like teriparatide are effective to treat osteoporosis by stimulating bone formation but could not completely restore bone quality (Rachner, Khosla, & Hofbauer, 2011). Prevention of osteoporosis from happening could be the best option. Nutrients, including minerals, vitamins, fatty acids, and dietary fibers, play important roles in bone remodeling. Calcium combined with vitamin D is conventionally used for prevention of osteoporosis but the efficacy is still controversial (Grant et al., 2005). Many food components or plant extracts (e.g. e.g. lactoferrin from milk or phenolic compounds from green tea) are reported to inhibit osteoporotic bone resorption or promote bone formation; however, the efficacy still needs confirmation while some of them might raise safety concerns (Setchell, 2001).

Food proteins or bioactive peptides are also reported to affect bone remodeling and can be potential candidates for osteoporosis treatment/management due to relatively high safety. Lactoferrin and casein phosphopeptides are both derived from milk and reported to promote bone growth (Cornish et al., 2004; Naot, Grey, Reid, & Cornish, 2005; Bennett et al., 2002). These proteins/peptides might be involved in the bone remodeling by modulating functions of bone cells. Current evidences also suggest oxidation and inflammation cytokines are associated with bone loss; therefore, food ingredients with antioxidant and anti-inflammation activities might have the potential to suppress bone loss (Mundy, 2007; Shen, Yeh, Cao, & Wang, 2009).

Phosvitin is the major phosphoprotein in egg yolk, having ~50% of the amino acids esterified to phosphate groups (Clark, 1984). The unique structure is the key for phosvitin's function to carry metal ions from plasma to the oocyte and provide calcium/phosphate for embryo development (Hiramatsu, Cheek, Sullivan, Matsubara, & Hara, 2005). Phosvitin and

phosphopeptides derived from phosvitin have been studied for their antioxidant, anti-inflammation, antimicrobial activities (Khan, et al., 2000; Nakamura, Ogawa, Nakai, Kato, & Kitts, 1998; Xu, Yang, Yin, Liu, & Mine, 2012). Recently, phosvitin was reported to promote bone formation in tissue culture and cell culture experiments (Liu et al., 2013; Liu, Li, Geng, Huang, & Ma, 2017). It was proposed that in these experiments phosvitin provided antioxidant capacity to mirror the physiological role of ascorbic acid, which cannot be obtained during the embryo development to support skeleton formation (Liu et al., 2013).

1.2 Hypotheses and objectives

It was hypothesized that 1) phosvitin and phosvitin phosphopeptides can modulate osteoblast and osteoclast activities and thus prevent bone loss; and 2) phosvitin and phosvitin phosphopeptides can suppress production of inflammatory proteins from bone cells under inflammatory stimulation. To investigate the above hypotheses, the following objectives were addressed:

1) to develop a scalable method to prepare enough phosvitin for animal study and for potential commercial applications (Chapter 3).

2) to study the effects of phosvitin/phosvitin hydrolysate on the function of osteoblast and osteoclast cells and the production of inflammatory proteins under stimulation (Chapters 4 and 5).

3) to study the effects of phosvitin hydrolysate on bone microstructure and bone turnover markers in ovariectomized rats (Chapter 6).

CHAPTER 2 - Literature Review

This review chapter will provide some background information for the present thesis. Beginning with a brief introduction of bone structure and bone remodeling, osteoporosis, the most prevalent bone disease, is then presented together with other common bone diseases. The role of inflammation in bone health is also discussed. Nutrients and food components such as minerals, vitamins, fatty acids, prebiotics, proteins, phenolic compounds and bioactive peptides are beneficial for bone health and are summarized in the next section. The last section of this review chapter is an review on phosvitin, including its structure, origin, functions in egg yolk, and bioactivities, including current evidence on bone health.

2.1. Introduction of bone and bone remodeling

2.1.1. Introduction of bone

Bone is the major constituent of the vertebrate skeleton. It is primarily composed of calcium and phosphate in the form of hydroxyapatite deposited onto a three-dimensional matrix of type I collagen. This structure makes bone both rigid and flexible to prevent fractures (Cunniffe & O'Brien, 2011). Bone can be classified into four general categories based on different shapes: long bones, short bones, flat bones, and irregular bones (Clarke, 2008). The structure of long bone is introduced here as an example. Anatomically, a long bone is composed of three sections: the hollow shaft called the diaphysis in the middle, two rounded distal epiphyses at each end, and two connecting sections called metaphyses between the diaphysis and epiphyses (Clarke, 2008). According to their functions, the dense and compact outer shell of the bone is also called cortical bone (compact bone); inside of the cortical bone is the trabecular

bone (spongy bone), which has a honeycomb-like network of trabecular plates and rods surrounding the bone marrow as shown in Figure 2.1 (Kini & Nandeesh, 2012).

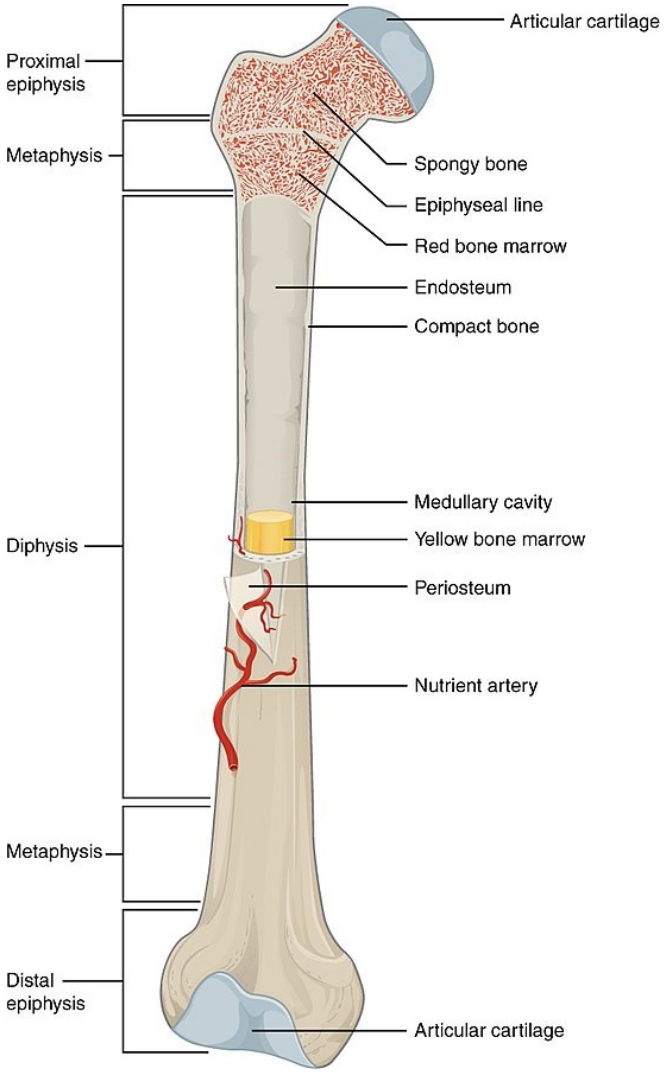

Figure 2.1. Structure of long bone (Reprinted with permission from OpenStax (2014); Attribution: the OpenStax name, OpenStax logo, OpenStax book covers, OpenStax CNX name, and OpenStax CNX logo are not subject to the creative commons license and may not be reproduced without the prior and express written consent of Rice University. For questions regarding this license, please contact support@openstax.org.)

Trabecular bone is a reservoir for different elements, especially calcium and phosphorus. It has a high turnover rate so that it can resorb or absorb minerals for maintaining mineral

homeostasis. Cortical bone can also provide minerals but only when there are severe deficits (Hadjidakis & Androulakis, 2006). The major function of cortical bone is to provide mechanical strength and protect internal tissues (Seeman & Delmas, 2006). Cortical bone consists of overlapping parallel osteons; blood vessels and nerves exist in the Haversian canals of cortical bone (Kini & Nandeesh, 2012).

Bone has multiple functions. It protects vital organs in the human body, accommodates marrow that produces blood cells, stores minerals and maintains mineral homeostasis, produces a series of growth factors and cytokines, and supports the whole body with mobility (Kini & Nandeesh, 2012). Maintaining bone health is very important. Loss of bone mass increases the risk of fractures (Kanis et al., 1994), which could cause hospitality or even mortality for elderly people.

Bone is metabolically active, undergoing remodeling (reshaping) all the time. The bone mass increases until peak bone mass (PBM) is reached at thirty years of age, and then starts to decline (Post, Cremers, Kerbusch, & Danhof, 2010). Bone metabolism is regulated by both internal and external factors. These factors include declines in mineral intake or absorption, deficiency of other nutrients such as vitamins, interrupted hormones secretion (e.g. parathyroid hormone (PTH) or estrogen), elevated inflammatory cytokines by chronic diseases, use of drugs (e.g. glucocorticoids), and even physical activity (Hahn, 2009).

2.1.2. Bone remodeling and bone cells

2.1.2.1 Bone remodeling

Bone homeostasis is regulated by bone remodeling, in which old bone tissue is resorbed by osteoclast cells and new bone tissue is formed by osteoblast cells. This process maintains the integrity of skeletal system by resorbing old and damaged bone and forming new bone tissue

(Seeman, 2009). Bone remodeling is carried out by the basic multicellular unit (BMU), which consists of osteoclasts, osteoblasts, and osteocytes located in a bone remodeling cavity (Hauge, Qvesel, Eriksen, Mosekilde, & Melsen, 2001). Bone remodeling includes five distinct and sequential phases: activation, resorption, reversal, formation, and termination as shown in Figure 2.2 (Raggatt & Partridge, 2010).

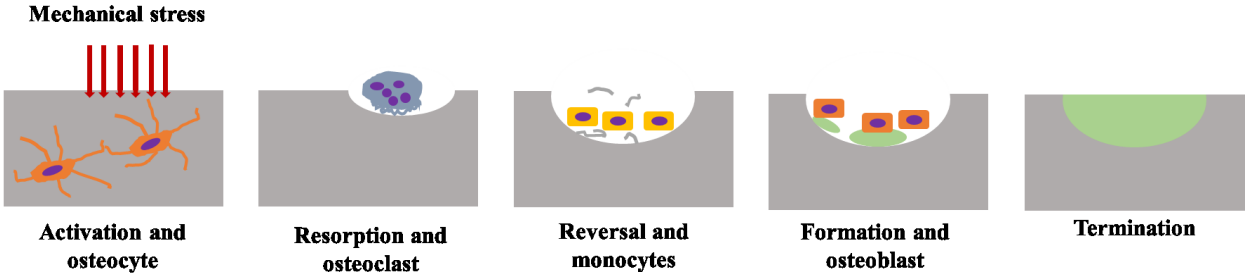

Figure 2.2. Scheme of bone remodeling cycle (Created according to the description of Raggatt & Partridge (2010))

In the activation phase, many factors could trigger bone remodeling. For example, bone damage caused by mechanical loading leads to apoptosis of osteocytes, and osteoclastogenesis due to reduced formation of inhibitory factor of transforming growth factor beta (TGF-β) produced by osteocytes (Aguirre et al., 2006). In resorption phase, macrophage-colony stimulating factor (M-CSF) and receptor activator of nuclear factor kappa-B ligand (RANKL) stimulate progenitors to form mature osteoclasts and maintain the resorption activities. Following resorption is the reversal phase, when the reversal cells (heterogenic monocytes probably derived from osteoblastic lineage) remove the debris of degraded bone matrix from the cavity (Abdelgawad et al., 2016). In the formation phase, osteoblast progenitors are recruited at the site of the cavity to form mature osteoblasts and synthesize new bone. When the resorbed bone is completely replaced, osteoblasts start to differentiate into bone lining cells attached to the surface

of the matrix, or differentiate into osteocytes embedded in the matrix, and the remodeling cycle is complete (Raggatt & Partridge, 2010).

2.1.2.2. Osteoblast cells

Osteoblasts have a critical role in bone remodeling. One of the major functions of osteoblasts is to synthesize matrix proteins and mineralize the matrix for new bone formation or fracture repair. More importantly, osteoblasts also regulate osteoclast differentiation and resorption activities by expressing osteoclastogenic factors (Karsenty, 2008). Osteoblasts are differentiated from pluripotent mesenchymal stem cells (Pittenger et al., 1999). Mesenchymal stem cells have the potential to differentiate into multiple cell lineages with different functions, but with stimulation of a series of specific transcription factors, such as runt-related transcription factor 2 (RUNX2), they will commit to osteoblasts (Komori et al., 1997; Komori, 2011). This differentiation process consists of three different stages: mesenchymal progenitor, pre-osteoblasts and osteoblasts. The mature osteoblasts produce several specific extracellular proteins including bone sialoprotein (BSP), osteopontin (OPN), osteocalcin (OCN), alkaline phosphatase (ALP) and type I collagen (Long, Berry, Zhao, Sun, & Humphrey, 2012). Type 1 collagen forms the protein frame of bone matrix while BSP mediates nucleation of hydroxyapatite crystallization (Vincent & Durrant, 2013). OCN induces calcification of bone matrix and bone specific ALP is osteoblast-specific protein reflecting bone formation activity of osteoblasts (Kanazawa et al., 2009).

The extracellular signal–regulated kinases (ERK) cascade is one of the cascades from mitogen-activated protein kinases (MAPK) signaling pathway that responds to extracellular stimuli. The MAPK/ERK signaling generally regulates cell proliferation, differentiation, migration, senescence and apoptosis (Johnson & Lapadat, 2002). In osteoblasts, MAPK/ERK

regulates phosphorylation and transcriptional function of RUNX2, and RUNX2 also mediates the response of MAPK/ERK pathway (Franceschi & Xiao, 2003). The phosphoinositide-3-kinase/protein kinase B (PI3K/AKT) pathway regulates cell proliferation and survival (Vivanco & Sawyers, 2002). It can be activated by many factors, including growth factors like platelet-derived growth factor (PDGF), insulin or insulin-like growth factor-1 (IGF-1), and several cytokines (Cantley, 2002). The activation of PI3K/AKT is essential for osteoblast differentiation: inhibition of IGF-1 led to reduction in ALP activity while expression of constitutively active AKT restored ALP activity and bone mineralization in MC3T3-E1 cells (Suzuki et al., 2014). It is also noteworthy that RUNX2 promoted osteoblast differentiation by coupling with PI3K/AKT pathway: arresting IGF-1 by its antibody completely inhibited RUNX2 induced osteoblast differentiation (Fujita et al., 2004). These results indicated that RUNX2 and PI3K/AKT pathways are mutually dependent on each other during osteoblast differentiation (Fujita et al., 2004).

2.1.2.3. Osteoclast cells

Osteoclasts are the giant multinucleated cells derived from progenitors in the monocyte/macrophage lineage and are responsible for resorption of mineralized bone matrix (Teitelbaum & Ross, 2003). Lack of osteoclasts will result in osteopetrosis in which the bone marrow cavity is filled with un-resorbed bone matrix. The osteoclast progenitors circulate in the blood and are recruited at the sites of bone remodeling (Muto et al., 2011). RANKL and M-CSF are the two master cytokines required for osteoclastic differentiation. These cytokines can bind to the receptor activator of nuclear factor kappa-B (RANK, receptor of RANKL) and macrophage colony-stimulating factor receptor (M-CSFR, also called c-FMS), respectively, expressed in osteoclast cell membrane, to stimulate osteoclasts differentiation and maintain their resorption

activities (Boyle, Simonet, & Lacey, 2003). Osteoblasts produce most of M-CSF, while a number of cells including stromal cells, osteoblasts and osteocytes could produce RANKL. Traditionally, RANKL was considered to be secreted from mature osteoblasts on the bone surface, or osteoblast progenitors in bone marrow (Sims & Gooi, 2008). However, *in vivo* studies proved that changes in the number of osteoblasts or osteoblast progenitors had no effect on osteoclast number (Corral et al., 1998; Galli et al., 2009). New evidence suggests the source of RANKL that regulates osteoclasts in bone remodeling might come from osteocytes buried in the bone matrix. It was reported that the deletion of RANKL from osteoblasts did not affect bone development or remodeling in mice; however, deletion of RANKL from osteocytes increased bone mass, which suggests that the RANKL from osteocytes controls osteoclasts' bone resorption activity (Xiong et al., 2011). On the other hand, osteoclasts might negatively regulate osteoblast differentiation through a semaphorin 4D (Sema4D) mediated pathway (Negishi-Koga et al., 2011). The binding of osteoclast secreted Sema4D to its receptor of Plexin-B1 on osteoblasts inhibits IGF-1 signaling via activation of Ras homolog gene family, member A (RhoA) (Sahai & Marshall, 2002). IGF-1 promotes osteoblast activity through activation of PI3K and the MAPK/ERK pathways (Guntur & Rosen, 2013), and thus osteoclast secreted Sema4D suppressed osteoblast activity by inhibition of IGF-1 signaling. This was further supported by the fact that administration of Sema4D antibody significantly prevented bone loss in postmenopausal women (Negishi-Koga et al., 2011).

The binding of RANKL to RANK triggers cytoplasmic domain of RANK to recruit tumor necrosis factor (TNF) receptor-associated factor 6 (TRAF6); many osteoclast-related signaling pathways, including p50/65 nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and MAPKs (p-38 and c-Jun N-terminal kinase (JNK)), are activated afterwards.

These pathways are believed to play an important role in osteoclast differentiation (Boyle et al., 2003).

The inhibitors of MAPK/JNK and p38 markedly inhibited the osteoclast differentiation (Ikeda et al., 2004; Takayanagi et al., 2002). It was reported that the p38 pathway was essential for RANKL-induced osteoclast differentiation, but not for RANKL-induced osteoclast function (Li et al., 2002). JNK are a group of serine/threonine kinases generally important for cell growth, differentiation, and apoptosis (Zhang & Liu, 2002). In osteoclasts, RANKL elevated expression of c-Jun (Wagner, 2002), which forms activator protein 1 (AP-1) with c-Fos and thus regulates nuclear factor of activated T-cells, cytoplasmic 1 (NFATc1) expression (Asagiri et al., 2005). NFATc1 is the master transcription regulator of osteoclast differentiation (Takayanagi et al., 2002), regulating osteoclast specific genes such as tartrate-resistant acid phosphatase (TRAP), cathepsin K and calcitonin receptor (Takayanagi et al., 2002; Kim, Day, & Morrison, 2005).

NF-κB is a family of several transcription factors that recognize a common gene sequence called κB. There are five NF-κB proteins: Rel (cRel), RelA (p65), RelB, NF-kappaB1 (p50) and NF-kappaB2 (p52). In the classical pathway of NF-κB, RANKL will induce the formation and translocation of p65/p50, NFATc1 to regulate gene transcription (Hayden and Ghosh, 2004; Takatsuna et al., 2005).

2.2. Osteoporosis, the most common bone disease

2.2.1. Osteoporosis

Osteoporosis is a progressive bone disease characterized by a loss of bone mass and bone density, leading to increased bone fragility and risk of fracture (Kanis, Melton, Christiansen, Johnston, & Khaltaev, 1994). It afflicts about 25% of the population of 50 years and above worldwide (Berger et al., 2010). In 2010, 10.2 million people in US and 27.6 million people in

Europe were diagnosed with osteoporosis (Wright et al., 2014; Hernlund et al., 2013). This prevalence tends to increase with the aging of world population. The treatment of osteoporosis and osteoporotic fracture related complications cost billions of dollars annually in Europe and North America, which is a heavy burden to the patient and society (Kanis & Johnell, 2005; Burge et al., 2007; Tarride et al., 2012).

Osteoporosis is a silent disease. Most of the people would not realize it until an osteoporotic fracture accurs. Osteoporotic fractures are the most serious clinical consequence of osteoporosis, which usually happen in the forearm, hip, or spine (Laster et al., 2011). Women are more susceptible to forearm fracture but less susceptible to spine fracture. On the other hand, men have higher rates of mortality caused by osteoporotic fractures and the assocaited complications (Hernlund et al., 2013). Most of the fractures can be fully healed but with elevated risk of future fractures, functional impairment, chronic pain, disability or even mortality (Colón-Emeric & Saag, 2006). Vertebral fractures sometimes come with chronic, disabling pains and increased incidence of morbidity such as height loss, deformity, disability, and mortality (Johnell et al., 2004). For hip fractures, the mortality risk within the first year of a fracture is increased by 10-20% and the risk of future fractures is increased by 2.5-fold (Colón-Emeric et al., 2003). About 20% of hip fracture patients require long-term hospitalization, and most of the patients did not completely recover their mobility (US Department of Health and Human Services, 2004). This significantly affects life quality of the patients and sometimes leads to psychological issues like depression or anxiety (Gold 2001).

2.2.2. Postmenopausal osteoporosis

Osteoporosis can be classified into two types: primary or secondary osteoporosis. Secondary osteoporosis is often caused by nutrient deficiency, use of certain drugs, and chronic

diseases. Primary osteoporosis is a result of the aging process, which usually happens in women after menopause or men in later life (Mayes, 2007).

Women are more susceptible to osteoporosis than men (Berger et al., 2010). It was not until the 1940s that researchers linked the lack of estrogen after menopause to the incidence of osteoporosis, and since then estrogen therapy has been used as a treatment for postmenopausal osteoporosis (Albright, Smith, & Richardson, 1941). For a long time, the postmenopausal bone loss was considered as a result of the declining bone formation activity based on histological observations (Arlot, Edouard, Meunier, Neer, & Reeve, 1984). However, examination of bone turnover biomarkers suggests that both bone formation and resorption activities are elevated after menopause, and the postmenopausal bone loss is probably due to bone resorption rate being faster than formation rate (Szulc & Delmas, 2008). It was reported that after menopause the levels of bone resorption markers (type 1 collagen cross-linked C-telopeptide (CTX) and cross-linked N-telopeptide (NTX)) increased by 79-97%, but levels of bone formation markers (OCN, bone ALP, C-propeptide of type 1 collagen (PICP)) only increased by 37-45% (Garnero, Sornay-Rendu, Chapuy, & Delmas, 1996; Garnero, Vergnaud, & Hoyle, 2008).

Postmenopausal bone loss has two stages: an acute bone loss in trabecular bone in the first 4-8 years followed by a slower but long-term bone loss mainly in cortical bone. The latter happens in both sexes and is sometimes regarded as age-related bone loss (Manolagas, 2010). Estrogen deficiency primarily induces bone loss in the first stage. Estrogen regulates bone remodeling through interaction with estrogen receptors (ERs) located within osteoclasts and osteoblasts. ERs are nuclear receptors that can directly regulate bone cells differentiation, function and apoptosis; ERs also alter inflammatory production by binding targeted genes. Estrogen withdrawal activates production of inflammatory cytokines such as interleukin-1 (IL-

1), IL-6 and tumor necrosis factor alpha (TNF-α), which stimulate osteoclast functions and extend the resorption duration within each remodeling cycle (Manolagas, 2010). After menopause the lack of estrogen also increases the number of BMUs and directly prolongs the lifespan of osteoclasts by suppressing apoptosis (Hughes et al., 1996; Eriksen, Langdahl, Vesterby, Rungby, & Kassem, 1999). Estrogen deficiency stimulates osteoblastogenesis, but also induces apoptosis of osteoblasts. Generally, estrogen deficiency increases cell numbers of both osteoclasts and osteoblasts, upregulates osteoclast activity and lifespan, but induces osteoblast apoptosis. Therefore, the elevated bone resorption exceeds bone formation (Kousteni et al., 2001). The above interactions result in an elevated bone turnover rate with an overall net bone loss in the first stage of postmenopausal women.

2.2.3. Osteoporosis and inflammation

Osteoporosis is a complicated process, which is affected by endocrine factors (imbalanced secretion of PTH and calcitonin, menopause), nutritional factors (deficiency of vitamin D and calcium), mechanical factors (immobility), and medications (Yun & Lee, 2004). Recent evidence indicates that inflammation also plays an important role in the development of osteoporosis (Mundy, 2007).

Inflammation is an immune response to protect the body from injury or harmful stimuli. Most inflammation is considered as a sign of the body's own healing. However, chronic inflammation with excessive production of inflammatory cytokines can induce bone loss. The earliest evidence of this was the discovery of soluble mediators from human peripheral blood leukocytes that could increase calcium release and the number of active osteoclasts in a bone tissue culture (Horton, Raisz, Simmons, Oppenheim, & Mergenhagen, 1972). One of these mediators was identified as IL-1 (Dewhirst, Stashenko, Mole, & Tsurumachi, 1985). Now, it is

widely accepted that many cytokines secreted during inflammation can stimulate bone resorption. These cytokines include IL-1, IL-6, IL-11, IL-15, IL-17, IL-23, and TNF-α (Mundy, 2007). A relationship has been observed between inflammation and bone loss. Although mechanisms differ, many inflammatory diseases such as inflammatory joint disease, inflammatory bowel disease, coeliac disease, lung inflammation, renal disease and disease affecting nerve and muscle may be associated with bone loss (Hardy & Cooper, 2009). Besides, some inflammatory cells e.g. lymphocytes and fibroblasts could express and release RANKL and thus promote bone resorption independent of osteoblasts (Kotake et al., 2001). TNF-α is the most studied proinflammatory cytokine. Estrogen deficiency resulting from ovariectomy could stimulate the secretion of TNF-α and IL-1 and enhance bone resorption activity (Kimble et al., 1995). Intervention with TNF-α receptor antagonist and IL-1 binding protein can completely abolish the reduction in bone mass (Kimble et al., 1995). Mice without TNF-α receptor or IL-1 receptor did not experience a significant bone loss after ovariectomy (Lorenzo et al., 1998; Ammann et al., 1997). These results demonstrate that proinflammatory cytokines probably play an important role in the pathogenesis of osteoporosis.

Many age-related diseases are associated with inflammation (Luc et al., 2003), and inflammation results in bone loss under pathological conditions such as periodontal disease, multiple myeloma and rheumatoid arthritis (Di Benedetto, Gigante, Colucci, & Grano, 2013). In postmenopausal women, estrogen deficiency results in excessive expression of RANKL in osteoblasts, and RANKL will in turn trigger osteoclasts to produce inflammatory proteins. Cytokines e.g. IL-1, IL-6 and TNF-α can promote osteoclasts differentiation/activities and thus enhance bone resorption (Steeve, Marc, Sandrine, Dominique, & Yannick, 2004); chemokines e.g. monocyte chemoattractant protein 1 (MCP-1) and regulated upon activation normal T cell

expressed and secreted (RANTES), could accelerate differentiation and resorption activities of osteoclasts and may cause further bone loss (Kim et al., 2005). Osteoblasts are also responsible for expressing inflammatory chemokines including IL-8, MCP-1, RANTES and macrophage inflammatory proteins-1 (MIP-1) under stimulation of inflammatory cytokines (Garlet et al., 2008; Lisignoli et al., 2002). These chemokines play an important role in bone remodeling by recruiting osteoclast progenitors and stimulating osteoclastogenesis (Wright et al., 2005).

2.2.4. Other common bone diseases

Osteopetrosis is a type of disease characterized by increased bone density caused by abnormalities in osteoclast differentiation or function (Royce & Steinman, 2003). The major function of osteoclasts is to resorb minerals and collagen matrix, to maintain mineral homeostasis and maintain the integrity of the skeleton. A large portion of osteopetrosis is due to defects in osteoclast gene transcription. For example, proton pump vacuolar ATPase and chloride-specific ion channel, chloride channel 7 facilitate proton transport during the acidification process, loss of these proteins due to gene mutations results in osteopetrosis (Kornak et al., 2001). Osteoclast cells secrete many enzymes including matrix metalloproteinases (MMPs) and lysosomal cathepsins to resorb collagen matrix; deletion of these enzymes also results in osteopetrosis (Saftig et al., 1998).

Named after Sir James Paget, Paget's disease of bone is characterized with excessive bone resorption and skeletal deformity caused by unorganized new bone formation (Tuck, Layfield, Walker, Mekkayil, & Francis, 2017). Paget's disease is the second most common bone disease after osteoporosis, and affects 1% of the population of 40 years and above (Hahn, 2009). This disease will result in extra-large and unorganized bone structure that not only brings pain to patients but also increases susceptibility to fractures. Although the pathogenesis remains unclear,

it is generally believed that mutation of related genes contributes to the development of this disease.

Hormones play a critical role in regulating bone turnover; therefore, any disruption in hormone secretion may affect bone turnover and cause bone diseases. For example, parathyroid gland disorders might cause inadequate PTH secretion and therefore low serum calcium levels. This is called hypoparathyroidism with symptoms like pain, seizures, and arrhythmias. On the contrary, hyperparathyroidism is a disease featured with high serum PTH and abnormal serum calcium level (can be higher or lower than normal). The symptoms include pain, hypertension and osteoporosis (Imel, DiMeglio, & Burr, 2014).

2.3. Nutrients and functional foods/nutraceuticals to improve bone health

Various drugs and therapies have been developed for osteoporosis prevention and treatment. Bisphosphonates are the most commonly used drugs to treat osteoporosis by suppressing bone resorption thus improving bone health (Weinstein, Roberson, & Manolagas, 2009). Hormone treatments are also applied to treat osteoporosis. They are aimed to regulate hormone metabolism associated with osteoclasts and thus inhibit osteoclastogenesis. Teriparatide is a fragment of recombinant human PTH but exhibits the opposite effects of PTH to improve skeletal microarchitecture and reduce fracture rates (Hodsman et al., 2005). Although effective, drug treatments are associated with serious side-effects including nausea, abdominal pain and loose bowel movements, hot flashes, depression and headaches (Rachner, Khosla, & Hofbauer, 2011). However, current drugs and therapies can not completely reverse the osteoporotic bone loss, and the recovery from osteoporotic fracture is long and painful. Prevention of fracture from occurring is probably the best option. Most of the osteoporotic fractures are caused by low bone mineral mass (Prentice, 1997). Many factors may affect bone mass and they can be roughly

classified into two categories: factors that cannot be altered (gender, age, genetics and ethnicity) and factors that can be altered (hormones, lifestyles and diet) (Cashman, 2006). Although genetics are responsible for most of the bone mass variation among population (Morrison et al., 1994), it is not possible to be altered according to current technology. On the other hand, diet could be easily intervened, and the nutrients/food components in diet also play an important role in bone health (Cashman, 2004). These nutrients/food components include minerals, vitamins, fatty acids, prebiotics, proteins, and functional ingredients derived from food commodities such as phenolic compounds and bioactive peptides.

2.3.1. Minerals

Calcium and phosphorus are the most abundant elements in bones. About 99% of calcium and 80% of phosphate exist in the human skeleton in the form of hydroxyapatite ($Ca_{10}(PO_4)_6(OH)_2$) (Bonjour, Guéguen, Palacios, Shearer, & Weaver, 2009). Calcium alone, or more frequently in combination with vitamin D, has been frequently used to prevent osteoporotic bone loss. Some of the earlier studies reported that calcium and vitamin D in combination could increase bone mineral density (Dawson-Hughes, Harris, Krall, & Dallal, 1997) and reduce incidence of osteoporotic fractures (Chapuy et al., 1992). In postmenopausal women, the decrease of estrogen secretion will lead to decreased calcium transportation through the intestinal epithelial membrane and reabsorption of calcium in kidney tubule (Prince et al., 1994; Christakos et al., 2003). Therefore, supplementation of calcium could partially compensate the calcium loss caused by estrogen deficiency and may be able to reduce risk of fractures (Shea et al., 2002). However, there were inconsistent reports and more recent studies suggest that increased calcium supplementation (with or without vitamin D) is not associated with decreased risk of fractures (Bolland et al., 2015). One possible explanation of the inconsistency is that the

subjects/patients used in these studies were under different conditions (different gender/age, post menopause or hospitalized), which greatly affect the outcomes of the experiments. Thus, it is difficult to reach a general conclusion whether calcium supplementation could benefit bone health or not.

Phosphate is the counterpart of calcium to form bone tissue and positively regulate bone growth (Spina et al., 2013). Unlike calcium, phosphorus exists in a broad category of foodstuffs, and the deficiency of phosphorus is rare. In fact, in most of the developed countries in Europe and North America, the intake of phosphorus is around 1,000-1,500 mg/day which largely exceeds the recommended daily intake of 700 mg/day (Willett & Buzzard, 1998). This is due to the increasing consumption of processed foods and beverages that contain phosphate salts as food additives (Bonjour et al., 2009). Although there is still some controversy, it is generally believed that phosphorus intake beyond requirement would result in secondary hyperparathyroidism and bone loss (Huttunen, Pietilä, Viljakainen, & Lamberg-Allardt, 2005).

Other elements, such as magnesium, are not part of hydroxyapatite crystals but they also affect bone metabolism (Wallach, 1990). Most of the magnesium in human body exists in bones and is localized at the surface of hydroxyapatite crystals (Glimcher, 2006). Magnesium deficiency is considered as a risk factor for osteoporotic bone loss (Rude, 2008). Low magnesium diet results in low bone formation activity and elevated resorption activity and osteoclast number. Although the exact mechanism remains unclear, the bone loss might be due to elevated secretion of inflammatory cytokines and impaired production of PTH and 1,25-dihydroxy vitamin D caused by magnesium deficiency (Rude & Gruber, 2004).

The role of strontium in bone health has been studied for many years since strontium lactate was reported in the 1950s to increase calcium deposition and bone mass in human

subjects (Shorr & Carter, 1952; McCaslin & Jane, 1959). It is interesting that strontium in the form of strontium ranelate shows both antiresorptive and anabolic activities at the same time in bone remodeling (Marie et al., 1993; Marie, Ammann, Boivin, & Rey, 2001). At the cellular level, strontium ranelate upregulates the expression of the osteoblast markers of ALP, BSP and OCN, and meanwhile suppresses osteoclast formation and resorption ability (Bonnelye, Chabadel, Saltel, & Jurdic, 2008).

Other minerals also play a role in bone metabolism. It is well known that fluoride could prevent dental caries, and an appropriate concentration of fluoride in drinking water might be associated with lowered incidence of osteoporosis evidenced by the formation of larger size of hydroxyapatite crystals (Jones, Riley, Couper, & Dwyer, 1999; Grynpas, 1990). Both iron and zinc are positively involved in collagen synthesis as enzyme cofactors (Prockop, 1971; Beattie & Avenell, 1992).

