The Potential of Egg White Ovotransferrin as a Functional Food Ingredient against Osteoporosis

by

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ABSTRACT

Ovotransferrin, an iron-binding glycoprotein, accounting for ~12% of egg white protein, is a member of the transferrin family. As a component of innate immunity, ovotransferrin has antimicrobial, anti-viral, anti-oxidant, and immunomodulatory activities. The overall objectives of this thesis were to understand the role of ovotransferrin on bone cells (osteoblasts and osteoclasts) and to explore its potential application as a bioactive protein against osteoporosis.

Using mouse preosteoblast MC3T3-E1 cells, ovotransferrin significantly promoted cell proliferation via regulation of the cell cycle at concentrations of 100 and 1000 μg/mL. Ovotransferrin also significantly stimulated osteoblast differentiation evidenced by increased expression of type I collagen and alkaline phosphates (ALP), as well as mineralization (increased bone matrix deposits) in a dose-dependent manner. Furthermore, ovotransferrin inhibited the production of receptor activator of nuclear factor kappa-B (RANKL), while increased osteoprotegerin (OPG), indicating the potential role of ovotransferrin on preventing osteoclastogenesis. The effect of ovotransferrin on transcription factors involved various pathways were studied. The extracellular signal-regulated kinase 1/2 (ERK1/2) antagonist, but not the c-Jun N-terminal kinase (JNK) antagonist, significantly attenuated ovotransferrin-induced increase of alkaline phosphatase (ALP), runt-related transcription factor 2 (Runx2), β-catenin, bone morphogenetic protein 2 (BMP-2), type I collagen synthesis and mineralization; p38 antagonist only decreased ovotransferrin-induced increase of ALP and Runx2. This research further showed that ovotransferrin activated the ERK1/2 cascade, including the phosphorylation of upstream proto-oncogene serine/threonine-protein kinase c-Raf and mitogen-activated protein kinase kinase

MEK1/2, as well as the downstream ribosomal s6 kinase p90RSK and mitogen- and stress-activated protein kinase 1 (MSK1). In addition, phosphoinositide-3-kinase (PI3K)-protein kinase C (Akt) pathway was also involved in ovotransferrin induced osteogenesis as adding ovotransferrin increased the expression of PI3K subunits p85 and p110, which further stimulated the phosphorylation of downstream kinase Akt. Low-density lipoprotein receptor-related protein 1 (LRP1) partially regulated ovotransferrin-stimulated ALP expression and mineralization, while independent to ERK1/2 and PI3K-Akt pathways.

Ovotransferrin was also investigated for its regulatory role on osteoclastogenesis. Macrophage RAW 264.7 was used to differentiate into osteoclasts with RANKL stimulation. Ovotransferrin significantly inhibited osteoclastogenesis, due to the attenuation of RANKL-induced activation of nuclear factor kappa B (NF-κB) and mitogen-activated protein kinase (MAPK) pathways. Also, the expression of osteoclastogenesis-associated proteins TNF receptor associated factor 6 (TRAF6), c-Fos, nuclear factor of activated T-cells cytoplasmic 1 (NFATc1), and cathepsin K (CathK), were significantly decreased in the presence of ovotransferrin. Ovotransferrin significantly preserved the calcium-phosphate (CaP) coating against osteoclastic resorption and induced cell apoptosis of mature osteoclast by regulating Bcl-2 family.

The potential role of ovotransferrin in slowing the progression of osteoporosis was studied using ovariectomized (OVX) Sprague-Dawley rats. Osteoporosis was developed in OVX rats, mimicking to postmenopausal osteoporosis. Oral administration of ovotransferrin did not affect body weight, food intake and organ weight. After 12 weeks of treatment, feeding ovotransferrin at 1% (w/w) in the diet prevented OVX-induced bone loss and maintained relatively high bone

mineral density and integrated bone microarchitecture. Bone marrow cells extracted from OVX rat fed with ovotransferrin produced less osteoclast when stimulated with RANKL *ex vivo*. Ovotransferrin feeding also decreased the production of serum cytokine tumor necrosis factor α (TNF-α) and interleukin 6 (IL-6), two stimulators for osteoclast differentiation. In addition to its direct regulatory role on bone turnover, ovotransferrin supplementation might benefit osteoporosis prevention by inhibiting adipogenesis, regulating immune response and stimulating short-chain fatty acid production.

In summary, this research demonstrated the potential of ovotransferrin as a functional food ingredient for bone health due to its role in promoting osteoblastic activity and inhibiting osteoclastic activity.

PREFACE

This thesis contains original work done by Nan Shang and has been written according to the guidelines for a paper format thesis of the Faculty of Graduate Studies and Research at the University of Alberta. The concept of this thesis originated from my supervisor Dr. Jianping Wu and the research was funded by a Discovery Grant from the Natural Science and Engineering Research Council of Canada (NSERC). The experimental protocol for the animal study was approved by the Animal Care and Use Committee at the University of Alberta (Protocol AUP 00001960) in accordance with the guidelines issued by the Canada Council on Animal Care.

The thesis consists of seven chapters: Chapter 1 provides a general introduction on the context and the objectives of the thesis; Chapter 2 is a literature review on several topics relevant to this thesis, including egg white ovotransferrin, bone physiology and metabolism, osteoporosis and current therapies, and the potential use of bioactive proteins in osteoporosis prevention; Chapter 3 contains the work on *in vitro* osteogenic activity of egg white ovotransferrin, which has been published as Nan Shang and Jianping Wu, "Egg White Ovotransferrin Shows Osteogenic Activity in Osteoblast Cells" in *Journal of Agricultural and Food Chemistry (2018, vol. 86, issue 11, 2775-2782);* Chapter 4 explores the underlying mechanisms of ovotransferrin-stimulated osteogenic activity; Chapter 5 investigates the effects of ovotransferrin on inhibition of osteoclastogenesis and osteoclastic bone resorption; Chapter 6 further validates the *in vivo* effects of ovotransferrin in preventing ovariectomy-induced osteoporosis; and Chapter 7 gives concluding remarks and discusses future research directions.

Dr. Jianping Wu greatly contributed to the experimental design, data interpretation, thesis preparation and edits. Dr. Lynn McMullen and Dr. Jonathan Curtis helped with the thesis review and evaluation. I was responsible for literature search relevant for the above studies, designing and performing laboratory experiments, data collection and analysis, and drafting the manuscript and thesis. Dr. Michael Doschak and Mrs. Ashely Quinton have provided substantial guidance and assistance for the micro-CT scanning, image construction and data analysis. Mrs. Nicole Coursen has provided technical assistance in animal studies including animal husbandry and tissue sampling upon termination. Dr. Aja Rieger and Mrs. Sabina Baghirova provided guidance for flow cytometry and data analysis.

DEDICATION

Dedicated to my beloved parents,

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LIST OF ABBREVIATIONS

α-MEM: Minimum essential eagle medium alpha modification

ACE: Angiotensin converting enzyme

Akt (PKB): Protein kinase B

ALP: Alkaline phosphatase

AP-1: Activator protein 1

APP: Acute-phase protein

BCAP: Bovine colostrum acidic protein

Bcl-xL: B-cell lymphoma-extra large

Bim: BH3-only family member

BMD: bone mineral density

BMP: Bone morphogenetic protein

BSA: Bovine serum albumin

CAGR: Compound annual growth rate

CathK: Cathepsin K

Cbfα1: Core binding factor alpha 1

CEF: Chicken embryo fibroblasts

CPP: Casein phosphopeptides

DC-STAMP: Dendritic cell-specific transmembrane protein

DMEM: Dulbecco's modified eagle medium

DMSO: Dimethyl sulfoxide

DNA: Deoxyribonucleic acid

DTT: Dithiothreitol

EAA: Estrogen agonist/antagonist

c-Raf: Proto-oncogene serine/threonine-protein kinase

EDTA: Ethylenediamineteraaceitc acid

ELISA: Enzyme-linked immunosorbent assay

ERK: Extracellular signal-regulated kinase

FasL: Factor associated suicide ligand

FBS: Fetal bovine serum

FDA: Food and drug administration

FGF: Fibroblast growth factor

GI: Gastrointestinal

GIO: Glucocorticoid-induced osteoporosis

GSK3: Glycogen synthase kinase 3

HSV-1: *Herpes simplex* virus

IgE: Immunoglobulin E

IGF-2: Insulin-like growth factor 2

IGFBP-2: Insulin-like growth factor binding protein 2

IOM: Institute of medicine

ITLN: Intelectin

JNK: c-Jun N-terminal kinase

LF: Lactoferrin

LGR4: Leucine-rich repeat-containing G-protein coupled receptor 4

LPA: Lysophosphatidic acid

LPS: Lipopolysaccharide

LRP: Low-density lipoprotein receptor-related protein

LRP: Low-density lipoprotein receptor-related protein

M-CSF: Macrophage colony stimulating factor

MAPK: Mitogen-activated protein kinase

MAPKAPK: MAPK-activated protein kinase

MBP: Milk basic protein

Mcl-1: Myeloid cell leukaemia sequence 1

MCP-1: Monocyte chemoattractant protein 1

MCS: Mesenchymal stem cell

MDV: Marek's disease virus

MMP9: Matrix metalloproteinase 9

mTOR: Mammalian target of rapamycin

NF-κB: Nuclear factor kappa B

NFATc1: Nuclear factor of activated T-cells cytoplasmic 1

Nrp: Neuropilin

OPG: Osteoprotegerin

PAGE: Polyacrylamide gel electrophoresis

PBS: Phosphate-buffered saline

PCR: Polymerase chain reaction

PHA: Phytohemagglutinin

PI3K: phosphoinositide-3-kinase

PKC: Protein kinase C

PMO: Postmenopausal osteoporosis

PST: Proline/serine/threonine-rich

PTH: Parathyroid hormone

PTH: Parathyroid hormone

RANKL: Receptor activator of nuclear factor kappa-B ligand

Ras: Small GTP-binding protein

RNA: Ribonucleic acid

RTK: Receptor tyrosine kinase

Runx2: Runt-related transcription factor 2

SD: Standard deviation

SDS: Sodium dodecyl sulfate

SEM: Standard error of mean

Sema3A: Semaphoring 3A

Sema4D: Semaphoring 4D

SERM: Selective estrogen inhibitors modulator

SOD: Superoxide dismutase

TRAF6: TNF receptor associated factor 6

TFBP: Transferrin binding protein

TFR: Transferrin receptor

TGF-β1: Transforming growth factor beta 1

TRAP: Tartrate-resistant acid phosphatase

Wnt: Wingless

X/XOD: Xanthine/xanthine oxidase

ICAM-1: Intercellular adhesion molecule 1

VCAM-1: Vascular cell adhesion protein 1

CHAPTER 1 – General Introduction and Objectives

1.1 General Introduction

Osteoporosis is one of the most serious bone disorders, defined as a decreased bone mass and structural deterioration (US Department of Health and Human Services, 2004). It is estimated that ~20% of elderly men and ~30% of elderly women are at elevated risk of osteoporotic fracture. With the rapidly increasing aging population, osteoporosis is becoming a serious health concern. Pharmacological therapies are available for osteoporosis treatment, such as the anti-resorption agents (e.g. bisphosphonates and estrogen) and anabolic agents (e.g. teriparatide) (Salari Sharif, Abdollahi, & Larijani, 2011). However, due the limited efficacy and inevitable side effects, those approved drugs are far from satisfactory.

Functional foods and nutraceuticals are a growing trend in the food and nutrition industry for promoting well-being and helping reduce the risk of disease (AAFC, 2015). Bioactive proteins constitute an important category of health-promoting nutrients that convey physiological benefits, such as promoting bone health (Mine, Li-Chan, & Jiang, 2010). Milk proteins, such as milk basic proteins (Aoyagi et al., 2010), bovine colostrum acidic protein (BCAP) (Du et al., 2011), and lactoferrin (Naot, Grey, Reid, & Cornish, 2005), have been studied for their bone health promoting activity. In comparison, there is limited research on the potential of egg proteins, other than egg yolk water-soluble protein and phosvitin (Ji, Leem, Kim, & Kim, 2007; Kim, Kim, & Leem, 2008; Liu, Li, Geng, Huang, & Ma, 2017).

Ovotransferrin, an iron-binding glycoprotein, accounts for ~12% of egg white protein. As a member of transferrin family (Wu & Acero-Lopez, 2012), ovotransferrin shares ~ 50% sequence similarity with lactoferrin, a well studied milk protein that was reported to stimulate bone formation and prevent bone resorption (Cornish et al., 2004; Naot et al., 2005). Ovotransferrin is also considered as a member of chick innate immunity (Giansanti, Leboffe, Angelucci, & Antonini, 2015), and its expression was increased during chondrogenesis and endochondral bone formation in developing chick embryo (Gentili et al., 1994; Gentili et al., 1994), indicating its involvement in chick bone development. Thus, ovotransferrin can be possibly developed as functional foods and nutraceuticals in promoting bone health.

1.2 Objectives and Hypothesis

Given the above background, we hypothesized that egg white ovotransferrin can promote bone health through regulating the activity of bone cells. The overall objectives of this research are to understand the regulatory roles of ovotransferrin on bone cells and to explore its potential application as a bioactive protein against osteoporosis. The specific objectives of the research are:

1) To investigate the *in vitro* regulatory role of ovotransferrin on osteoblastic activity and osteogenesis

2) To study the mechanisms involved in the ovotransferrin-stimulated osteogenesis

3) To investigate the *in vitro* effects of ovotransferrin on inhibiting osteoclastogenesis and bone resorptive activity; and

4) To study the *in vivo* efficacy of ovotransferrin on osteoporosis treatment in an ovariectomized (OVX) rat model

1.3 Chapter Format

There are seven chapters in the thesis and the brief description of each chapter are given as follows:

Chapter 1 gives a brief introduction on the current status of osteoporosis, and the growing interests in developing functional foods and/or nutraceuticals to promote bone health. Following with the general introduction, the thesis objectives and hypothesis are descried and the chapter format is presented.

Chapter 2 provides a literature review on the current knowledge of egg white ovotransferrin, bone physiology and metabolism, osteoporosis and current therapies, as well as the studies on bioactive proteins and osteoporosis prevention/treatment. In the end, the perspectives on developing bioactive proteins on health management as well as the potential challenges are summarized.

Chapter 3 reports the *in vitro* effect of ovotransferrin on stimulating osteoblastic activity. Murine preosteoblast MC3T3-E1 was used to investigate the stimulatory effects of ovotransferrin on cell proliferation, cell differentiation and mineralization. The effects on cell proliferation were characterized by BrdU incorporation assay, and cell cycle analysis. The effects on cell differentiation were tested by the expression of differentiation biomarkers using western blot and immunofluorescence. The effects on mineralization were evaluated by Alizarin Red assay. Objective 1 is addressed in this chapter.

Chapter 4 elucidates the signaling pathways underlying the ovotransferrin-stimulated osteoblastic activity and the possible receptors in response to ovotransferrin. Western blot was used to study the activation of signaling pathways, include MAPKs and PI3K-Akt pathway. Signaling antagonists were used to validate the involvement of the target pathways by blocking the activation. Biomarkers used in Chapter 3 were used to evaluate the osteogenic activity. qPCR was used to screen the receptors of ovotransferrin. Objective 2 is addressed in this chapter.

Chapter 5 reports the *in vitro* effects of ovotransferrin on inhibiting osteoclastogenesis and resorptive activity. Murine macrophage RAW 264.7 was used to generate osteoclast. The production and formation of osteoclast were measured by TRAP staining. The resorption of osteoclast was tested with calcium-phosphatase (CaP) resorption assay. Western blot, cell apoptosis assay and qPCR were used to elucidate the mechanism underlying the inhibitory effect of ovotransferrin. Objective 3 is addressed in this chapter.

Chapter 6 describes the *in vivo* effects of ovotransferrin on osteoporosis prevention. Ovariectomized Sprague-Dawley rats were used as the disease model representing the postmenopausal osteoporosis. The body weight, food intake and organ characteristics were recorded for a preliminary safety evaluation. The impact of dietary supplement of ovotransferrin on osteoporosis prevention was evaluated by bone mineral density, bone microarchitecture and serum biomarkers expression. The mechanisms underlying the prevention effects were investigated by serum cytokine production, osteoclastogenesis, as well as the adipogenesis, immune response, and short-chain fatty acid production. Objective 4 is addressed in this chapter.

Chapter 7 presents a general summary on the key findings and the significance of this study. The recommended future studies are outlined according to the limitations and challenges of this research.

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CHAPTER 2 - Literature Review

2.1 Egg White Ovotransferrin

Hen egg has a long history of use as a human food and is one of the few foods that are consumed throughout the world regardless of religion and ethnic group (Miranda et al., 2015). It is considered as the best source of high quality protein, which is only inferior to breast milk (Kuang et al., 2018; Miranda et al., 2015). Egg white accounts for 60% of whole egg and contains 11% of protein (Abeyrathne, Lee, & Ahn, 2013). Ovotransferrin is the second major protein in egg white that has many functional properties and can be separated from egg white using various approaches (Abeyrathne et al., 2013). In this section, the structure and biological properties of ovotransferrin are reviewed to highlight its potential as a nutraceutical and functional food ingredient.

2.1.1 Structure and composition of ovotransferrin

Hen egg white ovotransferrin was initially called conalbumin and then renamed ovotransferrin after being recognized as an iron-binding protein and a member of transferrin family (Williams, 1968). It is a single glycopeptide chain consisting of 686 amino acids and contains 15 disulfide bonds and no free sulfhydryl groups (Abeyrathne et al., 2013). This single chain is folded into two globular lobes linked by an alpha helix structure (Lee, McKnight, & Palmiter, 1980; Mazurier et al., 1983). Each lobe has the capability to reversibly bind one Fe^{3+} ion concomitantly with one bicarbonate anion (Vliegenthart et al., 1979; Williams, 1968; Williams, 1982). Studies confirmed that egg white ovotransferrin shows ~50% homology with human transferrin and lactoferrin, but differs from its isoelectric point (pI=6.0 for ovotransferrin and pI=8.8 for lactoferrin), glycosylation pattern (galactose and sialic acid are absent in ovotransferrin), and disulfide bonds

(Lee, McKnight, & Palmiter, 1980; Mazurier et al., 1983; Thibodeau, Lee, & Palmiter, 1978; Vliegenthart et al., 1979; Williams, 1968; Williams, 1982). Similarly to other transferrin proteins, ovotransferrin is responsible for iron scavenging and iron delivery due to the capability of reversibly binding two Fe^{3+} ions per molecular (Abeyrathne et al., 2013; Superti, Ammendolia, Berlutti, & Valenti, 2007). The ovotransferrin molecule comprises two homologous globular lobes and each lobe contains one iron-binding site. Based on the iron-binding situation, ovotransferrin can be defined as apo- (iron free) form and holo- (iron bound) form. Different physical and chemical properties have been found in each form. Basically, the holo-form is more resistant to chemical and physical conditions than the apo-form (Abeyrathne et al., 2013).

2.1.2 Bioactives of Egg White Ovotransferrin and Its-derived Peptides

Although egg white ovotransferrin shares great similarity to lactoferrin, research on the bioactivity of ovotransferrin is limited compared to the extensive studies on lactoferrin. The currently recognized biological properties of egg white ovotransferrin and its-derived peptides are summarized in Table 2.1.

2.1.2.1 Antimicrobial activity

Antimicrobial activity was the first recognized bioactivity of ovotransferrin. Ovotransferrin exhibits a strong antimicrobial activity against a broad spectrum of bacteria (Gram-positive and Gram-negative), fungi, and yeasts (Valenti et al., 1983; Valenti & Visca, 1985); thus it has been used as an antimicrobial agent in food products (Del Giacco, Leone, & Ferlazzo, 1985; Valenti et al., 1983). This antimicrobial property is believed due to the iron-binding ability of ovotransferrin that deprive the iron necessary for the growth of microorganisms (Alderton, Ward, & Fevold, 1946; Bullen, Rogers, & Griffiths, 1978). The species most sensitive to iron deprivation effect of

ovotransferrin are *Pseudomonas* spp., *Escherichia coli*, and *Streptococcus mutants*, while the *Streptococcus aureus*, *Proteus* spp., and *Klebsiella* spp. are more resistant (Valenti et al., 1981, 1982, 1983). Furthermore, this bacteriostatic activity of ovotransferrin can be enhanced by adding carbonate ion (MacGillivray et al., 1998; Valenti et al., 1981), increasing the pH (Baron et al., 2014) and immobilizing ovotransferrin by covalent linkage to Sepharose 4B (Valenti et al., 1982). The antimicrobial activity of ovotransferrin is also reported in metal ion saturated ovotransferrin, suggesting a more complex mechanism might be involved in the antimicrobial activity independent to iron deprivation (Tranter & Board, 1982; Valenti et al., 1983b). Both holo- and apo-ovotransferrin showed bactericidal activities against *S. aureus*, *Bacillus cereus* and *E. coli* (Ibrahim, 1996). The antimicrobial ability of holo-ovotransferrin was thought to be a result of the permeable ability of ovotransferrin that allows it to access the inner membrane and leads to both ion leakage from bacteria and the uncoupling of the respiration-dependent energy production (Aguilera, Quiros, & Fierro, 2003).

Additionally, isolation of a bactericidal domain in ovotransferrin further indicated the mechanisms independent of iron-binding ability. OTAP-92, a 9.9kDa peptide, showed strong sequence similarity with defensins and exhibited antibacterial activity (Ehret-Sabatier et al., 1996; Strahilevitz, Mor, Nicolas, & Shai, 1994; Zhou & Smith, 1990). This 92-resudue peptide contains six cysteines engaged in the form of three intrachain disulfide bonds to maintain the tertiary structure, which is essential to the antimicrobial activity. Its antibacterial action is due to the relatively high alkalinity and cysteine array that involves the blocking of the voltage-dependent K^+ channels (Ehret-Sabatier et al., 1996; Galvez et al., 1990; Ibrahim, Iwamori, Sugimoto, & Aoki, 1998; Ibrahim, Sugimoto, & Aoki, 2000; Strahilevitz et al., 1994).

2.1.2.2 Antioxidant activity

Ovotransferrin is a superoxide dismutase (SOD)-mimicking protein exhibiting antioxidant activity through scavenging superoxide radical ($O_2^{\cdot-}$) under the natural xanthine/xanthine oxidase (X/XOD) coupling system (Ibrahim, Hoq, & Aoki, 2007; Wu & Acero-Lopez, 2012). The overall antioxidant activity could be promoted by increasing the proximity of amino acid residues that scavenge free radicals and chelating pro-oxidative metal (Elias, Kellerby, & Decker, 2008). Thus, compared to intact ovotransferrin, self-cleaved ovotransferrin showed greater $O_2^{\cdot-}$ scavenging capacity and the N-terminal lobe was more efficient (Ibrahim et al., 2007; Ibrahim, Haraguchi, & Aoki, 2006). Accordingly, ovotransferrin digested with proteolytic enzymes, such as thermolysin and pepsin, resulted a dramatically increased oxygen radical absorption capacity compared to natural ovotransferrin (Huang et al., 2010; Shen, Chahal, Majumder, You, & Wu, 2010). Two tetrapeptides (Trp-Asn-Ile-Pro and Gly-Trp-Asn-Ile) have the highest antioxidant activity after digestion, indicating the important role of the motif Trp-Asn-Ile in response to the high antioxidant activity (Jahandideh, Chakrabarti, Davidge, & Wu, 2016; Shen et al., 2010). Furthermore, conjugation of ovotransferrin with polyphenols, such as catechin, also improved the oxygen radical absorbance capacity (You, Luo, & Wu, 2014).

2.1.2.3 Anti-inflammatory activity

Ovotransferrin is known to be an acute-phase protein (APP) in avian species, which is upregulated during inflammation (Chamanza et al., 1999; Gabay & Kushner, 1999; Hallquist & Klasing, 1994; Tohio et al., 1995; Xie, Huff, Huff, Balog, & Rath, 2002). Thus, the blood level of ovotransferrin is considered as an infection and inflammation marker in chicken (Rath et al., 2009).

The anti-inflammatory activity has been found in several ovotransferrin-derived peptides (Majumder, Chakrabarti, Davidge, & Wu, 2013; Wang et al., 2017). Dipeptide CR (Cys-Arg), FL (Phe-Leu), HC (His-Cys), LL (Leu-Leu) and MK (Met-Lys) significantly inhibited TNF-α-

induced inflammation in Caco-2 cells (Wang et al., 2017). Similarly, tripeptide IRW (Ile-Arg-Trp) and IQW (Ile-Gln-Trp) attenuated TNF-α-induced inflammatory response and oxidative stress in vascular endothelial cells (Majumder, Chakrabarti, Davidge, et al., 2013). Furthermore, the anti-inflammatory activity of IRW was evaluated *in vivo* using spontaneously hypertensive rats. IRW treatment significantly reduced the inflammatory marker, such as the expression of intercellular adhesion molecule 1 (ICAM-1) and vascular cell adhesion protein 1 (VCAM-1) (Majumder et al., 2015; Majumder, Chakrabarti, Morton, et al., 2013).

2.1.2.4 Immunomodulatory activity

Similar to other transferrin family members, ovotransferrin is a component of innate immunity (Giansanti et al., 2002; Giansanti, Giardi, Massucci, Botti, & Antonini, 2007). The immunomodulatory effect of ovotransferrin was found on macrophages and heterophils (Hang Xie et al., 2002). Also, ovotransferrin inhibited the lipopolysaccharide (LPS)- and phytohemagglutinin (PHA)-induced proliferation of mouse spleen lymphocytes (Otani & Odashima, 1997).

2.1.2.5 Other activities

The antiviral activity of ovotransferrin has been reported to the avian herpesvirus Marek's disease virus (MDV) (Giansanti et al., 2002). Although the mechanism is not clear, the antiviral efficacy was not correlated to iron saturation of ovotransferrin (Giansanti et al., 2002). It has been hypothesized that the antiviral activity to MDV is associated with two ovotransferrin fragments DQKDEYELL and KDLLFK, which can block the MDV infection in chicken embryo fibroblasts (CEF) (Giansanti et al., 2005). These fragments share the sequence homology with two lactoferrin-derived antiviral peptides that are effective against Herpes simplex Virus (HSV-1) (Siciliano et al., 1999).

Additionally, partially hydrolyzed ovotransferrin produced by thiol-linked auto-cleavage after reduction showed strong anti-cancer activity by remarkably inhibiting the proliferation of human colon (HCT-116) and breast (MCF-7) cancer cells in a dose-dependent manner (Ibrahim & Kiyono, 2009).

Ovotransferrin also showed promotion effect on chick embryo development. A transiently high expression of ovotransferrin and its receptor was found during the differentiation of hypertrophic chondrocytes into osteoblast-like cells in the chick embryo (Gentili et al., 1993; Gentili et al., 1994). This finding suggested the existence of autocrine and paracrine loops of ovotransferrin and its receptor during chondrogenesis and endochondral bone formation (Cancedda et al., 2000; Gentili et al., 1993; 1994). Furthermore, ovotransferrin showed significant improvement of mitochondrial activity and differentiation in embryonic chick brain and neural retina cells (Bruinink, Sidler, & Birchler, 1996).

Ovotransferrin has been developed as a drug carrier to improve the solubility of water-insoluble antibiotics and to facilitate the specific delivery into microbial or infected cells (Ibrahim et al., 2015).

Lastly, several antihypertensive peptides have been isolated from ovotransferrin hydrolysate via the inhibition to angiotensin converting enzyme (ACE), a key enzyme responsible for regulating hypertension (Hartmann & Meisel, 2007; B. Murray & FitzGerald, 2007). Three ovotransferrin-derived tripeptides IRW (Ile-Arg-Trp), LKP (Leu-Lys-Pro) and IQW (Ile-Gln-Trp) have been characterized with ACE inhibitory activity and potent in preventing hypertension (Majumder et al., 2015; Majumder, Chakrabarti, Morton, et al., 2013; Majumder, Chakrabarti, Davidge, et al., 2013).

2.1.3 Current Applications of Ovotransferrin

The functional properties and biological activities of ovotransferrin have been widely investigated; however, the practical use of ovotransferrin by industry is highly limited. Similar to bovine lactoferrin, ovotransferrin is suggested for use as an iron-supplementing agent in infant formula (Abeyrathne et al., 2013; Huang et al., 2006). Meanwhile, ovotransferrin is also used as an antibacterial agent in infant formula to protect against pathogen infection (Del Giacco et al., 1985; Valenti et al., 1983b). A study confirmed the safety of supplement ovotransferrin into infant formula by feeding 15 babies with ovotransferrin-treated milk. Ovotransferrin did not sensitize ovotransferrin-fed infants and the total immunoglobulin E (IgE) remained within the normal range (Del Giacco et al., 1985). In addition to incorporation into infant formula, ovotransferrin has been suggested to be combined into κ-carrageenan-based edible film to protect food products and to extend shelf life (Seol, Lim, Jang, Jo, & Lee, 2009).

In addition to promoting human health, ovotransferrin has been used in turkey farms for its antimicrobial activity to prevent respiratory disease. Respiratory pathogens are difficult to control in large-scale turkey production and cost a lot for antibiotic uses. Studies reported that use of a ovotransferrin aerosol on a large Belgian turkey farm significantly reduced infection with the respiratory pathogen *Chlamydia psittaci* and resulted in a prevention of respiratory disease, mortality and lowered antibiotic use (Van Droogenbroeck & Vanrompay, 2013; Van Droogenbroeck et al., 2011).

Ovotransferrin and its-derived hydrolysates/peptides have high potential for use in the pharmaceutical, nutraceutical, and food industries; however, the industrial applications are in the start-up stage even though a great number of researches on the use of ovotransferrin have been published in recent years. Future research on egg white ovotransferrin focusing on characterization

and validation of ovotransferrin functions by animal and clinical trials, as well as the development of ovotransferrin application based on food systems will benefit the development of ovotransferrin-based products and improving the sustainability of the egg industry.

2.2 Bone Physiology and Metabolism

2.2.1 Bone Composition

Bone mainly consists of two compounds: the inorganic mineral and the organic components (Morgan, Barnes, & Einhorn, 2008; Rosen, 2013). The inorganic is in the form of crystalline hydroxyapatite $[Ca_3(PO_4)_2]_3Ca(OH)_2$, which mainly provide strength and rigidity (Heaney, 2009; Morgan et al., 2008). It also contains impurities in addition to calcium and phosphate, such as carbonate, potassium, magnesium, chloride, fluoride and etc. (Brommage & Neuman, 1979; McConnell, 1964; McConnell, Foreman, Drew, Perkins, & Daly, 1971; Ou-Yang, Paschalis, Mayo, Boskey, & Mendelsohn, 2001). The organic component comprises more than 30 proteins with type I collagen being the most abundant (~90%) (Heaney, 2009; Morgan et al., 2008). While the type I collagen represent the major structural component of the bone matrix to offer flexibility, other proteins present in small quantities significantly contribute to the biological functions (Heaney, 2009; Morgan et al., 2008).

2.2.2 Bone remodeling

Throughout life, bone constantly undergoes a cycle of remodeling (Kini, Usha, Nandeesh, 2012). The remodeling process plays a fundamental role in bone metabolism, which is a prerequisite for a proper execution of bone functions (Parfitt, 2008). In a homeostatic equilibrium bone remodeling, the old and micro-damaged bone is continuously replaced by new and mechanically

sound bone, so that to preserve bone strength and adapt to mechanical load (Kini, Usha, Nandeesh, 2012). To manage the remodeling process, three types of bone cells are involved, osteoblast, osteocyte, and osteoclast cells. The osteoblast, known as the bone formation cell, is responsible for the production of new bone (Crockett, Rogers, Coxon, Hocking, & Helfrich, 2011; Feng, 2009; Kini, Usha; Nandeesh, 2012; Lian et al., 2012). After the matrix secretion, ~15% of mature osteoblasts are entrapped in the new bone matrix and differentiated into osteocytes, while other cells remain on the bone surface becoming flat lining cells (Crockett et al., 2011; Feng, 2009; Kini, Usha; Nandeesh, 2012). The osteoclast is a giant multinucleated cell lining on the bone surface and is responsible for bone resorption (Crockett et al., 2011; Feng, 2009; Kini, Usha; Nandeesh, 2012). Osteoclasts and osteoblasts closely collaborate in the remodeling process to replace old bone with new bone (Figure 2.1). Briefly, the remodeling cycle consists of three consecutive phases: resorption, reversal, and formation. First, partially differentiated preosteoclasts migrate to the bone surface and are further differentiated into multinucleated osteoclasts. Once the osteoclastic resorption is complete, the reversal phase starts. The main responsibility of the reversal phase is to prepare the bone surface for new bone formation and provide signals for osteoblast differentiation and migration. Following the completion of reversal phase, osteoblasts start producing new bone until the resorbed bone is completely replaced (Crockett et al., 2011; Feng, 2009; Kini, Usha; Nandeesh, 2012; Lian et al., 2012). The remodeling may be in balance when the amount of new-formed bone equals to the amount of resorbed, or maybe be imbalanced when bone formation is either more or less than the bone resorption. Once the remodeling appears to be imbalanced, different kinds of bone disorders will develop, such as osteoporosis.

2.2.3 Osteoblast and Bone Formation

Bone formation by osteoblasts occurs in three successive phases: the production and maturation of the osteoid matrix, followed by mineralization of the matrix. Initially, osteoblasts produce osteoid by rapidly depositing collagen. Then, collagen synthesis decreases and osteoids become fully mineralized. Bone formation by osteoblasts is controlled both locally and systemically. Studies have demonstrated the crucial importance of local bone formation control by bone morphogenetic protein (BMP) (Cao & Chen, 2005) and Wingless (Wnt) (Day, Guo, Garrett-Beal, & Yang, 2005) signaling pathways for osteoblast differentiation and function. BMP2 can act as a potent stimulator of osteoblast differentiation from mesenchymal stem cell (MSC), osteoprogenitor cell expansion, and osteoblastic bone formation activity, which is also used clinically to enhance bone formation, such as fracture repair (Chau, Leong, & Li, 2009). BMPs bind to the cell surface BMP receptors and elicit the canonical BMP-Smad pathway and/or the non-canonical mitogen-activated protein kinase (MAPK) pathway and phosphoinositide-3-kinase – protein kinase B (PI3K-Akt) pathway (Chau et al., 2009; Derynck & Zhang, 2003; Dijke & Hill, 2004). The activation of these pathways regulates the expression of Runt-related transcription factor 2 (Runx2) (Celil & Campbell, 2005; Fujita et al., 2004; Sharff et al., 2009), also known as core binding factor alpha 1, which is an important transcription factor indispensable for osteoblast differentiation (Javed, Chen, & Ghori, 2010; Kerstetter et al., 1997; Lin & Hankenson, 2011; Yavropoulou & Yovos, 2007).

In addition to BMP signaling, Wnt signaling is a key pathway in bone formation. Wnt-protein ligand binds to cell surface receptor Frizzled and co-receptors low-density lipoprotein receptor-related proteins 5 and 6 (LRP5, LRP6) to activate the downstream glycogen synthase kinase 3 (GSK3)/β-catenin pathway (Chau et al., 2009; He, Semenov, Tamai, & Zeng, 2004). The

accumulation of β-catenin in nucleus subsequently turns on Wnt target genes that might play role in osteoblast turnover, such as Myc, cyclin D, c-Jun, Fra-1, BMPs, fibroblast growth factor (FGF) 18 and Runx2 (Chau et al., 2009). Also, Wnt can also activate non-canonical pathways, such as c-Jun N-terminal kinase (JNK) (Qiu, Chen, & Kassem, 2011) and protein kinase C (PKC) (Luna-Ulloa, Hernández-Maqueda, Castañeda-Patlán, & Robles-Flores, 2011). The schematic presentation indicating the osteoblast differentiation and key regulation pathways are shown in Figure 2.2 and Figure 2.3.

2.2.4 Osteocyte and Bone Formation

Osteocytes are the most abundant cell type in bone and are distributed throughout the mineralized bone matrix (Goldring, 2015). They have the function to sense and respond to local biomechanical and systemic stimuli to regulate bone remodeling (Goldring, 2015). Meanwhile, osteocytes mediate the cell-cell interactions with osteoblast and osteoclast to regulate the production of RANKL and OPG (Kramer et al., 2010). Osteocytes also regulate bone remodeling via the release of soluble mediators, such as prostanoids, nitric oxide, and nucleotides (Schaffler et al., 2014; Cheng et al., 2001; Caballero-Alias et al., 2005). They are also the source of a broad spectrum of cytokines and growth factors such as insulin-like growth factor-1 (IGF-1) and vascular endothelial cell growth factor (VEGF) (Sheng, Lau, & Baylink, 2004; Heino, Hentunen, Vaananen, 2002). The recognition that osteocytes possess such diverse capacities in regulating bone remodeling suggests a novel approach to treat skeletal disorders.

2.2.5 Osteoclast and Bone Resorption

Bone resorption by osteoclast is a multistep process initiated by the proliferation of osteoclast precursors, the differentiation of precursors to mature osteoclasts, and finally degradation of the bone matrix (Teitelbaum, 2000). Osteoclast differentiation initially depends on the receptor for macrophage colony stimulating factor (M-CSF) regulated expression of receptor activator of nuclear factor kappa-B (RANK) (Crockett, Mellis, Scott, & Helfrich, 2011) and its ligand RANKL produced by osteoblasts and stromal cells (Leibbrandt & Penninger, 2008). Signaling through M-CSF and RANK leads to activation of the transcription factors nuclear factor kappa B (NF-κB), activator protein 1 (AP-1) and nuclear factor of activated T-cells cytoplasmic 1 (NFATc1) (Humphrey, Lanier, & Nakamura, 2005). This following regulates the expression of essential osteoclast genes, such as dendritic cell-specific transmembrane protein (DC-STAMP), tartrate-resistant acid phosphatase (TRAP), cathepsin K (CathK), matrix metalloproteinase 9 (MMP9) and β3 integrin, which allow the final differentiation and fusion of the precursors and function of the resulting multinucleated osteoclast. In addition to the essential role of M-CSF and RNAKL in osteoclastogenesis, osteoclast formation is closely associated with immune system and the osteoclastogenesis can be stimulated in inflammatory conditions, such as rheumatoid arthritis. The stimulation effect is through pro-inflammatory cytokines, such as tumor necrosis factor α (TNF-α) and interleukin 6 (IL-6) (Teitelbaum & Ross, 2003). The schematic presentation indicating the osteoclast differentiation and key regulation pathway are shown in Figure 2.4 and Figure 2.5.

Osteoclast numbers in bone are not only controlled by osteoclastogenesis process, but also through the regulation of their lifespan. Decreased osteoclast apoptosis lead to increased bone loss, such as in ovariectomy-induced osteoporosis (Hughes et al., 1996; Hughes & Boyce, 1997). Generally, osteoclast apoptosis is regulated by apoptotic factors, such as B-cell lymphoma-extra large (Bcl-

xL) (Woo, Kim, & Ko, 2002; Zhang et al., 2005), the BH3-only family member Bim (Akiyama et al., 2003) and myeloid cell leukaemia sequence 1 (Mcl-1) (Masuda et al., 2014). The expression of these apoptotic factors is regulated by signaling pathways including extracellular signal regulated kinase (ERK) (Ramos, 2008), the Akt (Kawamura et al., 2007) and mammalian target of rapamycin (mTOR) (Hadji, Coleman, & Gnant, 2013).

2.2.6 Interaction Between Osteoblasts and Osteoclasts

Bone remodeling is a tightly coupled process between osteoblast and osteoclast activity. Osteoblasts and osteoclasts can communicate and affect each other through direct cell-cell contact, cytokines and extracellular matrix interaction. The schematic presentation indicating the interaction between osteoblast and osteoclast is shown in Figure 2.6.

2.2.6.1 The role of osteoblasts in bone resorption

In general, osteoblasts can affect osteoclast formation, differentiation and apoptosis through several pathways. Osteoblasts can communicate with osteoclast via direct contact and forming gap junctions to allow small water-soluble molecules passage between two cells (Everts et al., 2002; Matsuo & Irie, 2008). However, the most common interaction between these two cells is through cytokines. Among all, the RANK/RANKL/OPG axis is the most well studied. RANKL, produced by osteoblasts, plays an essential role in initiating the osteoclastogenesis. It can bind with its receptor RANK, expressed on osteoclast progenitors, and actives the down signaling pathways related to osteoclast differentiation (Boyle, Simonet, & Lacey, 2003). Osteoblasts also produce an osteoclastogenesis inhibitory factor named osteoprotegerin (OPG). OPG serves as a decoy receptor that can bind with RANKL and block its ability to bind to and activate RANK (Boyle et al., 2003). So far, several other cytokines have been investigated to play important role in the osteoblast

affected osteoclastogenesis, such as Ephrin2/ephb4 (Zhao et al., 2006), M-CSF/MCP-1 (monocyte chemoattractant protein 1) (Kim & Kim, 2016; Li et al., 2007; Sambandam et al., 2010; Wiktor-Jedrzejczak et al., 1990), LGR4 (leucine-rich repeat-containing G-protein coupled receptor 4)/RANKL/RANK (Luo et al., 2016), Sema3A (semaphoring 3A)/Nrp (neuropilin) (Hayashi et al., 2012; Negishi-Koga et al., 2011) and lysophosphatidic acid (LPA) (David et al., 2010; Lapierre et al., 2010; McMichael, Meyer, & Lee, 2010; Panupinthu et al., 2008; Sims, Panupinthu, Lapierre, Pereverzev, & Dixon, 2013). In addition to affecting osteoclast formation, osteoclast apoptosis could also be induced by osteoblasts. Estrogen affects osteoclast survival through the upregulation of factor associated suicide ligand (FasL) in osteoblast leading to the apoptosis of pre-osteoclast (Garcia et al., 2013; Krum et al., 2008; Wang et al., 2015). Osteoporosis drugs, tamoxifen and reloxifene, can also induce pre-osteoclast apoptosis by the same osteoblast-dependent mechanism (Krum et al., 2008).

2.2.6.2 The role of osteoclasts in bone formation

Recently, studies also described the possible influence of osteoclasts in regulating osteoblastic bone formation. Although it is still not well understood, studies reported that osteoclasts might secrete some molecules, such as semaphoring 4D (Sema4D) (Negishi-Koga et al., 2011), sclerostin (Kusu et al., 2003) and MicroRNA/exsomes, to affect osteoblastic bone formation. Sema4D is highly expressed in osteoclasts and showed potential inhibition of bone formation through its receptor plexin-B1 expressed by osteoblasts (Negishi-Koga et al., 2011). For sclerostin, it was thought only expressed in osteocyte and has anti-anabolic effects on bone formation (Winkler et al., 2003); however, recently a study found that sclerostin is also expressed in osteoclast and could inhibit the activity of BMPs (Kusu et al., 2003). It has been shown that microRNAs also regulate osteogenic activity and osteoblastic bone formation (Lian et al., 2012). A recent study reported

that exosomal miR-214-3p produced by osteoclasts might negatively affect the osteoblastic bone formation (Li et al., 2016).

2.3 Osteoporosis and Current Therapies

2.3.1 Osteoporosis

Osteoporosis is defined as low bone mass, structural deterioration, and porous bone, leading to an increased fracture risk. Osteoporosis is by far the most common bone disease all over the world. Osteoporotic fracture can result in disability, diminished function, loss of independence, and premature death (US Department of Health and Human Services, 2004). Hip fracture, the leading cause of osteoporotic-related disability and death, occur predominantly in the aging populations and the incidence increases exponentially with age (Zeng et al., 2013). With the rapid increase in aging people and life expectancy world-wide, the estimated number of hip fractures is predicted to rise from 1.7 million in 1990 to 6.3 million in 2025 (Zeng et al., 2013). Not surprisingly, bone diseases will take a significant financial toll on both society and individuals who suffer from it. The direct care expenditures for osteoporotic fracture alone range from $12.2 to $17.9 billion USD each year (US Department of Health and Human Services, 2004). Recognizing that osteoporosis can have a significant impact on the individual health and the whole society, there is an urgent need to extend knowledge of bone pathology, improve diagnosis method, as well as to develop effective and safer treatment approaches.

2.3.2 Current Osteoporosis Therapies

As one of the most serious bone disease worldwide, several approaches have been developed for osteoporosis treatment and prevention. Non-pharmacological management includes adequate

calcium and vitamin D intake, weight-bearing exercising, smoking cessation, limitation of alcohol/caffeine consumption, and fall-prevention techniques (Buckley et al., 2017; Camacho et al., 2016; Cosman et al., 2014; Crockett & Das, 2013; NIH, 2001; Tosteson, Burge, Marshall, & Lindsay, 2008; US Department of Health and Human Services, 2004; Watts et al., 2012). The Institute of Medicine (IOM) recommends that dietary calcium intake should be limited to 1000 mg daily for men 50 to 70 years of age and to 1200 mg daily for women 51 years of age and older and for men 71 years of age and older (Taylor, Yaktine, & Del Balle, 2011). The relationship between calcium intake and several diseases, such as cardiovascular risk and kidney stone, has been debated (Moe & Chen, 2008). Therefore, dietary calcium intake is recommended to meet the requirements, rather than using calcium supplements (Cosman et al., 2014). Vitamin D is a key component in calcium absorption and bone health. Although some evidence supports using vitamin D supplementation to reduce fracture risk, recent studies have shown that higher monthly doses of vitamin D are associated with an increased risk of falls (Bischoff-Ferrari et al., 2016; Chung et al., 2011).

The cause of osteoporosis is due to the imbalance of bone remodeling which finally result in more bone resorption than formation. The goal of pharmacological therapies is categorized as either antiresorptive agents (i.e., bisphosphonates, estrogen agonist/antagonists [EAAs], estrogens, calcitonin, and denosumab) or anabolic agents (i.e., teriparatide) (Salari Sharif, Abdollahi, & Larijani, 2011). Antiresorptive medications primarily decrease the rate of bone resorption, while anabolic medications increase bone formation. Although several medications have overlapping indications, it is important to note that not all osteoporosis medications are approved by the Food and Drug Administration (FDA) to treat postmenopausal osteoporosis (PMO), osteoporosis in men, and/or glucocorticoid-induced osteoporosis (GIO) (Table 2.2). Recommendation for

treatment options are based on different characteristics, such as gender, degree of fracture risk, and additional risk factors, such as comorbid disease or medications (Buckley et al., 2017; Camacho et al., 2016; Cosman et al., 2014; Watts et al., 2012).

2.3.3 Emerging Therapies and Investigational Drugs

Due to the limitations and side effects of approved drugs, new therapies and investigational drugs are under investigations and looking for approval.

Sclerostin, a protein produced by osteoclasts, interferes with the proliferation and function of osteoblasts. Several humanized monoclonal antibodies that inhibit sclerostin have been developed, named Romosozumab (Evenity, Amgen/UCB) (MacNabb, Patton, & Hayes, 2016; Medscapte, 2017), Blosozumab and BPS 804 (MacNabb et al., 2016). As blosozumab and BPS 804 are still under the process of development and clinical testing, human trials confirmed the osteoporosis prevention effects of romosozumab. However, the FDA rejected approval of romosozumab due to a higher rate of serious adverse cardiovascular events compared with other current drugs, such as alendronate (MacNabb et al., 2016; Medscapte, 2017). Additionally, cathepsin K (CatK), a protease released by osteoclasts, can promote the degradation of bone matrix, especially type I collagen; thus, inhibiting CatK is assumed to prevent bone resorption. Merck & Co. developed a selective CatK inhibitor Odanacatib showed anti-osteoporosis effects, but will significantly increase the risk of stroke (Mullard, 2016). Therefore, Merck & Co. decided to not continue the development of Odanacatib in 2016 (Mullard, 2016).

In addition to antiresorptive agents, novel anabolic agents have been investigated. Ronacaleret, a calcium-sensing receptor antagonist, was suggested to stimulate transient endogenous parathyroid hormone (PTH) release and increase bone formation. However, the efficacy of Ronacaleret was

not sufficient to be pursued (Balan et al., 2009; Fitzpatrick et al., 2011, 2012). New calcilytic compounds have been developed subsequently and additional studies are continuing because of a lack of an orally active anabolic agent (Balan et al., 2009; Widler, 2011). Another approach is to develop glycogen synthase kinase 3 beta (GSK-3β) inhibitors. GSK-3β, a multifunctional serine/threonine kinase, is central to Wnt signaling, which is one of the most important regulation pathways of bone formation (Baron & Rawadi, 2007). Treatment of ovariectomized rats with a nonspecific GSK-3α and -3β inhibitor (LY603281-31-8) (Kulkarni et al., 2006) and treatment of mice with selective GSK-3β inhibitors lithium chloride or 6-bromo-indirubin-3'-oxime resulted in enhanced bone formation and increased bone mass (Clement-Lacroix et al., 2005). However, GSK-3β is thought to be related to pathogenesis of Alzheimer's disease and noninsulin-dependent diabetes (Baron & Rawadi, 2007), so, the clinical use of selective GSK-3β inhibitors in osteoporosis may be limited (Wilting et al., 2007).

