THE UNIVERSITY of ADELAIDE

TARGETING NF-κB AND NFATc1 SIGNALLING TO INHIBIT BONE RESORPTION IN PERI-PROSTHETIC OSTEOLYSIS

Muhamad Syahrul Fitri ZAWAWI

Discipline of Anatomy and Pathology
School of Medical Sciences
The University of Adelaide

December 2014

Final Thesis Lodgement June 2015

A thesis submitted in fulfilment of the requirement for the
Degree of Doctor of Philosophy in Medicine

TABLE OF CONTENT

TABLE OF ABBREVIATION

αvβ3	Alpha-v-beta-3 integrin
3D	Three dimensional
AnnVIII	Annexin 8
AP-1	Activator protein-1
ATPase	Adenosine triphosphatase
Atpv0d2	D2 isoform of vacuolar (H^+) atpase (V-atpase) V0 domain
BMD	Bone mass density
BMMs	Bone marrow macrophages
BMU	Basic multicellular unit
C	Carbon
CaMKs	Calcium/calmodulin-activated kinase
CAPE	Caffeic acid phenethyl ester
CathK	Cathepsin K
ChIP	Chromatin immunoprecipitation
CIA	Collagen-induced arthritis
CoCr	Cobalt chrome alloy
Co-Cr-Mo	Cobalt-chromium-molybdenum alloy
cpTi	Commercially pure titanium
CsA	Cyclosporin A
CSF-1	Colony stimulating factor 1
CTR	Calcitonin receptor
CTX-1	Serum type-1 carboxy-terminal collagen crosslinks
DAG	Diacylglycerol
DAP12	DNAX-activating protein 12kda
DC-STAMP	Dendritic-cell transmembrane protein
EU	Endotoxin units
FBGCs	Foreign-body giant cells
FcRγ	Fc receptor common gamma subunit
FK506	Tacrolimus
FKBP12	FK506-binding protein 12
GFP	Green fluorescent tagged
GIT	Gastrointestinal tract
GM-CSF	Granulocyte-macrophage colony-stimulating factor
H^+	Hydrogen ions
HCl	Hydrochloric acid
HMEECs	Human middle ear epithelial cells
ID2	Inhibitor of differentiation 2
Ig	Immunoglobulin
IL	Interleukin
INF-γ	Interferon-γ
IP3	Inositol-1,4,5-trisphosphate
ITAM	Immunoreceptor tyrosine-based activation motif
ITIM	Immunoreceptor tyrosine-based inhibitory motif
JNK	C-Jun terminal kinase

LPS	Lipopolysaccharides
LRC	Leukocyte receptor complex
MAGPHPVIVITGPHEE	Commercially available calcineurin inhibitory peptide motif VIVIT
M-CSF	Macrophage-colony stimulating factor
MITF	Microphtalmia transcription factor
MMPs	Lysosomal proteases matrix metalloproteinases
NC100	Natural compound 100
NFATc1	Nuclear factor of activated T-cells, cytoplasmic, calcineurin-dependent 1
O	Oxygen
OCIF	Osteoclasts inhibitory factor
ODF	Osteoclast differentiation factor
OPG	Osteoprotegerin
OPG-Fc	OPG-fusion protein
OPGL	Osteoprotegerin ligand
OSCAR	Osteoclast-associated receptor
OVX	Ovariectomized
PAR	Parthenolide
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate-buffered saline
P-C-P	Bisphosphonates
PD	Periodontal diseases
PE	Polyethylene particles
PGE2	Prostaglandin E2
PIAS3	Protein inhibitor of activated STAT 3
PIP2	Phospholipid phosphatidylinosotol-4,5-bisphosphate
PIR-A	Paired Ig receptor-A
PLCγ	Phospholipase C gamma
PLOSL	Polycystic lipomembranous osteodysplasia with sclerosis leukoencephalopathy
PMMA	Polymethylmethacrylate
P-O-P	Pyrophosphate
PTK	Src family protein tyrosine kinase
RA	Rheumatoid arthritis
RANK	Receptor activator of NF-κB
RANKL	Receptor activator of NF-κB ligand
RUNX-2	Runt-related transcription factor 2
SDF-1	Stromal-cell derived factor-1
SH2	Src homology 2
siRNA	Small interfering RNA
SS	Stainless steel
STA	Arthritic serum transfer
Syk	Spleen tyrosine kinase
Ti	Titanium alloy

TiAlV	Titanium-6-aluminium-4-vanadium alloy
TNF	Tumor necrosis factor
TRAF	TNF receptor activating factor
TRANCE	TNF-related induced cytokine
TRAP	Tartrate-resistant acid phosphatase
TREM2	Triggering receptor expressed in myeloid cells
TYROBP	Tyrosine kinase binding protein
UHMWPE	Ultra-high-molecular-weight PE
VIVIT	11R-VIVIT peptide
μCT	Micro-computed tomography

ABSTRACT

Peri-prosthetic osteolysis is a bone loss disease involving granulomatous inflammation in the soft tissues around prostheses characterised by excessive bone resorption adjacent to implants. Macrophages phagocytose particles of prosthetic material, thereby inducing persistent release of pro-inflammatory osteoclastogenic cytokines, such as, receptor activator of NF-κB ligand (RANKL). RANKL interacts with its receptor RANK, to activate key transcription factor in osteoclastogenesis, NFATc1. This activates an inflammatory response leading to bone erosion at the implant bone interface and subsequent prosthetic failure. Other bone cells are affected by particles. For example, osteoblasts have reduced bone formation activity, and osteocytes, undergo apoptosis, whereby in this process they release cytokines that stimulate bone loss.

Apart from the RANKL/RANK-NFATc1 system, immunoreceptor tyrosine-based activation motif (ITAM)-dependent pathway has been identified as a co-stimulatory pathway in osteoclasts. Osteoclast-associated receptor (OSCAR) and TREM2 are ITAM-containing receptors pairing with adaptor molecules FcRγ and DAP12, respectively. Our group has demonstrated the increased expression of NFATc1 and ITAM-related molecules adjacent to sites of bone loss in human peri-prosthetic tissues and polyethylene (PE) particle-stimulated osteoclasts *in vitro*. Soluble OSCAR has been proposed as a potential regulator of osteoclast activity in osteolysis. Considering the importance of NF-κB and NFATc1 we hypothesize that their inhibition of will abrogate osteoclast bone resorption.

The data presented here showed that inhibition of NFATc1 by calcineurin-NFAT inhibitors, FK506 and VIVIT, and inhibition of both NFATc1 and NF-κB by NC100, significantly suppressed osteoclast formation and activity in normal human-derived RANKL-induced osteoclast differentiation *in vitro*. mRNA expression analysis showed OSCAR was inhibited by FK506, VIVIT and NC100 at later stages of osteoclastogenesis. Together, this demonstrates potential benefits of targeting NFATc1 and NF-κB to suppress osteoclastogenesis and modulate ITAM-containing molecules in human osteoclasts.

In the murine calvarial model of PE-induced peri-prosthetic osteolysis, live animal micro-computed tomography analyses showed that PE particles significantly induced localised osteolysis in mice implanted with PE particles compared to controls. PAR strongly reduced surface bone resorption but not local bone volume. However, CAPE

treatment reduced local PE-induced calvarial osteolysis at both surface resorption and volumetric change. Additionally, PE particles significantly increased serum levels of bone resorptive marker CTX-1 and soluble OSCAR. However, neither PAR nor CAPE regulated CTX-1 and OSCAR in PE-implanted mice. Neither PE nor CAPE affected the gastrointestinal tract, a potential side effect of their treatments.

Interestingly, PE particles strongly enhanced osteocyte death, supporting previous reports that osteocytes undergo apoptosis in response to PE particles. PAR significantly decreased osteocyte apoptosis in PE-implanted mice, as assessed by reduced number of osteocytes with empty lacunae within the calvarial tissues. It could be important to determine the possible mechanism by which PE particles activate NF-κB in osteocytes as this could be an important mechanism inducing osteolysis in response to prosthetic particles.

Overall the results demonstrate that targeting NFATc1 and NF-κB signalling suppresses osteoclast differentiation and resorption induced by PE particles. Future studies are necessary to fully understand this pathology.

STUDENT DECLARATION

I certify that this work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution in my name and, to the best of my knowledge and belief, contains no material published or written by another person, except where due reference has been made in the text. In addition, I certify that no part of this will, in the future, be used in a submission in my name, for any other degree or diploma in any university without the prior approval of the University of Adelaide and where applicable, any partner institution responsible for the joint-award of this degree.

The author acknowledges that copyright of published works contained within this thesis resides with the copyright holder(s) of those works.

I, undersigned, the author of this thesis, give consent to this copy of my thesis when deposited in the University Library, being made available for loan and photocopying, subject to the provisions of the Copyright Act 1968. I understand that the University of Adelaide will make it available for use within the University library. I also give permission for the digital version of my thesis to be made available on the web, via the University's digital research repository, the Library catalogue and also through web search engines, unless permission has been granted by the University to restrict access for a period of time.

Beyond this, I do not wish to place any restriction on access to this thesis.

……..…

(Muhamad Syahrul Fitri ZAWAWI)

1158647 The University of Adelaide

Date: 14/12/2014

LIST OF JOINTLY AUTHORED PAPERS INCLUDED IN THESIS

CHAPTER 2

Regulation of ITAM adaptor molecules and their receptors by inhibition of calcineurin-NFAT signalling during late stage osteoclast differentiation

***M.S.F. Zawawi**, A.A.S.S.K. Dharmapatni, M.D. Cantley, K.P. McHugh, D.R. Haynes, T.N. Crotti.*

Biochemical and Biophysical Research Communications 2012; 427(2):404-9.

CHAPTER 3

Molecular mechanisms and therapeutic effects of NC100 on osteoclastic bone resorption and osteoporosis

*Qian Liu, Tao Wang, **Muhamad Syahrul Fitri Zawawi**, Tania Crotti, An Qin, Zhen Lin, Jinbo Yuan, Huagang Liu, Minghao Zheng, Jiake Xu, Jinmin Zhao.*

Journal of Cellular Physiology (manuscript in preparation).

CHAPTER 4

Parthenolide inhibits osteocyte apoptosis and osteoclastic bone surface resorption induced by polyethylene particles in a murine calvarial model of peri-implant osteolysis

***Muhamad S F Zawawi**, Victor Marino, Egon Perilli, Melissa D Cantley, Jiake Xu, P. Edward Purdue, Anak A S S K Dharmapatni, David R Haynes, Tania N Crotti.*

Journal of Biomedical Materials Research Part A – accepted April 2015 as "Parthenolide reduces empty lacunae and osteoclastic bone surface resorption induced by polyethylene particles in a murine calvarial model of peri-implant osteolysis"

CHAPTER 5

Caffeic acid phenethyl ester abrogates bone resorption in a murine calvarial model of polyethylene particle-induced osteolysis

***M.S.F. Zawawi**, E. Perilli, R.L. Stansborough, V. Marino, M.D. Cantley, J. Xu, A.A.S.S.K., Dharmapatni, D.R. Haynes, R.J. Gibson, and T.N. Crotti.*

Calcified Tissue International - accepted March 2015.

SCIENTIFIC COMMUNICATIONS

INTERNATIONAL:

2015

Work presented to Prof Heinz Redl at the Ludwig Boltzman Institute, Vienna, Austria.

2014

The 2nd Asia-Pacific Bone & Mineral Research (APBMR) Meeting in conjunction with the 26th Spring Scientific Congress of Korean Society for Bone and Mineral Research and the 2nd Seoul Symposium on Bone Health, Seoul, South Korea – Oral.

2013

American Society for Bone Mineral Research (ASBMR) Annual Meeting – Poster.

Hospital for Special Surgery (HSS), New York – Oral.

2011

International Osteoporosis Federation (IOF) 2nd Asia-Pacific Osteoporosis and Bone Meeting: Australian & New Zealand Bone & Mineral Society (ANZBMS) Annual Scientific Meeting with Japanese Society for Bone and Mineral Research (JSBMR), Queensland, Australia – Poster.

NATIONAL AND LOCAL:

2014

Florey International Postgraduate Research Conference, Faculty of Health Sciences (FHS) Postgraduate Research Conference 2014, The University of Adelaide. Adelaide, Australia – Poster.

20th Annual Conference of Australian & New Zealand Orthopaedic Research Society (ANZORS), Adelaide, Australia – Oral.

Invited presentation and laboratory visit. School of Pathology and Laboratory Medicine, The University of Western Australia. Sir Charles Gairdner Hospital, The University of Western Australia, Perth. – Oral.

School of Medical Sciences, The University of Adelaide. PhD Progress Seminar – Oral.

1st Australasian Bruker Skyscan User Meeting. SA, Australia – Oral.

2013

Australian & New Zealand Bone & Mineral Society (ANZBMS) 23rd Annual Scientific Meeting, Melbourne, Australia - Poster.

19th Annual Conference of Australian & New Zealand Orthopaedic Research Society (ANZORS), Sydney, Australia – Oral.

Faculty of Health Sciences (FHS) Postgraduate Research Conference 2013, Adelaide, Australia - Poster.

Australian Society for Medical Research (ASMR) South Australia Annual Scientific Meeting – Oral.

1st Malaysian Postgraduate Student Symposium of South Australia 2013, Adelaide, Australia – Oral.

Australian Rheumatology Association (ARA) South Australia Annual Scientific Meeting, Adelaide, Australia – Oral.

2012

Australian & New Zealand Bone & Mineral Society (ANZBMS) 22nd Annual Scientific Meeting in conjunction with 1st Asia-Pacific Bone and Mineral Research meeting, Perth, Australia – Poster.

Faculty of Health Sciences (FHS) Postgraduate Research Conference 2012, Adelaide, Australia - Poster.

Australian Society for Medical Research (ASMR) South Australia Annual Scientific Meeting, Adelaide, Australia - Poster.

ADDITIONAL ACHIEVEMENTS DURING CANDIDATURE

2014

Winner of MASCA-MONASH University Science Excellence Award (National Level), Australia. Malaysian Summit of Australia (MASA) 2014, Malaysian Students' Council of Australia (MASCA) National, Australia.

Travel award of The School of Medical Sciences Postgraduate Travel Award (International Conference Attendance). Faculty of Health Science (FHS), The University of Adelaide, SA, Australia.

Invited speaker (panelist). Postgraduate Forum, organized by Malaysian Postgraduate Student Association of South Australia Incorporated (MyPSA SA Inc.) and Adelaide University Malaysian Students Association (AUMSA), SA, Australia.

Role-Model Exemplary Excellence Award 2014 (Anugerah Murabbi Terbaik 2014). Australian-Malaysian Muslim Solidarity, South Australia Chapter (ISMA SA). South Australia, Australia.

2013

A Faculty Prize (Best Poster Presentation). Faculty of Health Sciences (FHS) Postgraduate Research Conference 2013. The University of Adelaide, SA, Australia.

A Travel Grant Award to attend a national conference. The 19th Annual Conference of Australian & New Zealand Orthopaedic Research Society (ANZORS). New South Wales, Australia.

Invited Main Speaker for an Academic Motivational Talk. Title "From Zero to Hero." Pursuit of Knowledge (Program Kembara Ilmu) by Australian-Malaysian Muslim Solidarity, South Australia Chapter (ISMA SA), SA, Australia.

2012

Finalist for the Adelaide Research & Innovation Pty Ltd (ARI) Award. Faculty of Health Sciences (FHS) Postgraduate Research Conference 2012. The University of Adelaide, SA, Australia.

Academic and Character Excellence Award 2012 (Anugerah Kecemerlangan Akademik dan Sahsiah (AKASIA)). Australian-Malaysian Muslim Solidarity, South Austalia Chapter (ISMA SA). South Australia, Australia.

2011

RLKA Scholarship (Full), School of Medical Sciences, Universiti Sains Malaysia (USM).

SLAB/SLAI (PhD) Scholarship (Full), Ministry of Education (MoE), Malaysia.

ACKNOWLEDGEMENTS

Supervisors

The highest gratitude to my principal supervisor, **David Haynes** for all the opportunities, guidance and inspiration. The massive sincere thanks to **Tania Crotti** and **Kencana Dharmapatni** for endless ideas, communications, your understanding, helps and many others. I wish to thank you all for the opportunities to embark my PhD journey here, for understanding the way I do my works and my other responsibility and commitments, etc etc etc – everything! including family support. You make us feel like home! Thank you, thank you and thank you.

Scholarship / sponsors

Special thanks to **Universiti Sains Malaysia (USM)** for accepting my application and sponsoring my family to Australia. Big thanks to the **Ministry of Education (MoE), Malaysia** for the scholarship and all.

Bone and joint research group

Thank you very much to **Melissa Cantley** for being so helpful and inspiring, **Kent Algate** for being the coolest guy every time, **Sylvia Saad** for your donut (sharing passions in food and cooking, I mean LOL), **Emma Murison** for your assistance in Nanozoomer and camera, **Roxanne Coleman**, **Hannah Kamitakahara** and **Ekram Alias** for awesome friendship.

The amazing people

Egon Perilli for your guidance in microCT, **Dale Caville** for imaging, **Victor Marino** for your helps with animal experiments and ethics, **Jiake Xu** for the input regarding the inhibitors, **Histology Lab** (Nadia, Emily, Chris Leigh), **Animal House staff** in IMVS and Adelaide Uni, **Adelaide Microscopy** (Ruth, Ken), **School Office** (Andrea Spedding, Martin Hutchens), Gut Microbiome Group **(Romany Stansborough** and **Rachel Gibson)**, collaborators especially **Ed Purdue, Kevin McHugh, Liu Qian**. Everyone!!

Family in Malaysia

I wholeheartedly thank my mother, **Fatimah Mat Noor (Umi)** for your love and smiles and father, **Hj Zawawi Derahman (Abah)** for your love and care. My strongest thanks to sister, **Nurul Husna (Adik)** for being soooooo amazingly strong taking care of Umi and Abah when Abe was not around. In addition thanks to my mother-in-law **Dawiyah Yajid (Ma)** for your love, hope, faith

and thiqah. Also thanks to all members in my family and in-laws whose names I don't mention here. You know who you are.