2.3.2. Vitamins

Supplementation of calcium along with vitamin D has been widely used for the prevention of osteoporosis (Jackson et al., 2006), although there is a controversy regarding the efficacy (Grant et al., 2005). The active metabolite of vitamin D (calcitriol) stimulates synthesis of calbindin, which actively transports calcium in the intestines (Ilichs & Kerstetter, 2000). Vitamin K is a co-enzyme involved in the expression of osteocalcin and matrix Gla-protein (MGP), the major bone proteins regulating bone growth (Ducy et al., 1996). A low level of serum vitamin K is associated with increased rates of osteoporotic fractures and low bone mass (Hodges, Akesson, Vergnaud, Obrant, & Delmas, 1993; Conway et al., 2005). Supplementation of vitamin K could stimulate osteoblast activity, reduce urinary calcium loss,

and improve bone strength in postmenopausal women (Knapen, Jie, Hamulyák, & Vermeer, 1993; Knapen, Schurgers, & Vermeer, 2007).

2.3.3. Polyunsaturated fatty acids

The polyunsaturated fatty acids (PUFA), including omega-3 fatty acids and omega-6 fatty acids, have been a primary research topic in nutrition for many years due to their biological and physiological effects on human health. Many reports suggest that omega-3 fatty acids might have positive effects on bone health while omega-6 fatty acids might have negative effects. An inverse association between higher ratios of omega-6/omega-3 fatty acids and bone mineral density (BMD) was also reported in a study with elderly men and women (Weiss, Barrett-Connor, & von Mühlen, 2005). PUFA might be involved in bone metabolism via different pathways. For example, peroxisome proliferator activator receptor gamma (PPARγ) is a transcription factor that inhibits osteoblast differentiation (Wan, 2010). Arachidonic acid (AA), an omega-6 fatty acid, was reported to stimulate PPARγ expression and thus inhibit proliferation of osteoblast (Maurin, Chavassieux, & Meunier, 2005). Eicosapentaenoic acid (EPA) is an omega-3 fatty acid; dietary EPA was reported to reduce bone loss in postmenopausal women, probably by inhibiting osteoclast activities (Terano, 2001). Such effects might be attributed to the fact that omega-3 fatty acids can inhibit release of inflammatory cytokines (Endres et al., 1989), and some of these cytokines, like TNF-α and IL-1, are elevated in postmenopausal osteoporosis and contribute to bone loss (Kimble et al., 1995). This can be further supported by the observation that omega-3 fatty acids inhibited bone resorption in ovariectomized (OVX) rats, with decreased secretion of TNF-α, IL-2, and interferon gamma (IFNγ) (Sun et al., 2003). Another study with young human subjects also mentioned that omega-3 fatty acids, especially docosahexaenoic acid (DHA), were positively associated with BMD accumulation and peak BMD (Högström, Nordström, &

Nordström, 2007). This might be explained by the fact that omega-3 fatty acids potentiated osteoblastogenesis by incorporating into mesenchymal cell membrane to produce compositional and structural aspects towards the osteoblastic phenotype (Levental et al., 2017). In fact, the mechanisms of action of omega-3 and omega-6 fatty acids on bone health are complicated and far from being fully understood.

2.3.4. Phenolic compounds

Recent studies suggest that oxidative stress and chronic inflammation are involved in the development of age-related osteoporosis by directly or indirectly regulating the balance between osteoclast and osteoblast (Manolagas & Parfitt, 2010). Therefore, functional foods rich in antioxidants, like green tea, are effective in the prevention of osteoporosis (Shen, Yeh, Cao, & Wang, 2009). Grapefruit pulp and citrus extract were also reported to improve bone quality in orchidectomized male rats due to their antioxidant properties (Mandadi et al., 2009). In another study, phenolic compounds isolated from *Curculigo orchioides* showed anti-osteoporotic activity in a bone tissue culture by stimulating osteoblast proliferation and ALP activity, and meanwhile downregulating bone resorption area, osteoclastogenesis and tartrate-resistant acid phosphatase (TRAP) activity (Jiao et al., 2015). The low incidence of osteoporosis in Mediterranean countries may be related to the high content of olives, vegetables and fruits in traditional Mediterranean foods (Rivas et al., 2013; Romero & Rivas, 2014). The phenolic compounds in olive oil have been widely studied for their antioxidant and anti-inflammation activities. Both olive oil and oleuropein (the main polyphenol in olive oil) prevented bone loss in talc (magnesium silicate)-induced inflammation in OVX rats with exacerbated bone loss in metaphysis of the femur (Puel et al., 2004). The effect of oleuropein on bone health may be attributed to its anti-inflammation activity but not antioxidant activity, as the antioxidant capacity of the plasma remained the same

(Puel et al., 2008). In another report with osteoblast cell culture, dried plum polyphenols restored expression of ALP, RUNX2, osterix and IGF-1 that were suppressed by TNF-α; these polyphenols downregulated expression of RANKL but not OPG, leading to inhibition of osteoclastogenesis (Bu, Hunt, & Smith, 2009). Although a lot of phenolic compounds are reported to improve bone health, the detailed mechanisms and the common functional groups still need to be explored.

2.3.5. Probiotics and prebiotics

Probiotics are a group of live microorganisms with an ability to improve the host's health by changing the microflora (Schrezenmeir & Vrese, 2001). Prebiotics are natural or synthetic food ingredients that can promote human health by stimulating the growth and activity of certain probiotics (Roberfroid, 2000). Most prebiotics are carbohydrates or dietary fibers (Gibson et al., 2010). Non-digestible oligosaccharides (NDOs) such as lactulose, galactooligosaccharides (GOS), fructooligosaccharides (FOS), oligofructose, and inulin are the most promising prebiotics for bone health (Whisner & Castillo, 2018). It is generally believed that the prebiotics are degraded by microbiota into short chain fatty acids, which would decrease pH and thus increase solubility and absorption of minerals in the gut (Scholz-Ahrens & Schrezenmeir, 2002). The gut microbiota modulates host metabolism, especially the immune system. It might affect bone metabolism as well. It was reported that germ-free mice developed better trabecular bone microarchitecture, higher BMD, less number of osteoclasts and lower expression of inflammatory cytokines in bone marrow than conventionally-raised mice. However, colonization of the microbiota from conventionally-raised mice to germ-free mice eliminated these differences (Sjögren et al., 2012). These results suggested that the gut microbiota could be a target when developing antiosteoporosis therapies.

2.3.6. Proteins

Milk is widely considered as an important food for enhancing bone health due to the presence of calcium and several active proteins (Uenishi et al., 2007). Lactoferrin, the iron binding glycoprotein from milk, has been widely studied and recognized as a bone growth factor (Cornish et al., 2004; Naot, Grey, Reid, & Cornish, 2005). Oral administration of lactoferrin was reported to prevent bone loss and improve bone microarchitecture in OVX rats (Guo et al., 2009; Hou, Xue, & Lin, 2012); meanwhile, the production of the pro-inflammatory cytokines TNF-α and IL-6 caused by ovariectomy was also suppressed (Guo et al., 2009). Several proposals are raised to explain the effects of lactoferrin on bone health. Lactoferrin may directly inhibit osteoclastogenesis by reducing calcitonin receptor expression in osteoclast (Lorget et al., 2002), or by suppressing RANKL expression by bone marrow cells (Cornish et al., 2004). Lactoferrin also promotes bone formation activity by stimulating osteoblast proliferation, differentiation and cell survival; these effects are probably mediated via activation of MAPK/ERK and AKT signaling pathway (Naot et al., 2005). Lactoferrin also exerts preventive effects on postmenopausal bone loss by modulating immune functions (Pfeilschifter, Köditz, Pfohl, & Schatz, 2002). This is supported by the fact that lactoferrin administration in OVX rats reduced dendritic and B cells population, inhibited T cells activation and proinflammatory cytokine (TNF-α) release (Blais, Malet, Mikogami, Martin-Rouas, & Tomé, 2009; Blais, Malet, Mikogami, & Tomé, 2010).

2.3.7. Bioactive peptides

Bioactive peptides are the small fragments derived from parent proteins by hydrolysis or other physical or chemical reactions; these fragments exert various physiological functions and eventually promote human health (Korhonen & Pihlanto, 2006). Numerous peptides have been

discovered from food proteins having positive effects on the functions of cardiovascular, digestive, immune and nervous systems (Hartmann & Meisel, 2007). Casein phosphopeptides (CPP) are the phosphorylated fragments released from casein proteins by enzymatic hydrolysis during intestinal digestion of dairy products (Meisel et al., 2003; Miquel, Alegría, Barberá, & Farré, 2006), commercial production of casein hydrolysate (Kitts 1994), or even during the processing of fermented products or cheese making (Kawahara, Aruga, & Otani, 2005). These phosphorylated amino acid residues could enhance calcium absorption by forming soluble phosphate salt with calcium ions during intestinal absorption (Meisel et al., 2003). The phosphate ions esterified to the serine residues of the CPP sequence is the critical functional groups, which are responsible for the calcium absorption promoting ability (Sato, Noguchi, & Naito, 1986; Gerber & Jost, 1986). This was supported by the fact that dephosphorylated casein protein diet resulted in lower concentration of soluble calcium ions in the small intestine than that of native casein protein diet (Gerber & Jost, 1986). However, the biological function of CPP is also determined by the N- and C-terminal structures of these peptides. Synthesized phosphopeptides without the N-terminus showed no effect on calcium absorption (Ferraretto, Gravaghi, Fiorilli, & Tettamanti, 2003). Other than calcium absorption, CPP has numerous beneficial effects on human health such as antioxidant effect (Sakanaka, Tachibana, Ishihara, & Juneja, 2005), immune-modulating effect (Otani, Watanabe, & Tashiro, 2001), and mineral absorption promoting effect (Bouhallab et al., 2002; Bennett et al., 2002).

2.4. Phosvitin and phosvitin hydrolysate as functional food ingredients to improve bone health

There is an increasing interest in functional foods and nutraceuticals for the prevention and management of osteoporosis. Bioactive proteins and peptides have great potential for this

application due to their high safety and various physiological activities, including bone formation promoting ability (Guo et al., 2009; Hou et al., 2012).

2.4.1. Phosvitin

Phosvitin, the major phosphoprotein from egg yolk, is the most phosphorylated proteins in nature; nearly all the serine residues, accounting for half of the amino acids in phosvitin, are phosphorylated (Clark, 1985). The molecular weight of phosvitin ranges from 37 to 45 kDa (Abe, Itoh, & Adachi, 1982). Phosvitin originates from vitellogenin, which is synthesized in the liver and cleaved into a heavy chain lipovitellin, light chain lipovitellin, phosvitin, and some other minor proteins in the developing embryo (Finn, 2007). Due to the large amount of negatively charged phosphoserine residues, phosvitin chelates most of the metal ions in the yolk (Greengard, Sentenac, & Mendelsohn, 1965). Among all the different hypotheses, phosvitin is generally believed to transport calcium and phosphorus to growing follicles to support bone formation (Hiramatsu, Cheek, Sullivan, Matsubara, & Hara, 2005). But actually, the calcium and phosphorus released from phosvitin are involved in many cellular events like activation of membrane fusion of the endosomes (Leabu, 2006) or providing substrate for nucleic acid synthesis before they are utilized for bone formation (Finn, 2007).

When consumed as part of a food, egg yolk phosvitin is considered as nutritionally negative due to the strong metal binding ability and resistance to digestion (Grogan, Shirazi, & Taborsky, 1990). On the other hand, phosvitin has raised great research interests among food scientists due to its anti-bacterial, antioxidant, and bone health promoting properties (Khan, et al., 2000; Nakamura, Ogawa, Nakai, Kato, & Kitts, 1998; Albright, Gordon, & Cotterill, 1984; Chung & Ferrier, 1992; Liu et al., 2013).

2.4.2. Current study of phosvitin and phosvitin hydrolysate on bone health

Current knowledge of phosvitin and phosvitin hydrolysate on bone health is still limited, probably due to the lack of a scalable method to extract enough phosvitin required to carry out an animal feeding study. It was reported that adding phosvitin hydrolysate to the diet increased calcium-to-ash ratios, bone mineral density and bone mineral content in femurs and tibias of Sprague Dawley (SD) rats (Choi, Jung, Choi, Kim, & Ha, 2005). In a study with mouse calvarial organ culture, phosvitin promoted bone formation activities by upregulating collagen synthesis, calcium deposition, and several biomarkers of bone formation (Liu et al., 2013). Phosvitin probably mirrors the role of ascorbic acid under physiological conditions since the activity of phosvitin on bone formation is related to its antioxidant activity or reducing ability (Liu et al., 2013). This was confirmed by the fact that both ascorbic acid-treated and phosvitin-treated osteoblasts produced similar levels of osteogenic gene markers: collagen type I, OCN, RUNX2, and bone morphogenetic protein-2 (BMP-2) (Liu, Li, Geng, Huang, & Ma, 2017).

Phosvitin has great potential in bone engineering applications by enhancing biomineralization. Bone is composed of collagen fiber scaffolds with highly organized hydroxyapatite crystals oriented to it (Hulmes, Wess, Prockop, & Fratzl, 1995). Phosphoproteins secreted by osteoblast play a key role in the biomineralization process by initiating nucleation of hydroxyapatite (Gorski, 1992). As a highly phosphorylated protein, phosvitin was also reported to enhance nucleation of hydroxyapatite crystals on a collagen sheet with collagen fibers (Kobayashi, Onuma, Oyane, & Yamazaki, 2004). Phosvitin also accelerates the phase transformation from di-calcium phosphate dihydrate to hydroxyapatite crystals, which could assist mineralization (Jie et al., 2017).

2.4.3. Extraction of phosvitin for industrial application

A scalable method to extract phosvitin from egg yolk with high purity and high recovery is a prerequisite for any potential industrial application. There are various extraction protocols reported in the literature but with limited success to scale up. Most methods use large amounts of organic solvents to remove lipids from yolk (Losso & Nakai, 1994), followed by use of high concentration of salts to break the phosphocalcic bridge between phosvitin and lipovitellin, and then precipitate phosvitin with metal ions or under extreme acidic pH (pH 1.5) (Tsutsui & Obara, 1984; Castellani, Martinet, David-Briand, Guerin-Dubiard, & Anton, 2003). Thus, dialysis is needed to remove excessive salt at the end of extraction. These methods are impractical for industrial use because they are tedious and low in recovery and purity, and use large amounts of organic solvents.

An aqueous method with water dilution and salt extraction was reported to obtain phosvitin with a recovery of 82.7%, but with a low purity of 54.5% (Ren & Wu, 2014). Heating at 90 °C for 20 min during extraction was reported to denature the contaminating lipoproteins and further improve the purity to 88.0% with a recovery of 58.8% (Albright et al., 1984; Ren & Wu, 2015). Although purity and yield are high, the heating process is energy demanding.

2.4.4. Other activities of phosvitin and phosvitin hydrolysate

2.4.4.1. Antimicrobial

The antimicrobial activity of phosvitin is a relatively new research topic and largely remains unexploited. One of the earliest publications revealed that phosvitin decreased the concentration of *Escherichia coli* from 10^5/mL to almost 0/mL after heating at 50 ^oC in 20 min. The antimicrobial activity of phosvitin is greatly reduced when the phosphoserine residues are saturated by metal ions or the hydrophobic C- and N-terminus of phosvitin are cleaved *(*Khan et

al., 2000). These findings suggest that phosvitin exhibits antimicrobial activity due to not only its extraordinary affinity to metal ions but also the hydrophobicity off its C- and N-terminus. Phosvitin also exhibits antimicrobial activity against *Escherichia coli* with high hydrostatic pressure treatment (Jung, Nam, Ahn, Kim, & Jo, 2013), or when combined with chitosan on cellulose mat (Zhou et al., 2014). A peptide derived from the C-terminal of phosvitin possessed similar antimicrobial activity as phosvitin. It increased the survival rate of animals and reduced the number of pathogen in various tissues when challenged by *Aeromonas hydrophila* (Ding, Liu, Bu, Li, & Zhang, 2012). More interestingly, this same peptide was found to induce membrane permeabilization and depolarization and cause apoptosis and necrosis of several multi-drug resistance bacteria (Li, Wang, Jiang, Cui, & Zhang, 2016).

The antimicrobial activity of phosvitin may originate from its role in early embryos to prevent pathogenic attacks by binding and disrupting potential pathogens. In the developing embryo of a chicken, injection of recombinant phosvitin enhanced its resistance to *Aeromonas hydrophila* challenge, but such effect was attenuated by co-injection of phosvitin antibody (Wang, Wang, Ma, Ding, & Zhang, 2011).

2.4.4.2. Anti-inflammation

Phosvitin hydrolysate or peptides, rather than native phosvitin protein, are more frequently reported for their anti-inflammation effects. Phosvitin can suppress TNF-α production in lipopolysaccharide (LPS) challenged RAW264.7 macrophages/mice and increased the survival rate of the animals (Ma, Wang, Wang, & Zhang, 2013). A peptide from the C-terminal of phosvitin demonstrates similar effects by suppressing production of inflammatory cytokines in LPS- or bacteria-challenged RAW264.7 cells or mice (Hu, Sun, Wang, Su, & Zhang, 2013; Ding et al., 2012). Phosvitin hydrolysate containing various peptides also exerts anti-inflammatory

activities by inhibiting expression of IL-8, MCP-1 and IL-12 in HT29 cells and expression of TNF-α, IL-1β, IL-6, and inducible nitric oxide synthases (iNOS) in RAW264.7 cells (Xu et al., 2012).

2.4.4.3. Antioxidant

For a long time, phosvitin has been regarded as an impressive antioxidant agent due to its strong metal chelating ability. Phosvitin inhibits metal-catalyzed oxidations in an egg yolk phospholipid emulsion system. Such activity is not affected by pasteurization but is greatly reduced by autoclaving, which causes phosvitin starts to lose phosphate groups (Lu & Baker, 1987). Phosvitin exerts significant inhibitory effect on Fe (II)-and H_2O_2-catalyzed DNA degradation, whereas ethylenediaminetetraacetic acid (EDTA), another strong metal chelator, promotes the DNA degradation. These results suggest that the *in vivo* antioxidant activity of phosvitin may be related to its molecular structure but not its metal chelating ability (Maheswari, Ramadoss, & Krishnaswamy, 1997).

Peptides derived from phosvitin inherit the antioxidant ability and are more frequently studied in cell cultures. Phosvitin hydrolysate was reported to suppress the production of proinflammatory cytokine (IL-8) and formation of malondialdehyde but elevated intracellular glutathione levels and glutathione reductase activity in H_2O_2-stimulated Caco-2 cells. Interestingly, the intact phosvitin or free phosphoserine residue does not show such antioxidative effects in H_2O_2-stimulated Caco-2 cells (Katayama, Xu, Fan, & Mine, 2006). The peptide structure may play a role in the oxidation activity. This is supported by the fact that phosvitin hydrolysate shows stronger ability of inhibiting lipid oxidation and scavenging 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radicals than intact phosvitin, although the hydrolysate possesses lower metal chelating capacity (Xu, Katayama, & Mine, 2007).

2.5. General conclusions

Osteoporosis is a disease with increasing prevalence as the population ages, costing billions of dollars for treating osteoporosis and related fractures. Moreover, osteoporotic fractures could cause disability, which brings great inconvenience/pain to the patients. There are effective drugs for treating osteoporosis but they are associated with severe side-effects and can not reverse the bone loss. Therefore, there is increasing interest in functional foods and nutraceuticals as alternatives for the prevention and management of osteoporosis. Calcium and vitamin D are conventionally used for the prevention of osteoporosis, but the efficacy is still questionable. The market for osteoporosis prevention is huge but there is a lack of affordable functional foods and nutraceuticals with proven efficacy and safety.

Phosvitin and phosvitin hydrolysate derived from chicken egg showed great potential for prevention/treatment of osteoporosis (Liu et al., 2013; Liu et al., 2017). Phosvitin promotes osteoblast functions but the mechanism is largely unknown. Besides, whether phosvitin affects osteoclast functions has not been studied. Phosvitin hydrolysate is the enzymatically-degraded phosvitin fragments, with a high density of phosphate groups similar to phosvitin but with much smaller molecular weights (Ren, Li, Offengenden, & Wu, 2015). Whether phosvitin hydrolysate inherits the activities from native phosvitin needs to be investigated. More importantly, proper animal experiments are needed to confirm the bone health benefits of phosvitin and phosvitin hydrolysate from cell cultures. The lack of a scalable method to prepare enough phosvitin for animal experiment poses great difficulty to the researchers.

CHAPTER 3 - Pilot-scale Extraction of Phosvitin from Egg Yolk Using the pH Shifting Method

3.1 Introduction

Phosvitin, the major phosphoprotein in egg yolk, is one of the most phosphorylated proteins in nature; nearly all the serine residues, accounting for half of the amino acids in phosvitin, are phosphorylated (Clark, 1985). Two variants of phosvitin were reported: β-phosvitin as the major component contains 10% phosphorus, and α-phosvitin as the minor component contains 3% phosphorus (Abe et al., 1982). Phosvitin is reported to possess anti-bacterial, antioxidant, and bone health promoting properties (Khan et al., 2000; Nakamura et al., 1998; Albright, Gordon, & Cotterill, 1984; Chung & Ferrier, 1992; Liu et al., 2013), indicating its great potential for its use as a functional food ingredient. There are various extraction protocols reported in the literature but with limited success for industrial application. Most methods first use large amounts of organic solvents to remove lipids from yolk (Losso & Nakai, 1994), followed by the use of 10% salt to break the phosphocalcic bridges between phosvitin and lipovitellin, and then precipitate phosvitin with metal ions or under extreme acidic pH (pH 1.5) (Tsutsui & Obara, 1984; Castellani, Martinet, David-Briand, Guerin-Dubiard, & Anton, 2003). Thus, dialysis is needed to remove excessive salt at the last step of extraction. These methods are tedious and are low in recovery and yield, making them impractical for industrial uses. Ting et al. (2010) used ultrafiltration membrane to extract phosvitin from hexane-delipidated egg yolk proteins but the recovery and purity were low. Lee, Abeyrathne, Choi, Suh, & Ahn (2014)

developed an aqueous method to extract phosvitin with a purity of 97.2% but a yield of 12.5 g kg^{-1} egg yolk dry mass, which is low.

To develop an environmentally friendly method without using organic solvents, Ren & Wu (2014) reported a method to extract phosvitin directly from the egg yolk granules using 10% NaCl, where a recovery of 82.7% phosvitin was achieved, but with a low purity of 54.5%; decreasing pH could further increase phosvitin purity but would result in a significant loss of yield. Using anion exchange chromatography, a recovery of 42.0% and a purity of 97.1% were obtained (Ren & Wu, 2014). Although the purity was high, the corresponding recovery was low, probably due to loss of phosvitin during ion exchange chromatography. Given the different thermal stabilities of phosvitin and other egg yolk proteins (mainly high-density lipoproteins (HDL)), a thermo-aided extraction method was developed bt Ren & Wu (2015). Heating denatures lipoproteins but leaves phosvitin unaffected since phosvitin is a linear protein (Albright et al., 1984). This thermo-aided method resulted in a purity of 88.0% and a yield of 23.5 g kg^{-1} yolk dry matter (58.8% recovery) at 90 °C (Ren & Wu, 2015). The purity and yield of this method were comparable to or higher than most of the previous methods. However, heating up to 90 °C is energy demanding.

There is no commercial method of preparing egg yolk phosvitin for food or functional food uses. Although the methods developed by Ren & Wu (2015) showed potential for scale-up production, the low centrifugal force of typical industrial centrifuges might result in inefficient separation of fractions and thus potentially decrease the purity and recovery in the final product. Besides, desalting by dialysis is not applicable to industrial production due to its small capacity and the use of a large amount of water. Therefore, the aim of this study was to improve the above phosvitin extraction method and test the feasibility of scale-up extraction. The specific objectives

of this study were 1) to study the effects of low centrifugal force with short duration of centrifugation on phosvitin extraction; 2) to investigate the effects of ultrafiltration (UF) on phosvitin desalting and different water dilution factors on phosvitin extraction; 3) to study the effects of using industrial centrifugation (decanter/disc stack centrifuge) on phosvitin extraction.

3.2. Materials and methods

3.2.1. Chemicals

Sodium chloride, sodium hydroxide, and hydrochloric acid were purchased from Fisher Scientific (Nepean, ON, Canada). Phosvitin standard (P1253, from chicken egg yolk) was purchased from Sigma-Aldrich, Ltd. (Oakville, ON, Canada). The deionized distilled water (DD water) used for this study was produced by a Barnstead water purification system (Thermo Scientific, Asheville, NC, USA).

3.2.2. Egg yolk

Eggs were purchased from a local supermarket. Egg shell was manually broken to separate egg yolk from egg white. The yolk was rolled on a Whatman #1 filter paper (Whatman Inc., Florham Park, NJ, USA) to remove adhering egg white. The vitellin's membrane was punctured by a needle to collect egg yolk into a beaker cooled with ice. The collected egg yolk was free of egg white and vitellin's membrane.

3.2.3. Granules preparation

Granules were prepared according to McBee & Cotterill (1979) with minor modifications. Briefly, yolk was 1/1 (wt/wt) diluted with deionized water, and then stirred for 1 h at 4 ^{o}C to mix yolk and water. The mixed yolk solution was then centrifuged at 10,000 g for 45 min at 4 ^{o}C to precipitate granules.

3.2.4. Effect of pH of granules/NaCl suspension on phosvitin extraction

In this experiment, the pH of granules/NaCl suspension was decreased from 4.5 to 1.5 by adding 1 M HCl to examine the effect of pH on phosvitin extraction. Granules prepared in section 3.2.3 were suspended in 10 times weight of 10% NaCl solution, keeping the pH to 7.25 by adding 3 M NaOH with magnetic stirring at 4 ºC overnight (Castellani et al., 2003). The pH of the granules/NaCl suspension was then sequentially adjusted to 4.5, 4.0, 3.5, 3.0, 2.5, 2.0 or 1.5 by using 1 M HCl; after 1 h stirring, the granules/NaCl suspension was centrifuged at 10,000 g, 4 ºC for 25 min. The supernatants containing phosvitin were dialyzed against DD water (24 h, with 4-5 water changes), centrifuged at 10,000 g, 4 ºC for 25 min and then lyophilized for analysis. The procedure was shown in Figure 3.1.

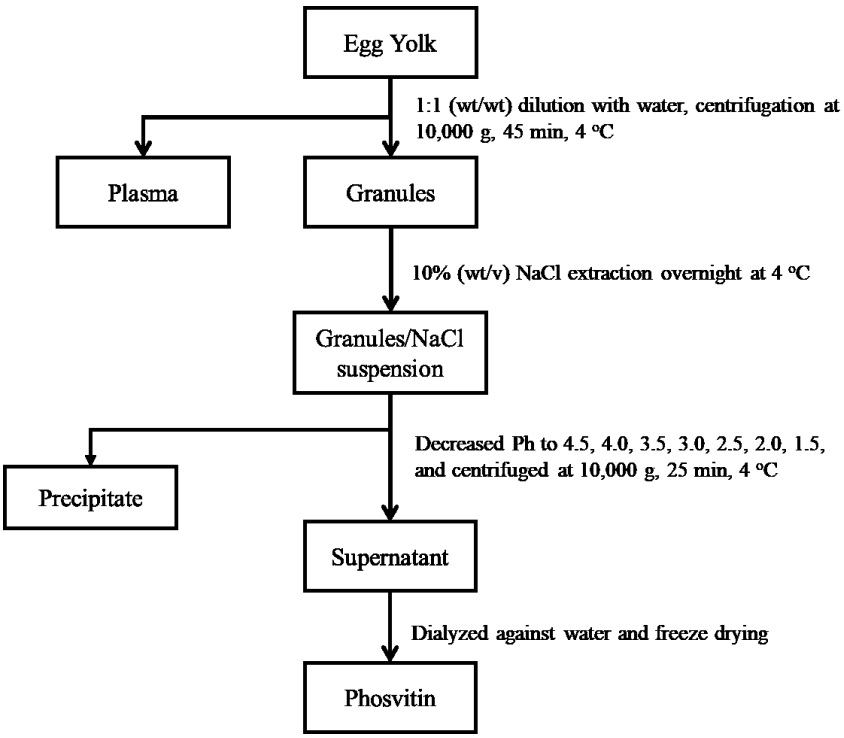

Figure 3.1. Phosvitin extraction protocol by using pH-shifting method.

3.2.5. Desalting by ultrafiltration

Ultrafiltration module of Prep/Scale Spiral Wound TFF-6 Module PLBC (10 kDa molecular weight cut-off (MWCO), 0.23 m^2) was purchased from Millipore (Etobicoke, ON, Canada) and fixed on a Prep/Scale-TEF cartridge holder and run by a pump (Model XX80EL005, Millipore, Etobicoke, ON, Canada). The supernatant of granule/NaCl suspension prepared at pH 3.0 (section 3.2.4) or prepared by heating at 90 ºC (Ren & Wu, 2015) was concentrated to a volume concentration ratio (VCR) of 10X and then diafiltrated by using 10 diavolumes (DV) of water while trans membrane pressure (P_T) was maintained at 137.9 kPa. The final retentate, which was a mixture of soluble phosvitin and insoluble lipovitellin, was centrifuged at 10,000 g, 4 ºC for 25 min. The supernatant containing phosvitin was lyophilized and stored at -20 ºC until use.

3.2.6. Effects of low centrifugal force and short duration on phosvitin extraction

The industrial separators' centrifugal force is lower and duration is shorter than the lab-scale ones. Therefore, it is necessary to study the effects of low centrifugal force and duration on phosvitin extraction before scale-up trial.

Briefly phosvitin was extracted as describe in section 3.2.4 but with slight modifications: phosvitin was extracted at pH 3.0 from granules obtained at centrifugal forces of 2,000 g, 3,000 g, 4,000 g, 5,000 g and 6,000 g, respectively, for 5 min at 4 ºC. The aliquots of granules obtained under these conditions were defatted by organic solvent and then analyzed by high-performance liquid chromatography (HPLC) as described in section 3.2.9 to determine the purity of phosvitin, and then the recovery was calculated as described in section 3.2.10. The rest of the granules were used to extract phosvitin as described in section 3.2.4 at pH 3.0, and the phosvitin recovery, yield and purity in final extracts were calculated as described in sections 3.2.9 and 3.2.10.

3.2.7. Effect of egg yolk dilution factors on phosvitin extraction

This experiment was designed to study the effect of different water dilution factors on phosvitin extraction. Briefly, phosvitin was extracted as described in section 3.2.4 at pH 3.0 with slight modifications: egg yolk was diluted with water at different ratios of 1/1, 1/2, 1/4, 1/8, 1/10 (weight/weight); phosvitin was extracted from granules obtained at centrifugation of 6,000 g for 5 min at 4 ºC. The plasma (supernatant) and granules (precipitate) after centrifugation were defatted by 10 times (wt/wt) of hexane and then dried to determine phosvitin purity, recovery and yield as described in sections 3.2.9 and 3.2.10.

3.2.8. Scale up production using industrial decanter or disc stack separators

Two commonly used industrial separators were used in this experiment to replace the lab centrifugation. Briefly, phosvitin was extracted as described in section 3.2.4 but with modifications: Un-pasteurized egg yolk was obtained from Egg Processing Innovations Cooperative (EPIC) (Lethbridge, AB, Canada). Egg yolk was diluted 1, 4, and 9 times (wt/wt) with de-ionized water to a final weight of 200 kg, and then centrifuged by GEA Westfalia decanter separator CA220-010 (GEA Inc., Drummondville, QC, Canada) at 3000 rpm and a feeding rate of 200 L/h, or by Alfa Laval High speed disk stack separator LAPX 404SGP-31G/TGP-61G (Alfa Laval Inc., Toronto, ON, Canada) at 9000 rpm and a feeding rate of 250 L/h to prepare granules. Then, the granules were used to extract phosvitin at pH 3.0 as described by section 3.2.4, and phosvitin purity, recovery and yield were calculated as described in sections 3.2.9 and 3.2.10.

3.2.9. Gel filtration chromatography

The purity of phosvitin extracts was analyzed using a TSK-Gel G3000SWxL stainless still column (0.78 x 30 cm, Tosoh Bioscience, Inc., South San Francisco, CA, USA) on a Waters

HPLC system (Waters, Milford, MA, USA). The phosvitin extracts were prepared with the same volume of running buffer (0.1 M sodium phosphate buffer containing 0.2 M NaCl, pH 7.0). After filtration with 0.45 μm PVDF filter (13 mm x 0.20 μm, MANDEL, Guelf, ON, Canada), 30 μL of samples were injected and eluted at a flow rate of 0.5 mL/min. The experiment was controlled by Empower II Software (Waters, Milford, MA, USA) and chromatogram was recorded at 215 nm. The Sigma standard phosvitin was used to prepare a standard curve at concentrations ranging from 0.1 to 5.0 mg/mL and the content of phosvitin in the extracts was thus calculated.

3.2.10. Calculation of phosvitin purity, recovery and yield

The purity of phosvitin was expressed as the percentage of phosvitin peak in the total integrated peak area in the HPLC chromatograms. Phosvitin yield was expressed as the amount of phosvitin extracted from 100 g yolk solids. Phosvitin recovery was calculated as the ratio of extracted phosvitin to the total phosvitin in egg yolk. Phosvitin accounted for overall 4% of egg yolk solids (Joubert & Cook, 1958).

3.2.11. Protein and lipid analysis

Nitrogen was determined in duplicate by using the Leco-N nitrogen determinator (Model FP-428, Leco Corporations, St. Joseph, MI, USA) and the crude protein was calculated by multiplying nitrogen content by a factor of 6.25.

Lipid content was determined according to the method of Hara & Radin (1978) and the lipid content was expressed as g/100 g dry weight.

3.2.12. Statistical analysis

All experiments in each section were repeated three times independently. The Statistical Analysis System software (SAS, version 9.0; SAS Institute, Cary, NC) was used to analyze the data. Values are reported as mean ± standard deviation (SD). The differences between values

were determined by one-way analysis of variance (ANOVA) with the Tukey post hoc test. A $p < 0.05$ was considered statistically significant. The different letters in the tables indicate significant differences in the values.

3.3. Results and discussion

3.3.1 Effect of pH of granules/NaCl suspension on phosvitin extraction

In egg yolk, phosvitin exists in the form of lipovitellin-phosvitin complex through phosphocalcic bridges (Burley & Vadehra, 1989). This phosphocalcic bridge can be disrupted by 10% NaCl and phosvitin can be separated from lipovitellin by their difference in solubility (Castellani et al., 2003). Both α-lipovitellin and β-lipovitellin are soluble at alkaline pH, but precipitate at acidic pHs (Sugano, 1958). Castellani et al. (2003) observed that lipovitellin was completely precipitated at pH 5.5 while phosvitin remained soluble at pH levels below 5.5. Considering that the isoelectric point (pI) of phosvitin is around pH 4 (Ternes, 1989), it may be possible to separate phosvitin from lipovitellin by solubility difference when the pH was adjusted from 7.0 to around 5.5. However, Ren & Wu (2014) reported that decreasing pH from 7.0 to 3.5 could only increase phosvitin purity to a limited extent at the cost of significant loss of recovery. This might be due to the co-precipitation of phosvitin with lipovitellin at acidic pH. Since the lipovitellin-phosvitin complex can be disrupted by 10% sodium chloride (Burley & Vadehra, 1989), it is not known if decreasing pH during NaCl extraction might avoid the co-precipitation of phosvitin with lipovitellin and thus possibly lead to separation of phosvitin from lipovitellin.