The best approach for osteoporosis prevention is to develop agents with dual actions on bone resorption and formation. Strontium ranelate is currently used in Europe (except UK) for the treatment of osteoporosis, although it is not licensed in the US (Lewiecki et al., 2007; Rodrigues et al., 2018). Strontium ranelate is reported to inhibit osteoclast activity, while stimulating osteoblast activity at the same time (Reginster et al., 2005). However, strontium ranelate has been found to be associated with increased risk of venous thromboembolism (Stevenson et al., 2007). More importantly, the change in bone mineral density should be monitored as strontium could be incorporated into bone (Blake, Lewiecki, Kendler, & Fogelman, 2007; Fogelman & Blake, 2005). Table 2.3 summarizes the emerging therapies and drug.

2.4 Bioactive Proteins and Prevention of Osteoporosis

Due to the low efficacy and associated side effects of pharmacological drugs, there is an increasing interest in developing dietary intervention and food-derived bioactive compounds on osteoporosis prevention. Studies have indicated the importance between protein intake and bone health management, especially in the aging population. Recent reports also pointed out the potential use of several food-derived bioactive proteins and peptides in prevention of osteoporosis. In this section, the major findings with regard to using food-derived bioactive proteins in prevention of osteoporosis are reviewed.

2.4.1 Dairy Protein

Dairy foods are considered as a vital component for improving bone health, as well as a great source of bioactive protein/peptides for investigation. Although the beneficial role of dairy products on bone health management was considered to be a result of the rich calcium content, the effects of bioactive proteins and peptides have been proposed and studied (Table 2.4). Milk whey protein, especially the fraction with alkaline isoelectric point (milk basic protein, MBP), suppressed bone resorption and prevented bone loss in ovariectomized aged rats (Toba et al., 2000). In addition, similar protection effects were also observed in young women (Aoe et al., 2001; Itabashi, 2006; Uenishi et al., 2007; Yamamura et al., 2002), menopausal women (Aoe, Koyama, Toba, Itabashi, & Takada, 2005) and healthy older women (>65 years old) (Aoyagi et al., 2010) who supplemented with MBP. Supplementation with MBP significantly increased bone mineral density and suppressed osteoclast-mediated bone resorption. Bovine colostrum acidic proteins (BCAPs) are able to prevent osteoporosis in ovariectomized rats by stimulating bone mineralization and growth (Du et al., 2011). The effective components in the bovine colostrum

acidic proteins were mainly osteopontin, lactoferrin, epidermal growth factor and insulin-like growth factor-2 (IGF-2) (Du et al., 2011). With further study, lactoferrin has been suggested as an anabolic factor for bone formation. Lactoferrin injected over the right hemicalvarium of adult male mice stimulated the osteoid deposition and mineralization (Cornish et al., 2004). Meanwhile, orally administration of lactoferrin also showed prevention of ovariectomy-induce bone loss and deteriorate of bone microarchitecture (Blais, Malet, Mikogami, Martin-Rouas, & Tomé, 2009; Guo et al., 2009; Malet et al., 2011). Among bioactive peptides, which can be derived from milk proteins, casein phosphopeptides (CPPs) are carriers for minerals, especially calcium (FitzGerald, 1998). CPPs showed to enhance calcium solubility and calcium uptake as well as to stimulate differentiation of human osteoblast cells (Donida et al., 2009; Tulipano et al., 2010).

2.4.2 Soy Protein

Soybeans and their constituents have been extensively investigated for their role in preventing chronic disease, such as osteoporosis and cardiovascular disease (Alekel, Weaver, Ronis, & Ward, 2013). Observational studies such as the low rates of hip fractures in Asians (Greendale et al., 2002; Zheng, Lee, & Chun, 2016) suggested that soybeans may contribute to bone health. Results of many human studies have indicated the protective role of soy protein, its isoflavones, or the combination on ovariectomy-induced osteoporosis prevention (Table 2.5). However, it is still not clear whether the protection effect of soy protein is due to its amino acid composition (Omi, Aoi, Murata, & Ezawa, 1994), non-protein constituents like isoflavones (Arjmandi, Birnbaum, et al., 1998; Arjmandi, Getlinger, et al., 1998), or a combination of these factors (Arjmandi, Getlinger, et al., 1998). While the importance of isoflavones in preserving bone has been demonstrated by animal studies (Anderson, Ambrose, & Garner, 1998; Fanti et al., 1998; Picherit et al., 2001),

whether isoflavones can exert similar bone protective effects independently of soy protein still remain controversial (Picherit et al., 2001; Picherit et al., 2001). Thus, more studies need to be conducted to investigate the functional components in soy protein and mechanisms of action.

2.4.3 Egg Protein

Egg, another good protein source, is reported to be of benefit for pediatric bone health (Coheley et al., 2018). Several bioactive proteins have been found in both egg white and yolk, and are suggested to be able to prevent osteoporosis (Table 2.6). Although most experiments were conducted *in vitro*, studies have claimed a positive relation between bone health and several egg white proteins. Ovalbumin has been suggested to stimulate osteoblast proliferation and differentiation, and further promote bone formation (Farrar, Barone, & Morgan, 2010). Ovotransferrin was also reported to play important role in chondrogenesis and endochondral bone formation in chick embryos (Gentili et al., 1993; Gentili et al., 1994). Meanwhile, egg white protein cystatin was found to exhibit inhibition activity on osteoclastic resorption, which can be used to prevent bone loss (Brand et al., 2004). Moreover, in addition to the direct stimulation on bone cells, egg white proteins can also be developed as protein-based scaffold for bone generation, or protein-based delivery system for osteoporosis drugs. Ovalbumin-, ovomucin- and lysozyme-incorporated scaffolds promoted bone formation by supporting cell adhesion and regulating osteogenic activity (Carpena, Abueva, Padalhin, & Lee, 2017; Farrar et al., 2010; Martins et al., 2009). Ovomucoid was developed for calcitonin delivery system which could protect calcitonin against breakdown for oral drug delivery (Shah & Khan, 2004).

Compared to bioactive proteins from egg white, several experiments investigated the bioactivities of egg yolk proteins on bone health with animal studies. Leem et al. (2004) studied the effects of

egg yolk proteins on longitudinal bone growth of adolescent male rats. Egg yolk proteins were extracted with ethanol and fractionated into yolk water-soluble protein and yolk water-insoluble protein. Adolescent male rats were fed with whole egg yolk proteins, yolk water-soluble protein and yolk water-insoluble protein all promoted longitudinal bone growth and the height of growth plates (Leem et al., 2004). The protective effect of these proteins was further reported due to the stimulation on bone formation as well as the inhibition on osteoclastogenesis (Ji, Leem, Kim, & Kim, 2007; Kim, Kim, & Leem, 2008). Continuously, the effects of egg yolk water-soluble protein-derived peptide was prepared and showed stronger effects on preventing bone resorption than egg yolk water-soluble protein (Kim, Lee, & Leem, 2011). Similarly, Kobayashi et al. investigated the effects of food enriched with egg yolk hydrolysate on bone metabolism in orchidectomized dogs (Kobayashi et al., 2015) and developed products Bonepep® (Pharma Foods International Co., Ltd., Kyoto, Japan), a commercial bone peptide produced by enzymatic treatment of yolk water-soluble component of defatted chicken eggs. Orchidetomized dogs supplemented with Bonepep® showed significantly promotion on bone formation immediately after orchiectomy (Kobayashi et al., 2015). Among all egg yolk protein, phosvitin is the most promising candidate to maintain bone health because of its strong ability to bind calcium (Hatta, Kapoor, & Juneja, 2008). Phosvitin has been reported to play important role in bone formation of chicken embryos through dephosphorylation (Li, Geng, Huang, Ma, & Zhang, 2014). Liu et al. found that phosvitin stimulated osteoblasts differentiation in the absence of ascorbic acid (Liu, Li, Geng, Huang, & Ma, 2016), which is known to be essential for bone formation. Although the underlying mechanisms are still under study, it might because phosvitin has similar function as ascorbic acid to induce type I collagen and osteocalcin expression during osteoblasts differentiation (Liu et al., 2016).

2.5 Perspectives and Potential Challenges

2.5.1 Current Osteoporosis Drug Market

In the US, an estimated 19% of older men (≥65 years old) and 30% of older women (≥65 years old) are at elevated risk of osteoporotic fracture and considered to be eligible for treatment. The burden of osteoporosis is similar in Europe and is projected to raise worldwide, with increasing aging populations and urbanization. Thus, rising prevalence of osteoporosis is a serious public health concern. The global osteoporosis treatment market was valued at 11.5 billion USD in 2015, and is expected to expand at a CAGR (compound annual growth rate) of 3.3% from 2017 to 2025 (Grand View Research, 2017). The global osteoporosis treatment market is segmented by drug type into bisphosphonates, parathyroid hormone therapy, calcitonin, selective estrogen inhibitors modulator (SERM) and RANKL inhibitors. Bisphosphonates are extensively used for prevention and treatment of osteoporosis, thus dominating the osteoporosis treatment market. However, a 50% decrease of using bisphosphonates in postmenopausal women from 2008 to 2012 was documented in US (Jha, Wang, Laucis, & Bhattacharyya, 2015; Khosla & Shane, 2016). The reason is probably multifactorial, but the public awareness about the devastating side effects is the most important one. Also, FDA highlighted a statement claiming the anti-fracture efficacy of bisphosphonates after 5 years of use is scarce and perhaps conflicting (Khosla et al., 2007; Shane et al., 2014). Anabolic agents are likely to further revolutionize the market with the arrival of a novel sclerostin inhibitor, romosozumab, and teriparatide biosimilars. Although this diversification of the anabolic offering will help to bolster the treatment paradigm and increase the completion in the market, the associated side effects, insufficient efficacy and high price tag still remains an unmet need in the osteoporosis market.

2.5.2 Market Opportunity of Bioactive Proteins

In the past few decades, growing consciousness among consumers regarding their health and proper diet is expected to increase the research interests in the promising use of foods to promote well-being and to help with the disease prevention. The global market of functional food and natural health products reached approximately 129.4 billion USD in 2015 and is expected to continue growing (AAFC, 2015; Grand View Research, 2016).

Bone health is closely related to diet and nutrition. Changing dietary patterns and/or providing supplemented nutrients have been suggested to play outstanding role in preventing osteoporosis. Currently, calcium and vitamin D are still the predominant supplementations in bone health management market. However, with the growing concern on the increasing risk of cardiovascular disease and kidney disease by supplementing calcium-vitamin D, there are emerging opportunities for other nutraceuticals, such as bioactive proteins. Bioactive proteins and peptides are an important category of nutraceuticals food sector, which is reported to valued at 75 billion USD per year currently (Mine, Li-Chan, & Jiang, 2010). The use of bioactive proteins in disease intervention is suitable for long-term use, and can provide additional nutritional value compare to pharmaceutical drugs. Currently, a number of bioactive-based functional foods or nutraceuticals are commercially available carrying health claims, such as the colostrum powder, whey protein powder, and collagen products. However, a further investigation and development of bioactive proteins application in osteoporosis is still needed to meet the growing increased market requirements.

2.5.3 Potential Challenges of Bioactive Protein Application

To further broaden the application of bioactive proteins in health promoting, a major challenge is to ensure the efficacy and convenient delivery. In general, bioactive proteins are susceptible to gastrointestinal (GI) digestion, in which the proteins are hydrolyzed into small peptides, and further digested to produce amino acid (Matthews & Laster, 1965). Thus, development of bioactive proteins poses high challenges for stability against GI digestion to ensure the intact proteins can be delivery to proper cellular site of action. Additionally, the mechanisms underlying the effects of bioactive proteins in disease prevention is difficult to elucidate. Even though bioactive proteins successfully go through the GI system and remain intact, the protein absorption and transportation to the target site can be also challenging. Although research reported that the intact lactoferrin has been found in the circulation (Kitagawa et al., 2003; Takeuchi, Kitagawa, & Harada, 2004), proteins with high molecular weight and size are hardly absorbed in small intestine. So, mechanism research is needed to provide the theoretical basis to support the beneficial effects of bioactive proteins. Finally, most of the current studies on bioactive proteins were conducted with only cells or animals. Therefore, further studies to establish the efficacy of bioactive proteins by clinical trials is inevitable.

2.6 General conclusions

There is growing interest in using bioactive compounds for health management and disease prevention. Osteoporosis is one of the most serious chronic bone disorders worldwide, while the therapies are far from satisfactory. Bone health is greatly affected by remodeling; thus, opportunities exist to identify bioactive compounds that can modulate the remodeling process. Currently, food-derived bioactive proteins have been suggested to play important roles in

prevention of osteoporosis. Although a number of research reports provided exciting results using bioactive proteins to promote bone health, much of the work is still in its infancy. The degradation of proteins through the gastrointestinal (GI) tract is one of the most important reasons that has limited the application of bioactive proteins. GI digestion may significantly affect the activity of bioactive proteins; therefor, the bioavailability and efficacy of orally administration of bioactive proteins should be further evaluated with animal and human study. Meanwhile, the mechanisms should be further studied to elucidate the principles underlying the beneficial action, which may provide more possible targets to exert the function of bioactive proteins. It may also provide hints to develop novel delivery methods in addition to orally administration, which can minimized the negative influence of GI digestion. Although there are still difficulties need get over, the great potential of food-derived bioactive proteins are expected to be a promising approach to benefit for human health as well as to add value to food industry.

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Figure 2.1 Bone remodeling cycle

Bone remodeling cycle mainly occurs in three phases: resorption, reversal, and formation. In the first phase, osteoclast precursors are stimulated to differentiation and adherence to bone surface to initiate the resorption. During the resorption, the bone matrix (old bone) is digested. Reversal phase is considered as a preparation stage for osteoblast to form new bone at the resorption site. Osteoblasts appear at the resorption site to synthesize new bone matrix and become either lining cells resting on the bone surface or osteocytes embedding into the new bone matrix. This figure was made according to Kapinas & Delany, (2011)

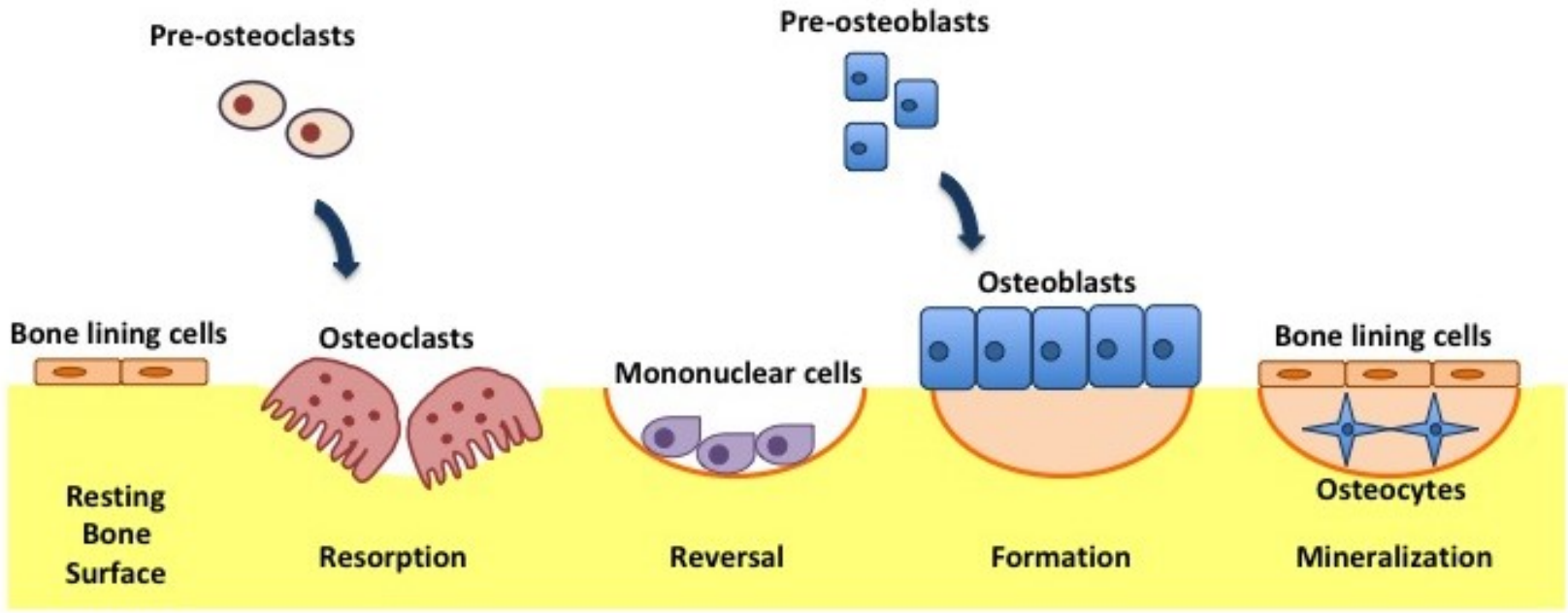

Figure 2.2 Osteoblast differentiation

Signalings by Runx2, BMP, and Wnt direct the mesenchymal stem cell differentiated to the osteoblast lineage. Fully differentiated osteoblasts have potential to express alkaline phosphatase, type I collagen, osteocalcin, osteopontin, and bone sialoprotein as well as to incorporate the hydroxyapatite into the newly deposited osteoid. At the end of bone formation, a subset of osteoblast further differentiated into osteocytes (embedded into bone matrix) or become inactive bone lining cells. The remaining osteoblasts undergo apoptosis. In addition to osteoblasts, mesenchymal stem cells can also differentiate into adipocytes, myoblasts, fibroblasts and chondrocytes. This figure was made according to Arboleya & Castañeda, (2013)

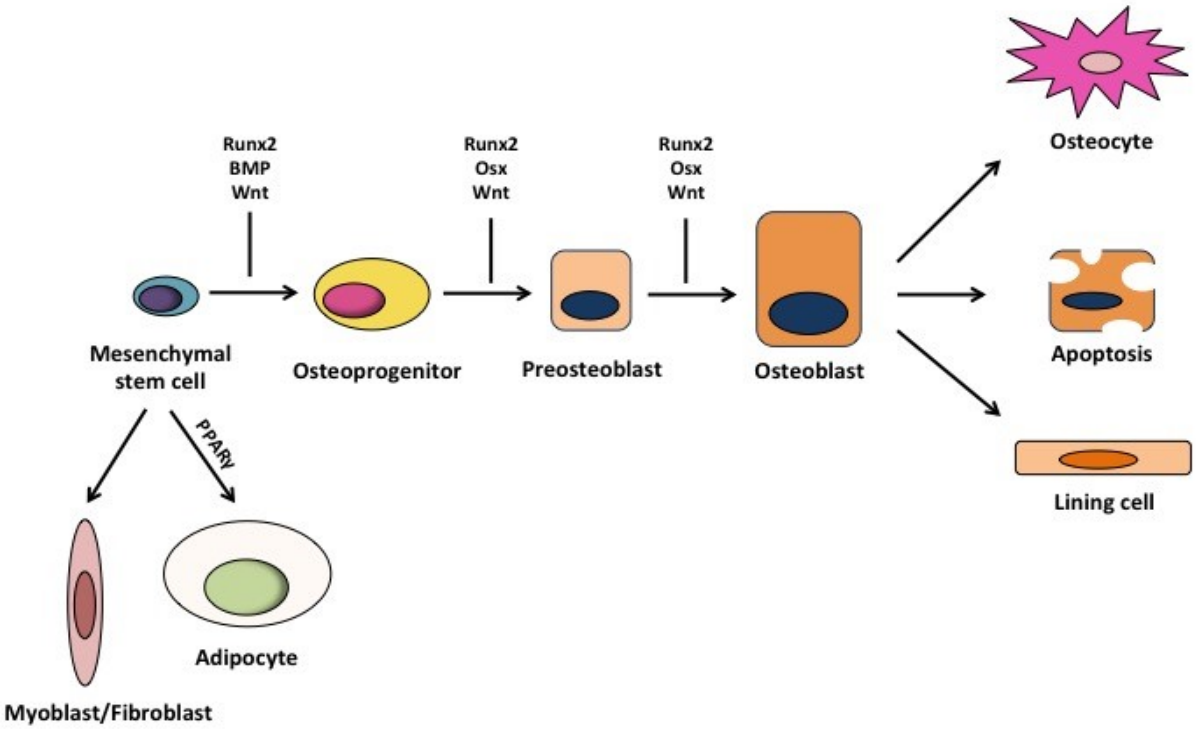

Figure 2.3 Key regulation pathways in osteoblast differentiation

Three key signaling pathways regulate the osteoblast differentiation: the BMP signaling, Wnt signaling and FGF signaling. (a) Smad-dependent-BMP signaling activated by binding to BMP receptor and then transducing signaling to Samds (Smad 1/5/8). Activated Smads form a complex with Smad4 then translocate into nucleus and interact with other transcription factors to trigger target gene expression. (b) Wnt signaling activated by binding to a receptor complex including a frizzled receptor coupled with LRP5/6 as co-receptor. The Wnt signaling induces a cascade of intracellular events that stabilize β-catenin and facilitate the transportation into nucleus, where it binds transcription factors and modulate target gene expression. (c) FGF signaling diverse roles in osteoblast differentiation; however, the intracellular signaling cascades responsible to FGF-regulated osteoblast differentiation remain unclear. So far, FGF signaling is known to active MERKs, Akt and PKC pathways to affect osteoblast differentiation.

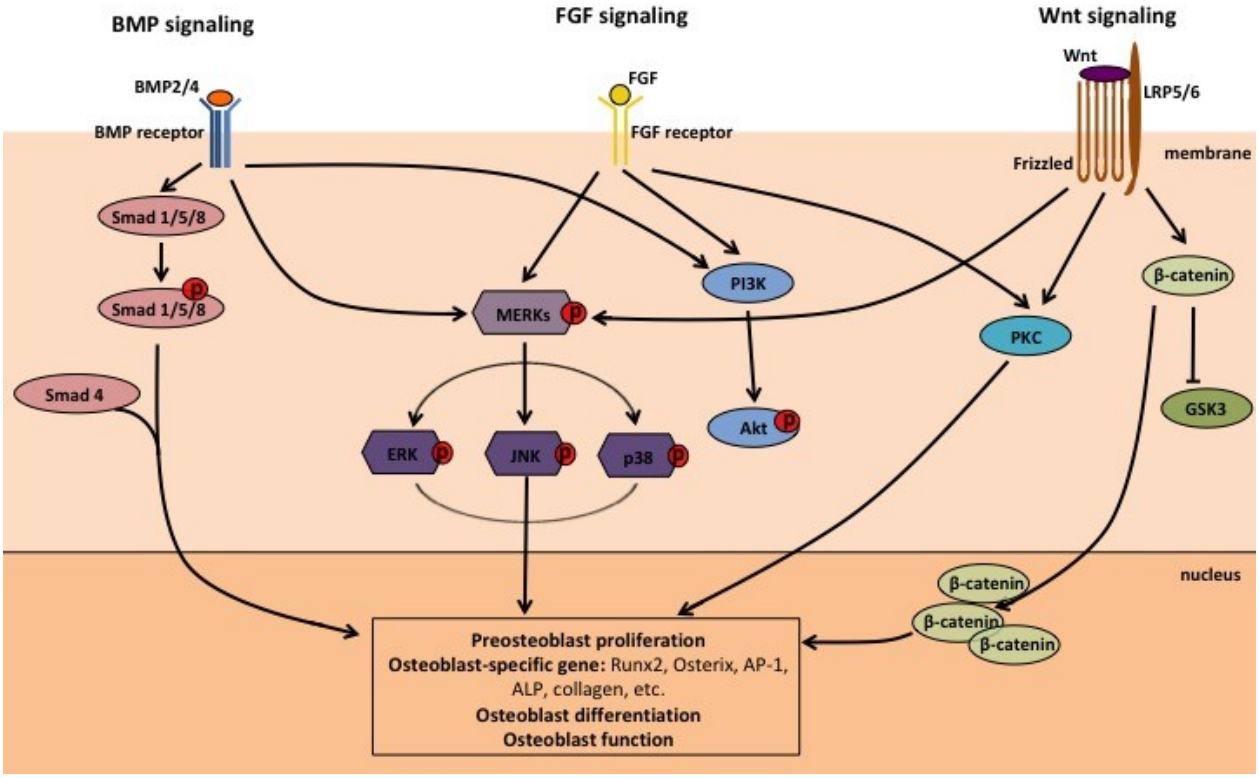

Figure 2.4 Osteoclast differentiation

In response to M-CSF, hematopoietic stem cells differentiated into macrophage colony-forming units (CFU-M), which are the common precursor cells of macrophages and osteoclasts. RANKL is essential for the differentiation step from CFU-M to multinucleated osteoclasts by cell-cell fusion. Finally, multinucleated osteoclasts undergo maturation and acquire bone-resorbing activity.

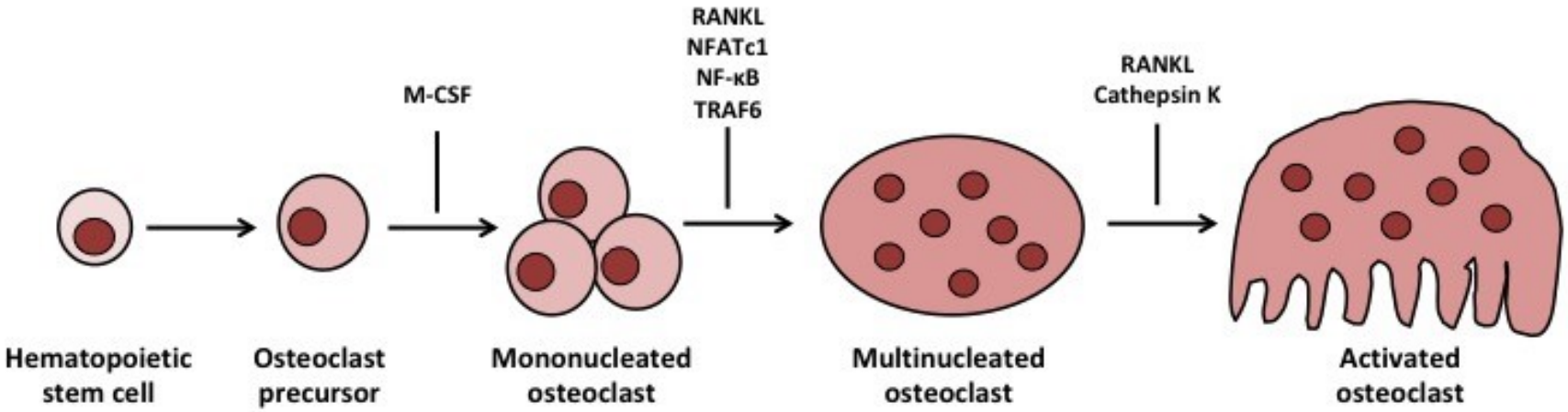

Figure 2.5 Key regulation pathways in osteoclast differentiation

M-CSF and RANKL are essential for osteoclast differentiation. M-CSF binds to its receptor c-Fms and activates ERK and Akt pathways to induce cell proliferation and survival of osteoclast precursors. Meanwhile, RANKL recruits TRAF6 to activate MAPKs, Akt and NF-κB pathways to promote differentiation of osteoclast precursors to osteoclasts. Additionally, various pro-inflammatory cytokines, such as TNF-α, IL-6 and IL-1, cooperatively orchestrate enhanced osteoclastogenesis. For example, TNF-α stimulates osteoclastogenesis via activate NF-κB and MAPKs pathways.

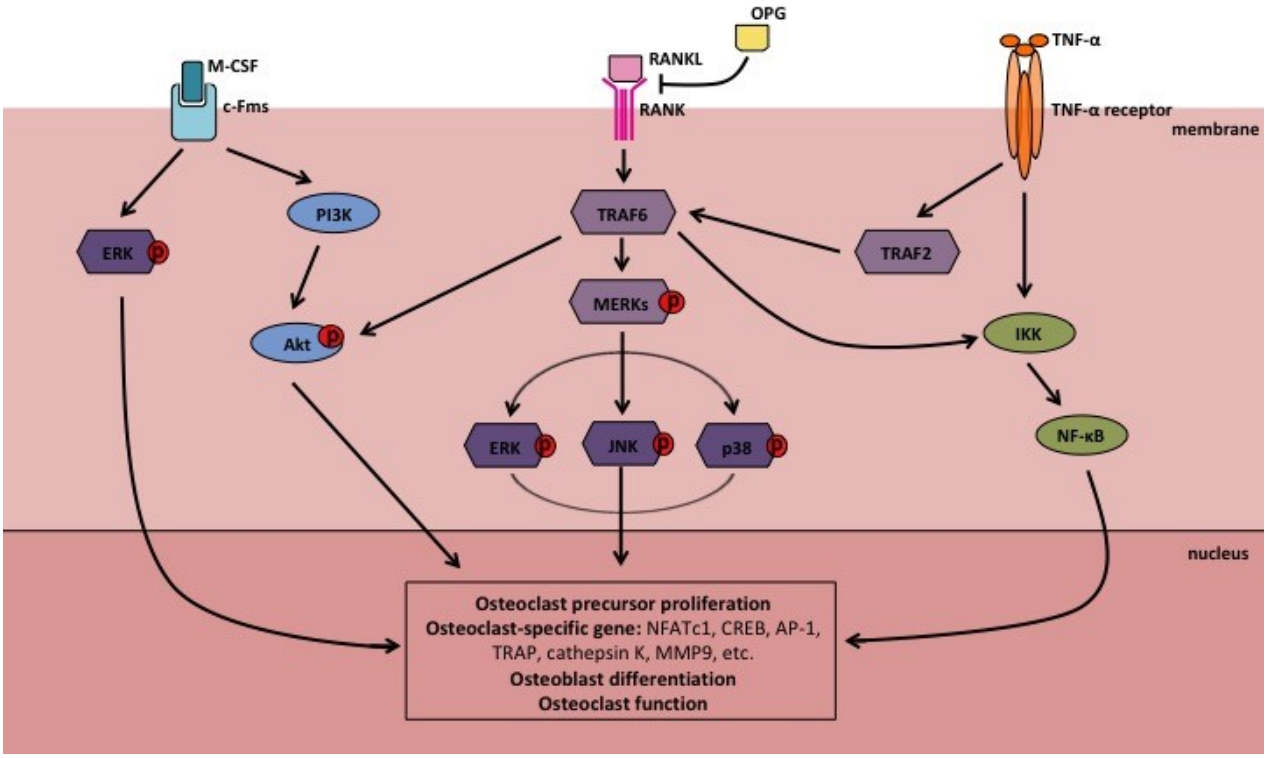

Figure 2.6 Interaction between osteoblast and osteoclast

Osteoblasts stimulate proliferation, differentiation, survival and apoptosis of osteoclasts by M-CSF/c-Fms, RANK/RANKL, LPA/LPA receptor, and Fas/FasL. Osteoblast also inhibits the proliferation, differentiation of osteoclasts by OPG, RANKL/LGR4 and Sema3A/Nrp. Meanwhile, osteoclasts produce sclerostin, Sema4D and MicroRNAs or exsomes and inhibit the differentiation or activity of osteoblast. This figure was made according to Matsuo & Irie, (2008)

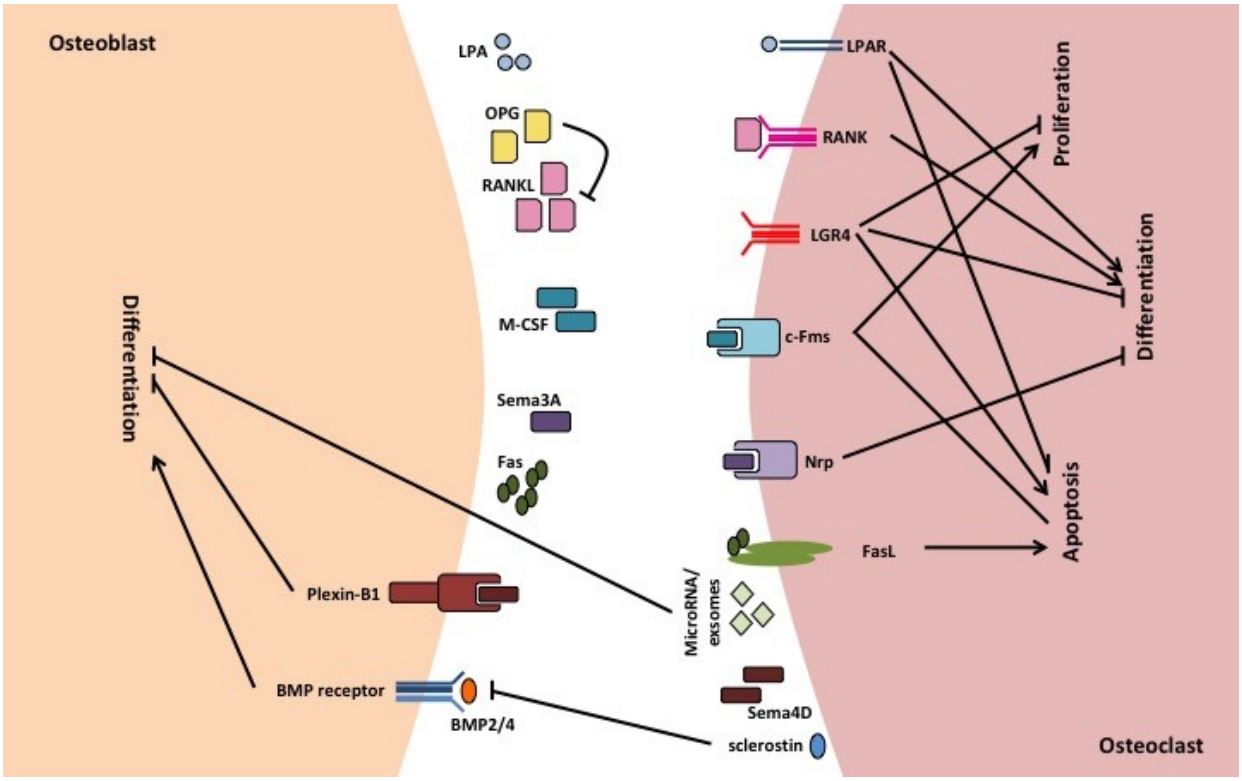

Table 2.1 Physiological and pharmacological activities of ovotransferrin and its peptides

Activity	Protein/peptide	Mechanisms	Reference
Antimicrobial Activity			
	Ovotransferrin	Iron deprivation necessary for the growth of microorganisms	Alderton, Ward, & Fevold (1946); Valenti et al. (1983); Valenti, Antonini, Fanelli, Orsi, & Antonini (1982)
		Bicarbonate addition increases antibacterial activity by forming a "protein-ferric-bicarbonate" complex	Valenti et al. (1981)
		Zn^{2+}-ovotransferrin complex enhances antibacterial activity	Valenti, Visca, Antonini, Orsi, & Antonini (1987)
		EDTA and/or lysozyme prevents *E. coli* O157:H7 or *L. monocytogenes* proliferation	Ko, Mendoncam, Ismail, & Ahn (2009)
		Ion leakage and uncoupling of the respiration-dependent energy production (holo-form)	Alderton et al. (1946); Valenti et al. (1983); Wellman-Labadie, Picman, & Hincke (2008)
	Ovotransferrin peptide OTAP-92 (Leu109-Asp200)	Block the voltage-dependent K^+ channel	Ibrahim, Iwamori, Sugimoto, & Aoki (1998); Ibrahim, Sugimoto, & Aoki (2000)
Antiviral Activity			
	Ovotransferrin	Antiviral activity against Marek's disease virus (MDV)	Giansanti et al. (2005)
		Viral adsorption inhibition	Giansanti et al. (2002)
	Ovotransferrin peptide DQKDEYELL and KDLLFK	Inhibit viral infection	Giansanti et al. (2002); Giansanti et al. (2005)
Antioxidant activity			

	Ovotransferrin	Superoxide dismutase (SOD)-like activity O_2^- scavenge and O_2^- dismutation capacity	Ibrahim, Hoq, & Aoki (2007) Ibrahim et al. (2007)
	Ovotransferrin peptide DLLFKDSAIMLK and FFSASCVPGATIE	Oxygen radical absorbance capacity	(Huang, Majumder, & Wu, 2010)
	Ovotransferrin peptide WNIP and GWNI	Motif WNI is responsible for antioxidant activity	Shen, Chahal, Majumder, You, & Wu (2010)
Anti-cancer activity			
	Reduced autoclaved ovotransferrin	Inhibit the cancer cell proliferation	Ibrahim & Kiyono (2009)
Anti-inflammation activity			
	Ovotransferrin	Acute-phase protein (APP) in chicken	Rath et al. (2009); Tohjo et al. (1995); Xie et al. (2002); Xie et al. (2002); Xie, Huff, Huff, Balog, & Rath (2002)
	Ovotransferrin peptide IRW and IQW	Attenuate TNF-α-induced inflammation	Majumder, Chakrabarti, Davidge, & Wu (2013)
Anti-hypertensive activity			
	Ovotransferrin peptide IRW, IQW and LKP	ACE inhibition	Majumder et al. (2015); Majumder et al. (2013)
	Ovotransferrin peptide KVREGT	ACE inhibition	Lee, Cheng, Enomoto, & Nakano (2006)
Immunomodulating activity			

	Ovotransferrin	Component of innate immunity	Giansanti et al. (2002); Giansanti, Giardi, Massucci, Botti, & Antonini (2007)
		Modulation of macrophages and heterophils functions	Xie et al. (2002)
		Inhibit LPS and PHA-induced lymphocyte proliferation	Otani & Odashima, (1997)
Growth Promotion			
	Ovotransferrin	Involve in chondrogenesis and endochondral bone formation in chicken	Cancedda, Castagnola, Cancedda, Dozin, & Quarto (2000); Gentili et al. (1993, 1994)
		Improve mitochondrial activity and differentiation in chick brain and neural retina cells	Bruinink, Sidler, & Birchler (1996)
		Myotrophic activity	Shimo-Oka, Hagiwara, & Ozawa (1986)
Drug delivery			
	Ovotransferrin	- Binding and delivery of water insoluble antibiotics (sulphantibiotics)	Ibrahim et al. (2015)

Table 2.2 Current FDA-approved osteoporosis drugs

Drug	Brand, Manufacturer	Treatment			Prevention	
		Postmenopausal osteoporosis	Osteoporosis in men	Glucocorticoid-induced osteoporosis	Postmenopausal osteoporosis	Glucocorticoid-induced osteoporosis
Alendronate	Fosamax, Merck	Yes	Yes	Yes	Yes	Yes
Alendronate/cholecalciferol	Fosamax Plus D, Merck	Yes	Yes	No	No	No
Alendronate effervescent	Binosto, Mission Pharmacal	Yes	Yes	No	No	No
Risedronate IR	Actonel, Warner Chilcott	Yes	Yes	Yes	Yes	Yes
Risedronate DR	Atelvia, Warner Chilcott	Yes	No	No	No	No
Ibandronate injection	Boniva, Genentech	Yes	No	No	No	No
Ibandronate tablets	Boniva, Genentech	Yes	No	No	Yes	No
Zoledronic acid	Reclast, Novartis	Yes	Yes	Yes	Yes	Yes
Denosumab	Prolia, Amgen	Yes	Yes	No	No	No
Raloxifene	Evista, Lilly USA	Yes	No	No	Yes	No

Conjugated estrogens/bazed oxifene	Duavee, Pfizer	No	No	No	Yes	No
Teriparatide	Forteo, Lilly USA	Yes	No	Yes	No	No
Abaloparatide	Tymlos, Radius Health	Yes	No	No	No	No
Calcitonin-salmon	Miacalcin injection, Novartis Swiss	Yes (When alternative treatments are not suitable)	No	No	No	No

This table was made according to Tu et al., (2018) and Waalen, (2010)

Table 2.3 The emerging therapies and investigational drugs for osteoporosis

Drug	Manufacturer	Mechanism	Investigation process	Side effects	Reference
Antiresorptive Agent					
Romosozumab	Evenity, Amgen/UBC	Inhibit sclerostin	Rejected by FDA, but will submit application again with updated data	Increase risk of cardiovascular diseases	Medscapte (2017)
Blosozumab	Eli Lilly	Inhibit sclerostin	Complete phase II clinical trials	No serious adverse effects reported	MacNabb, Patton, & Hayes (2016)
BPS 804	Novartis	Inhibit sclerostin	Complete phase II clinical trials	Increase risk of chronic kidney disease	MacNabb et al. (2016)
Odanacatib	Merck & Co.	Inhibit cathepsin K	Discontinued	Increase risk of stroke	Mullard (2016)
Lasofoxifene	Sermonix	SERM	Approved in Europe, but not in U.S. yet	Increase risk of breast cancer, coronary heart disease and stroke	
Anabolic Agents					
Ronacaleret	N/A	Stimulate PTH	Clinical trial is in progress	N/A	Balan et al. (2009); Fitzpatrick et al. (2011, 2012)
GSK-3β inhibitor	N/A	Inhibit GSK-3β	Less clinical data reported	Associate with Alzheimer's disease and diabetes	Baron & Rawadi (2007); Clement-Lacroix et al. (2005); Kulkarni et al. (2006); Wilting et al. (2007)

Dual Action Agents					
Strontium ranelate	N/A	Inhibit osteoclast activity, while stimulating osteoblast activity	Approved in Europe (except UK), but not in U.S. yet	Increase risk of venous thromboembolism	Blake, Lewiecki, Kendler, & Fogelman (2007); Reginster, Sarlet, Lejeune, & Leonori (2005); Stevenson, Davis, Lloyd-Jones, & Beverley (2007)

Table 2.4 Summary of current research on dairy bioactive proteins/peptides and their role in osteoporosis treatment

	Experimental design	Outcomes	Reference
Milk basic protein (MBP)			
Animal study			
21 51-week-old OVX rats	0.01% or 0.1% MBP for 17 weeks	- Increased BMD - Decreased urinary deoxypyridinoline	Toba et al. (2000)
75 6-month-old OVX rats	3 g/kg MBP for 4 months	- Recovered OVX-induce bone loss - Decreased plasma C-telopeptide of type I collagen - Preserved bone architecture - Increased organic matter in rat femurs	Kruger et al. (2017)
34 Healthy adult women	40 mg BMP per day for 6 months	- Increased BMD - Decreased urinary cross-linked N-teleopeptides of type I collagen and deoxypyridinoline	Aoe et al. (2001)
33 Healthy adult women	50 mL experimental beverage containing 40 mg MBP for 6 month	- Significantly increase BMD	Yamamura et al. (2002)
35 healthy young women	40 mg BMP per day for 6 months	- Increased BMD - Decreased urinary cross-linked N-telopeptides of type I collagen - Increased serum osteocalcin	Uenishi et al. (2007)
100 healthy menopausal women	50 mL experimental beverage containing 40 mg MBP for 12 month	- 1.5% increased BMD - Decreased urinary cross-linked N-teleopeptides of type I collagen	Aoyagi et al. (2010)
32 healthy menopausal women	40 mg MBP per day for 6 months	- Increased BMD - Decreased urinary cross-linked N-teleopeptides of type I collagen	Aoe, Koyama, Toba, Itabashi, & Takada (2005)
Bovine colostrum acid proteins (BCAPs)			
Animal study			

48 3-month OVX SD rats	50 mg/day, 10 mg/day or 2 mg/day BCAP for 12 weeks	- Increased BMC and BMD in a dose-depended manner	Du et al. (2011)
48 OVX rats	0.4 g/day, 0.2 g/day or 0.04 g/day BCAP for 12 weeks	- Increased femur weight and length - Increased BMD	Du, Wang, Wu, & Zhang (2009)
Lactoferrin (LF)			
Animal study			
45 sexually mature male mice	4 mg, 0.4 mg, or 0.4 mg LF injected into right hemicalvaria for 5 days	- Increased bone formation	Cornish et al. (2004)
42 6-week-old OVX mice	20 g/kg, 10 g/kg, 5 g/kg or 1 g/kg LF for 27 weeks	- Increased BMD and femoral failure load	Blais, Malet, Mikogami, Martin-Rouas, & Tomé (2009)
100 12-week-old OVX mice	10 g/kg LF for 1, 2, and 4 months	- Prevented OVX-induced bone loss	Malet et al. (2011)
60 3-month-old OVX rat	85 mg/kg, 8.5 mg/kg or 0.85 mg/kg LF for 3 months	- Increased BMD - Increased mechanical strength - Decreased serum TNF-αand IL-6	Guo et al. (2009)
Casein phosphopeptides (CPPs)			
Animal study			
20 OVX rat	100 mg/kg eggshell-CPPs for 4 weeks	- Increased calcium absorption - Increased femoral bone weight - Rescued serum osteocalcin and BMD	Kim et al. (2013)
16 5-week-old OVX rat	28 mg/100g CPP tofu or 28 mg/100 dephospho-CPP tofu for 4 weeks	- Prevented OVX-induced bone loss	Yamaguchi, Tezuka, Yoshihiro Tukada, & Kish (1998)

BMD: Bone mineral density

OVX: Ovariectomy

Table 2.5 Summary of human trials on the impact of soy consumption on bone health

Participants	Dietary Intervention	Outcomes	Reference
Healthy, peri-menopausal women	40g soy protein for 6 months	- Bone-specific alkaline phosphatase - N-terminal telopeptide of type I collagen	Alekel et al. (2000)
Healthy, post-menopausal women	25g soy protein for 12 months	- Bone mineral density - Total body - Bone mineral content - Deoxypyridinoline - Bone-specific alkaline phosphatase	Arjmandi et al. (2005)
Healthy, post-menopausal woman	25.6g soy protein for 9 months	- Bone-specific alkaline phosphatase - C-terminal telopeptide of type I collagen	Evans, Racette, Van Pelt, Peterson, & Villareal (2007)
Healthy, post-menopausal woman	18g soy protein for 12 months	- Bone mineral density - N-terminal telopeptide of type I collagen - Bone-specific alkaline phosphatase	Kenny et al. (2009)
Healthy, post-menopausal woman	25.6g soy protein for 12 months	- Bone mineral density - Bone-specific alkaline phosphatase	Kreijkamp-Kaspers et al. (2004)
Healthy, post-menopausal woman	38g soy protein for 6 months	- N-terminal telopeptide of type I collagen	Murray et al. (2003)
Healthy, post-menopausal woman	25g soy protein isolate for 24 months	- Bone mineral density - N-terminal telopeptide of type I collagen	Vupadhyayula, Gallagher, Templin, Logsdon, & Smith (2009)

Table 2.6 Summary of research on egg protein/peptides and their impact on bone health

Protein/Peptide	Research Model	Outcomes	Reference
Egg white protein/Peptide			
Ovalbumin	- Ovalbumin-based scaffolds - In vitro study with MC3T3-E1 cells	- Improved proliferation of MC3T3-E1 - Increased expression of alkaline phosphatase (ALP) and osteocalcin	Farrar, Barone, and Morgan (2010)
Ovotransferrin	- In vitro study with MC3T3-E1	- Stimulation of cell proliferation - Increased expression of type I collagen and ALP - Promoted cell differentiation	Shang and Wu (2018)
Ovomucoid	- Ovomucoid-based delivery system - N/A	- Protection of calcitonin breakdown for oral drug delivery - Homology between ovomucoid and osteonectin - The potential on binding collagen, calcium and hydroxyapatite	Shah and Khan (2004) Bolander et al. (1988)
Ovomucin	- Ovomucin-based scaffolds - In vitro study with bone marrow-derived mesenchymal stem cell	- Supported cell adhesion and growth	Carpena et al. (2017)
Lysozyme	- N/A - Lysozyme incorporated scaffolds - In vitro study with rat marrow stromal cells	- The regulation of collagen degradation during bone remodeling - Improved osteogenic activity - Increased production of mineralized extracellular matrix	Sakamoto et al. (1974) Martins et al. (2009)
Cystatin	- N/A	- Inhibited bone matrix degradation - Inhibited osteoclastic bone resorption	Brand et al. (2004)

Avidin	- N/A	- The expression of avidin is related to growth plate hypertrophic cartilage develop in chick embryos	Zerega et al. (2001)
	- Avidin-biotin binding system - In vitro study with human osteoblast cell line MG-63	- Improved osteoblast-like cell adhesion and proliferation - Increased the expression of type I collagen, osteopontin, ALP, and osteocalcin	Kim et al. (2015)
Egg Yolk Protein/Peptide			
Egg yolk proteins	- In vivo study with adolescent male rats	- Promoted longitudinal bone growth and height of growth plates	Leem et al. (2004)
Egg yolk water-soluble protein	- In vitro study with MC3T3-E1 cells	- Promoted cell proliferation and differentiation	Ji et al. (2007)
	- In vitro study with bone marrow cells from male ICR mice	- Inhibited TNFα-induced osteoclastogenesis	Kim, Kim, and Leem (2008)
Egg yolk water-soluble peptide	- In vivo study with ovariectomized rats	- Increased bone mineral density and cortical thickness in tibia - Prevented ovariectomy-induced cancellous bone loss	Kim, Lee, and Leem (2011)
Egg yolk water-soluble peptide product Bonepep®	- In vivo study with orchiectomized dogs	- Promoted bone formation after orchiectomy immediately	Kobayashi et al. (2015)
Phosvitin	- In vitro study with live calvarial bone organ culture models	- Stimulated osteoblast differentiation in the absence of ascorbic acid - Inhibited parathyroid hormone-induced osteoclastogenesis	Liu et al. (2016)

CHAPTER 3 - Egg White Ovotransferrin Shows Osteogenic Activity in Osteoblast Cells

3.1 Introduction

Osteoporosis, defined as a skeletal disorder, is characterized by an impairment of bone mass, strength, and microarchitecture, and is caused by an imbalanced bone formation and bone resorption, which is regulated respectively by osteoblast and osteoclast (WHO, 2003). As a serious public health concern, osteoporosis affects over 200 million people worldwide, leading to severe and localized pain, increased bone fractures, and reduced height (US Department of Health and Human Services, 2004). Moreover, osteoporosis is also a major cause of morbidity and health expenditure in ageing populations (Naot, Grey, Reid, & Cornish, 2005). Currently, antiresorptive medications such as bisphosphonates, selective estrogen receptor modulators and hormones are the main pharmacological treatment options for osteoporosis (Chen & Sambrook, 2012). Teriparatide, a recombinant fragment of parathyroid hormone (PTH) has been used as an anabolic agent in osteoporosis treatment by stimulating the bone formation (Rosen, 2004). Despite their great therapeutic value, these agents are limited in their ability to restore bone mass, and are associated with side effects such as nausea, irregular heartbeat, loose bowel movement, and even increased risk of cancer (Sharif, Abdollahi, & Larijani, 2011). Therefore, there is an increasing interest in using natural components as the alternatives for the prevention and treatment of osteoporosis (Jillian Cornish et al., 2004).