My wonderful family! <3

No words can describe how much I thank you, my wonderful **zaujah (wife), Rahimah Ab Rahman** - you're my absolute everything EVERYTHING! Yes, my most trustworthy thanks for everything. To my beautiful daughters, **Sumayya Tasneem** you're my biggest strength and **Iynas Yusra** you're my highest inspiration. Love you all <3 <3 <3

Bestest friends

I can't thank you enough, **Farhan Salmi** for being patient with my craziness (LOL) and for being there, always there! Thanks to **Fikri Abd Rahim** for your help and encouragements, **Annas Yahya** for your thought and concerns. And also to **Qamarus Salikin**, **Muslim Roslan** and **Sufian Ishak** for being my best friends since school age and supporting my undertakings.

Friends, ikhwah and akhawat

Special thanks to **Arshad Sideek**. Thanks to Zulhazri Isera, Naim Yusof, Aizat Farid, Ashraf Azman, 'Izzat Almi, Nizam Zakaria, Abg Zaki Ibrahim, Akmal Ghani, Saifuddin Khalid, Arif Fitri, Umair Abdullah, Ammar Muhd, Khair Razlan, …… and all ikhwah and friends (rumah Robert, rumah ET, rumah Meredith, everybody!) Thanks to **Siti Mariam Noor Din, Atiqah Azis** and all akhawats. All including ISMA, MyPSA SA, MASCA, KUASA and all. No enough space to write everyone's names. You know who you are.

A saying (hadith)[Sunan Abu Dawud 4811] Abu Huraira reported: The Prophet Muhamad (ﷺ) said:

"He who does not thank people does not thank the almighty God (Allah)."

Therefore I wish to say; thank you. Whoever you may be, who have helped me in some way or another, some which I am aware of, some which I might not be aware of; help, smiles, wishes, prayers including my online friends, especially on Facebook, for your likes, comments, replies, posts, and supports. Maybe I have not said thank you to you before, maybe I have, but now I am saying **thank you** to you. Jazakumullah khairan kathiran.

CHAPTER 1. Inhibition of NF-κB and NFATc1 signalling in peri-prosthetic osteolysis

1.1.Local inflammation-mediated bone loss

Bone loss occurs when there is a net change in overall bone because of the imbalance in bone formation and resorption. In general, there are two main categories of bone loss characterised by defective bone remodelling. They are the systemic and localised bone loss. Osteoporosis is one example of the systemic bone loss [1]. It is an ageing-related disease that results in bone fragility because of low bone mineral density [2]. Some examples of localised bone loss associated with inflammation are peri-prosthetic osteolysis [3], periodontal diseases (PD) [4, 5] and rheumatoid arthritis (RA) [6].

The term osteoimmunology has been used to describe the close interrelationship between bone homeostasis and immune systems [7]. Specifically osteoimmunology refers to the complex crosstalk between immune system and skeletal systems involving coordination of lymphocytes, mast cells and macrophages on osteoclasts, osteoblasts and osteocytes [2, 8-10]. It is important to understand the normal physiology in order to also understand the processes that lead to bone pathologies. Understanding these processes is also important in the context of inflammation-mediated bone loss, such as particle-induced peri-prosthetic osteolysis, PD and RA where there is local bone erosion [11-14].

This thesis specifically focuses on the osteoimmunology of localised bone loss that occurs in particle-induced peri-prosthetic osteolysis. In this thesis the signalling pathways important in osteoclast formation and activity *in vitro* as well as *in vivo* using an animal model, have been investigated. Regulation of immunomodulatory factors that play role in bone homeostasis is the main focus of the study. The aim is to identify a therapeutical approach that modulates both the immune system and bone metabolism in peri-prosthetic osteolysis. This approach may also be relevant to other diseases of local inflammation-mediated bone loss.

1.1.1.Particle-induced peri-prosthetic osteolysis

Prosthetic failure occurs due to the loss in bone stock around the prostheses, leading to loss of fixation of the prostheses. Prosthetic failure has been a major concern since recent reports that 5 to 30% of total joint replacement requires revision surgery within 15 to 20

years after prostheses implantion [15-17]. Hip and knee replacement has been increasing at 5 to 10% year after year for the past 10 years, with a combined increase in hip and knee replacement of 13.4% in the past year [15]. This may result in a dramatic increase of revision surgery which is more complicated and costly than primary joint replacement[18]. In addition, prosthetic failure affects people at all ages. Younger people, below 59 years of age, have a 27% risk of prosthetic failure compared to a 13% risk for people at 59-65 years old and 7.5% risk for 65-70 years old people [19]. It is very alarming that, despite new materials, prosthetic failure may greatly accelerate to become a major health burden. There are several factors promoting prosthetic failure including poor surgical technique, stress shielding, mechanical failure, infection and prosthetic fracture [20-22]. Literature, including the 2010 Australian National Joint Replacement Registry, have identified particle-induced peri-prosthetic osteolysis as the most common factor causing prosthetic failure requiring revision surgery [15].

Peri-prosthetic osteolysis, also known as peri-implant osteolysis or aseptic loosening, is a bone loss disease involving granulomatous inflammation in the soft tissues around prostheses [23]. It is often characterised by an increase of bone resorption adjacent to implants induced by wear particles of prosthetic materials [24]. The prosthetic particles are phagocytosed by macrophages and other phagocytic cells such as foreign-body giant cells (FBGCs) [20, 24-26], fibroblasts and dendritic cells resulting in granulomatous lesions predominated by macrophages at the interface of bone-cement around loosening implants [23]. This induces persistent release of inflammation-triggering mediators, chemoattractants and osteoclastogenic pro-inflammatory cytokines, such as receptor activator of NF-κB ligand (RANKL) and tumor necrosis factor (TNF) [10, 20, 27-34]. This leads to tissue and bone breakdown and subsequent prosthetic failure [10, 35].

1.1.2.Polyethylene (PE) particles

There are several types of materials used in and around prostheses [36]. Commonly used materials are polyethylene (PE) [20], polymethylmethacrylate (PMMA) cement [37], titanium alloy (Ti) [24, 38], ceramics [39] and metals [39-42]. The particles of cast and forged cobalt chrome alloy (CoCr), 316L stainless steel (SS) and titanium-6-aluminium-4-vanadium alloy (TiAlV) [24, 40], and commercially pure titanium (cpTi) [41] can elicit

significantly different biologic responses. Only slight differences in the composition of particles has been shown to stimulate higher levels of osteolytic agents interleukin-1 (IL-1), interleukin-6 (IL-6), TNF and prostaglandin E2 (PGE2) expressed in human monocytes [38]. These *in vitro* studies show particles of different prosthetic materials may induce different cytokines and thus have different effects on bone remodelling [24, 26, 40, 43-46]. Consistent with this, studies using animal models have also shown different effects to different biomaterials used in prostheses [47] (described in **Section 1.1.3**). Additionally, a combination of prostheses PMMA and ultra-high-molecular-weight PE (UHMWPE) resulted in a synergistic increase in membrane thickness suggesting that multiple biomaterials markedly increase the inflammatory reaction [47]. Together these studies support the idea that the extent of osteolysis is highly dependent on the materials used [41, 47].

This thesis investigates the effects of PE particles on osteolysis, as PE is one of the most frequently used materials and a common source of wear debris in clinical practice [48-54]. Many studies have illustrated a strong correlation between PE wear rates and the extent of osteolysis [55-58]. It has been found that PE is actively liberated from prostheses with evidence that the highest concentration of PE particles is present at the sites of osteolysis [49, 59-62]. It was reported that more PE particles are detected in peri-prosthetic tissues with osteolysis when compared to the tissues without osteolysis [49]. Patients with PE-containing prostheses suffer a higher incidence of peri-implant osteolysis compared to the patients with metal-on-metal hip arthroplasty [63, 64]. The significance of PE particles as a causative agent for osteolysis has also been demonstrated *in vitro* [28, 30, 31, 65]. Taken together these studies show that PE may be the most damaging types of wear particles associated with peri-prosthetic osteolysis, therefore, a key factor inducing bone loss in peri-prosthetic osteolysis [31, 49, 51-55, 66].

Apart from the local accumulation of wear particles inducing local osteolysis, particles have also been shown to travel to other regions, such as lymph nodes [67]. This in turn can induce distal bone resorption directly or indirectly via inflammatory factors [68]. It is possible that distal inflammatory factors may also affect other organs such as the gastrointestinal tract (GIT). This thesis investigates the effects of PE wear particles locally, and also systemicly. The systemic effects of wear particles can be measured by studying

the biochemical marker of bone breakdown, serum type-1 carboxy-terminal collagen crosslinks (CTX-1) [69-71], which reflects overall bone resorption activity *in vivo* [72]. The systemic effects of distal inflammatory factors on GIT can be determined by analyzing jejunal and colonic tissue structures and cell components including identification of apoptotic cells based on histopathology [73-75].

1.1.3.Murine calvarial model of PE-induced peri-prosthetic osteolysis

Complementing the *in vitro* models of particle-induced osteoclastogenesis, several animal prosthetic implant models have been developed to investigate the pathogenesis of aseptic loosening and to mimic the clinical situation [76]. Animal models have also directly demonstrated the significance of PE particles as the causative agent for osteolysis *in vivo* [54, 77, 78].

One example is the murine air pouch model that has been developed to investigate the number of cells infiltrating the air pouch in response toPE compared to other biomaterials TiAlV, CoCr and PMMA [47]. The cellular infiltration and mediators of inflammation generated in this model closely resemble the peri-prosthetic tissues associated with aseptic loosening [79, 80]. PE, TiAlV, CoCr and PMMA particles tested increased the absolute number and percentage of macrophages in the membrane when compared to controls [47]. Importantly, PE particles induced the largest numbers of macrophages within the tissue [47]. This air pouch model has also been used to investigate the effects of PE particle shape in adverse inflammatory reactions [81]. When compared to globular particles, elongated PE particles result in larger increase in IL-1β and TNFα levels. In addition, more severe apoptotic changes within the inflammatory membrane, membrane proliferation and inflammatory cellular infiltration [81]. Even though this air pouch mouse model provides copious and sufficient tissue space for particle injections and multiple analyses [81] and biomaterials are deeply embedded within the tissue and surrounded by macrophages [47, 79], this model does not include bony tissue [47, 81]. Bone is needed to accurately assess the osteolytic component of particle-induced aseptic loosening [47, 81].

A more relevant *in vivo* model to study peri-prosthetic osteolysis that enables bony tissue assessment is calvarial mouse model which was first reported in 1999 by Merkel *et al* [82]. Briefly, the model involves a surgery to implant the particles [82, 83] on

periosteum over the calvarium and the incision closed. The osteolysis was mostly investigated and quantitated through histology of the calvarial tissues [83] and volumetric analysis in micro-computed tomography (μCT) [67, 84]. The model has been modified and improved to further understand the pathogenesis of particle-induced peri-prosthetic osteolysis. Different types of wear particles which have been used as prostheses in clinical situation [76] such as PMMA [82], Ti [83, 85] and PE [67, 84-88] have now been tested in the model.

In vivo studies using murine calvarial model have shown the implantation of PE particles at a concentration of 2×10^8 particles/mL significantly induced osteolysis as shown by μCT after 7 days [86], 12 days [84] and 14 days [67, 87-89] after administration of PE particles. The PE particles used were commercially pure polyethylene particles (UHMWPE, Ceridust VP 3610) obtained from Clariant Company (Gersthofen, Germany) [67, 84, 86-88]. Greater than 35% of the particles were smaller than 1μm, with a mean particle size (given as equivalent circle diameter) of 1.75±1.43μm (range 0.05-11.6) [67, 84, 87, 88, 90, 91]. It has been shown that phagocytosis and cell mortality increase with particle size and concentration [92]. It has also been shown that the concentration of particles within a critical size range was important for biological responses [93]. This is accordant with a previous report that the severity of the cellular response in peri-prosthetic tissues was related to the number of wear particles [94]. Together these studies clearly depict the role of the number and size of particles to determine the extent of particle-induced osteolysis [81]. To be consistent with previous *in vivo* studies, this thesis investigates the effects of PE particles on the calvarial mouse model of peri-prosthetic osteolysis using similar sized PE particles and implanted at similar concentrations to those previously used.

In order to investigate particle-induced osteolysis, it is important to eliminate any other possible factors contributing to osteolysis. This includes a common contaminant, endotoxins [95, 96]. Endotoxins are lipopolysaccharides (LPS) derived from cell membrane of Gram-negative bacteria [96, 97], often found in water, tissue culture media and serum used for tissue culture and other materials used to grow cells in culture [96, 97]. Endotoxins stimulate monocytes and macrophages to release osteoclastic pro-

inflammatory mediators, such as TNF, IL-1 and IL-6. This also activates the immune system [96, 97] and induces endotoxin shock, tissue injury and death [98, 99].

The term endotoxin units (EU) have been used to describe the biological activity of an endotoxin in pharmaceutical and biological products. The maximum levels have been set to 5 EU per kg of body weight per hour by all pharmacopoeias [100]. LPS can contaminate particles that have been handled or in contact with bacteria and/or LPS itself [96]. Washing particles in 100% ethanol for 48 hours [86, 101] successfully decontaminates endotoxin from particles to <0.1 EU/ml, as detected by a commercial detection kit (Chromogenic End-Point TAL with Diazo Coupling Kit; Xiamen Houshiji Ltd., Xiamen, China) [86, 101]. Following this, particles are washed, centrifuged and resuspended in phosphate-buffered saline (PBS) to completely eliminate ethanol and stored in sterilized endotoxin-free tubes at 4°C till use [86, 101].

Another possible source of LPS is direct animal contact with LPS/bacteria. Macrophages are extremely sensitive to LPS [96] and can induce osteoclast formation and resorption activity via the activation of NF-κB signalling (described in **Section 1.3.3**) [95]. Therefore, it is crucial that *in vivo* studies are carried out using LPS-free animals. As a result of *Lps* gene mutations, LPS signal transduction in C57BL and C3H/HeJ mouse strains was impeded, thereby, inhibiting bone resorption and osteolysis [102]. Based on this the LPS-resistant C57BL [27, 67, 84, 86-88, 103-105] and C3H/HeJ [82, 106, 107] mice have been used as the murine models of particle-induced peri-prosthetic osteolysis. In this thesis C3H/HeJ mice were used due to access and availability as approved by the ethics committee at The University of Adelaide (M-2001-070) and SA Pathology (106/10).

It has been shown that the extent of osteolysis in a particle-induced murine calvarial osteolysis in young 4 week old mice peaks 7 days after the particle implantation and returns to baseline levels after 13 days [108]. This was 17-fold less than that in aged mice (104 weeks old) [108]. Importantly osteolysis returned to near normal levels in these young mice and this would reduce susceptibility to aseptic loosening in young patients [108]. Additionally adult mice at 12 to 64 weeks old of age only showed a moderate response to particles and treatments due to their normal skeletal growth [108]. Our research group has established a 14-day murine calvarial model of PE-induced peri-prosthetic osteolysis based on a model developed by Wedemeyer *et al*. [67, 87, 88] using

6- to 8-week-old LPS-resistant C3H/HeJ mice. The age 6- to 8-week-old can eliminate the factors in bone growth in mice that have not yet reached adult age at the end of the three weeks of experiment. Considering the findings that PE-induced osteolysis was at significant levels after 7 days [86], 12 days [84] and 14 days [67, 87, 88] after administration of particles, this project aims to determine the extent of osteolysis at day 14, which is a later stage of the osteolysis compared to early stage (day 7) where the process of osteolysis was reported as at its peak. Our model comprises histological and μCT analyses and is further described in Chapter 4 and 5 in this thesis.

Most previous studies only quantified the calvarial volumetric bone loss between animal groups by the *ex vivo* μCT scans at the final day of experiments [67, 82-84, 86-88]. Quantification of the volumetric osteolysis would be more specific if the volumetric change of each animal was determine throughout time. Therefore, studies carried out in this thesis involved the live-animal μCT volumetric assessment at baseline (day 0) being subtracted from the *ex vivo* μCT volumetric assessment at the final day of the experiment. This can take into account the bone growth throughout time and the volumetric differences between controls, diseased and treated/untreated animal groups over time. To our knowledge there are only limited studies investigating the calvarial volumetric osteolytic change over time [85]. The major advantage of the model used in this thesis is that osteolysis over time was used with each mouse being its own control. This increases statistical reliability and reduces the number of animals that need to be used for statistical significance.

It has been demonstrated that resorption near prostheses occurs on the bone surface at the interface [109] and at sites where there are high concentrations of PE particles are present near the surface tissues [20, 110, 111]. From this study the surface erosions are considered highly important in peri-prosthetic osteolysis because this better reflects the *in vivo* human situation. In support of this, recent studies have shown the effect of particles, on other bone cells, such as osteoblasts and osteocytes (described in Section 1.4 and 1.5 respectively), may also contribute to osteolysis [112, 113]. Whether bone surface resorption is due to osteoclast, osteoblast or osteocyte response to particles or activities is yet to be determined. In this thesis the aim is to develop a novel technique to both analyze and numerically quantify bone surface resorption by adapting μCT-derived three-

dimensional (3D) images in a murine calvarial model of osteolysis.

1.2.Bone and normal bone remodelling

Bone is a rigid yet dynamic specialised connective tissue with its microstructure patterned to have maximal strength but with minimal mass [114-116]. Bone is composed of osteocytes, bone forming osteoblasts, bone-resorbing osteoclasts, non-mineral matrix of collagen and non-collagenous osteoid proteins, with inorganic mineral salts deposited within the matrix. Formed by a mineralised matrix to maximise elasticity and strength [66], bone protects the vital organs, such as brain and lungs, and gives mechanical support to the body [117]. Bone provides an environment for blood cell formation in the marrow and acts as a storage site for calcium, phosphorus, proteins and fat [1].