The extraction protocol developed in this study by using pH-shifting was shown in Figure 3.1. Phosvitin obtained at pH 4.5, 4.0, 3.5, 3.0, 2.5, 2.0, 1.5 during NaCl extraction was analyzed by HPLC. As shown in Figure 3.2, in addition to α- and β-phosvitin, six contaminant peaks A, B, C, D, E and F were detected in the extract at pH 4.5 where the precipitation started to occur.

Peaks D and E were the major contaminants; at decreasing pHs, all contaminated peaks were significantly decreased, and peaks A, B, C, and F almost disappeared. Phosvitin extracts at pHs 3.5 to 2.0 showed less impurities than at other pHs. This might be due to the fact that phosvitin is soluble at these acidic pHs while the impurities (mainly lipovitellins) precipitated at acidic pHs from 3.5 to 2.0. Usually β-phosvitin is more abundant than α-phosvitin. Peak A might be low-density lipoproteins (LDL) as it showed much larger MW than HDL detected in the phosvitin standard. It is noteworthy that HDL peak was not detected in all phosvitin extracts, suggesting that decreasing pHs to acidic range in the presence of 10% NaCl could efficiently separate phosvitin from HDL. High ionic strength provided by 10% NaCl could break the phosphocalcic bridge between phosvitin and HDL (Causeret, Matringe, & Lorient, 1991); HDL will precipitate at acidic pHs while phosvitin is extremely water soluble at a wide range of pHs (Castellani et al., 2003).

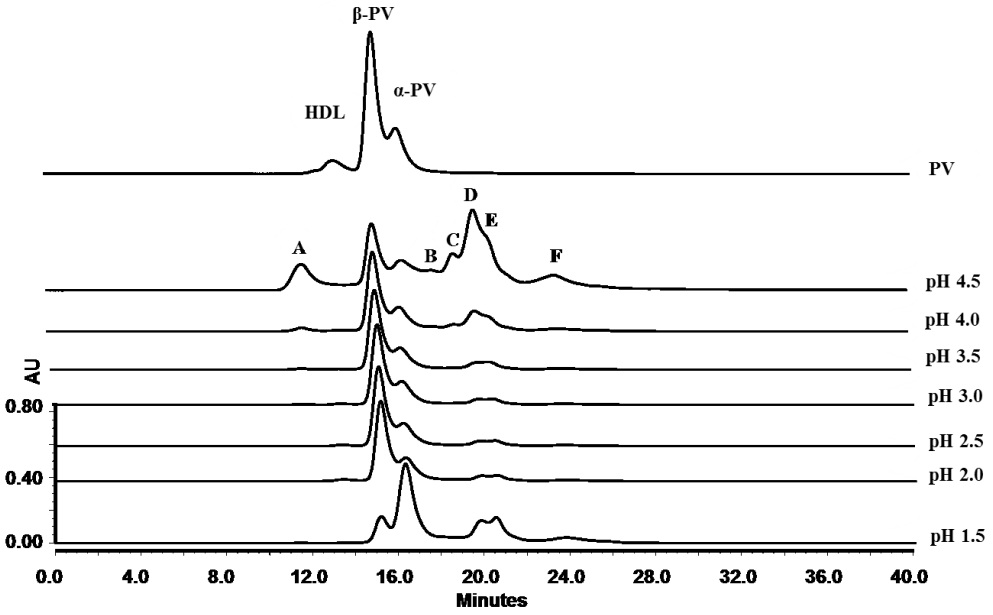

Figure 3.2. Gel filtration chromatograms of phosvitin extracts at different pHs. (Phosvitin was extracted at pH 4.5, 4.0, 3.5, 3.0, 2.5, 2.0, 1.5 as described in section 3.2.4. Phosvitin standard was purchased from Sigma. PV, phosvitin.)

The purity of phosvitin extracts increased from 33.1% at pH 4.5 to 90.7% at pH 2.5, and then decreased to 65% at pH 1.5 (Table 3.1). The recovery and yield increased from 15.5% to 68.1%, 0.6 to 2.7 g/100 g of yolk solid, at decreasing pH from 4.5 to 3.5, and then started to decline at pH 2.5 and below. The appropriate recovery and yield were observed at pHs 3.0 and 3.5 (there was no significant difference).

Table 3.1. Recovery and purity of phosvitin extracts obtained at different pHs

pH	PV purity (%)	PV yield (g/100 g yolk solids)	PV recovery (%)
4.5	33.1±4.0^{e}	0.6±0.1^{e}	15.5±2.0^{e}
4.0	67.9±5.0^{cd}	1.6±0.0^{d}	40.3±0.9^{d}
3.5	83.5±1.2^{b}	2.7±0.0^{a}	68.1±0.3^{a}
3.0	86.3±0.1^{ab}	2.7±0.0^{a}	67.3±0.6^{a}
2.5	90.7±0.0^{a}	2.5±0.0^{b}	62.8±1.1^{b}
2.0	90.1±0.5^{a}	2.2±0.0^{c}	55.8±0.2^{c}
1.5	65.0±0.8^{c}	0.6±0.0^{e}	16.0±0.1^{e}

Data with different letters within each column are significantly different at $p < 0.05$. PV, phosvitin.

3.3.2 Desalting by ultrafiltration

In most published reports, dialysis was used to remove the salt from phosvitin extracts. This experiment was proposed to test the feasibility of using ultrafiltration membrane to desalt instead of dialysis. As shown in Table 3.2, phosvitin extracted by pH-shifting method at pH 3.0 showed a comparable purity, yield and recovery with those obtained with dialysis (Table 3.1); however, phosvitin extracted by the heating method showed lower purity, yield and recovery compared to those of dialysis. This reduced extraction performance was probably due to the denaturation of lipoproteins during heating, which led to rapid fouling of membrane during

ultrafiltration. These results suggested that the pH method could be a better option than the heating method for potential scale-up extraction of phosvitin.

Table 3.2. Phosvitin purity, yield and recovery obtained with ultra filtration

Extraction methods	PV purity (%)	PV yield g/100g yolk solids	PV recovery (%)
pH 3.0	88.6±0.2[a]	2.5±0.1[a]	63.6±1.6[a]
90°C	69.5±3.7[b]	1.5±0.2[b]	38.0±6.1[b]

Data with different letters within each column are significantly different at $p < 0.05$. PV, phosvitin.

3.3.3. Effects of low centrifugal force and duration on phosvitin extraction

Since the centrifugal force of industrial centrifuges/separators is much lower than those used in the lab, it is important to study the effects of low centrifugal force and duration on the extraction before the scale-up production. The effect of centrifugal force (2,000 g, 3,000 g, 4,000 g, 5,000 g and 6,000 g) and short duration (5 min) on phosvitin extraction was studied. The extraction was conducted as described in Section 3.2.4 at pH 3.0. As shown in Table 3.3, the phosvitin recovery increased with increasing centrifugal force, ranging from 11.4% at 2,000 g to 39.1% at 6,000 g, and to 67.3% at 10,000 g (Ren & Wu, 2014). The yield showed a similar trend. A high centrifugal force seemed to be critical to obtain high recovery and yield of phosvitin. This is probably due to the fact that a high centrifugal force could separate granules more efficiently from plasma, and therefore more phosvitin could be extracted from the granules fraction in the next step. As shown in Table 3.3 (second column), the obtained granules increased from 1.9 g at 3,000 g to 6.6 g at 6,000 g based on 100 g yolk dry mass, which showed a similar trend with those of phosvitin recovery and yield. The phosvitin purity was not affected by centrifugal force, ranging from 84.7% to 86.7%, which was close to the phosvitin purity (86.3%) obtained at 10,000 g for 45 min (Ren & Wu, 2014). These results suggested that this extraction protocol

could extract phosvitin from granules obtained from a low centrifugal force with high purity, but the recovery and yield were low probably due to insufficient separation of granules under low centrifugal force.

Table 3.3. Effects of centrifugation speed (2,000 g to 10,000 g) on granules weight, phosvitin recovery in granules, and the recovery, yield and purity of extracted phosvitin from granules

Centrifugation speed (g)	Granules dry weight (g)	PV recovered in granules (%)	PV purity in the extracts (%)	PV recovery in the extracts (%)	PV yield in the extracts (g/100g yolk dry matter)
2,000	1.9±0.0^{a}	13.2±0.3^{a}	84.7±2.6^{a}	11.4±1.0^{a}	0.5±0.0^{a}
3,000	3.6±0.6^{b}	25.1±4.7^{b}	86.7±0.6^{a}	21.5±3.3^{b}	0.9±0.1^{b}
4,000	5.0±0.3^{c}	33.5±1.7^{bc}	86.1±1.4^{a}	28.4±0.9^{bc}	1.1±0.0^{bc}
5,000	6.1±0.5^{d}	41.0±1.5^{cd}	85.9±1.6^{a}	33.2±1.7^{cd}	1.3±0.1^{cd}
6,000	6.6±0.1^{d}	44.8±0.7^{de}	85.8±1.9^{a}	39.1±4.3^{de}	1.6±0.2^{de}
10,000 *	6.2±0.0 ^{d}	117.3 ± 8.1^{f}	86.3±0.1^{a}	67.3±0.6^{f}	2.7±0.0^{f}

Data with different letters within each column are significantly different at $p < 0.05$. Experiment started with 50 g native egg yolk. *The 117% phosvitin recovered in granules was cited from Ren & Wu (2014), and used as a reference for comparison; phosvitin in the rest of the table was prepared using low centrifugal forces as described in section 3.2.4. at pH 3.0. PV, phosvitin.

3.3.4 Effect of dilution factor on phosvitin extraction

The above results suggested that insufficient separation of egg yolk granule from egg yolk significantly affected phosvitin recovery and yield. Therefore, this experiment aimed to enhance granule separation by using different levels of water dilution and eventually increase phosvitin recovery and yield. Granules can be easily separated from plasma by water dilution method followed by centrifugation due to density difference. The granule fraction, containing 70% lipovitellin, 16% phosvitin and 12% LDL, has a higher density than the plasma fraction, which contains 85% LDL and 15% livetins (Joubert and Cook, 1958; Mccully, Common, &

Mok, 1962). It was previously reported that increasing water dilution resulted in more proteins being precipitated from yolk and thus might enhance granule separation (Kwan, Lichan, Helbig, & Nakai, 1991). Therefore, the effect of different water dilution factors on granule separation and phosvitin extraction was studied. The composition of the supernatant and pellets were analyzed as high water dilution might also cause precipitation and aggregation of plasma proteins with granule proteins (Akita & Nakai, 1992), and thus increase difficulty for phosvitin extraction. Besides, the lipid, protein and solid contents in these fractions were critical for their functional properties and potential future applications (Sirvente et al., 2007)

The extraction was conducted as described in Section 3.2.4 at pH 3.0 with different water dilution. Table 3.4 summarizes dry mass, lipid, protein and solid contents and phosvitin recovery from egg yolk to supernatant in the supernatant fraction. Phosvitin recovery in supernatant was decreased from 11.1% at 1/1 (yolk/water, wt/wt) dilution to 5.5% at 1/4 dilution. At 1/8 and 1/10 dilution, there was no phosvitin detected by HPLC in the supernatant, suggesting that most phosvitin was precipitated in the pellet. Accompanying with a decrease in phosvitin recovery in the supernatant, there was also a decrease in dry mass from 10.1 g at 1/1 dilution to 2.8 g at 1/10 dilution. This agreed with the results of Akita & Nakai (1992) and Kwan et al. (1991), who reported that plasma proteins and lipids tended to aggregate and precipitate into the pellets at increasing water dilution; at 10 times water dilution, most of the plasma proteins and lipids precipitated into the pellets and only livetins and LDLs existed in the plasma and supernatant fraction (Kwan et al., 1991). The lipid content of supernatant slightly increased from 65.7% at 1/1 dilution to 67.4% at 1/4 dilution and then decreased to 53.4% at 1/10 dilution. The protein content of supernatant slightly decreased from 23.8% at 1/1 dilution to 22.5% at 1/4 dilution and then increased to 33.7% at 1/10 dilution. In general, the protein and lipid contents of pellets at

1/1 to 1/4 dilution were close to those of granules obtained at high centrifugal force (22.3% protein and 67.2% lipids, Table 3.4). The solid content of supernatant greatly decreased at increasing water dilution, from 23.7% at 1/1 dilution to 1.1% at 1/10 dilution. The solid contents at 1/8 and 1/10 dilution were 2.1% and 1.1%, respectively, which were much lower than the 22.5% of the plasma obtained at high centrifugal force (Table 3.4). The decreasing solid contents were mainly due to precipitation of plasma lipids/proteins into pellets (Akita & Nakai, 1992). The low solid/lipid contents might have negative effects on plasma's emulsifying properties and limit its potential application in the food industry.

Table 3.4. Effect of dilution factors on the distribution of mass, lipids, protein in the plasma/supernatant of egg yolk after centrifugation.

Dilution factors (wt/wt)	Plasma dry mass (g)	Plasma lipid (%)	Plasma protein (%)	Plasma solid content (%)	PV recovered in plasma (%)
1/1	10.1±0.1^{a}	65.7±0.8^{a}	23.8±0.3^{a}	23.7±0.0^{a}	11.1±0.2^{a}
1/2	10.0±0.1^{a}	65.0±0.3^{a}	23.3±0.3^{a}	15.0±0.1^{b}	8.8±0.1^{b}
1/4	9.2±0.0^{b}	67.4±1.5^{a}	22.5±0.4^{a}	8.2±0.0^{c}	5.5±0.4^{c}
1/8	4.3±0.0^{c}	63.7±3.2^{a}	28.0±1.4^{b}	2.1±0.0^{d}	N.D.
1/10	2.8±0.0^{d}	53.4±2.9^{b}	33.7±2.5^{c}	1.1±0.0^{e}	N.D.
1/1 (10,000 g)*	9.6±0.0^{e}	67.2±2.4^{a}	22.3±0.0^{a}	22.5±0.0^{f}	N.D.

Data with different letters within each column are significantly different at $p < 0.05$. Experiment started with 25 g native egg yolk. N.D. not determined by HPLC; all the data were calculated on dry base. *Here phosvitin was prepared at 10,000 g centrifugation for 45 min at 4 ºC as describedin section 3.2.4 at pH 3.0, and used as a reference for comparison; phosvitin in the rest of the rows was prepared at 6,000 g 5 min at pH 3.0 as described in section 3.2.4. PV, phosvitin.

Table 3.5 shows the distribution of dry mass, lipids, proteins, solid contents and phosvitin recovery in pellets, as well as phosvitin purity and recovery in the phosvitin extracts. The pellet fraction was used for phosvitin extraction so the phosvitin recovery from egg yolk to pellets was critical for the extraction. Nearly all phosvitin in the egg yolk can be recovered into pellets at

10,000 g centrifugation at a dilution factor of 1/1 (yolk/water, wt/wt; Ren & Wu, 2014). In the current study, phosvitin recovery from yolk to pellets increased at increasing water dilution factors, from 50.1% at 1/1 (yolk/water, wt/wt) dilution to 74.8% at 1/4 dilution factor then decreased to 67.2% at 1/8 dilution and 69.8% at 1/10 dilution. At dilution factors of 1/8 and 1/10, there was no phosvitin detected in the supernatant as shown in Table 3.4, indicating the highest phosvitin recovery could be expected in the corresponding pellets. However, the phosvitin recovery from yolk to pellets at 1/8 and 1/10 dilution were ~70%. This discrepancy was probably due to the phosvitin loss when defatting pellets using organic solvent for HPLC analysis. The phosvitin extracts prepared by this method showed a purity ranging from 85.8% at 1/1 dilution to 90.8% at 1/4 dilution, which was also comparable to the Sigma standard. The phosvitin recovery in the extracts increased at increasing water dilution from 39.1% at 1/1 dilution to 53.9% at 1/4 dilution. The results suggested that increasing water dilution from 1/1 to 1/4 (yolk/water, wt/wt) could increase phosvitin recovery in the extracts; however, pellets obtained at 1/8 and 1/10 dilution were not suitable to extract phosvitin due to the interference of too many proteins/lipids in the pellets. The lipid content of pellet prepared at 1/1, 1/2 and 1/4 dilution were all around 30%, close to the lipid content of granules (26.0%); at dilution factors of 1/8 and 1/10, the lipid contents in pellets increased to around 57%, which were close to that of native egg yolk (Navidghasemizad, Temelli, & Wu, 2014). The protein contents of pellets at 1/1, 1/2 and 1/4 dilution were all around 55% and close to the protein content of granules (60.3%). But at dilution factor of 1/8 and 1/10, protein contents in pellets were decreased to around 30%, which was also close to that of native egg yolk (Navidghasemizad et al., 2014). These results suggested that at 1/8 and 1/10 dilution, most of the egg yolk proteins/lipids would precipitate into the pellet fraction and make it not suitable for phosvitin extraction due to the complexity.

Table 3.5. Effect of dilution factors on the distribution of mass, lipid, protein and phosvitin contents in the pellets of egg yolk after centrifugation.

Dilution factor (wt/wt)	Pellets dry mass (g)	Pellets lipid (%)	Pellets protein (%)	Pellets solid (%)	PV recovered in pellets (%)	PV purity (%)	PV recovery (%)
1/1	2.7±0.1^{a}	31.5±1.1^{a}	55.8±1.4^{ab}	36.8±0.3^{a}	50.1±2.7^{a}	85.8±1.9^{a}	39.1±4.3^{a}
1/2	2.8±0.1^{a}	28.3±0.8^{b}	57.3±0.3^{a}	32.7±0.7^{b}	57.4±3.0^{b}	90.3±0.4^{b}	53.3±3.0^{b}
1/4	3.5±0.0^{b}	32.4±1.0^{ac}	54.6±0.8^{b}	27.9±0.8^{c}	74.8±2.4^{c}	90.8±0.8^{b}	53.9±7.1^{b}
1/8	8.3±0.1^{c}	56.7±1.0^{d}	32.8±0.1^{c}	37.0±0.1^{ad}	67.72±1.5^{d}	N.D.	N.D.
1/10	9.8±0.1^{d}	57.9±1.0^{de}	30.1±0.3^{d}	39.4±0.1^{e}	69.8±1.7^{cde}	N.D.	N.D.
1/1*	3.1±0.0^{e}	26.0±0.7^{bf}	60.3±0.9^{e}	42.0±0.5^{f}	117.3±8.1^{f}	86.3±0.1^{ac}	67.3±0.6^{b}

Data with different letters within each column are significantly different at $p < 0.05$. Experiment started from 25 g native egg yolk. N.D. not determined by HPLC; all the data were calculated on dry basis. *Here phosvitin was prepared at 10,000 g centrifugation for 45 min at 4 ºC as described in section 3.2.4 at pH 3.0, and used as an reference; phosvitin in the rest of the rows was prepared at 6,000 g 5 min at pH 3.0 as described in section 3.2.4. PV, phosvitin.

3.3.5 Scale up production of phosvitin using industrial centrifugation decanter or disc stack separator

The results suggested increasing water dilution (up to 4) could improve granule separation and thus increase phosvitin recovery at low centrifugal force. Further scale-up extraction was designed to test the feasibility to extract phosvitin by using an industrial decanter separator or disk stack separator.

The extraction was conducted as described in Section 3.2.4 at pH 3.0 and egg yolk was diluted 1/4 (wt/wt) with water. The results are summarized in Table 3.6. In general, neither decanter separator nor disc stack separator used in the current study could produce a firm precipitate (granule fraction). Compared with lab centrifugation, both separators produced a

relatively low phosvitin recovery and low purity no matter what dilution factors were applied. But the disc stack separator performed better than decanter for this extraction as it could produce a relatively higher recovery. For both separators, the phosvitin purity at 1/1 and 1/4 dilution was higher than that of 1/9 dilution, which agreed well with the observation in the previous experiment. For the decanter separator, phosvitin recovery decreased from 7.7% at 1/1 dilution to 2.7% at 1/9 dilution. The maximum recovery of 7.7% obtained at 1/1 dilution was lower than that obtained using lab-scale centrifugation. For the disk stack separator, the recovery was higher than that from decanter separator, but still at a relatively low level compared with the 67.3% recovery obtained in lab-scale. The highest recovery of 28.7% was obtained at 1/4 dilution. It seemed that the recovery was related to the centrifugal force of the separator used. However, the extracted phosvitin purity seemed to be more related to dilution factors. The purity of phosvitin extracted by the decanter/disc stack separators at 1/1 and 1/4 dilution was both around 70%, which was much higher than the ~30% purity at 1/9 dilution. This could be explained by the fact that higher dilution factor could separate more mass into the precipitate but as previously observed at 1/8 or 1/10 dilution, nearly everything was precipitated (Akita & Nakai, 1992), and the complexity of the pellets increased the difficulty for phosvitin extraction and thus decreased the purity and recovery.

Table 3.6. Phosvitin recovery, yield and purity under pilot scale extraction using different centrifugation and dilution factors

Centrifuge type	Dilution factor (wt/wt, yolk/water)	PV purity (%)	PV yield (g/100 g yolk dry matter)	PV recovery (%)
Decanter	1/1	68.7±0.8[a]	0.3±0.0[a]	7.7±0.3[a]
	1/4	74.7±1.4[b]	0.2±0.0[b]	5.7±0.0[b]
	1/9	29.6±0.1[c]	0.1±0.0[c]	2.7±0.0[c]
Disk stack	1/1	69.5±1.0[ad]	0.7±0.0[d]	18.5±0.4[d]
	1/4	77.7±0.2[be]	1.2±0.0[e]	28.7±0.6[e]
	1/9	33.7±0.4[f]	0.8±0.0[f]	19.0±0.2[df]

Data with different letters within each column are significantly different at $p < 0.05$. PV, phosvitin.

3.4. Conclusions

Although various extraction methods were reported, large-scale extraction of phosvitin remains a challenge due to its tedious procedures, use of non-food compatible chemicals, and low purity and recovery. Based on previous progress on phosvitin extraction (Ren & Wu, 2015), this study tested the feasibility of scaling-up extraction of phosvitin. The results showed that both phosvitin purity and recovery increased at decreasing pHs from neutral to acidic range; a recovery of 67.3% was obtained at pH 3.0 with a purity of 86.3%, which is quite comparable with the Sigma standard (~88% pure). The purity and recovery were consistent when ultrafiltration was used to replace dialysis for desalting. However, phosvitin recovery was significantly affected by centrifugal force, reduced from 67.3% at 10,000 g to 11.4% at 2,000 g, although the purity remained relatively stable (84.7% at 2,000 g vs 86.3% at 10,000 g). Increasing water dilution ratio (yolk/water) from 1/1 to 1/4 increased phosvitin recovery, but reduced at water dilution of 1/8 and 1/10 (yolk/water, wt/wt) due to the co-precipitation of other

proteins with phosvitin. Pilot studies were performed at three dilution trials (1/1, 1/4, 1/9) using two industrial separators (decanter and disc stack separators); both the purity and recovery were lower than the lab conditions. The disk stack separator seemed better than decanter separator for phosvitin extraction with a yield of ~20-30% at all water dilution ratios and a relatively stable purity of ~70%.

Chapter 4 - Phosvitin and Its Hydrolysate Promote Differentiation and Inhibit TNF-α Induced Inflammation in MC3T3-E1 Cells

4.1. Introduction

Bone remodeling is the dynamic process to maintain the integrity of skeletal system in which old or damaged bone tissues are resorbed by osteoclasts and new bone tissues are synthesized by osteoblasts (Raggatt & Partridge, 2010). An imbalance between bone formation and bone resorption activities leads to skeletal abnormalities such as osteoporosis (Lerner, 2004). Osteoporosis is a skeletal disorder characterized by loss of bone mass, strength, degradation of bone micro-architecture and elevated risk of fractures (Kanis, Melton, Christiansen, Johnston, & Khaltaev, 1994). Most drugs and therapies to treat osteoporosis are antiresorptive agents that target osteoclasts. Current research provides further molecular insights into the communication between osteoblasts and osteoclasts, and the orchestrating signaling network, which offers novel molecular targets against osteoporosis (Rachner, Khosla, & Hofbauer, 2011).

Phosvitin (PV) is a highly phosphorylated protein from egg yolk (Byrne et al., 1984). Phosvitin and phosvitin hydrolysate (PVH) derived from it were reported to exert various physiological activities such as anti-inflammatory activity (Young, Nau, Pasco, & Mine, 2011; Xu, Yang, Yin, Liu, & Mine, 2012; Hu et al., 2013), antioxidant activity (Katayama, Xu, Fan, & Mine, 2006; Xu, Katayama, & Mine, 2007) and antimicrobial activity (Wang, Wang, Ma, Ding, & Zhang, 2011; Ma et al., 2013). Addition of phosvitin hydrolysate in animal diet was reported to increase calcium-to-ash ratio, bone mineral density and bone mineral content in femurs and tibias of Sprague Dawley (SD) rats (Choi et al., 2005). In a study with mouse calvarial organ

culture, phosvitin promoted bone formation activities by upregulating collagen synthesis, calcium deposition, and several biomarkers of bone formation (Liu et al., 2013). It was proposed that phosvitin mirrored the role of ascorbic acid under physiological conditions and the activity of phosvitin was related to its antioxidant activity or reducing ability (Liu et al., 2013). This was confirmed by the fact that both ascorbic acid-treated and phosvitin-treated osteoblastic cells produced similar expression levels of osteogenic gene markers, collagen type I, osteocalcin (OCN), runt-related transcription factor 2 (RUNX2), and bone morphogenetic protein-2 (BMP-2; Liu, Li, Geng, Huang, & Ma, 2017). This hypothesis was further supported by other studies that oxidative stress and chronic inflammation were involved in the development of osteoporosis by directly or indirectly regulating the balance between osteoclasts and osteoblasts (Manolagas & Parfitt, 2010); while functional foods with antioxidant/anti-inflammation activities, like green tea, grapefruit pulp and citrus extract were reported to increase bone mass or prevent bone loss *in vivo* (Shen, Yeh, Cao, & Wang, 2009; Mandadi et al., 2009). Inflammation plays a critical role in many chronic diseases. It was reported that the elevation of inflammation status is related to bone loss in arthritis and osteoporosis (Di Benedetto, Gigante, Colucci, & Grano, 2013). Tumor necrosis factor alpha (TNF-α) is an inflammatory cytokine that was previously believed to inhibit osteoblast differentiation (Gilbert et al., 2002; Abbas, Zhang, Clohisy, & Abu-Amer et al., 2003). However, depending on the concentration, cell type and duration of treatment, TNF-α could also induce osteogenic differentiation by upregulating expression of gene markers for osteoblast differentiation (Huang et al., 2011; Lu, Wang, Dunstan, & Zreiqat, 2012). Osteoblasts are responsible not only for forming new bone tissue but also for expressing inflammatory chemokines, including interleukin (IL)-8, growth-regulated oncogene-alpha (GRO-α), monocyte chemoattractant protein-1 (MCP-1), regulated on activation, normal T cell expressed and

secreted (RANTES), macrophage inflammatory proteins-1 (MIP-1) alpha and MIP-1β under stimulation of inflammatory cytokines (Zhu, Valente, Lorenzo, Carnes, & Graves, 1994; Lisignoli et al., 2002). These chemokines play an important role in bone remodeling by recruiting osteoclast progenitors and stimulating osteoclastogenesis (Graves, Jiang, & Valente, 1999).

These studies suggested that PV or PVH might promote bone formation activities and/or inhibit bone resorption activities probably due to its antioxidant and anti-inflammation activities; however, the underlying mechanism remains unknown. Furthermore, it is unclear if PVH shows better activity than that of PV or not. The objectives of this study were to study the effects of PV and PVH on osteoblast differentiation as well as the possible mediating pathways. It was also interesting to investigate whether PV or PVH show any anti-inflammation activities in TNF-α induced osteoblast cells as inflammatory cytokines/chemokines play an important role in bone remodeling.

4.2. Materials and methods

4.2.1 Chemicals and reagents

Dulbecco's phosphate buffered saline (PBS), Minimum Essential Medium alpha (MEM-α) and fetal bovine serum (FBS) were obtained from Gibco/Invitrogen (Carlsbad, CA, USA). Triton-X-100 was purchased from VWR International (West Chester, PA, USA). Alamar blue was purchased from Fisher Scientific (Nepean, ON, Canada). Recombinant Mouse TNF-αlpha (AA 80-235) was purchased from R&D Systems, Inc. (Minneapolis, USA). Pancreatin (p7545-25G, from porcine pancreas) purchased from Sigma-Aldrich, Ltd. (Oakville, ON, Canada). All the other chemicals were purchased from Sigma-Aldrich, Ltd. (Oakville, ON, Canada) or Fisher Scientific (Nepean, ON, Canada) otherwise specified.

4.2.2 Cell culture

The MC3T3-E1 cells (subclone 4, ATCC CRL-2593) were purchased from ATCC (Manassas, VA, USA) and cultured in MEM-α supplemented with 10% heat-inactivated FBS and 1% of penicillin-streptomycin. The cells were incubated in an incubator at 37 ^{o}C under 95% air and 5% CO_2. MC3T3-E1 cells were grown in 24 well plates under the above described conditions until confluence, then 10 mM β-glycerophosphate and 50 μg/mL ascorbic acid were added to the medium to induce differentiation. The differentiated cells were treated with 100, 200, 500 μg/mL phosvitin (PV) or phosvitin phosphopeptides (PVH) for 72 h with or without 5 ng/mL TNF-α to induce inflammation, and then subjected to Western blot and immunofluorescence, or the supernatant was analyzed by enzyme-linked immunosorbent assay (ELISA) as described below. In another experiment, MC3T3-E1 cells were cultured to ~80% confluency and then serum-starved in MEM-α with 1% FBS for 18 h. The cells were incubated with PV or PVH (500 μg/mL) for 90 min or 10 min to detect activation of extracellular signal-regulated kinases (ERK) pathway and protein Kinase B (AKT) pathway by Western blot. Toxicity assay was carried out by using Alamar blue (Fisher Scientific, Nepean, ON, Canada) according to the manufacturer's instructions, and the results indicated that PV and PVH did not increase cell death at the concentrations used in these experiments (data not shown).

4.2.3 Preparation of PV and PVH

PV and PVH were prepared according to a protocol developed previously (Ren & Wu, 2015). PVH was obtained by enzymatic hydrolysis using pancreatin. Partial dephosphorylation was performed before digestion by dissolving phosvitin into 0.2 M NaOH solution at 5% (wt/wt) at room temperature (25 °C) with magnetic stirring for 0.5 h. Partially dephosphorylated PV (with 29.8% phosphate removed) was incubated with pancreatin (40 ^{o}C, pH 7.5), at a ratio of

1/50 (enzyme/phosvitin, wt/wt) for 3 h. Hydrolysates (PVH) were centrifuged and lyophilized for the following assay (Ren & Wu, 2015).

4.2.4 Western blot

The cell lysates for Western blot were prepared as previously described (Chakrabarti & Davidge, 2016). Briefly, the cells were incubated with PV and PVH at different concentrations for 72 h. After incubation, the culture medium was removed and the cells were lysed by boiling hot Laemmli's buffer containing 50 μM dithiothreitol (DTT, a reducing agent) and 0.2% Triton-X-100. These cell lysates were first loaded to 10% tris-glycine gel to obtain separated protein bands, then the protein bands were transferred to a nitrocellulose membrane and immunoblotted with specific antibodies against alkaline phosphatase (ALP; mouse monoclonal antibody from Santa Cruz Biotechnologies, Santa Cruz, CA, USA), Runt-related transcription factor 2 (RUNX2) (rabbit polyclonal antibody from R&D Systems), Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and cyclooxygenase-2 (COX-2) (rabbit polyclonal antibody from Abcam, Cambridge, MA, USA). Goat anti-rabbit and donkey anti-mouse fluorochrome-conjugated secondary antibodies were purchased from Licor Biosciences (Lincoln, NB, USA). The immunoblotted protein bands were detected by a Licor Odyssey BioImager and quantified by densitometry using corresponding software (Licor Biosciences). The results were expressed as percentage of the treated groups to the corresponding untreated controls.

4.2.5 Detection of cytokines/chemokines in MC3T3-E1 cells by ELISA

Rat osteocalcin (OCN) ELISA kit (60-1505) was purchased from Immutopics Inc. (Clemente, CA, USA). Rat regulated on activation, normal T cell expressed and secreted (RANTES) ELISA kit (MMR00) and monocyte chemoattractant protein 1 (MCP-1) ELISA kit

(KA1831) were purchased from R&D Systems, Inc. (Minneapolis, USA). The assay was performed according to the manufacturer's instructions.

4.2.6 Immunofluorescence

The immunofluorescence analysis was carried out as previously described (Majumder et al., 2013). Briefly, cells were fixed by 4% formalin, permeabilized by 0.1% Triton-X-100 in PBS and incubated overnight with a rabbit polyclonal antibody against type I collagen (Novus Biologicals, Littleton, CO, USA). The goat anti-rabbit secondary antibody conjugated with Alexa Fluor546 (red) (Molecular Probes, Eugene, OR, USA) was used to stain type I collagen and the Hoechst33342 nuclear dye (1:10000; Molecular Probes Eugene, OR, USA) was used to stain the cell nuclei. Unbound antibody/dye was washed off by PBS. The images of stained cells were obtained by an Olympus IX81 fluorescence microscope (Olympus, Tokyo, Japan) equipped with a Metamorph imaging software (Molecular Devices, Sunnyvale, CA, USA). Fluorescence intensity was calculated from the red fluorescent signal by using Adobe Photoshop Elements 2.0 software (Adobe Systems Inc., San Jose, CA, USA). Mean values of fluorescence were obtained from three random areas.

4.2.7 Statistical analysis

All data are presented as mean ± standard error of mean of 3 independent experiments. Western blot results of treated groups are expressed as percentage of the untreated groups, and were analyzed by one way analysis of variance (ANOVA) with Dunnett's post-hoc test to compare with untreated groups. The PRISM 6 statistical software (GraphPad Software, San Diego, CA) was used for the analysis and to produce the graph. A $p < 0.05$ was considered statistically significant.