Ovotransferrin, a 78 kDa iron-binding glycoprotein found in avian egg white and in avian serum, is a member of transferrin family (Giansanti, Leboffe, Pitari, Ippoliti, & Antonini, 2012). Egg ovotransferrin displays several bioactivities, including antimicrobial, antioxidant, and

immunomodulating activities (Giansanti et al., 2012). Likewise, ovotransferrin is a component of innate immunity (Xie, Huff, Huff, Balog, & Rath, 2002). In chicken macrophages, ovotransferrin was reported to stimulate the production of IL-6, nitrite and Matrix Metallo-proteinase, suggesting that ovotransferrin can modulate macrophage and heterophil function (Xie et al., 2002). More recently, oral administration of egg ovotransferrin reduced the levels of inflammatory cytokines (Andersen, 2015). Since inflammation is known to play an important role in bone metabolism (Redlich & Smolen, 2012), ovotransferrin may have beneficial effects on bone health. Previous study also suggested that the expression levels of ovotransferrin and its receptor were increased during initial stages of bone formation in developing chick embryo (Gentili et al., 1994), further indicating its significant role on cartilage and bone growth. Lactoferrin, another member of transferrin family, was reported to increase osteoblast activity while decreasing osteoclast activity *in vitro* (Jillian Cornish et al., 2004). Injection of lactoferrin increases bone growth in adult male mice (Jillian Cornish et al., 2004; Naot et al., 2005). Guo et al. (2009) further proved that orally administrated lactoferrin increases bone mineral density and mechanical strength on ovariectomized-induced osteoporosis (Guo et al., 2009). In this research, we investigated the effects of ovotransferrin on cell proliferation and differentiation of bone osteoblast cell MC3T3-E1, a widely used cell model in osteoblastic activity study.

3.2 Materials and methods

3.2.1 Reagents

Ovotransferrin (Conalbumin from chicken egg white, at a purity of 98%) and Alizarin-S red stain were bought from Sigma Chemicals Co (St Louis, MO, USA). α-minimum essential medium (α-MEM) and Fetal Bovine Serum (FBS) were obtained from Gibco/Invitrogen (Carlsbad, CA, USA).

AlamarBlue cell viability reagent and BrdU labeling reagent were bought from ThermoFisher Scientific Inc. (Waltham, MA, USA). Propidium iodide flow cytometry kit was bought from Abcam plc. (Toronto, ON, Canada). Collagen I alpha antibody was bought from Novus Biological Canada ULC (Oakville, ON, Canada). Hoechst 33342 (Trihydrochloride), rabbit anti-mouse IgG (H+L) secondary antibody, and goat anti-rabbit IgG (H+L) secondary antibody were bought from Molecular Probes (Waltham, MA, USA). BrdU mouse antibody was bought from Cell Signaling Technology Inc. (Danvers, MA, USA). Triton-X-100 was from VWR International (West Chester, PA, USA). Goat anti-rabbit and donkey anti-mouse fluorochrome-conjugated secondary antibodies were purchased from Licor Biosciences (Lincoln, NB, USA).

3.2.2 Cell Culture

The murine osteoblastic cell line MC3T3-E1 (subclone 4, ATCC CRL-2593) was purchased from ATCC (Manassas, VA, USA). Cells were cultured in α-MEM supplemented with 10% FBS and penicillin-streptomycin in an incubator under 95% air and 5% CO_2 at 37 °C. Cells were subcultured using 0.25% trypsin every 2 to 3 days. All experiments were performed on 80-90% confluent cells grown in tissue culture grade plastic 96 or 48 well plates. To examine the activity of egg white ovotransferrin, the cells were incubated with different concentrations of ovotransferrin or 1000 μg/mL lactoferrin (the positive control) for different time periods prior to BrDU incorporation, western blotting, and immunofluorescence.

3.2.3 BrDU incorporation assay

The cells were seeded on 48 well tissue culture plates at a concentration of 1×10^4 cell/well, and incubated in α-MEM with 10% FBS. After 4 h of incubation, the medium was changed and different concentrations of ovotransferrin were added. Lactoferrin was also used as a positive control. Following 24 h of incubation, the cells were washed with PBS and placed in fresh α-MEM

with 1% FBS, containing 1% bromodeoxyuridine (BrDU) for 1 h. The cells were then fixed in 70% ethanol for 20 min, treated with 1N hydrochloric acid (HCl) for 20 min to antigen exposure, then permeabilized with 0.1% Triton-X-100 in phosphate buffered saline for 5 min and blocked in 1% bovine serum albumin (BSA) in phosphate buffered saline for 60 min, and finally incubated with mouse monoclonal antibody against BrDU (1:1000) at 4 °C. All the steps except addition of primary antibody were performed at room temperature. Following overnight incubation with the primary antibody, the cells were treated with anti-mouse secondary antibody for 30 min in the dark. Nuclei were stained with the Hoechst33342 nuclear dye. Cells were visualized under an Olympus IX81 fluorescent microscope. For each data point, 3 random fields were chosen. The percentage of nuclei positive for BrDU staining was noted in each field and the mean calculated.

3.2.4 Cell cycle analysis

Cell cycle distribution was analyzed by measuring DNA content using flow cytometer (FACS Canto II, BD Bioscience, CA). The cells were seeded on 6 well tissue culture plates at a concentration of 1×10^4 cells/well, and incubated in α-MEM with 10% FBS until confluence. After being treated with different concentrations of ovotransferrin or 1000 μg/mL lactoferrin for 24 h the adherent cells were washed once with PBS, then trypsinized, and centrifuged at 500 g for 5 min. The cell pellets collected were gently suspended in 400 μL PBS, slowly added 800 μL ice-cold 100% ethanol at 4 °C and mixed for at least 2 h. After centrifugation at 500 g for 5 min, the cell pellets were incubated with 200 μL staining solution (9.45 mL PBS containing 500 μL 20-fold diluted propidium iodide stock solution and 50 μL 200×RNase) at 37 °C in the dark for 30 min prior to the measurement.

3.2.5 Immunofluorescence

The immunofluorescence studies were performed similar to our previous studies (Liao, Chakrabarti, Davidge, & Wu, 2016). Briefly, cells were fixed in 4% formalin, permeabilized with 0.1% Triton-X-100 in PBS and immunostained overnight with a rabbit polyclonal antibody against type I collagen. Cells were treated with Alexa Fluor546 (red) conjugated goat anti-rabbit secondary antibody for 30 min in the dark. Nuclei were stained with the Hoechst33342 nuclear dye (1:10000) for 10 min. After washing to remove unbound antibody/dye, the immunostained cells were observed under an Olympus IX81 fluorescence microscope (Olympus, Tokyo, Japan). Images were obtained using Metamorph imaging software (Molecular Devices, Sunnyvale, CA, USA). Mean fluorescence intensity was calculated from the intensity of the red fluorescent signal (determined by Adobe Photoshop Elements 2.0 software; Adobe Systems Inc., San Jose, CA, USA) from 3 randomly selected fields per group.

3.2.6 Western blot

The cells were seeded on 48 well tissue culture plates at a concentration of 1×10^4 cells/well, and incubated in α-MEM with 10% FBS. The cells were treated with different concentrations of ovotransferrin or 1000 μg/mL lactoferrin for 72 h. After incubation, the culture medium was removed and the cells lysed in boiling hot Laemmle's buffer containing 50 μM dithiothreitol (DTT) and 0.2% Triton-X-100 to prepare samples for western blot as described previously (Liao et al., 2016). These cell lysates were run in SDS-PAGE, blotted to nitrocellulose membranes and immunoblotted with antibodies against ALP, RANKL, OPG and the loading control α-tubulin. Anti-tubulin was used at 0.4 μg/mL, while the others were used at 1 μg/mL. The protein bands were detected by a Licor Odyssey BioImager and quantified by densitometry using corresponding software (Licor Biosciences, Lincoln, NB, US). Each band of ALP, RANKL and OPG was normalized to its corresponding band of loading control. Cell lysates from untreated cells were

loaded onto every gel. The results were expressed as percentage of the corresponding untreated control.

3.2.7 Mineralization

The mineralization studies were performed similar to previous studies (Reinholz et al., 2000). The degree of mineralization was determined in the 12-well plates using Alizarin Red staining. Cells were incubated with different concentrations of ovotransferrin for 10 days. The medium was removed and cells were rinsed twice with PBS. The cells were fixed with ice-cold 70% (v/v) ethanol for 1 h. The ethanol was removed by aspiration and cells were washed twice with Milli-Q water. The cells were then stained with 1% (w/v) Alizarin-S Red in Milli-Q (pH 4.2) for 10 min at room temperature. After washing with Milli-Q water, the samples were observed under light and pictures were photographed.

3.2.8 Statistics

All data are presented as mean±SEM (standard error of mean) of between 4 and 7 independent experiments. Data were analyzed using One Way analysis of variance (ANOVA) with Dunnett's post-hoc test for comparisons to control. The PRISM 6 statistical software (GraphPad Software, San Diego, CA) was used for the analyses. $P < 0.05$ was considered significant.

3.3 Results

3.3.1 Ovotransferrin promotes cell proliferation of osteoblasts

Ovotransferrin did not show cytotoxicity to osteoblasts at concentrations up to 1000 µg/mL. To investigate the effect of ovotransferrin on cell proliferation, osteoblasts were incubated with thymidine analog BrDU, and the incorporation of BrDU into newly synthesized DNA was detected by fluorescence microscopy. As shown in Figure 3.1, the percentage of BrDU positive cells

significantly increased after adding lactoferrin (positive control, 1000 μg/mL) or ovotransferrin at concentrations of 100 μg/mL and 1000 μg/mL. At the concentration of ovotransferrin (1000 μg/mL), the percentage of BrDU-incorporated cells increased to 18.3±0.7%, which was twofold increment of the untreated group (10.4±1.2%). These results indicated that ovotransferrin could promote osteoblast proliferation, suggesting the potential role of ovotransferrin as an osteoinductive factor in bone formation.

3.3.2 Ovotransferrin inhibits the G0/G1 arrest but promotes S and G2/M arrest

To determine whether the promotion of cell proliferation can be attributed to cell cycle arrest, cell nuclei were stained with propidium iodide and cell cycle was analyzed by flow cytometry. As shown in Figure 3.2, the percentage of cells in G0/G1 phase decreased dose-dependently in the ovotransferrin groups, while increased in the lactoferrin group. At the concentration of 1000 μg/mL ovotransferrin, the percentage of cells in the G0/G1 phase decreased significantly to 74.3±0.6%, as compared to 86.4±0.4% in the untreated group and 87.6±3.0% in the lactoferrin group. On the contrary to G0/G1 phase, the percentage of cells in S and G2/M phases increased in the ovotransferrin groups while decreased in lactoferrin group; the highest percentage was shown at 100 μg/mL ovotransferrin treatment instead of 1000 μg/mL ovotransferrin group. The percentage of cells in the S phase was increased significantly to 15.9±0.3% in 100 μg/mL ovotransferrin treatment compared to 6.6±0.2% in the untreated group and 4.8±0.3% in the lactoferrin group. And the percentage of cells in G2/M phase was not affected by ovotransferrin addition.

3.3.3 Ovotransferrin increases expression of type I collagen in osteoblasts

Type I collagen expressed by osteoblasts play an important role as the structural component in bone formation. A high level of type I collagen expression, biosynthesis, and secretion is a typical indicator of osteoblasts differentiation. Thus, the expression of type I collagen was also examined in this study to investigate the effect of ovotransferrin on cell differentiation. As shown in Figure 3.3, the expression of type I collagen increased after treatment with lactoferrin (positive control) and ovotransferrin; at a concentration of 100 μg/mL ovotransferrin, type I collagen showed 1.5-fold increase compared with the untreated group. These results suggested that ovotransferrin could stimulate the expression of bone matrix component type I collagen, supporting further a beneficial role of ovotransferrin on promoting cell differentiation.

3.3.4 Ovotransferrin promotes expression of ALP in osteoblasts

Differentiation of osteoblast precursor to postmitotic matrix-producing osteoblasts is one of the most important stages in bone formation. Determination of alkaline phosphatase (ALP) expression is a widely used indicator for both osteoblast differentiation and bone formation. As shown in Figure 3.4, the expression of ALP was significantly increased after treatment with lactoferrin (positive control) and ovotransferrin (100 μg/mL and 1000 μg/mL) for 72 h. Interestingly, the maximal stimulation was observed at 100 μg/mL, not at the concentration of 1000 μg/mL. The expression level of ALP increased to 149.3±21.5% when treated with 100 μg/mL ovotransferrin, compared to 138.3±13.7% for 1000 μg/mL treatment. Our results suggested ovotransferrin could promote ALP expression when treated with ovotransferrin, further supporting the ability of ovotransferrin on stimulating osteoblast differentiation.

3.3.5 Ovotransferrin promotes mineralization of osteoblasts

Mineralization is the most important step of osteoblast differentiation and is frequently used as a marker to characterize bone formation. To examine the effect of ovotransferrin on mineralization in osteoblasts, MC3T3-E1 cells were incubated with ovotransferrin for 5 days, 10 days, 15 days and 20 days, and mineralization assay was carried out using Alizarin red S staining. The magnitude of the absorbance for the developed color is proportional to the quantity of calcium deposit, which is a direct indication of cell mineralization. As shown in Figure 3.5, at increasing incubation time, the calcium deposits were increased in MC3T3-E1 in all groups. In the first 5 days, there was no significant difference between untreated group and ovotransferrin treatment. At 10 days, a significantly increase of absorbance was observed at 1000 μg/mL ovotransferrin group, indicating ovotransferrin stimulated the calcium nodules and deposits in osteoblasts. At 20 days, the absorbance was significantly increased at both 100 μg/mL and 1000 μg/mL ovotransferrin groups as compared with the untreated group. These results indicated that ovotransferrin could promote the calcium deposit and calcium nodules production, suggesting that ovotransferrin could enhance mineralization.

3.3.6 Ovotransferrin inhibits expression of RANKL in osteoblasts

Besides the effect on bone formation, we also investigated the effect of ovotransferrin on bone resorption. RANKL, produced by osteoblasts, acts as a chemoattractant to initiate the osteoclastogenesis and contributes to osteoclast differentiation; therefore expression of RANKL is critical in regulating osteoclasts formation and bone resorption (Rucci, 2008). As shown in Figure 3.6, expression of RANKL decreased after treated with lactoferrin (positive control) and ovotransferrin in a dose-dependent manner. The expression of RANKL was significantly

decreased to 60.3±1.9% (100 μg/mL ovotransferrin), or to 55.4±8.6% (1000 μg/mL ovotransferrin), or to 58.8±2.1% (lactoferrin). These results indicated that ovotransferrin could inhibit the expression of RANKL, suggesting its potential role on preventing osteoclastogenesis.

3.3.7 Ovotransferrin promotes expression of OPG in osteoblasts

In addition to RANKL, osteoblasts also release OPG to counteract the function of RANKL and to prevent osteoclast-mediated bone resorption (Rucci, 2008). Thus, to confirm the effect of ovotransferrin on preventing bone resorption, expression of OPG was also determined by western blot. As shown in Figure 3.7, the expression of OPG was increasing in a dose-dependent manner, showing a 1.5-fold increase at 1000 μg/mL ovotransferrin treatment, compared with the untreated group. These results suggested that ovotransferrin could also promote the expression of OPG, which further supported the protective role of ovotransferrin on preventing osteoclast-induced bone resorption.

3.4 Discussion

Although a potential positive role of high-quality food protein intake on bone health has been suggested (Shams-White et al., 2017), effect of egg proteins has not been studied. In addition to their excellent essential amino acid profile and high digestibility, egg proteins are known to impart a wide range of physiological benefits including antimicrobial, anticancer, antioxidant and immunomodulation activities (Mine, Li-Chan. E., & Jiang, 2010). In this study, we showed for the first time that ovotransferrin could directly stimulate osteoblasts proliferation and differentiation as an osteogenic agent. In addition, ovotransferrin also contributes to inhibit osteoclastogenesis. The effects of ovotransferrin on both proliferation and differentiation of osteoblasts are profound,

being comparable to lactoferrin, a well-accepted nutraceutical for bone formation. Lactoferrin, extracted from milk belonging to transferrin family, is one of the most well studied bioactive proteins that acts as a promoter of osteoblasts growth and increases calvarial formation *in vivo* (Naot et al., 2005). Some reports claimed that the effects of lactoferrin on proliferation and survival of osteoblasts even exceed other established anabolic factors such as transforming growth factor-β, parathyroid hormone (PTH), amylin, and insulin (Cornish et al., 1999). In this study, we showed that ovotransferrin promoted osteoblast proliferation in a dose-depend manner, and reached significance at concentrations of 100 and 1000 μg/mL; in comparison, lactoferrin showed the same trend and the same magnitude (Cornish et al., 2004). In general, transferrin family is recognized to have stimulatory effects on proliferation. In addition to the stimulation effects on osteoblasts, lactoferrin was also reported to induce the growth and proliferation of human enterocytes (Buccigrossi et al., 2007), human endometrial stromal cells and human lymphoblastic T cells (Bi, Lefebvre, Duś, Spik, & Mazurier, 1997). Laskey et al. (1988) gave the evidence that transferrin supported cell proliferation by supplying iron for DNA synthesis (Laskey, Webb, Schulman, & Ponka, 1988). And then transferrin has been studied in different cell lines and showed simulative effects on lymphoblast cells (Laskey et al., 1988), osteoblast cells (Tsunoi et al., 1984), and oligodendrocytes progenitor cells (Silvestroff, Franco, & Pasquini, 2013) with different magnitude, while showed suppression effects on human hepatoma cells (A. W. M. Lee, Oates, & Trinder, 2003). Richie et al. (1987) reported that human milk-derived lactoferrin inhibited the mitogen and alloantigen induced human lymphocytes proliferation (Richie, Hilliard, Gilmore, & Gillespie, 1987). Zhang, Lima, & Rodrigues (2015) also described the potential anticancer activity of lactoferrin by inhibiting the growth of breast cancer cells (Zhang, Lima, & Rodrigues, 2015). Similarly, ovotransferrin has been successfully used in inhibiting the proliferative response of

mouse spleen lymphocytes stimulated by lipopolysaccharide and phytohemagglutinin (Otani & Odashima, 1997). Mizunoya et al. (2015) studied the growth promoting activity of several egg white proteins in C2C12 myoblast cell line and observed that ovalbumin and ovomucoid could stimulate the proliferation of myoblasts while ovotransferrin decreased cell proliferation (Mizunoya, Tashima, Sato, Tatsumi, & Ikeuchi, 2015). Therefore, it seems that the effect of ovotransferrin on cell proliferation as well as the sensitivity of the cells to respond to ovotransferrin vary depending on the cell types.

Cell growth to high density often reflects loss of cell cycle control (Smith et al., 2000). Thus, cell cycle analysis was performed to investigate the effect of ovotransferrin on cell cycle control. Treatment of osteoblasts with ovotransferrin showed controlled cell cycle, stimulated more cells at the S phase but less at G0/G1 phase, indicating active DNA synthesis and cell proliferation, which is consistent with BrDU incorporation results. In addition to osteoblasts, controlled cell cycle also occurs in adipocytes (Richon, Lyle, & McGehee, 1997; Yeh, Bierert, & Mcknightt, 1995), as well as mesenchymal cells (Hall & Miyake, 2000). Cell cycle progression has been described as an important mechanism contributing to bone cell activity. Glucocorticoids, a widely used immunosuppressive and anti-inflammatory drug, has been reported to inhibit bone formation by inhibiting cell cycle progression in osteoblasts and finally lead to osteoporosis and increased fracture risk in chronic glucocorticoid-treated patients (Smith et al., 2000; Smith, Coetzee, & Frenkel, 2002). San Martin et al. (2009) proposed that deregulation of cell cycle may contribute to the pathogenesis of osteosarcoma. Several osteogenic compounds have also been reported to stimulate cell proliferation through affecting cell cycle (San Martin et al., 2009). Cao et al. (2012) reported that icarii, a prenyl flavonoid from Epimedium considered as a strong candidate used in

bone tissue engineering, increased the percentage of S phase cell and decreased G1 phase in MC3T3-E1 cells (Cao et al., 2012). Polygonum amplexicaule var. sinense, a traditional herb, also showed simulative effects on osteoblasts by affecting cell cycle progression (Xiang, Su, Hu, & Yan, 2011).

The osteoinductive activity of ovotransferrin is also showed by its capacity to substantially promote osteoblast differentiation. The progress of bone formation was classified into three stages: preosteoblast recruitment characterized by cell proliferation and type I collagen secretion, followed by osteoblast precursor differentiation into postmitotic matrix-producing osteoblasts accompanied with downregulated replication, and osteoid mineralization by terminally differentiated osteoblasts associated with increased extracellular matrix accumulation and ALP expression and activity (Lee et al., 2004; Owen et al., 1990). As the most abundant protein among all bone matrixes, type I collagen is conductive to mineral deposition and also binds to non-collagenous matrix proteins to initiate and regulate mineralization (Ehara et al., 2003). Also, ALP is involved in bone matrix formation before crystallization of the calcium and phosphate ions (Lee et al., 2004)and may play a role in transport of phosphate (Millán & Whyte, 2016). **T**herefore, type I collagen and ALP are considered as the most important biomarkers to study osteoblastic activity. Lactoferrin was reported to enhance osteoblast differentiation by increasing the expression and activity of type I collagen and ALP in both mouse osteoblasts MC3T3-E1 and human osteoblast-like cells SaOS-2 (Cornish et al., 2004; Fritsche et al., 2001). Calcium phosphates such as α-tricalcium phosphate (α-TCP) and tera-calcium phosphate (TetCP) have been clinically applied as components of bone-substitutes materials. Ehara et al. (2003) reported that α-TCP and TetCP stimulated MC3T3-E1 differentiation through increasing ALP activity and type I collagen

expression (Ehara et al., 2003). In this study, ovotransferrin promoted both type I collagen and ALP expression, respectively, in the early and late phases, suggesting that, in addition to promoting matrixes production and osteoblasts differentiation in the early stage, it also affected late phase of calcification of bone matrix.

Proteins from transferrin family are suggested to play an important role in cell differentiation. Lactoferrin was reported to stimulate cell differentiation not only in osteoblasts, but also other cell lines such as stromal cells ST2 (Cornish et al., 2004), mesenchymal cells (Yagi et al., 2009), and human adipose stem cells (Ying et al., 2012). Macedo et al. (2004) studied the possible involvement of transferrin in T lymphocyte differentiation with hypotransferrinaemic mice and found transferrin itself or a pathway triggered by the interaction of transferrin with its receptor is crucial for normal early T-cell differentiation (Macedo et al., 2004). Paez et al. (2005) overexpressed human transferrin in two oligodendroglial cell lines and found transferrin is required for a more complete maturation of oligodendroglia (Paez, García, Campagnoni, Soto, & Pasquini, 2005).

As the most direct evidence of calcium deposit capability, mineralization was also investigated to evaluate the effect of ovotransferrin on bone formation. Ovotransferrin was found to increase bone-like tissue formation by MC3T3-E1 cells in a dose-dependent manner. This might be, in part, due to the increased cell activity and increased production of extra-cellular matrix like type I collagen or other non-collagenous proteins. The other possible reason for the enhanced formation of calcified tissue is that ovotransferrin may function as suppliers of original materials (e.g. amino acids) for bone formation. Ehara et al. (2003) also implied the possible function of α-TCP and

TetCP as calcium and phosphate materials for bone formation (Ehara et al., 2003). Above all, ovotransferrin acts to expand the pool of osteoblasts by exerting mitogenic effects as well as driving differentiation of precursors to produce more mature osteoblastic phenotypes capable of promoting bone matrix deposition and mineralization. This anabolic potency suggests that ovotransferrin or its analogs should be explored as therapies for osteoporosis that can restore skeletal strength because most current interventions merely arrest further structural decline.

Bone mass is regulated by continuous remodeling, which is based on a delicate balance between osteoblastic bone formation and osteoclastic bone resorption. The maturation and activation of osteoclasts is dependent on the RANK/RANKL/OPG system. Osteoblasts are the primary source of receptor activator of nuclear factor κB ligand (RANKL), which stimulates osteoclast differentiation and activates bone resorption after binding to RANK on osteoprogenitor cells (Rucci, 2008). Thus, the osteoblastic bone resorption depends on a delicate balance between the expression of RANKL and OPG. Ovotransferrin inhibited RANKL expression while promoted OPG expression in osteoblasts, indicating its inhibitory effect on bone resorption. Bone resorption prevention was also reported for milk protein (Lorget et al., 2002), soybean protein and soybean isoflavone (Messina & Messina, 2000). Lactoferrin reduced bone-resorbing activity was due to inhibited RANKL expression while promoted OPG expression (Lorget et al., 2002). Some drugs, like denosumab, showed prevention of bone resorption by inhibiting RANKL expression as well (Hamdy, 2008). Besides the regulation of RANK/RANKL/OPG system, ovotransferrin was shown anti-inflammatory activity (Xie et al., 2002). Given the inflammatory nature of osteoporotic condition, ovotransferrin may play a role in counterbalancing the inflammation-induced bone

resorption. The anti-inflammatory property of ovotransferrin may complement with its osteogenic effect on bone cells.

3.5 Conclusion

In conclusion, this study demonstrated for the first time that the egg white protein ovotransferrin could function as an osteogenic agent by stimulated proliferation and differentiation of osteoblasts as well as inhibited osteoclastogenesis. These findings suggested an important role of ovotransferrin in mediating bone remodeling and rebuilding the balance between bone formation and resorption. Although further research is warranted to test its *in vivo* efficacy, ovotransferrin shows the potential as a complementary therapeutic target for alleviation of bone disorders such as osteoporosis as well as an anabolic agent for promotion of bone repair.

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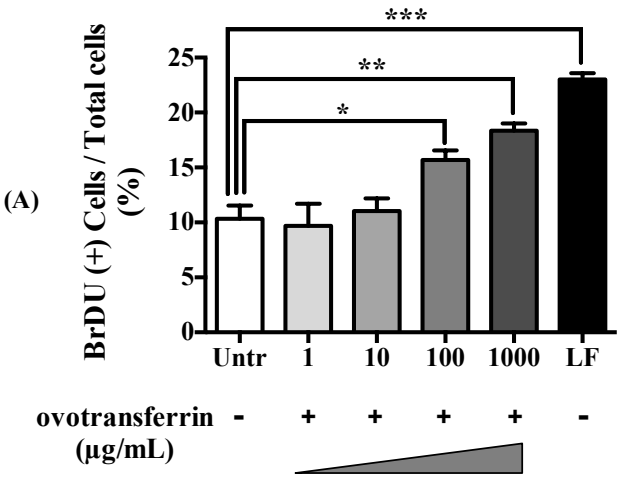
Figure 3.1 Effects of egg white ovotransferrin on cell proliferation in osteoblast cell MC3T3-E1

MC3T3-E1 cells were seeded into 48-well plate for 4 h and treated with 1000 μg/mL lactoferrin or different concentrations (1000, 100, 10, and 1 μg/mL) of ovotransferrin for 24 h prior to adding BrDU reagent, fixed, permeabilized and immunostained. Cell nuclei were counter-stained with Hoechst33342 dye. Percentage of BrDU(+) nuclei were counted in 3 random fields per group and their mean value determined. Data were expressed as mean±SEM. *, ** and *** indicate $p<0.05$, $p<0.01$ and $p<0.001$ respectively; as compared to the untreated control group.



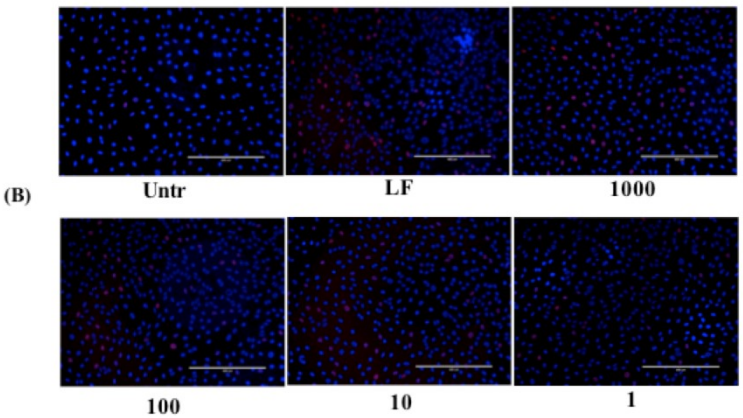
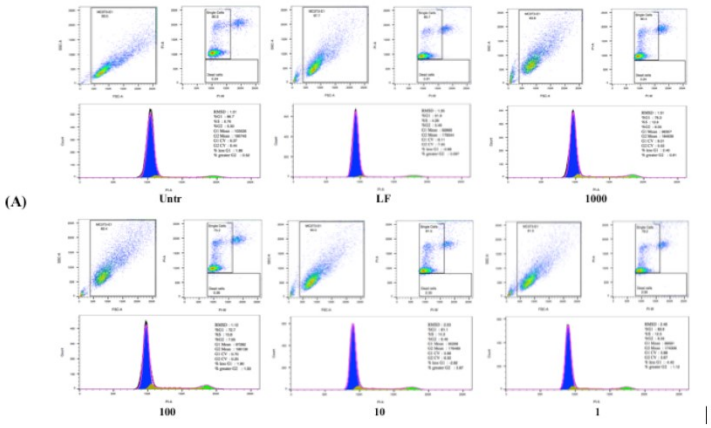


Figure 3.2 Effects of egg white ovotransferrin on cell cycle in osteoblast cell MC3T3-E1

Confluent monolayers of MC3T3-E1 cells were treated with different concentrations (1000, 100, 10, and 1 μg/mL) of ovotransferrin for 24 h prior to being trypsinized, collected, fixed and subjected to PI flow cytometry. Representative panels are shown. Percentage of each cell cycle phase was measured. Data were expressed as mean±SEM. * indicates $p<0.05$ as compared to the untreated control group.



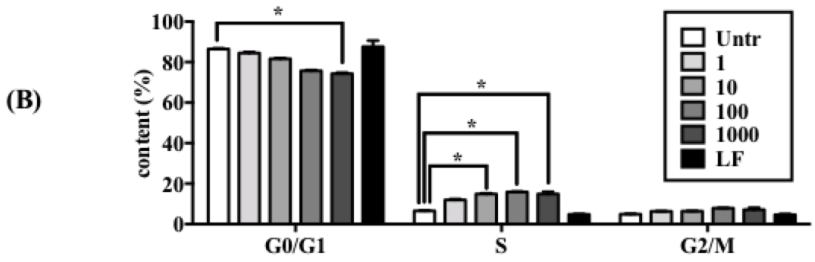
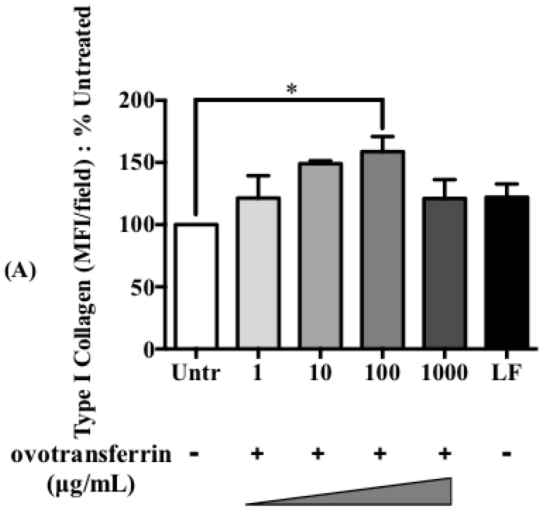


Figure 3.3 Effects of egg white ovotransferrin on type I collagen expression in osteoblast cell MC3T3-E1

MC3T3-E1 cells were seeded into 48-well plate for 4 h and treated with 1000 μg/mL lactoferrin or different concentrations (1000, 100, 10, and 1 μg/mL) of ovotransferrin for 72 h prior to being fixed, permeabilized and immunostained for type I collagen. Cell nuclei were counter-stained with Hoechst33342 dye. Mean fluorescence intensity was measured as the average of total fluorescent intensity from 3 randomly selected fields per group and expressed as percentage of the untreated control. Data were expressed as mean±SEM. * indicates $p<0.05$ as compared to the untreated control group.



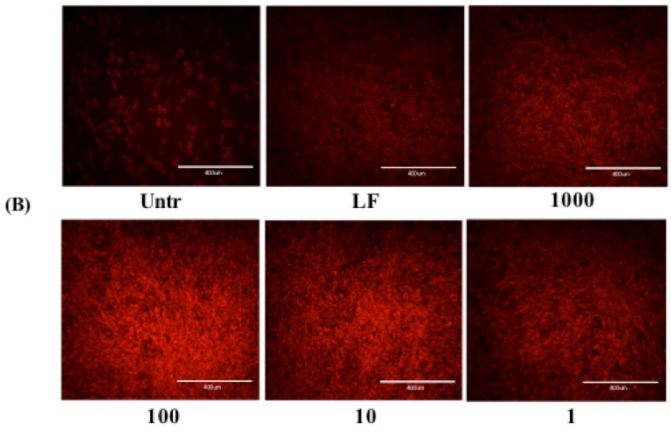


Figure 3.4 Effects of egg white ovotransferrin on ALP expression in osteoblast cell MC3T3-E1

Confluent monolayers of MC3T3-E1 cells were treated with 1000 μg/mL lactoferrin or different concentration (1000, 100, 10, and 1 μg/mL) of ovotransferrin for 72 h prior to being lysed and immunoblotted for ALP. A representative set of immunoblots is shown. Fluorescence intensity of the immunoblots was measured. Data were expressed as mean±SEM. * and ** indicate $p<0.05$ and $p<0.01$ respectively; as compared to the untreated control group.

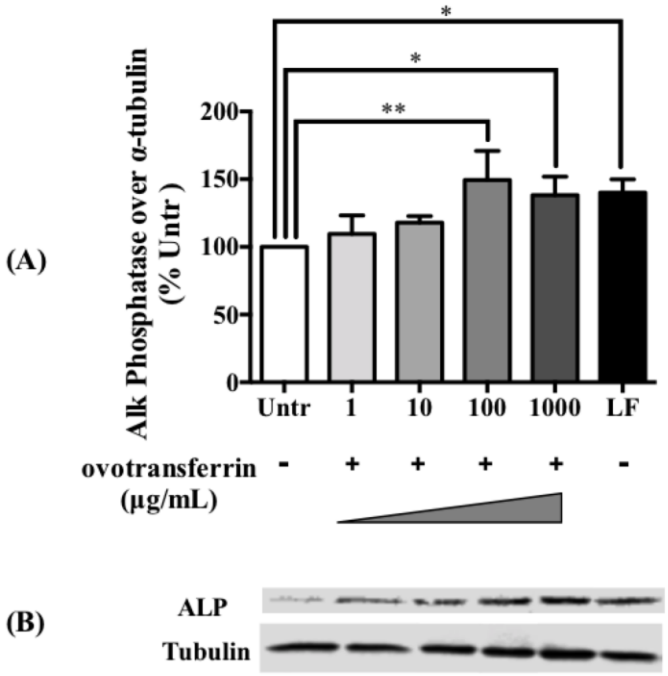


Figure 3.5 Effects of egg white ovotransferrin on mineralization in osteoblast cell MC3T3-E1

Confluent monolayers of MC3T3-E1 cells were treated with different concentrations (1000, 100, 10, and 1 μg/mL) of ovotransferrin with medium contain ascorbic acid and β-glycerophosphate for different days (5, 10, 15 20 days) prior to being stained with Alizarin Red S. Representative images of mineralization are shown. Absorbance at 562 nm was measured after cell distained with cetylpyridinium chloride in sodium phosphate. Data were expressed as mean±SEM. * indicates $p<0.05$ as compared to the untreated control group.

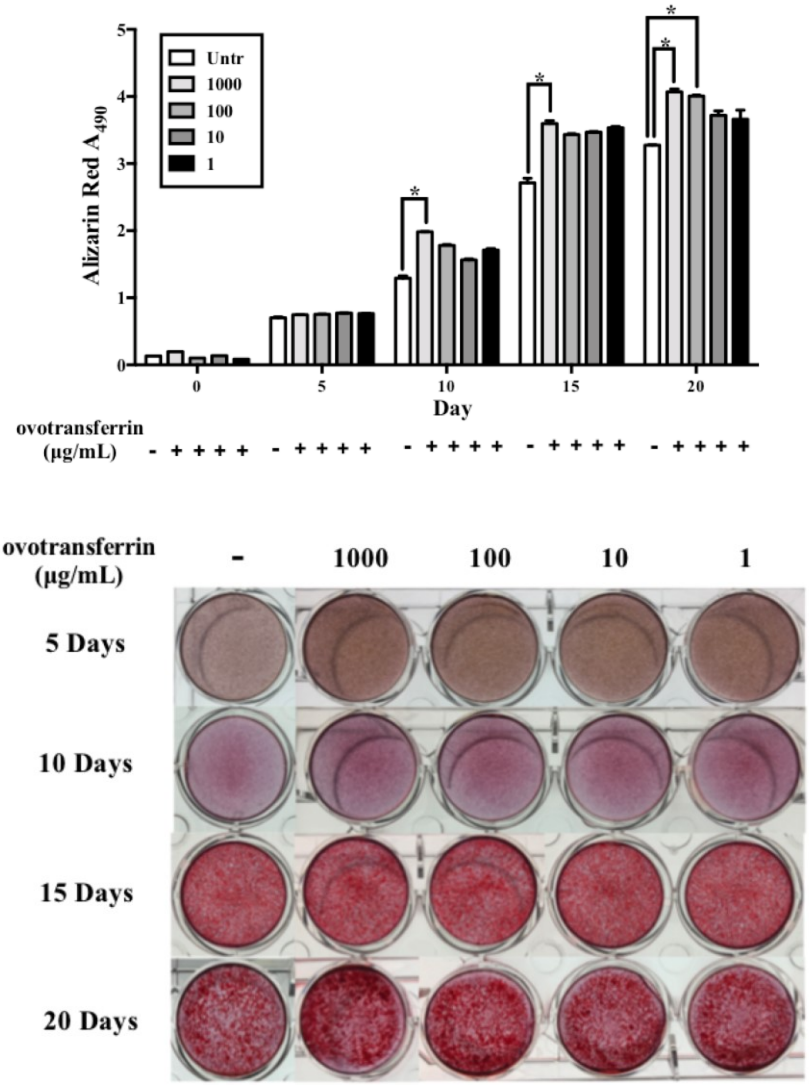


Figure 3.6 Effects of egg white ovotransferrin on RANKL expression in osteoblast cell MC3T3-E1

Confluent monolayers of MC3T3-E1 cells were treated with 1000 µg/mL lactoferrin or different concentrations (1000, 100, 10, and 1 µg/mL) of ovotransferrin for 72 h prior to being lysed and immunoblotted for RANKL. A representative set of immunoblots is shown. Data were expressed as mean±SEM. * indicates $p<0.05$ as compared to the untreated control group.

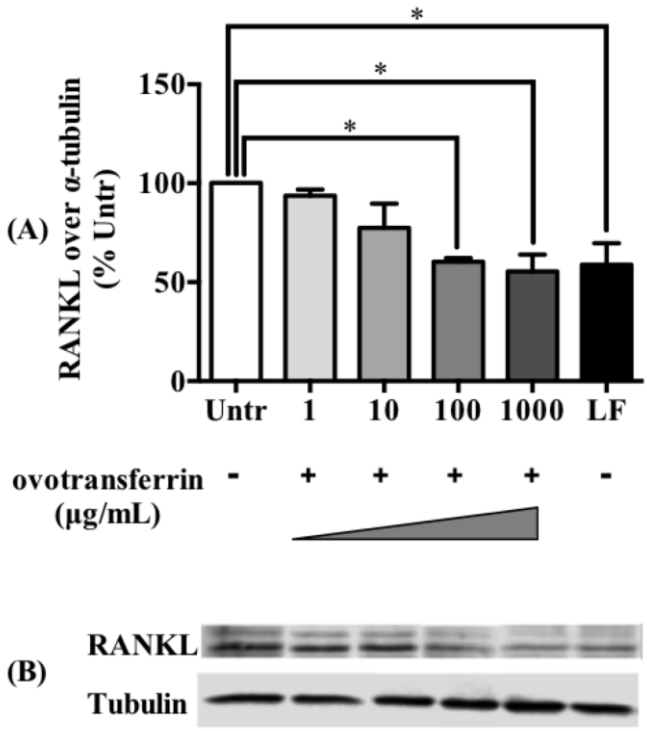
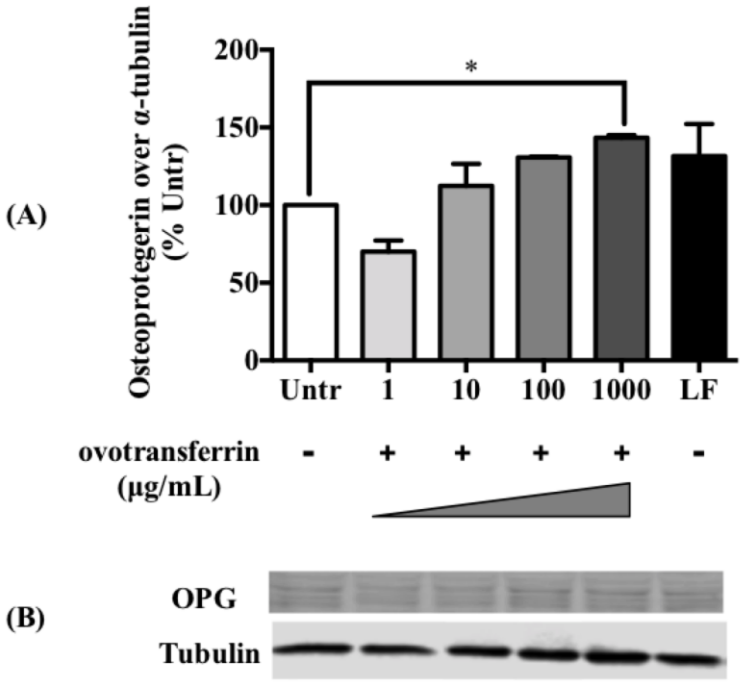


Figure 3.7 Effects of egg white ovotransferrin on OPG expression in osteoblast cell MC3T3-E1

Confluent monolayers of MC3T3-E1 cells were treated with 1000 µg/mL lactoferrin or different concentrations (1000, 100, 10, and 1 µg/mL) of ovotransferrin for 72 h prior to being lysed and immunoblotted for OPG. A representative set of immunoblots is shown. Data were expressed as mean±SEM. * indicates $p<0.05$ as compared to the untreated control group.



CHAPTER 4 - Ovotransferrin Stimulated Osteogenesis via activation of ERK1/2 and PI3K-Akt pathways independent to low-density lipoprotein receptor-related protein 1 (LRP1)

4.1 Introduction

Bone is a highly dynamic organ whose homeostasis is maintained through continuously remodeling (Kini, Usha; Nandeesh, 2012; Wozney et al., 1988). Bone remodeling is a delicate process controlling the balance between bone formation and resorption to keep bone integrity and quality (Kini, Usha; Nandeesh, 2012; Shahi, Peymani, & Sahmani, 2017). The bone-forming osteoblasts and the bone resorbing osteoclasts are two major components involving in and mediating this process (Kini, Usha; Nandeesh, 2012; Shahi et al., 2017). Osteoporosis is the most serious bone disorder worldwide, which leads to a systemic impairment of bone mass, strength, micro-architecture and an increased risk of osteoporotic fractures (Bates, 2007). The development of osteoporosis is mainly due to an imbalance between osteoblast and osteoclast activity, as well as an uncoupling of bone resorption and formation, which finally results in a greater bone resorption than formation (Bates, 2007). Two strategies have been introduced to osteoporosis prevention by either inhibiting bone resorption or promoting bone formation (Crockett & Das, 2013). The antiresorptive agents have been widely used in osteoporosis treatment, but their low efficacy, associated side effects and concurrent comorbidities challenged the use of these drugs (Crockett & Das, 2013). Compare to various antiresorptive agents, parathyroid hormone (PTH) is the only available drug to stimulate the osteoblast-controlled bone formation currently (Crockett & Das, 2013; Morley, Whitfield, & Willick, 2001). However, the high cost and short-term efficacy limits the application of this anabolic agent (Morley et al., 2001; Ponnapakkam, Katikaneni,

Sakon, Stratford, & Gensure, 2014). Thus, it is desirable to identify and develop natural anabolic agents to enhance bone formation by stimulating osteoblast activity.

Several lines of evidence have shown that food proteins, especially bioactive proteins, may contribute to bone health management (Bharadwaj, Naidu, Betageri, Prasadarao, & Naidu, 2009; Cornish et al., 2004; Eaton-Evans, 1994; Marshall, 2004; Messina & Messina, 2000). Among all, milk-derived lactoferrin is one of the most well studied bioactive proteins that has been reported to be a potent bone growth factor both *in vitro* and *in vivo* (Cornish et al., 2004; Guo et al., 2009; Hou, Xue, & Lin, 2012). Our previous work reported a similar effect of egg-derived ovotransferrin as lactoferrin in stimulating osteoblastic activity (Shang & Wu, 2018). Ovotransferrin, a 78-kDa iron-binding glycoprotein, belonging to the same transferrin family as lactoferrin, shares similar structures and biological activity. Egg white ovotransferrin was demonstrated to promote osteoblast proliferation and to inhibit osteoclastogenesis *in vitro*. In particular, ovotransferrin exerted beneficial effect on osteoblast differentiation and extracellular matrix calcification. Osteoblast differentiation is a prerequisite of bone formation *in vivo*. However, the molecular mechanisms underlying the effect of ovotransferrin on osteoblast differentiation remain poorly understood.

Mitogen-activated protein kinases (MAPKs) are serine/threonine kinases that are well-known mediators converting extracellular stimuli into cellular responses, including many of the extracellular ligands relevant to osteoblasts (Cargnello & Roux, 2011). The mammalian MAPK family includes extracellular signal-regulated kinase (ERK), c-Jun NH2-terminal kinase (JNK) and p38 (Cargnello & Roux, 2011). ERK MAPK pathway was reported to be responsible for the

lactoferrin induced mitogenic effects (Grey et al., 2004), as well as the collagen peptide induced osteogenic activity in osteoblast (Kim et al., 2013). Also, ERK1/2 was described to be implicated in bone morphogenic protein 2 (BMP-2) (Jaiswal et al., 2000) and $1,25(OH)_2D_3$-induced osteoblast differentiation (Chae et al., 2002). In skeletal biology, alternation in JNK activity is responsible for altered osteoblast activity (Yamashita et al., 2005). Treatment with a JNK inhibitor was reported to reduce mineralization and expression of mature osteoblast markers, while osteoblast differentiation was enhanced by overexpression of JNK2 (Matsuguchi et al., 2009a). Compare to ERK and JNK pathway, more evidence indicated the critical role of p38 MAPK pathway in osteoblast differentiation. The p38 MAPK was required for both natural (Greenblatt et al., 2010) and BMP-2 - induced differentiation in osteoblasts (Hu, Chan, Wang, & Li, 2003). Moreover, an increasing amount of research has shown that p38 MAPK is indispensable for osteoblast differentiation induced by various osteotropic factors, such as epinephrine (Suzuki, Palmer, Bonjour, & Caverzasio, 1999) and PTH (Rey, Manen, Rizzoli, Ferrari, & Caverzasio, 2007). In addition to MAPK signaling, the PI3K-Akt pathway plays a central role in the control of cell survival, growth, and proliferation (McGonnell et al., 2012). Both *in vitro* and *in vivo* studies also indicated that the phosphoinositide 3-kinase (PI3K)-protein kinase B (Akt) pathway is involved in regulation of osteoporosis through promoting osteoblast proliferation, differentiation and bone formation (Xi et al., 2015). More importantly, study reported that Akt is a molecular switch for transforming growth β-1 (TGF-β1)-induced osteoblastic differentiation. TGF-β1 could be either an inducer or an inhibitor of osteoblastic differentiation, which is determined by the state of Akt phosphorylation in MC3T3-E1 cells (Suzuki et al., 2014).