Bone tissues undergo physiological remodelling throughout life even though the skeletal growth, both organic and mineral components [118], has completed [66]. Approximately, 10% of the total bone content is replaced per year in adult vertebrates [2, 119, 120]. There are two major kinds of bones, trabecular and cortical. Trabecular bone is spongy and composed of a honeycomb-like network in the bone marrow compartment, giving the strength to stand stresses and weight-bearing bone. Cortical bone is dense and solid, surrounding the bone marrow space [1]. Bone components are continuously removed (bone resorption) and replaced (ossification or bone formation) by a homeostatic process called bone remodelling [1, 121].

Bone remodelling is essential for the renewal of skeleton, tooth eruption, fracture healing [122], maintenance of the structure and mineral homeostasis [121]. It also enables adaptation to mechanical constraints and maintenance of substantial calcium levels [66] providing structural support for the body and protection for organs in the body, as well as facilitating the movement of the body and materials in the intracellular processes.

Bone remodelling is a complex process dependent upon two tightly coupled mechanisms of bone resorption by osteoclasts and bone formation by osteoblasts [2]. This highly regulated process occurs in the bone basic multicellular unit (BMU) consisting of osteoclasts, osteoblasts and their precursors [119, 123] functioning in a complete coordinated sequence of mechanisms [124]. The bone constituents are continuously turned over and restructured by the delicate balance of bone deposition and bone resorption [125].

In healthy mature bone, bone formation and bone resorption counter balances each other to maintain a constant bone mass [126] by the integrated activities of two bone-related cells; bone-forming osteoblasts and bone-resorbing osteoclasts [127]. Abnormalities and imbalance of bone remodelling occur when one process occurs at a greater or lesser rate than the other leading to various skeletal disorders including bone loss [127].

1.3.Osteoclasts

Osteoclasts are multinucleated giant cells generally with 10 to 15 nuclei in each osteoclast, but the number of nuclei per cell can also vary from 2 to 100 [128]. Therefore, the cell size may also vary [129]. Within the bone osteoclasts are specialized cells to resorb bones in both normal and pathological bone remodelling and destruction [118] and usually found on the surface of bone resorption sites within shallow hollow structures called resorption cavities or Howship's lacunae (see **Figure 1.1**) [130].

Osteoclastogenesis or osteoclast differentiation is a 4-stage process that allows osteoclast precursor cells, including monocytes or CD14-positive cells [131], macrophages [24, 132] and dendritic cells [133] to develop into mature and functioning osteoclasts [115, 122]. Osteoclasts are highly motile cells, however, active differentiated osteoclasts *in vivo* are only found in close locality to mineralised bone and they are infrequently found in blood circulation [118]. Throughout this process osteoclasts express different types of osteoclast-associated markers, which enable determination of the stage of osteoclast differentiation. The step-by-step osteoclastogenesis is illustrated in **Figure 1.1**. The first stage of osteoclastogenesis is the recruitment of precursor cells from hematopoeitic lineage [134] and monocyte-macrophage lineage [135]. These cells are believed to belong to the leukocyte family of blood cells [118], that are likely to mature towards the osteoclastic lineage in the presence of RANKL and macrophage-colony stimulating factor (M-CSF) [2, 136]. The second stage is the multinucleation, a fusion process between mononuclear pre-osteoclasts to form non-functional polykaryons where by they express dendritic-cell transmembrane protein (DC STAMP), an important protein for osteoclast multinucleation. Following the third stage of the differentiation, which is the maturation and activation of polykaryons, the polykaryons undergo the fourth stage of osteoclastogenesis to become functional osteoclasts. This final stage is an activation of mature osteoclasts involving

cytoskeletal rearrangement for the cell motility and attachment to the bone matrix only occurring at or in close proximity to the site of bone resorption [137, 138]. Taken together the osteoclast differentiation pathway is common to that of macrophages and dendritic cells where a pro-myeloid precursor can develop into either an osteoclast or a macrophage or a dendritic cell [115, 120], depending on the type of receptor activator it is exposed to [122].

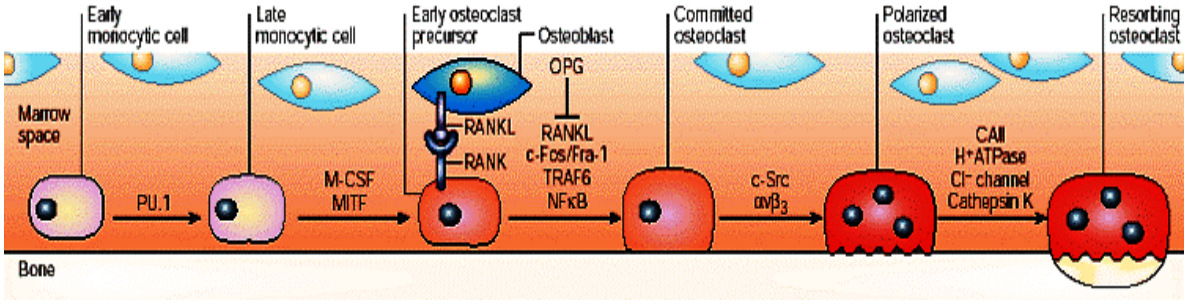

Figure 1.1 Regulation of osteoclast formation and function, and the factors involved [139].

Tartrate-resistant acid phosphatase (TRAP), a type of enzyme encoded by Acp5 [131, 140, 141], is one of the prominent osteoclast-associated cell markers widely used for the detection of osteoclasts in histochemistry [142]. TRAP is a reliable marker of osteoclast number and bone resorbing activity [131]. However, it is not only highly expressed by osteoclasts, but also inflammatory macrophages, dendritic cells [140] and FBGCs [143] and is found in human blood circulation in adults and children [144]. TRAP knock-out mice showed osteoclast formation and reduced bone resorption activity [140]. Overexpression of TRAP increased bone turnover [145]. However, TRAP has now been shown to be reflective of osteoclast number rather than activity [146, 147].

Another prominent osteoclast-associated cell marker is cathepsin K (CathK), a mammalian protease enzyme encoded by ctsk [148] and highly expressed in osteoclasts [148]. CathK is also found in other cell types including fibroblasts [149], macrophages and smooth muscle cells in atheroma [150]. The expression of CathK increases throughout osteoclastogenesis [151]. CathK degrades collagen type I at both sides of in and outside helical region [152], collagen type II and bone matrix protein osteonectin [152, 153].

CathK has been shown as a bone-matrix degrading enzyme. Humans with a CathK gene mutation developed osteopetrotic condition where bone hardens and becomes denser as opposed to osteoporosis and it is called as pycnodysostosis disease [79, 154, 155]. This osteopetrotic condition is also observed in CathK-deficient mice [156, 157]. CathK-specific inhibition using peptidyl vinyl sulfones as selective cysteine protease inactivators decreased bone resorption by 80% in a dose-dependent manner [158]. These studies clearly show that CathK plays an important role in bone resorption and is a reliable marker for osteoclast resorption activity.

Alpha-v-beta-3 (αvβ3) integrin (the vitronectin receptor) [160], which is expressed on bone resorbing osteoclasts [161] and regulated by expression of β3 subunit during osteoclast differentiation [162, 163], is a late-stage osteoclast-associated cell marker [125] involved in the attachment of osteoclasts to bone [164-166]. Studies have shown osteoclasts express five integrins α2β1, αvβ1, αvβ3, αvβ5 [165] and α9β1 [167]. High expression levels of integrin αvβ3 occurs at late stage osteoclast formation *in vitro* and is found at the ruffled membrane [165] associated with adhesion [164] and cell spreading [168, 169]. The requirement for αvβ3 in normal osteoclast function is demonstrated by β3 gene genetic deletion in mice [168]. Murine β3-null osteoclasts failed to form actin rings and did not form ruffled membranes, thereby, failing to resorb bone ensuing in osteosclerosis [168].

More recently, Annexin 8 (AnnVIII) has been identified as an osteoclast-specific marker important at late stage osteoclast differentiation and activation [170]. The suppression of AnnVIII expression retards actin ring formation, therefore, impeding cytoskeletal reorganization in osteoclasts [170, 171]. Taken together β3 and AnnVIII play essential roles in terminal osteoclast differentiation and activation and are reliable as osteoclast-specific markers. Importantly all these features including formation of actin rings and ruffled borders, attachment to bone and cytoskeletal reorganization are important for osteoclastic bone resorption mechanism which will be described further in **Section 1.3.1** in this thesis.

Since osteoclasts can be generated from mononuclear phagocytes of diverse tissue sources there are several methods to generate osteoclasts using *in vitro* models from tissues allowing experimental studies of osteoclast development and activity [135]. Additionally

the ability of alveolar macrophages differentiating into osteoclasts as shown by TRAP-positive colonies on mouse bone marrow-derived stromal cell lines [172] suggests that osteoclasts can be derived from mature cells of the monocyte-macrophage lineage. More importantly, osteoclast features characterized by CathK [148, 156-158], TRAP [173], calcitonin receptor (CTR) [174], OSCAR [159], β3 [164, 168, 175, 176] and AnnVIII [170, 171] during the progression of macrophage colony units into osteoclasts enables identification of the stages of the development of osteoclasts from human peripheral blood mononuclear cells (PBMC) *in vitro* and the differentiation can be monitored by measuring the expression of these features.

The multinucleation of osteoclasts is highly important for the bone-resorbing capability and efficiency [129, 177-180]. Murine-derived osteoclasts, incapable of multinucleation *in vitro* have a remarkably reduced resorption capacity [181, 182]. In support of this, murine cells lacking DC-STAMP exhibited a reduction in resorption efficiency *in vivo* leading to osteopetrosis, a condition associated with decreased bone resorbing capacity [178]. Murine cells deficit in d2 isoform of vacuolar (H^+) adenosine triphosphatase (V-ATPase) V0 domain (Atpv0d2), another important protein for osteoclast multinucleation, demonstrated impaired osteoclast fusion and maturation [183]. Osteoclasts possess a high number of mitochondria in their nuclei to generate high amounts of energy needed by the ATPase pump for the secretion of protons [184], thereby, providing synergy in terms of resorption efficiency in the bone resorption mechanism [179, 185]. Taken together, this shows that defects in osteoclast multinucleation reduce bone-resorbing activity. In addition bone resorption activity increases with the number of nuclei in osteoclasts [129, 179, 180] and cell size [129].

Osteoclast bone resorption activity is necessary for many skeletal processes and maintenance of an appropriate blood calcium level. It is obligatory during bone growth, tooth eruption and fracture healing [122]. Likewise, continuous physiological remodelling of bone is dependent on bone resorption [114, 122].

1.3.1.Mechanism of bone resorption

Bone resorption is a cell specific sequential multistep process involving the degradation of bone tissues by mature osteoclasts [114, 166]. The process or mechanism of

bone resorption begins with recruitment, adherence and attachment of mature multinucleated osteoclasts to bone surface [186]. Osteoclasts move to the bone surface via chemotaxis. The osteoclasts recognize the site of attachment by the aid of membrane-bound molecules known as integrins to form a 'sealing zone', which has high number of filamentous actin. αvβ3 integrin has high affinity for adherence to denatured collagen [165], therefore, playing a significant role in the attachment of osteoclasts to bone for resorption [138, 164-166, 168, 187].

Figure 1.1 represents a functioning osteoclast attached to the bone surface with formation of a sealing zone' and Howship's lacuna crucial for bone resorption. Surrounded by ruffled membranes, the sealing zone is formed by a complex cytoskeletal reorganization to enable osteoclast polarization at the resorption site on bone surface [188]. This in turn enables osteoclasts to firmly adhere to bone surface forming Howship's lacunae [130]. The ruffled border and Howship's lacunae increase the surface area of osteoclast-bone contact, thereby, enhancing the efficiency of osteoclastic bone resorption activity.

The first phase of bone resorption mechanism, the inorganic phase (bone demineralisation), begins with the release of bone degrading factors such as H^+ ions and matrix degrading enzymes [189]. Via exocytosis [190] osteoclasts release hydrogen ions (H^+) in the form of hydrochloric acid (HCl) and acidify the sealed region. This is facilitated by vacuolar H^+ATPase pump located in the osteoclast ruffled membrane [191, 192] to dissolute bone minerals [114]. H^+ produced by cytoplasmic carbonic anhydrase II within the osteoclasts [193] is released into the area between the cells and the bone [194]. In this acidic microenvironment, bone demineralises when the solid hydroxyapatite $Ca_{10}(PO_4)_6(OH)_2$ releases Ca^{2+}, HPO_4^{2-} and water, H_2O [195]. As a result of H^+ release this intracellular environment becomes alkaline, chloride/bicarbonate exchange pump neutralizes the microenvironment [194, 196]. Importantly sealing prevents leakage of all these factors involved in this phase [114, 189].

The second and final phase of bone resorption mechanism is the organic matrix degradation phase. Matrix degrading enzymes are released by osteoclasts via exocytosis. Lysosomal proteases matrix metalloproteinases (MMPs) such as MMP-9 [197, 198] and CathK [148] degrade the organic components of the bone forming lacuna pits on the surface of the bone as a result of bone resorption activity [114]. This bone resorption

mechanism terminates itself as osteoclasts have limited lifespan. The accumulation of high concentration of calcium from the bone demineralisation [199] or products from bone matrix including TGF-β cytokine [200] may also stop the resorption activity of osteoclasts.

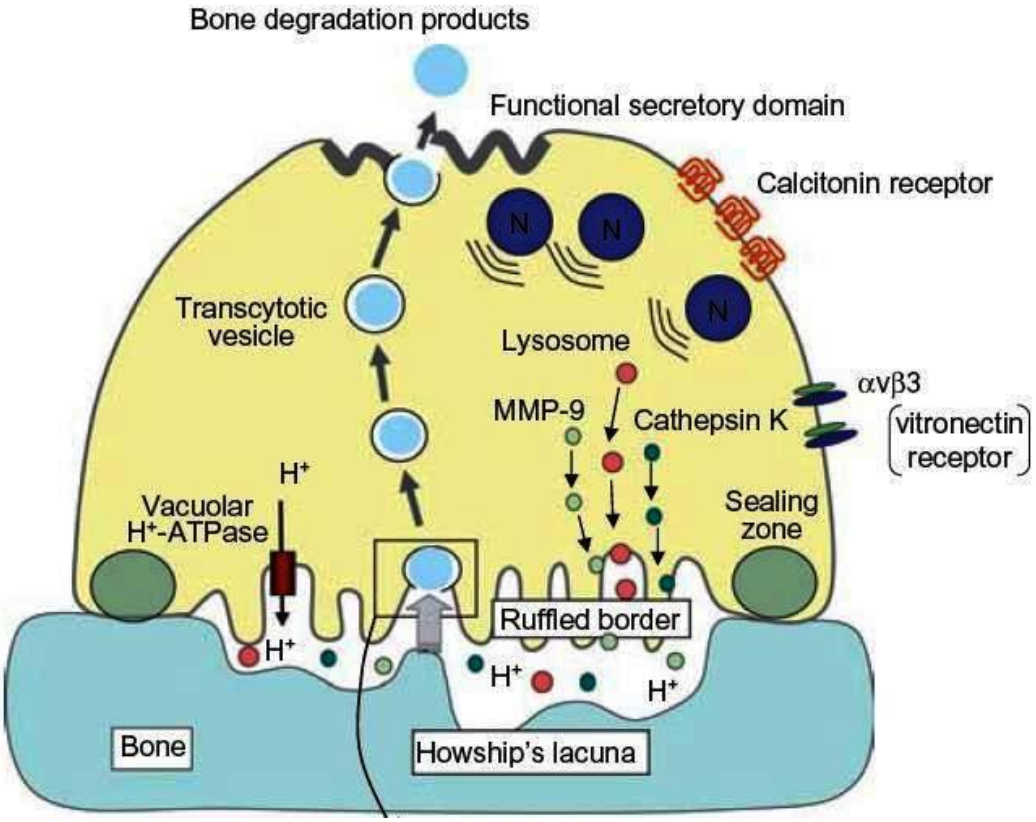

Figure 1.1 A functioning osteoclast at the site of resorption with the Howship's lacuna, sealing zone, ruffled border and other features completely formed to induce resorption activity [188].

1.3.2.M-CSF

M-CSF, also known as colony stimulating factor 1 (CSF-1) [201], is key cytokine promoting cell proliferation in osteoclast development and survival [202, 203]. Within the bone, M-CSF is usually expressed by stromal cells or osteoblasts [204, 205]. As illustrated in **Figure 1.5,** M-CSF binds to its receptor c-Fms on the surface of precursor cells [114, 173, 202] which in turn stimulates the proliferation of osteoclast precursors [201, 206]. Depending on the types of signals received, precursor cells may differentiate into

osteoclasts or dendritic cells. The MCSF-c-Fms binding acts as a crucial lineage-fate determining factor that leads precursor cells to differentiate into osteoclasts [206]. M-CSF exerts an autocrine effect to upregulate RANKL [207] and M-CSF induces its natural receptor, receptor activator of NF-κB (RANK) to effectively respond to RANKL [2]. The RANKL-RANK interaction further stimulates pre-osteoclasts to differentiate into mature osteoclasts [208]. It has been shown that M-CSF is required for cell proliferation of monocyte/macrophage-associated cells into osteoclasts *in vitro* [202]. M-CSF knock-out mice developed osteopetrosis [204] and these osteopetrotic mice had recovered their bone volume when treated with M-CSF in a further experiment [201]. M-CSF has been shown as important for bone resorption *in vivo* [201, 204].

1.3.3.RANKL/RANK-dependent pathway in osteoclast formation and activity

RANKL/RANK signaling pathway is the key pathway to regulate osteoclast formation and bone resorption activity in normal bone remodelling and pathological diseases. Excessive osteoclastogenesis results in bone loss. Osteoprotegerin (OPG) binds to RANKL preventing binding to its receptor, RANK to protect the skeleton from excessive bone resorption. Therefore RANKL/RANK/OPG system (**Figure 1.3**) is important in the maintenance of bone homeostasis [173].