4.3. Results and discussion

4.3.1 Effects of phosvitin and phosvitin hydrolysate on MC3T3-E1 differentiation

MC3T3-E1 is a pre-osteoblast cell line that can differentiate into osteoblasts in the presence of ascorbic acid (Franceschi et al., 1994). Ascorbic acid is an essential molecule that is involved in many physiological functions including collagen synthesis (Franceschi, 1992). PV was reported to mirror the role of ascorbic acid in developing chicken embryo when ascorbic acid could not be obtained or synthesized due to its antioxidant/reducing ability (Liu et al., 2013). However, there is no report on the effect of PV on osteoblast differentiation markers and associated signaling pathways. Besides, PVH was also reported to have various physiological functions, including antioxidant and anti-inflammatory ability (Katayama et al., 2006; Young et al., 2011), whether PVH retains PV's activity on MC3T3-E1 differentiation is unknown. Therefore, both PV and PVH were used to treat MC3T3-E1 cells during differentiation and the expressions of RUNX2, ALP, OCN and type 1 collagen synthesis were analyzed by using western blot, ELISA or immunofluorescence methods, respectively. As shown in Figure 4.1, PV (500 μg/mL) and PVH (100-500 μg/mL) significantly upregulated expression of RUNX2; PVH (500 μg/mL) increased ALP expression while PV (500 μg/mL) increased OCN released into the cell culture medium; both PV (500 μg/mL) and PVH (500 μg/mL) promoted type-1 collagen synthesis. RUNX2 is the master transcription factor that regulates osteoblast's differentiation. Deficiency of RUNX2 led to complete lack of bone formation because of the absence of mature osteoblasts (Komori et al., 1997). RUNX2 regulates a series of osteoblast specific gene expression including Col1a1, Col1a2, Spp1, Ibsp/BSP, Bglap2, Fn1/fibronectin, MMP13, and Tnfrsf11b/Opg (Komori, 2009). The upregulation of RUNX2 in this experiment could be related to the increased expression of ALP, OCN and type-1 collagen, which are all featured markers for

bone formation activities. PV was reported to upregulate mRNA of ALP (Liu et al., 2017) and enhance ALP activities in bone tissue culture (Liu et al., 2013), but this study failed to detect effects of PV on ALP expression in this experiment. OCN expression was always low in MC3T3-E1 cells (Zhang et al., 2014), and that might be one of the reasons that PVH showed no effects on OCN expression under current conditions.

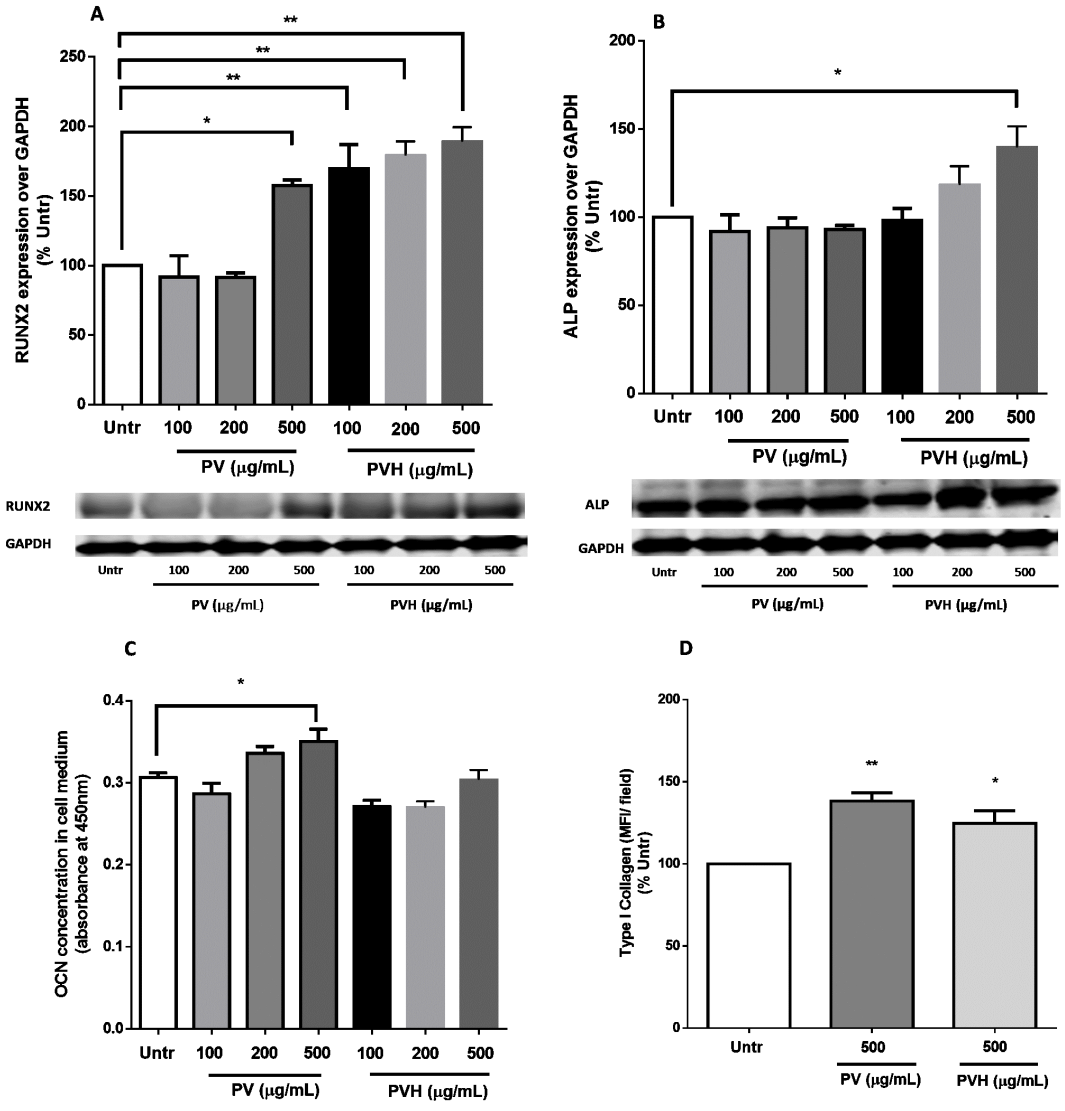

Figure 4.1. Effects of PV and PVH on MC3T3-E1 cells differentiation. MC3T3-E1 cells were incubated until confluency and then treated with PV or PVH for 72 h. The cells were lysed and analyzed for RUNX2 expression (A) and ALP expression (B) by Western blot, and set of

representative images are shown; the cell culture medium was collected and the secretion of OCN (C) was determined by ELISA; In another set of experiment following incubation with PV or PVH (500 μg/mL), cells were immune-stained for type I collagen. Fluorescence intensity was measured as the average of total fluorescent intensity, and expressed as percentage of the untreated control (D). *, ** and *** indicate $p<0.05$, $p<0.01$ and $p<0.001$ respectively, as compared to the group of untreated control. (Untr, untreated; PV, phosvitin; PVH, phosvitin hydrolysate; RUNX2, runt-related transcription factor 2; ALP, alkaline phosphatase; OCN, osteocalcin; GDAPH, glyceraldehyde 3-phosphate dehydrogenase)

4.3.2 Effects of phosvitin and phosvitin hydrolysate on TNF-α induced MC3T3-E1 cells.

Osteoblastic cells produce many chemokines such as RANTES, MCP-1, MIP-1, stromal-derived factor-1 (SDF-1) under stimulation of inflammatory factors such as IL-1α or TNF-α (Yu, Huang, Collin-Osdoby, & Osdoby, 2003). The released RANTES and MCP-1 stimulate bone resorption activities by promoting recruitment of osteoclast progenitors, RANKL induced osteoclastogenesis and migration of mature osteoclasts (Kim, Day, & Morrison, 2005; Graves et al., 1999). In this experiment, addition of 5 ng/mL TNF-α significantly increased RANTES and MCP-1 production by MC3T3-E1 cells (Figures 4.2-A and 4.2-B). However, adding PV at a concentration of 500 μg/mL significantly reduced the expression of RANTES/MCP-1 to a level of normal control; adding PVH could not affect RANTES production but significantly reduced MCP-1 production at lower concentrations (Figures 4.2-A and 4.2-B). Both osteoblasts and osteoclasts can produce RANTES under certain pathological conditions (Yu, Schneiderhan-Marra, Hsu, Bachmann, & Joos, 2009). Osteoblast is the major cell to produce MCP-1 in inflamed bones and MCP-1 plays an important role in the development of inflammation related bone disorders, including rheumatoid arthritis (Graves et al., 1999; Deshmane, Kremlev, Amini, & Sawaya, 2009). MCP-1 was inhibited by both PV and PVH, while that of RANTES was only inhibited by PV. The above results also suggested that PVH did not retain the same anti-

inflammation effect as that of PV on osteoblastic cells. It was also interesting to note that PVH did not show any effect at the high concentration of 500 μg/mL while PV showed activity only at this high concentration.

TNF-α was reported to increase COX-2 expression in MC3T3-E1 cells probably through nuclear factor B (NF-κB) and/or nuclear factor-interleukin-6 pathways (Yamamoto, Arakawa, Ueda, & Yamamoto, 1995). COX-2 is a widely studied pro-inflammatory protein. However, in osteoblast cells, inhibition of COX-2 might impair the healing process of bone fractures (Naik et al., 2009). COX-2 is an enzyme that catalyzes the production of prostaglandin E2 (PGE2), and PGE2 was reported to inhibit OPG secretion in osteoblasts and enhance RANK expression in osteoclasts (Liu, Kirschenbaum, Yao, & Levine, 2006). OPG is the decoy receptor for RANKL that can downregulate RANKL induced osteoclast differentiation (Khosla, 2001). Inhibition of OPG could be probably related to elevated bone resorption activities. However, the exact role of COX-2/PGE2 in bone metabolism is not clearly elucidated yet. It was also reported that the inhibition of COX-2 was associated with upregulation of osteoblast differentiation (Ryu, Qian, & Kim, 2010). In this experiment, TNF-α promoted expression of COX-2 in MC3T3-E1 cells, but the promoting effect was arrested by PV or PVH at all the concentrations (Figure 4.2-C). These results proved anti-inflammation activities of PV or PVH in TNF-α induced MC3T3-E1 cells. It was noted that inhibition of COX-2 by PV or PVH did not affect the expression of OPG and ALP (Figures 4.2-D and 4.2-E), which are the markers of osteoblast functions. Knock down of endogenous expression of RANTES could lead to reduction of ALP expression in osteoblasts (Liu et al., 2014). However, in this experiment, PV treatment greatly reduced RANTES secretion in TNF-α induced MC3T3-E1 cells but the expression of ALP was not affected (Figure 4.2-D). In fact, ALP/OPG production was regulated by many factors not solely by COX-2/PGE2 or

RANTES; therefore, it is possible that PV or PVH regulated osteoblast functions and inflammation responses through different pathways.

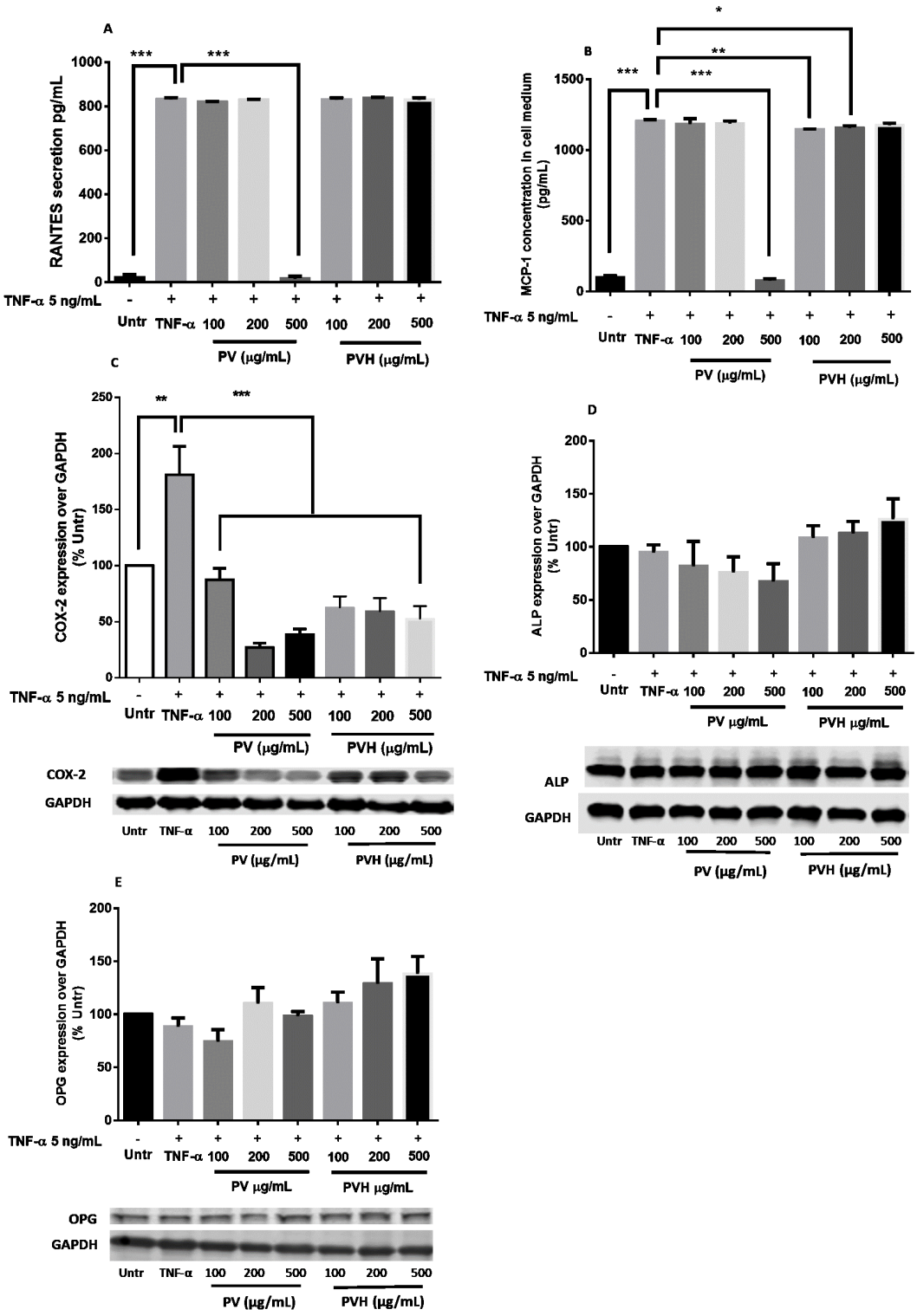

Figure 4.2. Effects of PV or PVH on TNF-α induced inflammation in MC3T3-E1 cells. MC3T3-E1 cells were incubated until confluency and then treated with PV or PVH for 72 h in the presence of 5 ng/mL TNF-α. The cell culture medium was collected and the concentrations of RANTES (A) and MCP-1 (B) were determined by ELISA; the cells were lysed and expression of COX-2 (C), ALP (D) and OPG (E) were analyzed by Western blot. A set of representative images is shown. *, ** and *** indicate $p<0.05$, $p<0.01$ and $p<0.001$ respectively, as compared to the group of TNF-α. (Untr, untreated; PV, phosvitin; PVH, phosvitin hydrolysate; RANTES, regulated upon activation normal T cell expressed and secreted; MCP-1, monocyte chemoattractant protein-1; COX-2, cyclo-oxygenase-2; ALP, alkaline phosphatase; OPG, osteoprotegerin; GDAPH, glyceraldehyde 3-phosphate dehydrogenase)

4.3.3 Phosvitin and phosvitin hydrolysate activated ERK and AKT pathways in MC3T3-E1 cells.

Both ERK and AKT pathways were activated by PV or PVH treatments as shown in Figure 4.3. This probably suggested the effect of PV or PVH on osteoblast differentiation and inflammation responses might be mediated through these pathways. ERK is one of the cascades from mitogen-activated protein kinases (MAPK) signaling pathway that responds to extracellular stimuli. The MAPK/ERK signaling generally regulates cell proliferation, differentiation, migration, senescence and apoptosis (Johnson & Lapadat, 2002). In osteoblasts, MAPK/ERK regulates phosphorylation and transcriptional function of RUNX2, and RUNX2 also mediates the response of MAPK/ERK pathway (Franceschi & Xiao, 2003). The phosphoinositide-3-kinase/protein kinase B (PI3K/AKT) pathway regulates cell proliferation and survival (Vivanco & Sawyers, 2002). It can be activated by many factors including growth factors like platelet-derived growth factor (PDGF), insulin or insulin-like growth factor (IGF)-1, and several cytokines (Cantley, 2002). The activation of PI3K/AKT is essential for osteoblast differentiation: inhibition of IGF-1 led to reduction in ALP activity while expression of constitutively active

AKT restored ALP activity and bone mineralization in MC3T3-E1 cells (Suzuki et al., 2014). It is also noteworthy that RUNX2 promoted osteoblast differentiation by coupling with PI3K/AKT pathway: arresting IGF-1 by its antibody completely inhibited RUNX2 induced osteoblast differentiation (Fujita et al., 2004). These results indicated that RUNX2 and PI3K/AKT pathways are mutually dependent on each other during osteoblast differentiation (Fujita et al., 2004). These results can also be used to explain the activation of ERK and AKT pathways, and elevated RUNX2 expression in this experiment.

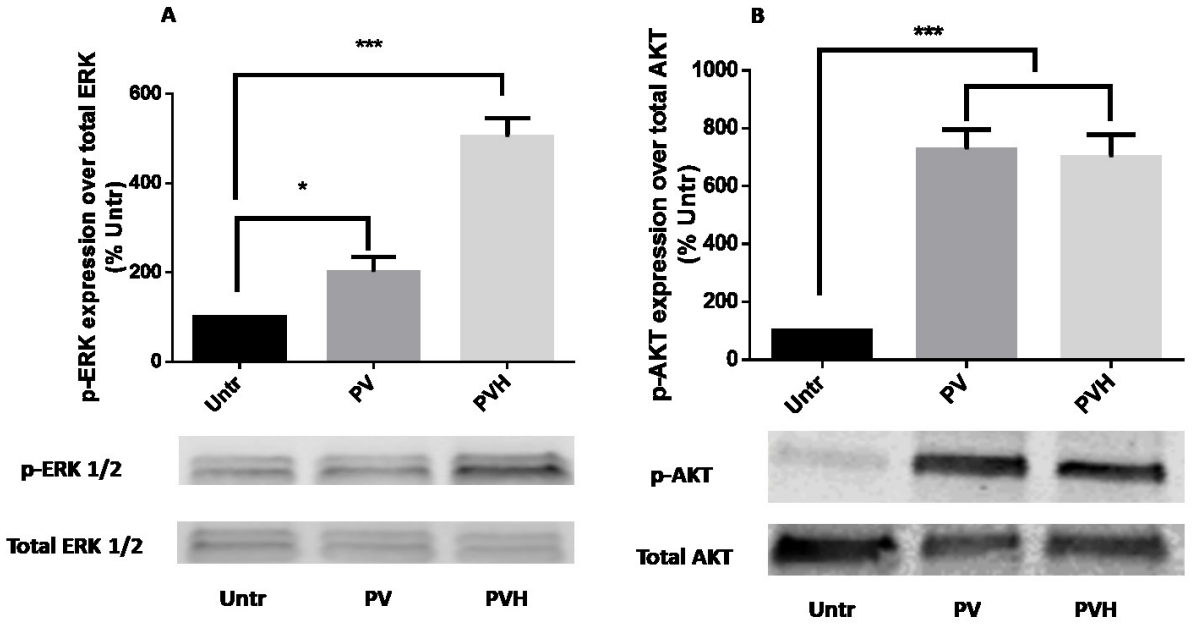

Figure 4.3. Activation of ERK/AKT pathways by PV or PVH treatments. MC3T3-E1 cells were cultured to ~80% confluency and then serum-starved in MEM-α with 1% FBS for 18 h. The cells were incubated with PV or PVH (500 μg/mL) for 90 min to detect ERK activation (A) or 10 min to detect AKT activation (B). A set of representative images is shown. *, ** and *** indicate $p<0.05$, $p<0.01$ and $p<0.001$ respectively, as compared to the group of untreated. (Untr, untreated; PV, phosvitin; PVH, phosvitin hydrolysate; ERK, extracellular signal-regulated kinases; AKT, protein Kinase B; GDAPH, glyceraldehyde 3-phosphate dehydrogenase)

4.4. Conclusions

Osteoporosis is a progressive bone disease caused by an imbalance between bone formation and bone resorption. Most current therapeutics target at inhibition of osteoclast-mediated bone resorption. However, osteoporosis is often coupled with diminished osteoblast activity and function; inhibition of excessive bone resorption is necessary but it is also of great importance to regain osteoblast's function and restore bone quality, possibly by regulating osteoblasts. Given the biological function of osteoblasts, recent research attention has focused on developing anabolic drugs. Strontium ranelate and teriparatide are two anabolic drugs targeting at G protein-coupled calcium-sensing and parathyroid hormone-1 receptors to promote bone formation activities (Diepenhorst et al., 2017).

PV and PVH have drawn great research interest due to their various physiological functions. PV was also reported to promote bone formation in tissue culture and PVH promoted bone mineral density *in vivo* (Liu et al., 2013; Choi, Jung, Choi, Kim, & Ha, 2005). Considering that oxidation and inflammation also play a role in bone loss under pathological conditions, PV and PVH are promising candidates with bone formation promoting ability.

The findings of this study indicated that PV or PVH promoted osteoblast differentiation by upregulating expression of RUNX2, ALP, OCN and collagen synthesis. PV or PVH also inhibited TNF-α induced inflammation by reducing the production of RANTES and MCP-1, which are critical for recruiting osteoclast progenitor and maintaining osteoclast functions. COX-2 is a proinflammatory protein and its expression in osteoblasts was inhibited by PV or PVH. The inhibition of COX-2 did not affect expression of OPG and ALP, which suggested that differentiation of osteoblast was probably not affected. PV or PVH might promote osteoblast differentiation and exert anti-inflammation effects by mediating ERK and AKT pathways.

CHAPTER 5 - Phosvitin and Its Hydrolysate Inhibit RAW264.7 Cell Differentiation and Inflammation

5.1. Introduction

Bone metabolism is a dynamic process in which old or damaged bone tissue is resorbed by osteoclasts and new bone tissue is formed by osteoblasts. This process is also known as bone remodeling which repairs the micro damages or fractures and maintains the integrity of skeletal system (Seeman, 2009). In the resorption phase of bone remodeling, macrophage-colony stimulating factor (M-CSF) and receptor activator of nuclear factor kappa-B ligand (RANKL) stimulate osteoclast progenitors to form mature osteoclasts and increase the resorption activity. Following resorption, osteoblast progenitors are then recruited on the site of the cavity to form mature osteoblasts and synthesize new bones (Raggatt & Partridge, 2010).

Osteoclasts play a critical role in the process of bone remodeling by resorbing the mineralized bone matrix (Teitelbaum & Ross, 2003). Lack of osteoclasts will result in osteopetrosis which is the bone marrow cavity being filled with un-resorbed bone tissues (Boyce, Yao, & Xing, 2009). Osteoclasts are a group of giant multinucleated cells that are derived from monocyte/macrophage lineage. A lot of factors could affect osteoclast differentiation/activities including calcitriol, parathyroid hormone (PTH) and some inflammatory cytokines (Mundy, 2007). RANKL is one of the most critical factors that regulates osteoclastogenesis. It can bind the receptor activator of nuclear factor kappa-B (RANK, receptor of RANKL) to stimulate osteoclasts differentiation and maintain their resorption activities by triggering a series of signaling pathways (Boyle, Simonet, & Lacey, 2003). Once bound by RANKL, the cytoplasmic

domain of RANK will recruit tumor necrosis factor (TNF) receptor-associated factor 6 (TRAF6), which will lead to activation of osteoclast-related signaling pathways such as c-Jun N-terminal kinase (JNK), p50/65 nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and p38 mitogen-activated protein kinases (MAPK) (Boyle et al., 2003). Osteoprotegerin (OPG) is the soluble decoy receptor that competitively binds RANKL over RANK and thus negatively regulates osteoclastogenesis. This RANKL/RANK/OPG interaction dominantly regulates osteoclast differentiation (Khosla, 2001).

It was also well known that many age-related diseases e.g. osteoporosis are associated with inflammation (Luc et al., 2003); inflammation results in bone loss under pathological conditions such as periodontal disease, multiple myeloma and rheumatoid arthritis (Benedetto et al., 2013). In postmenopausal women, estrogen deficiency results in excessive expression of RANKL in osteoblasts, and RANKL will trigger osteoclasts to produce inflammatory cytokines. Some of these cytokines e.g. interleukin-1 (IL-1), interleukin-6 (IL-6) and tumor necrosis factor-alpha (TNF-α) could promote osteoclasts differentiation/activities and thus elevate bone resorption (Steeve, Marc, Sandrine, Dominique, & Yannick, 2004); other chemokines e.g. monocyte chemoattractant protein 1 (MCP-1) and regulated upon activation normal T cell expressed and secreted (RANTES), could stimulate osteoclast cell to accelerate its differentiation and resorption activities in an autocrine loop (Kim et al., 2005).

Phosvitin (PV) is the major phosphorylated protein in egg yolk in which ~50% amino acids are phosphorylated (Byrne et al., 1984). Phosvitin and phosvitin hydrolysate (PVH) have raised considerable research attention due to antioxidant, anti-inflammation and calcium absorption promoting activities (Young, Nau, Pasco, & Mine, 2011; Xu, Yang, Yin, Liu, & Mine, 2012; Hu, Sun, Wang, Su, & Zhang, 2013; Choi et al., 2005). In a study of *ex vivo*

embryo's development, phosvitin was proven to upregulate bone formation activities (elevated collagen synthesis, mineralization, and bone formation markers) and inhibit bone resorption biomarkers (tartrate-resistant acid phosphatase activity) (Liu et al., 2013). Another study reported similar results that phosvitin promotes mineralization of osteoblasts with consistent changes in corresponding regulatory genes (Liu, Li, Geng, Huang, & Ma, 2017). However, there is scanty knowledge in the literature on the effect of PV or PVH on osteoclast and bone resorption activities. Therefore, the objectives of this study were 1) to investigate the effects of PV or PVH on osteoclast differentiation as well as their possible pathways, and 2) to investigate the effect of PV or PVH on inflammation in osteoclasts.

5.2. Materials and methods

5.2.1. Chemicals and reagents

Dulbecco's Modified Eagle's medium (DMEM), penicillin-streptomycin (10,000 U/mL) and fetal bovine serum (FBS) were obtained from Gibco/Invitrogen (Carlsbad, CA, USA). Triton-X-100 was purchased from VWR International (West Chester, PA, USA). Alamar blue was purchased from Fisher Scientific (Nepean, ON, Canada). Recombinant mouse RANKL (462-TEC-010) was purchased from R&D Systems, Inc. (Minneapolis, USA). Pancreatin (p7545-25G, from porcine pancreas) was purchased from Sigma-Aldrich, Ltd. (Oakville, ON, Canada) to prepare phosvitin hydrolysate. All the other chemicals were purchased from Sigma-Aldrich, Ltd. (Oakville, ON, Canada) or Fisher Scientific (Nepean, ON, Canada) otherwise specified. Phosvitin and phosvitin hydrolysate were prepared according to a protocol previously described (Ren & Wu, 2015).

5.2.2. Cell culture

The RAW 264.7 cells (ATCC TIB-71) were purchased from ATCC (Manassas, VA, USA) and cultured in DMEM supplemented with 10% heat-inactivated FBS and 1% of penicillin-streptomycin. The cells were incubated in an incubator at 37 ^{o}C under 95% air and 5% CO_2. RAW 264.7 cells were grown in 24 well plates under the above described conditions with 50 ng/mL RANKL to induce osteoclastogenesis. The differentiated cells were treated with 50, 100, 500 μg/mL of PV or PVH for different time periods as indicated in the fellowing sections, and then subjected to Western blot, and the supernatant was analyzed by enzyme-linked immunosorbent assay (ELISA) as described below. Toxicity assay was carried out by using Alamar blue (Fisher Scientific, Nepean, ON, Canada) according to the manufacturer's instructions, and the results indicated that PV and PVH did not increase cell death at the concentrations used in these experiments (data not shown).

5.2.3. Determination of tartrate-resistant acid phosphatase (TRAP) activity and staining of TRAP positive cells

RAW264.7 cells were seeded at a concentration of 2×10^4 cells/well into a 48-well culture plate in the presence of 50 ng/mL RANKL. After 24 h incubation, 50, 100, 500 μg/mL of PV or PVH were then added to the plate. The medium and PV or PVH were changed after 3 days. After 5 days of incubation, the medium was removed and the cells were washed with PBS and then lysed with 300 mL of 0.2% Triton X-100. TRAP activity in cell lysate was determined by using Sigma's TRAP kit (CS0740). Briefly, an aliquot of 25 μL cell lysate (50 times diluted) was added to 50 μL of substrate solution and then incubated at 37^{o}C for 30 min. The reaction was arrested by adding 200 μL of 0.1 N NaOH. Absorbance at 405 nm was measured by using a Molecular Devices SpectraMax M3 microplate reader (Molecular Devices LLC, San Jose, CA,

USA). In another experiment, cells were not lysed but stained with Sigma's TRAP staining kit (387A-1KT) following the instruction. Images were obtained from a Zeiss Primovert microscope equipped with an Axiocam 105 color camera and Zen 2 software.

5.2.4. Preparation of cell lysate for RANKL induced signaling pathways study

RAW264.7 cells were seeded at a concentration of 2×10^4 cells/well into a 48-well culture plate. To study the effects of PV or PVH on NF-κB pathway, cells were treated with PV or PVH (500 μg/mL) for 48 h, followed by treatment with RANKL (50 ng/mL) for 30 min. To study the effects of PV or PVH on mitogen-activated protein kinase (MAPK) activation, cells were treated with PV or PVH (500 μg/mL) for 48 h, followed by treatment with RANKL (50 ng/mL) for 45 min. To determine the changes in nuclear factor of activated T-cells, cytoplasmic 1 (NFATc1) and c-Fos, cells were treated with RANKL (50 ng/mL) for 24 h and then PV or PVH (500 μg/mL) was added for another three days (Rahman, Bhattacharya, & Fernandes, 2006). Cell lysate was collected and analyzed by Western blot as described in section 5.2.7.

5.2.5. Inflammatory cytokines/chemokines production in RANKL stimulated RAW264.7 cells by ELISA

RAW264.7 cells were seeded at a concentration of 1×10^5 cells/well into a 24-well culture plate and incubated for 24 h. Then 50, 100, 500 μg/mL of PV or PVH were added to the plate to incubate for another 24 h. RANKL (50 ng/mL) was added to the medium to stimulate production of cytokines/chemokines. After 3 days, the medium was collected and the concentrations of tumor necrosis factor alpha (TNF-α), regulated on activation, normal T expressed and secreted (RANTES) chemokine and monocyte chemoattractant protein-1 (MCP-1) were analyzed by their respective kits, TNF-α ELISA kit (NBP1-92681), RANTES ELISA kit (MMR00) and MCP-1 ELISA kit (KA1831), purchased from R&D Systems, Inc. (Minneapolis, USA).

5.2.6. Western blot

The cell lysate from section 5.2.5 was subjected to Western blot according to a previously published method (Chakrabarti & Davidge, 2016). Briefly, the medium was removed and the cells were lysed by boiling hot Laemmli's buffer containing 50 µM dithiothreitol (DTT, a reducing agent) and 0.2% Triton-X-100. These cell lysates were first loaded to a 10% tris-glycine gel to separate proteins based on molecular weights, then the protein bands were transferred to a nitrocellulose membrane and immunoblotted with specific antibodies: JNK (mouse monoclonal antibody from R&D Systems Inc., cat# mab2076; Minneapolis, MN, USA), p-JNK (rabbit monoclonal antibody from R&D Systems Inc., cat# mab1205; Minneapolis, MN), phospho-p65 (rabbit polyclonal antibody from Santa Cruz Biotechnology, cat# sc-3033; Santa Cruz, CA, USA), p65 (mouse monoclonal antibody from Santa Cruz Biotechnology, cat# sc-8008; Santa Cruz, CA, USA),NFATc1 (Rabbit antibody from Santa Cruz Biotechnology, cat# mab8032; Santa Cruz, CA, USA), inducible nitric oxide synthase (iNOS) (rabbit polyclonal anti-iNOS from Abcam Inc., cat# ab-3523; Cambridge, MA, USA), and the loading control α-tubulin (rabbit polyclonal antibody from Abcam, cat# ab15246; Cambridge, MA, USA), Phospho-p38 (p-p38) (Rabbit antibody from NOVUS Biologicals, cat# NB500-138; Littleton, CO, USA), c-Fos (Rabbit antibody from NOVUS Biologicals, cat# NB110-; Littleton, CO, USA). Goat anti-rabbit and Donkey anti-mouse fluorochrome-conjugated secondary antibodies were purchased from Licor Biosciences (Lincoln, NB, USA). The immunoblotted protein bands were detected by a Licor Odyssey BioImager and quantified by densitometry using image studio lite 5.2 software (Licor Biosciences). The results were expressed as percentage of the treated groups to the corresponding untreated controls.

5.2.7. Statistical analysis

All data are presented as mean ± standard error of mean of three independent experiments. Western blot results of treated groups are expressed as percentage of the untreated groups and were analyzed by one way analysis of variance (ANOVA) with Dunnett's post-hoc test to compare with untreated groups. The PRISM 6 statistical software (GraphPad Software, San Diego, CA) was used for the analysis and to produce graph. A $p < 0.05$ was considered statistically significant.

5.3. Results and discussion

5.3.1. Effects of phosvitin/phosvitin hydrolysate on RANKL-stimulated RAW264.7 cells differentiation

RANKL is widely used to differentiate RAW264.7 cells into tartrate-resistant acid phosphatase (TRAP)-positive osteoclast-like cells (Shevde, Bendixen, Dienger, & Pike, 2000). TRAP is a marker for osteoclast function (Minkin, 1982). In this experiment, 50 ng/mL RANKL was used to stimulate RAW264.7 cells for up to 5 days as described under Materials and Methods. The TRAP activities were analyzed as shown in Figures 5.1-A and 5.1-1B. Representative images of RAW264.7 cells under each treatment were shown in Figure 5.1-C.