Critical cellular decisions such as proliferation, migration and differentiation, are regulated by stimulatory cues from the extracellular environment and convert to a cellular response through binding to membrane receptors that can trigger the signal transduction pathway inside the cells (Groves & Kuriyan, 2010). Both MAPK pathway and PI3K-Akt pathway can be activated by several receptors, such as RTKs (receptors tyrosine kinases) and G-protein-coupled receptors (Katz, Amit, & Yarden, 2007; Naor, Benard, & Seger, 2000). It is believe that most of the bioactive macromolecules exert effects though binding specific receptors, rather than directly transport into cell plasma. Lactoferrin receptors are thought to have pivotal roles in mediating multiple functions of lactoferrin. Studies indicated that lactoferrin promoted osteoblast proliferation through low-density lipoprotein receptor-related protein 1 (LRP1) - mediated ERK1/2 MAPK activation (Grey et al., 2004), whereas lactoferrin-induced osteoblast survival and differentiation is LRP1-independent (Grey, Zhu, Watson, Callon, & Cornish, 2006; W. Zhang et al., 2014).

In this chapter, the signaling pathways that transduce the stimulating signal of ovotransferrin during osteoblast differentiation were examined. Meanwhile, the involvement of possible ovotransferrin receptors, especially LRP1, in ovotransferrin-treated osteoblast was evaluated.

4.2 Material and Methods

4.2.1 Reagents and antibodies

Ovotransferrin (Conalbumin from chicken egg white, at a purity of 98%) and Alizarin-S red stain were bought from Sigma Chemicals Co. (St Louis, MO, USA). α-minimum essential medium (α-MEM) and Fetal Bovine Serum (FBS) were obtained from Gibco/Invitrogen (Carlsbad, CA, USA). Triton-X-100 was bought from VWR International (West Chester, PA, USA). CytoSelect wound

healing assay kit was purchased from Cell Biolabs Inc., (San Diego, CA, USA). Collagen I alpha antibody was bought from Novus Biological Canada ULC (Oakville, ON, Canada). Mouse monoclonal primary antibody against ALP, β-catenin, BMP-2/4, PI3-kinase p85α, PI3-kinase p110α, phosphory-CREB-1 and CREB-1 were purchased from Santa Cruz Biotechnology (Mississauga, ON, Canada). Mouse monoclonal primary antibody against Runx2 and transferrin receptor, and rabbit monoclonal primary antibody against TFR1, ITLN1/2, LRP1, α-Tubulin and GAPDH were purchased from Abcam (Toronto, ON, Canada). Phospho-ERK1/2 pathway sampler kit and rabbit monoclonal primary antibody against phosphor-JNK (ser73), JNK, phosphor-p38 MAPK (Thr180/Tyr182) and p38 were purchased from Cell Signaling Technology (Whitby, ON, Canada). Rabbit monoclonal primary antibody against phosphor-Akt (Ser473) and Akt were purchased from Cell Signaling Technology (Danvers, MA, USA). EKR1/2 antagonist U0126, JNK antagonist SP600125, p38 antagonist SB203580, Akt antagonist API-1 and LRP1 antagonist Rap were purchased from Tocris (Oakville, ON, Canada). Hoechst 33342 (Trihydrochloride), rabbit anti-mouse IgG (H+L) secondary antibody, and goat anti-rabbit IgG (H+L) secondary antibody were bought from Molecular Probes (Waltham, MA, USA). Goat anti-rabbit and donkey anti-mouse fluorochrome-conjugated secondary antibodies were purchased from Licor Biosciences (Lincoln, NB, USA).

4.2.2 Cell Culture

The murine pre-osteoblastic cell line MC3T3-E1 (subclone 4, ATCC CRL-2593) was purchased from ATCC (Manassas, VA, USA). Cells were cultured under the same condition described in Chapter 3. Briefly, cells were cultured in α-MEM supplemented with 10% FBS and penicillin-streptomycin in an incubator under 95% air and 5% CO_2 at 37°C. All experiments were performed on 80-90% confluent cells grown in tissue culture grade plastic 48 or 24 well plates. To investigate

the involvement of the signaling pathways in ovotransferrin-induced osteoblastic activity, cells were incubated with ovotransferrin in different conditions prior to western blotting, immunofluorescence and mineralization assay as described below.

4.2.3 Western blot

The cells were seeded on 48 well tissue culture plates at a concentration of 1×10^4 cells/well counted on a haemocytometer, and incubated in α-MEM with 10% FBS. After treatment, the culture medium was removed and the cells lysed in boiling hot Laemmle's buffer containing 50 μM dithiothreitol (DTT) and 0.2% Triton-X-100 to prepare samples for western blot as described previously (Shang & Wu, 2018). These cell lysates were run in SDS-PAGE, blotted to nitrocellulose membranes and immunoblotted with primary antibodies. The protein bands were detected by a Licor Odyssey BioImager and quantified by densitometry using the corresponding software (Licor Biosciences, Lincoln, NB, US). Each target band was normalized to the corresponding band of loading control. Cell lysates from untreated cells were loaded onto every gel. The results were expressed as percentage of the corresponding untreated control.

4.2.4 Immunofluorescence

The immunofluorescence studies were performed similar to our previous studies (Shang & Wu, 2018). Briefly, cells were fixed in 4% paraformaldehyde, permeabilized with 0.1% Triton-X-100 in PBS and immunostained overnight with a rabbit polyclonal antibody against type I collagen. Cells were treated with Alexa Fluor546 (red) conjugated goat anti-rabbit secondary antibody for 30 min in the dark. Nuclei were stained with the Hoechst33342 nuclear dye (1:10000) for 10 min. After washing with PBS to remove unbound antibody/dye, the immunostained cells were observed under an Olympus IX81 fluorescence microscope (Olympus, Tokyo, Japan). Images were obtained using Metamorph imaging software (Molecular Devices, Sunnyvale, CA, USA). Mean

fluorescence intensity was calculated from the intensity of the red fluorescent signal (determined by Adobe Photoshop Elements 2.0 software; Adobe Systems Inc., San Jose, CA, USA) from 3 randomly selected fields per group.

4.2.5 Mineralization Assay

The mineralization studies were performed similar to previous studies (Shang & Wu, 2018). The degree of mineralization was determined in the 12-well plates using Alizarin Red staining. Cells were incubated with different concentrations of ovotransferrin for 15 days. The medium was removed and cells were rinsed twice with PBS. The cells were fixed with ice-cold 70% (v/v) ethanol for 1 h. The ethanol was removed by aspiration and cells were washed twice with Milli-Q water. The cells were stained with 1% (w/v) Alizarin-S Red in Milli-Q (pH 4.2) for 10 min at room temperature. After washing with Milli-Q water, the samples were observed under light microscopy and photographed.

4.2.6 Cell Migration Assay

Migration of osteoblast MC3T3-E1 cells were evaluated by a CytoSelect wound healing assay kit (Cell Biolabs, Inc., San Diego, CA, USA) according to the manual. Briefly, cells were seeded into a 24-well plate with a wound healing insert in the middle of each well. Cells were cultured overnight until the monolayer formed. The wound healing insert was removed to create a 0.9 mm wound healing area. Cells were pre-treated with 50 μM Akt inhibitor for 1h and then treated with 1000 μg/mL ovotransferrin and Akt inhibitor together for 12 h. Afterward, cells were fixed with 4% of paraformaldehyde solution and cell nuclei were stained with Hoechst33342 nuclear dye (1:10000) for 10 min. Cells were visualized by an Olympus IX83 fluorescence microscope (Olympus, Shinjuku, Tokyo, Japan). The wound healing area was measured by Image J software.

A smaller area indicated a higher migration ability of the cells. Results of each treatment group were normalized to the corresponding untreated group.

4.2.7 Statistics

All data are presented as mean±SEM (standard error of mean) of 6 independent experiments. Data were analyzed using One Way analysis of variance (ANOVA) with Dunnett's post-hoc test for comparisons to control. The PRISM 6 statistical software (GraphPad Software, San Diego, CA) was used for the analyses. $P<0.05$ was considered significant.

4.3 Results

4.3.1 Ovotransferrin stimulates osteogenesis associated transcription factors

To investigate the mechanism underlying the ovotransferrin stimulated osteogenesis, we first evaluated several transcription factors that play vital role in regulating osteoblastic activity including osteoblast differentiation and mineralization. In general, ovotransferrin does-dependently affected the expression of Runx2, BMP-2, β-catenin and phosphorylation of CREB on osteoblast MC3T3-E1 cells (Figure 4.1A, B, C, D, E). The strongest stimulation on the expression of BMP-2 (~1.5 folds) and β-catenin (~2.5 folds) was found at the concentration of 1000 μg/mL, while the expression of Runx2 (~2 folds) and phosphorylation of CREB (~2.5 folds) increased more at the concentration of 100 μg/mL.

4.3.2 Ovotransferrin stimulates osteogenesis via activate MAPK pathways

Previous studies demonstrated that the MAPK pathways are involved in the regulation of osteoblast differentiation and osteogenesis. Thus, we investigated the effect of ovotransferrin on three MAPK members, including ERK, JNK and p38. Our results showed that ovotransferrin

treatment significantly activated all three MAPK pathways (Figure 4.2). ERK1/2 phosphorylation was significantly increased during the time treated but the highest expression was observed at 20-30 min and then started to decline (though remained in a high level of phosphorylation); once the old medium was replaced by new medium (containing ovotransferrin), the phosphorylation of ERK1/2 was dramatically increased again, which suggesting that ovotransferrin could continuously induce the activation of ERK1/2 pathway (Figure 4.2A). Compared with ERK1/2, ovotransferrin only increased the phosphorylation of JNK at the initial 20 to 30 min period and then declined to basal level; replenishment with new medium (containing ovotransferrin) after 12 h did not change the level of phosphorylation of JNK (Figure 4.2B). The stimulation of p38 phosphorylation was time-response, increased at prolonged incubation and peaked during 30 min to 1 h and then stated to decrease afterwards.(Figure 4.2C); replenishment with new medium (containing ovotransferrin) at 12 h did not increase further phosphorylation of p38. These results suggest that ovotransferrin may activate JNK and p38 pathways in an acute manner, while can continuously activate ERK1/2 pathway thus exhibiting long-term effect in regulating osteogenesis.

4.3.3 ERK pathway is involved in ovotransferrin-stimulated osteogenesis, but no JNK and p38 pathways

To further validate the involvement of MAPK pathways in ovotransferrin-stimulated osteogenesis, different antagonists were used to inhibit the activation of its corresponding pathways. Three different antagonists were used to inhibit ERK, JNK and p38 pathways, at the concentrations not toxic to cells (Figure 4.7.1). Several biomarkers were used to indicate the effects of ovotransferrin-stimulated osteogenesis, including the expression of ALP, Runx2, β-catenin, BMP-2, type I collagen, and the mineralization level. ERK1/2 antagonist alone showed no significant influence

on the expression of the biomarkers (Figure 4.3), while it significantly attenuated the increase of ovotransferrin-induced expression of ALP (Figure 4.3A), Runx2 (Figure 4.3B), β-catenin (Figure 4.3C), BMP-2 (Figure 4.3D), and type I collagen (Figure 4.3E). More importantly, ERK1/2 antagonist obviously attenuated the stimulation of mineralization induced by ovotransferrin treatment (Figure 4.3F), which provided direct evidence that the ERK pathway plays an important role in ovotransferrin-regulated osteogenesis.

However, JNK antagonist did not show such significant effects (Figure 4.4). Pre-treatment with antagonist of JNK did not attenuate the high expression of neither ALP (Figure 4.4A) nor Runx2 (Figure 4.4B). For p38 pathway, p38 antagonist significantly decreased ovotransferrin-induced expression of ALP (Figure 4.5A) and Runx2 (Figure 4.5B); however, this decrease might be due to the inhibition of basal expression level as the treatment with p38 antagonist alone showed a significant inhibition in ALP and Runx2 expression.

4.3.4 Ovotransferrin stimulates the phosphorylation and activation of the ERK cascade

ERK1/2 cascade acts downstream of Ras (small GTP-binding protein) and usually involves sequential phosphorylation and activation of Raf, MEK1/2 (MAPK/ERK kinases), ERK1/2 and MAPKAPKs (MAPK-activated protein kinases) (Wortzel & Seger, 2011). Although ERK1/2 are considered as the core components, MAPKAPKs components (such as p90RSK and MSK1/2) phosphorylate a large number of substrates that eventually regulate most cellular processes, including proliferation, differentiation, survival, apoptosis and more (Wortzel & Seger, 2011). Thus, the phosphorylation of several ERK1/2 upstream and downstream kinases was investigated to evaluate the role of ovotransferrin in activating the ERK cascade. Ovotransferrin at concentrations of 100 and 1000 μg/mL significantly stimulated the phosphorylation of ERK

upstream kinase c-Raf (Figure 4.6A) and MEK1/2 (Figure 4.6B). Meanwhile, the phosphorylation of downstream MAPKAPK p90RSK (Figure 4.6C) and MSK1 (Figure 4.6D) were also increased by ovotransferrin. The activation of p90RSK and MSK1 may further regulate the transcription factors like Runx2, β-catenin, and CREB, therefore stimulating osteogenesis.

4.3.5 Ovotransferrin stimulates osteogenesis via activate PI3K-Akt pathways

In addition to MAPK pathways, activation of PI3K-Akt pathway by many molecules is known to play a vital role in osteogenesis was studied. Here, the effects of ovotransferrin in activating PI3K-Akt pathway. The phosphorylation of Akt was dramatically increased within the initial 6 h, and then declined to basal level until new medium was replaced at 24 h (Figure 4.7A). As the downstream signaling of PI3K, the increased phosphorylation of Akt might be due to the activation of PI3K. The current study found that 1000 μg/mL ovotransferrin stimulated the expression of two PI3K subunits, p85 and p110 (Figure 4.7B). These results showed that ovotransferrin could activate PI3K-Akt pathway in osteoblast cells and contribute to osteogenesis.

To further confirm the involvement of the PI3K-Akt pathway in ovotransferrin stimulated osteoblastic activity, Akt antagonist was used to evaluate the expression of Runx2 and BMP-2 while the Akt pathway was inhibited. This study showed that Akt antagonist significantly attenuated ovotransferrin induced expression of Runx2 (Figure 4.8A) and BMP-2 (Figure 4.8B). The PI3K-Akt pathway has been found to play an important role in regulating osteoblast migration via mediating Runx2 and BMP-2 expression; thus, the cell migration assay was performed to evaluate the influence of Akt inhibition on ovotransferrin promoted cell migration. Ovotransferrin treatment significantly reduced the area of migration as well as the distance of the migration area, while pre-treatment with Akt antagonist before ovotransferrin significant diminished the positive

effects (Figure 4.8C). The area of migration and the distance of the migration area were significantly larger in Akt antagonist added group than ovotransferrin treatment alone. Finally, the role of PI3K-Akt pathway on ovotransferrin-induced osteogenesis was confirmed directly by mineralization assay. Reduced mineralization was observed when the cells treated with both Akt antagonist and ovotransferrin, compared with cell treated only with ovotransferrin (Figure 4.8E).

4.3.6 LRP1 is not involved in mediating the osteoinductive effect of ovotransferrin

Although our previous results revealed the involvement of ERK MAPK and PI3K-Akt pathway in ovotransferrin-induced osteogenesis, the receptors that respond to ovotransferrin and activate the corresponding signaling still remain unclear. Thus, in this study, we investigated the receptors that may act in response to ovotransferrin. According to the literatures, 7 different receptors that respond to ovotransferrin, transferrin family and lactoferrin, were selected for study (Table 4.7.1). PCR results showed that only TFBP (transferrin binding protein), TFR1 (transferrin receptor 1), ITLN1 (intelectin-1) and LRP1 (low-density lipoprotein receptor-related protein 1) were expressed in osteoblast MC3T3-E1 (Figure 4.7.2). The expression of TFR1, ITLN1 and LRP1 was further confirmed by western blot (Figure 4.9). Cells treated with ovotransferrin significant increase in the expression of ITLN1 and LRP1 (Figure 4.9), suggesting the response of these 2 receptors to ovotransferrin.

To further validate the participation of LRP1 in the osteoinductive effects of ovotransferrin, a natural LRP1 antagonist Rap was used to block the binding of all known ligand to receptor (Herz & Strickland, 2001; Zhang et al., 2014). Rap was added before ovotransferrin treatment to block the interaction between ovotransferrin and LRP1. With Rap pretreatment, ovotransferrin-induced high expression of ALP (Figure 4.10A) was inhibited, while the expression of Runx2 (Figure

4.10B) and phosphorylation of ERK1/2 (Figure 4.10C) and Akt (Figure 4.10D) were not affected. Interestingly, the mineralization assay showed that Rap pre-treatment inhibited ovotransferrin-induced mineralization, at least partly (Figure 4.10E). These results suggested the involvement of LRP1 in response to ovotransferrin stimulated osteoblastic activity, but not via the activation of ERK and Akt pathways.

4.4 Discussion

Ovotransferrin has been recognized as a new anabolic factor that potently promotes bone formation and inhibits bone resorption (Shang & Wu, 2018). In this chapter, we showed that ovotransferrin stimulated osteoblast differentiation mainly through LRP1-independent ERK1/2 MAPK and PI3K-Akt pathways. This work, to the best of our knowledge, is the first evidence of the signaling mechanism underlying the beneficial effect of ovotransferrin on osteoblast differentiation.

Osteoblast differentiation is tightly controlled by several master regulators, such as Runx2, Wnt/β-catenin and BMPs (Qi et al., 2003). In this study, ovotransferrin significantly increased the expression of Runx2, BMP-2, β-catenin and the phosphorylation of CREB, suggesting that the stimulatory effect of ovotransferrin on osteoblast differentiation is through these master regulators (Figure 4.1). Runx2 is the most extensively studied master regulator in osteogenesis (Rutkovskiy, Stensløkken, & Vaage, 2016). It activates the genetic pathways controlling osteoblast differentiation as well as the expression of osteopontin, bone sialoprotein, osteocalcin, osteoprotegerin, RANKL, and many others (Li et al., 2012; Rutkovskiy et al., 2016). BMPs are another key molecule in the regulation of osteoblast differentiation as well as act as strong inducers of bone formation (Bae, Kim, Won, Min, & Hwang, 2017; Chen, Deng, & Li, 2012). In particular,

BMPs activate the phosphorylation and translocation of specific proteins to increase the transcription of Runx2, osteocalcin, type I collagen, and ALP (Bae et al., 2017; Yamaguchi, Komori, & Suda, 2000). BMP signaling also activates β-catenin, a signal transducer of Wnt/Notch system, to induce osteoblast differentiation (Bain, Müller, Wang, & Papkoff, 2003). Normally, cytoplasm β-catenin translocates to the nucleus and activates by binding to its cofactors and results in a subsequent regulation of Runx2, as well as other pro-osteogenic genes (Takada, Kouzmenko, & Kato, 2009). Upon BMP stimulation, more β-catenin accumulates and translocates to the nucleus, and accelerate osteoblast differentiation (Bae et al., 2017). Furthermore, BMPs promote Runx2 phosphorylation and bind to coactivator CREB to induce the osteogenic differentiation (Lee, Hong, & Bae, 2002), at least three CREB proteins (CREB1, CREB 3, and CREB 5) have been found Runx2-responsive (Teplyuk et al., 2008).

Mitogen-activated protein kinase (MAPK) pathways provide a major link between the external environment and nucleus to control cell proliferation and differentiation in different cell types, including osteoblasts. The critical role of ERK MAPK pathway in osteoblast differentiation and mineralization has been well established *in vivo*. Mice with a deletion of *Erk1* and *Erk2* in osteoblasts display substantially reduced bone mineralization, demonstrating the importance of ERK for osteoblast mineralization (Matsushita et al., 2009). More importantly, the activation of ERK pathway has been suggested to be involved in several potent bone formation anabolites, such as drug PTH (Chen et al., 2004; Swarthout, Doggett, Lemker, & Partridge, 2001), and natural functional components lactoferrin (Grey et al., 2004; Liu et al., 2018) and isoflavone (Bhargavan et al., 2009). In this study, ovotransferrin treatment significant increased the phosphorylation of ERK and other important components involved in ERK signaling pathway, such as c-Raf,

MEK1/2, p90RSK and MSK1 (Figure 4.2 and Figure 4.6). Meanwhile, blocking ERK1/2 with its antagonist U0126 inhibited ovotransferrin-induced increase of bone formation biomarkers (ALP, Runx2, BMP-2, β-catenin and type I collagen) and mineralization. These findings are consistent with other studies using different cell types. A decrease of ALP activity was reported when ERK activity was inhibited in human osteoblasts HOB and bone marrow stromal cells (Lai et al., 2001). Meanwhile, the inhibition of ERK also decreased the matrix mineralization by regulating the bone matrix proteins, such as type I collagen, osteopontin, and bone sialoprotein (Lai et al., 2001). ERK inhibition impaired expression and activities of matrix proteins due to the regulation of transcription factors, especially Runx2 (Kanno, Takahashi, Tsujisawa, Ariyoshi, & Nishihara, 2007; G Xiao et al., 2000). Recently, MEK, the up-stream effector of MAPK, has been shown to regulate the activity of Runx2 (Xiao et al., 2000). Stimulation of MAPK by constitutively active MEK enhances, whereas the MEK inhibitor PD98059 inhibits, the expression of osteocalcin (Shimo et al., 2007). Notably, studies also suggested that active ERK may bind to the C-terminal proline/serine/threonine-rich (PST) domain of Runx2 and contribute to Runx2 transcriptional activity (Ge et al., 2012; Guozhi Xiao, Jiang, Gopalakrishnan, & Franceschi, 2002). Moreover, ERK has been found to bind to the promoter of osteocalcin and bone sialoprotein in osteoblast via the association between ERK and Runx2, intriguing the possibility that ERK activity may be differentially regulated in the context of specific osteoblast-relevant promoters (Ge et al., 2012; Guozhi Xiao et al., 2002).

In addition to ERK signaling, our study found that the phosphorylation of JNK was also increased by ovotransferrin treatment; however, blocking JNK with inhibitor SP600125 did not affect ovotransferrin-induced expression of ALP and Runx2 (Figure 4.4). Currently, the regulatory role

of JNK pathway in osteoblast differentiation remains unclear. Treatment with JNK inhibitor was reported to reduce the expression of osteocalcin and bone sialoprotein, two biomarkers of last-stage of differentiation (Matsuguchi et al., 2009b). ALP, however, is considered as an early-stage biomarker; thus, adding JNK inhibitor did not affect the expression of ALP as JNK only functions at late-stage. Furthermore, Runx2 may not be the target in response to JNK pathway, although Runx2 is considered as the most important factor required in osteoblast differentiation and bone formation. Several other transcription factors also play indispensable role in osteoblast differentiation, such as AP-1 family members (JunB and Fra-1) (Eferl et al., 2004; Kenner et al., 2004) and CREB/ATF family member ATF4 (Yang et al., 2004). For example, inactivation of JNK did not inhibit the expression of Runx2, but significantly inhibited the induction of ATF4, (Matsuguchi et al., 2009a). Additionally, activation of JNK kinase cascade was also found to mobilize the AP-1/ATF transcription factors and regulate osteoblastic activity (Yamashita et al., 2005). Based on these, some late-stage biomarkers and transcription factors in addition to ALP and Runx2 should be tested to better understand the involvement of JNK pathway in response to ovotransferrin stimulation.

p38 MAPKs have been shown to play an important role in osteoblast commitment and maturation as well as proliferation (Greenblatt et al., 2010; Rodríguez-Carballo et al., 2014; Thouverey & Caverzasio, 2012). Studies have found that p38 MAPK was activated as the calvarial osteoblast differentiates along with ERKs (Hu et al., 2003). When p38 was inhibited, the expression of ALP and the mineral deposition were significantly reduced (Hu et al., 2003). Meanwhile, osteoblast MC3T3-E1 expressing dominant-negative p38 MAPK also displayed sign of delay in mineralization (Hu et al., 2003). Similar results were observed in our study. The p38 inhibitor

SB203580 significantly inhibited the basal expression of ALP and Runx2, indicating the importance of p38 in osteoblast differentiation (Figure 4.5). Interestingly, in this study, we found that ovotransferrin significantly increased the phosphorylation of p38 within 24 h, suggesting the stimulatory role of ovotransferrin in activating p38 signaling. Adding p38 inhibitor together with ovotransferrin reduced the expression of ALP and Runx2 compared with ovotransferrin alone, but remained significantly higher than control (Figure 4.5). These results suggested that ovotransferrin might affect the expression of ALP and Runx2 via pathways other than p38 MAPK pathway, such as the BMP pathway.

The PI3K-Akt signaling pathway plays a central role in the control of cell survival, growth, and proliferation throughout the body. With regard to bone, there is an increasing amount of evidence suggesting the potential osteoblast-specific effect of PI3K-Akt pathway. The Akt knockout animals showed impairment of skeletal development and functional integrity, indicating the essential role of Akt activation in bone health (Easton et al., 2005; Garofalo et al., 2003; Kawamura et al., 2007; Peng et al., 2003). Recent evidence suggests that Runx2 functions are not only regulated by phosphorylation of ERK and p38 MAPK, but also affected by PI3K-Akt signaling. The master factor Runx2 was reduced in Akt-1 knockout mice, indicating that the Runx2 might be a downstream target of Akt pathway (Kawamura et al., 2007). However, studies further informed that Runx2-Akt interactions are unlikely to occur via direct phosphorylation because Runx2 lacks an Akt consensus phosphorylation site (Kawamura et al., 2007). Thus, it is believed that Akt may regulate Runx2 indirectly via affecting the activity of GSK3β, an inhibitor of Runx2 DNA binding ability (Kugimiya et al., 2007), or FoxO1, a repressor of Runx2-dependent osteocalcin transcription (Yang et al., 2011; Zhang et al., 2011). In our study, ovotransferrin significantly

stimulated the phosphorylation of Akt and resulted in an increase of Runx2 expression, BMP-2 expression, osteoblast migration activity and mineralization (Figure 4.7). More importantly, these enhancements induced by ovotransferrin were attenuated after blocking with Akt inhibitor API-1, indicating a key role of Akt activation in ovotransferrin-induced osteogenesis (Figure 4.8). As activation of Akt is always considered via PI3K, our study further investigated the effect of ovotransferrin on PI3K expression. PI3K itself consist of a regulatory p85 subunit and a catalytic p110 subunit (McGonnell et al., 2012). The recruitment and phosphorylation of p85 and p110 subunit is required for activation of Akt (McGonnell et al., 2012). In our study, ovotransferrin significantly increased the expression of both p85 subunit and p110 subunit of PI3K, indicating a key role of activation of PI3K-Akt signaling in response to ovotransferrin treatment.

There are studies reported the ability of ovotransferrin in regulating different signaling pathways, such as MAPK pathways (Lee, Ahn, & Paik, 2018) and NF-κB pathway (Giansanti et al., 2002), to exert the immune-enhancing activity and anti-inflammatory activity. However, to our best knowledge, there is no study published to elucidate the possible receptors involved in response to ovotransferrin. As ovotransferrin is a macromolecule, we believed that the most likely way to trigger signal transduction by ovotransferrin is through binding to membrane receptors. Thus, based on our findings that ovotransferrin exert osteogenesis activity by activating the MAPK and PI3K-Akt pathways, we tentatively investigated the possible receptors that may be involved in eliciting the signal transduction. In this study, 7 possible receptors were selected based on their ability to bind overall transferrin family members and lactoferrin-specific binding receptors, including ovotransferrin receptor TFBP (Gentili et al., 1993; 1994), transferrin family receptors TFR1 (Kawabata, 2018), TFR2 (Kawabata, 2018), Culilin/LRP2, IGFBP-2 (Miljuš et al., 2015;

Weinzimer et al., 2001) and lactoferrin receptors ITLN1 (Akiyama et al., 2013; Shin et al., 2008) and LRP1 (Grey et al., 2004, 2006). Currently, TFBP is the only ovotransferrin receptor reported. Our study showed expression of TFBP was increased by ovotransferrin; a previous study also found that the expression of ovotransferrin receptor was increased during chondrogenesis and endochondral bone formation in developing chick embryo (Gentili et al., 1994). Unfortunately, there is no commercial TFBP antibody or inhibitor available currently (Figure 4.9); thus it is difficult to investigate relation the role of TFBP on ovotransferrin-induced osteoblast differentiation. Our study found that the expression of ITLN1 and LRP1 was significantly increased, while the expression of TFR1 did not change (Figure 4.9). Intelectin, also called small intestine lactoferrin receptor, is the well documented lactoferrin receptor that specifically recognizes lactoferrin in human small intestine (Suzuki, Lopez, & Lönnerdal, 2005). However, there is no evidence available for the functions of intelectin in osteoblast activity to date. LRP1 is the only known lactoferrin receptor that mediates the action of lactoferrin in osteoblasts (Naot et al., 2011). Studies have shown that lactoferrin stimulated osteoblast proliferation through LRP1-meidated activation of ERK pathway (Grey et al., 2004). Meanwhile, LRP1 not only acts as a membrane receptor of lactoferrin, but also regulates the endocytosis to transport lactoferrin into cytoplasm (Grey et al., 2004). Thus, LRP1 could be the most promising receptor for ovotransferrin to mediate different physiological processes of osteoblasts. Blocking LRP1 with its inhibitor Rap did not affect the expression of Runx2, ERK and Akt pathways, while ALP and mineralization was partially attenuated (Figure 4.10). These results suggested that LRP1 was independent to ovotransferrin-induced expression of Runx2 as well as the activation of ERK and Akt pathway; but LRP1 might play roles in regulating ovotransferrin induced ALP expression and

mineralization. Therefore, further studies are needed to identify the role of LRP1 in ovotransferrin promoted osteoblast differentiation.

4.5 Conclusion

In conclusion, this study found that ovotransferrin stimulated osteoblast differentiation mainly through LRP1-independent c-Raf-MEK-ERK and PI3K-Akt signaling pathways. Ovotransferrin activated ERK and PI3K-Akt signaling transduction and regulated several bone-specific factors, such as Runx2, β-catenin and BMP-2, leading to an enhanced osteoblast differentiation. Our study also indicated that LRP1 could partially regulate the function of ovotransferrin via ERK- and AKT-independent signaling pathways.

4.6 References

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Figure 4.1 Effects of ovotransferrin on the expression of master factors involved in osteoblast differentiation.

MC3T3-E1 cells treated with different concentration of ovotransferrin for 24 h and proteins were extracted for western blot analysis. (A) The expression of Runx2; (B) The expression of BMP-2; (C) The expression of β-catenin; (D) The phosphorylation of CREB; (E) The western blot for each of the biomarkers. Data were expressed as Mean±SEM (n=6). Means without a common letter indicated $p<0.05$.

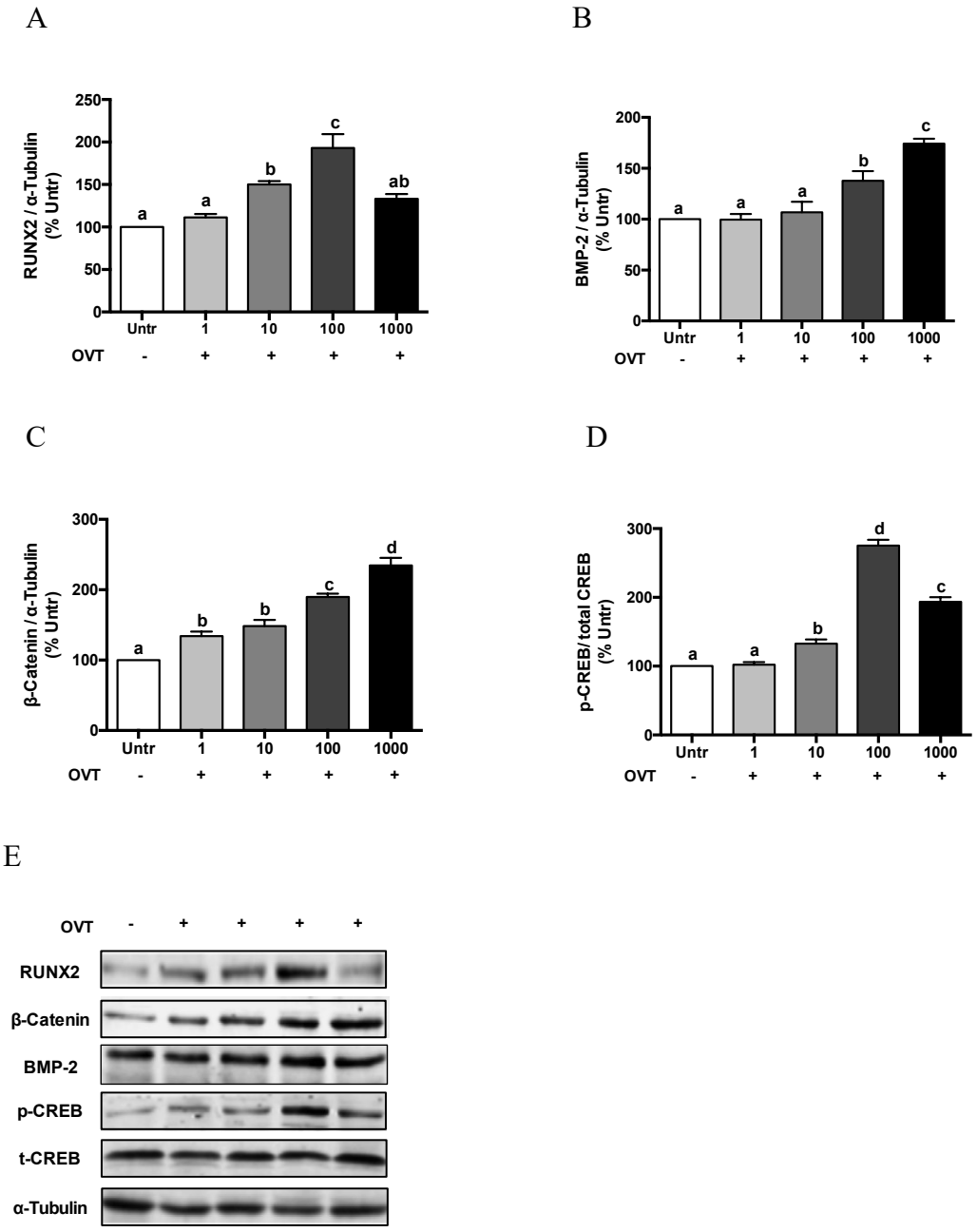
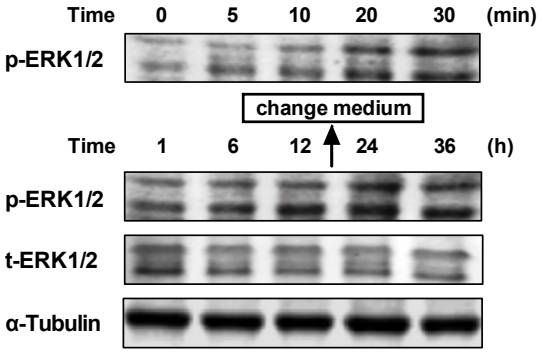


Figure 4.2 Effects of ovotransferrin on activation of MAPK pathways.

MC3T3-E1 cells treated with 1000 μg/mL of ovotransferrin for different time (indicated in figures) and proteins were extracted for western blot analysis. (A) The time course of ERK1/2 phosphorylation; (B) The time course of JNK phosphorylation; (C) The time course of p38 phosphorylation.

A



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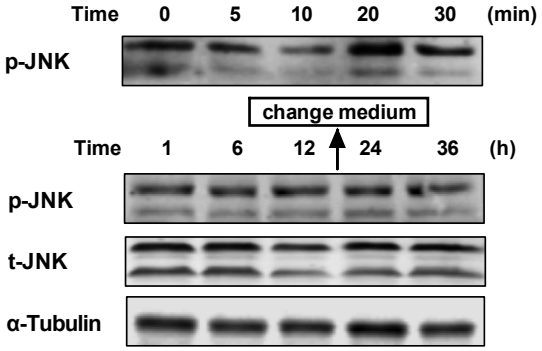

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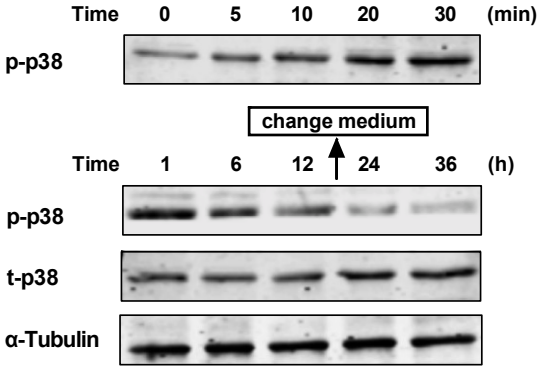
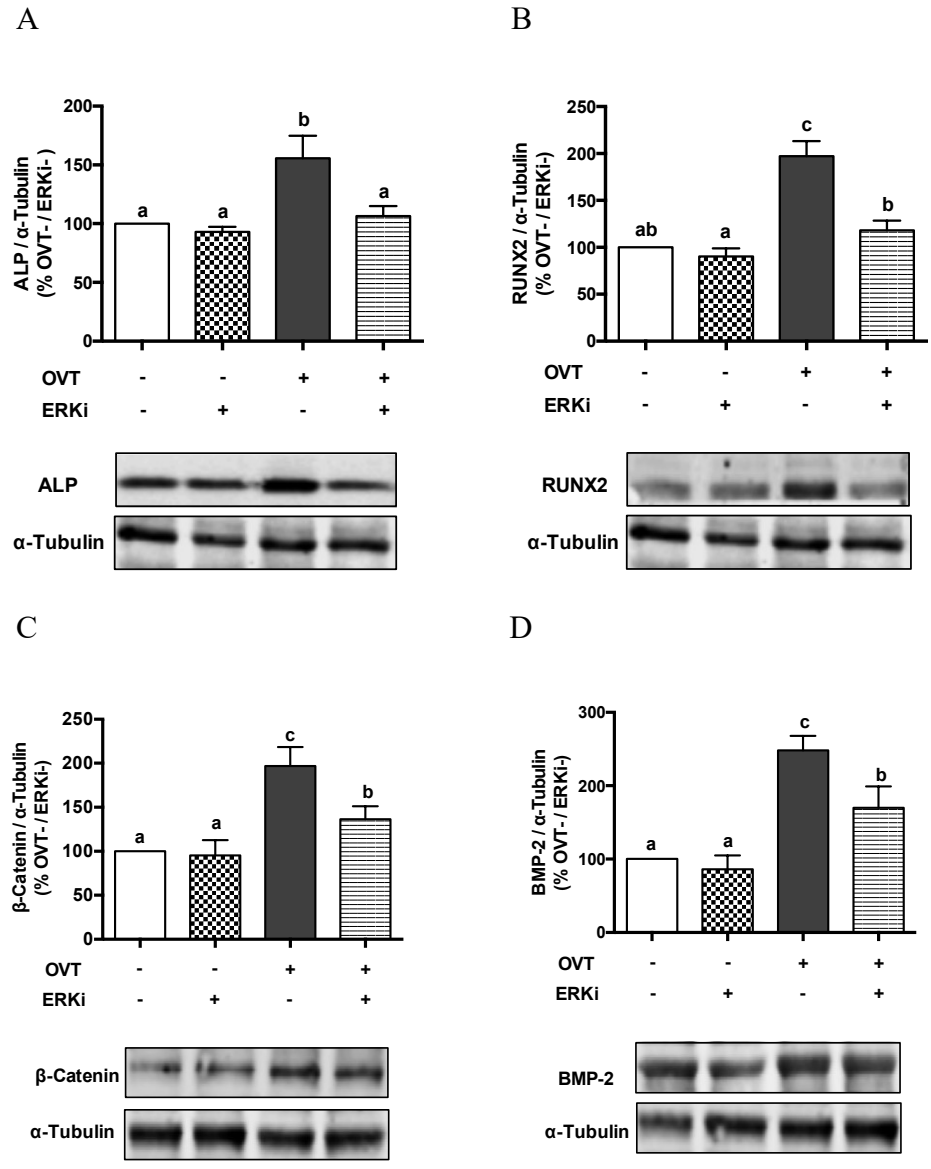
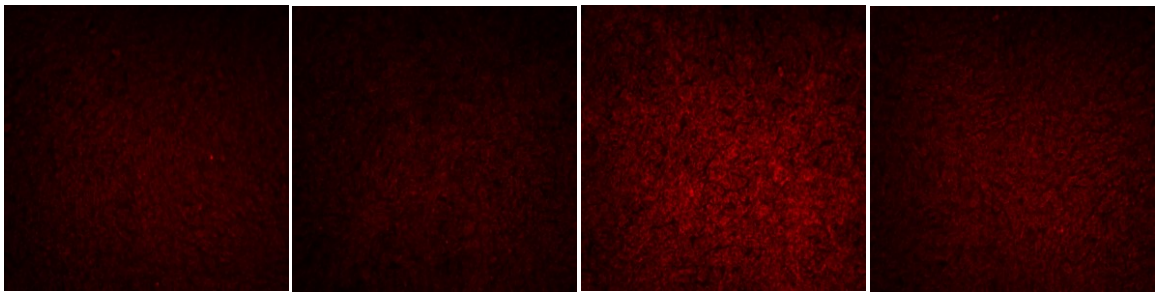


Figure 4.3 Effects of ERK inhibitor on ovotransferrin-induced osteoblast differentiation.

MC3T3-E1 cells were pre-treated with ERK inhibitor for 1 h and then treated with 1000 μg/mL of ovotransferrin for western blot analysis (24 h treatment), immunofluorescence analysis (36 h) and mineralization (15 d). (A) The expression of ALP; (B) The expression of Runx2; (C) The expression of β-catenin; (D) The expression of BMP-2; (E) The expression of type I collagen; (F) The osteoblast mineralization. Data were expressed as Mean±SEM (n=6). Means without a common letter indicated $p<0.05$.



E



OVT - / ERKi - | OVT - / ERKi + | OVT + / ERKi - | OVT + / ERKi +

F

OVT - / ERKi - | OVT - / ERKi + | OVT + / ERKi - | OVT + / ERKi +

Figure 4.4 Effects of JNK inhibitor on ovotransferrin-induced osteoblast differentiation.

MC3T3-E1 cells were pre-treated with JNK inhibitor for 1 h and then treated with 1000 μg/mL of ovotransferrin for 24 h and proteins were extracted for western blot analysis. (A) The expression of ALP; (B) The expression of Runx2. Data were expressed as Mean±SEM (n=6). Means without a common letter indicated $p<0.05$.

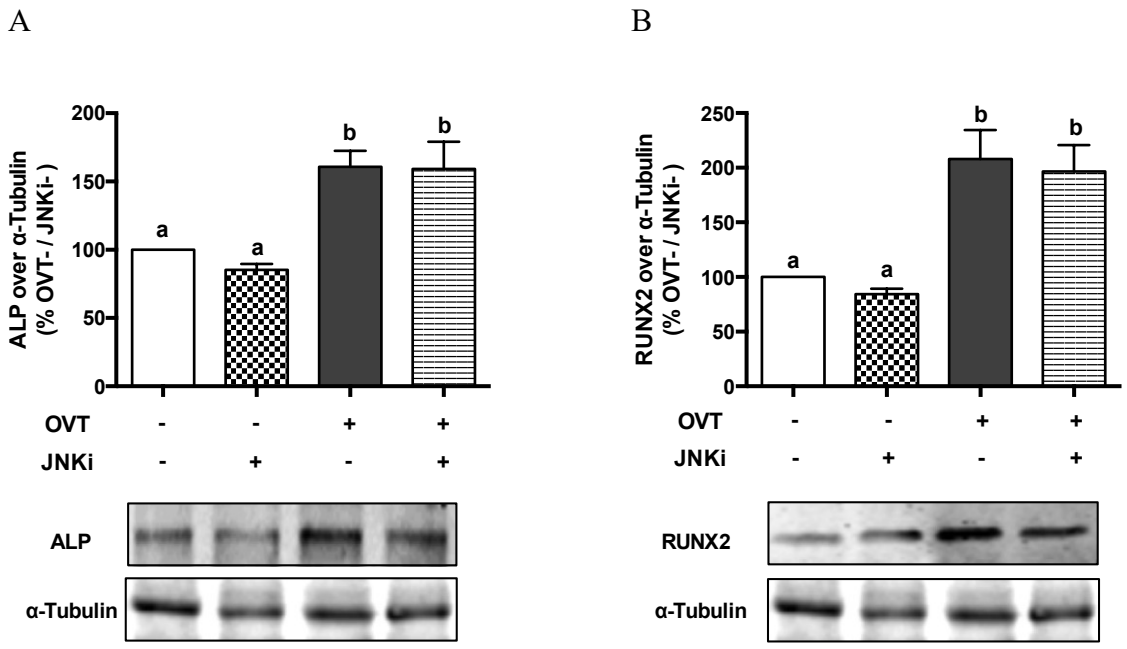


Figure 4.5 Effects of p38 inhibitor on ovotransferrin-induced osteoblast differentiation.

MC3T3-E1 cells were pre-treated with p38 inhibitor for 1 h and then treated with 1000 μg/mL of ovotransferrin together for 24 h and proteins were extracted for western blot analysis. (A) The expression of ALP; (B) The expression of Runx2. Data were expressed as Mean±SEM (n=6). Means without a common letter indicated $p<0.05$.

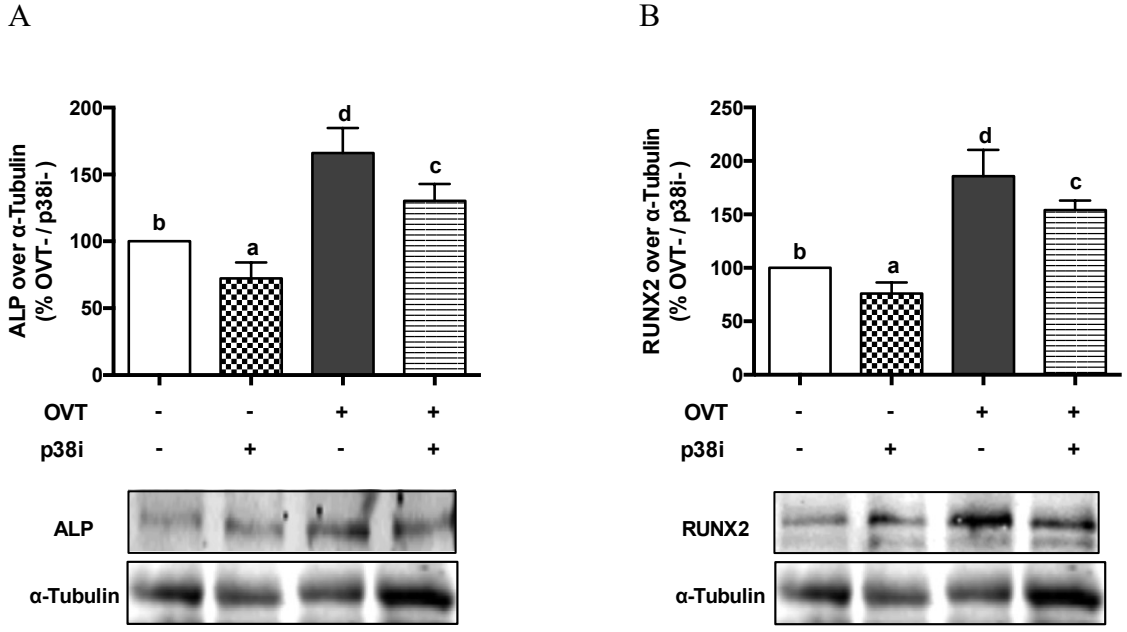


Figure 4.6 Effects of ovotransferrin on activation of the ERK MAPK pathway.

MC3T3-E1 cells were treated with 100 μg/mL and 1000 μg/mL of ovotransferrin together for 24 h and proteins were extracted for western blot analysis. (A) The phosphorylation of c-Ras; (B) The phosphorylation of MEK1/2. (C) The phosphorylation of p90RSK; (D) The phosphorylation of MSK1. Data were expressed as Mean±SEM (n=6). Means without a common letter indicated $p<0.05$. Untr: cells without ovotransferrin treatment.