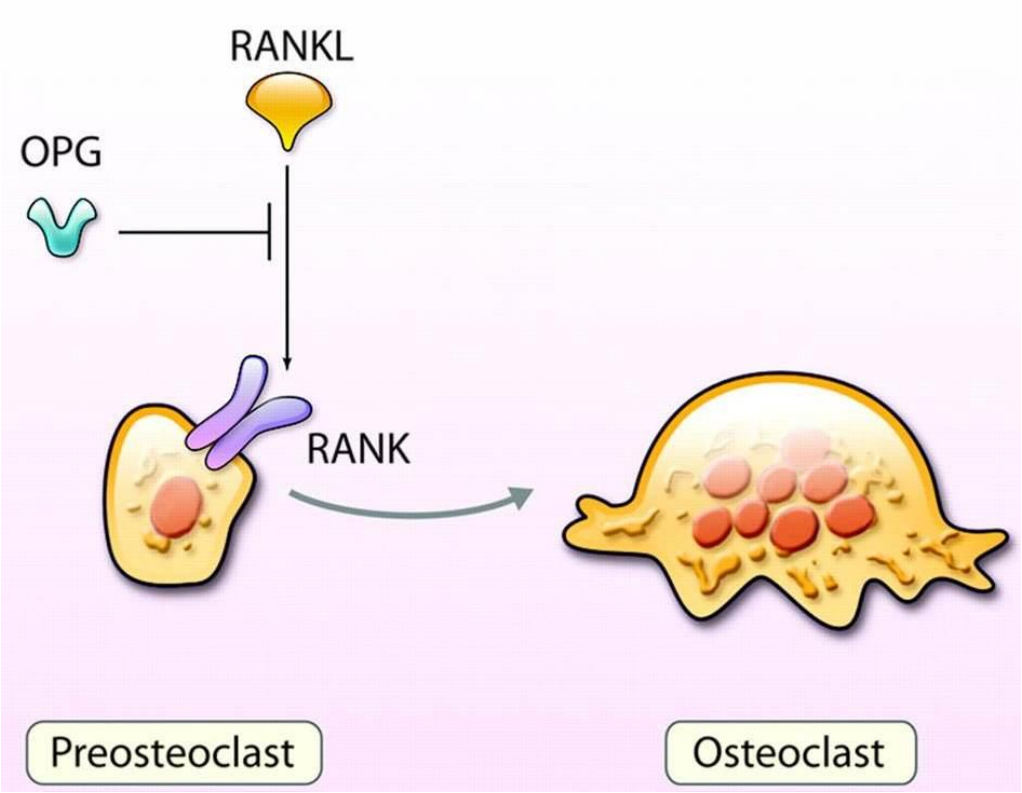

Figure 1.2 RANKL/RANK/OPG interactions [209].

1.3.3.1.RANKL/RANK

RANKL [210] is also known as osteoclast differentiation factor (ODF) [211], TNF-related induced cytokine (TRANCE) [173, 212] and osteoprotegerin ligand (OPGL) [173, 213-215]. RANKL is highly expressed in osteoblasts [115], bone marrow, lymphoid tissue [216], fibroblasts [217] and dendritic cells [218]. RANKL is a cytokine in the TNF superfamily, which is expressed as a 317-amino acid long protein containing a receptor binding core domain in a cysteine-rich extracellular domain [219]. It is mostly released from the cell surface as a soluble molecule following proteolytic cleavage by MMPs such as MMP-14 [220, 221]. RANKL is found in membrane-bound and soluble form [222]. Membrane-bound RANKL binds more efficiently to its receptor RANK when compared to the soluble RANKL [222].

In bone, RANKL induces osteoclast differentiation and resorption activity as well as prevents osteoclast apoptosis *in vitro* [219]. The presence of M-CSF and RANKL can induce differentiation of pre-osteoclasts into multinucleated osteoclasts in human, mouse and rat *in vitro* [216, 223]. Consistent with this, *in vivo* studies with transgenic mice

lacking functional RANKL developed osteopetrosis [213, 224] whilst mice administered with excessive recombinant RANKL developed osteoporosis [216]. These studies highlight the importance and role of RANKL in osteoclast differentiation and maturation [225].

RANKL is also found in autoimmune system, hence, has a major role in osteoimmunology. It is greatly expressed in activated T-cells [219] where RANKL induces the activation of c-Jun terminal kinase (JNK) in T-cells and in turn accelerates the proliferation of T-cells. Other than osteoporosis RANKL knock-out mice are accompanied by immune system abnormalities including deficient mature T and B cells [225].

RANK is a natural receptor for RANKL [226, 227] present on the surface of osteoclast precursors. The ligation between RANKL and RANK is important to activate NF-κB to complete the development of functional osteoclasts [227]. Small interfering RNA (siRNA) targeting RANK significantly inhibits both osteoclast formation and bone resorption in RAW 264.7 and primary bone marrow cell cultures *in vitro* [228]. Mice with a disruption of RANK or RANKL show severe osteopetrosis due to failure to form osteoclasts *in vivo* [213, 229] as well as accompanied by a defect in tooth eruption [220]. RANK knock-out mice exhibited severe bone sclerosis due to lack of osteoclasts, suggesting RANK is a key factor in the differentiation, maturation and functioning osteoclasts [230]. These findings clearly demonstrate that RANK/RANKL is essential for osteoclastogenesis [220].

1.3.3.2. OPG

OPG is a TNF receptor superfamily member which possesses four cysteine-rich domains, two death domain homologous regions and a high net positive charge heparin-binding domain so that it can dimerise with heparin to be functional [120]. OPG is secreted as a soluble protein [214, 231] by osteoblasts [214], marrow stromal cells, monocytes/marophages, dendritic cells, fibroblasts, both T and B lymphocytes [214, 215], endothelial cells [137, 232-234] and mesenchymal stem cells [235]. OPG is identical to osteoclasts inhibitory factor (OCIF) [214, 231, 236]. OPG is an effective negative regulator of osteoclastogenesis where it binds to RANKL [214, 215] preventing RANKL from binding to RANK (**Figure 1.2**) [237]. This RANKL/OPG binding inhibits osteoclastogenesis as well as promotes the apoptosis of osteoclasts both *in vitro* and *in vivo*

[216, 237]. In support of this, transgenic mice lacking OPG suffer severe osteoporosis, resulting from both an increased number and enhanced activity of osteoclasts [220, 221, 238]. Decreased osteoclast differentiation in transgenic mice and rats with over-expression of OPG exhibited suppression of bone resorption [239] and osteopetrosis [214]. Taken together RANK/RANK/OPG system plays a major role in osteoclast resorption activity and bone metabolism. Generally most of the known hormones and cytokines work on bone through this RANKL/RANK/OPG system and this signalling pathway is the target of antiresorptive inhibitors used in this study.

1.3.3.3.RANKL/RANK/OPG system

Briefly, RANKL-RANK binding triggers the intracellular pathway beginning with the activation of mitogen-activated kinases, including JNK and p38 [220, 221], TNF receptor activating factor (TRAF) including TRAF1, 2, 3, 5 and 6 [2, 218, 220, 240] which then activates NF-κβ [218]. TRAF6 plays a crucial role (**Figure 1.3**). The elevated amount of TRAF6 and activated NF-κβ further enhance the dephosphorylation and activation of nuclear factor of activated T-cells, cytoplasmic, calcineurin-dependent 1 (NFATc1) (described in **Section 1.3.3.4**) [241]. Mediated by JNK, p38 and NF-κB1 (p50/p105) and NF-κB2 (p52/p100) [125], dephosphorylated NFATc1 translocates specific transcription factors into the nucleus to enable expression of osteoclast-specific genes [175, 242, 243] including TRAP, OSCAR [244] and αvβ3 [175, 240, 242]. This in turn initiates osteoclast formation and differentiation. Similarly, the activated NF-κβ will induce c-Fos to relay the intracellular signal from RANKL stimulation (**Figure 1.4**) [245]. c-Fos heterodimerizes with c-Jun to form an important transcription factor, activator protein-1 (AP-1). c-Fos cooperates with NFATc1 [246] to bind to the promoter regions on osteoclast-specific genes and induce those genes in osteoclastogenesis [242].

TRAF6 knock-out mice developed severe osteopetrosis *in vivo* with defects in osteoclast function, impaired bone remodelling and tooth eruption [247, 248] and osteoclast precursor cells failed to differentiate when cultured *in vitro* [248]. TRAF6-null osteoclasts were also defective in cytoskeletal organization similar to RANK- and RANKL-null mice [247, 249]. However inhibition of TRAF6 interaction cannot fully suppress the activity of downstream factors such as JNK, indicating that the process of

osteoclast formation may be partially regulated by other molecules compensating TRAF6 deficiency [250].

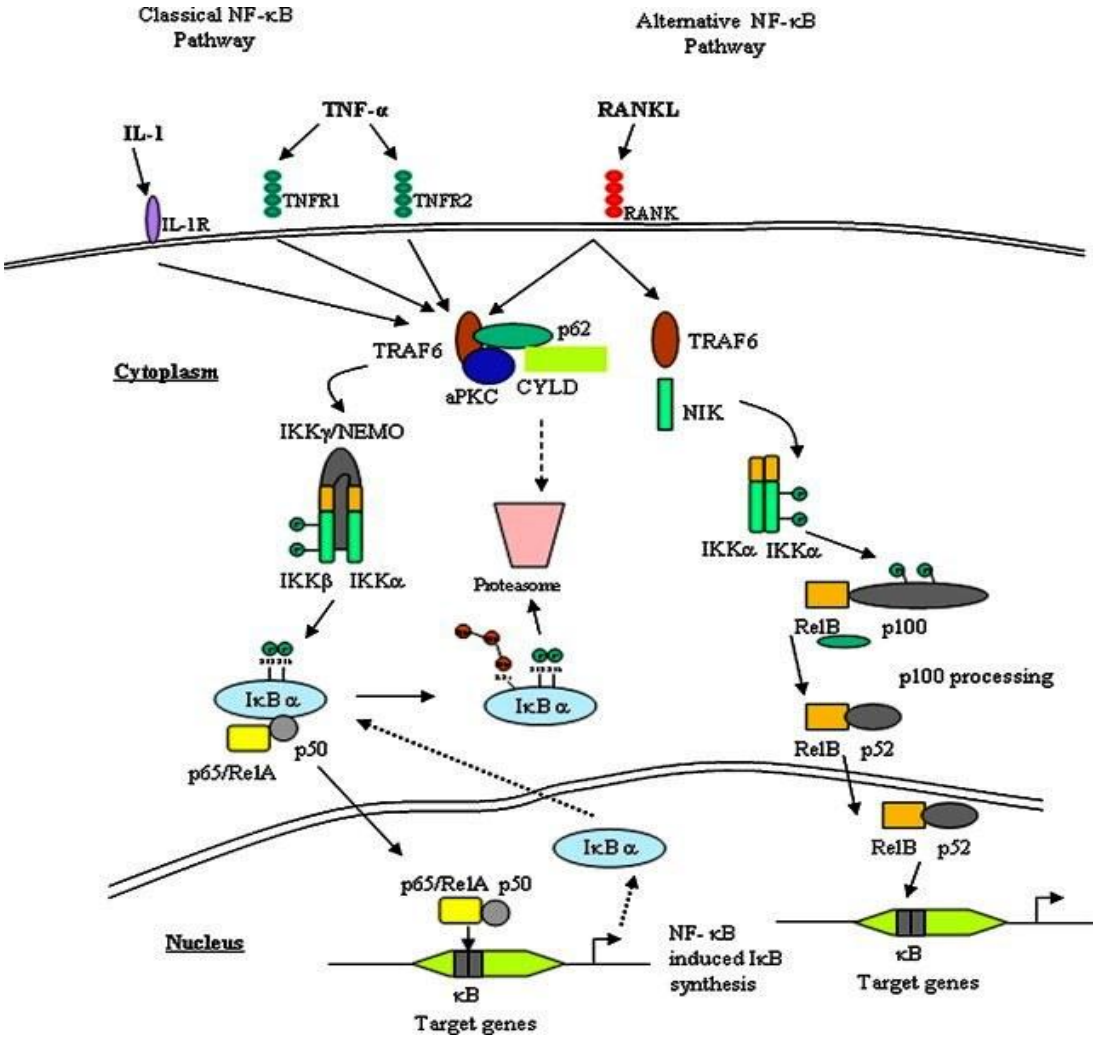

Figure 1.3 RANKL signaling via NF-κB pathway [251].

1.3.3.4.NF-κB

NF-κB is a nuclear factor or an omnipresent transcription factor that regulates the expression of genes encoding cytokines, chemokines, growth factors and cell adhesion molecules important in health and diseases [251]. Osteoclastic cytokines including IL-1, IL-6, RANKL and TNF-α have been shown to initiate osteoclastogenesis via induction of NF-κB signaling [208, 252]. NF-κB which exists in all eukaryotic cell types acts as a

regulator on various genes in many genes [95] and is a critical regulator of inflammation [253].

The NF-κB pathway, illustrated in **Figure 1.5,** is associated with RANKL/RANK-regulated osteoclast formation, differentiation and resorption activity [251]. Phosphorylation of NF-κB and the degradation of its inhibitory unit Iκβα are required to activate NF-κB. The activated NF-κB is a heterodimer consisting two proteins, most commonly seen is p65/p50 [254]. Activated NF-κB enhances cell proliferation and reduces apoptosis [255]. Of NF-κB, p50 and p52 are crucial for the normal development of osteoclasts. Gene-knock-out of both p50 and p52 led to osteopetrosis due to lack of osteoclasts, which suggests an essential role of the NF-κB molecule in mediating osteoclastogenesis [256, 257].

Apart from cytokines, bacterial products such as LPS activate NF-κB signaling [95]. It has been shown LPS-induced osteolysis was inhibited through the suppression of NF-κB signaling and activity *in vivo* by NF-κB inhibitors [103, 107]. This highlights the modification of NF-κB signalling pathway may be a potential therapeutic target for the treatment of many osteoclast-related osteolysis diseases. Taken together NF-κB inhibition has been shown to suppress osteoclastogenesis, therefore, understanding the RANKL/RANK-regulated NF-κB signaling pathway may serve as a therapeutic target for osteoclast-associated bone loss including treatment using anti-resorptive inhibitors.

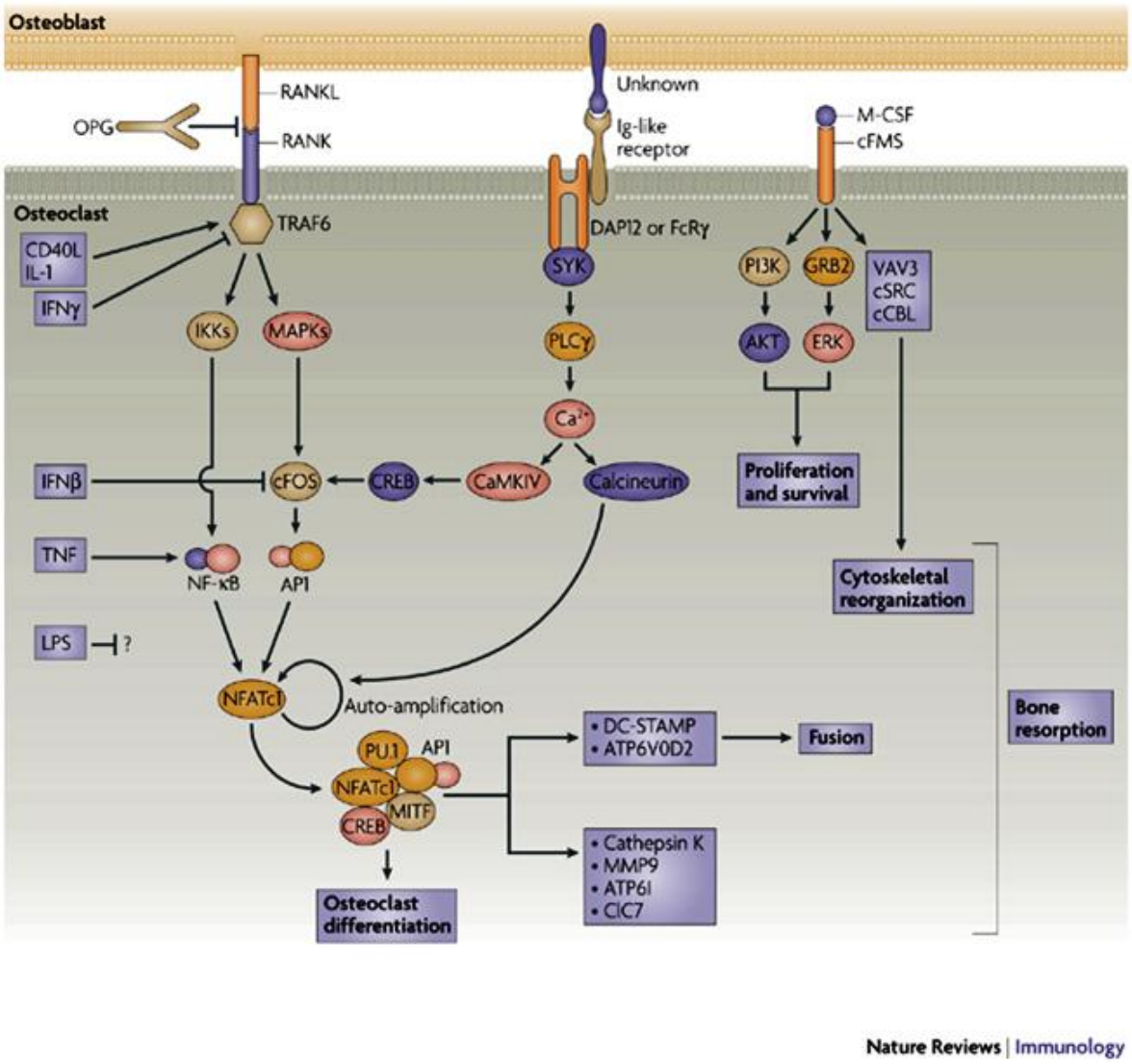

Figure 1.4 RANKL/RANK signaling system via NF-κB together with ITAM co-stimulatory system induces NFATc1 expression to regulate osteoclastogenesis in inflammation-mediated localised bone loss pathologies [2].