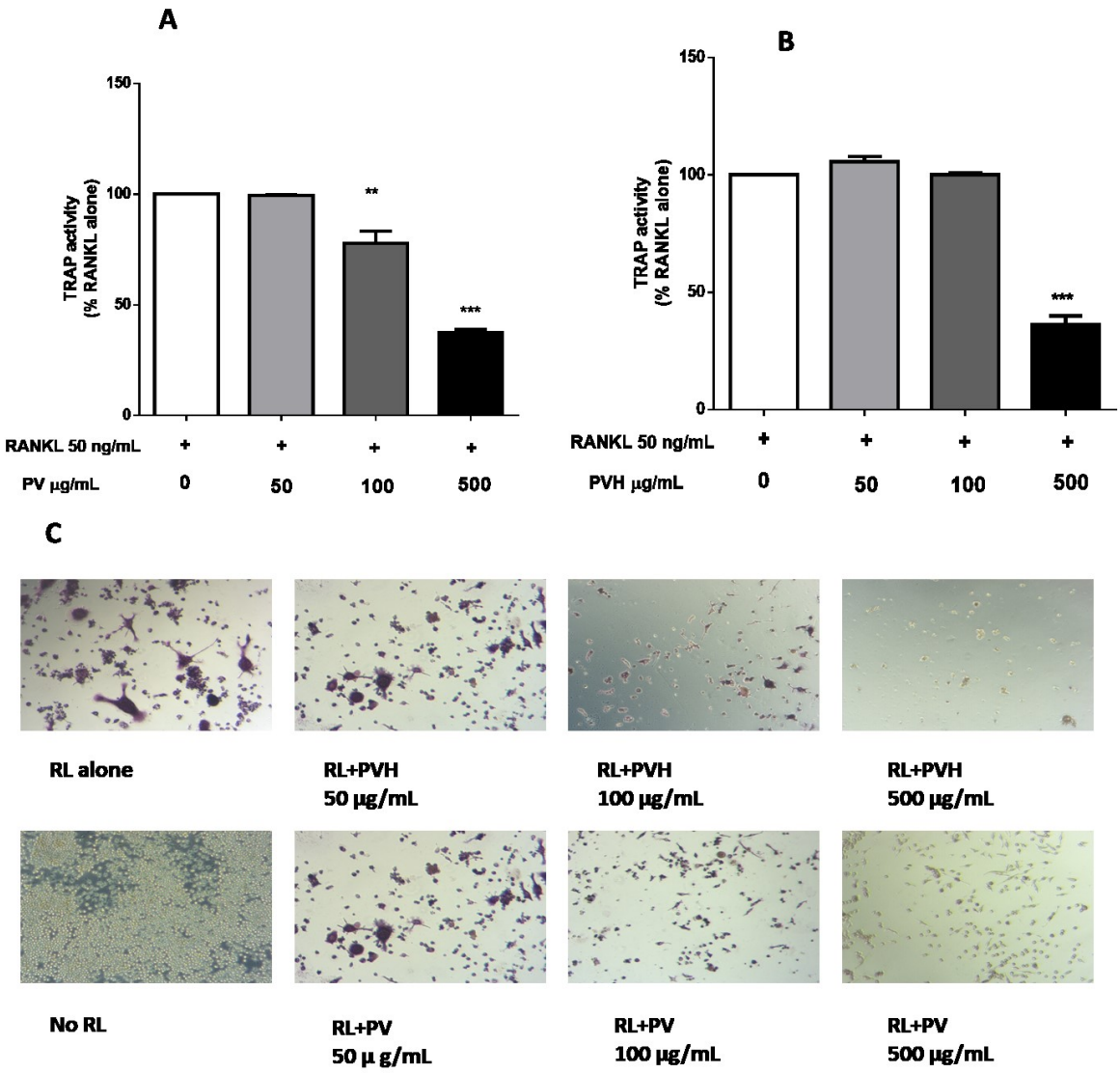

Figure 5.1. Effects of PV or PVH on TRAP-positive cells formation in RANKL-stimulated RAW264.7 cells. RAW264.7 cells were incubated with 50 ng/mL RANKL for 24 h followed by PV or PVH for another 5 days, then the cells were lysed by 0.2% Triton X-100. (A) TRAP activity of PV treated RAW264.7 cells; (B) TRAP activity of PVH treated RAW264.7 cells, (C) In another set of experiment, following incubation, cells were stained with Sigma's TRAP staining kit (387A-1KT). A set of representative images are shown. *, ** and *** indicate $p<0.05$, $p<0.01$ and $p<0.001$ respectively, as compared to the group of RANKL alone. (RL, RANKL; PV, phosvitin; PVH, phosvitin hydrolysate)

The TRAP activity was not affected by PV or PVH at concentrations of 50 and 100 μg/mL but was significantly suppressed at a concentration of 500 μg/mL. Accordingly, large, multi nucleated osteoclast cells (stained with purple color) were formed when RAW264.7 cells were treated with RANKL alone; adding PV or PVH at low concentrations (50 μg/mL) did not affect the differentiation, while it was significantly reduced at the concentrations of 100 and 500 μg/mL. The presence of mononuclear RAW264.7 cells stained as TRAP positive indicated that these cells were activated but not differentiated.

Binding of RANKL to RANK will activate several signalling pathways and expression of transcription factors including nuclear factor of activated T-cells, cytoplasmic 1 (NFATc1) and c-Fos, two important transcription factors for osteoclast differentiation. NFATc1 is the master transcription regulator of osteoclast differentiation (Takayanagi et al., 2002), regulating osteoclast specific genes such as TRAP, cathepsin K and calcitonin receptor (Takayanagi et al., 2002; Kim, Day, & Morrison, 2005). The mRNA expression of NFATc1 is selectively regulated by RANKL (Asagiri et al., 2005). Monocytes and macrophages are the precursors of osteoclasts. The role of c-Fos is to commit these precursors into osteoclasts instead of mature macrophages through the formation of activation protein 1 (AP1; Teitelbaum, 2000). Besides, c-Fos is also involved in the autoamplification of NFATc1 as NFATc1 expression was completely inhibited in c-Fos deficient cells (Matsuo et al., 2004). In this experiment, the presence of RANKL stimulated osteoclast differentiation and therefore upregulated the expression of NFATc1 and c-Fos; however, the treatment with PV or PVH significantly downregulated the expression of these two transcription factors to the level of untreated cells (Figure 5.2). These results were in good agreement with the observation in TRAP activity assay, and further proved the inhibitory effects of PV or PVH on osteoclast differentiation.

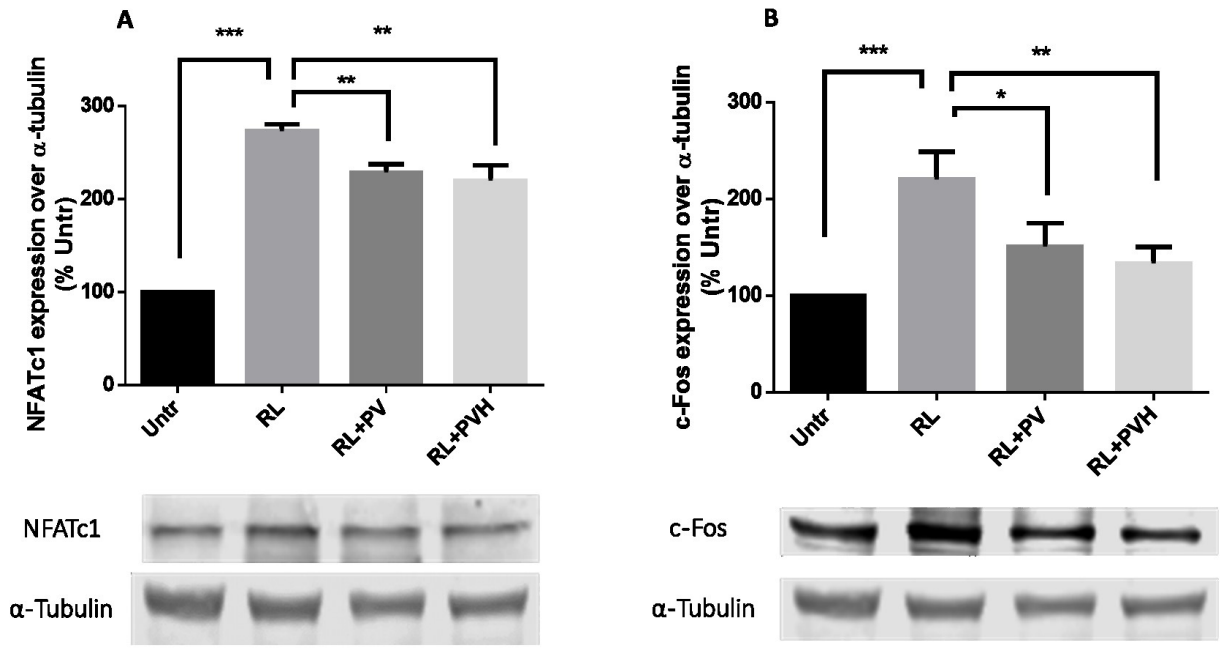

Figure 5.2. Effects of PV or PVH on osteoclastogenesis transcription factors in RANKL-stimulated RAW264.7 cells. RAW264.7 cells were cultured with RANKL (50 ng/mL) for 24 h. Then PV (500 µg) or PVH (500 µg) was added and cultured for another 72 h. Whole cell lysates were analyzed by Western blot using antibodies against NFATc1 (A) and c-Fos (B). A set of representative images was shown. *, ** and *** indicate $p<0.05$, $p<0.01$ and $p<0.001$ respectively, as compared to the group of RANKL alone. (Untr, untreated group without RANKL or PV or PVH; RL, RANKL; PV, phosvitin; PVH, phosvitin hydrolysate)

5.3.2. Effects of PV or PVH on inflammation products expression in RANKL stimulated RAW264.7 cells

RAW264.7 is a macrophage-like cell line and has been widely used in inflammation studies. In this experiment, addition of RANKL at 50 ng/mL not only induced osteoclastogenesis but also elevated inflammatory response. The production of inducible nitric oxide synthase (iNOS), tumor necrosis factor alpha (TNF-α), regulated on activation, normal T expressed and secreted (RANTES) and monocyte chemoattractant protein-1 (MCP-1) were all elevated by RANKL treatment. Adding 500 µg/mL PV or PVH however markedly reduced the production of

these inflammatory products, which proved the anti-inflammation activity of PV or PVH during osteoclast differentiation. One major function of iNOS is to catalyze the production of NO in response to stimuli (Surh et al., 2001), and constitutive production of NO within osteoclasts was proven to be essential for normal osteoclast function (Brandi et al., 1995). Thus downregulation of iNOS might result in reduced osteoclast activities (Ralston et al., 1995). Many inflammatory cytokines can cause bone loss in rheumatoid arthritis (Tracey, Klareskog, Sasso, Salfeld, & Tak, 2008). It was reported that excessive TNF-α formation might have negative effects on bone remodelling by stimulating osteoblast to express RANKL and directly working on osteoclasts to accelerate bone resorption (Kim et al., 2005). RANTES and MCP-1 were produced when osteoclast precursor differentiated into mature osteoclasts; the released RANTES and MCP-1 also stimulated osteoclast differentiation and formed an autocrine loop (Kim et al., 2005). PV or PVH have been reported to exert anti-inflammatory activities with other cell lines (Young et al., 2011; Xu et al., 2012; Hu et al., 2013). The results of this study suggested PV or PVH also possessed anti-inflammatory ability in RANKL stimulated osteoclast differentiation, which might have potential implications in the studies on inflammation related bone diseases.

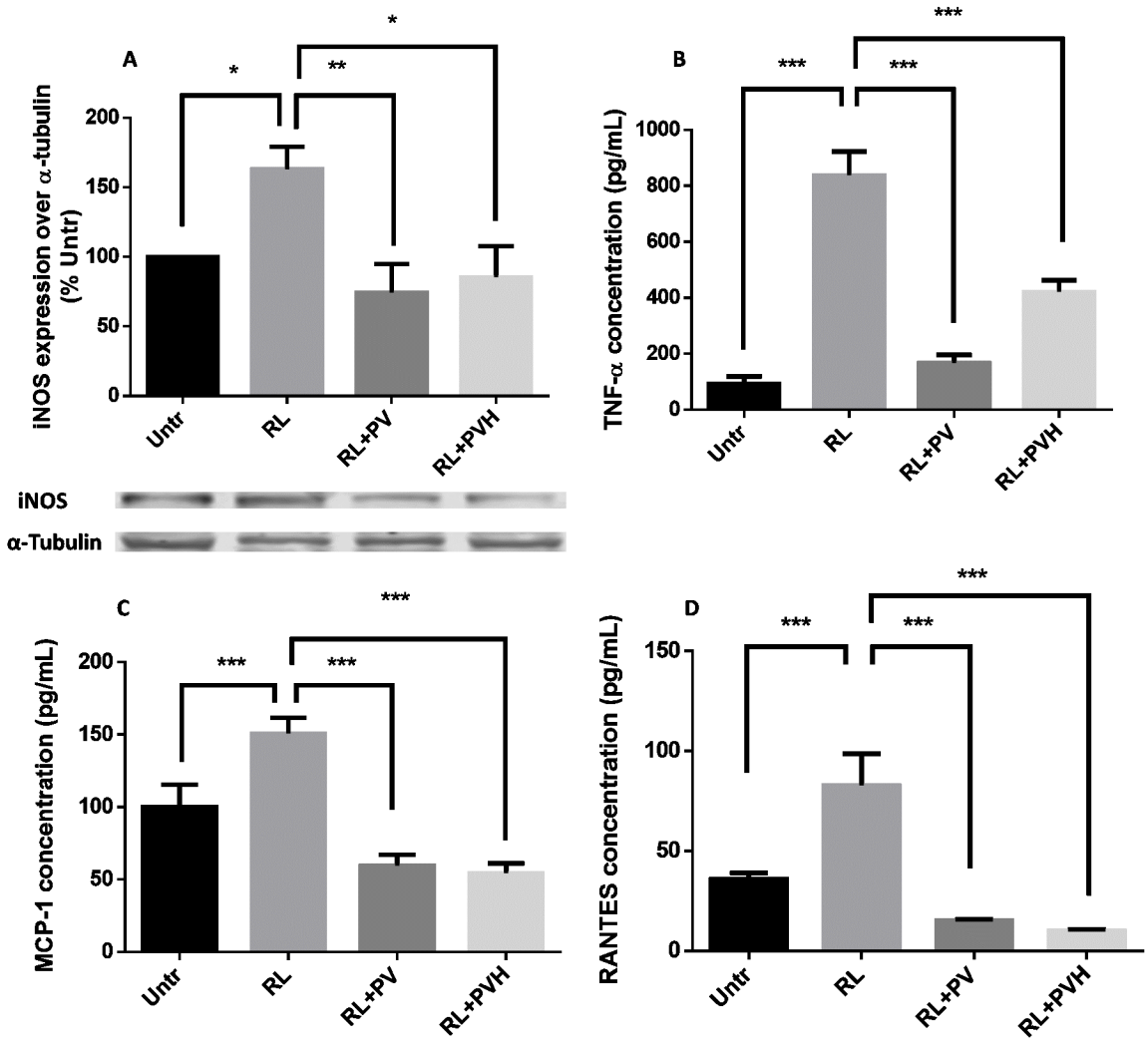

Figure 5.3. Effects of PV or PVH on inflammation protein expression/secretion in RANKL-stimulated RAW264.7 cells. RAW264.7 cells were cultured for 24 h. Then, cells were incubated with either PV (500 µg) or PVH (500 µg) for 24 h. RANKL (50 ng/mL) was then added to stimulate inflammatory cytokines/chemokines secretion for 16 h. At the end of culture, medium was collected and analyzed by using corresponding ELISA kits or the cell lysates were analyzed by Western blot. A: Whole cell lysates were analyzed by Western blot using antibodies against iNOS. A set of representative images was shown. B: TNF-α secretion in cell medium. C: MCP-1 secretion in cell medium. D: RANTES secretion in cell medium. *, ** and *** indicate $p<0.05$, $p<0.01$ and $p<0.001$ respectively, as compared to the group of RANKL alone. (Untr,

untreated group without RANKL or PV or PVH; RL, RANKL; PV, phosvitin; PVH, phosvitin hydrolysate)

5.3.3. Possible signalling pathways via which PV or PVH affect RAW264.7 cells

The above results indicated that PV or PVH probably involved in the signalling pathways that were activated during osteoclast differentiation by RANKL. The binding of RANKL to RANK triggered cytoplasmic domain of RANK to recruit tumor necrosis factor (TNF) receptor-associated factor 6 (TRAF6); many osteoclast-related signaling pathways, including p50/65 nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and mitogen-activated protein kinases (MAPKs) (p-38 and c-Jun N-terminal kinase (JNK)), were activated afterwards, which are the most important and widely studied pathways for osteoclast differentiation (Boyle et al., 2003).

The MAPK p38 and JNK pathways are believed to play an important role in osteoclast differentiation as the JNK inhibitor SP600125 and the p38 MAPK inhibitor SB203580 markedly inhibited the differentiation (Ikeda et al., 2004; Takayanagi et al., 2002). It was reported that the p38 pathway was essential for RANKL-induced osteoclast differentiation, but not for RANKL-induced osteoclast function (Li et al., 2002). JNK are a group of serine/threonine kinases generally important for cell growth, differentiation, and apoptosis (Zhang & Liu, 2002). In osteoclasts, RANKL elevated expression of c-Jun (Wagner, 2002), which forms activator protein 1 (AP-1) with c-Fos and thus regulates NFATc1 expression (Asagiri et al., 2005). In this experiment, RANKL treatment elevated phosphor-p38 and phosphor-JNK expression in RAW264.7 cells. Pre-treatments with PV or PVH for 48 h significantly reduced phosphor-p38 and phosphor-JNK expression (Figures 5.4-A and 5.4-B). These results indicated PV or PVH could inhibit RANKL-induced p38 and JNK activation.

NF-κB is a family of several transcription factors that recognize a common gene sequence called κB. There are five NF-κB proteins: Rel (cRel), RelA (p65), RelB, NF-kappaB1 (p50) and NF-kappaB2 (p52). In the classical pathway of NF-κB, RANKL will induce the formation of active subunit of p65/p50 and translocation to nucleus to change gene transcription (Hayden & Ghosh, 2004). The transcription factor of NFATc1 was regulated by NF-κB pathway since it was observed that NF-κB inhibitor could reduce NFATc1 expression in RANKL stimulated osteoclasts (Takatsuna et al., 2005). NF-κB is also a classical pathway that regulates cellular response to a stimulus (Gilmore, 2006). NF-κB pathway mediated iNOS expression has been widely reported (Surh et al., 2001). RANKL induced inflammatory responses in this experiment could be explained as RANKL activated NF-κB pathway. RANKL elevated NF-κB phosphor-p65 expression but adding PV or PVH significantly reduced phosphor-p65 expression especially for PVH ($p<0.001$) (Figure 5.4-C). This suggested that the NF-κB pathway was suppressed by PV or PVH treatment, and this might contribute to the reduction in NFATc1 as well as the inflammatory markers determined in Figure 5.3.

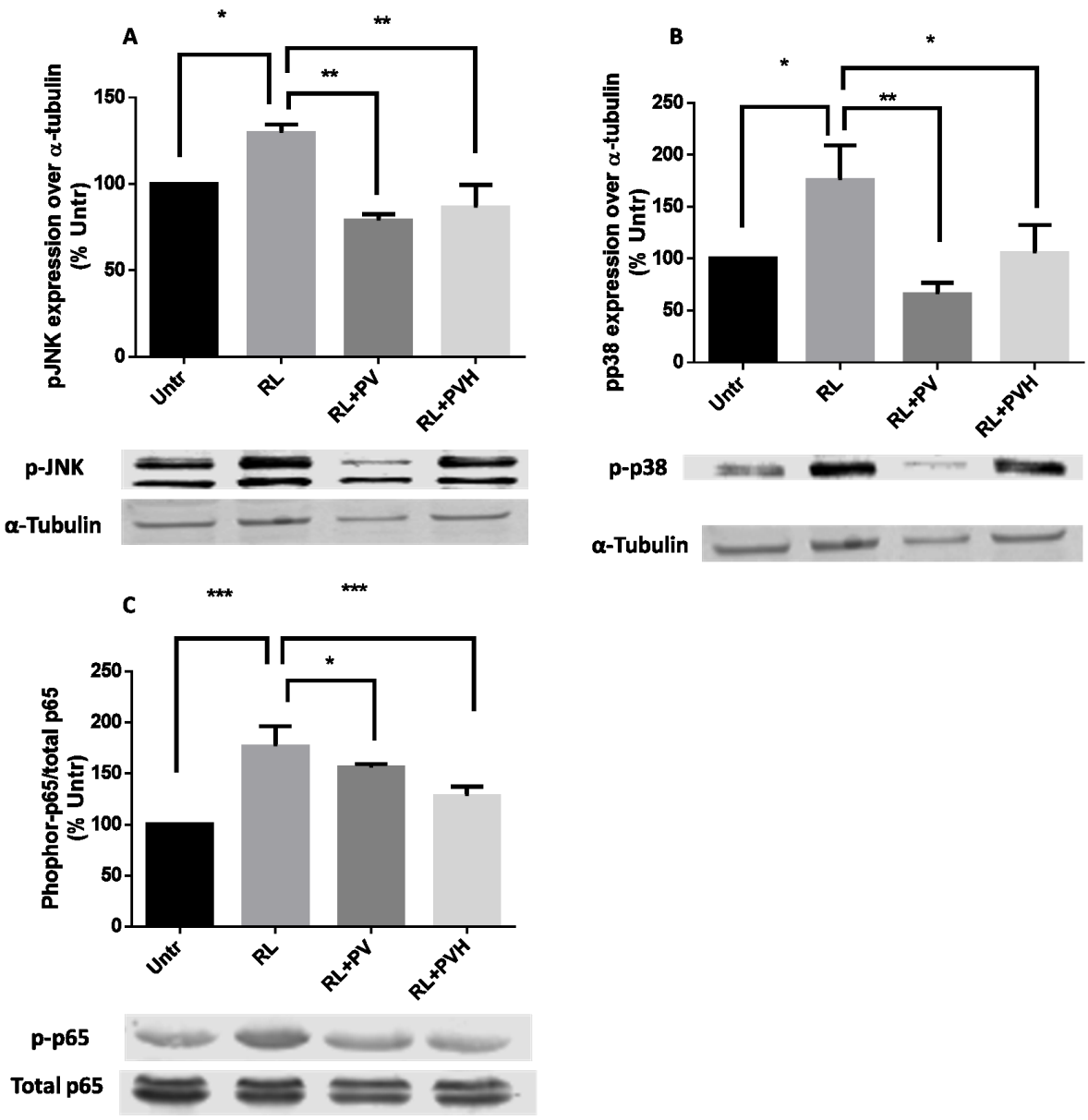

Figure 5.4. Effects of PV or PVH on different signaling pathways in RANKL-stimulated RAW264.7 cells. RAW264.7 cells were incubated with either PV (500 μg) or PVH (500 μg) for 48 h, followed by treatment with RANKL (50 ng/ml) for 30 min for JNK/p38, or 45 min for p65 expression. Whole cell lysates were analyzed by Western blot using antibodies against JNK and phosphor-JNK (A), p38 and phosphor-p38 (B), and p65 and phosphor-p65(C). A set of representative images was shown. *, ** and *** indicate $p<0.05$, $p<0.01$ and $p<0.001$ respectively as compared to the group of RANKL alone. (Untr, untreated group without RANKL or PV or PVH; RL, RANKL; PV, phosvitin; PVH, phosvitin hydrolysate)

5.4. Conclusions

Phosvitin is a highly phosphorylated protein in egg yolk and it was believed to serve as a reservoir of calcium and phosphate during embryo development (Moran, 2007). Phosvitin has become a popular research topic due to its various physiological functions including anti-inflammation (Young et al., 2011; Xu et al., 2012; Hu et al., 2013), antimicrobial (Ding, Liu, Bu, Li, & Zhang, 2012), and bone formation promoting abilities (Liu et al., 2013). These findings suggested that phosvitin's role in egg embryo development was not only as a reservoir but also regulated bone formation and immune functions. Phosvitin has been reported to stimulate bone formation activities by upregulating collagen synthesis, mineralization, and bone formation markers (Liu et al., 2013), but whether phosvitin plays a role in osteoclast differentiation/functions remains unclear.

In this study, PV or PVH reduced TRAP activity and expression of transcription factors of NFATc1/c-Fos in RANKL induced RAW264.7 cells. These results indicated that PV or PVH could inhibit RANKL induced osteoclastogenesis. PV or PVH treatments also supressed the secretion of inflammatory cytokines/chemokine (TNF-α, MCP-1, RANTES) and expression of iNOS. The MAPK and NF-κB pathways were essential for osteoclastogenesis. In this study, the activation of MAPK JNK/p38 and NF-κB p65 was suppressed by PV or PVH treatments. The inhibitory effects of PV or PVH on RANKL induced osteoclastogenesis and inflammatory response were probably mediated through MAPK JNK/p38 or NF-κB p65 pathways, or both.

CHAPTER 6 - Egg Yolk Phosvitin Hydrolysate Prevents Bone Loss in OVX Rats

6.1. Introduction

Osteoporosis is a progressive bone disease characterized by loss of bone mass/density and degradation of bone micro structure (Raisz, 2005). It afflicts about 25% of the population of 50 years old and above worldwide (Berger et al., 2010). The prevalence will increase as the world population is aging rapidly. Osteoporosis increases the risk of fracture due to reduced bone mass and declined bone microarchitectures (Raisz, 2005). Bone metabolism is a dynamic process in which old or damaged bone tissue is resorbed by osteoclast cells and new bone tissue is formed by osteoblast cells (Raggatt & Partridge, 2010). Various therapeutics have been developed to treat osteoporosis, most of which are anti-resorptive agents aimed to inhibit activities of osteoclasts and thus slow down bone loss (Weinstein, Roberson, & Manolagas, 2009). Although effective, these treatments are associated with serious side-effects including nausea, abdominal pain and loose bowel movements, hot flashes, depression, headaches etc. (Wysowski & Chang, 2005). Hormone replacement therapy (estrogen and parathyroid hormone) may increase the risk of breast cancer and cardiovascular disease (Women's Health Initiative, 2002). Supplementation of calcium along with vitamin D has been widely used for prevention of osteoporosis (Jackson et al., 2006); however, the efficacy is still controversial (Grant & Holick, 2005; Zhao, Zeng, Wang, & Liu, 2017).

There is an increasing interest in food bioactives as safer alternatives for the prevention and management of osteoporosis. Isoflavones are known as dietary estrogen and have been

demonstrated to prevent postmenopausal osteoporosis by modulating estrogen receptors (Potter et al., 1998; Lecomte, Demay, Ferrière, & Pakdel, 2017). Oxidative stress and chronic inflammation are involved in development of osteoporosis by directly or indirectly regulating the balance between osteoclasts and osteoblasts (Manolagas & Parfitt, 2010). Functional foods rich in antioxidants, like green tea, grapefruit pulp, citrus extract, and blueberry might be effective to prevent osteoporotic bone loss (Shen, Yeh, Cao, & Wang, 2009; Mandadi et al., 2009; Devareddy et al., 2008). Lactoferrin was reported to stimulate osteogenesis and inhibit osteoclastogenesis *in vitro* and *in vivo* (Cornish 2004; Naot, Grey, Reid, & Cornish, 2005). Casein phosphopeptide (CPP) was considered to promote bone health due to enhanced intestinal calcium absorption (Kitts & Yuan, 1992). However, recent studies revealed CPP might also modulate osteoclasts or osteoblast differentiation (Matsui, Yano, Awano, Harumoto, & Saito, 1994; Tsuchita, Goto, Yonehara, & Kuwata, 1995; Donida et al., 2009).

Phosvitin (PV) is the major phosphoprotein from egg yolk with over 50% of the amino acids being phosphorylated (Byrne et al., 1984). Phosvitin and the phosphopeptides derived from its hydrolysate (PVH) were reported to exert a variety of bioactivities, including antioxidant activity (Katayama, Xu, Fan, & Mine, 2006; Xu, Katayama, & Mine, 2007), calcium solubilizing ability (Jiang & Mine, 2000), anti-inflammatory activity (Young, Nau, Pasco, & Mine, 2011) and calcium absorption-promoting ability (Choi, Jung, Choi, Kim, & Ha, 2005). PV was reported to promote collagen synthesis and mineralization in a live calvarial bone organ culture model (Liu et al., 2013). The cell culture experiments in Chapters 4 and 5 also suggested that PV or PVH could promote osteoblastic cells differentiation and collagen synthesis, and inhibit microphages differentiation into osteoclasts by mediating MAPK or NF-κB signalling. PV and PVH exhibited strong anti-inflammatory activities in cell cultures (Young et al., 2011; Xu, Yang, Yin, Liu, &

Mine, 2012). Whether PV or PVH could prevent bone loss and reduce inflammation *in vivo* remains unknown. The objectives of this study were to investigate the effects of PV or PVH on the trabecular bone microarchitecture, bone turnover markers and inflammatory proteins secretion, and the phenotypes of spleen cells in ovariectomized (OVX) rats (an osteoporosis model).

6.2. Materials and methods

6.2.1. Preparation of phosvitin and phosvitin hydrolysate

PV was prepared according to the protocol described in Chapter 3. Briefly, 400 kg egg yolk was 1/4 (wt/wt) diluted with water and centrifuged by disc stack separator separator to obtain granules. During NaCl extraction, granules were heated at 90 ^{o}C for 20 min to further precipate impurities. Salts were removed by ultrafiltration. PVH was prepared according to Ren, Li, Offengenden, & Wu (2015). Briefly, PV was partially dephophorylated by dissolving in 0.2 M NaOH solution for 0.5 h. Partially dephosphorylated PV (with 70.2% phosphate remaining) was incubated with pancreatin (40 ^{o}C, pH 7.5, 3 h) at a ratio of 1/50 (wt/wt) to prepare phosvitin hydrolysate.

6.2.2. Animals

Ovariectomized (OVX) Sprague-Dawley (SD) rats (10 weeks old) were obtained from Charles River Laboratories Inc. (Wilmington, MA, USA) and kept at the University of Alberta Health Sciences Laboratory Animal Services (HSLAS) facility. All the procedures in the experimental protocol were approved by the Canada Council on Animal Care. A pilot study was first conducted to determine the effectiveness of different forms of phosvitin (native phosvitin, partial dephosphorylated phosvitin, phosvitin hydrolysate) for treating OVX rats (3 rats in each group), and phosvitin hydrolysate was selected for the formal study due to more significant

results in maintaining trabecular bones measured by Micro CT scanning. In the formal study, 24 rats were randomly assigned to 3 diet groups (n=8): untreated control fed AIN-93M diet (TD94048, ENVIGO, Madison, WI), low dosage fed 0.2% (wt/wt) phosvitin hydrolysate in AIN-93M diet, high dosage fed 2% (wt/wt) phosvitin hydrolysate in AIN-93M diet. During the 12-week-long experiment, the bone mineral density and micro-architecture were tested at 0, 4, 8, and 12 weeks by non-invasive micro-computed tomography imaging. At the end of the experiment, animals were euthanized, and serum and spleen were collected for further analysis.

6.2.3. Micro-CT imaging

During the 12-week treatment, the right tibia of each rat was scanned *in vivo* by micro-CT (Skyscan 1076, Kontich BE) at 18 μm resolution with 100 kV, 100 μA using a 1.0 mm aluminum filter at four time points of baseline (zero week), 4, 8 and 12 weeks. The image was taken by using the vendor-supplied imaging control software (Version 2.6.0; Skyscan N.V., Kontich, Belgium), reconstructed by using NRecon software Version 1.4.4 (SKYSCAN, Kartulzersweg 3B 2990 Kentich, Belgium), and finally quantified for trabecular bone thickness, trabecular bone number, trabecular bone separation and bone volume fraction (bone volume/tissue volume) by using image analysis software (CT-An, Skyscan NV, Belgium).

6.2.4. Detection of bone turn over markers, inflammatory cytokines and chemokines by ELISA

The serum concentrations of six commonly used bone turnover markers were examined by enzyme-linked immunosorbent assay (ELISA). Rat osteocalcin (OCN) ELISA kit (Cat 60-1505) was purchased from Immutopics Inc. (Clemente, CA, USA). Rat bone alkaline phosphatase (ALP) ELISA kit (ABX256730), rat C-Telopeptide of type I collagen (CTX) ELISA kit (ABX255739), rat procollagen I N-terminal propeptide (P1NP) ELISA kit (ABX573268), rat

tartrate-resistant acid phosphatase 5b (TRACP-5b) ELISA kit (ABX256730) were purchased from Abbexa Ltd (Cambridge. United Kingdom). Inflammation markers were analyzed by using ELISA kits including normal T expressed and secreted (RANTES) (MMR00) and monocyte chemoattractant protein-1 (MCP-1) (MJE00) from Research and Diagnostic systems Inc. (Minneapolis, MN, USA), and macrophage inflammatory proteins 1 (MIP-1) (ab213916) from Abcam (Massachusetts, CT, USA). Analysis was performed according to the manufacturer's instructions.

6.2.5. Assessment of immune functions of spleen cells by flow cytometry

The assessment of immune functions was carried out according to Yu, Field, & Wu (2018). After euthanasia, OVX rat spleens were removed aseptically and placed in sterile Krebs-Ringer-HEPES buffer (pH 7.4) supplemented with 0.5% (w/v) bovine serum albumin. Immune cells and red blood cells were isolated by pushing spleens through a sterile nylon mesh screen (100 μm). The phenotypes of the isolated immune cells from spleens were analyzed by a direct immunofluorescence assay with pre-labelled monoclonal antibodies. The antibodies were purchased from Cedarlane Laboratories (Burlington, ON, Canada) and the following combinations were used: CD3/CD51/CD11b/c, CD25/CD3/CD8/CD4, CD28/CD8/CD4, CD71/OX12/CD8/CD4 and OX62/CD80/OX6/CD11b/c. Cells were fixed in 1% (w/v) paraformaldehyde in phosphate buffered saline. The proportion of positive cells for each antibody was determined according to the fluorescence intensity by flow cytometry (LSR-Fortessa; BD Biosciences, Mississauga, ON, Canada) using the Kaluza software (Beckman Coulter Inc., Mississauga, ON, Canada).

6.2.6. Statistical analysis

All data are presented as mean ± standard deviation (SD) and were analyzed by one-way analysis of variance (ANOVA) with Dunnett's post-hoc test or two-way ANOVA with Tukey's multiple comparisons test. The PRISM 6 statistical software (GraphPad Software, San Diego, CA) was used for the analysis and to produce graphs. A $p < 0.05$ was considered statistically significant.