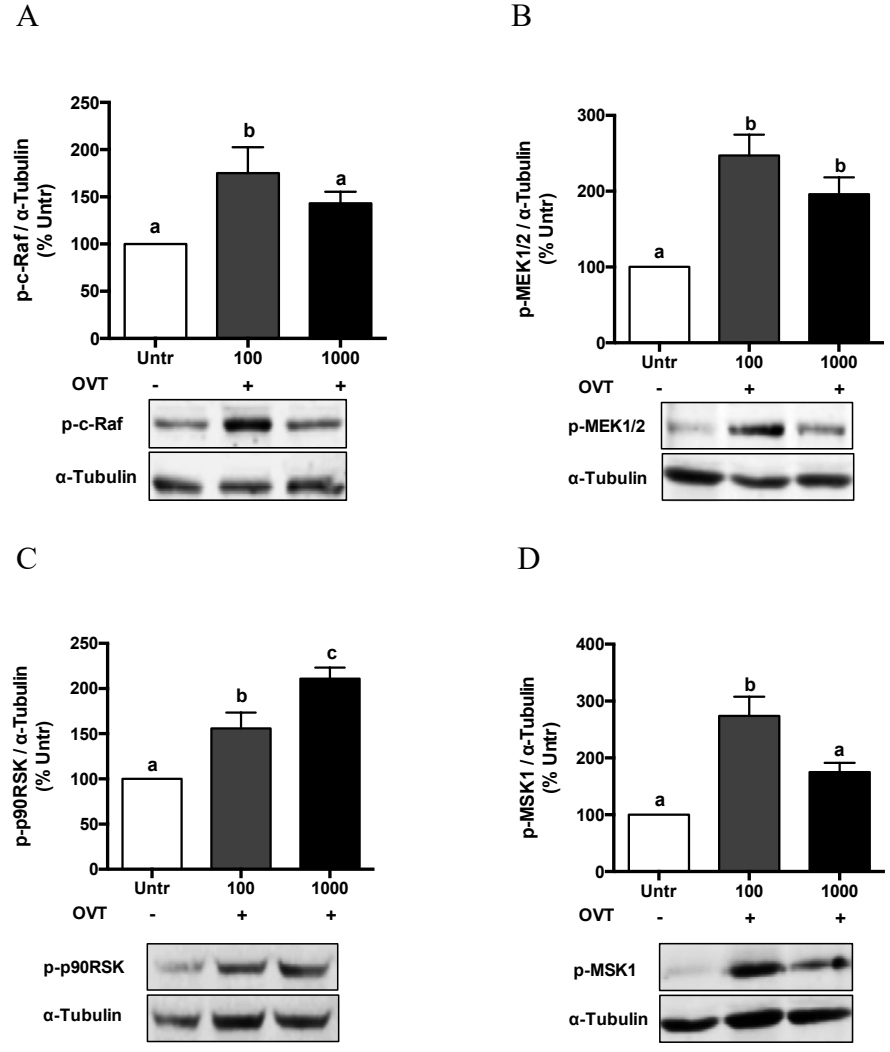
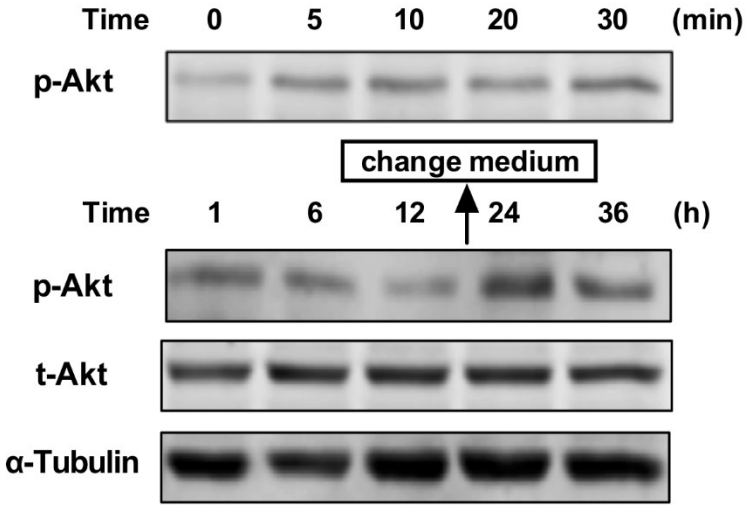


Figure 4.7 Effects of ovotransferrin on activation of PI3K-Akt pathways.

MC3T3-E1 cells treated with ovotransferrin and proteins were extracted for western blot analysis. (A) The time course of Akt phosphorylation (MC3T3-E1 cells treated with 1000 μg/mL ovotransferrin for different times). (B) The expression of PI3K p85 and p110 subunit (MC3T3-E1 cells treated with 100 or 1000 μg/mL ovotransferrin for 24 h). Data were expressed as Mean±SEM (n=6). Means without a common letter indicated $p<0.05$. Untr: cells without ovotransferrin treatment.

A



B

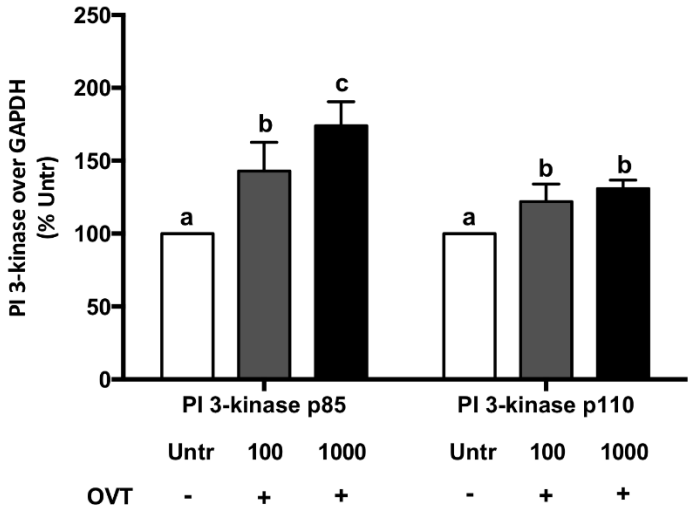
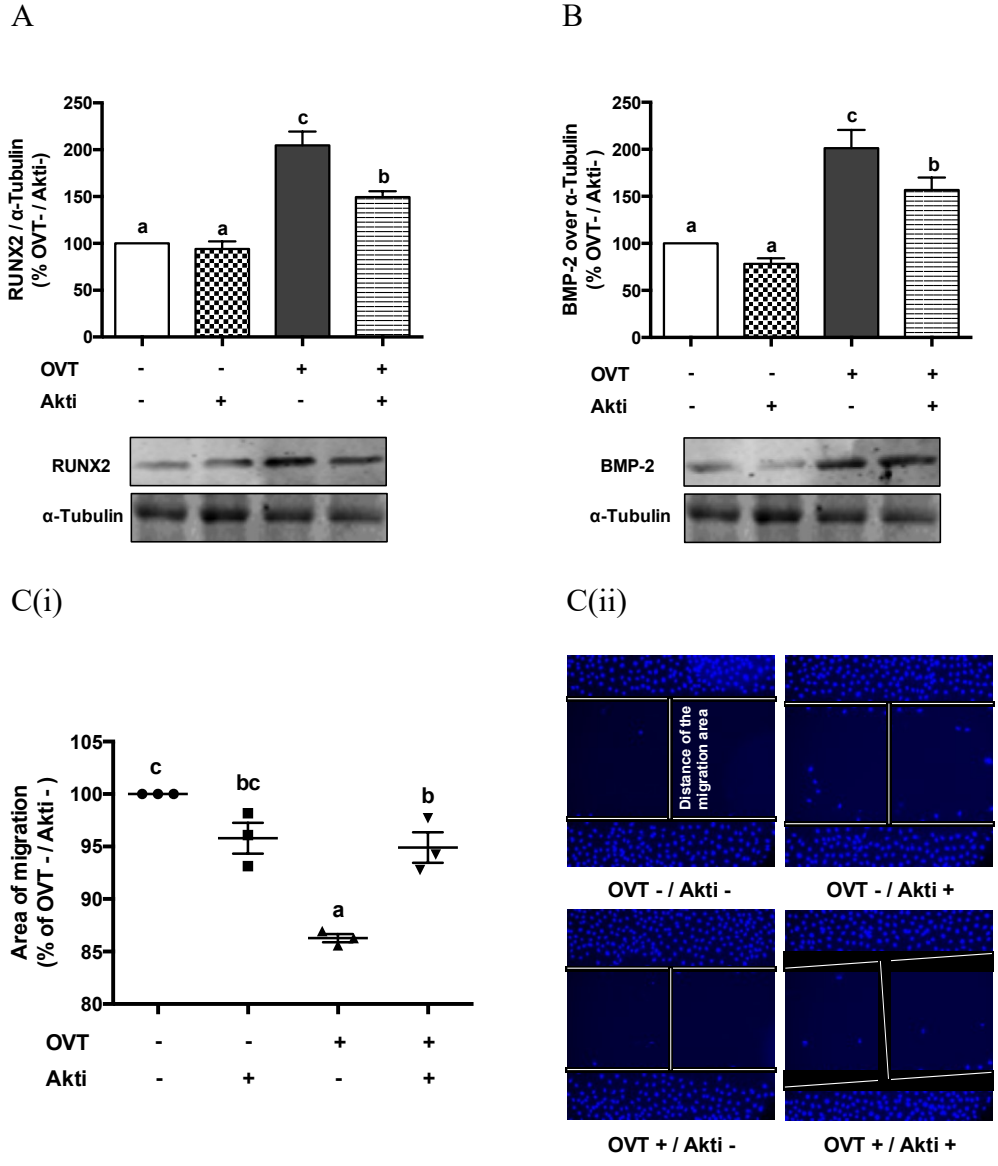


Figure 4.8 Effects of Akt inhibitor on ovotransferrin-induced osteoblast differentiation.

MC3T3-E1 cells were pre-treated with Akt inhibitor for 1 h and then treated with 1000 μg/mL of ovotransferrin together for western blot analysis (24 h treatment), cell migration (12 h) and mineralization (15 d). (A) The expression of Runx2; (B) The expression of BMP-2; (C) (i) The area of migration, (ii) The distance between the migration area; (D) The osteoblast mineralization. Data were expressed as Mean±SEM (n=6). Means without a common letter indicated $p<0.05$.



D

OVT - / Akti - OVT - / Akti + OVT + / Akti - OVT + / Akti +

Figure 4.9 The effects of ovotransferrin on the receptor expression.

MC3T3-E1 cells were treated with 1000 μg/mL of ovotransferrin and proteins were extracted for western blot analysis. The expression of TFBP, TFR1 and LRP1. Data were expressed as Mean±SEM (n=6). ** indicated $p<0.01$ and *** $p<0.001$.

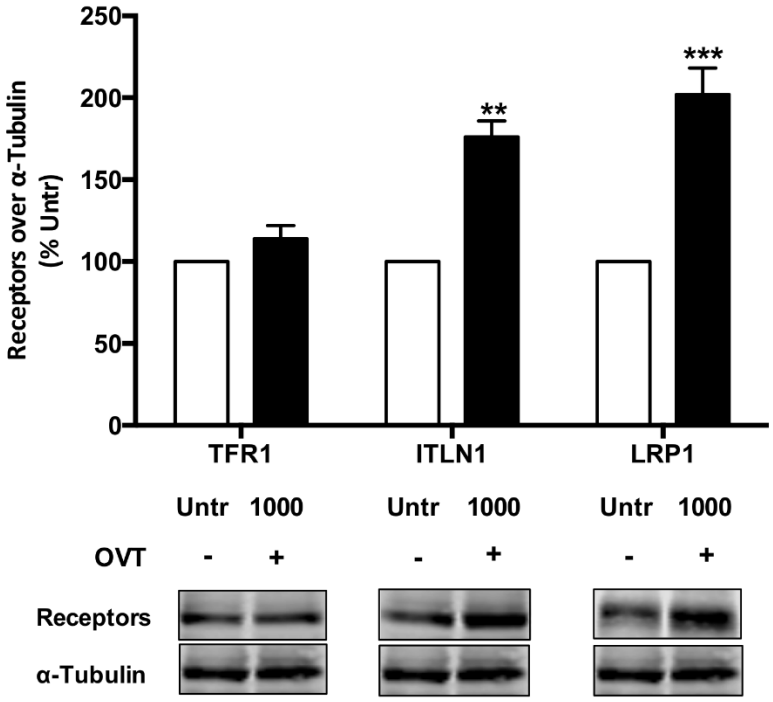
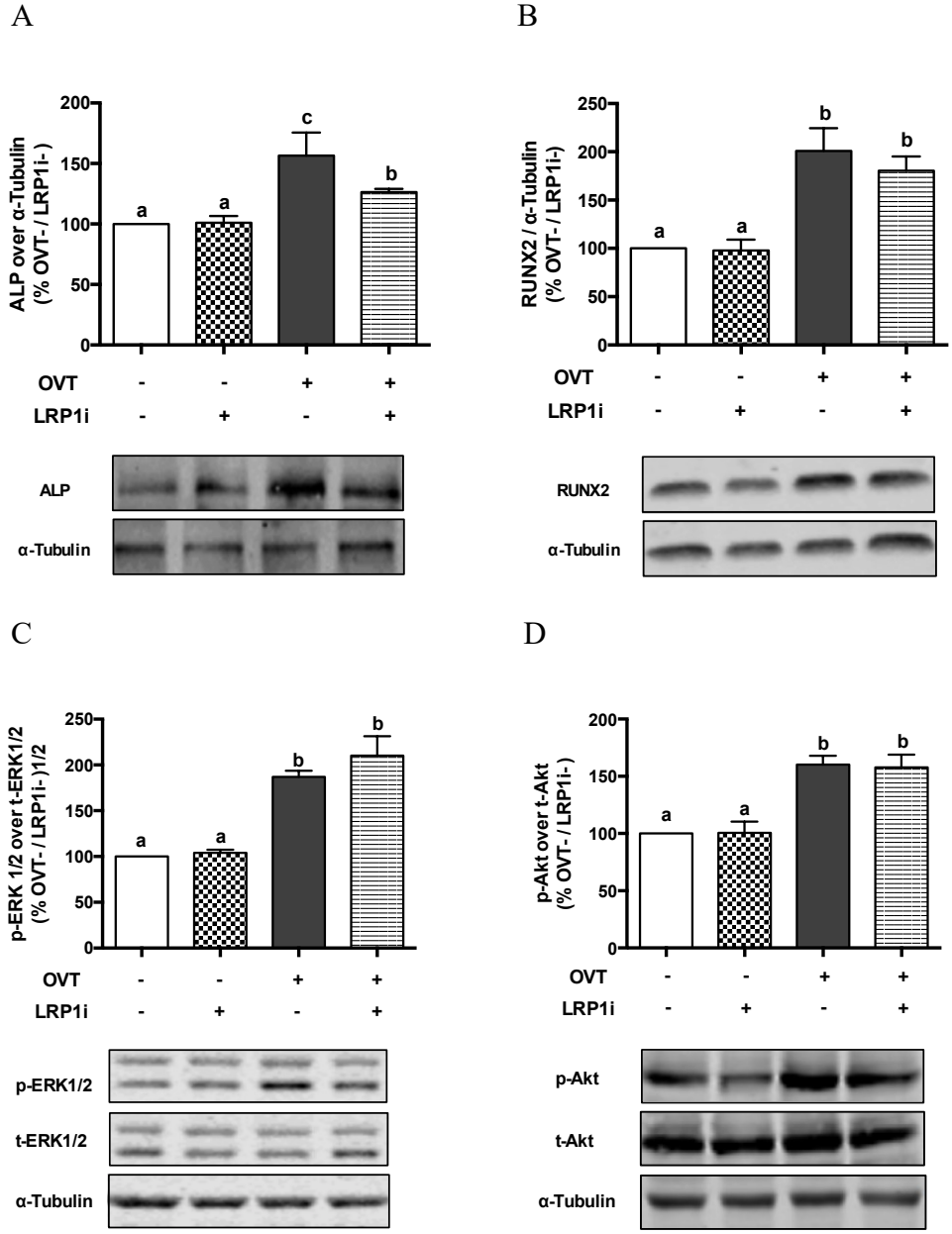


Figure 4.10 Effects of LRP1 inhibitor on ovotransferrin-induced osteoblast differentiation.

MC3T3-E1 cells were pre-treated with LRP1 inhibitor for 2 h and then treated with 1000 μg/mL of ovotransferrin for western blot analysis (24 h treatment) and mineralization (15 d). (A) The expression of ALP; (B) The expression of Runx2; (C) The phosphorylation of ERK1/2; (D) The phosphorylation of Akt; (E) The osteoblast mineralization. Data were expressed as Mean±SEM (n=6). Means without a common letter indicated $p<0.05$.

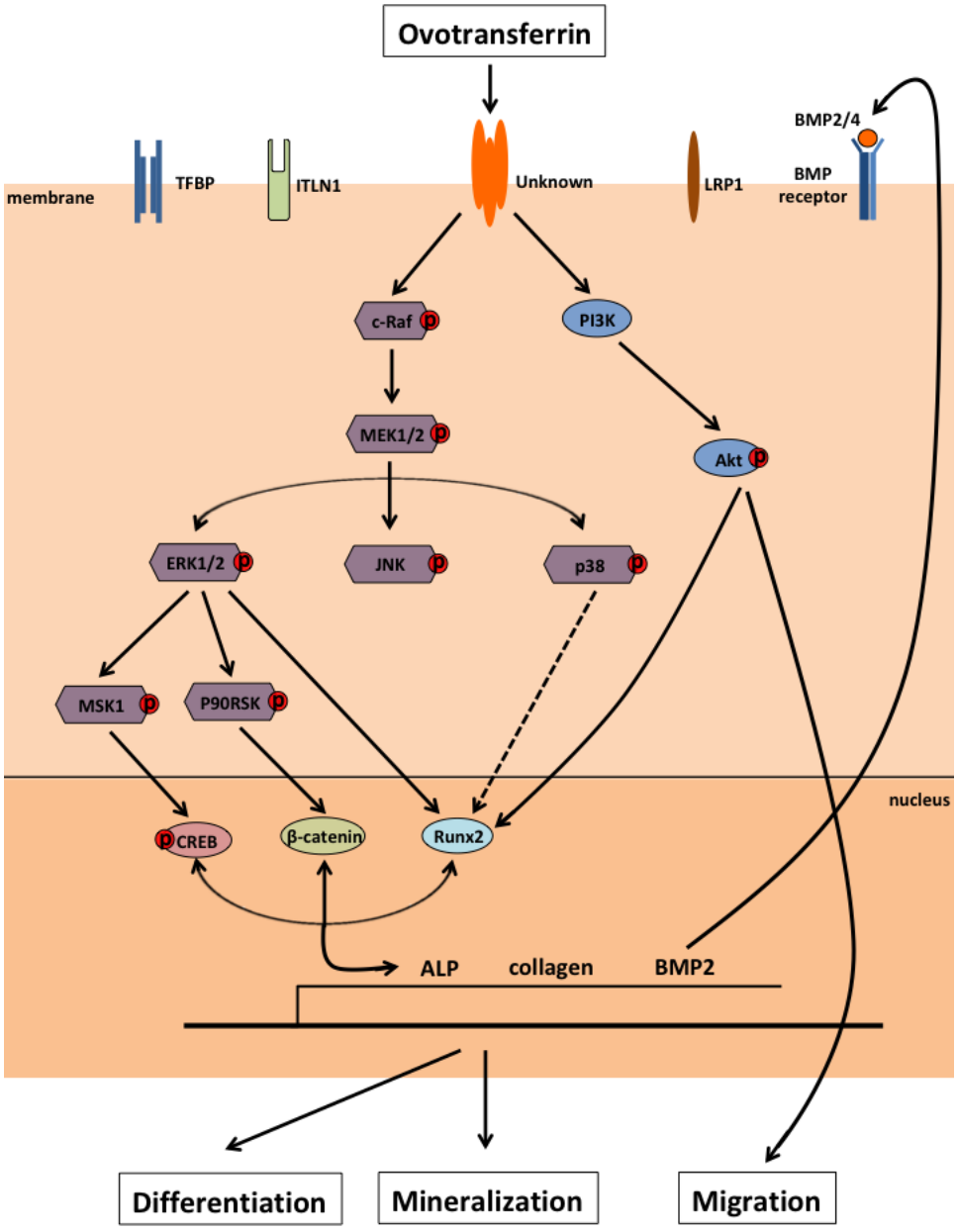


E

OVT - / LRP1i - OVT - / LRP1i + OVT + / LRP1i - OVT + / LRP1i +

Figure 4.11 Scheme summary of ovotransferrin activated signaling pathways.

Ovotransferrin stimulation activated the MAPK pathways, including ERK1/2, JNK and p38. The activation of ERK1/2 cascade further regulated the expression of Runx2, β-catenin, and phosphorylation of CREB and stimulated osteoblast differentiation and mineralization. Ovotransferrin also activated PI3K-Akt pathway and regulated osteoblast differentiation via Runx2. The activation of PI3K-Akt pathway also regulated the migration of osteoblast.



4.7 Supplementary data and information

Figure 4.7.1 Toxicity of antagonists

Ovotransferrin treated with different signaling antagonist for 24h and Alarma Blue assay was used to evaluate the cell toxicity of each antagonist.

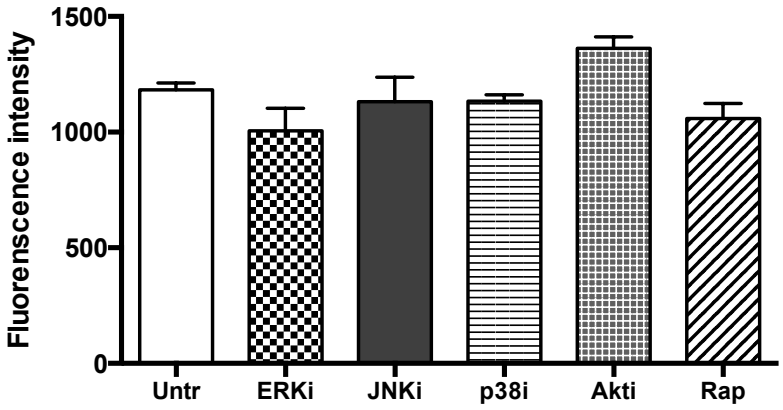


Figure 4.7.2 The PCR amplify of the selected receptors

The mRNA was extracted from MC3T3-E1 and PCR amplify was sued to investigate the expression of selected receptors.

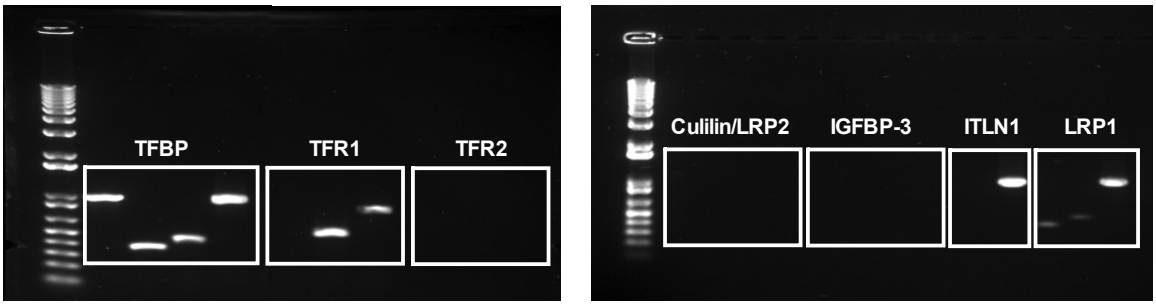


Table 4.7.1 List of the selected receptors

Name	Abbreviation	Functions	Reference
Transferrin binding protein	TFBP	Ovotransferrin receptor	Gentili et al., 1993; 1994
Transferrin receptor 1	TFR1	Primary target of transferrin in the iron transport system	Kawabata, 2018
Transferrin receptor 2	TFR2	Target for transferrin family members, while the expression is restricted in cell type	Kawabata, 2018
Cubilin/LRP2 complex	N/A	Function as a receptor for transferrin and also acts as endocytic receptors for a variety of proteins including hemoglobin, albumin, vitamin-carrier and lipoprotein	Miljuš et al., 2015; Weinzimer et al., 2001
Insulin-like growth factor (IGF)-binding protein-3	IGFBP-3	Has been reported to bind transferrin	Miljuš et al., 2015; Weinzimer et al., 2001
Intelectin-1	ITLN1	Intestinal lactoferrin receptor	Akiyama et al., 2013; Shin et al., 2008
Low-density lipoprotein receptor-related protein 1	LRP1	Lactoferrin receptor that expressed on osteoblast, chondrocyte and cartilage tissue that involved in various biological processes	Grey et al., 2004; 2006

Table 4.7.2 The primers designed in this chapter

Gene	Primer Sequences (5'-3')	
TFBP-1	Forward	GGAAGGAGTTCGGCACGAATA
	Reverse	TCCACACGGGATTCATAGCG
TFBP-2	Forward	GGAAGGAGTTCGGCACGAATA
	Reverse	TCCTTCTCTGTTGCTTCCCG
TFBP-3	Forward	CGCCACAACAGCCAACTATT
	Reverse	TGGTCCCTGGTCCAGATGAT
TFBP-4	Forward	GAACAGCGCCAGTTCAACAA
	Reverse	GGCTCTGGAAGAGTGTTGCT
TFR1-1	Forward	TTGCCATGGGGTCAGTTCAA
	Reverse	GGTTGGCAAGTCCAAACCAAA
TFR1-2	Forward	AGCATCTGCTAATGAGACCCA
	Reverse	ACCCTGAGAGCCTAGTGACAT
TFR1-3	Forward	TCTAGCATGAACCAGGGGGA
	Reverse	GTAGTGCGTGTCAGTCCACA
TFR2-1	Forward	GTTGCTGAGCTGAACTTGGC
	Reverse	TAGTGCGTGTCAGTCCACAC
TFR2-2	Forward	AGATCTTTCTGGACCTGGCTGC
	Reverse	GGGATCCTGGGAGAAGTCTGA
TFR2-3	Forward	ATTGTTTTGCAGTCTGCCCG
	Reverse	TAGTGCGTGTCAGTCCACAC
Culilin-1	Forward	GAACAGCGCCAGTTCAACAA
	Reverse	GGCTCTGGAAGAGTGTTGCT

Culilin-2	Forward	CGCCACAACAGCCAACTATT
	Reverse	TGGTCCCTGGTCCAGATGAT
LRP2	Forward	TAACCCCAGCTGTGACCCT
	Reverse	AGCAACTGCTCAGAAGAGCC
IGFBP-3-1	Forward	AGATGCGAGCTTAGAGCGG
	Reverse	GCGCGCACTGGGACA
IGFBP-3-2	Forward	CAGTGCGCGCCTCCG
	Reverse	ATTATGTGGCACGGAGCATCT
IGFBP-3-3	Forward	GCTGTGCATGTCCAACACAA
	Reverse	ACGGCTCCCTCATACTCCTT
ITLN1-1	Forward	GGAGCACACAAAGGCACAAG
	Reverse	GAGAAGTCAGGGCCAATCCC
ITLN1-2	Forward	TGATTGCCACGAGAGTGTG
	Reverse	CTGGGCCATTGTCAGTCCAA
LRP1-1	Forward	GAGTGTTCCGTGTATGGCAC
	Reverse	GATGCCTTGGATGATGGTC
LRP1-2	Forward	TATGAAGGTGGAGAGCCCGA
	Reverse	CAGCTTCCAGGGGTATGCTC
LRP1-3	Forward	CGTGCCTACCTTCCCGAC
	Reverse	CGGACTTGAGTGAGCCAGG

CHAPTER 5 - Egg White Ovotransferrin Attenuates RANKL-induced Osteoclastogenesis and Bone Resorption

5.1 Introduction

Bone is a dynamic organ, which undergoes continuously remodeling to changes its mass and form (Kini, Usha; Nandeesh, 2012). Normal bone metabolism relies on a balance between bone resorption and formation that regulated by osteoclasts and osteoblasts, respectively (Takatsuna et al., 2004). When this balance turns to favour of bone resorption, it results in osteolytic conditions and leads to bone diseases such as osteoporosis (Takatsuna et al., 2004). The reduction in bone mass and its architectural integrity are considered to result from the massive recruitment of osteoclasts and the excessive osteoclastic activity (Takatsuna et al., 2004; Wei et al., 2018). Thus, inhibition of osteoclastic activity and the subsequent bone resorption has profound effect on regulating abnormal remodeling process and is therapeutically important for osteoporosis prevention.

Osteoclasts are giant, multinucleated cells derived from monocytic/macrophage lineage, which are responsible for bone resorption (Tanaka et al., 2006). The essential step of osteoclasts generation is the binding of receptor-activator of NF-κB ligand (RANKL) to its receptor RANK in osteoclast precursors. This binding triggers the trimerization of TNF receptor-associated factor 6 (TRAF6), leading to the activation of downstream signaling pathways, such as NF-κB, Scr and mitogen-activated protein kinases (MAPKs) pathways. The MAPKs include p38 MAPK, c-jun-N-terminal kinase (JNK), and extracellular signal-regulated kinase (ERK) (Boyle, Simonet, & Lacey, 2003; Thummuri et al., 2015). Activation of NF-κB and MAPKs subsequently results in the expression

of transcription factors such as proto-oncogene c-Fos (c-Fos) and nuclear factor of activated T-cells, cytoplasmic 1 (NFATc1), which are crucial for the differentiation of mature osteoclasts from the precursors (Thummuri et al., 2015). Hence, targeting NF-κB and MAPKs/AP-1 signaling to decrease osteoclastogenesis has been a promising strategy for treating osteolytic disorders.

In addition to impairing osteoclast formation, the induction of apoptosis of matured osteoclasts is another strategy for osteoporosis treatment. The significance of osteoclast apoptosis in bone disorders has been identified in several genetically modified animal models (Xing & Boyce, 2005). Decreased osteoclast apoptosis in general leads to increased bone loss, which is seen after ovariectomy due to estrogen deficiency (Hughes et al., 1996; Hughes & Boyce, 1997). Decreased osteoclast apoptosis in inflamed joints may contribute in part to reduced efficacy of bisphosphonate therapy to prevent local bone loss in patients with rheumatoid arthritis, compared to those patients with osteoporosis (Zhang et al., 2005). The apoptosis process is triggered by two different pathways: the intrinsic pathway through mitochondrial and the extrinsic pathway by ligand activation via death receptors (Elmore, 2007; Roux et al., 2005; Xing & Boyce, 2005). Although the extrinsic pathway in osteoclast apoptosis has several features differing from those in other cell types (for example, TNF promotes survival in osteoclasts rather than death in other cell types (Glantschnig, Fisher, Wesolowski, Rodan, & Reszka, 2003; Lee et al., 2001; Zhang et al., 2005)), osteoclasts contain the general apoptosis machinery of the intrinsic pathway (Xing & Boyce, 2005). Caspases are one of the main adhesion molecules involved in bone cell apoptosis (Mollazadeh, Fazly Bazzaz, & Kerachian, 2015). The release of protein-activated caspase through the mitochondria is governed by Bcl-2 family (Hung & Chow, 1997). Bad, Bax, and Bid are the apoptotic members while Bcl-2 and Bcl-xL are considered as anti-apoptotic members (Elmore,

2007). Among Bcl-2 family members, Bcl-xL has been mostly studied in osteoclasts. M-CSF, RANKL and TNF all increase Bcl-xL protein level in mature osteoclasts (Woo, Kim, & Ko, 2002; Xing & Boyce, 2005). Over-expression of Bcl-xL in osteoclasts *in vitro* prolongs their life span and protects them from bisphosphonate-induced cell death (Zhang, Badell, Schwarz, Boyce, & Xing, 2004). Bcl-2 expression and Bcl-2: Bax ratio are significantly increased in pagetic osteoclasts, which have more nuclei than normal osteoclasts. Abnormally high levels of Bcl-2 protein delayed the apoptosis of matured osteoclasts in pagetic patients (Brandwood et al., 2003; Mee, 1999).

To date, most FDA-approved drugs against osteoporosis are anti-resorptive agents such as bisphosphonates. These anti-resorptive agents prevent bone loss by inhibiting osteoclasts formation and/or inducing apoptosis in matured osteoclasts, but are associated with severe side effects such as renal impairment and osteonecrosis of the jaw (Drake, Clarke, & Khosla, 2008). Food-derived natural products are gaining the momentum towards the development of alternative against osteoporosis. Ovotransferrin is an iron-binding glycoprotein, which is rich in egg white. Egg ovotransferrin is found to have profound pharmacological activities, such as antimicrobial, antioxidant, and immunomodulating activities (Wu & Acero-Lopez, 2012). Our previous study reported that ovotransferrin stimulated osteogenic activity (Shang & Wu, 2018) via down regulation of RANKL/OPG ratio, suggesting its possible additional effects on mediating osteoclastogenesis. In this study, we hypothesized that ovotransferrin could help prevent RANKL-induced osteoclastogenesis and induce apoptosis in matured osteoclasts. Hence, we tested the activity of ovotransferrin in cell model of osteoclastogenesis and illustrated the underlying molecular mechanisms. We found that ovotransferrin significantly inhibits RANKL-induced

osteoclastogenesis accompanied with the inhibition of NF-κB and MAPK signaling. Furthermore, ovotransferrin induced cell apoptosis in matured osteoclasts via intrinsic apoptosis pathway by regulating Bcl-2 family members.

5.2 Materials and methods

5.2.1 Reagents and antibodies

Recombinant mouse soluble RANKL was purchased from R&D Systems (Oakville, ON, Canada). Dulbecco modified Eagles medium (DMEM), fetal bovine serum (FBS), penicillin-streptomycin (1000 U/mL) and TRIzol Reagent was purchased from Thermo Fisher Scientific (Burlington, ON, Canada). Ovotransferrin and TRAP-staining kit was purchased from Sigma-Aldrich (St. Louis, MO, USA). Annexin V-FITC Apoptosis Staining/Detection Kit was purchased from Abcam (Toronto, ON, Canada). NF-κB pathway sampler kit was purchased from Cell Signaling Techology (Whitby, ON, Canada).

5.2.2 Cell culture

The murine monocyte cell line RAW 264.7 was purchased from ATCC (Manassas, VA, USA). RAW 264.7 cells were cultured at 37°C in 5% CO_2 atmosphere in Dulbecco modified Eagles medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% pen-strep. All experiments were conducted using the cells between passage number 18 to 25.

5.2.3 Osteoclasts generation and TRAP staining

Osteoclast cells were generated from culture of murine monocyte cell line RAW 264.7. RAW 264.7 cells were seeded in 24-well plate at density of 1×10^4 cells/well and incubated for 6 h to allow the cells to attach to the surface. After 6h, the culture medium was replaced with medium contains 100 ng/ml RANKL. Osteoclasts were successfully induced by RANKL added every 48 h

over the course of 4 days. RAW 264.7 cells were treated with different concentration of ovotransferrin (1 μg/mL to 1000 μg/mL) for 4 days for its effects on osteoclastogenesis. To confirm and count the generation of multinucleated osteoclast-like cells, the cultured cells were fixed in 4% paraformaldehyde for 1 h at 4 °C and then stained for the enzyme tartrate-resistant acid phosphatase (TRAP) using the TRAP-staining kit according to the manufacturer instructions (0.1mg/mL naphthol AS-MX phosphate and 0.3 mg/mL Fast Red Violet LB staining). The cells were observed under a light microscope with the magnification of 10X (Olympus IX83) and the images were captured by Metamorphy (Olympus). TRAP-positive cells containing three or more nuclei were counted. The experiment was repeated 5 times.

5.2.4 Cell apoptosis assay

Osteoclasts were generated from RAW 264.7 cells stimulated with RANKL (100 ng/mL) for 4-6 days as described in *2.3* and identified by microscopy. Cells were treated with 1-1000 μg/mL ovotransferrin for 12 h and collected by centrifugation. The apoptosis detection was conducted using the apoptosis detection kit according to manufacturer's instruction. Briefly, the cells were re-suspended in 500 μL binding buffer, followed by the addition of 5 μL of Annexin V-FITC and 5 μL of propidium iodide, and then incubation for 5 min in the darkness at room temperature. The Annexin V-FITC/PI binding was analyzed by flow cytometry (FACSCanto II, BD Biosciences, Mississauga, ON, Canada).

5.2.5 Resorption assay

RAW 264.7 cells were seeded in a calcium phosphate (CaP)-coated 48-well plate (Cosmo Bio Co., Ltd, Tokyo, Japan) and cultured with 100 ng/mL RANKL for 6 days to generated multinucleated osteoclasts, and treated with 1-1000 μg/mL ovotransferrin for 5 days. An aliquot of 150 μL new medium was added into the well every 2 days without removing the old medium. The generated

multinucleated osteoclasts were identified with microscopy. An aliquot of 100 μL culture medium was used to identify the resorption by measuring the fluorescence intensity every 2 days. After 6 days, the adherent cells were removed by the treatment of 5% hypochlorite for 5 min and the plate was washed with water and then dried., Microscopic images of each well were captured using a microscope and the pit areas are measured by Image J.

5.2.6 Western blot analysis

The RAW 264.7 cells were seeded in 48 well tissue culture plates at a density of 1×10^4 cells/well, and incubated in α-MEM with 10% FBS. The cells were treated with different concentrations of ovotransferrin and RANKL. At the end of incubation, the culture medium was removed and the cells lysed in boiling Laemmle's buffer containing 50 μM dithiothreitol (DTT) and 0.2% Triton-X-100 to prepare samples for western blot as described previously. These cell lysates were run in SDS-PAGE, blotted to nitrocellulose membranes and immunoblotted with antibodies. The concentration of each antibody was according to the manufacturer's instruction. The protein bands were detected by a Licor Odyssey BioImager and quantified by densitometry using corresponding software (Licor Biosciences, Lincoln, NB, USA). Each band was normalized to its corresponding band of loading control. Cell lysates from untreated cells were loaded onto every gel. The results were expressed as percentage of the corresponding untreated control.

5.2.7 RNA extraction and quantitative PCR

Total RNA was extracted from the cells using TRIzol® reagent by following the manufacturer's instruction, and cDNA was synthesized using SuperScript II Reverse Transcriptase (Invitrogen, Burlington, ON, Canada) according to the manufacturer's instruction. Real-time PCR was performed using Fast SYBR Green PCR Master Mix (Applied Biosystem, Burlington, ON, Canada) and an ABI 7300 Sequencing Detection System (Applied Biosystem, Burlington, ON, Canada).

The amplification conditions were as follow: initial denaturation at 95°C for 10 min, followed by 35 cycles of 10 s at 95°C, 15 s at 60°C, and 10 s at 72°C. Real-time PCR primers used in this study are listed in Table 5.7.1.

5.2.8 Statistical analysis

All data are presented as mean±SEM (standard error of mean) from 4 and 8 independent experiments. Data were analyzed using One Way analysis of variance (ANOVA) with Dunnett's post-hoc test for comparisons to control. The PRISM 6 statistical software (GraphPad Software, San Diego, CA) was used for the analyses. $P< 0.05$ was considered significant.

5.3 Results

5.3.1 Ovotransferrin inhibits osteoclast formation from RAW 264.7

To examine the effect of ovotransferrin on osteoclast formation from mouse osteoclast precursors, RAW 264.7 were cultured in osteoclastogenic medium containing 100 ng/mL RANKL with various concentrations of ovotransferrin (1-1000 μg/mL) for 6 days. RANKL addition significantly increased osteoclast formation, whereas ovotransferrin addition significantly suppressed RANKL-induced osteoclast formation (Figure 5.1A). Further, count of TRAP-positive ($TRAP^+$) osteoclasts confirmed that ovotransferrin dose dependently inhibited RANKL-induced osteoclast differentiation (Fig. 5.1B).

5.3.2 Ovotransferrin inhibited RANKL-induced activation of NF-κB and MAPK pathway

RANKL-induced osteoclast differentiation involves activation of NF-κB and MAPK pathways. Phosphorylation of NF-κB enhances its transcriptional potential. We found that RANKL significantly increased the phosphorylation of IKKα/β (Ser 176/178), IκBα (Ser 32), and NF-κB

p65 (Ser 536), whereas ovotransferrin pre-treatment suppressed their phosphorylation (Fig. 5.2). During osteoclastogenesis, RANKL also increases the activation of MAPK signaling. So, we further investigated the effect ovotransferrin on MAPKs under RANKL stimulation in osteoclast precursors. Ovotransferrin treatment significantly inhibited RANKL-induced phosphorylation of MAPKs in a dose-depended manner (Fig. 5.3). These data indicate that ovotransferrin inhibits osteoclast formation and differentiation by attenuating NF-κB and MAPK pathways.

5.3.3 Ovotransferrin inhibited RANKL-induced expression of the factors involved in osteoclastogenesis

We further assessed the expression of the factors involved in osteoclastogenesis including TRAF6, c-Fos, NFATc1 and Cathepsin K. Western blotting showed that RANKL stimulation significantly increased the expression of TRAF6, NFATc1 and cathepsin K, while pre-treatment of ovotransferrin significantly decreased RANKL-induced expression of osteoclast-specific proteins (Fig. 5.4). Interestingly, the RANKL did not increase the expression of c-Fos, but pre-treatment of ovotransferrin significantly decreased the expression in a dose-dependent manner (Fig. 5.4C). These data indicate that ovotransferrin inhibits osteoclast differentiation by inhibiting several osteoclastogenesis-related factors.

5.3.4 Ovotransferrin inhibits resorptive activity of matured osteoclasts

Since ovotransferrin inhibited osteoclast formation and expression of osteoclastic markers, we further investigated whether ovotransferrin could inhibit the osteoclastic resorption *in vitro*. RAW 264.7 cultured in a fluoresceinated calcium phosphate (CaP)-coated plate were treated with 100 ng/mL RANKL and different concentrations of ovotransferrin (1-1000 μg/mL) for 6 days to

generate multinucleated osteoclasts. RANKL increased the fluorescent intensity of culture medium with time (Fig. 5.5A), indicating the generation of osteoclasts and osteoclastic resorption of CaP; whereas ovotransferrin treatment significantly decreased RANKL-induced osteoclastic resorption (Fig. 5.5A and 5.5B). After 6 days, the total areas of resorption pits were measured and the formation of resorption pits were substantially reduced in the presence of ovotransferrin (Fig. 5.5C and 5.5D). These data indicate that ovotransferrin inhibited not only osteoclast differentiation but also the bone-resorbing activity of mature osteoclasts *in vitro*.

5.3.5 Ovotransferrin stimulates the apoptosis of osteoclasts

We further studied ovotransferrin-induced cell apoptosis of osteoclasts using flow cytometry. Matured multinucleated osteoclasts were identified using microscope and then treated with different concentrations of ovotransferrin (1-1000 μg/mL). Flow cytometry revealed that ovotransferrin induced apoptosis of matured osteoclasts in a dose-depended manner (Fig. 5.6A and 5.6B). Next, we further assessed the expression of *Bcl-2* family, which is one of the major regulators for cell apoptosis. RT-PCR analysis showed that ovotransferrin treatment significantly increased the expression of *Bim and Bad*, while decreased *Bcl-2* and *Bcl-x* expression (Fig. 5.6C). These findings suggested that ovotransferrin regulates *Bcl-2* family, and may further acting on osteoclasts mitochondria and induce apoptosis.

5.4 Discussion

Bone is constantly remodelling by bone formation osteoblast cells and bone resorbing osteoclast cells (Kini, Usha; Nandeesh, 2012). The over-activation of osteoclasts leads to excessive bone resorption and is a characteristic of several osteolytic disorders, such as osteoporosis. Thus,

osteoclasts remain one of the key targets for the development of potential osteoporosis therapies. However, current treatments targeting osteoclasts to prevent bone loss are associated with some side-effects such as renal toxicity and osteonecrosis with bisphosphonates (Perazella & Markowitz, 2008) and endometrial cancer risk with selective estrogen receptor modulators (Brown, 2009). Diet is one of the most important factors that may contribute to a proper bone structure and function. Nutrients like vitamin D and calcium are well studied in respect to their effects on bone matrix production and mineralization (Alekel, Weaver, Ronis, & Ward, 2013). However, other nutritional factors may also be implied in the process, such as protein. It was reported that a proper increase of protein intake is associated with higher bone mineral density (Bonjour, 2005; Kerstetter & Insogna, 2003). Furthermore, natural compounds including bioactive protein have been a source of therapeutic in bone health management. For instance, soy proteins have been suggested to improve bone quality by inhibiting osteoclast activity (Alekel et al., 2000; Messina & Messina, 2000). Lactoferrin, a protein derived from milk was also reported to promote bone formation by stimulating osteoblastic activity (Cornish et al., 2004) while preventing bone resorption by inhibiting osteoclast differentiation and resorptive activity (Lorget et al., 2002).

Although a positive role of diet protein intake on bone health has been suggested, the effect of egg protein has not been well studied. In addition to their essential amino acid profile and high digestibility, egg proteins are known to impart a wide range of physiological benefits including antimicrobial, anticancer, antioxidant and immunomodulation activities (Wu & Acero-Lopez, 2012). In our previous study, we showed for the first time that egg white protein ovotransferrin could directly stimulate osteoblasts proliferation and differentiation as an osteogenic agent (Shang & Wu, 2018) Thus, the present study aims to characterize the cellular and molecular effects of egg

white ovotransferrin on mouse osteoclasts. In this study, we demonstrated that ovotransferrin exerts an anti-osteoclastogenesis effect via suppress RANKL-induced activation of NF-κB and MAPK signaling pathway. In addition, we also found that ovotransferrin could induce cell apoptosis in matured multinuclear osteoclasts by regulating the expression of Bcl-2 family members.

Osteoclastogenesis is a complex process, which is primarily regulated by RANKL and RANK. Binding of RANKL to RANK is the essential step in osteoclast development. It leads to recruitment of TRAF6 and activation of downstream signaling such as NF-κB and three MAPKs including ERK, JNK, and p38 (Karin, 2005; Matsumoto, Sudo, Saito, Osada, & Tsujimoto, 2000; Thummuri, Naidu, & Chaudhari, 2017). The role of NF-κB in RANKL/RANK pathway for osteoclast differentiation has been confirmed by both *in vitro* and *in vivo* studies (Abu-Amer, 2013). NF-κB signaling comprises a number of activation steps. Under normal conditions, NF-κB exists as a complex with the cytoplasmic inhibitory protein IκBα. With the stimulation of RANKL, IκB kinase (IKK) is activated and stimulates the phosphorylation of IκB proteins at Ser32 and 36 residues, which leads to the polyubiquitination of IκB and subsequent proteasomal degradation. IκB degradation increases nuclear translocation of NF-κB p65 and leads to the transcription of the genes involved in osteoclast differentiation (Thummuri et al., 2017). In our study, we found that ovotransferrin inhibits RANKL-induced phosphorylation of IKKα/β (Ser 176/180), IκBα (Ser 32), and NF-κB p65 (Ser 536), suggesting the regulatory role of ovotransferrin in NF-κB pathway. In addition to NF-κB pathway, activation of MAPK signaling is another pivotal regulation in RANKL-induced osteoclast activation and differentiation (Tanaka et al., 2006; Thummuri et al., 2017). MAPKs play an essential role in the transduction of extracellular stimuli into intracellular

responses and thereby contribute to the regulation of diverse cellular activities (Lee et al., 2016). Hence, in the current study, we also determined the effect of ovotransferrin on RANKL-mediated phosphorylation of MAPK and found that ovotransferrin markedly inhibits RANKL-induced phosphorylation of three MAPKs. These results indicate that the anti-osteoclastogenic effects of ovotransferrin are associated with the suppression of RANKL-induced NF-κB and MAPK signaling pathways.

The phosphorylation of NF-κB and MAPK signaling pathways is the early stage of osteoclastogenesis. Following the activation of NF-κB and MAPK signaling, the transcription of osteoclast differentiation factors such as c-Fos and NFATc1 are expressed and acts as the regulator for osteoclast differentiation at early or mid stage (Teitelbaum, 2004; Thummuri et al., 2017). Binding of NF-κB and NFATc1 to their promoter regulates the expression of various osteoclast-specific genes, including TRAP, cathepsin K, and DC-STAMP (Takatsuna et al., 2004; Thummuri et al., 2017). In our results, we observed that ovotransferrin decreases the expression of c-Fos, NFATc1 and cathepsin K in RANKL-stimulated osteoclast precursors. These results suggest that ovotransferrin inhibits osteoclastogenesis by decreasing the expression of c-Fos, NFATc1, and its downstream regulators that are crucial for osteoclastogenesis.