1.3.3.5.NFATc1

NFATc1 is a member of the NFAT transcription factor family and was first identified in T-cells. Currently there are 5 members identified in this transcription factor family, which are NFAT1 (NFATc2), NFAT2 (NFATc1), NFAT3 (NFATc4), NFAT4 (NFATc3) and NFAT5 [2]. Studies have shown RANKL highly stimulates osteoclastogenesis through NFATc1 [125, 242]. This induction depends on both the TRAF6-NF-κB and c-Fos pathway [220]. Since NFATc1 induction has been shown to be impaired in TRAF-null

cells [258], NFATc1 has been suggested as important in the early phase of osteoclastogenesis. This is supported by the findings that RANKL-stimulated induction of NFATc1 is suppressed by NF-κB inhibition [259] and by absence of p50/p52 [244]. NFATc1 mRNA and protein levels were undetectable in c-Fos-null [260] indicating that upregulation of NFATc1 expression takes place downstream of c-fos activation.

NFATc1 regulates a number of osteoclast-specific genes, such as CathK, TRAP, β3, MMP9 and OSCAR cooperatively with other transcription factors such as AP-1, PU.1 and microphtalmia transcription factor (MITF) [2, 139, 220, 261]. Various studies have identified the NFAT binding sites in the promoter region of osteoclast-specific genes. For example, NFAT binds to *acp5* (TRAP-encoding gene), *calcr* (calcitonin receptor), *ctsk* (an extracellular bone matrix protein-hydrolysing cysteine protease) [242] and β3 encoding-gene [175, 262].

NFAT signaling transduction cascade is a key pathway in osteoclastogenesis. Osteoclastogenesis is retarded in NFATc1 suppression [263] and knock-out embryonic stem cells *in vitro* [242]. Its relationship to RANKL is demonstrated when NFATc1 expression is upregulated in response to RANKL-stimulated osteoclast differentiation [125, 242, 263]. Over-expression [242] or ectopic expression of constitutively active [125] NFATc1 stimulates osteoclastogenesis itself even though in the absence of RANKL. Taken together these studies illustrate the essential role of NFATc1 in the induction of osteoclastogenesis.

In a further study, NFATc2 was found to be greatly expressed following RANKL stimulation and binds to the promoter of NFATc1 to upregulate the NFATc1 expression [264]. NFATc1 can also auto-regulate its own promoter during osteoclastogenesis thus enabling the strong induction of NFATc1 which lasts till the end of osteoclastogenesis [264]. AP-1 containing c-Fos together with continuous activation of calcium signalling is involved in this autoamplification [220, 242]. Comparing mRNA expression between NFATc1 and NFATc2, it appears that the regulation of NFATc1 mRNA expression is mainly controlled at the epigenetic level [264]. Auto-amplification of NFATc1 expression also indicates that NFATc1 plays a central role in the molecular mechanism of osteoclastogenesis.

1.3.4.ITAM-dependent signalling pathway

The immunoreceptor tyrosine-based activation motif (ITAM)-dependent signalling pathway has recently been shown to co-stimulate osteoclastogenesis along with RANK/RANKL system [9, 265-268]. ITAM signalling is downstream to RANK (**Figure 1.5**) and has been shown as fundamental for osteoclastogenesis [220, 269]. Additionally ITAM signalling system is also crucial in the regulation of effector immune cells proliferation, survival and differentiation [265, 266].

Molecules in the ITAM co-stimulatory pathway in pre-osteoclasts and osteoclasts discussed in this thesis are two adaptor proteins and two co-receptor proteins. The two ITAM-containing adaptor proteins are DNAX-activating protein 12kDa (DAP12) and Fc receptor common gamma subunit (FcRγ) expressed in osteoclast-lineage myeloid cells [267]. Murine DAP12$^{-/-}$ FcRγ$^{-/-}$ osteoclast precursor cells in a RANKL- and M-CSF-mediated osteoclast culture system cannot undergo osteoclast differentiation *in vitro* [265, 266]. Additionally, DAP12$^{-/-}$ FcRγ$^{-/-}$ mice exhibited severe osteopetrosis, with bone marrow filled with unresorbed bone *in vivo* [266]. This indicates the important role of ITAM-containing adaptor molecules to induce osteoclast formation.

Ligands cannot directly bind to these adaptor proteins due to lack and minimal extracellular domain in their structure [270-272]. Therefore, DAP12 and FcRγ pair with two co-receptor proteins in order to receive and send signals to the extracellular micro-environment [271, 272]. The two co-receptor proteins are triggering receptor expressed in myeloid cells (TREM2) and OSCAR [246, 267, 273, 274]. The positively charged transmembrane amino acid residues on the co-receptors binds through ionic interaction to the corresponding negatively charged transmembrane residues on the adaptor proteins [267, 271, 274-277].

Following ligand recognition and receptor clustering from the OSCAR-FcRγ and TREM2-DAP12 bindings respectively, tyrosine residues are phosphorylated by Src family protein tyrosine kinase (PTK) [267], thereby recruiting spleen tyrosine kinase (Syk) to the motif [265, 266] creating a docking site [278]. In turn, this activates Syk [265, 266, 279, 280] through the Src homology 2 (SH2) domain of the Syk's C-terminal binding to the ITAM motif [266, 281] and autophosphorylation of important effector molecules, such as phospholipase C gamma (PLCγ) [282] that in turn mediates several other intracellular

pathways that take place during osteoclastogenesis process. It has been reported that deficiency or inhibition of Syk tyrosine kinase resulted in impaired osteoclast differentiation and function [265, 266]. The activated Syk-PLCγ will increase the intracellular calcium concentration to activate the calcineurin [283] to dephosphorylate NFATc1 [240, 284], activate Ras resulting in stimulation of the ERK pathway and cellular activation and induce the NF-κB activation (**Figure 1.4**) [268]. NFATc1 translocation will then take place resulting in the stimulation of NFATc1 expression **(Figure 1.5**) as well as activated NF-κB will enhance osteoclastogenesis [264]. This important signalling cascade is illustrated in **Figure 1.6**. It has been suggested the stimulation on this ITAM signalling pathway is stopped (in a negative feedback loop) by stimulation on the ITAM-counter immunoreceptor tyrosine-based inhibitory motif (ITIM)-mediated pathway [268]. However, this is not discussed in this thesis.

Apart from inducing osteoclast formation, ITAM signalling pathway has also been found to play a significant role in regulation of osteoclast resorption activity and capacity. Osteoclasts from DAP12$^{-/-}$ FcRγ$^{-/-}$ mice failed to multinucleate *in vitro* [265, 266]. Osteoclasts from DAP12$^{-/-}$ FcRγ$^{-/-}$ mice also exhibited cytoskeletal dysfunction, failed to firmly adhere to bone surface and secure cell polarisation ensuing a failure to resorb bone [186, 279]. As described in **Section 1.3.1**, failure in multinucleation and cytoskeletal reorganization will retard bone resorption capacity in osteoclasts.

As illustrated in **Figure 1.5**, the activation of phospholipase C by TRAF6 produces inositol-1,4,5-trisphosphate (IP3) that enhances intracellular calcium levels which in turn leads to the activation of calcineurin to dephoshorylate NFATc1 [125]. This calcium signalling is initiated by the phosphorylation of PLCγ downstream of ITAM signalling [265], resulting in the cleavage of the cell membrane phospholipid phosphatidylinosotol-4,5-bisphosphate (PIP2) into the molecules of IP3 and diacylglycerol (DAG), which become the second messengers in the signalling system. PLCγ is believed to involve in osteoclastogenesis that phospholipase-deficient mice exhibited osteopetrosis and defective in osteoclastogenesis [285]. The IP3 induces calcium release from the endoplasmic reticulum, which leads to the increase in intracellular calcium concentration. This activates downstream effector proteins, phosphotase calcineurin and calcium/calmodulin-activated

kinase (CaMKs). Phosphatase calcineurin is the molecule reported to control the activity of NFATc1 [286, 287].

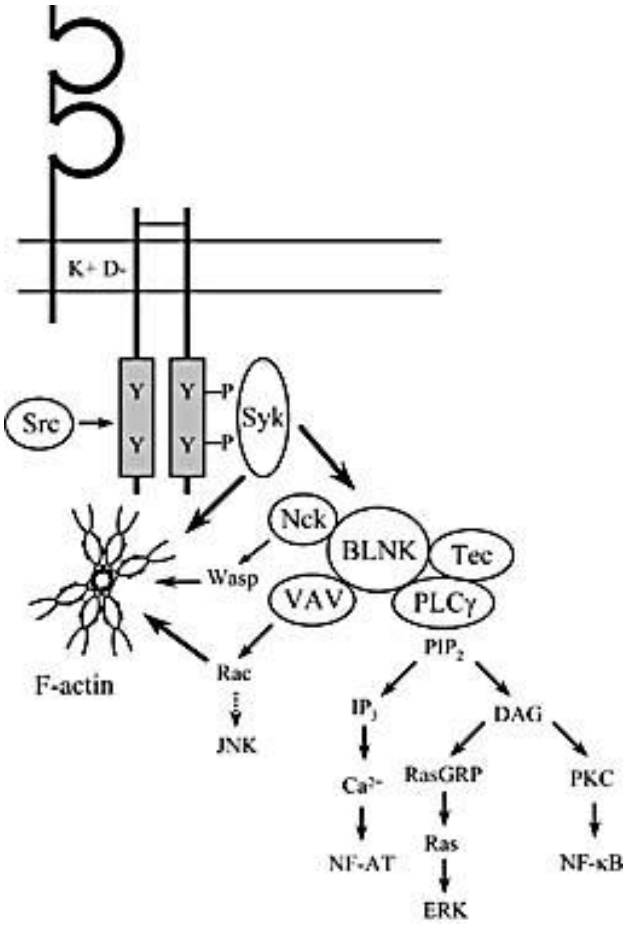

Figure 1.5 Haemopoietic cell ITAM signalling cascade. Ligand-induced receptor aggregation results in ITAM phosphorylation by Src, allowing recruitment and activation of Syk family PTK. Syk family PTK phosphorylate F-actin and the BLNK/SLP-76 family of adaptor proteins, leading to recruitment and activation of multiple signalling pathway [268].

1.3.4.1.DAP12

DAP12 also known as tyrosine kinase binding protein (TYROBP) or KARAP [275, 288], is expressed in osteoclasts, monocytes differentiating into macrophages [289] and dendritic cells [276], granulocytes as well as some T and B lymphocytes [181, 271, 290-292].

DAP12-null mice exhibited severe osteopetrosis with increased bone mass and trabecular thickness [181], and when cultured *in vitro* the osteoclast precursor cells failed to undergo differentiation [182, 265, 266, 272, 293] and multinucleation [294], similar to osteoclasts from DAP12$^{-/-}$ FcR$\gamma^{-/-}$ mice [265, 266]. Retroviral introduction of DAP12 into DAP12$^{-/-}$ FcR$\gamma^{-/-}$ mice restored the multinucleation and differentiation capability [181, 182, 265, 266]. Additionally DAP12 overexpression enhanced proliferation rate of the osteoclast progenitors.

Apart from osteoclast formation, DAP12 has been shown as important for osteoclast cyoskeletal reorganisation for bone resorption mechanism [186]. Deletion of Syk downstream of DAP12 [265] led to failure in cytoskeletal dysfunction that led to inability to resorb mineralised substrate *in vitro* [280]. This cytoskeletal dysfunction resulted in impaired formation of actin ring, ruffled border and attachment to bone surface thereby inhibiting ostecoclasts from resorbing bone [186]. Together these studies demonstrate that DAP12 by itself has significant roles played in osteoclast development in health and disease.

For example in the context of human pathology, DAP12 deficiency due to genetic mutation on chromosome-19 leading to premature termination of the protein is believed as the cause to an uncommon genetically recessive human disease called polycystic lipomembranous osteodysplasia with sclerosis leukoencephalopathy (PLOSL) or also known as Nasu-Hakola disease [182, 288, 295]. PLOSL is described in **Section 1.3.4.2.**

1.3.4.2.TREM2

Together with TREM1, TREM3 and TREM-like 4 [296], TREM2 is a member the TREM family [297]. TREM2 was first discovered in human monocyte-derived dendritic cells and mouse macrophage RAW264.7 cell line [276, 277] and is widely expressed by cells from myeloid lineage such as dendritic cells, macrophages, microglial cells and osteoclasts [277, 297]. TREM2 may be regulated by MITF and PU.1 since promoter region of TREM2 contains the binding sites for these transcription factors [246]. TREM2-Fc proteins, which bind to TREM2 ligand, inhibit osteoclast formation in an osteoclast-osteoblast *in vitro* co-culture [246]

Human TREM2-deficient pre-osteoclasts could not differentiate into functional osteoclasts *in vitro* [182]. When TREM2 was suppressed through RNA silencing [298] in RAW264.7 cell osteoclast cultures, osteoclastogenesis, bone resorbing activity and migrating capability were suppressed. More importantly, TREM2 stimulation in mouse pre-osteoclasts enhanced osteoclast multinucleation and chemotaxis [294, 299]. Meanwhile stimulation of TREM2 in RAW264.7 cells and murine primary bone marrow macrophages (BMMs) via antibody cross-linking enhanced osteoclast-like cells formation in the presence of RANKL, M-CSF and DAP12 [299].

The findings that there is no putative NFAT binding site on TREM2 promoter region and TREM2 gene expression is not significantly inhibited with inhibition of calcineurin/NFATc1 signalling give the idea that NFATc1 does not regulate TREM2 expression [246]. Indeed, there have been more recent *in vitro* studies showing that TREM2 is upstream of NFATc1 expression in the RANKL-induced CaMK-MEK-ERK the cell signalling pathway in PBMC-derived osteoclast culture [298, 300].

Studies have also shown that TREM2 is highly regulated by ligand binding [246]. When TREM2 was blocked from ligand binding in RAW264.7 cell osteoclast cultures osteoclastogenesis, bone resorbing activity and migrating capability were suppressed [299]. However, the ligand for TREM2 is still unknown. TREM2 has been shown to bind to anionic ligands possibly through pattern recognition [299, 301]. A more recent study suggested plexin-A1, a receptor for semaphorins, as a potential activating ligand in TREM2/DAP12-mediated signalling pathway during osteoclastogenesis. In the study, stimulation of osteoclast precursors with cells deficient in plexin-A1 led to reduction in osteoclast formation *in vitro* and *in vivo* mouse model lack of plexin-A1 exhibited osteosclerosis and increased bone mass [302]. Recent studies on characterizing the ligand of TREM2 reported that TREM2 binds to bacterial LPS [303] and it has been suggested to function as phagocytic receptor for bacteria in macrophages [304].

Mutations in DAP12 and TREM2 did not lead to complete loss of osteoclastogenesis but resulted in delayed osteoclast differentiation and decreased dentine resorption [182]. In humans, a mutation of TREM2 or DAP12 leads to PLOSL as discussed in **Section 1.3.4.1** [220, 305]. PLOSL is characterized by osteopenia and early onset of osteoporosis, lipid-filled bony cysts on wrists and ankles, trabecular bone loss and cortical bone

demineralisation, loss of brain white matter leading to presenile dementia disease, premature death before age 50 [305, 306] and fragile bones [295]. DAP12-null mice with PLOSL demonstrated bone and brain abnormalities suggesting PLOSL is associated with DAP12 deficiency [307]. However a very recent conflicting data reported that osteopenia in PLOSL was observed in transgenic mice overexpressing DAP12. Whether deficiency or overexpression of DAP12, mutation of DAP12 gene is shown as contributing to the development of PLOSL. Additionally, TREM2 mutation in PLOSL is believed to cause defect in neuronal apoptotic clearance by microglia leading to brain abnormality [308, 309]. Taken together these show that DAP12 and TREM2 have important roles in cellular regulation including osteoclast formation and resorption activity.

1.3.4.3.FcRγ

FcRγ is an adaptor protein for Fc receptors [265, 274]. The FcRγ chain has been demonstrated to facilitate or upregulate the expression of OSCAR on the cell surface [274] and ITAM signal transduction [265]. FcRγ has also been found to couple with other receptors such as FcRIII [310] and paired Ig receptor-A (PIR-A) [311].

It has been shown that pre-osteoclasts with double mutations in both DAP12 and FcRγ could not differentiate into multinucleated osteoclasts even in the presence of osteoblasts [265, 266, 293]. This suggests that ITAM-containing adaptor proteins DAP12 and FcRγ are cooperatively involved in transducing signals for osteoclastogenesis [246]. However, pre-osteoclasts which are deficient in DAP12, "DAP-null" were able to multinucleate in the presence of osteoblasts [265, 266]. This concept is supported by *in vivo* finding that double knock-out of both DAP12 and FcRγ mice develop severe osteopetrosis but it is less severe in DAP12 knock-out alone. Together these studies suggest the role of FcRγ in osteoclastogenesis could be compensated by other mechanism or pathway *in vivo* as FcRγ-deficient mice do not develop osteopetrosis [265, 266, 274].

Interestingly osteoclasts with DAP12 deficiency increase in size when treated with FcRγ [159, 265, 266]. Additionally activation of OSCAR-FcRγ increase osteoclast formation [186] and stimulation of OSCAR or FcRγ-mediated pathway was able to enhance osteoclastogenesis in DAP12-deficient cells [77]. Together these studies show

that FcRγ may compensate the osteoclastogenesis in DAP12 deficiency. Hence despite this redundancy, the role of FcRγ may still be important to osteoclast formation [265, 266].

1.3.4.4.OSCAR

OSCAR, an immunoglobulin (Ig)-like receptor, is a novel member of leukocyte receptor complex (LRC)-encoded protein found at both gene and protein levels in human and murine osteoclasts [159]. In mouse, OSCAR is only expressed by mature osteoclasts, found in bone tissues and murine osteoclast-like cell-line RAW264.7 cells [159]. Although sharing 73% similar sequence homology [159], in human OSCAR is expressed by many types of cells including monocytes, dendritic cells [312-315] and endothelial cells [9, 316]. The expression of OSCAR is usually found at the terminal stage of osteoclast differentiation after TRAP-positive pre-osteoclast formation [159] and NFATc1 expression [243] suggesting OSCAR as a reliable marker for mature osteoclasts [159, 181].