6.3. Results and discussion

6.3.1. Effects of phosvitin hydrolysate on bone microarchitecture of OVX rat

The initial body weight, final body weight and uterus weight are shown in Table 6.1. The untreated OVX control, OVX rats fed on 0.2% and 2% (wt/wt) PVH started with similar body weights. After the 12 week-long experiment, no significant difference was found in the final body weights of all the three groups. This suggested that PVH diet employed in this experiment did not affect the body weight of OVX rats. The uterus weight of 2% (wt/wt) PVH diet group was higher than that of the OVX control group. The procedure of OVX results in gain of body weight and atrophy of the uterus, which could be partially prevented by hormone replacement therapy (e.g. estrogen) (Guo et al., 2009). Such protective effects might be related to hormone changes since the atrophy of uterus was mainly due to estrogen deficiency (Zaid, Sulaiman, Sirajudeen, & Othman, 2010).

Table 6.1. Body and uterus weights in OVX control (2% casein in diet), low dose phosvitin hydrolysate group (0.2% phosvitin hydrolysate+1.8% casein in diet), high dose phosvitin hydrolysate group (2% phosvitin hydrolysate in diet)

Weight (g)	OVX control	OVX+0.2% PVH	OVX+2% PVH
Body (initial)	262.5±7.7 [a]	255.0±8.6 [a]	259.3±6.4 [a]
Body (final)	441.4±41.6 [a]	427.5±29.0 [a]	438.1±19.9 [a]
Uterus (final)	0.3±0.1 [a]	0.3±0.1 [ab]	0.4±0.1 [bc]

Data are presented as mean±SD, n=8. Within a given row, values that share the same superscript letter are not statistically different from each other. PVH, phosvitin hydrolysate.

Ovariectomy remarkably deteriorates bone microstructure and causes a significant loss in bone mass due to hormonal changes (Egermann, Goldhahn, & Schneider, 2005). In this experiment, the trabecular bone of OVX control group experienced significant deterioration during the 12 week period, as trabecular bone separation (Tb.Sp) and bone structure index (SMI) increased, and trabecular bone thickness (Tb.Th), trabecular bone number (Tb.N), trabecular volumetric bone mineral density (vBMD) and bone volume fraction (BV/TV) all decreased (Figure 6.1). Compared with the OVX control group, treatment with 0.2% (wt/wt) PVH did not show significant changes in any of the five parameters except for Tb.N at 4 weeks (Figure 6.1-B). However, treatment with 2% (wt/wt) PVH resulted in lower Tr.Sp (Figure 6.1-A, week 8) and SMI (Figure 6.1-C, week 8), but higher Tr.N (Figure 6.1-B, week 4, 8, 12), Tr.Th (Figure 6.1-E, week 8), BV/TV (Figure 6.1-D, week 8) and high vBMD (Figure 6.1-F, week 8 and 12) compared to those of OVX control group. Tr.Sp, Tr.N, Tr.Th and BV/TV were commonly used to describe the quality of bone microstructure. Usually lower Tr.Sp and higher Tr.N, Tr.Th and BV/TV are associated with better bone quality (Isaksson et al., 2011).

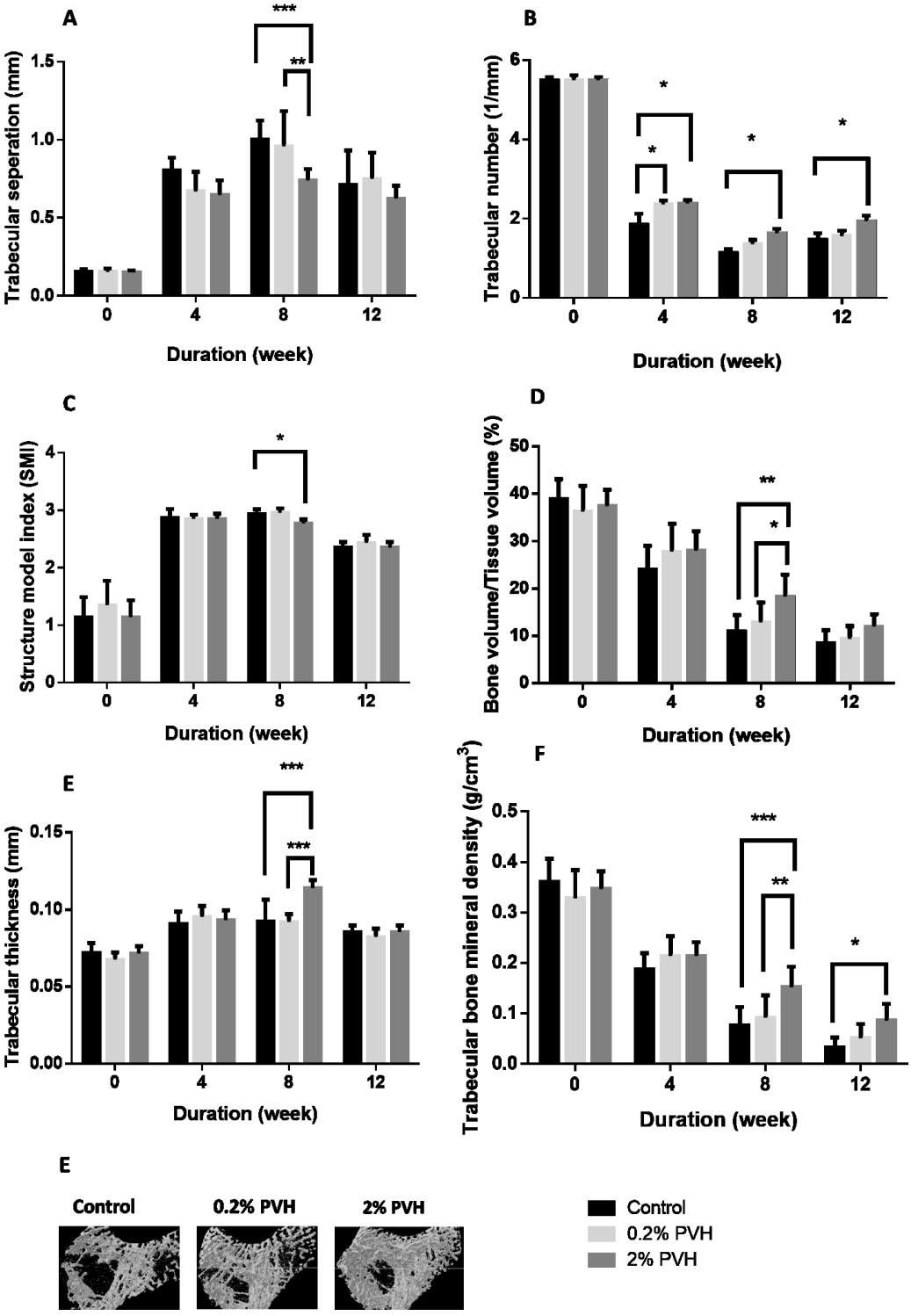

Figure 6.1. Effects of phosvitin hydrolysate on trabecular bone microstructure in OVX rats measured by Micro CT. A, changes in trabecular bone separation; B, changes in trabecular bone number; C, changes in trabecular bone structure model index (SMI) values; D, changes in

trabecular bone volume fraction; E, changes in trabecular bone thickness; F, trabecular bone mineral density; E, a representative MicroCT image of trabecular bone structure at eight weeks' time point. Data are presented as mean±SD, n=8. PVH, phosvitin hydrolysate.

Structure model index (SMI) is another widely used morphometric parameter to measure rod-like and plate-like geometry in the three-dimensional trabecular bone structure. The ratio of rods to plates (from 0 to 3) in the structure is important for the mechanical properties of the bone. Lower ratio is favorable for superior mechanical properties (Hara, Tanck, Homminga, & Huiskes, 2002). Traditionally, the areal bone mineral density (aBMD), including both cortical and trabecular bones is measured by dual-energy X-ray absorptiometry (DXA). However, recent studies indicate that trabecular vBMD is a very sensitive indicator of trabecular bone mass and it is the decrease of vBMD but not aBMD that correlates to the increased risk of fractures (Darelid et al., 2010). The above results suggested that 2% (wt/wt) PVH diet was able to suppress bone loss in OVX rats. A representative Micro CT image of trabecular bone was given in Figure 6.1-E to show the differences between control and PVH treated groups. It was also noted that most of the significant changes were detected at week 8, and none of the parameters could be restored to the levels of starting point. This suggested that the preventive effects of PVH on bone loss was limited and could not completely restore bone mass.

6.3.2. Effects of phosvitin hydrolysate on bone turnover markers

Osteoblasts and osteoclasts secrete many molecules into blood circulation, which could reflect bone turnover status. Analysis of these bone turnover markers could provide some insights to explain the observations from Micro CT imaging. In this experiment, six commonly used markers were examined by ELISA, including OCN, bone ALP, CTX, P1NP andTRACP-5b as shown in Table 6.2.

Table 6.2. Changes in bone related serum markers in OVX control (2% casein in diet), low dose phosvitin hydrolysate group (0.2% phosvitin hydrolysate+1.8% casein in diet), high dose phosvitin hydrolysate group (2% phosvitin hydrolysate in diet)

Bone markers	**OVX control**	**OVX+0.2% PVH**	**OVX+2% PVH**
OCN (ng/mL)	37.6±2.9 [a]	37.3±2.4 [a]	41.1±0.6 [b]
ALP (ng/mL)	59.6±13.7 [a]	59.4±9.5 [a]	59.9±21.9 [a]
CTX (ng/mL)	26.8±3.9 [a]	26.7±2.9 [a]	25.1±3.4 [a]
P1NP (ng/mL)	25.1±2.6 [a]	24.5±1.8 [a]	30.0±5.7 [b]
TRACP (ng/mL)	0.3±0.1 [a]	0.3±0.1 [ab]	0.2±0.0 [b]

Within a given row, values that share the same superscript letter are not statistically different from each other. Data are presented as mean±SD, n=8. PVH, phosvitin hydrolysate. Osteocalcin (OCN), bone alkaline phosphatase (ALP), C-Telopeptide of type I collagen (CTX), procollagen I N-terminal propeptide (P1NP), tartrate-resistant acid phosphatase 5b (TRACP-5b).

OCN, ALP and P1NP are markers for bone formation activities. Bone matrix mainly consists of type I collagen, which is derived from type I procollagen. During collagen fibers assembling, the C and N-terminal propeptides are cleaved and released to blood circulation. Therefore, the serum procollagen I N-terminal propeptide (P1NP) is positively corelated with the rate of collagen synthesis (Garnero, Vergnaud, & Hoyle, 2008; Vasikaran et al., 2011). OCN is exclusively synthesized by osteoblasts and is one of the most important non-collagenous proteins that induces calcification of bone matrix. The elevation of serum OCN is usually associated with increased bone mineral density (BMD) (Szulc et al., 1994). Bone ALP is an enzyme exclusively expressed by osteoblast cells. Higher concentration of bone ALP usually relates to higher number of osteoblasts and therefore it is commonly used as a maker for bone formation activity (Kushida, Takahashi, Kawana, & Inoue, 1995). In this experiment, the serum OCN and P1NP

levels were significantly higher in 2% (wt/wt) PVH group than that of OVX control group. These changes probably suggested a more active bone formation activity in PVH fed group than that of the control group. Serum ALP level showed no difference between the control and treated groups; this might be due to the large variation in the analysis.

TRACP-5b and CTX were makers for bone resorption activities. The concentration of TRACP-5b is positively correlated to the number of osteoclasts and bone resorption activity (Rissanen, Suominen, Peng, & Halleen, 2008). CTX is the sequence with eight amino acids cleaved from the C-terminal of type I collagen during bone resorption. Therefore, serum CTX is positively related to the bone resorption activities (Rosen et al., 2000). In this analysis, the serum TRACP 5b level in 2% (wt/wt) PVH group was lower than that of OVX control group, which suggested that osteoclast number or activities may be reduced by 2% (wt/wt) PVH diet. The corresponding serum CTX level was expected to be suppressed as well. However, no difference was detected in the CTX levels between the two groups. This discrepancy might be due to the high level of variation in the experiments or insufficient number of animals used.

6.3.3. Effects of phosvitin hydrolysate on inflammatory serum cytokines/chemokines

Cytokines are proteins released in response to infection, immune responses, inflammation, and trauma (Dinarello, 2000). Chemokines have similar structures with cytokines but with smaller molecular weights (Fernandez & Lolis, 2002). They are secreted in response to stimuli such as proinflammatory cytokines and thus start to recruit monocytes, neutrophils, and lymphocytes to the inflammatory sites (Deshmane, Kremlev, Amini, & Sawaya, 2009). Ovariectomy in mice or rats was reported to elevate production of many proinflammatory cytokines and chemokines such as regulated on activation, normal T cell expressed and secreted (RANTES), interleukin-10 (IL-10), IL- 18, Il-6, IL-1, monocyte chemoattractant protein-1

(MCP-1), TNF-β, RANK/RANKL and macrophage inflammatory proteins 1 (MIP-1) due to estrogen deficiency (Libouban, Moreau, Baslé, Bataille, & Chappard, 2003; Russell et al., 2017). In this study, OVX rats fed 2% (wt/wt) PVH showed lower serum levels of MCP-1 and RANTES, and both 2% (wt/wt) and 0.2% (wt/wt) PVH showed lower MIP-1α levels, compared with the OVX control animals fed on casein (Figure 6.2). The inflammatory cytokines tumor necrosis factor alpha (TNF-α), IL-6, IL-1 were also analyzed by ELISA but the concentrations were too low to be detected. The estrogen deficiency in postmenopausal women raises bone turnover rate; bone remodeling is imbalanced in favor of resorption due to prolonged lifespan of osteoclast than osteoblast (Manolagas, 2010). The elevated RANKL secretion induces production of MCP-1 and RANTES in osteoclast precursors, and these two chemokines in turn stimulate osteoclastogenesis (Kim, Day, & Morrison, 2005). MIP-1α strongly promotes bone resorption (Choi et al., 2000) and the decrease of MIP-1α production is associated with increased bone mineral density and suppressed osteoclast development in OVX rats (Vijayan, Khandelwal, Manglani, Gupta, & Surolia, 2014). In this study, the decrease of these chemokines could be a sign of suppressed bone resorption activity as measured by Micro CT imaging (Figure 6.1) and decreased bone resorption markers (Table 6.2). The previous experiments also observed the inhibitory effects of phosvitin and PVH on RANKL or TNF-α induced RANTES/MCP-1 production in osteoclast precursors (RAW264.7 cells) and osteoblast cells (MC3T3-E1) (Chapter 4 and 5). Together these results might suggest that PVH modulates osteoblast and osteoclast functions via its anti-inflammation activity.

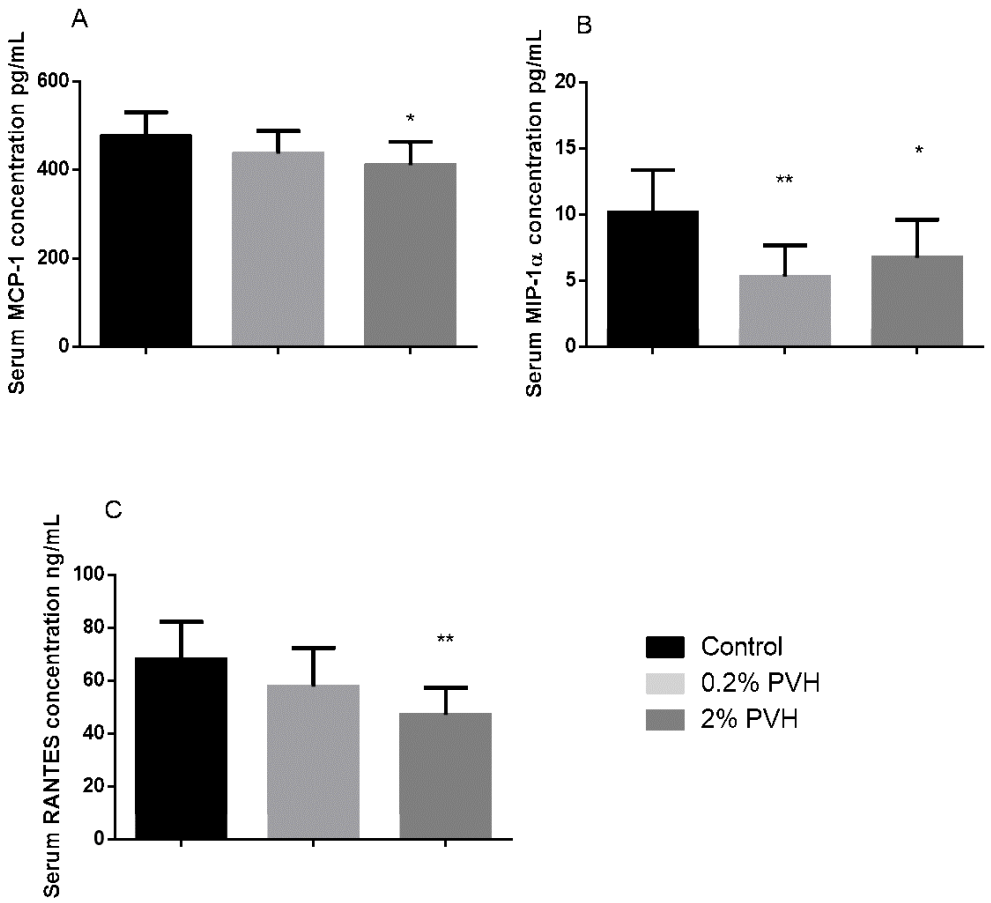

Figure 6.2. Effects of phosvitin hydrolysate on inflammatory cytokines/chemokines in OVX rats measured by ELISA. A, changes in serum MCP-1 concentration; B, changes in serum MIP-1α concentration; C, changes in serum RANTES concentration. (RANTES: normal T expressed and secreted; MCP-1: monocyte chemoattractant protein-1). Data are presented as mean±SD, n=8. PVH, phosvitin hydrolysate.

6.3.4. Effects of phosvitin hydrolysate on immune cell phenotype in spleen

The phenotype changes in immune cells were analyzed by flow cytometry and the results are summarized in Table 6.3. This experiment employed several antibodies that were generally related to immune functions, including total T cells (cluster of differentiation (CD) 3), T cell subsets of T helper cells (CD3+CD4+), T cytotoxic cells (CD3+CD8+), T cells subsets expressing IL-2 receptor (CD25+) and co-stimulatory factor (CD28+), dendritic cells (OX62+),

antigen presenting cells expressing major histocompatibility complex (MHC) II (OX6+) and CD11b/c+ cells (macrophages, granulocytes and dendritic cells) (Coico & Sunshine, 2015; Yu et al., 2018).

Table 6.3. Changes in spleen cells phenotypes in OVX control (2% casein in diet), low dose phosvitin hydrolysate group (0.2% phosvitin hydrolysate+1.8% casein in diet), high dose phosvitin hydrolysate group (2% phosvitin hydrolysate in diet)

Phenotypes	**OVX control**	**OVX+0.2% PVH**	**OVX+2% PVH**
CD3+	37.4±3.9 [a]	35.2±5.5 [a]	39.7±3.2 [a]
CD25+	4.9±1.0 [a]	4.6±0.9 [a]	4.3±0.8 [a]
CD3+CD4+	21.3±2.5 [a]	20.9±3.9 [a]	22.7±3.0 [a]
CD3+CD8+	15.5±1.9 [a]	14.0±1.5 [a]	17.0±2.6 [a]
CD28+	24.4±4.8 [a]	25.3±4.4 [a]	22.9±5.4 [a]
OX62+	12.8±2.6 [a]	14.8±4.6 [a]	12.6±4.1 [a]
OX6+	2.4±0.6 [a]	2.7±1.1 [a]	2.6±1.0 [a]
CD11b/c+	5.7±1.8 [a]	5.8±1.1 [a]	5.2±1.3 [a]

Data are presented as mean±SD, n=8. Within a given row, values that share the same superscript letter are not statistically different from each other. PVH, phosvitin hydrolysate.

PVH was reported to have anti-inflammatory effects *in vitro* (Young et al., 2011; Xu et al., 2012), and also reduced production of MCP-1, RANTES and MIP-1α in this experiment. Such effects may be related to changes in immune functions. However, this study failed to detect any phenotype changes between the PVH (0.2% and 2% (wt/wt)) groups and the control group. These results suggested that PVH probably did not affect immune functions in OVX rats. The inhibition of bone loss and inflammatory chemokine production in OVX rats may be attributed to

the direct modulatory effects of PVH on bone cell differentiation or functions as shown in the cell culture experiment (Chapter 4 & 5).

Egg is a nutritious food with various bioactive components. The research interests in egg yolk protein hydrolysate/peptides can be dated back to 1990s when it was first prepared and studied as a potent alternative protein source for infant food. Compared with soybean protein hydrolysate, which is widely accepted as an alternative protein source in infant food, egg yolk protein hydrolysate showed higher absorption rate, protein efficiency ratio, feed efficiency ratio, net protein utilization values and biological values (Gutierrez et al., 1998). Phosvitin represents 11% of yolk proteins (Burley & Cook, 1961). It is reasonable to assume that phosvitin hydrolysate generated during the digestion might contribute to the physiological activities of egg yolk hydrolysate as well (Goulas, Triplett, & Taborsky, 1996; Khan, Babiker, Azakami, & Kato, 1998). This study reported 2% (wt/wt) PVH in diet could significantly improve bone micro structure compared with control animals, elevate bone formation biomarkers, suppress bone resorption biomarkers and inflammatory chemokines (MCP-1, RANTES and MIP-1α).

6.4. Conclusions

This experiment for the first time reported that phosvitin hydrolysate could prevent bone loss in OVX rats. To be specific, 2% (wt/wt) PVH in animal diet resulted in significantly lower bone separation, SMI value, and higher bone thickness, trabecular bone number, trabecular vBMD and bone volume fraction than the control animals at certain time points. Correspondingly, some changes in bone turnover biomakers were also detected as higher concentrations of serum OCN and P1NP and lower TRACP-5 in animals fed with 2% PVH. The rise of serum OCN and P1NP levels suggested an increase in bone formation activities whereas a drop in TRACP-5b level indicated suppressed osteoclast number and activities. These results

suggested the prevention of PVH on OVX bone loss may be a combination of both anabolic and anti-resorptive effects.

Osteoporosis is a widely spread bone disease affecting about 1/4 of the senior population. The situation will be more serious with prolonged life span and increasing senior population. Due to the side-effects of drug treatments, functional foods to prevent/manage osteoporosis becomes increasingly popular. Although the detailed mechanisms remain unknown, the above preliminary results suggested that phosvitin hydrolysate has great potential to be developed into functional foods to reduce bone loss and/or promote bone growth.

CHAPTER 7 - Final Remarks and Future Works

7.1 Key findings

The overall purpose of this thesis was to study the potential of phosvitin as a functional food or nutraceutical ingredient towards bone health. To be specific, the effects of phosvitin and phosvitin hydrolysate on osteoblasts (**Chapter 4**) and osteoclasts (**Chapter 5**) and ovariectomized rats (**Chapter 6**) were studied. A method to extract phosvitin was developed and the possibility for scale up production was investigated in **Chapter 3**. The key findings are as follows:

1) Phosvitin is the principal phosphoprotein in egg yolk with the potential for various value-added applications. This potential has not been realized due to the lack of a suitable method of scale-up extraction. To develop a scale-up method of phosvitin extraction, effects of pH, low centrifugal force, ultra filtration desalting, use of industrial separators (decanter and disc stack separator) and different water dilution factors on the extraction were studied. The results suggested that decreasing pH of the granule in 10 NaCl from neutral to acidic range could increase phosvitin purity and recovery. The highest recovery of 67.3% was obtained at pH 3.0 with a purity of 86.3%. Desalting achieved by ultrafiltration was comparable to that of dialysis. Increasing water dilution ratio increased phosvitin recovery in the pellets, while that of purity and recovery were reduced at water dilution ratios of 1/8 and 1/10 (yolk/water, wt/wt), due to the co-precipitation of other impurities. However, phosvitin extraction was significantly affected by centrifugal force. Low centrifugal force resulted in low phosvitin recovery (11.4% at 2,000 g vs 67.3% at 10,000 g), although the purity remained high (84.7% at 2 ,000 g vs 86.3% at 10,000 g). Neither of the industrial separators (decanter and disc stack separators) were sufficient to

separate pellet from egg yolk and thus resulted in significantly lower yield (~20-30%), while the purity from both separators was comparable (~70%).

2) Phosvitin and its hydrolysate are reported to have antioxidant, anti-inflammation and antimicrobial activities. Given the critical role of inflammation on bone health, the work was thus designed to study the effects of phosvitin and its hydrolysate on osteoblast differentiation and inflammation in MC3T3-E1 cells. Both phosvitin and its hydrolysate promoted osteoblast differentiation by upregulating expression of runt-related transcription factor 2 (RUNX2), alkaline phosphatase, osteocalcin production and collagen synthesis. They also inhibited tumor necrosis factor alpha (TNF-α)-induced inflammation by reducing the production of regulated on activation, normal T cell expressed and secreted (RANTES) and monocyte chemoattractant protein-1 (MCP-1), which are crucial for recruiting osteoclast progenitor and maintaining osteoclast functions, and cyclooxygenase-2 (COX-2), a proinflammatory agent. However, the expression of osteoprotegerin (OPG) and alkaline phosphatase (ALP) were not affected. Adding phosvitin and its hydrolysate activated both extracellular signal–regulated kinases (ERK) and protein kinase B (AKT) signaling pathways, indicating that both phosvitin and its hydrolysate promote osteoblast differentiation and exert anti-inflammation effects via ERK and AKT pathways.

3) Phosvitin and its derived hydrolysate have been reported to have various physiological functions including anti-inflammation and bone formation promoting activities. However, whether phosvitin and phosvitin hydrolysate could affect bone resorption activities remains unknown. This study was thus designed to examine the effects of phosvitin and phosvitin hydrolysate on osteoclastogenesis in RAW264.7 cells. These results indicated that both phosvitin and phosvitin hydrolysate inhibited receptor activator of nuclear factor kappa-B ligand

(RANKL) induced osteoclastogenesis in RAW264.7 cells. The tartrate-resistant acid phosphatase (TRAP) positive cells and TRAP activity were both reduced upon treatment of phosvitin or phosvitin hydrolysate. The expression of osteoclastogenesis-associated transcription factors, c-Fos and nuclear factor of activated T-cells 1 (NFATc1) was downregulated. Inflammatory biomarkers, such as TNF-α, MCP-1, RANTES, and inducible nitric oxide synthases (iNOS), were significantly suppressed by phosvitin or phosvitin hydrolysate. These results suggested that the inhibitory effects of phosvitin and phosvitin hydrolysate on RAW264.7 cells differentiation might be mediated through c-Jun N-terminal kinase (JNK), p38 and nuclear factor kappa beta (NF-κB) pathways. These results indicated that phosvitin and its hydrolysate might act as inhibitory agents to bone resorption activities.

4) This study for the first time reported that phosvitin hydrolysate could improve bone micro structure and prevent bone loss in OVX rats. To be specific, 2% phosvitin hydrolysate in animal diet resulted in significantly lower bone separation, structure model index (SMI), and higher bone thickness, trabecular bone number, volumetric bone mineral density (vBMD) and bone volume fraction than the control animals at certain time points. Correspondingly, some changes in bone turnover markers were also detected as higher concentrations of serum osteocalcin (OCN) and N-terminal propeptide of type I collagen (P1NP) and lower serum band 5 tartrate-resistant acid phosphatase (TRACP-5) in animals fed with 2% PVH. The rise of serum OCN and P1NP levels are usually associated with an increase in bone formation activities whereas a drop in TRACP-5b levels could be a sign of suppressed osteoclasts number and activities. These results suggested the prevention of bone loss in OVX rats by phosvitin hydrolysate might be a combination of both anabolic and anti-resorptive effects. These

preliminary results suggested that phosvitin hydrolysate has good potential to be developed into functional foods to reduce bone loss and promote bone growth.

7.2 Significance of this research

Osteoporosis is a very common disease afflicting many elderly people worldwide (Berger et al., 2010). This prevalence tends to be increasing rapidly due to the aging of the world population. The cost to treat osteoporosis and related complications is a big burden for the society. Osteoporosis is a result of imblanced bone remodeling in which bone resorption activity excels bone formation activity (Seeman, 2009). Currently, most of the drugs and therapies have good performance to inhibit osteoporotic bone loss but some of the patients may experience serious side effects like nausea, abdominal pain and loose bowel movements, hot flashes, depression, and headaches (Rachner, Khosla, & Hofbauer, 2011). Besides, the loss of bone quality cannot be completely reversed. The concept to prevent osteoporosis from happening is accepted widely. Calcium combined with vitamin D is conventionally used for prevention of osteoporosis but the efficacy is still under debate (Grant et al., 2005). There are also side effects like nausea, vomiting, stomach pain, or constipation. Other products for osteoporosis treatments like isoflavones might raise safety concerns (thyroid diseases) (Setchell, 2001). There is an increasing demand for functional foods and nutraceuticals to prevent osteoporosis with high safety and efficacy.

Phosvitin and its hydrolysate have gained great research interest in the last two decades due to their multiple physiological functions. The bone health benefits of phosvitin and its hydrolysate might originate from their role in skeleton development in embryo or from their anti-inflammation activity (Hiramatsu, Cheek, Sullivan, Matsubara, & Hara, 2005; Liu et al., 2013; Mundy, 2007). This thesis for the first time reported that phosvitin hydrolysate prevented bone

loss in ovariectomized rats. In cell culture experiments, phosvitin and phosvitin hydrolysate promoted osteoblast differentiation but inhibited osteoclast differentiation. Besides, this is also the first time to reveal that phosvitin and phosvitin hydrolysate suppressed the production of inflammatory proteins in osteoclast and osteoblast stimulated by RANKL and TNF-α respectively. Since inflammation also plays a role in osteoporotic bone loss, the bone health benefits in animals might relate to the anti-inflammation activity of phosvitin and phosvitin hydrolysate. Phosvitin was reported to promote bone formation but the mechanism is unclear (Liu et al., 2013; Liu, Li, Geng, Huang, & Ma, 2017). This research further confirmed these effects by the observations that phosvitin and phosvitin hydrolysate promoted osteoblastogenesis. It was also suggested that these effects might be mediated via AKT and ERK pathways by phosvitin and phosvitin hydrolysate treatments. Whether phosvitin and phosvitin hydrolysate affect osteoclast differentiation remains unknown. **Chapter 5** indicated that phosvitin and phosvitin hydrolysate inhibited RAW264.7 cell differentiation into osteoclasts, probably via the JNK, p38 and NF-κB pathways. This research indicated possible pathways via which phosvitin and phosvitin hydrolysate affect bone cells differentiation and production of inflammatory proteins. These results might be linked to the observations in the animal study, in which phosvitin hydrolysate suppressed bone loss caused by ovariectomy (**Chapter 6**). To summarize, the results from this PhD research suggest phosvitin and its hydrolysate have great potential to be used as functional food ingredients with bone health benefits.

7.3 Limitations of this thesis and recommendations for future research

As it is always said, research work raises more questions than it answers. The limitations in this current thesis are discussed here, and to resolve these limitations, some recommendations are proposed for future research work:

1. In the animal study, phosvitin hydrolysate inhibited bone loss but could not completely restore it. This means the animals or potential consumers who intake phosvitin hydrolysate are still under the risk of osteoporotic fractures. Modification of phosvitin hydrolysate (e.g. use different enzymes) might enhance the efficacy. Besides, pretreatment with phosvitin hydrolysate for some time before ovariectomy might exert greater protective effects on bone microstructure in animal study.

2. Chronic inflammation is associated with bone loss. This research observed the anti-inflammation effects of phosvitin and phosvitin hydrolysate in bone cells but did not provide details to link these observations to the preventive effects on bone loss in OVX rats. Rheumatoid arthritis is a disease causing inflammation-induced bone loss. Since phosvitin and phosvitin hydrolysate suppressed release of RANTES and MCP-1in bone cells and animal serum, it will be interesting to further study the pathway how phosvitin/phosvitin hydrolysate affect RANTES and MCP-1 production and thus modulate bone cells functions.

3. Phosvitin and phosvitin hydrolysate were reported to have antioxidant and anti-inflammation activity. In the cell culture experiments, they both suppressed the production of inflammatory markers in osteoclasts and osteoblasts (Chapter 4 & 5). Therefore, it will be interesting to study the effects of phosvitin and phosvitin hydrolysate in an animal model with inflammation diseases such as inflammatory bowel disease (IBD) susceptible rats.

4. The recovery of the phosvitin from current method with industrial separators is ~20%, which is too low for potential industrial use. It is necessary to modify this method to increase the yield. Low centrifugal force from industrial separators could not effectively separate the granule fraction from the plasma fraction, which caused significant loss of phosvitin in the plasma fraction, and the impurities in granule fraction may co-precipitate with phosvitin and thus further

reduce the yield and purity. A custom-made separator for this extraction protocol might greatly increase the yield and purity, but the price will be high. It will be better to develop an extraction protocol without using centrifugation. Instead, filtration and sedimentation could be considered, and the extraction protocol needs to be substantially modified.

5. The promoting effects of phosvitin and phosvitin hydrolysate on osteoblast differentiation were observed with activation of ERK and AKT pathway; however, it is not known if these two pathways are the only two involved. Further study using specific antagonists of ERK and AKT or using their respective knock-down or knock-out cells will further elaborate the roles of these two pathways. Similarly the pathways responsible for suppressing osteoclastogenesis also need to be confirmed. Besides, the site of action of phosvitin and phosvitin hydrolysate remains unknown. There could be a receptor for phosvitin in cell membrane, or the negative charge of phosvitin might alter some cell membrane protein like ion channels and thus affect signaling pathways.

6. The animal study (Chapter 6) used a scale-up method to prepare phosvitin, which had a lower purity (~70% pure) than the phosvitin (~90%) used for cell culture experiments in Chapters 4 and 5. The high content of impurities (~30%) in this phosvitin preparation might affect the outcome of animal study. These impurities usually include livetins and high-density lipoproteins, which are also bioactive proteins and might contribute to the benefical effects on bones. It is necessary to repeat the animal experiment with highly purified phosvitin/phosvitin hydrolysate to confirm the bioactivites of phosvitin/phosvitin hydrolysate on bones. Besides, the purity of these phosvitin preparations determined by gel filtration high performance liquid chromatography (HPLC) should be double confirmed by using sodium dodecyl sulfate

polyacrylamide gel electrophoresis (SDS-PAGE); the α and β-phosvitin fractions from HPLC could be further analyzed by mass spectrometry (MS) for possible contaminants.

BIBLIOGRAPHY

Abdelgawad, M. E., Delaisse, J. M., Hinge, M., Jensen, P. R., Alnaimi, R. W., Rolighed, L., ... & Andersen, T. L. (2016). Early reversal cells in adult human bone remodeling: osteoblastic nature, catabolic functions and interactions with osteoclasts. *Histochemistry and Cell Biology*, *145*(6), 603-615.