In addition to inhibit the osteoclast formation, pharmaceutical agents such as bisphosphonates and tamoxifen also inhibit osteoclastic bone resorption, at least in part, by inducing osteoclast apoptosis via different mechanisms (Drake et al., 2008; Hughes et al., 1996). Bisphosphonates can bind to and inhibit the activity of farnesyl pyrophosphate synthase, a key regulatory enzyme in mevalonic acid pathway, and inhibited the posttranslational modification of several specific proteins (e.g., the

small guanosine triphosphate-binding proteins Rab, Rac, and Rho), which play central roles in the regulation of core osteoclast activation, therefore ultimately leading to osteoclast apoptosis (Luckman et al., 1998). Tamoxifen, acting as estrogen receptor agonist, prevents excessive bone loss by limiting osteoclast life span through the promotion of osteoclast apoptosis by mediating TGF-β (transforming growth factor β) (Hughes et al., 1996). Thus, in this study, we also investigated whether egg white ovotransferrin may affect osteoclast function by inducing apoptosis. Here, we found that ovotransferrin could induce cell apoptosis in matured osteoclasts via affecting the gene expression of *Bcl-2* family members. The expression of anti-apoptotic protein *Bcl-2* and *Bcl-xL* in osteoclasts was decreased, while the pro-apoptotic protein *Bim* and *Bax* was increased in the presence of ovotransferrin.

Generally, apoptotic process is triggered by two pathways. One is established by ligand activation via death receptor pathway (Xing & Boyce, 2005). These death receptors belong to tumor necrosis factor (TNF) receptor superfamily, including Fas, TNF-related apoptosis-inducing ligand (TRAIL), and TNF-R1 (Mollazadeh et al., 2015). However, the regulation of the death receptor pathway in osteoclasts has several features that differ from those in other cell types. For example, TNF promotes osteoclast survival rather than death in other cells (Glantschnig et al., 2003; Lee et al., 2001) The other pathway is regulated by pro- and anti-apoptotic Bcl-2 family members and involves mitochondrial release of cytochrome c, thus which is also known as mitochondrial pathway (Xing & Boyce, 2005). The anti-apoptotic Bcl-2 family members include Bcl-2, Bcl-xL, Bcl-w, Mcl-1, A1 and Boo/Diva, which all contain Bcl-2 homology (BH) domains (Xing & Boyce, 2005). The pro-apoptotic members, depending on the composition of their BH domains, are further divided in to the BH3-only pro-apoptotic members (Bim, Bad, and Bid) and other pro-apoptotic

proteins (Bak, Bax, and Bod) (Xing & Boyce, 2005). Although the regulatory mechanisms of the Bcl-2 family underlying osteoclasts apoptosis have not been fully identified and characterized, studies have demonstrated the significant relationship between the expression of Bcl-2 family and osteoclast apoptosis. Among all Bcl-2 family members, anti-apoptotic protein Bcl-xL and pro-apoptotic protein Bim have been the ones mostly studied in osteoclasts. Over-expression Bcl-xL in osteoclasts *in vitro* prolongs their life span and protects them from bisphosphonate-induced cell death, suggesting the adverse effects of Bcl-xL on inducing osteoclast apoptosis and prevent bone loss (Zhang et al., 2004). However, interesting results have been found *in vivo*. Iwasawa et al. found that the anti-apoptotic protein Bcl-xL positively regulates osteoclast survival, but negatively regulate osteoclast activity by down-regulating extracellular matrix (ECM) proteins production and c-Src kinase activity (Iwasawa et al., 2009). However the effects only showed the inhibitor treatment (ABT-737) in the study; thus more studies are warranted to validate the effects of Bcl-xL protein, or even the Bcl-2 family proteins on osteoclasts survival and their ultimate resorbing activity. Compared to Bcl-xL, the effect of pro-apoptotic protein Bim are consistent in different studies. The increased number of osteoclasts along with the elongated life span was observed in Bim-/- mice, which suggested the negative effect of Bim in osteoclast survival (Akiyama et al., 2003). Moreover, TGF-β1, an important functional modulator of osteoclasts, can induce osteoclast apoptosis by affecting the expression of Bim. The expression of Bim was increased in the presence of TGF-β1, which led to the upregulation of activated caspase 9 (Houde, Chamoux, Bisson, & Roux, 2009). In addition to Bcl-xL and Bim, Bcl-2 also plays role in osteoclast apoptosis and in the pathology of postmenopausal osteoporosis. The mRNA and protein levels of Bcl-2 in osteoclasts were distinctly increased in postmenopausal osteoporosis patients, leading to the inhibition of osteoclast apoptosis and excessive bone loss (Pang, Gong, Han, & Liu, 2018).

5.5 Conclusions

Our study established that egg white ovotransferrin could suppress RANKL-mediated osteoclastogenesis and resorption activity. Moreover, we demonstrated that the prevention effects of ovotransferrin occur through suppression of NF-κB and MAPK activation during the process of osteoclast differentiation along with the induction of cell apoptosis in matured osteoclasts. It is now clear that the balance among osteoclasts, osteoblasts and osteocytes is an important determinant of bone mass and strength. However, some of the drugs used for osteoporosis prevention by regulating osteoclastogenesis and osteoclast life span are affecting the survival of osteoblasts and/or osteocytes, and therefore is harmful to the skeleton's integrity in long term therapy. Our study found that egg white protein ovotransferrin inhibits osteoclasts differentiation and survival, suggesting its potential as a favorable nutritional approach for bone health management.

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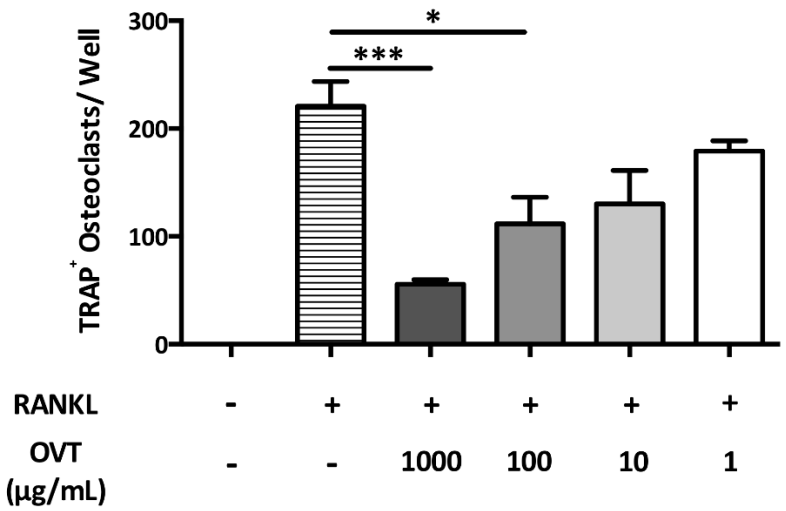
Figure 5.1 Effect of ovotransferrin on RANKL-induced osteoclastogenesis.

(A) RAW 264.7 cells were incubated with RANKL (100 ng/mL) and various concentrations of ovotransferrin (OVT) for 4 days and then TRAP staining was performed. (B) $TRAP^+$ multinucleated cells with at least three nuclei were counted as osteoclasts. Data were expressed as means ± SEM (n=8). * and *** indicated $p < 0.05$ and $p < 0.001$.

A



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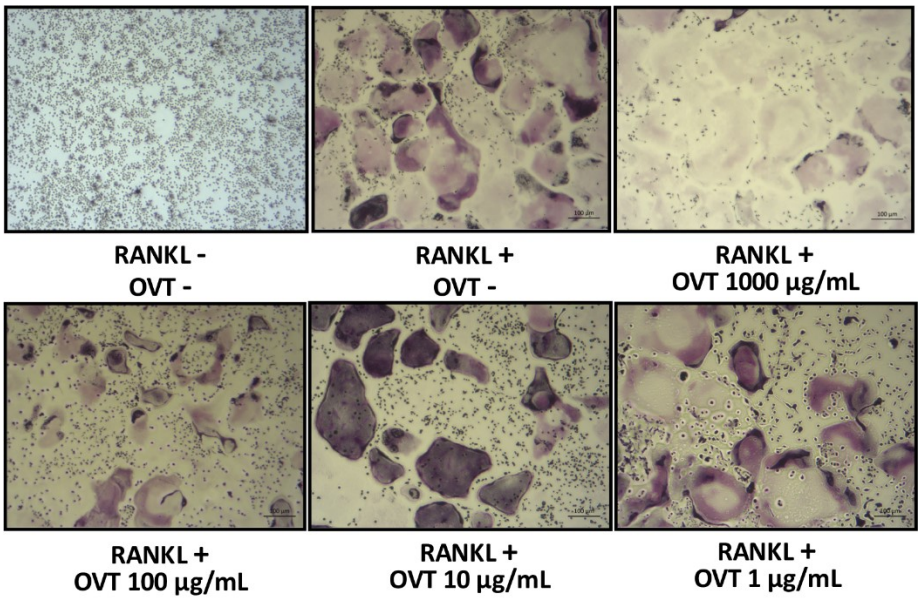

Figure 5.2 Effect of ovotransferrin on RANKL-induced NF-κB activation.

RAW 264.7 cells were pretreated with ovotransferrin (1-1000 μg/mL) for 30min prior to stimulation with RANKL (100 ng/mL). Then RAW 264.7 cells were treated with both RANKL and ovotransferrin for 4 h. Whole cell lysates were used for western blotting analysis of p-NF-κB p65 (ser 536)/NF-κB p65 (A), p-IκB (ser 32)/IκB (B), and p-IKKα/β (ser 176/180)/IKKα+IKKβ (C). All bands were shown in (D). Data were expressed as means ± SEM (n=6). Means without a common letter indicated $p<0.05$.

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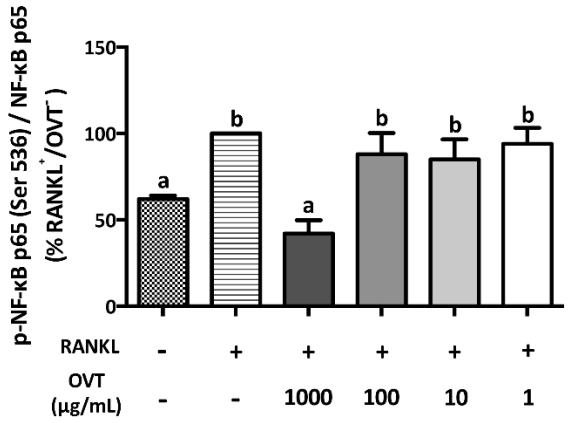

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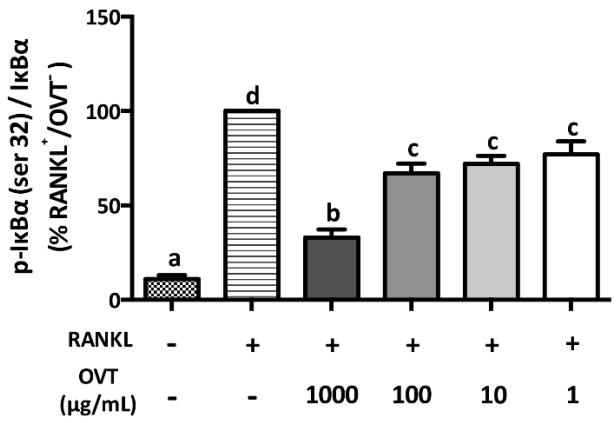

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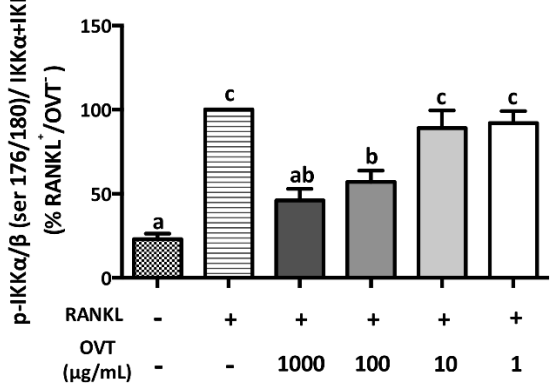

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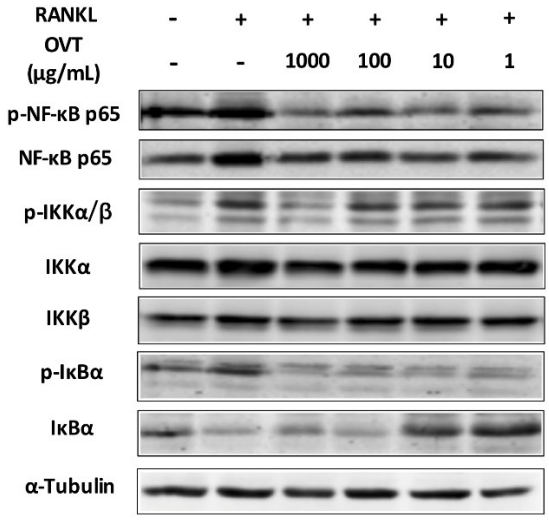


Figure 5.3 Effect of ovotransferrin on RANKL-induced MAPK activation.

RAW 264.7 cells were pretreated with ovotransferrin (1-1000 μg/mL) for 30min prior to stimulation with RANKL (100 ng/mL). Then RAW 264.7 cells were treated with both RANKL and ovotransferrin for 4 h Whole cell lysates were used for western blotting analysis of p-p44/42 Erk1/2 (Thr 202/Tyr 204)/p44/42 Erk1/2 (A), p-p38 (Thr 180/Tyr182)/p38 (B), and p-JNK (Thr 183/Tyr 185)/JNK (C). All bands were shown in (D). Data were expressed as means ± SEM (n=6). Means without a common letter indicated $p<0.05$.

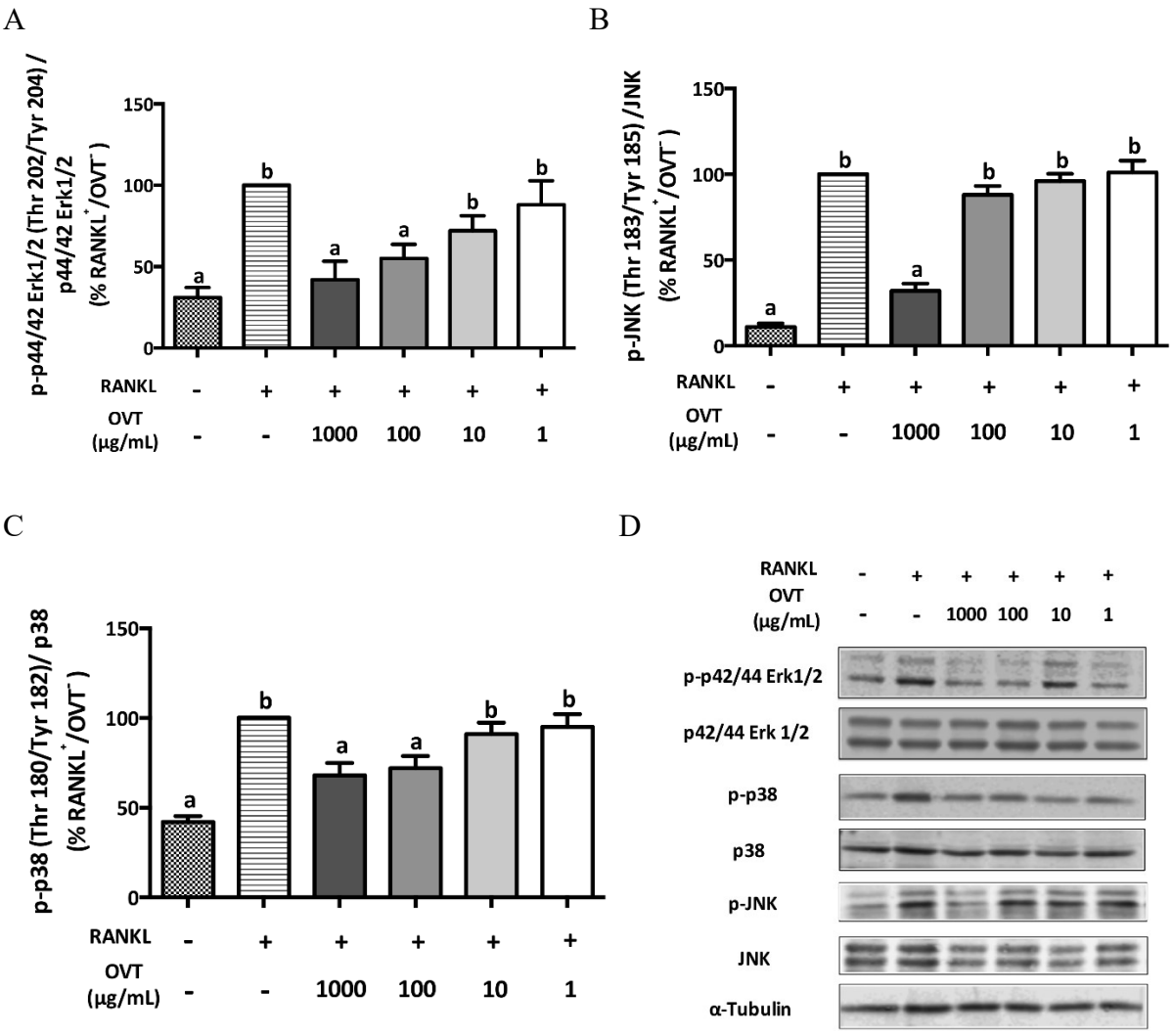
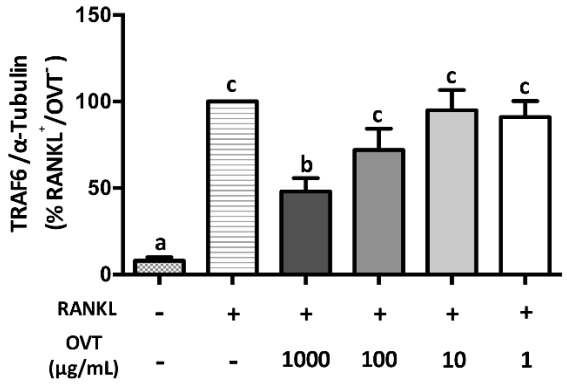


Figure 5.4 Effect of ovotransferrin on RANKL-induced expression of proteins involved in osteoclastogenesis.

RAW 264.7 cells were pretreated with ovotransferrin (1-1000 μg/mL) for 2 h prior to stimulation with RANKL (100 ng/mL). Then RAW 264.7 cells were treated with both RANKL and ovotransferrin for 12 h. Whole cell lysates were used for western blotting analysis of TRAF6 (A), cFos (B), NFATc1 (C) and cathepsin K (D). All bands were shown in (E). Data were expressed as means ± SEM (n=6). Means without a common letter indicated $p<0.05$.

A



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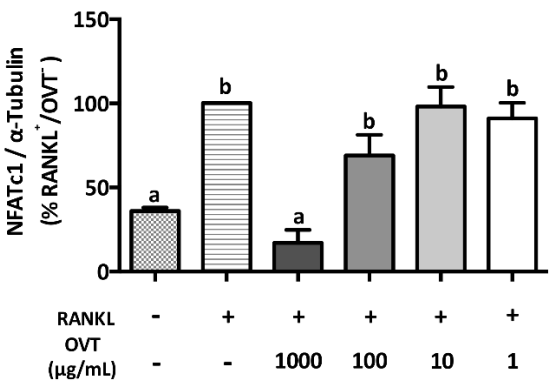

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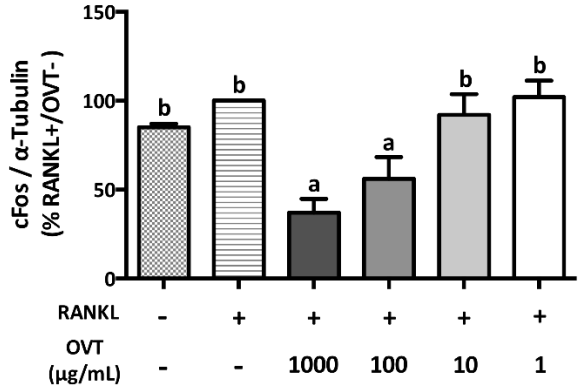

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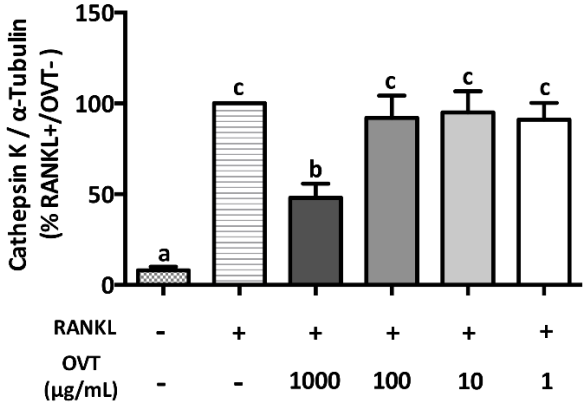

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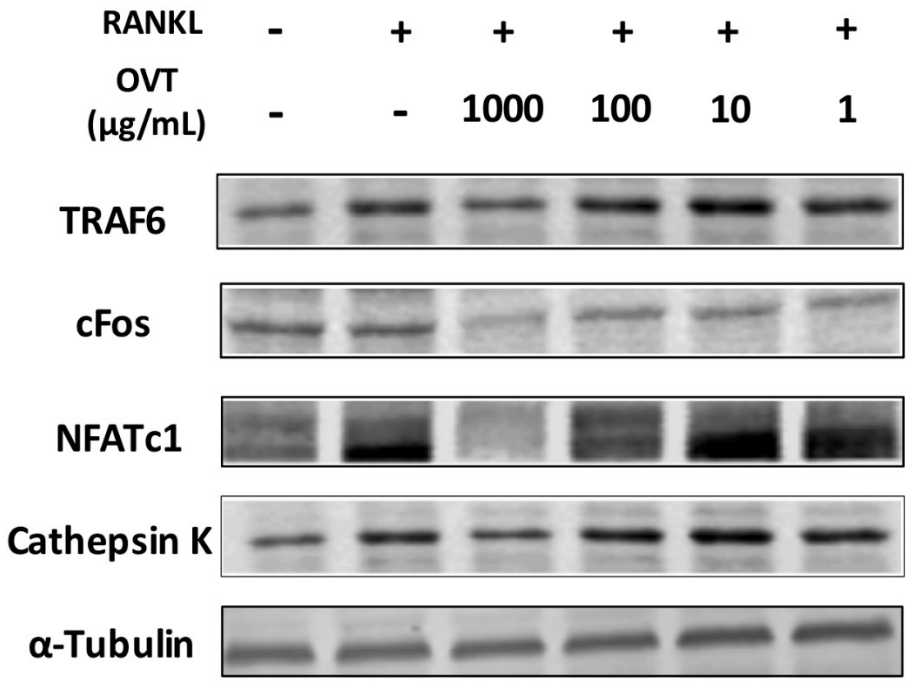
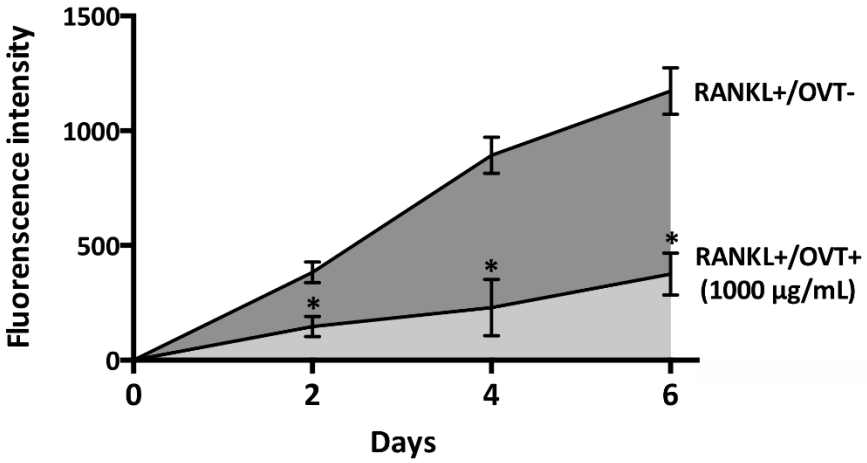

RANKL - + + + + +
OVT (μg/mL) - - 1000 100 10 1
TRAF6
cFos
NFATc1
Cathepsin K
α-Tubulin

Figure 5.5 Effect of ovotransferrin on RANKL-induced bone resorption *in vitro.*

RAW 264.7 were cultured onto fluoresceinated calcium phosphate (CaP)-coated plate and stimulated with RANKL (100 ng/mL). After 1day for adhesion, cells were treated with ovotransferrin (1-1000 µg/mL) and RANKL together for 6 days. (A) The fluorescence intensity of resorbed CaP during 6-day treatment. * indicated $p < 0.05$. (B) The total resobed CaP after 6 days. (C) The total area of resorption pits after 6 days. (D) The image of resorption area. Data were expressed as means ± SEM (n=4). Means without a common letter indicated $p<0.05$.

A



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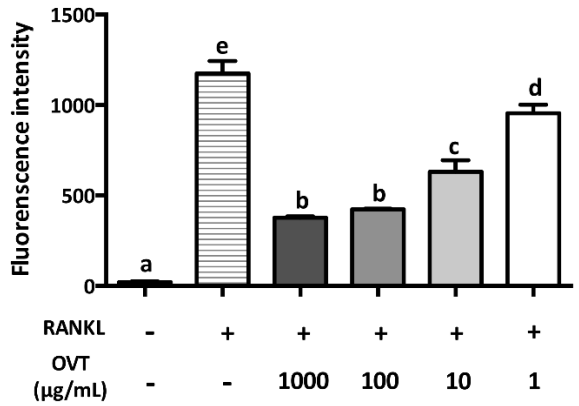

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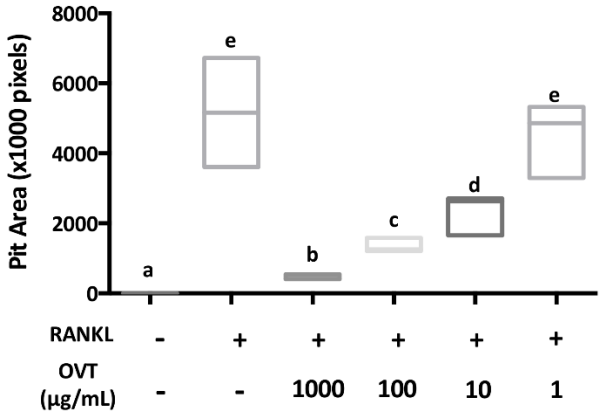


D

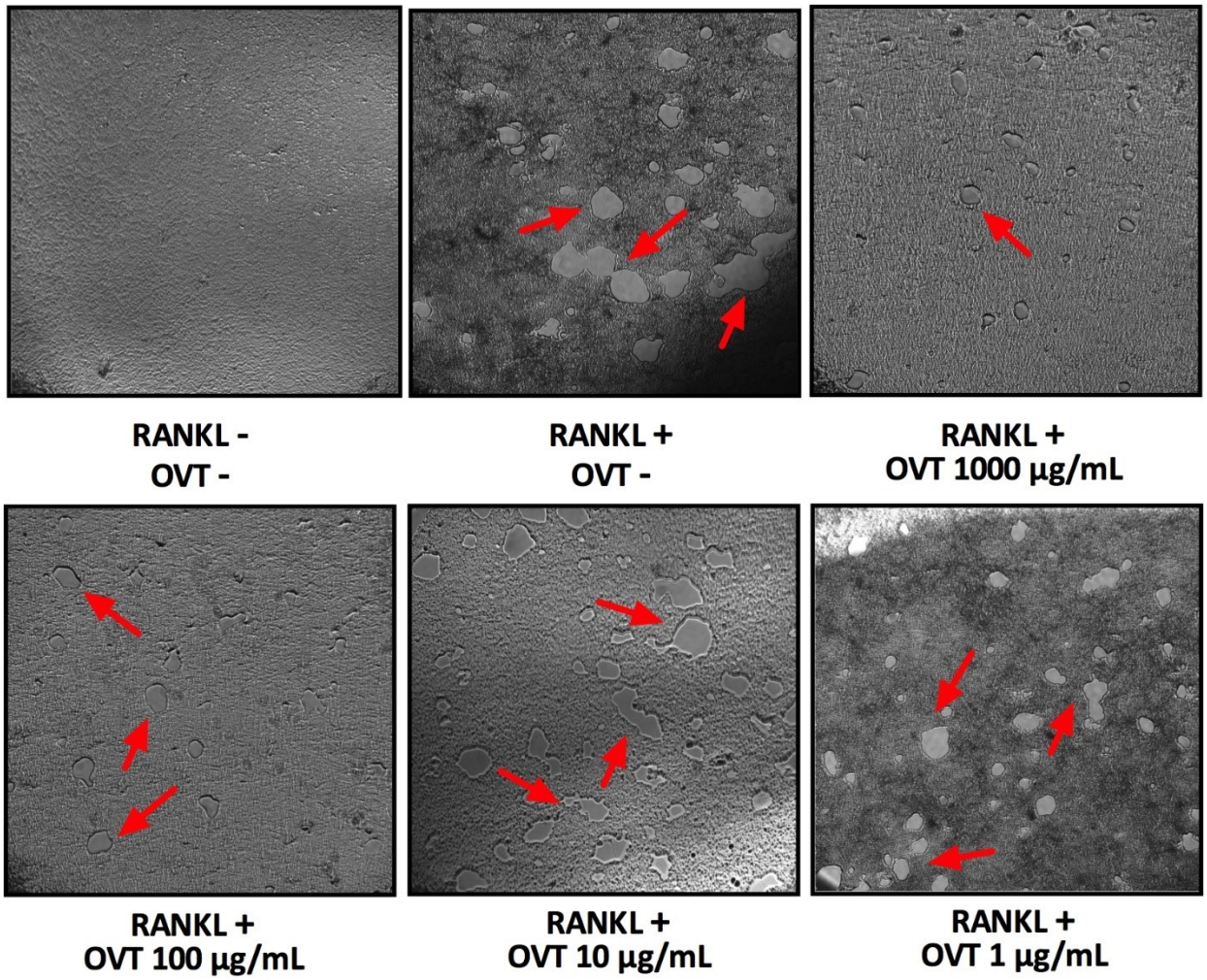
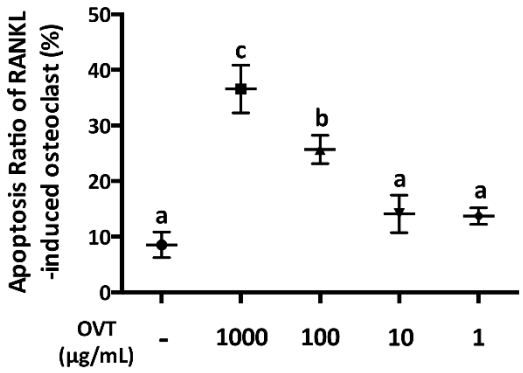

RANKL -
OVT -
RANKL +
OVT -
RANKL +
OVT 1000 µg/mL
RANKL +
OVT 100 µg/mL
RANKL +
OVT 10 µg/mL
RANKL +
OVT 1 µg/mL

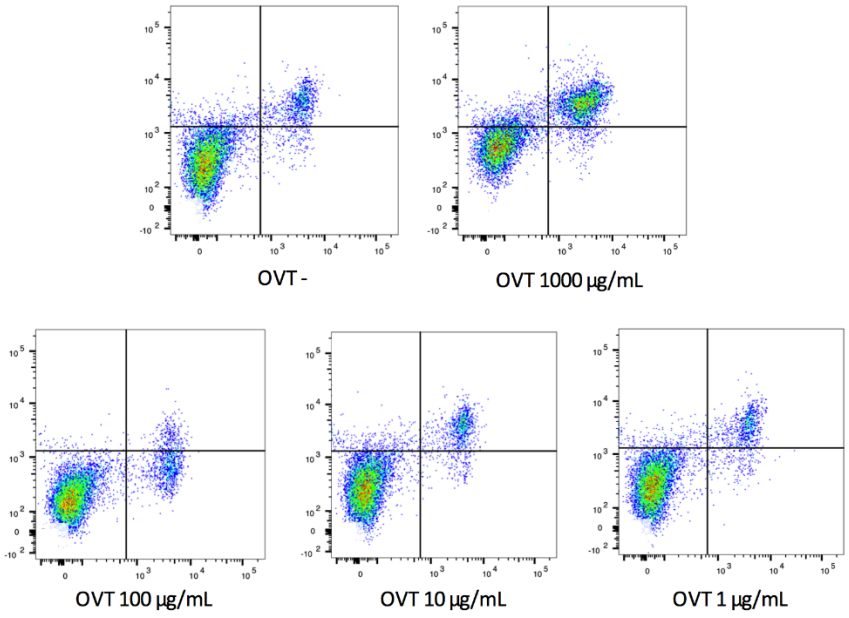
Figure 5.6 Effect of ovotransferrin on RANKL-induced osteoclasts apoptosis.

Osteoclasts were generated by stimulating RAW 264.7 with RANKL (100 ng/mL) for 4-6 days and multinucleated cells were identified by microscopy. Then cells were treated with ovotransferrin (1-1000 µg/mL) for 12 h. (A) The osteoclasts apoptosis percentage. (B) The osteoclasts apoptosis measured by flow cytometry. The lower left quadrand: PI-/V-FITC-; upper left quadrand: PI+/V-FITC-; lower right quadrand: PI-/V-FITC+; upper right quadrand: PI+/V-FITC+. (C) The gene expression of Bcl-2 family (*Bcl-2, Bcl-x, Bim, Bid*). Data were expressed as means ± SEM (n=4). Means without a common letter indicated $p<0.05$.* and ** indicated $p < 0.05$ and $p < 0.01$.

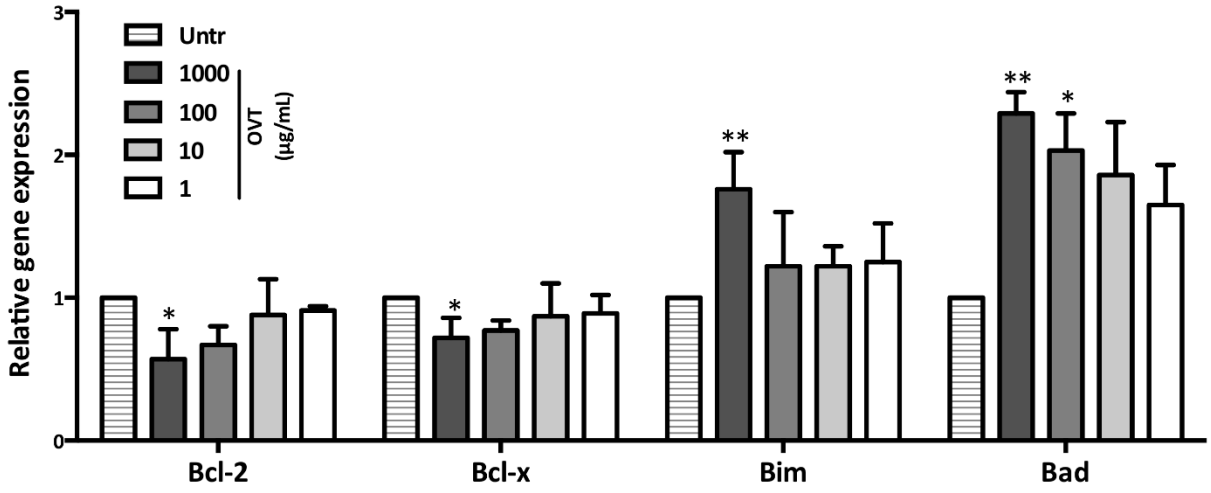
A



B



C


Relative gene expression
3
2
1
0
Untr
1000
100
10
1
OVT (µg/mL)
*
*
**
**
*
Bcl-2
Bcl-x
Bim
Bad

5.7 Supplementary data and information

Table 5.7.1 Primers designed for qPCR

Gene	**Primer Sequences (5'-3')**	
Bcl-2	Forward	TGAACCGGCATCTGCACAC
	Reverse	CGTCTTCAGAGACAGCCAGGAG
Bcl-x	Forward	GCTGGGACACTTTTGTGGAT
	Reverse	TGTCTGGTCACTTCCGACTG
Bax	Forward	ACCAGCTCTGAACAGATCATG
	Reverse	ACTTTAGTGCACAGGGCCTTG
Bim	Forward	CTTCCATACGACAGTCTC
	Reverse	AACCATTTGAGGGTGGTCTTC
β-actin	Forward	AGATGTGGATCAGCAAGCAG
	Reverse	GCGCAAGTTAGGTTTTGTCA

CHAPTER 6 – Oral Administration of Egg White Ovotransferrin Prevents Osteoporosis in Ovariectomized Rat

6.1 Introduction

Osteoporosis is a multifactorial systemic skeletal disease, characterized by decreased bone mineral density and micro-architectural deterioration of bone tissue, resulting in increased bone fragility and susceptibility to fracture (Tu et al., 2018). As an aging-related disease, osteoporosis is a major health and economic burden with the extension of average life span. Although both men and women will suffer from bone loss during their life, women are more susceptible to osteoporosis and osteoporotic fractures, especially postmenopausal women (Ji & Yu, 2015). Healthy bone requires continuous remodeling which is pivotal for bone density maintenance (Ji & Yu, 2015); however, the normal bone turnover cycle can be impaired and result in bone loss. Several factors are associated with osteoporosis such as decreased level of sex hormones (e.g. estrogen, testosterone) (de Villiers et al., 2013; Rahman, Bhattacharya, Banu, Kang, & Fernandes, 2009) and growth factors (e.g. insulin-like growth factor, fibroblast growth factor) (Fromigué, Modrowski, & Marie, 2004; Giustina, Mazziotti, & Canalis, 2008; Rosen, Donahue, & Hunter, 1994), drug side effects (e.g. glucocorticoid-induced osteoporosis) (Smith et al., 2000), lack of essential nutrients (e.g. mineral, vitamin) (Nieves, 2005), as well as acquired habit, such as smoking and alcohol intake (Wilsgaard et al., 2009).

Current osteoporosis therapies rely on either anti-resorptive agents like bisphosphonates, selective estrogen receptor modulator (SERMs), denosumab, or an anabolic agent like parathyroid hormone (PTH) analogs (Tu et al., 2018). However, these therapies are associated with side effects such as

gastrointestinal toxicity (Drake, Clarke, & Khosla, 2008), and increased risk of cancer, strokes and cardiovascular system diseases (Crockett & Das, 2013). The presence of numerous side effects have prompted further research to explore alternatives, especially food derived bioactive components.

In the recent decades, food-derived bioactive components are gaining the momentum towards the development of new therapies for osteoporosis prevention and treatment. The potential of soy isoflavones has been demonstrated in a number of studies (Arjmandi et al., 2005; Kenny et al., 2009; Messina & Messina, 2000). Evidence from epidemiologic studies also supported that dietary soy isoflavones attenuated menopause-induced osteoporotic bone loss by decreasing bone resorption and stimulating bone formation (Lauderdale et al., 1997; Silverman & Madison, 1988). Lactoferrin, an iron binding protein, has gained attention for its ability to prevent osteoporosis by stimulating bone formation while inhibiting bone resorption (Cornish et al., 2004; Grey, Zhu, Watson, Callon, & Cornish, 2006; Guo et al., 2009). Moreover, as a well-studied bioactive protein, lactoferrin is also able to benefit for bone health by promoting the differentiation of pluripotent mesenchymal cells into osteoblastic and chondroblastic lineage (Yagi et al., 2009) and inhibiting the production of inflammatory cytokines, such as IL-6 and TNF-α (Conneely, 2001), suggesting a multifunction of bioactive proteins in regulating bone health.

Egg white ovotransferrin, a 76 kDa iron-binding glycoprotein belonging to the transferrin family, has been found to offer similar capabilities as lactoferrin on bone health management (Shang & Wu, 2018; Wu & Acero-Lopez, 2012). Ovotransferrin not only potently stimulates the proliferation and differentiation of osteoblasts, but also inhibits osteoclastogenesis and resorption

activity. These results suggested the potential of ovotransferrin in preventing osteoporosis. Moreover, as a major functional protein in egg white, ovotransferrin has been widely studied for its multiple biological functions such as immunomodulatory, anti-inflammatory activity and antioxidative activity (Wu & Acero-Lopez, 2012), which may also play important roles in maintaining bone integrity. Although orally administered proteins are hydrolyzed in the gastrointestinal tract and thus may lose their biological activity, oral uptake of ovotransferrin has been found to exhibit similar physiologic effects *in vivo* as displayed *in vitro*, such as its anti-inflammatory activity (Kobayashi et al., 2015). However, all our previous studies were conducted in experiments that allowed direct contact of ovotransferrin with bone cells. To the best of our knowledge, the efficacy of orally administered ovotransferrin on bone health has yet to be examined.

The *in vivo* effects of egg white ovotransferrin on preventing menopausal-induced osteoporosis were investigated using ovariectomized (OVX) rats. The objectives of this work were 1) to evaluate the safety (based on organ measurements) and effects of ovotransferrin treatment on osteoporosis prevention by measuring bone mineral density and micro-architecture; 2) to investigate the regulatory role of ovotransferrin on bone metabolism by measuring the expression of bone formation/resorption biomarkers (representing the bone remodeling process), and the production of osteoclastogenesis associated cytokines; 3) to investigate the auxiliary functions of ovotransferrin on bone health management including the regulatory role on adipogenesis, phenotypes of immune cells in bone marrow cells (representing local immunity) and spleen (representing systemic immunity), and short chain fatty acid production (representing gut microbiota).

6.2 Materials and methods

6.2.1 Chemicals

Alendronate sodium was purchased from Sigma-Aldrich (Oakville, ON, Canada) and diluted in water for injection. Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), bovine serum albumin (BSA) and penicillin-streptomycin were purchased from Sigma-Aldrich (Oakville, ON, Canada). The Krebs-Ringer-HEPES (KRH) buffer and ACK lysis buffer was prepared according to the previous study (Gars et al., 2018). Fluorescent pre-labeled monoclonal antibodies CD3, CD4, CD80, CD28 were purchased from eBioscience (San Diego, CA, USA), antibodies CD8, CD25, CD71, OX62, CD11b/c, CD51 were purchased from Biolegend (Vindeland, ON, Canada), and antibodies RANK, OX12 and OX6 were purchased from BD Biosciences Pharmingen (Mississauga, ON, Canada). All primers were synthesized by Invitrogen (Burlington, ON, Canada) and the sequences are shown in Table 6.7.4. AIN-93M purified animal diets and casein were purchased from Envigo (Madison, WI, USA). The nutritional characteristics of experimental diets is shown in Table 6.7.5.

6.2.2 Experimental protocol

This experiment protocol was approved by the University of Alberta Animal Welfare Committee (Protocol AUP00001960) in accordance with the guidelines issued by the Canadian Council on Animal Care and also adhered to the Guide for the Care and Use of Laboratory Animals published by the United States National Institutes of Health. The experiment was carried out using 12-week-old virgin female Sprague-Dawley rats weighing 292 ± 20 g obtained from Charles River (Senneville, QC, Canada), in which 40 rats were ovariectomized (OVX) and 8 rats were sham-operated (Sham). Upon arrival, the total of 48 rats were housed at University of Alberta animal facility with a 12:12 h cycle of light: dark in a humidity and temperature controlled (60% RH, and

23°C) environment and maintained on diet and water *ad libitum*. In addition to the sham group (n=8), another 40 OVX rats were randomly divided into 5 dietary groups (n=8/group); 1) negative control (NC), 2) positive control (PC, injected alendronate sodium 3 times/week), 3) high ovotransferrin treatment (HOvt, a normal diet supplemented with 1% w/w ovotransferrin), 4) medium ovotransferrin treatment (MOvt, a normal diet supplemented with 0.2% w/w ovotransferrin and 0.8% w/w casein), 5) low ovotransferrin treatment (LOvt, a normal diet supplemented with 0.04% w/w ovotransferrin and 0.96% w/w casein). The doses of ovotransferrin were designed according to the published literatures (Blais, Malet, Mikogami, Martin-Rouas, & Tome, 2009; Guo et al., 2009; Hou, Xue, & Lin, 2012; Malet et al., 2011; Yoshimaki et al., 2013). Sham, NC and PC groups were provided with a normal diet supplemented with 1% w/w casein to exclude the nutritional effect of ovotransferrin as a protein. After 12 weeks, rats were sacrificed and tissues were collected. The diagram of an experimental protocol is shown in Fig. 1.

6.2.3 Sample collection

Rats were weighed every 2 days and food intake was measured 3 times per week. Serum and fecal samples were collected every 4 weeks and stored at -80°C. After 12 weeks of treatment, the rats were placed in metabolic cage for urine collection. During sacrifice, the serum was collected immediately after euthanasia. Tissues were removed, weighted and stored at -80°C until analysis.

6.2.4 Micro-computerized tomography analysis and bone mineral density determination

The right tibia was measured by micro-computerized tomography analysis (micro-CT) using Skyscan 1076 (Kontich BE, Belgium) at 18μm resolution with 70kV, 100 μA with a 1.0mm Aluminum filter. Briefly, inhalant isofluorane was used to induce animal anaesthesia by gradually increasing the concentration of isofluorane from 0.5% to 2% in 100% oxygen. Then animal was placed on the bed of micro-CT scanner face down and the right leg was straightened and

immobilized with tapes. Gently pushed the bed into the scanner and performed a Scout Scan of the animal to get the images of tibia. Projected images were reconstructed using vendor-supplied software Nrecon 1.6.1.5 (SkyScan NV, Kontich BE) with images oriented in axial plane of section. A circular region of interest (ROI) relative to the size of the tibia bone was used to sample a standardized amount of trabecular bone in the vertebral body in order to conduct standard histomorphometric analysis. The tibia growth plate was utilized as anatomical landmarks in order to consistently segment and sample the same region of trabecular bone from all samples. Vendor morphometric software CTAn 1.10.0.1 (SkyScan, Kontich BE) was then used to analyze tibia trabecular microarchitecture parameters (percentage bone volume, trabecular thickness, trabecular numbers and trabecular separation) and tissue mineral density against the vendor-supplied bone phantoms of known mineral density (0.25g/cm^3 and 0.75g/cm^3) to calibrate BMD values from Hounsfield units.

6.2.5 Bone biochemical markers and cytokine measurement

Serum and urinary calcium were measured in triplicate with a calcium assay kit (Abcam, Toronto, ON, Canada) and urinary creatinine was measured in triplicate with a creatinine assay kit (Abcam, Toronto, ON, Canada). Serum osteocalcin, parathyroid hormone (PTH), and urinary pyridinoline (PYD) were measured in duplicate with ELISA kit (Quidel, San Diego, CA, U.S.A.). Serum bone alkaline phosphatase (BALP) and procollagen I intact N-terminal (PINP) were measured in duplicate with ELISA kit (Abbexa, Houston, TA, U.S.A.). Serum TNFα and IL-6 were measured in triplicate with ELISA kit (Abcam, Toronto, ON, Canada).

6.2.6 Isolation of bone marrow cells

Bone marrow was harvested by inserting a syringe needle (23 gauge) into one end of the femur and flushed with DMEM containing 10% FBS and 5% pen-strep. Marrow cells were centrifuged

and treated with ACK lysis buffer for 5 min at room temperature, then washed and centrifuged. Cells were re-suspended in fresh DMEM and the total counting was determined with a haemocytometer. The extracted bone marrow cells were used for immune cells phenotype determination and osteoclast differentiation. Leftover cell pellets were transferred to 1.5 mL microcentrifuge tubes, rinsed with PBS and storage at -80°C for qRT-PCR and western blot analysis.

6.2.7 Isolation of spleen cells

At necropsy, spleens were removed and placed in sterile Krebs-Ringer-HEPES (KRH) buffer (pH 7.4) supplemented with 0.5% (w/v) BSA. Spleen cells were extracted by push spleen through the cell strainers (40 μm) using barrel of sterile syringe. Isolated spleen cells were treated with ACK lysis buffer to remove excess red blood cells for 5 min on ice. Cells were washed and re-suspended in the KRH buffer and counted on a haemocytometer. The extracted spleen cells were used for phenotype determination.

6.2.8 Bone marrow cells and spleen cells phenotype analysis

Freshly isolated immune cells from both bone marrow and spleen were analyzed using a direct four-color labelled immunofluorescence assay. Cells were incubated with pre-labelled monoclonal antibodies to identify and quantify the phenotypes. The 96 well V-bottom plate was pre-coated with 200 μL of 4% FBS in PBS for 30 min at room temperature. 20-40 μL (1×10^5~4×10^5 cells/well) bone marrow cells or spleen cells were added into V-bottom plate and stained with antibodies according to manufacturer's recommendations. The antibody combinations are shown in Table 6.7.5. After 30 min incubation in dark, cells were fixed in 4% paraformaldehyde, and the proportion of positive cells for each marker was determined according to the relative fluorescence

intensity by flow cytometry Canto II (BD Biosciences, Mississauga, ON, Canada) using the FlowJo software (FlowJo LLC, Ashland, OR, USA).

6.2.9 Osteoclast differentiation and resorptive activity analysis

Bone marrow cells were seeded in 24-well plate at a density of $1\times10^4 \sim 5\times10^4$ cells/well and cultured with DMEM containing 10% FBS, 5% pen-strep and 10 ng/mL M-CSF for 2 days. Then, the culture medium was replaced with DMEM containing 10 ng/mL M-CSF and 100 ng/ml RANKL to stimulate osteoclastogenesis. Osteoclasts were successfully differentiated by incubation with M-CSF and RANKL after 4 days. TRAP-staining kit was used to confirm and count the multinucleated osteoclast-like cells according to the manufacturer's instruction. Simply, cells were fixed in 4% paraformaldehyde for 1 h in 4°C and then stained with TRAP staining solution (0.1mg/mL naphthol AS-MX phosphate and 0.3 mg/mL Fast Red Violet LB staining). Cells were observed with a light microscope under the 10×lense (Olympus IX83, Richmond Hill, ON, Canada) and images were captured by Metamorphy (Olympus, Richmond Hill, ON, Canada). Any TRAP-positive multinucleated cells containing three or more nuclei were counted.