OSCAR has been also been shown to play role in immune system [313, 314] through antigen presentation as well as survival, maturation and activation of dendritic cells [269, 274, 313-315]. OSCAR elevates pro-inflammatory reaction in monocytes and neutrophils by inducing the secretion of cytokines such as granulocyte-macrophage colony-stimulating factor (GM-CSF) and IL-4 that sustain T-cell proliferation [312, 315]. Moreover, OSCAR reduced apoptotic cell number and increased anti-apoptotic molecule Bcl-2 expression in dendritic cells, leading to reduced apoptosis and prolonged cell survival [312, 313]. Ligation of human OSCAR on monocytes and neutrophils results in the induction of a pro-inflammatory cascade and the initiation of downstream immune responses [315].

With full length cDNA comprising 282 amino acids [9, 159], OSCAR is a type I transmembrane protein with a positively charged arginine residue in the transmembrane domain [159] suggesting OSCAR couples with other transmembrane proteins or ligands in the induction of ITAM signalling. Based on protein structure modelling, human OSCAR is expected to contain a signal peptide [9] and it has been speculated that its secretory function and supporting the idea of soluble isoform [269]. The presence of the soluble isoform is further supported by detection *in vivo* and *in vitro* studies [317].

A ligand for OSCAR has been identified as an extracellular matrix collagen [77]. The interaction between OSCAR and osteoblast-derived collagen ligand has been shown to enhance osteoclastogenesis *in vitro* in which this osteoclastogenesis was retarded when the OSCAR and ligand interaction was blocked using OSCAR-Fc fusion protein [77, 159, 269]. Interestingly this ligand was stained with OSCAR-Ig fusion protein [159] which suggests this OSCAR ligand might be expressed by osteoblasts [159]. This is supported by detection of OSCAR ligand on osteoblasts upon stimulation by osteotropic factors 1, 25-$(OH)_2$ vitamin D3 and PGE2 as well as RANKL [246]. In a study involving an interaction between mononucleated osteoclast expressing OSCAR and collagen expressed by osteoblasts, it was found that collagen type I is a ligand for OSCAR [77]. These suggest that OSCAR is responsible for osteoclast differentiation process in close proximity to bone in the presence of osteoblasts instead of in tissues and circulation.

OSCAR has a specific role in co-stimulating osteoclastogenesis [159, 265]. Upregulation of OSCAR expression has been observed during osteoclastogenesis [274] and inhibition of calcineurin/NFAT-mediated pathway downregulates OSCAR gene expression [246]. Therefore these suggest OSCAR gene expression could be directly regulated by NFATc1. This has been further demonstrated by a direct chromatin immunoprecipitation (ChIP) assay and gene reporter assay [243, 246] that RANKL/RANK binding induces NFATc1 expression [243, 246]. Consistently with this constitutively active NFATc1 was found to promote the expression of OSCAR [243]. While there are two NFATc1-binding sites identified on mouse OSCAR promoter region which indicates NFATc1 has the capability to directly induce OSCAR gene expression [243], NFATc1 has also been reported to upregulate downstream of calcium signalling mediated by OSCAR [265]. This implies that a positive feedback loop may be involved in the interaction between OSCAR and NFATc1 (**Figure 1.5**) [246, 265].

Importantly, transcription factors MITF, PU.1 [318], USF, inhibitor of differentiation 2 (ID2) [319], MafB and protein inhibitor of activated STAT 3 (PIAS3) [320] also regulate OSCAR expression. A study that mouse promoter region on the OSCAR gene indicates that MITF and PU.1 may bind to promoter site to initiate OSCAR expression [318], is supported by the finding that MITF and PU.1 transcription factors enhanced OSCAR gene expression together with TRAP [246], CathK [321] and NFATc1 [246, 318]. USF also

promotes OSCAR expression [322]. ID2 [319], MafB and PIAS3 [320] negatively regulate OSCAR expression. For example overexpression of PIAS3 recruited histone deacytelase 1 to the promoter of OSCAR reducing OSCAR and NFATc1 expression and eventually inhibited osteoclast differentiation [320].

1.4.Osteoblasts

Osteoblasts, the bone-forming cells [323], are cells of multipotent mesenchymal origin [1, 2]. Osteoblasts secrete bone-matrix proteins and promote subsequent mineralization within the bone [2], together with production of collagen and osteocalcin [323]. In contrast to osteoclasts, osteoblasts are mononucleated cells found in various shapes including flat and plump depending on their stages of cellular activity. Formation of osteoblasts are regulated by specific transcription factors such as runt-related transcription factor 2 (RUNX-2) and osterix [2], which are essential to determine the formation of osteoblasts [2, 324]. The similar factors could cross-inhibit the other factors that may differentiate mesenchymal progenitor cells into other cells such as adipocytes and chondrocytes [324].

Osteoblasts play significant function in the regulation of osteogenesis, ossification and bone formation. Briefly, when receiving bone-forming signals, mesenchymal cells cluster and differentiate into osteoblasts, forming an ossification center in the fibrous connective tissues within the bone. Following this osteoblasts secrete extracellular organic bone matrix (osteoid) therefore leading to matrix mineralization and bone formation [325]. Osteoblasts deposit osteoid as they undergo differentiation [325] and ultimately, some osteoblasts undergo apoptosis [325, 326] and some osteoblasts become trapped and embedded in bone matrix giving rise to osteocytes which gradually stop secreting osteoid [325]. Osteocytes are described in **Section 1.5.**

Apart from the responsibility in depositing bone matrix for bone formation, osteoblasts particularly mature osteoblasts, have been shown to regulate formation and activity of osteoclasts [1, 325]. Osteoblasts initiate the chemotactic mobilization of osteoclasts by releasing osteoclastogenic key regulator molecules, RANKL and M-CSF [237]. Osteoblasts also release stromal-cell derived factor-1 (SDF-1) important in the migration [323] and homing of osteoclasts [327]. Importantly osteoblasts secrete OPG to inhibit osteoclastogenesis [214]. Together with the regulation of osteoclastogensis, osteoblasts

indirectly modulate the calcium homeostasis in bone. It has now been established that osteoblasts play an important role in bone homeostasis to maintain composition of bone by promoting bone resorption via osteoclasts followed by performing bone formation and mineralization when necessary.

1.5.Osteocyte

Osteocytes are the most plentiful cells within bone matrix residing in lacunae and form a network throughout mineralized bone tissue, communicating through gap junctions with each other and with surface osteoblasts [325, 328]. Therefore osteocytes act as mechanosensors within bone in which they regulate the signals to instruct osteoclasts where and when to resorb bone and osteoblasts where and when to form bone [1, 119]. They do this via the formation of long dendritic processes travelling through canaliculi, a small tunnel structure connecting osteocytes within bone matrix or lacunae with cells on bone surface [329]. This syncytial network support and maintain bone structure and metabolism [1].

As described, osteoblasts mature into osteocytes, thus osteocytes represent terminally differentiated osteoblasts that stop releasing osteoid. Additionally, once they are embedded in the mineralised matrix, osteocytes secrete sclerostin, a negative regulator of the osteoblast [330] retarding bone-forming activity of osteoblasts [331, 332]. These show that osteocytes play a role in regulating osteoblasts for initiating and terminating bone formation.

Within bone, micro-damage may happen as a result of normal wear and tear [112]. It has been suggested that bone microdamage causes osteocyte injuries leading to disrupted osteocyte integrity and osteocyte apoptosis [333]. Moreover, it has been proposed that osteocytes can remove minerals from their lacunae and perilacunar matrix which may explain bone fragility [328] and the presence of empty lacunae in ageing bone suggests that osteocytes may eventually undergo apoptosis [1]. Together these give rise to the idea that osteocytes may also be involved in regulating bone remodelling.

It has been hypothesized that dying osteocytes in the close proximity to the microdamage transmit chemotactic signals to osteoclasts requesting for bone remodelling for fracture removal at particular sites of bone [112, 334]. The signal transmission would

likely involve osteopontin, an osteoclast chemoattractant important for initiating bone remodelling following mechanical loading [335-337]. It was found that the expression of osteopontin by osteocytes is upregulated in hypoxic condition [335]. In addition to microdamage responses osteocyte cellular network induces RANKL expression thereby increasing RANKL-mediated local osteoclast formation [113, 338, 339]. This recruitment of functional ostecolasts as well as induction of osteoclast formation around sites of microdamage due to osteocyte apoptosis indicate that osteocytes play an important role in regulating osteoclasts and bone resorption at bone erosion sites.

1.6.Regulation of particle-induced peri-prosthetic osteolysis

1.6.1.Osteoclasts in peri-prosthetic osteolysis

Immunohistochemical analyses of the pseudomembrane (as described in **Section 1.1.1**) surrounding the failed prostheses in peri-prosthetic tissues have identified populations of cells expressing osteoclast markers [340-342]. This is supported by observations of radiological examination in the form of radiographic hallmark features of aseptic loosening called radiolucent zones at the interface between failed prostheses and adjacent bone [111, 343, 344]. The progression of bone erosion in this disease appears to be dominated by excessive numbers of macrophages [345] believed to be the progenitors for osteoclasts [24, 132]. Consistent with this, macrophages isolated from the tissues were found to have capability to become osteoclasts *ex vivo* either with [41, 132] or without the support of osteoblasts or stromal cells [24]. Taken together these findings suggest that ingestion of prosthetic wear particles by macrophages results in expression of osteoclast-differentiating molecules [24] followed by increased macrophage-derived osteoclast formation and activity that eventually results in excessive bone loss [3, 23, 346].

As described in **Section 1.1.1**, the phagocytosis of prosthetic materials by phagocytic cells such as macrophages and FBGCs persistently releases osteoclastogenic pro-inflammatory mediators, including RANKL [28, 30, 31]. Consistent with this, all types of wear particles including TiAlV, CoCr and SS show stimulation and release of osteoclastogenic pro-inflammatory mediators such as IL-1β, IL-6 and TNF-α by human

monocytes *in vitro* even though there is variation in the levels and pattern of expression [24].

PE particles also induce upregulation of expression of IL-1β, IL-6, TNF-α [347], RANK [20, 28, 233], RANKL and M-CSF [24] in cells of the monocytic lineage. This release of osteoclastogenic pro-inflammatory molecules stimulated by PE particles in turn recruits osteoclast precursor cells such as dendritic cells [133] and macrophages [62, 65, 92] into the tissues [23, 111], thereby initiating and elevating osteoclast differentiation process in the tissues surrounding the prostheses [20, 27, 28, 31-34, 65, 345, 346]. This is supported by the finding that cells containing PE particles found in human peri-prosthetic tissues expressed osteoclast phenotypic markers TRAP, CathK and CTR [25]. Importantly introduction of PE into *in vivo* model has led to increased osteoclast-like formation and activity in a particle concentration-dependent manner [54, 348]. Both excessive osteoclast formation and activity appear to be responsible to promote PE-induced peri-prosthetic osteolysis [76] and modulation of molecules involved during osteoclastogenesis is potentially important to combat the development of osteolysis in the disease.

1.6.1.1.RANK-RANKL-OPG in peri-prosthetic osteolysis

RANKL/RANK/OPG system (**Figure 1.3**) is important in maintenance of bone homeostasis and normal bone physiology. Disruption in the balance between the levels and activity of these molecules results in enhanced osteoclastogenesis thereby excessive bone resorption [24, 115, 233, 234, 349-351] and suppression of osteoblast differentiation and bone formation activity [352] as shown in RA, periodontal disease [232, 349, 353] and peri-prosthetic osteolysis [3, 350, 354, 355].

In inflammation-mediated bone loss diseases, the RANKL/OPG imbalance is caused by elevated cytokine expression such as RANKL [20, 233, 349, 356], IL-1 [357, 358] and TNF-α [20] levels relative to OPG, or decreased OPG expression levels relative to RANKL [24, 234, 359]. This is shown by a positive correlation between severity of synovitis in RA patients and the increase in RANKL [349] accompanied with decreased OPG expression [232, 360]. However, studies have shown high RANKL expression is the most prominent factor. In RANKL-mediated *in vitro* osteoclast cultures with higher concentrations of RANKL (50ng/mL), dentine resorption pit formation was significantly

increased when compared to osteoclast culture with a lower concentration of RANKL (10ng/mL) [20]. Recruitment of pro-inflammatory cytokine TNF-α to RANKL-induced osteoclast culture also increased osteoclast resorption activity [20]. Together these findings show that imbalanced RANKL:OPG ratio contributes to osteoclastogenesis.

In order to reduce osteoclast bone resorption caused by excessive RANKL expression, studies have shown introduction of OPG may restore excessive RANKL expression to its normal relative levels. Treatment with OPG significantly reduced TNF-α-mediated osteoclast formation and bone destruction *in vitro* [361]. Similarly, treatment with OPG-fusion protein (OPG-Fc) *in vivo* significantly reduced osteoclast formation and bone erosion in a rat model of collagen-induced arthritis (CIA) [362]. Consistent with this treatment with a peptide that functions like OPG [363] and a molecule that modulates endogenous OPG expression [364] reduced bone resorption in ovariectomised mice [363, 364] and amine-induced adjuvant arthritic mice [364]. Taken together these show that promotion of OPG binding to RANKL restores the excessive RANKL expression levels *in vivo*.

When OPG/RANKL ratio is out of balance due to the lack of RANKL, introduction of RANKL in RANKL-deficient animals has shown improved symptoms in pathological bone loss diseases. For example, blocking RANKL-induced osteoclastogenesis was found to stop alveolar bone loss in an experimental model of periodontitis [365, 366]. Additionally, RANKL-knock-out mice failed to show signs of arthritis following arthritic serum transfer (STA) of RANKL [224, 361, 362].

Significantly higher levels of RANKL were found in the peri-prosthetic tissues of patients with implant failure than in similar tissues from osteoarthritic and healthy subjects [233]. In contrast, OPG protein levels were similar in all tissues with the net result higher RANKL:OPG ratio [233]. This shows that there is excessive RANKL molecules were found in peri-prosthetic osteolysis. In peri-prosthetic osteolysis, RANKL molecules are expressed by osteoblasts [367], fibroblasts [217, 368], activated T-lymphocytes, giant cells and macrophages [3, 24, 354, 355]. Macrophages, which have phagocytosed wear particles, have been shown as responsible for the high release of RANKL protein and mRNA in particle-induced osteolysis around the prostheses [3, 20]. This shows that the presence of wear particles stimulates the RANKL expression, thus osteoclast formation.

Wear particles, at low concentration, were able to increase the expression of RANKL and M-CSF by osteoblast culture *in vitro* [367]. Particles of prosthetic materials also stimulated human monocytes to express osteoclast-associated genes and osteoclast-mediating factors *in vitro* [350]. Complementing this, metal and PE wear debris have shown increased RANKL in mouse calvarial model *in vivo* [369]. These findings support the contention that high levels of RANKL in peri-prosthetic tissues of patients with prosthetic loosening may significantly contribute to aseptic implant loosening [233].

In the human situation, synovial fluid from peri-prosthetic patients showed excessive RANKL and insufficient OPG levels and this was able to stimulate osteoclast formation *in vitro* when added to an osteoclast cultures [370]. As described earlier, OPG may have an effect to counter this. Addition of exogenous OPG has counteracted the effect in that osteoclast culture [370]. Supporting this, treatment with OPG reduced osteolysis in a PE-induced osteolysis mouse calvarial model [371]. Similarly, treatment with RANK-fusion protein, RANK-Fc which is also an antagonist to RANKL composed of extracellular region of RANK with Fc portion of human IgG1, has reduced resorption in a particle-induced osteolysis animal model [372].

Together these studies indicate ratio of RANKL:OPG gene and protein expression is associated with enhanced differentiation and activity of the bone-resorbing osteoclasts, suggesting RANKL:OPG ratio as an important indicator for bone erosion. As RANKL and OPG are key molecules regulating osteoclastogenesis [232, 360], therapeutic interventions targeting these molecules and their signaling cascades are currently being studied to treat inflammation-mediated bone loss diseases particularly peri-prosthetic osteolysis as being discussed in this thesis.

1.6.1.2.NF-κB in peri-prosthetic osteolysis

The NF-κB signalling factor is involved in regulating osteoclastogenesis in normal bone homeostasis as well as in disease. In the context of peri-prosthetic osteolysis, wear particles have been found to induce NF-κB activation [373]. Ti particles activated NF-κB in peripheral blood monocytes from healthy donors and also induced the production of TNF-α and IL-6 cytokines which in turn enhanced osteoclastogenesis *in vitro* [373]. Within 15 minutes of exposure to Ti, NF-κB binding activity is rapidly induced by

particles in murine macrophage cell line, ANA-1 *in vitro* and this involves the degradation of p105, a precursor of p50 that binds to p65, in NF-κB signaling pathway. TNF-α induction is mediated, at least in part, through NF-κB binding to the κB2a, one of the four NF-κB binding sites in the TNF-α promoter [253]. In addition, as described above in **Section 1.3.3.3**, NF-κB inhibitors suppress NF-κB signalling pathway to reduce bone loss in LPS-induced osteolysis *in vivo* [103, 107]. Taken together the modulation of NF-κB may reduce osteoclast formation and activity therefore bone loss through reduced NF-κB activation in p0article-induced peri-prosthetic osteolysis.

1.6.1.3.NFATc1 in peri-prosthetic osteolysis

NFATc1 is the key transcription factor for osteoclastogenesis in normal bone homeostasis and is essential in pathological bone loss. NFATc1 at both mRNA and protein expression levels is higher in peri-prosthetic osteolysis than osteoarthritis tissues although levels difference did not reach significance [35]. This is consistent with low T lymphocyte numbers observed in these tissues [35]. This is further supported by the findings that NFATc1 was expressed in BMMs and multinucleated cells cultured with Ti [45] and PMMA particles [46]. Inactivation of NFATc1 in this culture potently impeded the Ti [45] and PMMA [46] particle-induced osteoclast formation and activity *in vitro*. Consistent with this inhibition of NFATc1 significantly suppressed volumetric bone resorption in a PE-induced osteolysis in a murine calvarial *in vivo* model [89]. Together these studies and many others illustrate the NFATc1 regulation and modulation may become a reliable therapeutic target in the prevention of bone loss in peri-prosthetic osteolysis. This thesis investigates the effects of NFATc1 inhibition on osteoclast formation and activity, ITAM-associated molecules *in vitro* and localized and systemic bone resorption in a PE-induced osteolysis *in vivo*.