Abbas, S., Zhang, Y. H., Clohisy, J. C., & Abu-Amer, Y. (2003). Tumor necrosis factor-α inhibits pre-osteoblast differentiation through its type-1 receptor. *Cytokine, 22*(1-2), 33-41.

Abe, Y., Itoh, T., & Adachi, S. (1982). Fractionation and characterization of hen's egg-yolk phosvitin. *Journal of Food Science, 47*(6), 1903-1907.

Akita, E. M., & Nakai, S. (1992). Immunoglobulins from egg-yolk - isolation and purification. *Journal of Food Science, 57*(3), 629-634.

Aguirre, J. I., Plotkin, L. I., Stewart, S. A., Weinstein, R. S., Parfitt, A. M., Manolagas, S. C., & Bellido, T. (2006). Osteocyte apoptosis is induced by weightlessness in mice and precedes osteoclast recruitment and bone loss. *Journal of Bone and Mineral Research*, *21*(4), 605-615.

Albright, K. J., Gordon, D. T., & Cotterill, O. J. (1984). Release of iron from phosvitin by heat and food-additives. *Journal of Food Science, 49*(1), 78-81.

Albright, F., Smith, P. H., & Richardson, A. M. (1941). Postmenopausal osteoporosis: its clinical features. *Journal of the American Medical Association*, *116*(22), 2465-2474.

Ammann, P., Rizzoli, R., Bonjour, J. P., Bourrin, S., Meyer, J. M., Vassalli, P., & Garcia, I. (1997). Transgenic mice expressing soluble tumor necrosis factor-receptor are protected against bone loss caused by estrogen deficiency. *The Journal of Clinical Investigation*, *99*(7), 1699-1703.

Arlot, M., Edouard, C., Meunier, P. J., Neer, R. M., & Reeve, J. (1984). Impaired osteoblast function in osteoporosis: comparison between calcium balance and dynamic histomorphometry. *British Medical Journal (Clinical Research Ed.)*, *289*(6444), 517-520.

Asagiri, M., Sato, K., Usami, T., Ochi, S., Nishina, H., Yoshida, H., ... & Takayanagi, H. (2005). Autoamplification of NFATc1 expression determines its essential role in bone homeostasis. *Journal of Experimental Medicine, 202*(9), 1261-1269.

Beattie, J. H., & Avenell, A. (1992). Trace element nutrition and bone metabolism. *Nutrition Research Reviews*, *5*(1), 167-188.

Bennett, T., Desmond, A., Harrington, M., McDonagh, D., FitzGerald, R., Flynn, A., & Cashman, K. D. (2000). The effect of high intakes of casein and casein phosphopeptide on calcium absorption in the rat. *British Journal of Nutrition*, *83*(6), 673-680.

Berger, C., Goltzman, D., Langsetmo, L., Joseph, L., Jackson, S., Kreiger, N., ... & Hanley, D. A. (2010). Peak bone mass from longitudinal data: implications for the prevalence, pathophysiology, and diagnosis of osteoporosis. *Journal of Bone and Mineral Research, 25*(9), 1948-1957.

Blais, A., Malet, A., Mikogami, T., Martin-Rouas, C., & Tomé, D. (2009). Oral bovine lactoferrin improves bone status of ovariectomized mice. *American Journal of Physiology-Endocrinology and Metabolism*, *296*(6), E1281-E1288.

Blais, A., Malet, A., Mikogami, T., & Tomé, D. (2010). Bovine lactoferrin improves bone status of ovariectomized mice via immune function modulation. *Bone*, *46*, S69.

Bolland, M. J., Leung, W., Tai, V., Bastin, S., Gamble, G. D., Grey, A., & Reid, I. R. (2015). Calcium intake and risk of fracture: systematic review. *British Medical Journal*, *351*, h4580.

Bonjour, J. P., Guéguen, L., Palacios, C., Shearer, M. J., & Weaver, C. M. (2009). Minerals and vitamins in bone health: the potential value of dietary enhancement. *British Journal of Nutrition*, *101*(11), 1581-1596.

Bonnelye, E., Chabadel, A., Saltel, F., & Jurdic, P. (2008). Dual effect of strontium ranelate: stimulation of osteoblast differentiation and inhibition of osteoclast formation and resorption *in vitro*. *Bone*, *42*(1), 129-138.

Bouhallab, S., Cinga, V., Aít-Oukhatar, N., Bureau, F., Neuville, D., Arhan, P., ... & Bouglé, D. (2002). Influence of various phosphopeptides of caseins on iron absorption. *Journal of Agricultural and Food Chemistry*, *50*(24), 7127-7130.

Boyle, W. J., Simonet, W. S., & Lacey, D. L. (2003). Osteoclast differentiation and activation. *Nature, 423*(6937), 337.

Boyce, B., Yao, Z., & Xing, L. (2009). Osteoclasts have multiple roles in bone in addition to bone resorption. *Critical Reviews™ in Eukaryotic Gene Expression, 19*(3), 171-180.

Brandi, M. L., Hukkanen, M., Umeda, T., Moradi-Bidhendi, N., Bianchi, S., Gross, S. S., ... & MacIntyre, I. (1995). Bidirectional regulation of osteoclast function by nitric oxide synthase isoforms. *Proceedings of the National Academy of Sciences, 92*(7), 2954-2958.

Bu, S. Y., Hunt, T. S., & Smith, B. J. (2009). Dried plum polyphenols attenuate the detrimental effects of TNF-α on osteoblast function coincident with up-regulation of Runx2, Osterix and IGF-I. *The Journal of Nutritional Biochemistry*, *20*(1), 35-44.

Burge, R., Dawson- Hughes, B., Solomon, D. H., Wong, J. B., King, A., & Tosteson, A. (2007). Incidence and economic burden of osteoporosis- related fractures in the United States, 2005–2025. *Journal of Bone and Mineral Research*, *22*(3), 465-475.

Burley, R. W., & Cook, W. H. (1961). Isolation and composition of avian egg yolk granules and their constituent α-and β-lipovitellins. C*anadian Journal of Biochemistry and Physiology, 39*(8), 1295-1307.

Burley, R. W., & Vadehra, D. V. (1989). *The Avian Egg: Chemistry and Biology*. New York: Wiley

Byrne, B. M., Van het Schip, A. D., Van de Klundert, J. A., Arnberg, A. C., Gruber, M., & Ab, G. (1984). Amino acid sequence of phosvitin derived from the nucleotide sequence of part of the chicken vitellogenin gene. *Biochemistry, 23*(19), 4275-4279.

Cantley, L. C. (2002). The phosphoinositide 3-kinase pathway. *Science, 296*(5573), 1655-1657.

Cashman, K. D. (2004). Diet and control of osteoporosis. *Functional foods, ageing and degenerative disease. Cambridge, UK: Woodhead Publishing Limited*, 83-114.

Cashman, K. D. (2006). Milk minerals (including trace elements) and bone health. *International Dairy Journal*, *16*(11), 1389-1398.

Castellani, O., Martinet, V., David-Briand, E., Guerin-Dubiard, C., & Anton, M. (2003). Egg yolk phosvitin: preparation of metal-Free purified protein by fast protein liquid chromatography using aqueous solvents. *Journal of Chromatography B-Analytical Technologies in the Biomedical and Life Sciences, 791*(1-2), 273-284.

Causeret, D., Matringe, E., & Lorient, D. (1991). Ionic-strength and pH effects on composition and microstructure of yolk granules. *Journal of Food Science, 56*(6), 1532-1536.

Chakrabarti, S., & Davidge, S. T. (2016). Analysis of G-protein coupled receptor 30 (GPR30) on endothelial inflammation. In *Estrogen Receptors* (pp. 503-516). Humana Press, New York, NY.

Chapuy, M. C., Arlot, M. E., Duboeuf, F., Brun, J., Crouzet, B., Arnaud, S., ... & Meunier, P. J. (1992). Vitamin D3 and calcium to prevent hip fractures in elderly women. *New England Journal of Medicine*, *327*(23), 1637-1642.

Choi, I., Jung, C., Choi, H., Kim, C., & Ha, H. (2005). Effectiveness of phosvitin peptides on enhancing bioavailability of calcium and its accumulation in bones. *Food Chemistry, 93*(4), 577-583.

Choi, S. J., Cruz, J. C., Craig, F., Chung, H., Devlin, R. D., Roodman, G. D., & Alsina, M. (2000). Macrophage inflammatory protein 1-alpha is a potential osteoclast stimulatory factor in multiple myeloma. *Blood, 96*(2), 671-675.

Christakos, S., & Prince, R. (2003). Estrogen, vitamin D, and calcium transport. *Journal of Bone and Mineral Research*, *18*(10), 1737-1739.

Chung, S. L., & Ferrier, L. K. (1992). pH and sodium-chloride effects on emulsifying properties of egg-yolk phosvitin. *Journal of Food Science, 57*(1), 40-42.

Clark, R. C. (1985). The primary structure of avian phosvitins - contributions through the edman degradation of methylmercaptovitins prepared from the constituent phosphoproteins. *International Journal of Biochemistry, 17*(9), 983-988.

Clarke, B. (2008). Normal bone anatomy and physiology. *Clinical journal of the American Society of Nephrology*, *3*(Supplement 3), S131-S139.

Coico, R., & Sunshine, G. (2015). *Immunology: A Short Course*. New York: John Wiley & Sons.

Colón-Emeric, C., Kuchibhatla, M., Pieper, C., Hawkes, W., Fredman, L., Magaziner, J., ... & Lyles, K. W. (2003). The contribution of hip fracture to risk of subsequent fractures: data from two longitudinal studies. *Osteoporosis International*, *14*(11), 879-883.

Colón-Emeric, C. S., & Saag, K. G. (2006). Osteoporotic fractures in older adults. *Best Practice & Research Clinical Rheumatology*, *20*(4), 695-706.

Conway, S. P., Wolfe, S. P., Brownlee, K. G., White, H., Oldroyd, B., Truscott, J. G., ... & Shearer, M. J. (2005). Vitamin K status among children with cystic fibrosis and its relationship to bone mineral density and bone turnover. *Pediatrics*, *115*(5), 1325-1331.

Cornish, J. (2004). Lactoferrin promotes bone growth. *Biometals, 17*(3), 331-335.

Cornish, J., Callon, K. E., Naot, D., Palmano, K. P., Banovic, T., Bava, U., ... & Chan, V. A. (2004). Lactoferrin is a potent regulator of bone cell activity and increases bone formation *in vivo*. *Endocrinology*, *145*(9), 4366-4374.

Corral, D. A., Amling, M., Priemel, M., Loyer, E., Fuchs, S., Ducy, P., ... & Karsenty, G. (1998). Dissociation between bone resorption and bone formation in osteopenic transgenic mice. *Proceedings of the National Academy of Sciences*, *95*(23), 13835-13840.

Cunniffe, G. M., & O'Brien, F. J. (2011). Collagen scaffolds for orthopedic regenerative medicine. *Journal of the Minerals, Metals, and Materials Society*, *63*(4), 66.

Darelid, A., Ohlsson, C., Rudäng, R., Kindblom, J. M., Mellström, D., & Lorentzon, M. (2010). Trabecular volumetric bone mineral density is associated with previous fracture during childhood and adolescence in males: the GOOD study. *Journal of Bone and Mineral Research, 25*(3), 537-544.

Dawson-Hughes, B., Harris, S. S., Krall, E. A., & Dallal, G. E. (1997). Effect of calcium and vitamin D supplementation on bone density in men and women 65 years of age or older. *New England Journal of Medicine*, *337*(10), 670-676.

Deshmane, S. L., Kremlev, S., Amini, S., & Sawaya, B. E. (2009). Monocyte chemoattractant protein-1 (MCP-1): an overview. *Journal of Interferon & Cytokine Research, 29*(6), 313-326.

Devareddy, L., Hooshmand, S., Collins, J. K., Lucas, E. A., Chai, S. C., & Arjmandi, B. H. (2008). Blueberry prevents bone loss in ovariectomized rat model of postmenopausal osteoporosis. *The Journal of Nutritional Biochemistry, 19*(10), 694-699.

Dewhirst, F. E., Stashenko, P. P., Mole, J. E., & Tsurumachi, T. (1985). Purification and partial sequence of human osteoclast-activating factor: identity with interleukin 1 beta. *The Journal of Immunology*, *135*(4), 2562-2568.

Di Benedetto, A., Gigante, I., Colucci, S., & Grano, M. (2013). Periodontal disease: linking the primary inflammation to bone loss. *Clinical and Developmental Immunology,* 2013.

Diepenhorst, N., Rueda, P., Cook, A. E., Pastoureau, P., Sabatini, M., & Langmead, C. J. (2017). G protein-coupled receptors as anabolic drug targets in osteoporosis. *Pharmacology & Therapeutics*.

Dinarello, C. A. (2000). Proinflammatory cytokines. *Chest, 118*(2), 503-508.

Ding, Y., Liu, X., Bu, L., Li, H., & Zhang, S. (2012). Antimicrobial–immunomodulatory activities of zebrafish phosvitin-derived peptide Pt5. *Peptides*, *37*(2), 309-313.

Donida, B. M., Mrak, E., Gravaghi, C., Villa, I., Cosentino, S., Zacchi, E., ... & Ferraretto, A. (2009). Casein phosphopeptides promote calcium uptake and modulate the differentiation pathway in human primary osteoblast-like cells. *Peptides, 30*(12), 2233-2241.

Ducy, P., Desbois, C., Boyce, B., Pinero, G., Story, B., Dunstan, C., ... & Bradley, A. (1996). Increased bone formation in osteocalcin-deficient mice. *Nature*, *382*(6590), 448.

Egermann, M., Goldhahn, J., & Schneider, E. (2005). Animal models for fracture treatment in osteoporosis. *Osteoporosis International, 16*(2), S129-S138.

Endres, S., Ghorbani, R., Kelley, V. E., Georgilis, K., Lonnemann, G., van der Meer, J. W., ... & Schaefer, E. J. (1989). The effect of dietary supplementation with n—3 polyunsaturated fatty acids on the synthesis of interleukin-1 and tumor necrosis factor by mononuclear cells. *New England Journal of Medicine*, *320*(5), 265-271.

Eriksen, E. F., Langdahl, B., Vesterby, A., Rungby, J., & Kassem, M. (1999). Hormone replacement therapy prevents osteoclastic hyperactivity: a histomorphometric study in early postmenopausal women. *Journal of Bone and Mineral Research*, *14*(7), 1217-1221.

Fernandez, E. J., & Lolis, E. (2002). Structure, function, and inhibition of chemokines. *Annual Review of Pharmacology and Toxicology*, *42*(1), 469-499.

Ferraretto, A., Gravaghi, C., Fiorilli, A., & Tettamanti, G. (2003). Casein-derived bioactive phosphopeptides: role of phosphorylation and primary structure in promoting calcium uptake by HT- 29 tumor cells. *Federation of European Biochemical Societies Letters*, *551*(1-3), 92-98.

Finn, R. N. (2007). Vertebrate yolk complexes and the functional implications of phosvitins and other subdomains in vitellogenins. *Biology of Reproduction*, *76*(6), 926-935.

Franceschi, R. T. (1992). The role of ascorbic acid in mesenchymal differentiation. *Nutrition Reviews, 50*(3), 65-70.

Franceschi, R. T., Iyer, B. S., & Cui, Y. (1994). Effects of ascorbic acid on collagen matrix formation and osteoblast differentiation in murine MC3T3-E1 cells. *Journal of Bone and Mineral Research, 9*(6), 843-854.

Franceschi, R. T., & Xiao, G. (2003). Regulation of the osteoblast-specific transcription factor, Runx2: Responsiveness to multiple signal transduction pathways. *Journal of Cellular Biochemistry, 88*(3), 446-454.

Fujita, T., Azuma, Y., Fukuyama, R., Hattori, Y., Yoshida, C., Koida, M., ... & Komori, T. (2004). Runx2 induces osteoblast and chondrocyte differentiation and enhances their migration by coupling with PI3K-Akt signaling. *Journal of Cell Biology*, *166*(1), 85-95.

Galli, C., Fu, Q., Wang, W., Olsen, B. R., Manolagas, S. C., Jilka, R. L., & O'Brien, C. A. (2009). Commitment to the osteoblast lineage is not required for RANKL gene expression. *Journal of Biological Chemistry 284,* 12654-12662.

Garlet, T. P., Coelho, U., Repeke, C. E., Silva, J. S., de Queiroz Cunha, F., & Garlet, G. P. (2008). Differential expression of osteoblast and osteoclast chemmoatractants in compression and tension sides during orthodontic movement. *Cytokine*, *42*(3), 330-335.

Garnero, P., Sornay- Rendu, E., Chapuy, M. C., & Delmas, P. D. (1996). Increased bone turnover in late postmenopausal women is a major determinant of osteoporosis. *Journal of Bone and Mineral Research*, *11*(3), 337-349.

Garnero, P., Vergnaud, P., & Hoyle, N. (2008). Evaluation of a fully automated serum assay for total N-terminal propeptide of type I collagen in postmenopausal osteoporosis. *Clinical Chemistry, 54*(1), 188-196.

Gerber, H. W., & Jost, R. (1986). Casein phosphopeptides: Their effect on calcification of *in vitro* cultured embryonic rat bone. *Calcified Tissue International*, *38*(6), 350-357.

Gibson, G. R., Scott, K. P., Rastall, R. A., Tuohy, K. M., Hotchkiss, A., Dubert-Ferrandon, A., ... & Macfarlane, S. (2010). Dietary prebiotics: current status and new definition. *Food Science & Technology Bulletin Functional Foods*, *7*(1), 1-19.

Gilbert, L., He, X., Farmer, P., Rubin, J., Drissi, H., Van Wijnen, A. J., ... & Nanes, M. S. (2002). Expression of the osteoblast differentiation factor RUNX2 (Cbfa1/AML3/Pebp2αA) is inhibited by tumor necrosis factor-α. *Journal of Biological Chemistry, 277*(4), 2695-2701.

Gilmore, T. D. (2006). Introduction to NF-κB: players, pathways, perspectives. *Oncogene, 25*(51), 6680.

Glimcher, M. J. (2006). Bone: nature of the calcium phosphate crystals and cellular, structural, and physical chemical mechanisms in their formation. *Reviews in Mineralogy and Geochemistry*, *64*(1), 223-282.

Gold, D. T. (2001). The nonskeletal consequences of osteoporotic fractures: psychologic and social outcomes. *Rheumatic Disease Clinics of North America*, *27*(1), 255-262.

Gorski, J. P. (1992). Acidic phosphoproteins from bone matrix: a structural rationalization of their role in biomineralization. *Calcified Tissue International*, *50*(5), 391-396.

Goulas, A., Triplett, E. L., & Taborsky, G. (1996). Oligophosphopeptides of varied structural complexity derived from the egg phosphoprotein, phosvitin. *Journal of Protein Chemistry, 15*(1), 1-9.

Grant, W. B., & Holick, M. F. (2005). Benefits and requirements of vitamin D for optimal health: a review. *Alternative Medicine Review, 10*(2), 94-111.

Graves, D. T., Jiang, Y., & Valente, A. J. (1999). The expression of monocyte chemoattractant protein-1 and other chemokines by osteoblasts. *Frontiers in Bioscience*, *4*, D571-D580.

Greengard, O., Sentenac, A., & Mendelsohn, N. (1964). Phosvitin, the iron carrier of egg yolk. *Biochimica et Biophysica Acta*, *90*, 406-407.

Grogan, J., Shirazi, A., & Taborsky, G. (1990). Phosphorus nuclear magnetic resonance of diverse phosvitin species. *Comparative biochemistry and physiology. B, Comparative Biochemistry*, *96*(4), 655-663.

Grynpas, M. D. (1990). Fluoride effects on bone crystals. *Journal of Bone and Mineral Research*, *5*(S1), S169-S175.

Guntur, A. R., & Rosen, C. J. (2013). IGF-1 regulation of key signaling pathways in bone. *BoneKEy Reports*, *2013*(2), 437.

Guo, H. Y., Jiang, L., Ibrahim, S. A., Zhang, L., Zhang, H., Zhang, M., & Ren, F. Z. (2009). Orally administered lactoferrin preserves bone mass and microarchitecture in ovariectomized rats. *The Journal of Nutrition, 139*(5), 958-964.

Gutierrez, M. A., Mitsuya, T., Hatta, H., Koketsu, M., Kobayashi, R., Juneja, L. R., & Kim, M. (1998). Comparison of egg-yolk protein hydrolysate and soyabean protein hydrolysate in terms of nitrogen utilization. *British Journal of Nutrition, 80*(5), 477-484.

Hadjidakis, D. J., & Androulakis, I. I. (2006). Bone remodeling. *Annals of the New York Academy of Sciences*, *1092*(1), 385-396.

Hahn, T. J. (2009). Metabolic bone disease. *Manual of Endocrinology and Metabolism. Baltimore, MD: Lippincott Willams and Wilkins*, 362-380.

Hara, A., & Radin, N. (1978). Lipid extraction of tissues with a low-toxicity solvent. *Analytical Biochemistry, 90*(1), 420-426.

Hara, T., Tanck, E., Homminga, J., & Huiskes, R. (2002). The influence of microcomputed tomography threshold variations on the assessment of structural and mechanical trabecular bone properties. *Bone, 31*(1), 107-109.

Hardy, R., & Cooper, M. S. (2009). Bone loss in inflammatory disorders. *Journal of Endocrinology*, *201*(3), 309-320.

Hartmann, R., & Meisel, H. (2007). Food-derived peptides with biological activity: from research to food applications. *Current Opinion in Biotechnology*, *18*(2), 163-169.

Hauge, E. M., Qvesel, D., Eriksen, E. F., Mosekilde, L., & Melsen, F. (2001). Cancellous bone remodeling occurs in specialized compartments lined by cells expressing osteoblastic markers. *Journal of Bone and Mineral Research*, *16*(9), 1575-1582.

Hayden, M. S., & Ghosh, S. (2004). NF-kB and Rel proteins: evolutionarily conserved mediators of immune responses. *Genes & Development, 18*, 2195-2224.

Hernlund, E., Svedbom, A., Ivergård, M., Compston, J., Cooper, C., Stenmark, J., ... & Kanis, J. A. (2013). Osteoporosis in the European Union: medical management, epidemiology and economic burden. *Archives of Osteoporosis*, *8*(1-2), 136.

Hiramatsu, N., Cheek, A. O., Sullivan, C. V., Matsubara, T., & Hara, A. (2005). Vitellogenesis and endocrine disruption. In *Biochemistry and Molecular Biology of Fishes* (Vol. 6, pp. 431-471). Elsevier.

Hodges, S. J., Akesson, K., Vergnaud, P., Obrant, K., & Delmas, P. D. (1993). Circulating levels of vitamins K_1 and K_2 decreased in elderly women with hip fracture. *Journal of Bone and Mineral Research*, *8*(10), 1241-1245.

Hodsman, A. B., Bauer, D. C., Dempster, D. W., Dian, L., Hanley, D. A., Harris, S. T., ... & Orwoll, E. (2005). Parathyroid hormone and teriparatide for the treatment of osteoporosis: a review of the evidence and suggested guidelines for its use. *Endocrine Reviews*, *26*(5), 688-703.

Högström, M., Nordström, P., & Nordström, A. (2007). n− 3 Fatty acids are positively associated with peak bone mineral density and bone accrual in healthy men: the NO2 Study. *The American Journal of Clinical Nutrition*, *85*(3), 803-807.

Horton, J. E., Raisz, L. G., Simmons, H. A., Oppenheim, J. J., & Mergenhagen, S. E. (1972). Bone resorbing activity in supernatant fluid from cultured human peripheral blood leukocytes. *Science*, *177*(4051), 793-795.

Hou, J. M., Xue, Y., & Lin, Q. M. (2012). Bovine lactoferrin improves bone mass and microstructure in ovariectomized rats via OPG/RANKL/RANK pathway. *Acta Pharmacologica Sinica*, *33*(10), 1277.

Hu, L., Sun, C., Wang, S., Su, F., & Zhang, S. (2013). Lipopolysaccharide neutralization by a novel peptide derived from phosvitin. *The International Journal of Biochemistry & Cell Biology, 45*(11), 2622-2631.

Huang, H., Zhao, N., Xu, X., Xu, Y., Li, S., Zhang, J., & Yang, P. (2011). Dose-specific effects of tumor necrosis factor alpha on osteogenic differentiation of mesenchymal stem cells. *Cell Proliferation, 44*(5), 420-427.

Hughes, D. E., Dai, A., Tiffee, J. C., Li, H. H., Mundy, G. R., & Boyce, B. F. (1996). Estrogen promotes apoptosis of murine osteoclasts mediated by TGF–β. *Nature Medicine*, *2*(10), 1132.

Hulmes, D. J. S., Wess, T. J., Prockop, D. J., & Fratzl, P. (1995). Radial packing, order, and disorder in collagen fibrils. *Biophysical Journal*, *68*(5), 1661-1670.

Huttunen, M. M., Pietilä, P. E., Viljakainen, H. T., & Lamberg-Allardt, C. J. (2006). Prolonged increase in dietary phosphate intake alters bone mineralization in adult male rats. *The Journal of Nutritional Biochemistry*, *17*(7), 479-484.

Ikeda, F., Nishimura, R., Matsubara, T., Tanaka, S., Inoue, J. I., Reddy, S. V., ... & Kukita, T. (2004). Critical roles of c-Jun signaling in regulation of NFAT family and RANKL-regulated osteoclast differentiation. *The Journal of Clinical Investigation, 114*(4), 475-484.

Ilich, J. Z., & Kerstetter, J. E. (2000). Nutrition in bone health revisited: a story beyond calcium. *Journal of the American College of Nutrition*, *19*(6), 715-737.

Imel, E. A., DiMeglio, L. A., & Burr, D. B. (2014). Metabolic Bone Diseases. In *Basic and Applied Bone Biology* (pp. 317-344).

Isaksson, H., Töyräs, J., Hakulinen, M., Aula, A. S., Tamminen, I., Julkunen, P., ... & Jurvelin, J. S. (2011). Structural parameters of normal and osteoporotic human trabecular bone are affected differently by microCT image resolution. *Osteoporosis International*, *22*(1), 167-177.

Jackson, R. D., LaCroix, A. Z., Gass, M., Wallace, R. B., Robbins, J., Lewis, C. E., ... & Bonds, D. E. (2006). Calcium plus vitamin D supplementation and the risk of fractures. *New England Journal of Medicine, 354*(7), 669-683.

Jiang, B., & Mine, Y. (2000). Preparation of novel functional oligophosphopeptides from hen egg yolk phosvitin. *Journal of Agricultural and Food Chemistry, 48*(4), 990-994.

Jiao, L., Cao, D. P., Qin, L. P., Han, T., Zhang, Q. Y., Zhu, Z., & Yan, F. (2009). Antiosteoporotic activity of phenolic compounds from *Curculigo orchioides*. *Phytomedicine*, *16*(9), 874-881.

Jie, Y., Cai, Z., Li, S., Xie, Z., Ma, M., & Huang, X. (2017). Hydroxyapatite nucleation and growth on collagen electrospun fibers controlled with different mineralization conditions and phosvitin. *Macromolecular Research*, *25*(9), 905-912.

Johnell, O., Kanis, J. A., Oden, A., Sernbo, I., Redlund-Johnell, I., Petterson, C., ... & Jönsson, B. (2004). Mortality after osteoporotic fractures. *Osteoporosis International*, *15*(1), 38-42.

Johnson, G. L., & Lapadat, R. (2002). Mitogen-activated protein kinase pathways mediated by ERK, JNK, and p38 protein kinases. *Science, 298*(5600), 1911-1912.

Jones, G., Riley, M., Couper, D., & Dwyer, T. (1999). Water fluoridation, bone mass and fracture: a quantitative overview of the literature. *Australian and New Zealand Journal of Public Health*, *23*(1), 34-40.

Joubert, F. J., & Cook, W. H. (1958). Preparation and characterization of phosvitin from hen egg yolk. *Canadian Journal of Biochemistry and Physiology, 36*(4), 399-408.

Jung, S., Nam, K. C., Ahn, D. U., Kim, H. J., & Jo, C. (2013). Effect of phosvitin on lipid and protein oxidation in ground beef treated with high hydrostatic pressure. *Meat Science*, *95*(1), 8-13.

Kanazawa, I., Yamaguchi, T., Yamamoto, M., Yamauchi, M., Yano, S., & Sugimoto, T. (2009). Serum osteocalcin/bone-specific alkaline phosphatase ratio is a predictor for the presence of vertebral fractures in men with type 2 diabetes. *Calcified Tissue International*, *85*(3), 228-234.

Kanis, J. A., & Johnell, O. (2005). Requirements for DXA for the management of osteoporosis in Europe. *Osteoporosis International*, *16*(3), 229-238.

Kanis, J. A. (1994). Assessment of fracture risk and its application to screening for postmenopausal osteoporosis: synopsis of a WHO report. *Osteoporosis International, 4*(6), 368-381.

Karsenty, G. (2008). Transcriptional control of skeletogenesis. *Annual Review of Genomics and Human Genetics*, *9*, 183-196.

Katayama, S., Xu, X., Fan, M. Z., & Mine, Y. (2006). Antioxidative stress activity of oligophosphopeptides derived from hen egg yolk phosvitin in Caco-2 cells. *Journal of Agricultural and Food Chemistry, 54*(3), 773-778.

Kawahara, T., Aruga, K., & Otani, H. (2005). Characterization of casein phosphopeptides from fermented milk products. *Journal of Nutritional Science and Vitaminology*, *51*(5), 377-381.

Khan, M. A. S., Nakamura, S., Ogawa, M., Akita, E., Azakami, H., & Kato, A. (2000). Bactericidal action of egg yolk phosvitin against *Escherichia coli* under thermal Stress. *Journal of Agricultural and Food Chemistry, 48*(5), 1503-1506.

Khosla, S. (2001). Minireview: The opg/rankl/rank system. *Endocrinology, 142*(12), 5050-5055.

Kim, M. S., Day, C. J., & Morrison, N. A. (2005). MCP-1 is induced by receptor activator of nuclear factor-κB ligand, promotes human osteoclast fusion, and rescues granulocyte macrophage colony-stimulating factor suppression of osteoclast formation. *Journal of Biological Chemistry, 280*(16), 16163-16169.

Kim, N., Kadono, Y., Takami, M., Lee, J., Lee, S. H., Okada, F., ... & Yeh, W. C. (2005). Osteoclast differentiation independent of the TRANCE–RANK–TRAF6 axis. *Journal of Experimental Medicine, 202*(5), 589-595.

Kim, Y., Sato, K., Asagiri, M., Morita, I., Soma, K., & Takayanagi, H. (2005). Contribution of nuclear factor of activated T cells c1 to the transcriptional control of immunoreceptor osteoclast-associated receptor but not triggering receptor expressed by myeloid cells-2 during osteoclastogenesis. *Journal of Biological Chemistry, 280*(38), 32905-32913.

Kimble, R. B., Matayoshi, A. B., Vannice, J. L., Kung, V. T., Williams, C., & Pacifici, R. (1995). Simultaneous block of interleukin-1 and tumor necrosis factor is required to completely prevent bone loss in the early postovariectomy period. *Endocrinology*, *136*(7), 3054-3061.

Kini, U., & Nandeesh, B. N. (2012). Physiology of bone formation, remodeling, and metabolism. In *Radionuclide and Hybrid Bone Imaging* (pp. 29-57). Springer, Berlin, Heidelberg.

Kitts, D. D. (1994). Bioactive substances in food: identification and potential uses. *Canadian Journal of Physiology and Pharmacology*, *72*(4), 423-434.

Kitts, D. D., & Yuan, Y. V. (1992). Caseinophosphopeptides and calcium biovailability. *Trends in Food Science & Technology,* 3, 31-35.

Knapen, M. H., Jie, K. S. G., Hamulyák, K., & Vermeer, C. (1993). Vitamin K-induced changes in markers for osteoblast activity and urinary calcium loss. *Calcified Tissue International*, *53*(2), 81-85.

Knapen, M. H. J., Schurgers, L. J., & Vermeer, C. (2007). Vitamin K2 supplementation improves hip bone geometry and bone strength indices in postmenopausal women. *Osteoporosis International*, *18*(7), 963-972.

Kobayashi, N., Onuma, K., Oyane, A., & Yamazaki, A. (2004). The role of phosvitin for nucleation of calcium phosphates on collagen. In *Key Engineering Materials* (Vol. 254, pp. 537-540). Trans Tech Publications.

Komori, T. (2009). Regulation of osteoblast differentiation by Runx2. In *Osteoimmunology* (pp. 43-49). Springer, Boston, MA.

Komori, T. (2011). Signaling networks in RUNX2-dependent bone development. *Journal of Cellular Biochemistry*, *112*(3), 750-755.

Komori, T., Yagi, H., Nomura, S., Yamaguchi, A., Sasaki, K., Deguchi, K., ... & Sato, M. (1997). Targeted disruption of Cbfa1 results in a complete lack of bone formation owing to maturational arrest of osteoblasts. *Cell, 89*(5), 755-764.

Korhonen, H., & Pihlanto, A. (2006). Bioactive peptides: production and functionality. *International Dairy Journal*, *16*(9), 945-960.

Kornak, U., Kasper, D., Bösl, M. R., Kaiser, E., Schweizer, M., Schulz, A., ... & Jentsch, T. J. (2001). Loss of the ClC-7 chloride channel leads to osteopetrosis in mice and man. *Cell*, *104*(2), 205-215.

Kotake, S., Udagawa, N., Hakoda, M., Mogi, M., Yano, K., Tsuda, E., ... & Saito, S. (2001). Activated human T cells directly induce osteoclastogenesis from human monocytes: possible role of T cells in bone destruction in rheumatoid arthritis patients. *Arthritis & Rheumatism: Official Journal of the American College of Rheumatology*, *44*(5), 1003-1012.

Kousteni, S., Bellido, T., Plotkin, L. I., O'brien, C. A., Bodenner, D. L., Han, L., ... & Roberson, P. K. (2001). Nongenotropic, sex-nonspecific signaling through the estrogen or androgen receptors: dissociation from transcriptional activity. *Cell*, *104*(5), 719-730.

Kushida, K., Takahashi, M., Kawana, K., & Inoue, T. (1995). Comparison of markers for bone formation and resorption in premenopausal and postmenopausal subjects, and osteoporosis patients. *The Journal of Clinical Endocrinology & Metabolism*, *80*(8), 2447-2450.