6.2.10 RNA extraction and qRT-PCR

Total RNA was extracted from the cells using TRIzol® reagent following the manufacturer's instruction, and cDNA was synthesized using SuperScript II Reverse Transcriptase (Invitrogen, Burlington, ON, Canada) according to the manufacturer's instruction. Real-time PCR was performed using Fast SYBR Green PCR Master Mix (Applied Biosystem, Burlington, ON, Canada) and an ABI 7300 Sequencing Detection System (Applied Biosystem, Burlington, ON, Canada). The amplification conditions were as follow: initial denaturation at 95°C for 10 min, followed by 35 cycles of 10 s at 95°C, 15 s at 60°C, and 10 s at 72°C.

6.2.11 Western blot

Whole-cell protein extracts were prepared from the bone marrow cells. The cells were lysed in boiling hot Laemmle's buffer containing 50 μM dithiothreitol (DTT) and 0.2% Triton-X-100 to prepare samples for western blot as described previously. The cell lysates were run in SDS-PAGE, blotted to nitrocellulose membranes and immunoblotted with antibodies. The concentration of each antibody was determined according to the manufacturer's instruction. The protein bands were detected by a Licor Odyssey BioImager and quantified by densitometry using corresponding software (Licor Biosciences, Lincoln, NB, USA). Each band was normalized to its corresponding band of a loading control. The results were expressed as percentage of the corresponding negative control or untreated control.

6.2.12 Short chain fatty acid analysis

Three to four replicates of frozen fecal samples (50 mg) were weighed into a 2 mL polypropylene tube and dissolved in 25% phosphoric acid. After centrifugation, 0.8 mL of supernatant sample was added a 1.8 mL GC vial, mixed 0.2 mL of 25% phosphoric acid, and 0.2 mL of internal standard solution. Standards were prepared by combining 1 mL of standard solution and 0.2 mL of internal standard solution (23.253 μmol/mL isocaproic acid). For GC analysis, 1 μL of the sample was injected to 1177 Split/Splitless injector or 0.2 μL for 1079 PVT injector on Stabliwax-DA meter, 0.53 mm ID, 0.5 μm df (Restek Corporation, Bellefonte, PA, USA). Injection temperature and detector temperature were 250°C. Helium was used as carrier gas at a constant flow rate of 20 mL/min.

6.2.13 Statistical analysis

Data are presented as mean±SEM (standard error of mean) of between 4 and 7 independent experiments. Data were analyzed using Two Way Analysis of Variance (ANOVA) with Tukey's

multiple comparisons test or One Way Analysis of Variance (ANOVA) with Dunnett's post-hoc comparisons test. The PRISM 6 statistical software (GraphPad Software, San Diego, CA) was used for the analyses. $P < 0.05$ was considered significant.

6.3 Results

6.3.1 Ovotransferrin showed no significant effects on body weight, food intake and tissue weight

A diagram of the experimental design is shown in Figure 6.1. All rats had increased body weight by the end of the experiment (Figure 6.2A). OVX rats gained significantly higher weight than that of the Sham rats. High dose ovotransferrin treatment prevented the OVX-induced weight gain. However, medium dosage and low dosage had no such influence on the body weight (Table 6.7.1). As expected, OVX caused atrophy of uterine tissue, indicating the success of the surgical procedure (Figure 6.2C and Table 6.7.2). However, treatment with ovotransferrin and drug alendronate sodium had no influence on the uterine weight. No significant differences in food intake were observed in different dietary groups (Figure 6.2B). Tissue weight was not affected by the treatments (Table 6.7.2)

6.3.2 Ovotransferrin administration prevented the decrease of bone mineral density in OVX rats

OVX rats successfully developed osteoporosis with a significant decrease in tibia trabecular bone mineral density with increasing time (Figure 6.3A), but did not affect the mineral density in tibia cortical bone (Fig. 6.3C, D). Over 12-week treatment, both PC and HOvt groups significantly retarded the decline of trabecular bone mineral density compared to negative control (NC) group

(Figure 6.3A, B), although the bone mineral density value was still less than Sham group. OVX rats supplemented with casein (NC) did not counteract the OVX-induced bone mineral density loss, suggesting that the protection role of ovotransferrin is not due to the nutritional role of food protein. OVX rats treated with medium dose and low dose of ovotransferrin did not show prominent effects on preventing BMD loss (Figure 6.3B).

6.3.3 Ovotransferrin administration preserved bone micro-architecture in OVX rats

In addition to improving bone mineral density, ovotransferrin also prevented the deterioration of bone micro-architecture in tibia trabecular bones caused by ovariectomy. Ovariectomy markedly reduced the percent bone volume, trabecular thickness, trabecular number, and increased the trabecular separation on tibia compare to the Sham rats (Figure 6.4A-D), indicating a serious damage to bone microstructure and integrity. Ovotransferrin administration significantly preserved the OVX-induced decrease of percent bone volume, trabecular thickness, and trabecular number, but not the elevation of trabecular separation (Figure 6.4A-D). Similar results were also observed in drug injection group (PC). Direct evidences were observed in the reconstructed three-dimensional trabecular images (Figure 6.4E). The plate-like structure mostly resolved into a rod-like structure, with lots of the connecting rods missing in OVX rats (NC), whereas in the ovotransferrin group, this loss of trabecular bone mass and connectivity was prevented (Figure 6.4E). Microstructure, which deteriorated substantially after OVX, was markedly improved by ovotransferrin.

6.3.4 Ovotransferrin administration regulated bone formation and bone resorption in OVX rats

To investigate the regulatory role of ovotransferrin on bone remodeling, serum and urine biomarkers, which indicate bone formation and bone resorption, were measured (Table 6.1). Serum calcium (Ca^{2+}) was not affected, while the urinary Ca^{2+} was significantly increased in OVX rats compared to the Sham group. Interestingly, ovotransferrin administration attenuated the increase of urinary Ca^{2+} in OVX rats without affecting serum Ca^{2+}. Additionally, the concentrations of serum bone alkaline phosphatase (BALP) and urinary pyridinoline (PYD), the markers of bone formation and bone resorption respectively, were higher in the OVX rats (NC) than those of the Sham rats. Due to the interplay between bone resorption and formation, the exceeding bone resorption caused by OVX will also lead to a stimulation of bone formation correspondingly, but this stimulation is trivial. Both ovotransferrin administration and drug injection (PC) showed a suppression in serum BALP and urinary PYD, suggesting the inhibitory effects on OVX-induced overactive bone turnover and therefore help rehabilitate normal bone remodeling. The effect of ovotransferrin on preventing bone resorption was also evidenced by a decreased serum osteocalcin level after ovotransferrin administration. Although osteocalcin is regarded as a maker of bone formation, when bone resorbed, osteocalcin fragments are released, and the serum concentration of osteocalcin fragments reflect bone resorption (Cundy, Reid, & Grey, 2014). Generally, there is a two-fold rise in plasma osteocalcin concentration at the menopause, consistent with our results. Serum N-terminal propeptide of type I collagen (PINP), a biomarker indicating collagen synthesis, was also evaluated as an indicator of bone formation. As expected, OVX showed significant inhibition in serum PINP, while ovotransferrin administration elevated the concentration. Serum PTH level in high dose ovotransferrin-treated OVX rats were

dramatically increased than other groups. PTH is the only available anabolic drug used to stimulate bone formation in osteoporosis patients. The increased serum PTH level with ovotransferrin treatment may also suggest the possible ability of ovotransferrin in promoting bone formation.

6.3.5 Ovotransferrin inhibited the production of serum cytokines in OVX rats

Bone resorption is tightly regulated by multiple cytokines, especially TNFα and IL-6, which could stimulate osteoclasts differentiation (Azuma, Kaji, Katogi, Takeshita, & Kudo, 2000; Wu, Zhou, Huang, Ji, & Kang, 2017). In our study, serum TNFα was markedly greater in the negative control group than in Sham group (Figure 6.5A). With 12-week of ovotransferrin administration, the serum TNFα level did not show significant increase compared to the Sham group, indicating that ovotransferrin is able to inhibit ovariectomy-caused high TNFα producing. Interestingly, ovariectomy did not cause a significant increase of IL-6. The serum IL-6 concentrations were similar in negative control (NC) and Sham groups; however, both high dose ovotransferrin treatment and drug intervention led to a lower serum IL-6 level (Figure 6.5B).

6.3.6 Osteoclast precursors derived from ovotransferrin-administrated OVX rats showed less differentiation ability and resorption activity

To examine the effect of ovotransferrin on the differentiation ability of osteoclast precursors, bone marrow cells extracted from different animal groups were cultured in osteoclastogenic medium and the differentiated osteoclasts were counted with TRAP staining. After 6 days of incubation, osteoclasts were successfully differentiated from bone marrow cells (Figure 6.6A). A greater number of osteoclasts were generated from bone marrow cells extracted from negative control group (NC), compared to the Sham group (Figure 6.6A, B). Importantly, bone marrow cells

extracted from the ovotransferrin administration groups showed less mature osteoclasts, especially in the high dose ovotransferrin group. Similar results were observed in drug injection group (PC). The absorption activity of differentiated osteoclasts was investigated using a calcium phosphate (CaP)-coated plate. Ovotransferrin administration not only reduced the number of differentiated osteoclasts, but also showed less absorptive activity compared to the negative group (NC) (Figure 6.6C-E). Compared with the Sham rats, OVX rats showed higher osteoclastic resorption; whereas, ovotransferrin administration attenuated OVX-induced excessive osteoclastic resorption.

To have a better understanding on the effects of ovotransferrin on bone marrow microenvironment, the expression of cathepsin K (CathK) and matrix metalloproteinase 9 (MMP9) were measured in bone marrow cells. Bone marrow CathK and MMP9 expression has been reported to replicate in the regulation of bone metastasis, suggesting the negative association between high expression of CathK, MMP9 and bone health (Ohshiba, Miyaura, Inada, & Ito, 2003; Podgorski et al., 2009). Ovariectomy significantly increased the expression of CathK and MMP9 compared to the Sham group, while both ovotransferrin administration and drug injection reduced their expression (Figure 6.7); only the HOvt group significantly decreased both CathK and MMP9 expression.

6.3.7 Ovotransferrin inhibited osteoclastogenesis by inhibiting the NF-κB pathway

In a previous *in vitro* study, ovotransferrin showed prevention on RANKL-induced osteoclastogenesis in RAW 264.7 cell by inhibiting NF-κB pathway (Chapter 5, this thesis). This study confirmed this finding with primary osteoclast precursors extracted from bone marrow. Osteoclasts were generated and treated with different concentration of ovotransferrin (1000-10mg/mL). Similar to a previous study, ovotransferrin treatment significantly inhibited the

expression of NF-κB p65 and IκBα (Figure 6.8), which are two most important components involved in the NF-κB pathway.

6.3.8 Ovotransferrin administration exerted a global regulatory effect in OVX rats

Postmenopausal leads to various health issues in addition to osteoporosis, for example obesity and inflammation. Recent studies have demonstrated an interlinked traid among postmenopausal osteoporosis, obesity and sarcopenia (Vaidya, 2014). Thus, to have a comprehensive understanding on the beneficial effects of ovotransferrin in OVX rats, the influences on adipogenesis (Figure 6.7.1), immune modulation (Figure 6.7.2, Table 6.7.3, Table 6.7.4) and short-chain fatty acid (Figure 6.7.3, Table 6.7.5) production with ovotransferrin supplementation were investigated (all results were shown on 6.7 Supplementary Data and Information)

For adipogenesis study, we investigated the expression of several major genes and proteins involved in adipogenesis. qRT-PCR results showed that ovariectomy significantly increased the expression of peroxisome proliferator-activated receptor γ (*PPARγ*), CCAAT-enhancer-binding protein α (*C/EBPα*), *perilipin* and adipocyte protein 2 (*aP2*) gene, indicating an activation of adipogenesis in estrogen deficiency situation (Figure 6.7.1A). Ovotransferrin administration significantly inhibited the gene expression of *PPARγ* and *C/EBPα*, but not *perilipin* and *aP2* (Figure 6.7.1A). The protein expression of PPARγ and C/EBPα was measured by western blot (Figure 6.7.1B, C). Increased protein expression of PPARγ and C/EBPα were observed in OVX rats, but was attenuated at the HOvt and PC groups.

For immune modulation study, we investigated the effect of ovotransferrin administration on immune alternation, including both local bone marrow cells and systemic splenocytes. Compared with the Sham group, OVX did not cause a significant change in spleenocyte phenotypes (Table

6.7.3). Meanwhile, ovotransferrin administration did not affect immune cell populations significantly either, suggesting a lack of effect on the systemic immune system (Table 6.7.3). The significant observations were made in the bone marrow compartment. Although the number of T-cells in bone marrow was relatively low, these cells are believed to play important roles in the local regulation of bone turnover. In general, ovariectomy caused relatively remarkable changes in both T-cells and B-cells in bone marrow compared to in spleen (Figure 6.7.2 and Table 6.7.4). The percentage of total T cells (CD3+) and T cytotoxic cells (CD8+) in bone marrow were significantly decreased after ovariectomy, while ovotransferrin administration attenuated this decrease (Figure 6.7.2A, B). Meanwhile, the population of T-cell subsets, including T-cell express IL-2 receptor (CD25+), T-cell express co-stimulatory factors (CD28+), T-cell express anti-integrin alpha (CD51+), dendritic cells (OX62+), and B-cells (OX12+) was increased in OVX rats, while those of CD25+, CD28+ and OX12+ cells were suppressed upon ovotransferrin administration (Figure 6.7.2C, D, H). Inspiring findings were observed on the population changes of monocytes/macrophages (CD11b/c+) and RANK+ cells, which are the precursor cells of osteoclasts. The population of CD11b/c+ and RANK+ cells were significantly increased in OVX rats, while high dose ovotransferrin treatment reduced them notably (Figure 6.7.2G, H). These results are consistent with our previous finding that fewer osteoclasts were differentiated from bone marrow cells derived from ovotransferrin treatment groups (Figure 6.6).

In this study, the concentrations of SCFAs were measured in fecal samples. Interestingly, 12-week of ovotransferrin administration significantly increased the fecal SCFAs content in OVX rats, including acetic acid, proprionic acid, isobutyric acid, butyric acid, isovaleric acid, valeric acid, and caproic acid (Figure 6.7.3 and Table 6.7.5). Although it is not known the contribution of these findings to bone outcomes, these results may help to explain the functions of ovotransferrin in an

overall perspective and provide a broader beneficial effect of ovotransferrin in postmenopausal women.

6.4 Discussion

The effect of egg white protein ovotransferrin on osteoporosis prevention of OVX rats was studied in this study. OVX rat is a well-accepted osteoporosis model that mimics the estrogen deficiency-induced osteoporosis in postmenopausal women. In current study, adding ovotransferrin up to 1% (w/w) did not affect food intake, weight gain, and tissue weight significantly, suggesting the potential of ovotransferrin to be developed as a functional food ingredient. Our study provides direct evidence that ovotransferrin can acts as an orally active agent to preserving bone mass and micro-architecture in a growing OVX rat model (aged 3 to 6 months). Our findings indicate that ovotransferrin at a dose of 1% (w/w) could attenuate estrogen-dependent bone loss and micro-architecture deterioration in OVX rats. The mechanical properties of bone are influenced by bone mass as well as bone size, bone quality, and bone micro-architecture. In this study, ovotransferrin treatment resulted in an increase of percent bone volume consistent with the improvement in bone mineral density, suggesting ovotransferrin could prevent the loss of bone mass after ovariectomy. Ovotransferrin administration improved trabecular architecture as it increases trabecular architecture. In addition, ovotransferrin appears to partially restore trabecular connectivity by increasing trabecular numbers. Ovotransferrin showed more effective protection against trabecular loss. Our findings suggest that ovotransferrin increases both the bone mineral density and microarchitectures, therefore, has the potential to be used as a treatment of osteoporosis.

To address the mechanisms involved, several bone remodeling biomarkers including BALP, PINP, PYD and PTH were measured. After ovariectomy, bone formation markers BALP and osteocalcin were elevated with a commensurate increase of the bone resorption marker PYD. This could be interpreted as a coupling between bone resorption and formation (Han & Wang, 2017; Sims, Morris, Moore, & Durbridge, 1996; Yokose et al., 1996). Accelerated bone resorption also leads to an trivial stimulation on bone formation. Thus, the process is dominated by bone resorption and results in bone loss. Administration of ovotransferrin, on the other hand, reduced BALP, osteocalcin and PYD, which suggested the possible effects of ovotransferrin on adjusting the imbalance of bone remodeling. Moreover, ovotransferrin may function as a stimulator on bone formation as indicated by a substantial increase in collagen synthesis (PINP) after ovotransferrin administration. Furthermore, we measured two osteoclastogenic cytokines and found that ovotransferrin down regulated the production of serum TNFα and IL-6 under the experimental conditions. TNFα, along with IL-6, plays a critical causal role in the rapid bone loss associated with estrogen deficiency (Riggs, 2000). They promote bone resorption by affecting osteoclast differentiation and activity. Ovotransferrin was suggested as an innate immunity of laying hen eggs (Hallquist & Klasing, 1994); ovotransferrin and its cationic fragment OTAP-92 could enhance intestinal and systemic immune response (Levy, Bulet, & Ehret-Sabatier, 2004; Zhu et al., 2018). Meanwhile, ovotransferrin hydrolysates and ovotransferrin-derived peptide showed anti-inflammatory activity by inhibiting the production of inflammatory cytokines, such as TNFα and IL-6 (Huang et al., 2010; Liao, Chakrabarti, Davidge, & Wu, 2016; Liu et al., 2017). Our results suggested that ovotransferrin might inhibit bone resorption as an anti-inflammatory agent, thus improving bone health.

A further study on the effect of ovotransferrin on osteoclasts formation and resorption was investigated using freshly extracted bone marrow cells. Under the same culture condition, bone marrow cells derived from OVX rats developed more osteoclasts compared to the Sham rats, indicating estrogen deficiency could activate osteoclastogenesis and promote bone loss in OVX rats. But oral administration of ovotransferrin significantly ameliorated the osteoclast formation. Furthermore, ovotransferrin treatment also prevented OVX-induced excessive osteoclastic resorption. These might be partly due to the effects of ovotransferrin on regulating osteoclast precursors in bone marrow. In our study, ovotransferrin treatment significantly suppressed the expression of CathK and MMP9 in bone marrow cells. CathK is the key enzyme responsible for osteolysis of bone, which is also the only known mammalian protease capable of degrading both helical and non-helical regions of type I collagen (Garnero et al., 2000). Overexpression of CathK has been demonstrated to result in accelerated bone turnover (Kiviranta et al., 2001; Saftig et al., 2002). Similarly, MMPs are a group of proteolytic enzymes involved in the degradation of extracellular matrix of various tissues including bone (Sundaram et al., 2007). Among all, MMP9 is highly expressed at early stages of osteoclast development and in mature osteoclasts (Reponen, Sahlberg, Munaut, Thesleff, & Tryggvason, 1994). Increased MMP9 activity has been detected in human osteoclastomas and osteoclasts in Paget's disease (Wucherpfennig, Li, Stetler-Stevenson, Rosenberg, & Stashenko, 2009). It has also been shown that upregulation of MMP9 plays an important role in the pathogenesis of dental pulp inflammation and destruction (Tsai, Chen, Huang, Su, & Chang, 2005). Cytokines such as IL-1, TNFα and granulocyte macrophage colony-stimulating factor (GM-CSF) have been shown to induce the upregulation of MMP9 and enhance the activity of MMP9, therefore contributing to bone degradation (Kusano et al., 1998; Sundaram et al., 2007; Zhou, Zhang, Ardans, & Wahl, 2003). Thus, the suppression of CathK and MMP9

expression in bone marrow cells might contribute to reduce osteoclastic bone resorption by preventing osteoclast formation and activity in OVX rats.

In the previous study, ovotransferrin was able to inhibit RANKL-induced osteoclastogenesis by regulating the activation of NF-κB pathway (Chapter 3). NF-κB is one of the important signaling pathways activated by the binding of RANKL to RANK. This binding triggers the translocation of NF-κB p65 into nucleus and foster the transcription of genes involved in osteoclast differentiation (Thummuri, Naidu, & Chaudhari, 2017). Using osteoclasts differentiated from bone marrow cells, ovotransferrin significantly inhibited RANKL-induced expression of NF-κB p65 and IκBα, indicating the involvement of NF-κB pathway in suppressing osteoclastogenesis. The inactivation of NF-κB has been reported interrupt osteoclastogenesis and osteoclasts function (Kim, Oh, & Lee, 2017). A2B adenosine receptor (A2BAR), a regulator of bone homeostasis, showed inhibition of NF-κB activation and suppressed the induction of osteoclast markers, including CathK and MMP9, which contribute to the decrease in osteoclast cell-cell fusion and bone resorption activity (Kim et al., 2017).

In addition to the direct effect on bone tissue and bone cells, our study also found an inhibition effect of ovotransferrin on adipogenesis in bone marrow cells. Osteoblasts and adipocytes originate from a common precursor, MSCs; preserving bone tissue requires adequate osteoblastic differentiation while minimizing adipogenesis (Pino, Miranda, Figueroa, Rodríguez, & Rosen, 2016). Increased bone marrow fat content has been demonstrated in osteoporotic individuals (Justesen et al., 2001; Meunier, Aaron, Edouard, & Vignon, 1971). Marrow adipogenesis promoted OVX-induced osteoporosis (Moerman, Teng, Lipschitz, & Lecka-Czernik, 2004; Zayzafoon,

Gathings, & McDonald, 2004). Meanwhile, the coupling between activation of adipogenic process and suppression of osteogenesis in mesenchymal marrow stroma/stem cells has also been revealed (Moerman et al., 2004). In our study, elevated expression of PPARγ and C/EBPα, two master transcription factors for adipogenic differentiation, was observed in OVX rats, and was down-regulated after ovotransferrin administration. Although more work is needed to further validate and reveal the association between adipogenesis and osteoporosis prevention upon ovotransferrin administration, this finding suggested a new therapeutic target to develop ovotransferrin as an anti-osteoporosis agent by inhibiting adipogenesis.

Over last several years, tight molecular and cellular links between immune activation and bone loss have been identified (Horowitz, Bothwell, Hesslein, Pflugh, & Schatz, 2005; Walsh et al., 2006). Evidences strongly proposed that immune system is involved in the pathogenesis of estrogen deficient osteoporosis (Safadi et al., 2000). Here, we examined the phenotypic response of immune cells in spleen and bone marrow after treated with ovotransferrin. Although the phenotype changes were observed with ovotransferrin administration, especially in bone marrow (e.g. CD3+, CD8+ and OX12+ population), it is not clear what role, if any, these changes plays in the local regulation of bone metabolism and hematopoiesis. Studies have demonstrated the involvement of CD8+ T-cells and OX12+ B-cells in regulating osteoclast differentiation by modulating the cytokines; however, the results still endure a lot of discussion and controversy (Breuil et al., 2010; John, Hock, Short, Glasebrook, & Galvin, 1996; Masuzawa et al., 1994; Onoe et al., 2010; Redoglia et al., 1990). Thus, further experiments should be conducted to explore the relationship between ovotransferrin-influenced alternations of immune system, especially in the local bone marrow microenvironment, and ovotransferrin-treatment in OVX rats.

As a food-derived compound, the role of ovotransferrin as a nutrient should be considered. Several revealing reports have highlighted the link between diet, gut microbiota and bone health (Lucas et al., 2018). High-fiber diet and its main metabolites after microbial fermentation, short-chain fatty acids (SCFAs), significantly increases bone mass and prevents postmenopausal and inflammation-induced bone loss (Lucas et al., 2018). Here we found an increase of fecal SCFA content in ovotransferrin treated OVX rats, especially acetic acid and proprionic acid. Furthermore, the concentration of isobutyric acid, isovaleric acid, valeric acid and caproic acid were significantly increased compare to the OVX rats without ovotransferrin supplementation. Although the main sources of SCFA are carbohydrates, but amino acids valine, leucine, and isoleucine obtained from protein breakdown can be converted into isobutyrate, isovalerate, and 2-methyl butyrate, known as branched-chain SCFA (Ríos-Covián et al., 2016). Thus, ovotransferrin or its derivatives after gastrointestinal digestion may benefit bone health by stimulating SCFAs production.

6.5 Conclusion

In summary, for the first time we reported the *in vivo* effects of ovotransferrin to treat osteoporosis in OVX rats. Rats supplemented with 1% w/w and 0.2% w/w of ovotransferrin significantly preserved OVX-induced loss of bone mineral density and deterioration of trabecular micro-architecture. Additionally, ovotransferrin administration suppressed the overactive bone remodeling by inhibiting osteoclastogenesis and osteoclastic bone resorption, while exerting considerable positive influence in bone formation. Ovotransferrin also inhibited marrow adipogenesis, modulated immune function (both systemic and local), and enhanced the formation of short-chain fatty acids in the gut. It is not known the contributions of these findings to bone

outcomes, which warrant further studies. Above all, these results suggested a possible application of ovotransferrin on osteoporosis treatment in postmenopausal women.

6.6 Reference

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Figure 6.1 The experimental design.

Rats were separated into 6 groups providing different diet for 12 weeks. Drug was injected in positive control group. NC: negative control (OVX rats without OVT supplementation); PC: positive control (OVX rats with Alendronate injection); HOvt: high ovotransferrin treatment (OVX rats supplied with 1% OVT); MOvt: medium ovotransferrin treatment (OVX rats supplied with 0.2% OVT); LOvt: low ovotransferrin treatment (OVX rats supplied with 0.04% OVT); OVX: ovariectomized; OVT: ovotransferrin; W: week.

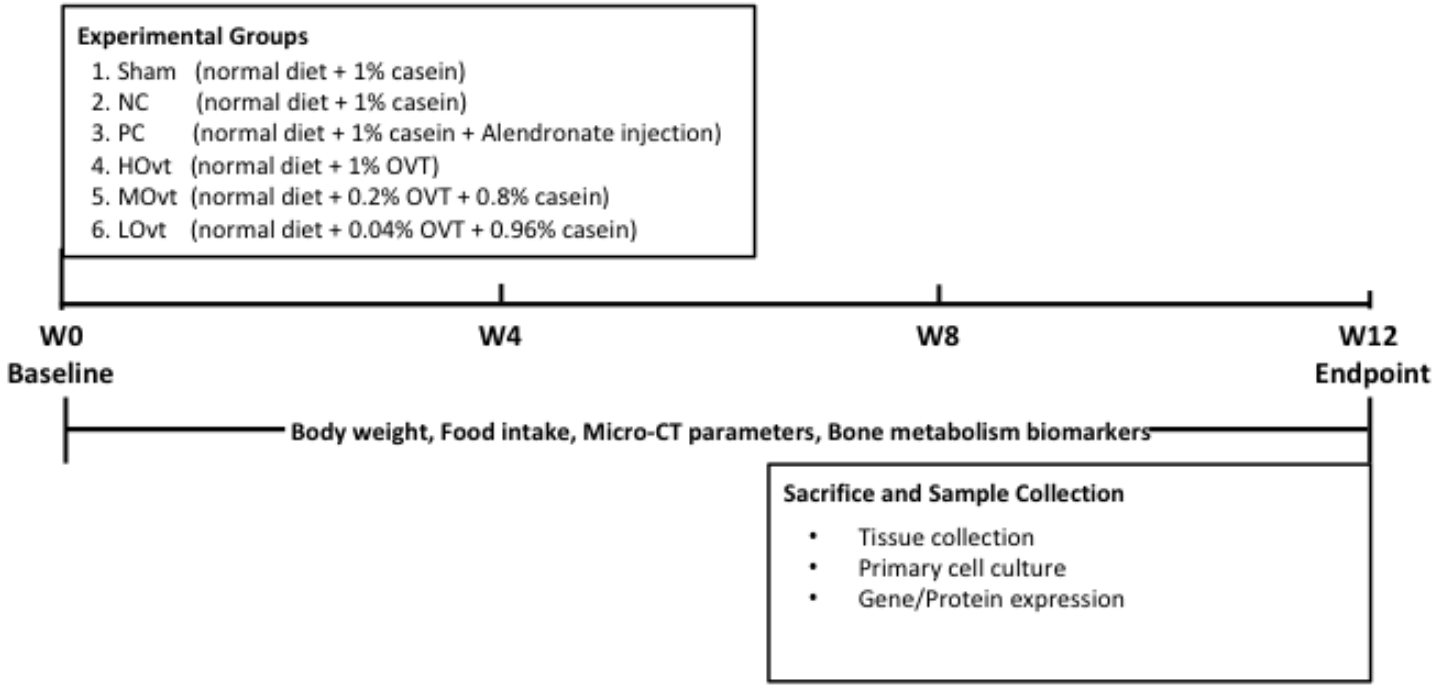
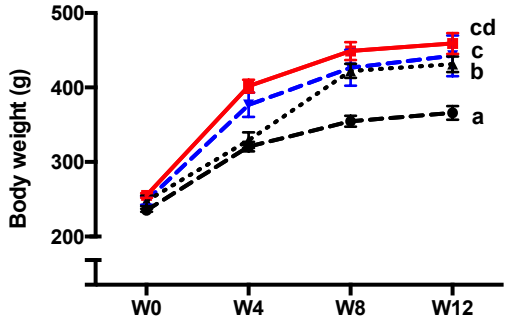


Figure 6.2 Effects of ovotransferrin diet on body weight, food intake, and uterus weight.

(A) The change of body weight during 12-week treatment. (B) The change of food intake during 12-week treatment, (C) Uterus weight after 12 weeks. W: week. Data were expressed as Mean±SEM (n=8 per group). Means without a common letter indicated $p<0.05$.

A



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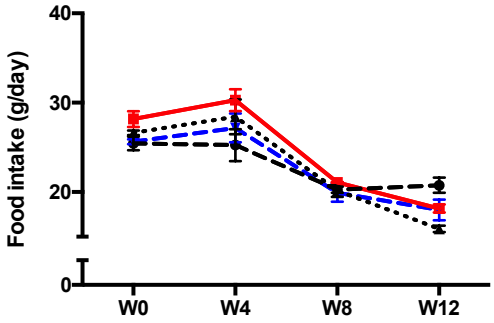
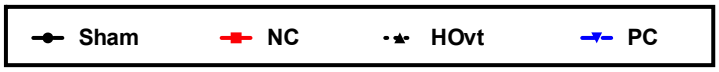




C

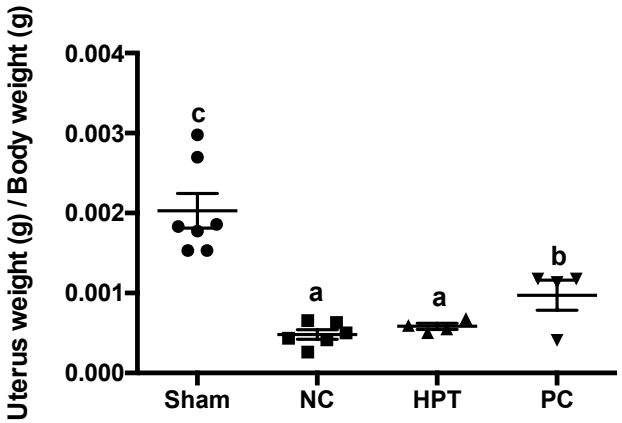


Figure 6.3 Effects of ovotransferrin diet on tibia trabecular bone mineral density and cortical bone mineral density.

A) The change of tibia trabecular bone mineral density during 12-week treatment, (B) The tibia trabecular bone mineral density in W0 and W12, (C) The change of tibia cortical bone mineral density during 12-weeks treatment, (D) The tibia cortical bone mineral density in W0 and W12. W: week. Data were expressed as Mean±SEM (n=8 per group). Means without a common letter indicated $p<0.05$.

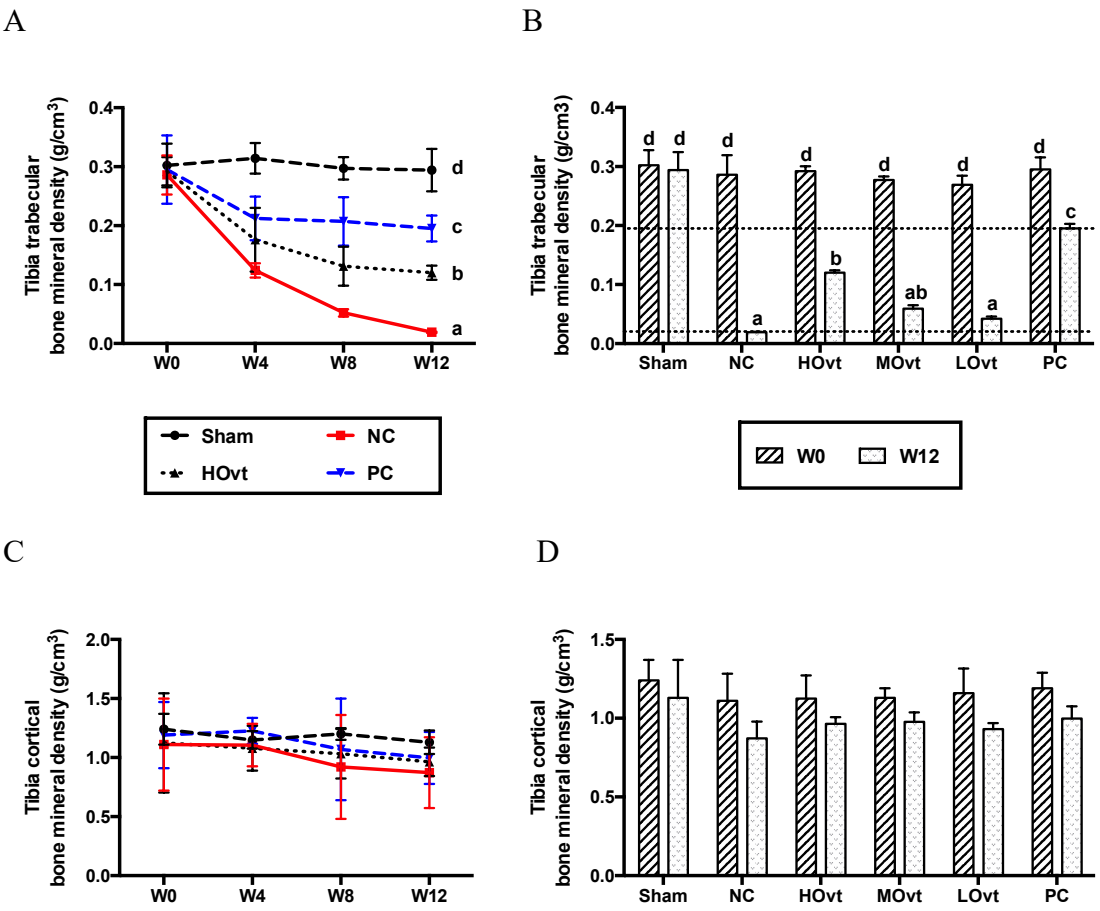
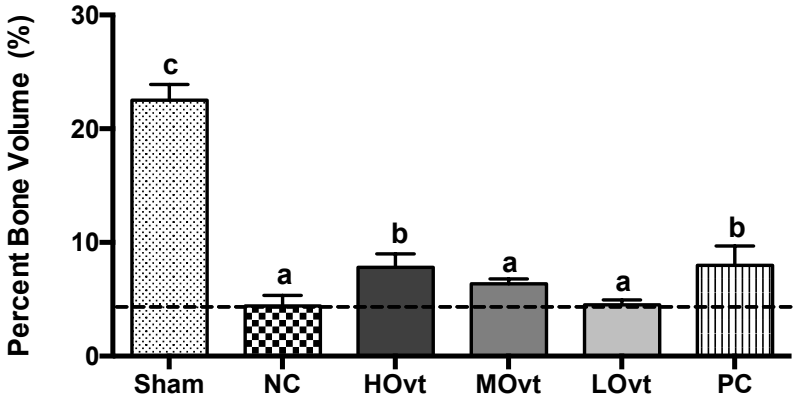


Figure 6.4 Effects of ovotransferrin diet on tibia trabecular micro-architecture.

(A) The tibia trabecular percent bone volume in W12, (B) The tibia trabecular thickness in W12, (C) The tibia trabecular number in W12, (D) The tibia trabecular separation in W12, (E) Three-dimensional trabecular images of vertical section (a) and transversal section (b) of proximal tibia and the volume of interest of proximal tibia (c) in W12. W: week. Data were expressed as Mean±SEM (n=8 per group). Means without a common letter indicated $p<0.05$.

A



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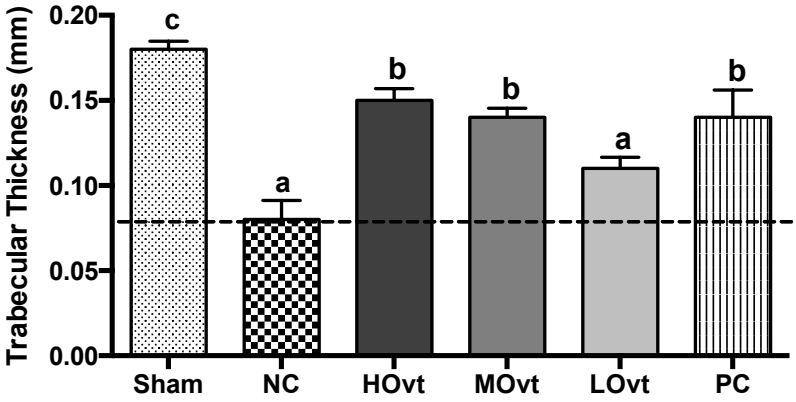

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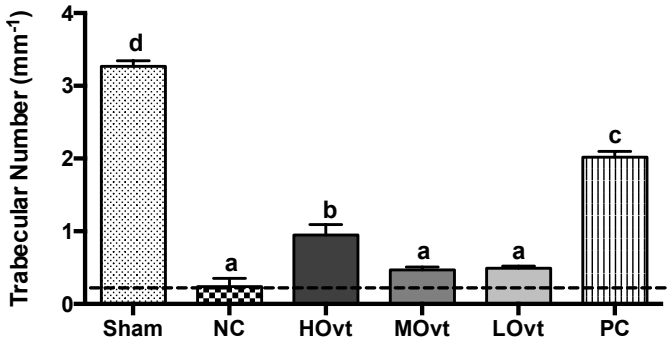
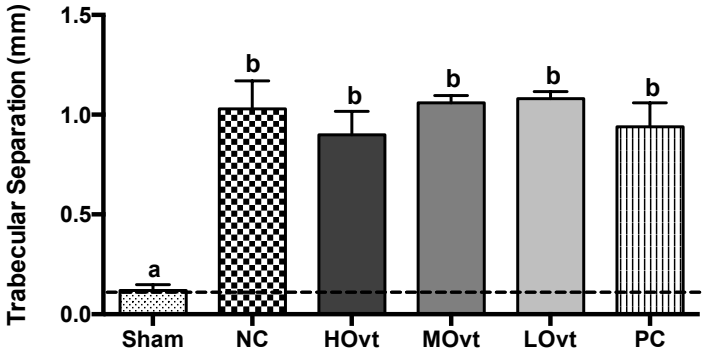

Trabecular Number (mm-1)
4
3
2
1
0
d
a
b
a
a
c
Sham
NC
HOvt
MOvt
LOvt
PC

D


Trabecular Separation (mm)
1.5
1.0
0.5
0.0
a
b
b
b
b
b
Sham
NC
HOvt
MOvt
LOvt
PC

E

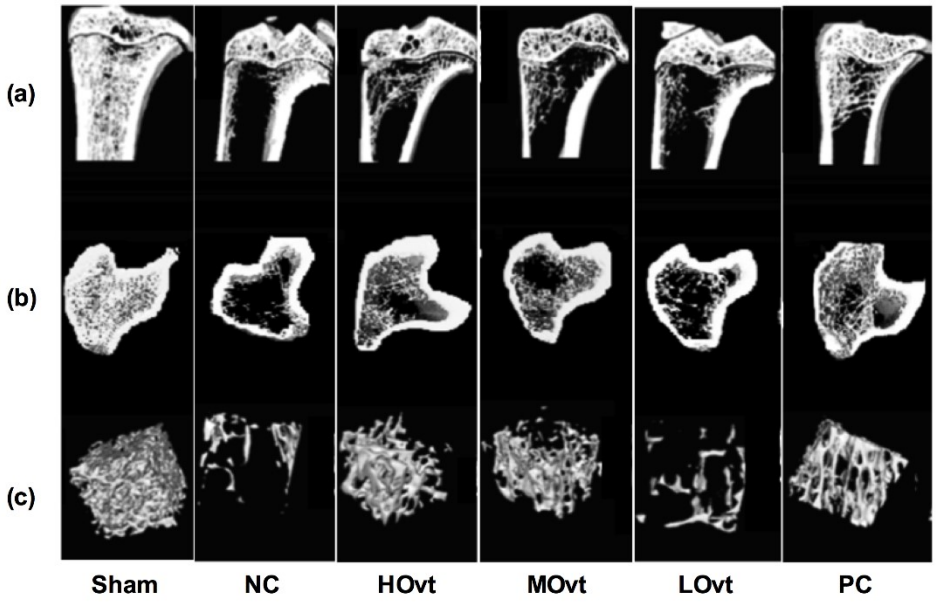
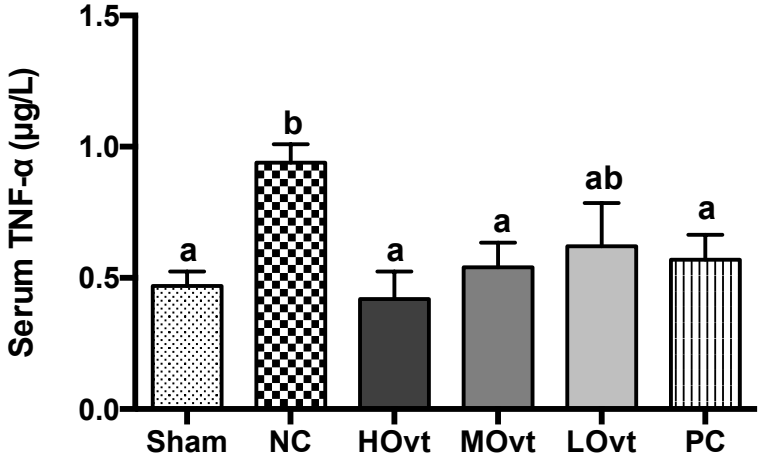

(a)
(b)
(c)
Sham
NC
HOvt
MOvt
LOvt
PC

Figure 6.5 Effects of ovotransferrin diet on serum cytokine production.

(A) The serum TNFα concentration in W12, (B) The serum IL-6 concentration in W12. W: week. Data were expressed as Mean±SEM (n=8 per group). Means without a common letter indicated $p<0.05$.

A



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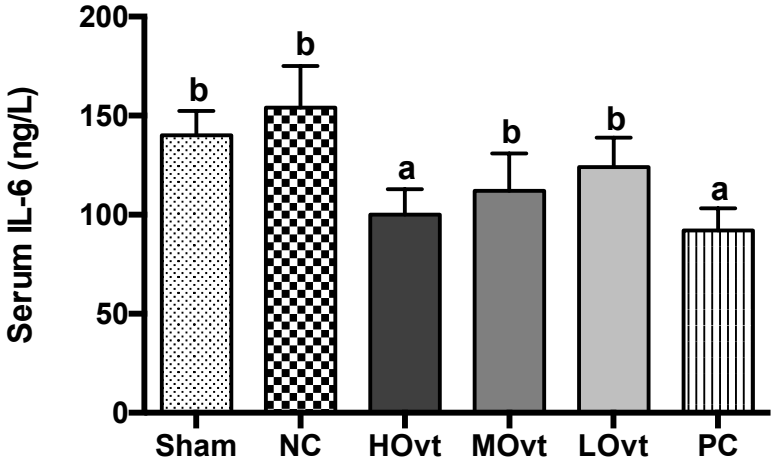
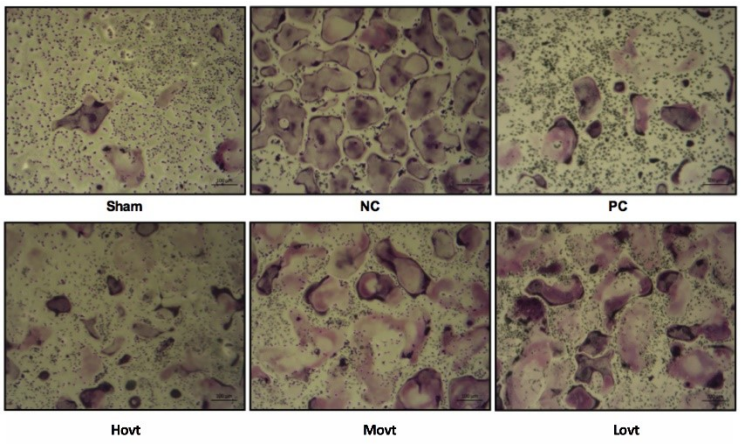


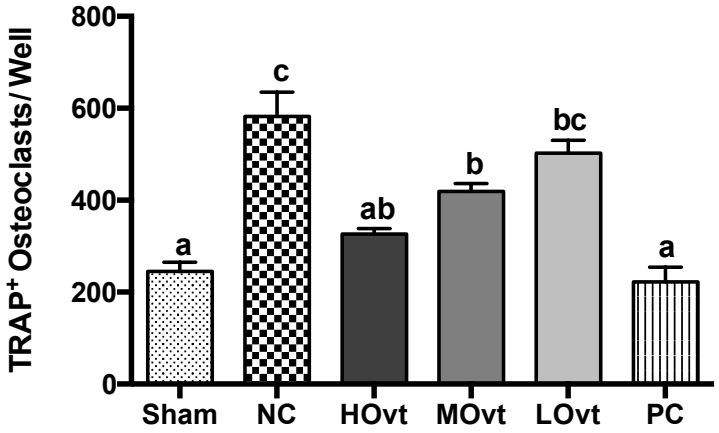
Figure 6.6 Effects of ovotransferrin diet on bone marrow cells-stimulated osteoclasts formation and resorption.

Bone marrow cells were extracted from rat left femur and culture with 10 ng/mL M-CSF and 100 ng/mL RANKL for 6 days to stimulate osteoclasts. (A) The osteoclasts after TRAP staining, (B) The number of TRAP positive cells differentiated from different animal groups, (C) The osteoclastic resorption activity, (D) The osteoclastic resorbed pit area, (E) The images of osteoclastic resorbed area. Data were expressed as Mean±SEM (n=8 per group). Means without a common letter indicated $p<0.05$.