1.6.1.4.ITAM signaling in peri-prosthetic osteolysis

While the importance of RANKL-RANK in localized inflammation-mediated bone loss has been reported, limited studies have shown the expression of ITAM factors in pathology. Our research group have shown the expression of ITAM factors was increased in synovial tissue and vasculature of rheumatoid arthritic joints [374] and in tissues

adjacent to bone erosion sites in peri-prosthetic osteolysis patients [35]. Therefore, it is suggested that ITAM-associated molecules may contribute to the pathogenesis and severity of RA, periodontal disease, peri-prosthetic osteolysis and osteoporosis [35, 269, 338, 374-376].

In PE-induced peri-prosthetic osteolysis, osteoclast-like cells containing PE particles in human tissues express high levels of ITAM molecules OSCAR, TREM2, DAP12 and FcRγ [35] and when assessed by a semi-quantitative scoring system, these are significantly higher when compared to the levels of OSCAR, TREM2, DAP12 and FcRγ, respectively, in osteoarthritic synovial control tissue [35]. This was consistent with the finding that the corresponding mRNA levels were also increased *in vitro* [35]. Additionally, PE particles added into human PBMC-derived osteoclast cell cultures *in vitro* upregulated ITAM expression [35].

This ITAM co-stimulatory immune pathway is also associated with increased osteoclast differentiation and activity [265, 377]. These ITAM molecules are important in osteoclastogenesis and human localized bone loss but there is only a limited number of studies investigating the expression of osteoclast ITAM-associated molecules in the context of pathogenic bone loss. Therefore, it is valuable to further understand the relationship between the co-stimulatory ITAM pathway in osteoclast regulation and particle-induced peri-prosthetic osteolysis.

16.1.4.1.OSCAR in peri-prosthetic osteolysis

Upregulation of OSCAR expression in RA occurs not only locally in mononuclear monocytes prior to differentiation into osteoclasts at the inflamed synovium, but also in the circulating monocytes before cells enter synovium [269]. Hence it is thought that the increase in the expression of OSCAR could also be associated with systemic inflammation [269]. However, it has been controversial as to whether soluble OSCAR increases in healthy individuals or as a result of erosive activity. Serum levels of soluble OSCAR are shown to inversely correlate with erosion and disease activity [338] as it has been found that higher serum levels of soluble OSCAR are detected in serum in healthy individuals with no history of arthritis when compared to RA patients [269, 338]. However, recently higher serum levels of OSCAR are found in RA patients rather than healthy individuals

hence soluble OSCAR indicates erosive activity [376]. Additionally our research group has shown sOSCAR in the synovial fluid of osteoarthritis patients has no significant difference to that in active RA patients [374].

OSCAR has also been investigated in the context of osteoarthritis [374], low bone mass density (BMD) [375] and osteoporosis [378]. Incubation of cells with synovial fluid taken from osteoarthritis patients *in vitro* increased OSCAR and NFATc1 mRNA expression, suggesting that there are unknown factors in synovial fluids stimulating OSCAR expression [374]. A single nucleotide polymorphism, OSCAR 2322A>G that affects CREB binding site in OSCAR promoter region, is associated with low BMD [375]. Moreover, $FcR\gamma^{-/-}$ $DAP^{-/-}$ in acute estrogen deficiency-induced osteoporotic ovariactomised mice improves bone loss [378].

We believe soluble OSCAR (sOSCAR) can potentially act as a decoy receptor for OSCAR ligand within the joint and affect osteoclast development in RA. It is possible successful treatment results in increased cleavage of cell associated OSCAR resulting in increased sOSCAR levels in the joint. In this way sOSCAR regulates osteoclastic bone resorption and is an early marker that predicts joint damage. Of note, the biological effect of serum and synovial fluid-derived OSCAR on osteoclastogenesis has not been investigated. Considering the OSCAR-encoded gene is a newly-discovered target of NFATc1 and OSCAR itself is a more recently discovered osteoclast-specific protein, little is known about OSCAR regulation and its role in osteoclastogenesis. In this thesis we investigated the effects of NFATc1 inhibition on OSCAR expression *in vitro*. To further understand this correlation to the context of pathological bone loss, this thesis investigated the serum levels of soluble OSCAR in normal mice and peri-prosthetic osteolysis *in vivo*.

1.6.2.Osteoblasts in peri-prosthetic osteolysis

Although the effects of PE particles have been proposed to be less on osteoblast bone formation when compared to osteoclastogenesis [379], it is important to understand the role played by osteoblasts in this pathology. Research has been reporting the involvement of osteoblasts in wear particle-induced osteolysis. PE particles promote osteoblast maturation to osteocytes [50]. It has been demonstrated that PE promote bone loss through regulation of osteoblasts by suppressing bone formation [50, 379, 380] or promoting

osteoclastogenesis through the production of pro-osteoclastogenesis mediators such as RANKL [379, 381]. In response to PE osteoblasts undergoing maturation produce nitric oxide which has been shown to be involved in mediating localized bone destruction [382]. The production of RANKL and associated cytokines may induce osteoclastic bone resorption in PE-induced osteolysis through these mechanisms.

1.6.3.Osteocytes in peri-prosthetic osteolysis

Osteocytes have been shown to contribute to osteoclastogenesis in pathology. One of the mechanisms osteocytes regulate osteoclast activity is through induction of osteocyte apoptosis [112]. It has been shown the microdamage may induce osteocyte injuries and apoptosis thereby generating one or more signals for recruitment of functional osteoclasts to resorb bone at the sites of bone containing the dead or dying cells [112]. Additionally it has been shown cobalt-chromium-molybdenum alloy (Co-Cr-Mo) particles *in vitro* has directed osteocytes to undergo apoptosis [383].

Another mechanism suggesting the involvement of osteocytes in osteoclast regulation is through expression of inflammatory cytokines to initiate osteoclast formation. In response to mechanical damage on the surface of the matrix, the osteocyte cellular network induces RANKL-mediated local osteoclast formation and resorption *in vitro* [113]. Additionally osteocytes also produce osteoclast-associated inflammatory cytokines including RANKL, IL-8 and M-CSF in response to Co-Cr-Mo particles *in vitro* [383]. PE particles induce RANKL production by osteocytes *in vitro* [50].

This micro-damage on the matrix surface which elevates osteocyte apoptosis therefore enhancing osteoclast activity around the damaged matrix surface may explain the importance to investigate the effects of wear particles implantation on bone resorption and osteocyte apoptosis. It is possible that the osteocyte response to wear particles can enhance surface bone resorption through similar mechanisms. This thesis investigates the effects of PE particles on osteocyte apoptosis together with local bone surface resorption on murine calvarial bone in PE-induced osteolysis.

1.7.Inhibition of bone resorption

The treatment of peri-prosthetic osteolysis has been improving with the use of novel anti-resorptive drugs as well as osteoanabolic drugs. Since osteoclasts have been shown as the main culprit in promoting bone resorption at the site of bone erosions, osteoclasts are becoming an important target for development of therapeutics to treat peri-prosthetic osteolysis. The treatments include reduction of osteoclast formation and number, inhibition of osteoclast resorption activity and increase of osteoclast apoptosis [384]. This thesis focuses on targeting the signalling pathways important in osteoclast formation and activity *in vitro* as well as using an animal model for *in vivo* studies.

1.7.1.Intercellular signalling pathway inhibitors

Most therapeutic approaches to lessen pathological bone loss have been focusing on the inhibition of osteoclast bone resorption using drugs or inhibitors. Several bone resorption inhibitors currently used are bisphosphonates and denosumab, which target molecules at the upstream signalling pathway of osteoclastogenesis.

1.7.1.1.Bisphosphonates

Bisphosphonates (P-C-P) are analogs of pyrophosphate (P-O-P) but with oxygen (O) replaced by carbon (C) [126]. Bisphosphonates, a class of anti-resorptive drugs, bind strongly to mineral crystals of bone thereby inhibiting osteoclast bone resorption activity [184, 385]. Bisphosphonates are effectively used in the treatment of various bone disorders, including osteoporosis, tumour-associated osteolysis, arthritis [386], Paget's disease and bone tumors [184]. Due to reduction in osteoclast precursors [387], there was a 50% reduction of bone resorption due to bisphosphonate treatment [184]. Despite having benefits of reducing generalized bone loss in osteoporosis, their effectiveness in suppressing focal bone erosions is questionable [388, 389]. There are also concerns about a side effect of upper gastrointestinal distress that the arrest of bone turn over may lead to low quality bone susceptible to fracture with long term use. However further studies have shown several types of bisphosphonates such as alendronate [390, 391], TRK-530 [392] and pamidronate [65, 393] inhibit PE-induced osteolysis in animals with some success *in*

vivo. This is further supported by reduced particle-induced osteolysis with reduced side effects [90, 394].

1.7.1.2.Denosumab

A humanised monoclonal immunoglobulin G (2) antibody [389], denosumab (formally known as AMG162), acts by binding to RANKL like natural OPG, thereby preventing RANKL interaction with its receptor, RANK [395]. This results in decreased osteoclast formation, activity and survival therefore bone resorption [396]. In osteoporotic postmenopausal women, denosumab treatment was shown to increase BMD by reducing bone turnover markers and reducing vertebral, hip and non-vertebral fractures [397]. Denosumab has a potent effect on osteoclast function but needs further studies in particle-induced osteolysis [398].

1.7.2.Intracellular signalling pathway inhibitors

In addition to RANKL/RANK signaling pathway in osteoclastogenesis, NFAT signal transduction pathway has drawn significant attention for its role in osteoclastogenesis. Calcineurin is the key mediator of NFAT signalling pathway. This calcineurin/NFAT signalling pathway is becoming an important target for treatment of pathological bone loss.

A well known inhibitor of calcineurin pathway, an immunosuppressant drug Cyclosporin A (CsA) isolated from the fungus *Hypocladium inflatum gams* that is used to treat allograft rejection and graft versus host disease [136], has been shown to retard RANKL-induced osteoclastogenesis *in vitro* [125, 263]. CsA forms a complex with an intracytoplasmic protein cyclophilin, which in turn inhibits intracellular phosphatase calcineurin [136, 399, 400] to exert its immunosuppressive effect. This calcineurin pathway inhibition in turn blocks nuclear localisation of NFATc1 and, therefore, osteoclastogenesis [125, 242, 263]. Tacrolimus (FK506) [400] and 11R-VIVIT peptide (VIVIT) demonstrated similar outcomes [125]. However, due to limited studies on calcineurin/NFAT signalling pathway, the mechanism of action of the calcineurin inhibitors including CsA and FK506, and how it affects a range of mediators downstream is not clear. In this thesis we aim to determine the effects of calcineurin/NFAT inhibition on ITAM signalling pathway downstream to RANKL/RANK signalling in

osteoclastogenesis in the context of inflammation. Calcineurin/NFAT inhibitors FK506 (**Section 1.7.2.1**, Chapter 2), VIVIT (**Section 1.7.2.2**, Chapter 2) and NC100 (**Section 1.7.2.3**, Chapter 3) have been investigated to better understand the role of ITAM-associated molecules in osteoclastogenesis in the context of pathogenic bone loss.

Importantly, NF-κB and NFATc1 downstream to calcineurin have been shown to be the key signalling molecules in osteoclastogenesis. The development of novel drugs or inhibitors that specifically target NF-κB and NFATc1 may be specifically useful to treat inflammation-mediated pathological bone loss diseases such as peri-prosthetic osteolysis. In this thesis the effects of NF-κB inhibitor parthenolide (PAR) (**Section 1.7.2.4**, Chapter 4) and NF-κB/NFATc1 inhibitor caffeic acid phenethyl ester (CAPE) (**Section 1.7.2.5**, Chapter 5) are being studied in an animal model of particle-induced peri-prosthetic osteolysis.

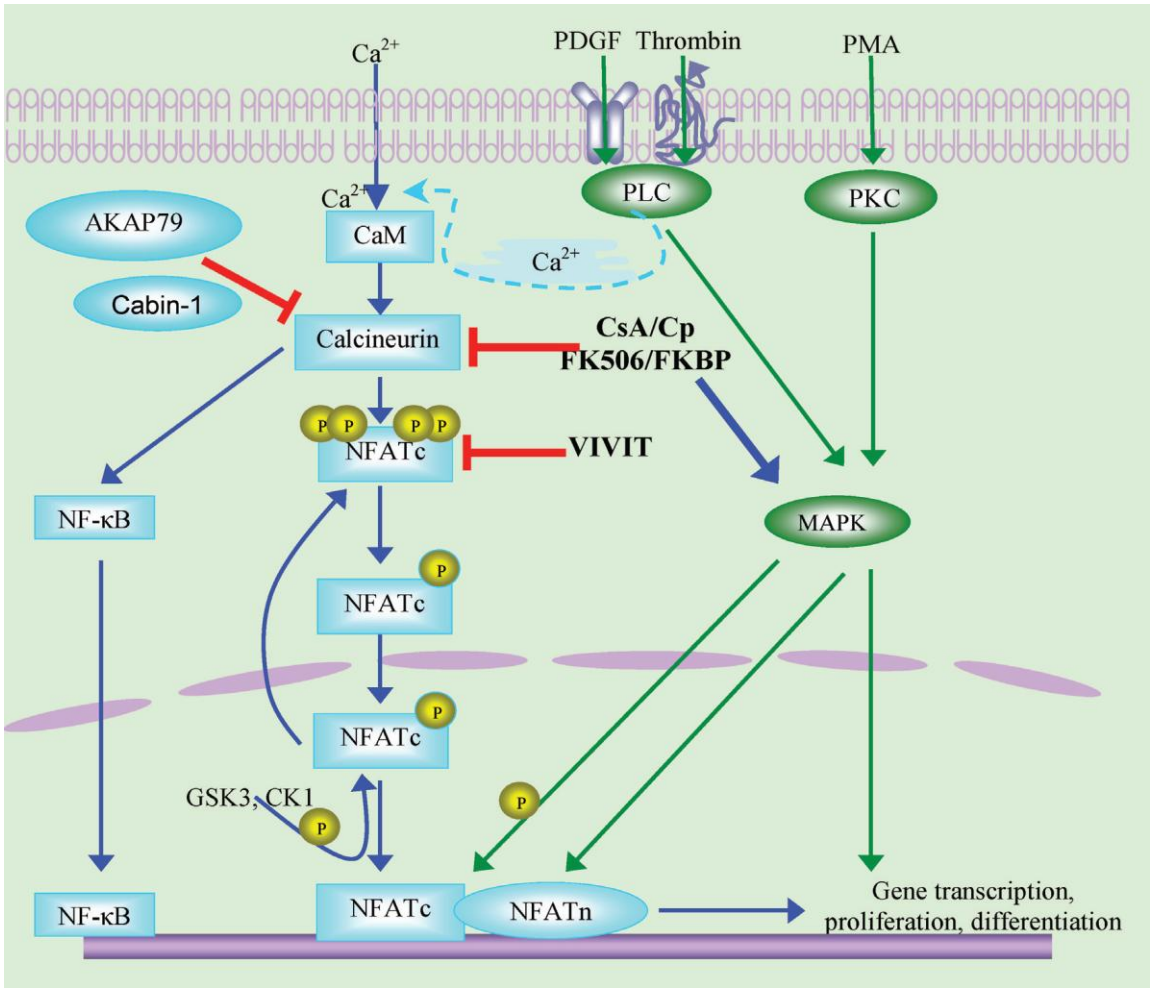

Figure 1.7 Calcineurin/NFAT inhibitors FK506, CsA and VIVIT target different points in the calcineurin pathway in osteoclastogenesis **[401].**

1.7.2.1.Tacrolimus (FK506)

FK506, a compound isolated from *Streptomyces tsukubaensis* bacteria, is a macrolide immunosuppressant drug used in organ transplantation, particularly liver donor transplantation [400] and atopic dermatitis [402]. FK506 has been identified as an effective treatment of DMARD-resistant or intolerant RA patients [403]. It binds exclusively to a member of intracellular cytosolic immunophilin proteins FK506-binding protein 12 (FKBP12) [136, 404]. The FK506-FKBP12 complex blocks calcineurin, thereby inhibiting immune function of osteoclastogenesis (**Figure 1.7**) [125, 136]. In addition FK506 also blocks T cell-derived pro-inflammatory cytokines such as TNF-α, IL-2 and interferon-γ

(INF-γ) [136] and reduces NFATc1 nuclear localisation [125] thereby inhibiting RANKL-induced osteoclastogenesis *in vitro* [402, 404].

Complemetary to this, FK506 at 0.1μM has been shown to more strongly supress osteoclastogenesis when compared to lower doses of FK506 (0.001 and 0.01μM) [402]. FK506 at 0.1μM inhibits human monocyte-osteoclast differentiation more effectively at the late stage of osteoclast differentiation [402]. FK506 also exhibits anabolic effects on the osteoblast *in vivo* and *in vitro* [405]. Together, these reports demonstrate and confirm the significant inhibition of calcinuerin/NFAT signalling by FK506 in osteoclastogenesis [136, 242]. Thus far no studies have shown specifically how inhibition of NFATc1 by FK506 modulates ITAM molecules. The current study investigates the modulatory effects of calcineurin/NFAT supression by FK506 on ITAM-signalling associated molecules in osteoclastogenesis *in vitro*.

1.7.2.2.11R-VIVIT peptide (VIVIT)

Previous studies have shown that a peptide, "SPRIEIT", encoding an amino acid sequence that has only a low affinity towards calcineurin pathway [406]. Further study has shown that the amino acid sequence, a commercially available calcineurin inhibitory peptide motif VIVIT (MAGPHPVIVITGPHEE) is 25 times more effective than the original SPRIEIT peptide, can selectively and potently inhibit calcineurin-NFAT interaction [399, 401]. The development of VIVIT may avoid side effects associated with treatment with FK506 [401].