Kwan, L., Lichan, E., Helbig, N., & Nakai, S. (1991). Fractionation of water-soluble and water-insoluble components from egg-yolk with minimum use of organic-solvents. *Journal of Food Science, 56*(6), 1537-1541.

Laster, A. J., & Tanner, S. B. (2011). Duration of treatment in postmenopausal osteoporosis: how long to treat and what are the consequences of cessation of treatment? *Rheumatic Disease Clinics*, *37*(3), 323-336.

Lecomte, S., Demay, F., Ferrière, F., & Pakdel, F. (2017). Phytochemicals targeting estrogen receptors: beneficial rather than adverse effects? *International Journal of Molecular Sciences, 18*(7), 1381.

Leabu, M. (2006). Membrane fusion in cells: molecular machinery and mechanisms. *Journal of Cellular and Molecular Medicine*, *10*(2), 423-427.

Lerner, U. H. (2004). New molecules in the tumor necrosis factor ligand and receptor superfamilies with importance for physiological and pathological bone resorption. *Critical Reviews in Oral Biology & Medicine, 15*(2), 64-81.

Levental, K. R., Surma, M. A., Skinkle, A. D., Lorent, J. H., Zhou, Y., Klose, C., ... & Levental, I. (2017). ω-3 polyunsaturated fatty acids direct differentiation of the membrane phenotype in mesenchymal stem cells to potentiate osteogenesis. *Science Advances*, *3*(11), eaao1193.

Li, X., Udagawa, N., Itoh, K., Suda, K., Murase, Y., Nishihara, T., ... & Takahashi, N. (2002). p38 MAPK-mediated signals are required for inducing osteoclast differentiation but not for osteoclast function. *Endocrinology, 143*(8), 3105-3113.

Li, Z., Wang, P., Jiang, C., Cui, P., & Zhang, S. (2016). Antibacterial activity and modes of action of phosvitin-derived peptide Pt5e against clinical multi-drug resistance bacteria. *Fish & Shellfish Immunology*, *58*, 370-379.

Libouban, H., Moreau, M. F., Baslé, M. F., Bataille, R., & Chappard, D. (2003). Increased bone remodeling due to ovariectomy dramatically increases tumoral growth in the 5T2 multiple myeloma mouse model. *Bone, 33*(3), 283-292.

Lisignoli, G., Toneguzzi, S., Grassi, F., Piacentini, A., Tschon, M., Cristino, S., ... & Facchini, A. (2002). Different chemokines are expressed in human arthritic bone biopsies: IFN-γ and IL-6 differently modulate IL-8, MCP-1 and RANTES production by arthritic osteoblasts. *Cytokine, 20*(5), 231-238.

Liu, J., Czernick, D., Lin, S. C., Alasmari, A., Serge, D., & Salih, E. (2013). Novel bioactivity of phosvitin in connective tissue and bone organogenesis revealed by live calvarial bone organ culture models. *Developmental Biology, 381*(1), 256-275.

Liu, Q., Li, C., Geng, F., Huang, X., & Ma, M. (2017). Hen egg yolk phosvitin stimulates osteoblast differentiation in the absence of ascorbic acid. *Journal of the Science of Food and Agriculture, 97*(13), 4532-4538.

Liu, Y. C., Kao, Y. T., Huang, W. K., Lin, K. Y., Wu, S. C., Hsu, S. C., ... & Lu, J. (2014). CCL5/RANTES is important for inducing osteogenesis of human mesenchymal stem cells and is regulated by dexamethasone. *Bioscience Trends, 8*(3), 138-143.

Liu, X. H., Kirschenbaum, A., Yao, S., & Levine, A. C. (2006). Interactive Effect of Interleukin-6 and Prostaglandin E2 on Osteoclastogenesis via the OPG/RANKL/RANK System. *Annals of the New York Academy of Sciences, 1068*(1), 225-233.

Long, C. L., Berry, W. L., Zhao, Y., Sun, X. H., & Humphrey, M. B. (2012). E proteins regulate osteoclast maturation and survival. *Journal of Bone and Mineral Research*, *27*(12), 2476-2489.

Lorenzo, J. A., Naprta, A., Rao, Y., Alander, C., Glaccum, M., Widmer, M., ... & Pilbeam, C. C. (1998). Mice lacking the type I interleukin-1 receptor do not lose bone mass after ovariectomy. *Endocrinology*, *139*(6), 3022-3025.

Lorget, F., Clough, J., Oliveira, M., Daury, M. C., Sabokbar, A., & Offord, E. (2002). Lactoferrin reduces *in vitro* osteoclast differentiation and resorbing activity. *Biochemical and Biophysical Research Communications*, *296*(2), 261-266.

Losso, J. N., & Nakai, S. (1994). A simple procedure for the isolation of phosvitin from chicken egg yolk. In *Egg Uses and Processing Technologies: New Developments*; Sim, J. S., Nakai, S., Eds.; Cab International: Oxon, U.K.

Lu, C. L., & Baker, R. C. (1987). Effect of pH and food ingredients on the stability of egg yolk phospholipids and the metal-chelator antioxidant activity of phosvitin. *Journal of Food Science*, *52*(3), 613-616.

Lu, Z., Wang, G., Dunstan, C. R., & Zreiqat, H. (2012). Short-term exposure to tumor necrosis factor-alpha enables human osteoblasts to direct adipose tissue-derived mesenchymal stem cells into osteogenic differentiation. *Stem Cells and Development, 21*(13), 2420-2429.

Luc, G., Bard, J. M., Juhan-Vague, I., Ferrieres, J., Evans, A., Amouyel, P., ... & Ducimetiere, P. (2003). C-reactive protein, interleukin-6, and fibrinogen as predictors of coronary heart disease: the PRIME study. *Arteriosclerosis, Thrombosis, and Vascular Biology, 23*(7), 1255-1261.

Ma, J., Wang, H., Wang, Y., & Zhang, S. (2013). Endotoxin-neutralizing activity of hen egg phosvitin. *Molecular Immunology, 53*(4), 355-362.

Maheswari, S. U., Ramadoss, C. S., & Krishnaswamy, P. R. (1997). Inhibition of Fe (II) catalyzed linoleic acid oxidation and DNA damage by phosvitin. *Molecular and Cellular Biochemistry*, *177*(1-2), 47-51.

Mandadi, K., Ramirez, M., Jayaprakasha, G. K., Faraji, B., Lihono, M., Deyhim, F., & Patil, B. S. (2009). Citrus bioactive compounds improve bone quality and plasma antioxidant activity in orchidectomized rats. *Phytomedicine, 16*(6-7), 513-520.

Manolagas, S. C. (2010). From estrogen-centric to aging and oxidative stress: a revised perspective of the pathogenesis of osteoporosis. *Endocrine Reviews*, *31*(3), 266-300.

Manolagas, S. C., & Parfitt, A. M. (2010). What old means to bone. *Trends in Endocrinology & Metabolism, 21*(6), 369-374.

Marie, P. J., Ammann, P., Boivin, G., & Rey, C. (2001). Mechanisms of action and therapeutic potential of strontium in bone. *Calcified Tissue International, 69*(3), 121-129.

Marie, P. J., Hott, M., Modrowski, D., De Pollak, C., Guillemain, J., Deloffre, P., & Tsouderos, Y. (1993). An uncoupling agent containing strontium prevents bone loss by depressing bone resorption and maintaining bone formation in estrogen-deficient rats. *Journal of Bone and Mineral Research*, *8*(5), 607-615.

Matsui, T., Yano, H., Awano, T., Harumoto, T., & Saito, Y. (1994). The influences of casein phosphopeptides on metabolism of ectopic bone induced by decalcified bone matrix implantation in rats. *Journal of Nutritional Science and Vitaminology, 40*(2), 137-145.

Matsuo, K., Galson, D. L., Zhao, C., Peng, L., Laplace, C., Wang, K. Z., ... & Wagner, E. F. (2004). Nuclear factor of activated T-cells (NFAT) rescues osteoclastogenesis in precursors lacking c-Fos. *Journal of Biological Chemistry, 279*(25), 26475-26480.

Maurin, A. C., Chavassieux, P. M., & Meunier, P. J. (2005). Expression of PPARγ and β/δ in human primary osteoblastic cells: influence of polyunsaturated fatty acids. *Calcified Tissue International*, *76*(5), 385-392.

Mayes, S. L. (2007). Review of postmenopausal osteoporosis pharmacotherapy. *Nutrition in Clinical Practice*, *22*(3), 276-285.

Mcbee, L. E., & Cotterill, O. J. (1979). Ion-exchange chromatography and electrophoresis of egg-yolk proteins. *Journal of Food Science, 44*(3), 656-660.

McCaslin Jr, F. E., & Janes, J. M. (1959). The effect of strontium lactate in the treatment of osteoporosis. In *Proceedings of Staff Meetings of the Mayo Clinic* (Vol. 34, pp. 329-334).

Mccully, K., Common, R., & Mok, C. (1962). Paper electrophoretic characterization of proteins and lipoproteins of hen's egg yolk. *Canadian Journal of Biochemistry and Physiology, 40*(7), 937-&.

Meisel, H., & FitzGerald, R. J. (2003). Biofunctional peptides from milk proteins: mineral binding and cytomodulatory effects. *Current Pharmaceutical Design*, *9*(16), 1289-1296.

Minkin, C. (1982). Bone acid phosphatase: tartrate-resistant acid phosphatase as a marker of osteoclast function. *Calcified Tissue International, 34*(1), 285-290.

Miquel, E., Alegría, A., Barberá, R., & Farré, R. (2006). Casein phosphopeptides released by simulated gastrointestinal digestion of infant formulas and their potential role in mineral binding. *International Dairy Journal*, *16*(9), 992-1000.

Moran Jr, E. T. (2007). Nutrition of the developing embryo and hatchling. *Poultry Science*, *86*(5), 1043-1049.

Morrison, N. A., Qi, J. C., Tokita, A., Kelly, P. J., Crofts, L., Nguyen, T. V., ... & Eisman, J. A. (1994). Prediction of bone density from vitamin D receptor alleles. *Nature*, *367*(6460), 284.

Mundy, G. R. (2007). Osteoporosis and inflammation. *Nutrition Reviews, 65*(suppl_3), S147-S151.

Muto, A., Mizoguchi, T., Udagawa, N., Ito, S., Kawahara, I., Abiko, Y., ... & Penninger, J. M. (2011). Lineage-committed osteoclast precursors circulate in blood and settle down into bone. *Journal of Bone and Mineral Research*, *26*(12), 2978-2990.

Naik, A. A., Xie, C., Zuscik, M. J., Kingsley, P., Schwarz, E. M., Awad, H., ... & Zhang, X. (2009). Reduced COX-2 expression in aged mice is associated with impaired fracture healing. *Journal of Bone and Mineral Research, 24*(2), 251-264.

Nakamura, S., Ogawa, M., Nakai, S., Kato, A., & Kitts, D. D. (1998). Antioxidant activity of a maillard-type phosvitin-galactomannan conjugate with emulsifying properties and heat stability. *Journal of Agricultural and Food Chemistry, 46*(10), 3958-3963.

Naot, D., Grey, A., Reid, I. R., & Cornish, J. (2005). Lactoferrin–a novel bone growth factor. *Clinical Medicine & Research, 3*(2), 93-101.

Navidghasemizad, S., Temelli, F., & Wu, J. (2014). Physicochemical properties of leftover egg yolk after livetins removal. *Lwt-Food Science and Technology, 55*(1), 170-175.

Negishi-Koga, T., Shinohara, M., Komatsu, N., Bito, H., Kodama, T., Friedel, R. H., & Takayanagi, H. (2011). Suppression of bone formation by osteoclastic expression of semaphorin 4D. *Nature Medicine*, *17*(11), 1473.

OpenStax. (2014, July 30). Anatomy & physiology. Retrieved from http://cnx.org/contents/14fb4ad7-39a1-4eee-ab6e-3ef2482e3e22@6.27.

Otani, H., Watanabe, T., & Tashiro, Y. (2001). Effects of bovine β-casein (1-28) and its chemically synthesized partial fragments on proliferative responses and immunoglobulin

production in mouse spleen cell cultures. *Bioscience, Biotechnology, and Biochemistry*, *65*(11), 2489-2495.

Pfeilschifter, J., Köditz, R., Pfohl, M., & Schatz, H. (2002). Changes in proinflammatory cytokine activity after menopause. *Endocrine Reviews*, *23*(1), 90-119.

Pittenger, M. F., Mackay, A. M., Beck, S. C., Jaiswal, R. K., Douglas, R., Mosca, J. D., ... & Marshak, D. R. (1999). Multilineage potential of adult human mesenchymal stem cells. *Science*, *284*(5411), 143-147.

Post, T. M., Cremers, S. C., Kerbusch, T., & Danhof, M. (2010). Bone physiology, disease and treatment. *Clinical Pharmacokinetics*, *49*(2), 89-118.

Potter, S. M., Baum, J. A., Teng, H., Stillman, R. J., Shay, N. F., & Erdman Jr, J. W. (1998). Soy protein and isoflavones: their effects on blood lipids and bone density in postmenopausal women. *The American Journal of Clinical Nutrition, 68*(6), 1375S-1379S.

Prentice, A. (1997). Is nutrition important in osteoporosis? *Proceedings of the Nutrition Society*, *56*(1B), 357-367.

Prince, R. L. (1994). Counterpoint: estrogen effects on calcitropic hormones and calcium homeostasis. *Endocrine Reviews*, *15*(3), 301-309.

Prockop, D. J. (1971). Role of iron in the synthesis of collagen in connective tissue. In *Federation Proceedings* (Vol. 30, No. 3, pp. 984-990).

Puel, C., Mathey, J., Agalias, A., Kati-coulibaly, S., Mardon, J., Obled, C., ... & Coxam, V. (2006). Dose–response study of effect of oleuropein, an olive oil polyphenol, in an

ovariectomy/inflammation experimental model of bone loss in the rat. *Clinical Nutrition*, *25*(5), 859-868.

Puel, C., Quintin, A., Agalias, A., Mathey, J., Obled, C., Mazur, A., ... & Coxam, V. (2004). Olive oil and its main phenolic micronutrient (oleuropein) prevent inflammation-induced bone loss in the ovariectomised rat. *British Journal of Nutrition*, *92*(1), 119-127.

Rachner, T. D., Khosla, S., & Hofbauer, L. C. (2011). Osteoporosis: now and the future. *The Lancet, 377*(9773), 1276-1287.

Raggatt, L. J., & Partridge, N. C. (2010). Cellular and molecular mechanisms of bone remodeling. *Journal of Biological Chemistry, 285*(33), 25103-25108.

Rahman, M. M., Bhattacharya, A., & Fernandes, G. (2006). Conjugated linoleic acid inhibits osteoclast differentiation of RAW264. 7 cells by modulating RANKL signaling. *Journal of Lipid Research, 47*(8), 1739-1748.

Raisz, L. G. (2005). Pathogenesis of osteoporosis: concepts, conflicts, and prospects. *The Journal of Clinical Investigation, 115*(12), 3318-3325.

Ralston, S. H., Ho, L. P., Helfrich, M. H., Grabowski, P. S., Johnston, P. W., & Benjamin, N. (1995). Nitric oxide: A cytokine-induced regulator of bone resorption. *Journal of Bone and Mineral Research, 10*(7), 1040-1049.

Ren, J., Li, Q., Offengenden, M., & Wu, J. (2015). Preparation and characterization of phosphopeptides from egg yolk phosvitin. *Journal of Functional Foods, 18*, 190-197.

Ren, J., & Wu, J. (2014). Preparation of high purity egg phosvitin using anion exchange chromatography. *Food Chemistry, 158*, 186-191.

Ren, J., & Wu, J. (2015). Thermal-aided phosvitin extraction from egg yolk. *Journal of the Science of Food and Agriculture, 95*(13), 2595-2600.

Rissanen, J. P., Suominen, M. I., Peng, Z., & Halleen, J. M. (2008). Secreted tartrate-resistant acid phosphatase 5b is a Marker of osteoclast number in human osteoclast cultures and the rat ovariectomy model. *Calcified Tissue International, 82*(2), 108-115.

Rivas, A., Romero, A., Mariscal-Arcas, M., Monteagudo, C., Feriche, B., Lorenzo, M. L., & Olea, F. (2013). Mediterranean diet and bone mineral density in two age groups of women. *International Journal of Food Sciences and Nutrition*, *64*(2), 155-161.

Roberfroid, M. B. (2000). Prebiotics and probiotics: are they functional foods? *The American Journal of Clinical Nutrition*, *71*(6), 1682S-1687S.

Romero Perez, A., & Rivas Velasco, A. (2014). Adherence to Mediterranean diet and bone health. *Nutricion Hospitalaria*, *29*(5), 989-996.

Rosen, H. N., Moses, A. C., Garber, J., Iloputaife, I. D., Ross, D. S., Lee, S. L., & Greenspan, S. L. (2000). Serum CTX: a new marker of bone resorption that shows treatment effect more often than other markers because of low coefficient of variability and large changes with bisphosphonate therapy. *Calcified Tissue International, 66*(2), 100-103.

Royce, P. M., & Steinmann, B. (Eds.). (2003). *Connective Tissue and Its Heritable Disorders: Molecular, Genetic, and Medical Aspects*. John Wiley & Sons.

Rude, R. K. (2008). Magnesium homeostasis. In *Principles of Bone Biology (Third Edition)* (pp. 487-513).

Rude, R. K., & Gruber, H. E. (2004). Magnesium deficiency and osteoporosis: animal and human observations. *The Journal of Nutritional Biochemistry*, *15*(12), 710-716.

Russell, A. L., Grimes, J. M., Cruthirds, D. F., Westerfield, J., Wooten, L., Keil, M., ... & Larco, D. O. (2017). Dietary isoflavone-dependent and estradiol replacement effects on body weight in the ovariectomized (OVX) rat. *Hormone and Metabolic Research, 49*(6), 457.

Ryu, B., Qian, Z. J., & Kim, S. K. (2010). Purification of a peptide from seahorse, that inhibits TPA-induced MMP, iNOS and COX-2 expression through MAPK and NF-κB activation, and induces human osteoblastic and chondrocytic differentiation. *Chemico-Biological Interactions, 184*(3), 413-422.

Saftig, P., Hunziker, E., Wehmeyer, O., Jones, S., Boyde, A., Rommerskirch, W., ... & Von Figura, K. (1998). Impaired osteoclastic bone resorption leads to osteopetrosis in cathepsin-K-deficient mice. *Proceedings of the National Academy of Sciences*, *95*(23), 13453-13458.

Sahai, E., & Marshall, C. J. (2002). RHO–GTPases and cancer. *Nature Reviews Cancer*, *2*(2), 133.

Sakanaka, S., Tachibana, Y., Ishihara, N., & Juneja, L. R. (2005). Antioxidant properties of casein calcium peptides and their effects on lipid oxidation in beef homogenates. *Journal of Agricultural and Food Chemistry*, *53*(2), 464-468.

Sato, R., Noguchi, T., & Naito, H. (1986). Casein phosphopeptide (CPP) enhances calcium absorption form the ligated segment of rat small intestine. *Journal of Nutritional Science and Vitaminology*, *32*(1), 67-76.

Scholz-Ahrens, K. E., & Schrezenmeir, J. (2002). Inulin, oligofructose and mineral metabolism—experimental data and mechanism. *British Journal of Nutrition*, *87*(S2), S179-S186.

Schrezenmeir, J., & de Vrese, M. (2001). Probiotics, prebiotics, and synbiotics—approaching a definition. *American Journal of Clinical Nutrition*, *73*, *361S-364S*.

Seeman, E. (2009). Bone modeling and remodeling. *Critical Reviews™ in Eukaryotic Gene Expression, 19*(3), 219-233.

Seeman, E., & Delmas, P. D. (2006). Bone quality—the material and structural basis of bone strength and fragility. *New England Journal of Medicine*, *354*(21), 2250-2261.

Setchell, K. D. (2001). Soy isoflavones—benefits and risks from nature's selective estrogen receptor modulators (SERMs). *Journal of the American College of Nutrition*, *20*(sup5), 354S-362S.

Shea, B., Wells, G., Cranney, A., Zytaruk, N., Robinson, V., Griffith, L., ... & Guyatt, G. (2002). VII. Meta-analysis of calcium supplementation for the prevention of postmenopausal osteoporosis. *Endocrine Reviews*, *23*(4), 552-559.

Shen, C. L., Yeh, J. K., Cao, J. J., & Wang, J. S. (2009). Green tea and bone metabolism. *Nutrition Research, 29*(7), 437-456.

Shevde, N. K., Bendixen, A. C., Dienger, K. M., & Pike, J. W. (2000). Estrogens suppress RANK ligand-induced osteoclast differentiation via a stromal cell independent mechanism involving c-Jun repression. *Proceedings of the National Academy of Sciences, 97*(14), 7829-7834.

Shorr, E. & CARTER A. C. (1952). The usefulness of strontium as an adjuvant to calcium in the remineralization of the skeleton in man. *Bulletin of the Hospital for Joint Diseases*, *13*, 59-66.

Sims, N. A., & Gooi, J. H. (2008, October). Bone remodeling: Multiple cellular interactions required for coupling of bone formation and resorption. In *Seminars in Cell & Developmental Biology* (Vol. 19, No. 5, pp. 444-451). Academic Press.

Sirvente, H., Beaumal, V., Gaillard, C., Bialek, L., Hamm, D., & Anton, M. (2007). Structuring and functionalization of dispersions containing egg yolk, plasma and granules induced by mechanical treatments. *Journal of Agricultural and Food Chemistry, 55*(23), 9537-9544.

Sjögren, K., Engdahl, C., Henning, P., Lerner, U. H., Tremaroli, V., Lagerquist, M. K., ... & Ohlsson, C. (2012). The gut microbiota regulates bone mass in mice. *Journal of Bone and Mineral Research*, *27*(6), 1357-1367.

Spina, A., Sorvillo, L., Esposito, A., Borgia, A., Sapio, L., & Naviglio, S. (2013). Inorganic phosphate as a signaling molecule: A potential strategy in osteosarcoma treatment. *Current Pharmaceutical Design, 19*(30), 5394-5403.

Statistics Canada. (2009). *Canadian Community Health Survey*. Retrieved from http://www.phac-aspc.gc.caSteeve, K. T., Marc, P., Sandrine, T., Dominique, H., & Yannick, F. (2004). IL-6, RANKL, TNF-alpha/IL-1: interrelations in bone resorption pathophysiology. *Cytokine & Growth Factor Reviews, 15*(1), 49-60.

Sugano, H. (1958). Studies on egg yolk proteins. 3. Preparation and properties of 2 major lipoproteins of the egg yolk, alpha-lipovitellin and beta-lipovitellin. *Journal of Biochemistry, 45*(6), 393-401.

Sun, D., Krishnan, A., Zaman, K., Lawrence, R., Bhattacharya, A., & Fernandes, G. (2003). Dietary n-3 fatty acids decrease osteoclastogenesis and loss of bone mass in ovariectomized mice. *Journal of Bone and Mineral Research*, *18*(7), 1206-1216.

Surh, Y. J., Chun, K. S., Cha, H. H., Han, S. S., Keum, Y. S., Park, K. K., & Lee, S. S. (2001). Molecular mechanisms underlying chemopreventive activities of anti-inflammatory phytochemicals: down-regulation of COX-2 and iNOS through suppression of NF-κB activation. *Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis, 480*, 243-268.

Suzuki, E., Ochiai-Shino, H., Aoki, H., Onodera, S., Saito, A., Saito, A., & Azuma, T. (2014). Akt activation is required for TGF-β1-induced osteoblast differentiation of MC3T3-E1 pre-osteoblasts. *PloS ONE, 9*(12), e112566.

Szulc, P., Arlot, M., Chapuy, M. C., Duboeuf, F., Meunier, P. J., & Delmas, P. D. (1994). Serum undercarboxylated osteocalcin correlates with hip bone mineral density in elderly women. *Journal of Bone and Mineral Research, 9*(10), 1591-1595.

Szulc, P., & Delmas, P. D. (2008). Biochemical markers of bone turnover: potential use in the investigation and management of postmenopausal osteoporosis. *Osteoporosis International*, *19*(12), 1683-1704.

Takatsuna, H., Asagiri, M., Kubota, T., Oka, K., Osada, T., Sugiyama, C., ... & Umezawa, K. (2005). Inhibition of RANKL-Induced Osteoclastogenesis by (−)-DHMEQ, a Novel NF-κB

Inhibitor, Through Downregulation of NFATc1. *Journal of Bone and Mineral Research, 20*(4), 653-662.

Takayanagi, H., Kim, S., Koga, T., Nishina, H., Isshiki, M., Yoshida, H., ... & Wagner, E. F. (2002). Induction and activation of the transcription factor NFATc1 (NFAT2) integrate RANKL signaling in terminal differentiation of osteoclasts. *Developmental Cell, 3*(6), 889-901.

Tarride, J. E., Guo, N., Hopkins, R., Leslie, W. D., Morin, S., Adachi, J. D., ... & Goeree, R. (2012). The burden of illness of osteoporosis in Canadian men. *Journal of Bone and Mineral Research*, *27*(8), 1830-1838.

Teitelbaum, S. L. (2000). Bone resorption by osteoclasts. *Science, 289*(5484), 1504-1508.

Teitelbaum, S. L., & Ross, F. P. (2003). Genetic regulation of osteoclast development and function. *Nature Reviews Genetics, 4*(8), 638.

Terano, T. (2001). Effect of ω3 polyunsaturated fatty acid ingestion on bone metabolism and osteoporosis. In *Fatty Acids and Lipids-New Findings* (Vol. 88, pp. 141-147). Karger Publishers.

Ternes, W. (1989). Characterization of water-soluble egg-yolk proteins with isoelectric-focusing. *Journal of Food Science, 54*(3), 764-765.

Ting, C. P., Bertrand, P., Pouliot, Y., Juneja, L. R., Okubo, T., Gauthier, S. F., & Mine, Y. (2010). On the use of ultrafiltration for the concentration and desalting of phosvitin from egg yolk protein concentrate. *International Journal of Food Science & Technology, 45*(8), 1633-1640.

Tracey, D., Klareskog, L., Sasso, E. H., Salfeld, J. G., & Tak, P. P. (2008). Tumor necrosis factor antagonist mechanisms of action: a comprehensive review. *Pharmacology & Therapeutics, 117*(2), 244-279.

Tsuchita, H., Goto, T., Yonehara, Y., & Kuwata, T. (1995). Calcium and phosphorus availability from casein phosphopeptides in male growing rats. *Nutrition Research, 15*(11), 1657-1667.

Tsutsui, T., & Obara, T. (1984). Preparation and characterization of phosvitin from hen's egg-yolk granule. *Agricultural and Biological Chemistry, 48*(5), 1153-1160.

Tuck, S., Layfield, R., Walker, J., Mekkayil, B., & Francis, R. (2017). Adult Paget's disease of bone: a review. *Rheumatology*, *56*(12), 2050-2059.

Uenishi, K., Ishida, H., Toba, Y., Aoe, S., Itabashi, A., & Takada, Y. (2007). Milk basic protein increases bone mineral density and improves bone metabolism in healthy young women. *Osteoporosis International*, *18*(3), 385-390.

US Department of Health and Human Services. (2004). Bone health and osteoporosis: a report of the Surgeon General. *Rockville, MD: US Department of Health and Human Services, Office of the Surgeon General*, 87.

Vasikaran, S., Eastell, R., Bruyère, O., Foldes, A. J., Garnero, P., Griesmacher, A., ... & Wahl, D. A. (2011). Markers of bone turnover for the prediction of fracture risk and monitoring of osteoporosis treatment: a need for international reference standards. *Osteoporosis International, 22*(2), 391-420.

Vijayan, V., Khandelwal, M., Manglani, K., Gupta, S., & Surolia, A. (2014). Methionine down-regulates TLR 4/MyD 88/NF-κ B signalling in osteoclast precursors to reduce bone loss during osteoporosis. *British Journal of Pharmacology, 171*(1), 107-121.

Vincent, K., & Durrant, M. C. (2013). A structural and functional model for human bone sialoprotein. *Journal of Molecular Graphics and Modelling*, *39*, 108-117.

Vivanco, I., & Sawyers, C. L. (2002). The phosphatidylinositol 3-kinase–AKT pathway in human cancer. *Nature Reviews Cancer, 2*(7), 489.

Wagner, E. F. (2002). Functions of AP1 (Fos/Jun) in bone development. *Annals of the Rheumatic Diseases*, *61*(suppl 2), ii40-ii42.

Wallach, S. (1990). Effects of magnesium on skeletal metabolism. *Magnesium and Trace Elements*, *9*(1), 1-14.

Wan, Y. (2010). PPARγ in bone homeostasis. *Trends in Endocrinology & Metabolism*, *21*(12), 722-728.

Wang, S., Wang, Y., Ma, J., Ding, Y., & Zhang, S. (2011). Phosvitin plays a critical role in the immunity of zebrafish embryos via acting as a pattern recognition receptor and an antimicrobial effector. *Journal of Biological Chemistry, 286*(25), 22653-22664.

Weinstein, R. S., Roberson, P. K., & Manolagas, S. C. (2009). Giant osteoclast formation and long-term oral bisphosphonate therapy. *New England Journal of Medicine, 360*(1), 53-62.

Weiss, L. A., Barrett-Connor, E., & von Mühlen, D. (2005). Ratio of n–6 to n–3 fatty acids and bone mineral density in older adults: the Rancho Bernardo Study. *The American Journal of Clinical Nutrition*, *81*(4), 934-938.

Whisner, C. M., & Castillo, L. F. (2018). Prebiotics, bone and mineral metabolism. *Calcified Tissue International*, 1-37.

Willett, WC., & Buzzard M. (1998) Nature of variation in diet. In: *Willett WC, ed. Nutritional Epidemiology, 2nd ed*, New York, Oxford University Press, 33–49.

Wright, N. C., Looker, A. C., Saag, K. G., Curtis, J. R., Delzell, E. S., Randall, S., & Dawson- Hughes, B. (2014). The recent prevalence of osteoporosis and low bone mass in the United States based on bone mineral density at the femoral neck or lumbar spine. *Journal of Bone and Mineral Research*, *29*(11), 2520-2526.

Wright, L. M., Maloney, W., Yu, X., Kindle, L., Collin-Osdoby, P., & Osdoby, P. (2005). Stromal cell-derived factor-1 binding to its chemokine receptor CXCR4 on precursor cells promotes the chemotactic recruitment, development and survival of human osteoclasts. *Bone*, *36*(5), 840-853.

Writing Group for the Women's Health Initiative Investigators. (2002). Risks and benefits of estrogen plus progestin in healthy postmenopausal women: principal results from the Women's Health Initiative randomized controlled trial. *Journal of the American Medical Association, 288*(3), 321-333.

Wysowski, D. K., & Chang, J. T. (2005). Alendronate and risedronate: reports of severe bone, joint, and muscle pain. *Archives of Internal Medicine, 165*(3), 346-347.

Xiong, J., Onal, M., Jilka, R. L., Weinstein, R. S., Manolagas, S. C., & O'Brien, C. A. (2011). Matrix-embedded cells control osteoclast formation. *Nature Medicine*, *17*(10), 1235.

Xu, X., Katayama, S., & Mine, Y. (2007). Antioxidant activity of tryptic digests of hen egg yolk phosvitin. *Journal of the Science of Food and Agriculture, 87*(14), 2604-2608.

Xu, C., Yang, C., Yin, Y., Liu, J., & Mine, Y. (2012). Phosphopeptides (PPPs) from hen egg yolk phosvitin exert anti-inflammatory activity via modulation of cytokine expression. *Journal of Functional Foods, 4*(4), 718-726.

Yamamoto, K., Arakawa, T., Ueda, N., & Yamamoto, S. (1995). Transcriptional roles of nuclear factor B and nuclear factor-interleukin-6 in the tumor necrosis factor-dependent induction of cyclooxygenase-2 in MC3T3-E1 cells. *Journal of Biological Chemistry, 270*(52), 31315-31320.

Young, D., Nau, F., Pasco, M., & Mine, Y. (2011). Identification of hen egg yolk-derived phosvitin phosphopeptides and their effects on gene expression profiling against oxidative stress-induced Caco-2 cells. *Journal of Agricultural and Food Chemistry, 59*(17), 9207-9218.

Yu, W., Field, C. J., & Wu, J. P. (2018). Spent hen muscle protein hydrolysate: a potential IL-10 stimulator in a murine model. *Food & Function, 9,* 4714-4719.

Yu, X., Huang, Y., Collin- Osdoby, P., & Osdoby, P. (2003). Stromal Cell-Derived Factor- 1 (SDF- 1) Recruits osteoclast precursors by inducing chemotaxis, matrix metalloproteinase- 9 (MMP- 9) activity, and collagen transmigration. *Journal of Bone and Mineral Research, 18*(8), 1404-1418.

Yu, X., Schneiderhan-Marra, N., Hsu, H. Y., Bachmann, J., & Joos, T. O. (2009). Protein microarrays: effective tools for the study of inflammatory diseases. In *Reverse Chemical Genetics* (pp. 199-214). Humana Press, Totowa, NJ.

Yun, A. J., & Lee, P. Y. (2004). Maldaptation of the link between inflammation and bone turnover may be a key determinant of osteoporosis. *Medical Hypotheses*, *63*(3), 532-537.

Zaid, S. S., Sulaiman, S. A., Sirajudeen, K. N., & Othman, N. H. (2010). The effects of Tualang honey on female reproductive organs, tibia bone and hormonal profile in ovariectomised rats-animal model for menopause. *BMC Complementary and Alternative Medicine, 10*(1), 82.

Zhang, W., & Liu, H. T. (2002). MAPK signal pathways in the regulation of cell proliferation in mammalian cells. *Cell Research*, *12*(1), 9-18.

Zhang, W., Guo, H., Jing, H., Li, Y., Wang, X., Zhang, H., ... & Ren, F. (2014). Lactoferrin stimulates osteoblast differentiation through PKA and p38 pathways independent of lactoferrin's receptor LRP1. *Journal of Bone and Mineral Research, 29*(5), 1232-1243.

Zhao, J. G., Zeng, X. T., Wang, J., & Liu, L. (2017). Association between calcium or vitamin D supplementation and fracture incidence in community-dwelling older adults: a systematic review and meta-analysis. *Journal of the American Medical Association, 318*(24), 2466-2482.

Zhu, J. F., Valente, A. J., Lorenzo, J. A., Carnes, D., & Graves, D. T. (1994). Expression of monocyte chemoattractant protein 1 in human osteoblastic cells stimulated by proinflammatory mediators. *Journal of Bone and Mineral Research, 9*(7), 1123-1130.