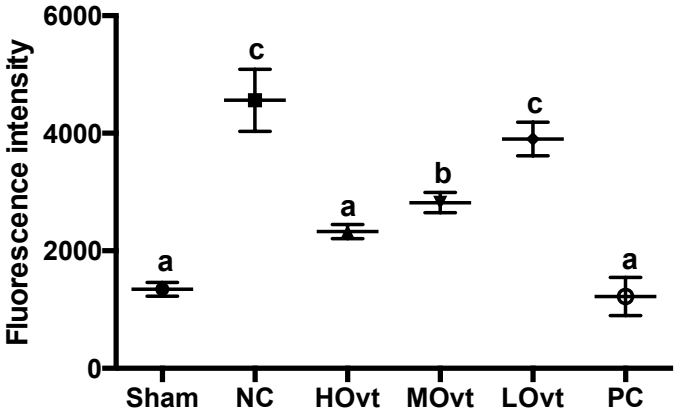
A



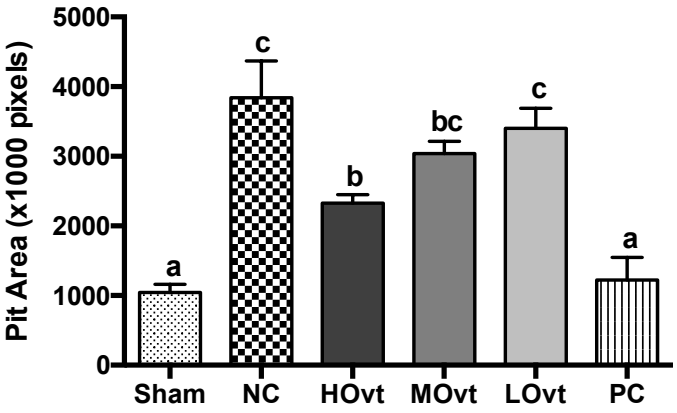
B



C


Fluorescence intensity
6000
4000
2000
0
a
c
a
b
c
a
Sham
NC
HOvt
MOvt
LOvt
PC

D


Pit Area (x1000 pixels)
5000
4000
3000
2000
1000
0
a
c
b
bc
c
a
Sham
NC
HOvt
MOvt
LOvt
PC

E

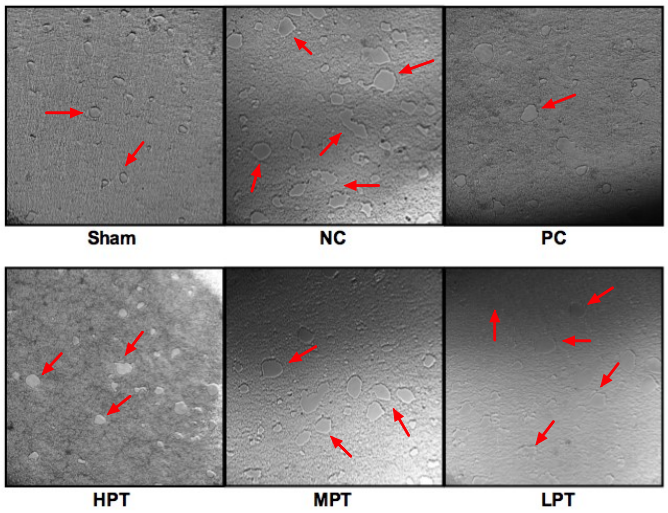
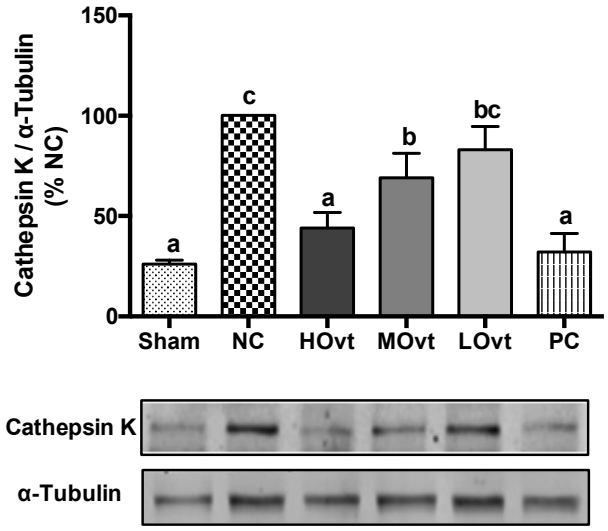

Sham
NC
PC
HPT
MPT
LPT

Figure 6.7 Effects of ovotransferrin diet on osteoclastogenesis-related protein expression in bone marrow.

Bone marrow cells were extracted from rat left femur and whole proteins were extracted for western blot analysis. (A) The protein expression of Cathepsin K, (B) The protein expression of MMP9. Data were expressed as Mean±SEM (n=8 per group). Means without a common letter indicated $p<0.05$.

A



B

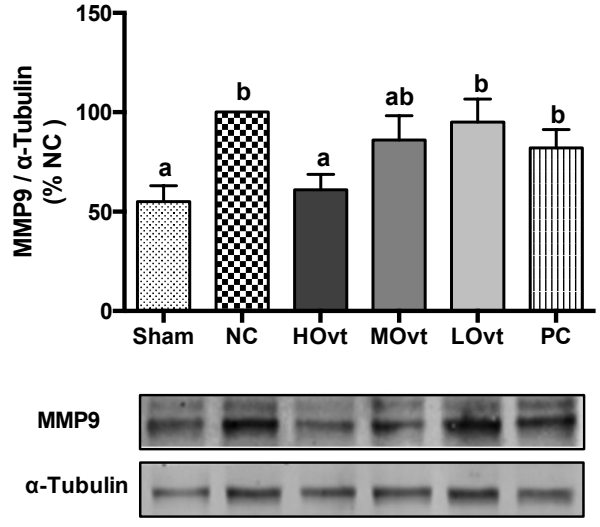
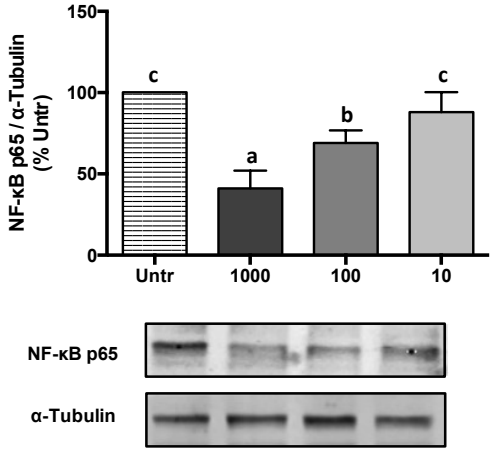


Figure 6.8 Effects of ovotransferrin treatment on osteoclast differentiation.

Bone marrow cells were extracted from left femur of Sham rats and cultured with 10 ng/mL M-CSF and 100 ng/mL and RANKL to stimulate osteoclasts. After 6 days, different concentrations of ovotransferrin were added and incubated for 24 h. Then cells were collected and protein was extracted for western blot analysis. (A) The protein expression of NF-kB p65, (B) The protein expression of IkBa. Data were expressed as Mean±SEM (n=8 per group). Means without a common letter indicated $p<0.05$.

A



B

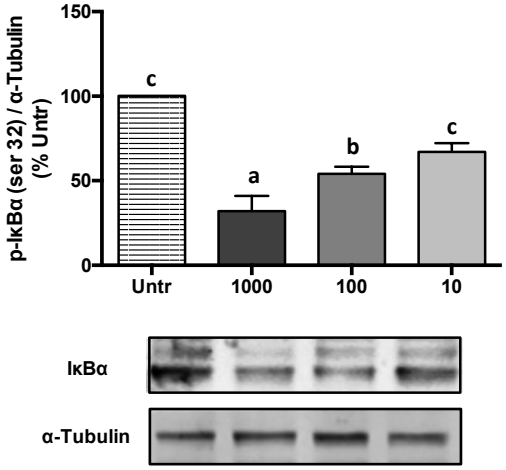
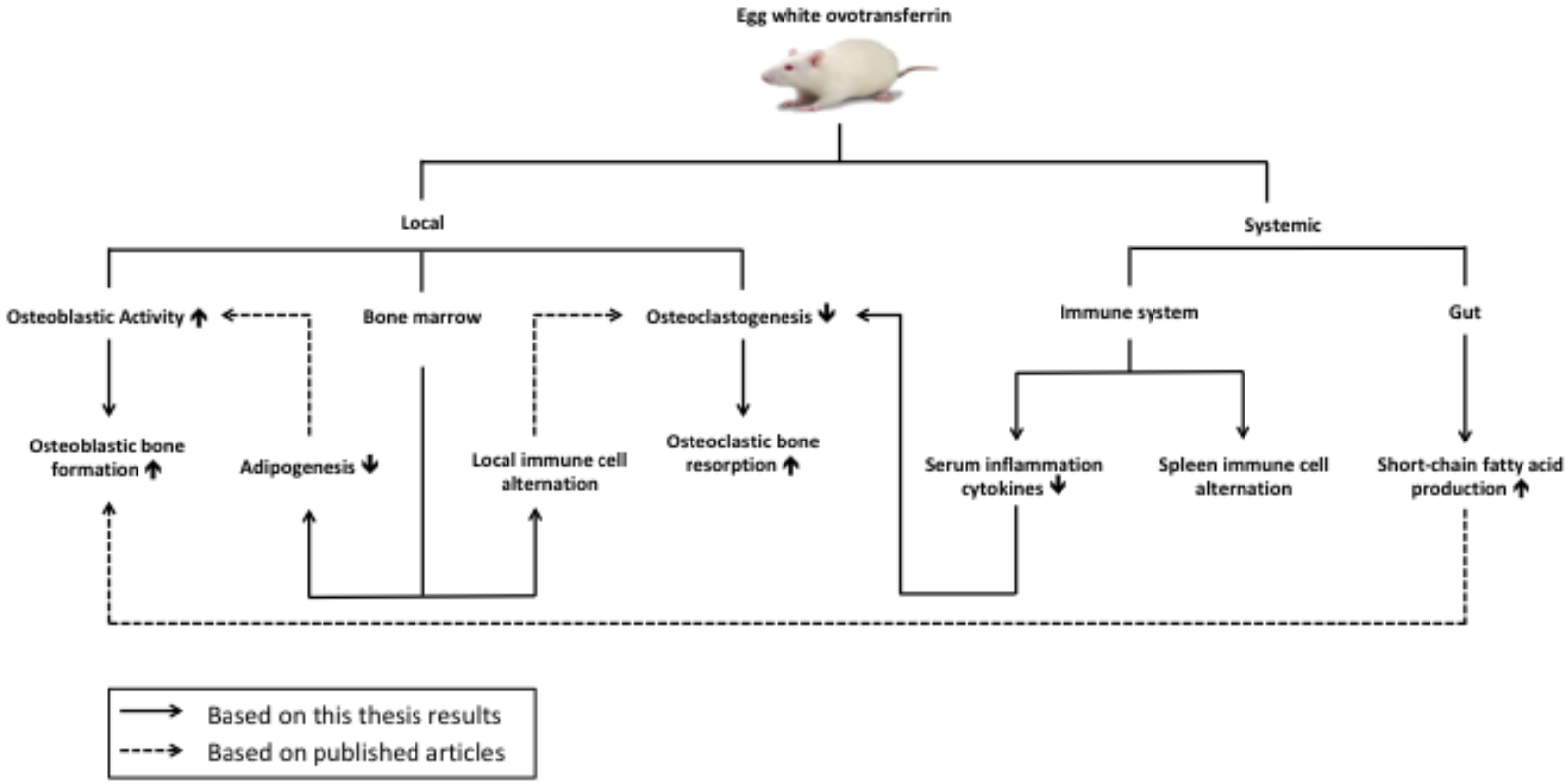


Figure 6.9 Regulatory role of ovotransferrin in OVX rats.

Ovotransferrin may be investigated as a treatment for osteoporosis to improve bone quality by regulating bone remodeling. Ovotransferrin inhibits the adipogenesis, which may lead to increases in the osteoblast lineage differentiation. Ovotransferrin also alters the local immune cell phenotypes, which may lead to decrease of osteoclast lineage differentiation. Ovotransferrin also shows regulatory role to decrease the production of inflammatory cytokines and to increase the production of short-chain fatty acid.

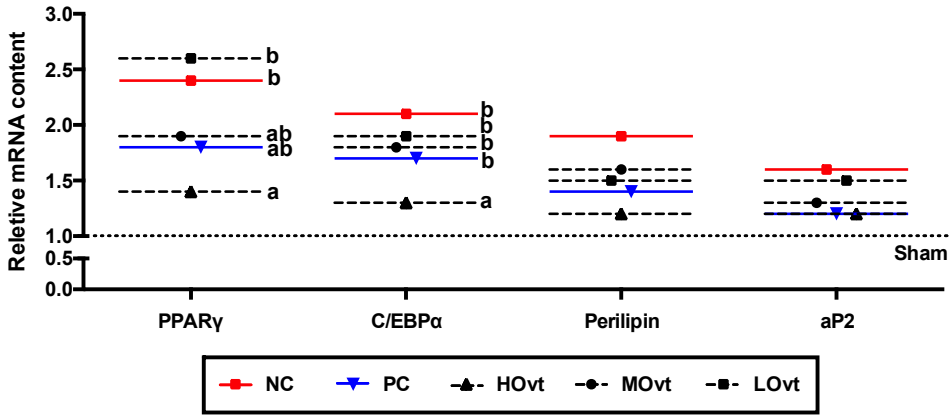


6.7 Supplementary data and information

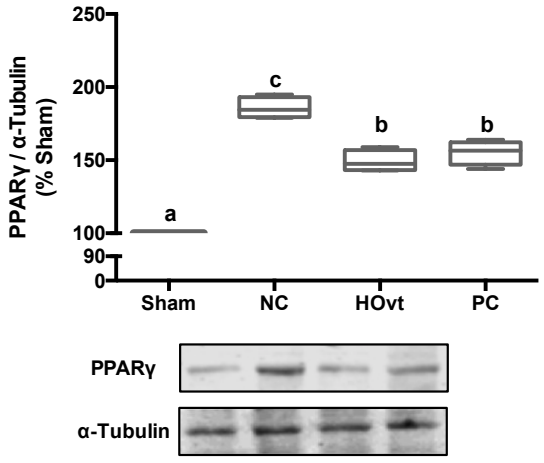
Figure 6.7.1 Effects of ovotransferrin diet on adipogenesis in bone marrow cells

Bone marrow cells were extracted from rat left femur. RNA was extracted for qRT-PCR analysis and whole proteins were extracted for western blot analysis. (A) The gene expression of adipogenesis associated gene, *PPARγ*, *C/EBPα*, *Perilipin*, and *aP2*, (B) The protein expression of PPARγ, (C) The protein expression of C/EBPα. Data were expressed as Mean±SEM (n=8 per group). Means without a common letter indicated $p<0.05$.

A



B



C

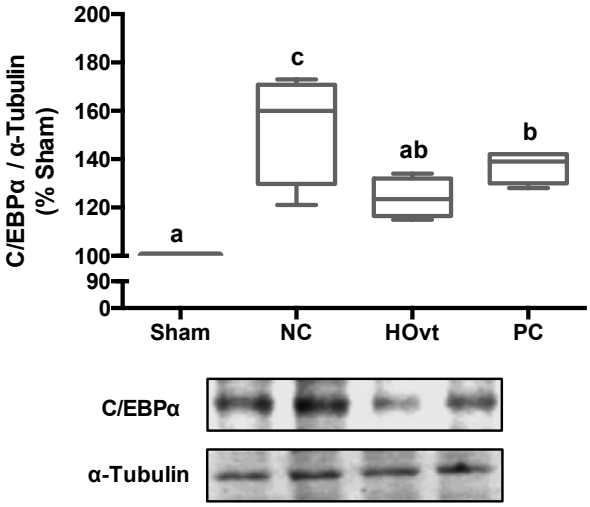

C/EBPα / α-Tubulin (% Sham)
200
180
160
140
120
100
90
0
a
c
ab
b
Sham
NC
HOvt
PC
C/EBPα
α-Tubulin

Figure 6.7.2 Phenotype analysis of freshly isolated immune cells

Values were percentage proportion of the total gate cells as determine by immunofluorescence. (A) The population of CD3+ cells, (B) The population of CD8+ cells, (C) The population of CD25+ cells, (D) The population of CD28+ cells, (E) The population of OX62+ cells, (F) The population of CD51+ cells, (H) The population of CD11b/c+ cells, (I) The population of OX12+ cells, (G) The population of RANK+ cells. Data were expressed as Mean±SEM (n=8 per group). Means without a common letter indicated $p<0.05$.

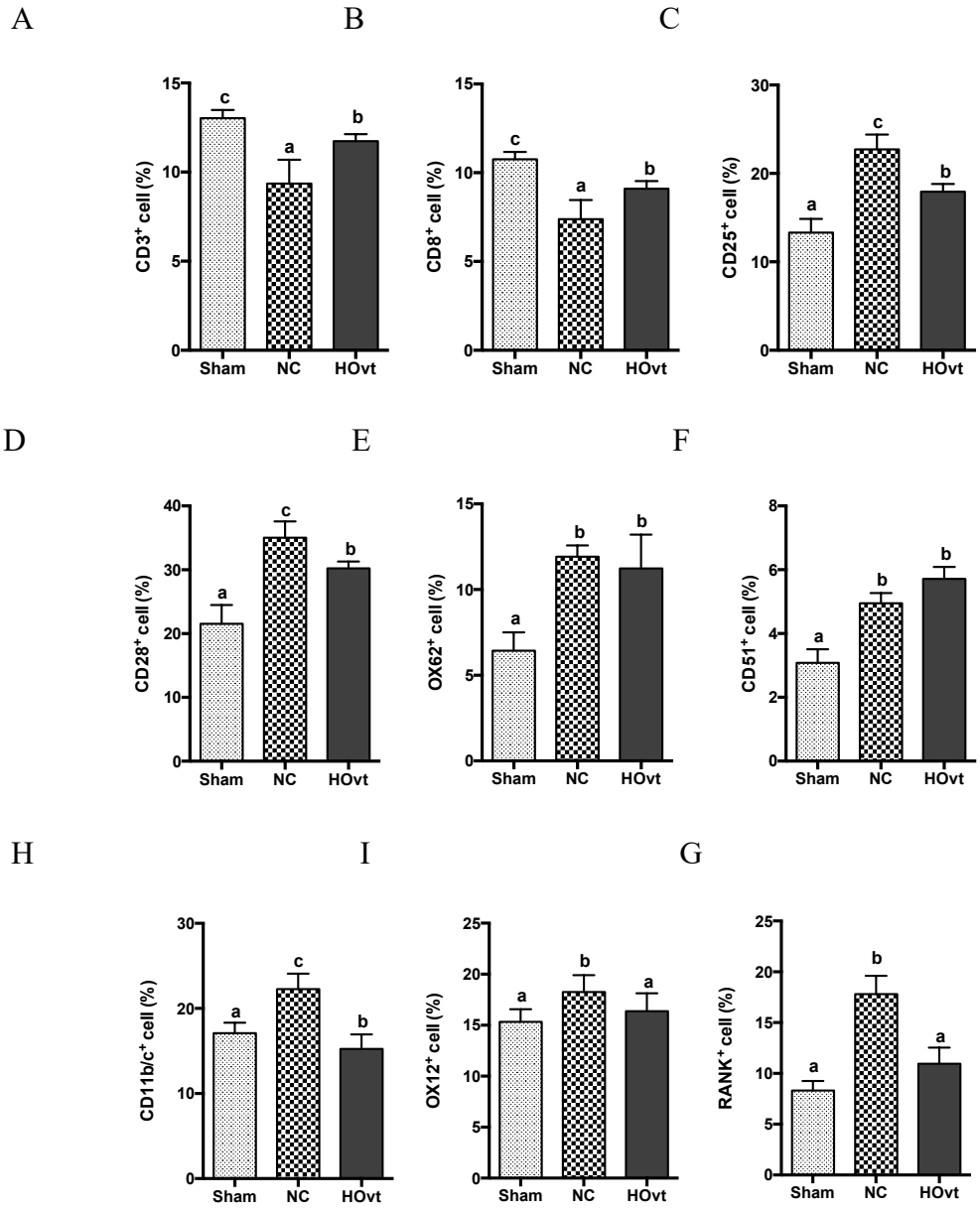


Figure 6.7.3 Effects of ovotransferrin diet on fecal short-chain fatty acids content

Concentration of 7 short-chain fatty acid (acetic acid, proprionic acid, isobutyric acid, butyric acid, isovaleric acid, valeric acid, caproic acid) in rat's fecal samples were analysis by GC. Data were expressed as Mean±SEM (n=8 per group). Means without a common letter indicated $p<0.05$.

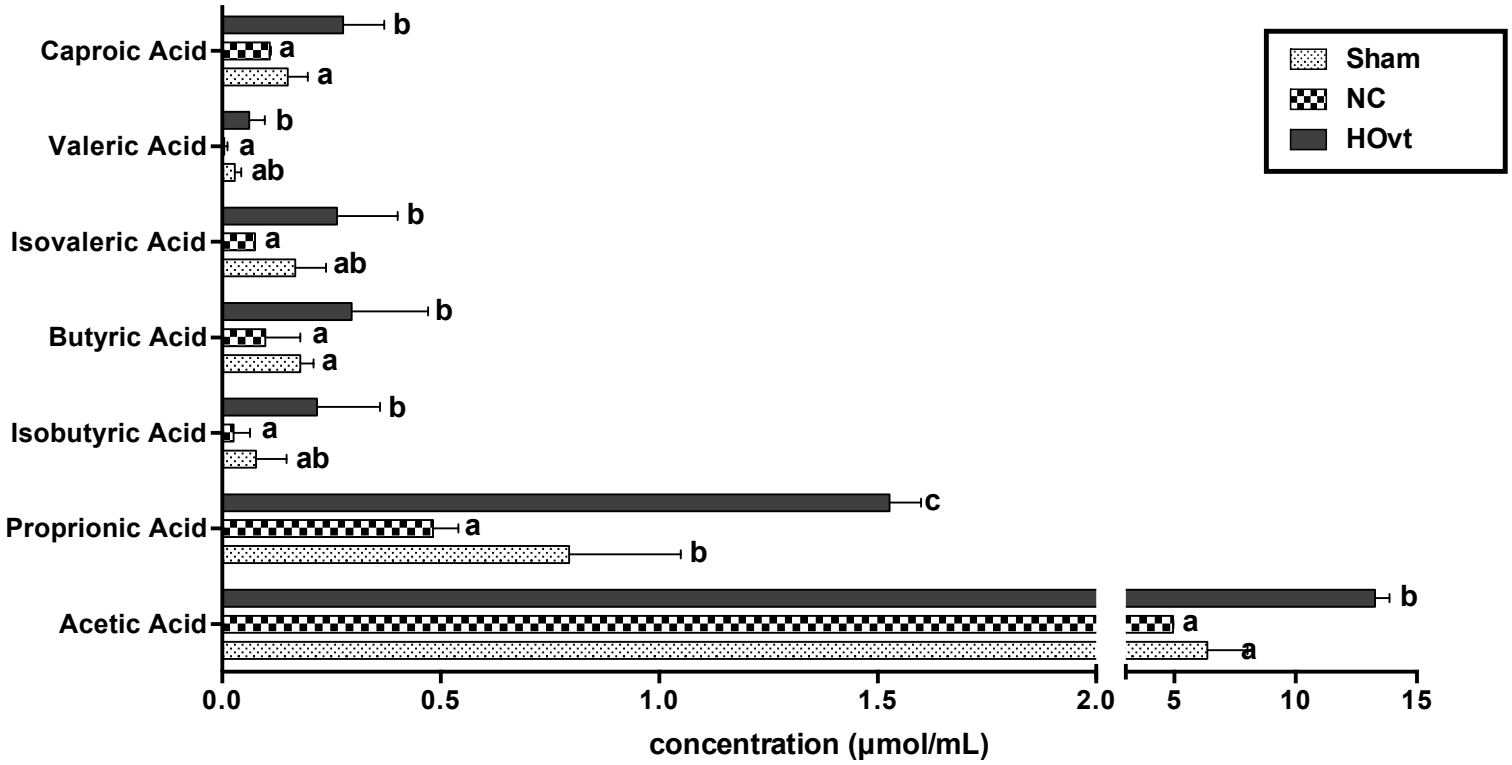


Table 6.7.1 The body weight (g) change in all treatment groups (n=8)

	Sham	NC	HOvt	MOvt	LOvt	PC
W0	235.26±5.56	255.10±7.53	248.02±14.17	250.17±8.27	248.30±14.31	248.11±10.94
W4	320.63±18.38	401.83±21.07	329.25±21.12	382.75±26.62	390.50±23.06	376.75±32.57
W8	354.63±20.65	449.00±29.49	422.25±19.07	422.88±31.34	441.38±28.87	426.50±48.14
W12	366.00±25.63	459.00±34.13	431.25±20.95	431.00±31.96	453.63±30.97	442.50±54.71

Data were expressed as Mean±SEM.

Table 6.7.2 The tissue weight (mg) in all treatment groups (n=8)

	Sham	NC	HOvt	MOvt	LOvt	PC
Heart	1.63±0.08	1.73±0.19	1.62±0.08	1.65±0.16	1.63±0.08	1.63±0.04
Liver	13.42±1.39	15.87±3.27	12.92±0.91	13.79±2.94	12.21±1.76	12.10±1.22
Kidney	2.37±0.21	2.46±0.18	2.28±0.23	2.47±0.14	2.43±0.14	2.32±0.19
Spleen	0.67±0.08	0.80±0.44	0.69±0.05	0.87±0.38	0.64±0.06	0.67±0.06
Uterus	0.90±0.42	0.22±0.07	0.27±0.03	0.40±0.18	0.44±0.14	0.43±0.17

Data were expressed as Mean±SEM.

Table 6.7.3 Phenotype analysis of freshly isolated spleen cells (n=8)

	Sham	NC	HOvt	MOvt	LOvt	PC
CD3+	44.8±4.9	43.7±5.0	39.8±2.1	40.7±5.4	38.6±4.0	37.0±3.8
CD4+	34.0±4.1	31.9±3.6	30.4±4.2	33.3±5.0	32.0±3.1	33.9±4.8
CD8+	27.0±3.8	26.7±2.8	24.7±4.8	22.0±1.8	23.6±2.3	22.7±2.4
CD25+	9.3±1.9	11.1±3.7	12.0±2.2	11.1±2.8	11.2±3.1	14.8±1.6
CD28+	4135±3.2	42.5±7.9	38.4±3.1	41.9±9.3	40.3±4.1	48.5±3.4
CD51+	8.7±4.9	5.9±2.7	7.3±1.7	6.0±1.7	5.8±1.6	8.9±2.3
CD71+	21.9±3.5	24.0±8.3	23.4±2.0	25.9±5.6	25.4±4.8	34.2±1.6
CD11b/c+	20.4±4.5	20.1±6.0	19.5±2.7	19.0±3.8	20.1±3.3	26.5±1.2
OX6+	37.8±6.4	37.1±3.5	40.3±1.1	43.2±2.5	40.3±3.9	42.6±2.7
OX12+	31.2±4.2	37.7±3.8	33.5±2.1	35.6±3.9	37.2±1.8	38.2±5.0
OX62+	7.8±1.9	7.7±2.6	9.4±1.9	8.9±2.4	8.9±2.1	12.6±2.9
RANK+	5.1±0.8	5.8±1.2	5.0±0.4	5.2±0.7	5.2±0.3	4.7±1.0
CD3+ CD4+	21.6±2.6	20.2±2.5	18.4±3.4	21.6±4.1	18.7±3.3	18.0±3.2

CD3+ CD8+	21.5±4.3	22.0±3.0	19.9±5.0	17.4±1.8	18.4±1.7	17.6±2.4
CD4+ CD25+	2.9±0.5	3.5±1.2	3.6±0.7	3.0±0.7	3.2±0.9	4.0±0.7
CD8+ CD25+	2.7±0.6	3.1±1.2	3.5±0.6	2.7±0.8	2.8±1.0	3.6±1.2
CD4+ CD28+	6.4±0.9	7.2±1.3	6.8±1.0	6.0±1.6	6.0±1.0	6.9±0.8
CD8+ CD28+	19.4±2.3	18.2±4.9	17.2±2.7	19.3±4.5	18.5±2.4	21.8±3.0
CD4+ CD71+	13.9±3.1	14.1±5.8	14.5±2.1	15.3±4.0	15.7±3.2	20.8±2.8
CD8+ CD71+	2.9±0.8	3.5±1.7	4.3±1.0	3.7±1.1	4.1±1.2	5.7±1.1
OX6+ OX62+	11.1±2.9	11.4±4.0	12.3±2.1	12.4±3.1	11.8±2.4	17.0±1.7
OX6+ CD11b/c+	6.4±1.8	6.4±2.7	7.5±1.4	7.4±2.2	7.5±2.2	11.3±2.5
CD3+ CD4+ CD25+	17.3±4.3	21.2±6.3	24.0±2.	16.9±4.3	20.1±4.3	21.2±1.9
CD3+ CD8+ CD25+	7.2±2.4	8.9±3.4	11.2±4.3	8.9±2.9	8.5±3.0	10.7±4.0

Values were percentage proportion of the total gated cells as determined by immunofluorescence.

Data were expressed as Mean±SEM.

Table 6.7.4 Phenotype analysis of freshly isolated bone marrow cells (n=8)

	Sham	NC	HOvt	MOvt	LOvt	PC
CD3+	13.0±1.3	9.4±3.3	11.7±1.1	12.2±1.90	14.1±1.3	20.7±1.4
CD4+	9.3±2.5	12.5±3.9	14.6±1.1	14.2±2.9	12.1±3.5	13.1±1.7
CD8+	10.8±1.2	7.4±2.6	9.1±0.9	10.1±1.7	11.6±1.8	17.8±2.1
CD25+	13.3±4.5	22.7±4.1	17.9±2.5	20.2±4.8	21.6±3.7	31.9±3.8
CD28+	21.5±8.3	35.0±6.3	30.2±2.1	33.3±4.5	34.5±2.8	48.6±2.6
CD51+	3.1±1.2	5.0±0.8	5.7±0.8	5.7±1.4	5.4±1.1	8.3±2.2
CD71+	27.0±3.4	24.7±8.6	26.5±2.9	26.3±5.0	20.2±5.4	25.5±4.8
CD11b/c+	17.1±3.5	22.3±4.4	15.2±3.3	18.3±4.4	18.4±3.4	21.2±4.1
OX6+	5.9±1.7	8.8±1.1	8.8±0.8	10.1±2.3	9.9±1.8	13.9±2.4
OX12+	15.3±3.5	18.3±4.0	16.4±3.5	17.3±5.0	18.6±2.8	18.7±2.5
OX62+	6.4±3.0	11.9±1.6	11.2±4.0	11.5±3.5	14.5±2.2	21.8±4.3
RANK+	8.3±2.7	17.8±4.4	11.0±3.2	16.4±2.8	15.2±2.9	6.4±1.4
CD3+ CD4+	2.1±0.5	3.0±0.3	3.8±0.7	3.3±0.62	3.4±0.7	4.2±0.9

CD3+ CD8+	2.5±0.8	2.5±0.4	2.6±0.3	2.7±0.7	2.8±0.5	3.8±0.4
CD4+ CD25+	4.3±2.0	7.3±2.5	8.4±0.7	7.9±1.5	6.5±2.5	8.1±2.1
CD8+ CD25+	2.8±1.2a	4.6±0.6	4.2±0.6	4.2±1.0	5.21±0.8	8.4±1.4
CD4+ CD28+	3.7±1.7	6.5±2.0	7.3±0.5	7.0±1.7	4.3±3.7	7.2±2.2
CD8+ CD28+	4.1±1.5	6.3±0.8	5.6±0.6	5.9±1.5	7.2±1.2	11.7±1.3
CD4+ CD71+	5.5±2.4	8.2±3.2	9.4±0.6	10.9±5.1	7.6±2.8	7.6±2.7
CD8+ CD71+	4.4±2.0	6.8±1.7	6.4±1.0	7.1±1.7	7.0±1.5	10.6±2.2
OX6+ OX62+	3.3±1.4	5.2±0.8	5.8±0.7	5.8±1.6	6.4±1.0	9.5±1.8
OX6+ CD11b/c+	0.2±0.1	0.3±0.1	0.3±0.1	0.3±0.1	0.2±0.1	0.3±0.1
CD3+ CD4+ CD25+	80.8±20.0	94.3±1.3	92.3±3.0	92.8±3.1	93.1±1.8	96.4±1.1
CD3+ CD8+ CD25+	37.6±12.0	56.1±5.9	58.6±11.9	52.5±15.8	62.7±9.6	61.7±5.2

Values were percentage proportion of the total gated cells as determined by immunofluorescence.

Data were expressed as Mean±SEM.

Table 6.7.5 The concentration of fecal short-chain fatty acids (μmol/mL) (n=8)

	Sham	NC	HOvt
Acetic acid	6.3654±1.6527	4.9498±0.0084	13.2733±0.6017
Proprionic acid	0.7940±0.2555	0.4821±0.0580	1.5274±0.0717
Isobutyric acid	0.0776±0.0694	0.0262±0.0370	0.2170±1.4442
Butyric acid	0.1786±0.0303	0.0988±0.0798	0.2967±0.1746
Isovaleric acid	0.1673±0.0703	0.0751±0.00002	0.2628±0.1389
Valeric acid	0.0288±0.0149	0.0050±0.0070	0.0620±0.0354
Caproic acid	0.1502±0.0459	0.1094±0.0020	0.2765±0.0943

Data were expressed as Mean±SEM.

Table 6.7.6 Sequences of primers used in qRT-PCR

Gene	**Primer Sequences (5'-3')**	
PPARγ	Forward	TCCTCCTGTTGACCCAGAGCAT
	Reverse	AGCTGATTCCGAAGTTGGTGG
C/EBPα	Forward	TGAACAAGAACAGCAACGAG
	Reverse	TCACTGGTCACCTCCAGCAC
Perilipin	Forward	GACACCACCTGCATGGCT
	Reverse	TGAAGCAGGGCCACTCTC
aP2	Forward	GCGTGGAATTCGATGAAATCA
	Reverse	CCCGCCATCTAGGGTTATGA
β-actin	Forward	AGTACCCCATTGAACACGGC
	Reverse	TTTTCACGGTTAGCCTTAGG

Table 6.7.7 Nutritional characteristics of experimental diet

Formula	g/kg
Casein	140.0
L-cystine	1.8
Corn starch	465.692
Maltodextrin	155.0
Sucrose	100.0
Soybean oil	40.0
Cellulose	50.0
Mineral Mix, AIN-93M-MX (64049)	35.0
Vitamin Mix, AIN-93-VX (94047)	10.0
Choline Bitartrate	2.5
TBHQ, antioxidant	0.008

Table 6.7.8 The antibody combination for immune cells phenotype analysis

1	2	3	4	5	6	7	8	9	10	11	12
cells	CD25 F CD3 PE CD8 PerCP CD4 APC	CD28 F RANK PE CD8 PerCP CD4 APC	CD71 F OX12 PE CD8 PerCP CD4 APC	OX62 F CD51 PE OX6 PerCP CD11b/c APC							

For each cell sample, 5 combinations were conducted according to the table above. Maximum 4 antibodies with different color-labeled were able to add into one well

CHAPTER 7 - General Summary and Discussion

7.1 Key Findings of the Present Research

The overall purposes of this research were to understand the regulatory roles of ovotransferrin in bone cell activity and to explore its potential application as a bioactive protein against osteoporosis. The key findings of each study are listed below:

1) The first objective was to investigate the regulatory role of ovotransferrin on osteoblastic activity and osteogenesis (Chapter 3)

Ovotransferrin, the second major protein in egg white, is an iron-binding glycoprotein belonging to transferrin family (Giansanti, Leboffe, Angelucci, & Antonini, 2015). In addition to its nutritional value, egg white ovotransferrin displays numerous biological properties, including antimicrobial, antioxidant, and immunomodulating activities (Xie, Huff, Huff, Balog, & Rath, 2002). A previous study also reported that the expression of ovotransferrin and its receptor were increased during the initial stage of bone formation in developing chick embryo, suggesting the importance of ovotransferrin in bone formation (Gentili et al., 1993; 1994). Moreover, another transferrin family member lactoferrin, derived from bovine milk, has been reported to promote bone formation by stimulating bone formation cell osteoblast activity (Cornish et al., 2004; Naot, Grey, Reid, & Cornish, 2005), and to provide benefits for bone health (Blais, Malet, Mikogami, Martin-Rouas, & Tomé, 2009; Guo et al., 2009; Hou, Xue, & Lin, 2012).

In this study, ovotransferrin directly stimulated osteoblast proliferation, differentiation and mineralization as an osteogenic agent. Osteoblasts treated with ovotransferrin exhibited increased

proliferation caused by cell-cycle arrest at the S and G2/M transition. Ovotransferrin also stimulated osteoblast differentiation by increasing the expression of type I collagen and alkaline phosphatase (ALP), and finally promoted mineralization to produce osteoid. Meanwhile, ovotransferrin also showed potent effect on inhibiting osteoclastogenesis. Ovotransferrin inhibited osteoblast-produced receptor activator of nuclear factor kappa-B (RANKL), while increased the expression of osteoprotegerin (OPG). Egg white ovotransferrin showed comparable activity to bovine lactoferrin, the well-established bioactive protein for bone formation, indicating the potential of ovotransferrin for promoting bone formation.

2) The second objective was to study the mechanisms involved in the ovotransferrin-stimulated osteogenesis (Chapter 4)

Osteoblast differentiation is a prerequisite of bone formation; thus, it is desirable to identify and develop anabolic agents to enhance bone formation by stimulating osteoblastic activity. However, regulation of osteoblast differentiation is through a complicated cooperation of various signal transductions, such as the activation of bone morphogenic proteins (BMPs) pathway (Cao & Chen, 2005), mitogen-activated protein kinases (MAPKs) pathway (Chau, Leong, & Li, 2009; Derynck & Zhang, 2003; Dijke & Hill, 2004), and phosphoinositide 3-kinases (PI3K)-protein kinase B (Akt) pathway (Chau et al., 2009; Derynck & Zhang, 2003; Dijke & Hill, 2004). Studies also reported the importance of these signaling pathways in bioactive compounds promoted osteogenesis (Grey, Zhu, Watson, Callon, & Cornish, 2006; Heo et al., 2018; Naot et al., 2011). In the previous chapter, we tested the stimulatory role of ovotransferrin on osteoblast differentiation; however, the molecular mechanism underlying the effect remains poorly understood. Therefore, the objective of this study was to explore the mechanisms involved in

ovotransferrin-stimulated osteogenesis, especially the activation of MAPK and PI3K-Akt pathways.

Ovotransferrin activated MAPK pathways by increasing phosphorylation of ERK1/2, JNK and p38, in a time-dependent manner; however, only the ERK1/2 pathway responded to ovotransferrin-induced osteogenesis. Antagonist of ERK1/2 blocked the activation of ERK1/2 pathway and attenuated ovotransferrin promoted osteoblast differentiation and mineralization, while antagonist of JNK showed no effects. Adding p38 inhibitor reduced the expression of ALP and Runx2 compared with ovotransferrin alone, but remained significantly higher than control. Ovotransferrin activated ERK1/2 pathway was further validated by investigating the phosphorylation of the core component involved in ERK cascade, including c-Raf, MEK1/2, p90RSK and MSK1. Ovotransferrin caused an increase in the phosphorylation of upstream Raf and MEK1/2, as well as the downstream p90RSK and MSK1, indicating the osteoblastic promotion was due to the activation of the ERK cascade. Ovotransferrin also increased the expression of two PI3K subunits p85 and p110, leading to increased phosphorylation of Akt and osteogenesis. Akt antagonist was also used to confirm the effect of PI3K-Akt in response to the stimulation of ovotransferrin; meanwhile we found that PI3K-Akt not only regulated ovotransferrin induced osteoblast differentiation, but also contributed to the migration of osteoblast. The activation of ERK1/2 and PI3K-Akt pathways further regulated the expression of Runx2, BMP-2, and β-catenin, which play vital roles in osteoblast differentiation, and benefit for osteogenesis and bone formation. In addition to the activated pathway associated with ovotransferrin stimulation, the possible receptors that response to ovotransferrin and initiate the signal transduction were studied in this chapter. Three receptors that might be able to bind with ovotransferrin in osteoblasts, named transferrin

binding protein (TFBP), transferrin receptor (TFR1), and low-density lipoprotein receptor-related protein 1 (LRP1), were significantly expressed in the presence of ovotransferrin. LRP1 antagonist pre-treatment reduced ALP and mineralization, but did not affect ovotransferrin-activated ERK1/2 or PI3K-Akt pathway, indicating LRP1 was independent to ERK1/2 and PI3K-Akt activation-associated osteoinduction.

3) The third objective was to investigate the in vitro effects of ovotransferrin on inhibiting osteoclastogenesis and bone resorptive activity (Chapter 5)

Bone metabolism relies on activities of bone formation and resorption. An excessive bone resorption will result in osteolytic bone conditions and lead to bone disorders, such as osteoporosis (Takatsuna et al., 2004; Wei et al., 2018). Osteoclast, derived from monocyte/macrophage lineage, is responsible for bone resorption (Tanaka et al., 2006). The development of osteoporosis is due to a massive recruitment of osteoclast and an excessive osteoclastic activity (Takatsuna et al., 2004; Wei et al., 2018). Thus, the inhibition agents targeting to attenuate osteoclastogenesis and osteoclastic resorption is the major therapy strategy for osteoporosis treatment (Sharif, Abdollahi, & Larijani, 2011). In previous chapters, we investigated the anabolic effect of ovotransferrin on promoting bone formation by stimulating osteoblastic activity; therefore, the objective of this study was to explore the effect of ovotransferrin on inhibiting osteoclastogenesis and resorptive activity, and its associated mechanisms.

Ovotransferrin showed significant inhibition on osteoclastogenesis and decreased the number of RANKL-induced osteoclasts. Calcium-phosphates resorption assay indicated that the resorptive activity of osteoclasts treated by ovotransferrin was significantly suppressed. Further studies found

the inhibition effect was due to the regulation of RANKL-activated NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells) and MAPK pathways. Ovotransferrin pre-treatment suppressed the phosphorylation of the major components in NF-κB cascade, including IKKα/β, IκBα, and NF-κB p65. Ovotransferrin also inhibited RANKL-induced phosphorylation of MAPKs in dose-depended manner. The suppression of these two pathways resulted in a down-regulation of several osteoclastogenesis-associated proteins, such as TRAF6 (TNF receptor associated factor 6), c-Fos, NFATc1 (nuclear factor of activated T-cells cytoplasmic 1), and cathepsin K. In addition to inhibiting osteoclastogenesis, ovotransferrin also accelerated cell apoptosis in mature osteoclast via regulating the Bcl-2 family members, in which the expression of pro-apoptotic *Bim* and *Bad* were increased while the anti-apoptotic *Bcl-2* and *Bcl-x* was decreased.

4) The fourth objective was to study the in vivo efficacy of ovotransferrin on osteoporosis prevention in an ovariectomized (OVX) rat model (Chapter 6)

Ovariectomized (OVX) rat, after ovary removal, represents the clinical features of estrogen deficiency-induced bone loss, including a rapid bone loss due to exceeding bone resorption, a greater loss of cancellous bone than cortical bone, and reduced calcium absorption (Jee & Yao, 2001; Kalu, 1991; Komori, 2015). Thus, OVX rat model is the most widely used animal model used in study of postmenopausal osteoporosis and assessment of osteoporosis therapies (Jee & Yao, 2001; Kalu, 1991; Komori, 2015). Previous research demonstrated that ovotransferrin enhanced osteoblastic activity and bone formation, and inhibited osteoclastogenesis and bone resorption; therefore, the objective of this study was to study the *in vivo* efficacy of orally administrated ovotransferrin in OVX rats.

Ovotransferrin administration for 12 weeks did not affect body weight, food intake and organ weight, but prevented the OVX-induced decrease of bone mineral density, and partially preserved OVX-induced deterioration of bone microarchitecture. This improvement was due mainly to the regulation of bone turnover, in which ovotransferrin inhibited bone resorption and stimulated bone formation. Decreased bone resorption after ovotransferrin administration was evidenced 1) decreased production of serum and urinary bone resorption biomarkers; 2) decreased production of serum cytokine TNF-α and IL-6, which are two stimulators of osteoclast differentiation; and 3) interference of the differentiation of osteoclast precursors-derived from bone marrow. Stimulated bone promotion was evidenced 1) improved mineral density and trabecular bone microarchitecture; and 2) increased production of serum bone formation biomarkers. These results supported our *in vitro* findings that ovotransferrin could regulate bone cells activity. In addition to its regulatory roles on bone cell (osteoblast, osteoclast and their precursors) and bone remodeling, ovotransferrin also inhibited adipogenesis, modulated immune function (both systemic and local), and enhanced the formation of short-chain fatty acids in gut. It is not known the contributions of these findings to the bone outcomes, which are warranted further studies.

In conclusion, this thesis demonstrated the potential use of ovotransferrin as functional foods and/or nutraceuticals for osteoporosis prevention through the management of bone remodeling via regulating both osteoblastic and osteoclastic activity.

7.2 Significance of This Research

Egg-derived bioactive proteins and peptides have been reported to have various biological functions, such as antimicrobial, anti-oxidant, anti-inflammation, immunomodulatory and among

others. (Mine, 2007). As the second major protein in egg white, ovotransferrin has been widely studied for its bioactivities (reviewed in Chapter 2). However, to our best knowledge, this is the first study reporting *in vitro* (in cells) and *in vivo* (in rodents) bone health promoting effect of ovotransferrin. This thesis has greatly contributed to the knowledge of ovotransferrin as a regulatory molecule on bone cell activity paving a new way of value addition to the egg industry. Thus, the study exhibits significance in both egg industry and bone health management.

1) Significance to the Egg Industry

In 2017, there were 1059 egg producers in Canada, generating in total $108 million farm cash receipts (AAFC, 2017a). Currently, the Canadian egg market is divided into table eggs and process eggs (AAFC, 2017b). The table egg market is presents approximately 73% of the Canadian market, while the processed egg market accounts for the remaining 27% (AAFC, 2017b). In addition to a small share of the market, processed egg is limited in the production of whole egg, albumen and egg yolks in frozen, dried or liquid form, which is mainly used for further manufacturing of bakery products, mayonnaise, noodles, etc. (AAFC, 2017b). Although some of the processed eggs are used for specialty items such as shampoo, pet foods and adhesives (AAFC, 2017b), the narrow applications of egg products limit the egg industry profile. Egg, as an inexpensive source of protein, can be utilized for producing bioactive proteins and/or peptides. Unfortunately, compared to other protein-rich foods such as dairy proteins, the value-added application of egg is negligible. Therefore, the potential of ovotransferrin in preventing osteoporosis and enhancing bone health will be of great interest to food, nutraceutical and pharmaceutical companies, which will eventually bring benefits to the egg industry by developing valued-add egg products.

2) Significance to Osteoporosis Treatment

According to Health Canada, osteoporosis affects ~ 1.4 million Canadians and costs over $2.3 billion to the Canadian healthcare system for treating osteoporosis and the fractures as of 2010 (Health Canada, 2018). Some pharmaceutical drugs used in osteoporosis treatment are reported to have associated risk: increase the risk of ovarian cancer and affect the risk of cardiovascular diseases. Therefore, there is a trend to use dietary intervention or food-derived components to treat osteoporosis for long-term therapy. The findings of this study provide a new approach for osteoporosis treatment with fewer side effects and be suitable for long-term use. Meanwhile, the mechanisms and signaling pathway study provided better understanding on the regulatory role of ovotransferrin and will help with the development of new therapeutic approach to maintain bone health.

7.3 Future Research Directions

It should be noted that there are limitations in the research reported in this thesis. Firstly, we used murine cell lines for the *in vitro* studies (preosteoblast MC3T3-E1 and macrophage RAW 264.7); validation of these effects of ovotransferrin should be made with primary cells and human cell lines, such as hFOB 1.19 and Saos-2. Additionally, it is not know the metabolism of ovotransferrin after oral administration and the responsible metabolites reach to the action site. Thirdly, the animal was double-housed during the husbandry and shared the diet together. Thus, it was difficult to precisely monitor the diet intake and ovotransferrin intake of each rat. As the ovotransferrin was mixed into the diet and could be taken *ad libitum*, the ovotransferrin intake was estimated, but not accurate. Finally, in this study, ovotransferrin exhibited osteoporosis inhibitory effect in ovariectomized (OVX) rats with preserved bone mineral density and microarchitecture; however, no direct evidence indicated the stimulation effect of ovotransferrin on osteogenesis and bone

formation *in vivo*. Based on the key findings and the limitations of the research, the recommended future studies are outlined below:

1. As a protein, orally administration of ovotransferrin will go through the gastrointestinal tract and be digested into small peptides. The bioavailability of ovotransferrin and the remaining intact ovotransferrin after digestion should be studied to estimate the effective dosage of ovotransferrin to exert bioactivity. Since pig's gastrointestinal tract shows almost the same physiology as human, a piglet model might provide insights on the metabolism of ovotransferrin. Also, it remains to be determined if the digested forms are responsible for the activity after orally administration. If not, strategies are needed to protect ovotransferrin against gastrointestinal digestion.

2. In studies using cell lines, ovotransferrin contacted directly with bone cells; however, the absorption of intact high molecular protein in intestinal and transportation to action site (bone cells) is not clear. Although studies have reported that intact lactoferrin could be absorbed into circulation and exert effect in bone cells (Kitagawa et al., 2003), *in vivo* evidence for ovotransferrin is lacking. Protein labeling can be used to track the transportation and circulation of exogenous proteins both *in vitro* and *in vivo*.

3. The action of ovotransferrin involves various pathways. The involvement of MAPK and Akt pathways in regulating ovotransferrin-induced osteogenesis should be confirmed *in vivo*, for example using knockout animal models. Additionally, MAPK and Akt are two signaling pathways controlling almost all cellular activities, including proliferation, differentiation, and apoptosis. Other bone-specific regulatory pathways, such as BMP and Wnt pathways should be included into

the study to further elucidate the bone health promotion role of ovotransferrin. Finally, the membrane receptors of ovotransferrin should be identified.

4. Ovotransferrin administration affected immune function, muscle synthesis, adipogenesis, and short chain fatty acid production in ovariectomized rat model. The contributions of these findings to bone health should be further explored. It remains to be established if these findings might provide additional benefits to other postmenopausal symptoms (e.g. sarcopenia, obesity).

5. In this study, ovariectomized rat model was used to study the role of ovotransferrin on osteoporosis treatment. It remains to be established if ovotransferrin shows similar benefits in other animal models (e.g. aged animal model, glucocorticoid-induced osteoporosis model, or rheumatoid arthritis model), which will broaden the application of ovotransferrin.

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