As illustrated in **Figure 1.8**, VIVIT has a more specific and selective profile than that of FK506 and CsA in its ability to inhibit the NFAT pathway. This specificity is confirmed by studies using green fluorescent tagged (GFP)-VIVIT where GFP-VIVIT only inhibited NFAT activation but not NF-κB [399]. The idea of specificity is supported by similar inhibition of calcineurin phosphatase activity by VIVIT which only needs a relatively small amount (10μM) compared to CsA [401]. VIVIT interferes selectively with calcineurin-NFAT interaction without affecting calcineurin phosphatase activity [399].

VIVIT affects osteoclast morphology and suppression of β3 integrin gene expression in murine bone marrow-derived osteoclasts [262]. VIVIT inhibits RANKL-induced osteoclastogenesis *in vitro* and reduces the nuclear localisation of NFATc1 [125]. VIVIT

significantly reduced resorption and completely inhibited NFATc1 expression in BMMs and multinucleated cell cultures [407]. In support of this, inactivation of NFATc1 by VIVIT potently impeded the Ti particle-induced TNF-α expression [46] and osteoclastogenesis in RANKL-induced BMM culture [45]. A similar observation was seen in PMMA-induced BMM-derived osteoclast cultures and inactivation of NFATc1 by VIVIT also reduced the resorption area and total number of resorption pits on ivory slices [46]. This further demonstrates the potency of VIVIT to target calcineurin pathway and inhibit osteoclast formation and differentiation. However, the effects of VIVIT treatment on the regulation of ITAM factors have not been investigated. This opens up a new area to develop strategies to inhibit the excessive bone resorption activities by osteoclasts in pathological bone loss. The current study investigates the effects of calcineurin/NFAT inhibition using VIVIT on the ITAM factors *in vitro*.

1.7.2.3.Natural compound 100 (NC100)

The development of natural compounds that can regulate osteoclast formation and bone resorption activity is potentially essential to prevent and treat bone loss diseases. NC100, a new calcineurin-inhibiting natural compound isolated from *Zanthoxylum nitidum (Rutacae)* and *Fagara zanthoxyloides* has not yet been tested in any bone-related studies. It has been shown as antifungal, antihypertensive and antileukemic [408]. NC100 has strong antitumor activity and anti-hypertensive properties [409]. NC100 possibly inhibits tumor cell proliferation via the promotion of T and B cell proliferation and differentiation to enhance NK cell and monocyte-macrophage cell activation [410]. NC100 has also been used as an active component to multidrug resistant tumor cells by inhibiting the growth and promoting the apoptosis of KBV200 cells [411]. In addition, NC100 has been used for toothpaste in China for 30 years. However, the role of NC100 in osteoclastic bone resorption is unknown. Recently, our collaborator Professor Jiake Xu (University of Western Australia) has demonstrated NC100 inhibits murine osteoclast development *in vitro* by reducing NFATc1 expression in cells (unpublished work). In order to understand the molecular mechanisms of NC100 in osteoclast formation, the effects of NC100 on osteoclast formation and activity must be investigated.

1.7.2.4.Parthenolide (PAR)

Recently, several natural and synthetic NF-κB inhibitors have been studied. They exert their suppressive effects by retarding NF-κB activitie at different sites [251]. PAR, an active ingredient of medicinal plant Feverfew [251, 412, 413], significantly inhibits *in vitro* osteoclastogenesis, particularly during the early stage of it [107]. PAR prevents NF-κB DNA binding by inhibiting thereby sequestering Ikβ kinase in the cytoplasm, as well as by directly modifying p65 protein [107]. Additionally PAR reduces LPS- [107] and PE-induced [86] osteolysis in mice. These findings suggest PAR as a treatment to target the NF-κB pathway in osteoclast formation for the treatment of human peri-implant bone lysis. The current study investigates whether PAR treatment reduces the PE particle-induced volumetric bone change over time in a murine calvarial model of osteolysis at day 14. Importantly, the current study assesses the effects of PE particles and PAR treatment on surface bone resorption of the calvariae and osteocyte apoptosis.

1.7.2.5.Caffeic acid phenethyl ester (CAPE)

A phenolic antioxidant derived from the propolis of honeybee hives, CAPE, is a potent and specific inhibitor of NFATc1 and NF-κB [414-416]. This natural compound has anti-viral [414] and anti-inflammatory properties [415, 416]. *In vitro* osteoclast assays demonstrate that CAPE inhibits RANKL-induced activation of NF-κB and NFAT via p65 nuclear translocation resulting in abrogation of osteoclastogenesis and induction of apoptosis [416], suggesting CAPE as a potential candidate for the prevention or treatment of osteolytic and arthritic bone diseases [416].

CAPE at high dose has been shown to have anti-inflammatory properties in a rat air pouch model (10-100mg/ml) [417] and LPS-induced inflammation of cultured human middle ear epithelial cells (HMEECs) (50-200μM) [418]. In a rat model of colitis, CAPE at 30mg/kg caused weight loss although it effectively reduced macroscopic colonic damage [419]. Importantly CAPE at low dose, 0.5mg/kg [420] and 1.0mg/kg (unpublished data from Jiake Xu) inhibits local bone loss in an ovariectomized (OVX) murine model of osteolysis. It is therefore important to ascertain the effects of CAPE on locally induced bone loss and systemic effects on bone resorption and GIT.

1.8.Conclusion and study outline

Many studies have shown activated RANKL-RANK signalling involving NF-κB enhances osteoclastogenesis via calcineurin and NFATc1 expression. Similarly, ITAM-related factors OSCAR, FcRγ, TREM2 and DAP12 have been found to induce NFATc1 expression, thereby, increasing osteoclast formation and activity. However, it is unclear whether inhibition of osteoclastogenesis through calcineurin/NFAT pathway modulates ITAM signaling and subsequent osteoclast numbers and activity.

Inhibition of NF-κB and NFATc1 has been effective in reducing bone resorption in osteoporotic animal model. However, the effects of this inhibition have not been studied in PE-induced peri-prosthetic osteolysis. In addition, the effects on ITAM-associated factors, such as OSCAR, are yet to be fully understood. It is important to elucidate the effects of PE particles on NF-κB/NFATc1 signalling as well as NF-κB/NFATc1 inhibition at local and systemic levels.

1.8.1.Hypotheses and aims

Hypothesis 1: Inhibition of NF-κB and NFATc1 modulates ITAM signalling and osteoclastogenesis in human osteoclast cultures *in vitro*.

Aim 1.0: To investigate the effects of calcineurin/NFAT inhibitors FK506, VIVIT and NC100 on ITAM expression, late stage osteoclast markers and osteoclast resorption in PBMC-derived human osteoclast culture *in vitro*.

Hypothesis 2: Inhibition of NF-κB supresses localized and systemic bone resorption in a murine calvarial model of particle-induced peri-prosthetic osteolysis.

Aim 2.0: To establish a novel technique to analyse local surface bone resorption by using μCT in a murine calvarial model of PE-induced peri-prosthetic osteolysis.

Aim 3.0: To investigate the effects of NF-κB inhibitor PAR on local and systemic bone resorption in a murine calvarial model of PE-induced peri-prosthetic osteolysis.

Aim 4.0: To determine the systemic effects of PE particles and PAR treatment on the soluble OSCAR levels in a murine calvarial model of PE-induced peri-prosthetic osteolysis.

Aim 5.0: To investigate effects of PE particles and PAR treatment on osteocytes in a murine calvarial model of PE-induced peri-prosthetic osteolysis.

Hypothesis 3: Inhibition of NF-κB and NFATc1 suppresses localized and systemic bone resorption in a murine calvarial model of particle-induced peri-prosthetic osteolysis.

Aim 6.0: To investigate the effects of NF-κB and NFATc1 inhibitor CAPE on local and systemic bone resorption in a murine calvarial model of PE-induced peri-prosthetic osteolysis.

Aim 7.0: To determine the systemic effects of PE particles and CAPE treatment on soluble OSCAR levels in a murine calvarial model of PE-induced peri-prosthetic osteolysis.

Aim 8.0: To investigate the systemic effects of PE particles and CAPE treatment on GIT in a murine calvarial model of PE-induced peri-prosthetic osteolysis.

1.9.References

[1] Kini U, Nandeesh BN. Physiology of Bone Formation, Remodeling, and Metabolism. In: Fogelman I, Gnanasegaran G, van der Wall H, editors. Radionuclide and Hybrid Bone Imaging: Springer Berlin Heidelberg; 2012. p. 29-57.

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

[3] Haynes DR, Crotti TN, Zreiqat H. Regulation of osteoclast activity in peri-implant tissues. Biomaterials 2004;25:4877-85.

[4] Cantley MD, Bartold PM, Marino V, Reid RC, Fairlie DP, Wyszynski RN, et al. The use of live-animal micro-computed tomography to determine the effect of a novel phospholipase A2inhibitor on alveolar bone loss in anin vivomouse model of periodontitis. Journal of Periodontal Research 2009;44:317-22.

[5] Taubman MA, Valverde P, Han X, Kawai T. Immune response: the key to bone resorption in periodontal disease. J Periodontol 2005;76(11 Suppl):2033-41.

[6] Cantley MD, Haynes DR, Marino V, Bartold PM. Pre-existing periodontitis exacerbates experimental arthritis in a mouse model. Journal of clinical periodontology 2011;38:532-41.

[7] Arron JR, Choi Y. Bone versus immune system. Nature 2000;408(6812):535-6.

[8] Walsh MC, Kim N, Kadono Y, Rho J, Lee SY, Lorenzo J, et al. Osteoimmunology: interplay between the immune system and bone metabolism. Annu Rev Immunol 2006;24:33-63.

[9] Nemeth K, Schoppet M, Al-Fakhri N, Helas S, Jessberger R, Hofbauer LC, et al. The role of osteoclast-associated receptor in osteoimmunology. J Immunol 2011;186(1):13-8.

[10] Goodman SB, E G, Yao Z. The basic science of periprosthetic osteolysis. 2013.

[11] Walsh NC, Gravallese EM. Bone remodeling in rheumatic disease: a question of balance. Immunol Rev 2010;233(1):301-12.

[12] Bogoch ER, Moran E. Abnormal bone remodelling in inflammatory arthritis. Can J Surg 1998;41(4):264-71.

[13] Goldring SR, Purdue PE, Crotti TN, Shen Z, Flannery MR, Binder NB, et al. Bone remodelling in inflammatory arthritis. Ann Rheum Dis 2013;72 Suppl 2:ii52-5.

[14] Sato K, Takayanagi H. Osteoclasts, rheumatoid arthritis, and osteoimmunology. Curr Opin Rheumatol 2006;18(4):419-26.

[15] Graves SE, Davidson D, Ingerson L, Ryan P, Griffith EC, McDermott BF, et al. The Australian Orthopaedic Association National Joint Replacement Registry. Med J Aust 2004;180(5 Suppl):S31-4.

[16] Fender D, Harper WM, Gregg PJ. Outcome of Charnley total hip replacement across a single health region in England: the results at five years from a regional hip register. J Bone Joint Surg Br 1999;81(4):577-81.

[17] Allami MK, Fender D, Khaw FM, Sandher DR, Esler C, Harper WM, et al. Outcome of Charnley total hip replacement across a single health region in England. The results at ten years from a regional arthroplasty register. J Bone Joint Surg Br 2006;88(10):1293-8.

[18] Harris WH. The problem is osteolysis. Clin Orthop Relat Res 1995;(311):46-53.

[19] Kavanagh BF, Wallrichs S, Dewitz M, Berry D, Currier B, Ilstrup D, et al. Charnley low-friction arthroplasty of the hip. Twenty-year results with cement. J Arthroplasty 1994;9(3):229-34.

[20] Holding CA, Findlay DM, Stamenkov R, Neale SD, Lucas H, Dharmapatni AS, et al. The correlation of RANK, RANKL and TNFalpha expression with bone loss volume and polyethylene wear debris around hip implants. Biomaterials 2006;27:5212-9.

[21] Aspenberg P, van der Vis H. Fluid pressure may cause periprosthetic osteolysis. Particles are not the only thing. Acta Orthop Scand 1998;69(1):1-4.

[22] Skripitz R, Aspenberg P. Pressure-induced periprosthetic osteolysis: a rat model. J Orthop Res 2000;18(3):481-4.

[23] Goldring SR, Schiller AL, Roelke M, Rourke CM, O'Neil DA, Harris WH. The synovial-like membrane at the bone-cement interface in loose total hip replacements and its proposed role in bone lysis. J Bone Joint Surg Am 1983;65(5):575-84.

[24] Haynes DR, Crotti TN, Potter AE, Loric M, Atkins GJ, Howie DW, et al. The osteoclastogenic molecules RANKL and RANK are associated with periprosthetic osteolysis. J Bone Joint Surg Br 2001;83:902-11.

[25] Shen Z, Crotti TN, McHugh KP, Matsuzaki K, Gravallese EM, Bierbaum BE, et al. The role played by cell-substrate interactions in the pathogenesis of osteoclast-mediated peri-implant osteolysis. Arthritis Res Ther 2006;8:R70.

[26] Howie DW. Tissue response in relation to type of wear particles around failed hip arthroplasties. J Arthroplasty 1990;5(4):337-48.

[27] Yamanaka Y, Abu-Amer W, Foglia D, Otero J, Clohisy JC, Abu-Amer Y. NFAT2 is an essential mediator of orthopedic particle-induced osteoclastogenesis. J Orthop Res 2008;26:1577-84.

[28] Baumann B, Rader CP, Seufert J, Noth U, Rolf O, Eulert J, et al. Effects of polyethylene and TiAlV wear particles on expression of RANK, RANKL and OPG mRNA. Acta Orthop Scand 2004;75(3):295-302.

[29] Horowitz SM, Gonzales JB. Inflammatory response to implant particulates in a macrophage/osteoblast coculture model. Calcif Tissue Int 1996;59(5)::392-6.

[30] Matthews JB, Green TR, Stone MH, Wroblewski BM, Fisher J, Ingham E. Comparison of the response of three human monocytic cell lines to challenge with polyethylene particles of known size and dose. J Mater Sci Mater Med 2001;12(3):249-58.

[31] Rader CP, Sterner T, Jakob F, Schutze N, Eulert J. Cytokine response of human macrophage-like cells after contact with polyethylene and pure titanium particles. J Arthroplasty 1999;14(7):840-8.

[32] Jiranek WA, Machado M, Jasty M, Jevsevar D, Wolfe HJ, Goldring SR, et al. Production of cytokines around loosened cemented acetabular components. Analysis with immunohistochemical techniques and in situ hybridization. J Bone Joint Surg Am 1993;75(6)::863-79.

[33] Murray DW, Rushton N. Macrophages stimulate bone resorption when they phagocytose particles. J Bone Joint Surg Br 1990;72(6):988-92.

[34] Ren W, Markel DC, Schwendener R, Ding Y, Wu B, Wooley PH. Macrophage depletion diminishes implant-wear-induced inflammatory osteolysis in a mouse model. J Biomed Mater Res A 2008;85(4):1043-51.

[35] Alias E, Dharmapatni ASSK, Holding AC, Atkins GJ, Findlay DM, Howie DW, et al. Polyethylene particles stimulate expression of ITAM-related molecules in peri-implant tissues and when stimulating osteoclastogenesis in vitro. Acta Biomaterialia 2012;8(8):3104-12.

[36] Havelin LI, Espehaug B, Vollset SE, Engesaeter LB. The effect of the type of cement on early revision of Charnley total hip prostheses. A review of eight thousand five hundred

and seventy-nine primary arthroplasties from the Norwegian Arthroplasty Register. J Bone Joint Surg Am 1995;77(10):1543-50.

[37] Jones LC, Frondoza C, Hungerford DS. Effect of PMMA particles and movement on an implant interface in a canine model. J Bone Joint Surg Br 2001;83(3):448-58.

[38] Rogers SD, Howie DW, Graves SE, Pearcy MJ, Haynes DR. In vitro human monocyte response to wear particles of titanium alloy containing vanadium or niobium. J Bone Joint Surg Br 1997;79(2):311-5.

[39] Kaufman AM, Alabre CI, Rubash HE, Shanbhag AS. Human macrophage response to UHMWPE, TiAlV, CoCr, and alumina particles: analysis of multiple cytokines using protein arrays. J Biomed Mater Res A 2008;84(2):464-74.

[40] Haynes DR, Boyle SJR, S. D., Howie DW, Vernon-Roberts B. Variation in cytokines induced by particles from different prosthetic materials. Clin Orthop Relat Res 1998;(352):223-30.

[41] Neale SD, Haynes DR, Howie DW, Murray DW, Athanasou NA. The effect of particle phagocytosis and metallic wear particles on osteoclast formation and bone resorption in vitro. J Arthroplasty 2000;15(5):654-62.

[42] Haynes DR, Rogers SD, Hay S, Pearcy MJ, Howie DW. The differences in toxicity and release of bone-resorbing mediators induced by titanium and cobalt-chromium-alloy wear particles. J Bone Joint Surg Am 1993;75(6):825-34.

[43] Shanbhag AS, Jacobs JJ, Black J, Galante JO, Glant TT. Macrophage/particle interactions: effect of size, composition and surface area. J Biomed Mater Res 1994;28(1):81-90.

[44] Shanbhag AS, Jacobs JJ, Black J, Galante JO, Glant TT. Human monocyte response to particulate biomaterials generated in vivo and in vitro. J Orthop Res 1995;13(5):792-801.

[45] Liu F, Zhu Z, Mao Y, Liu M, Tang T, Qiu S. Inhibition of titanium particle-induced osteoclastogenesis through inactivation of NFATc1 by VIVIT peptide. Biomaterials 2009;30:1756-62.

[46] Liu FX, Wu CL, Zhu Z-a, Li M-q, Mao Y-q, Liu M, et al. Calcineurin/NFAT pathway mediates wear particle-induced TNF-alpha release and osteoclastogenesis from mice bone marrow macrophages in vitro. Acta Pharmacol Sin 2013;34(11):1457-66. .

[47] Wooley PH, Morren R, Andary J, Sud S, Yang SY, Mayton L, et al. Inflammatory responses to orthopaedic biomaterials in the murine air pouch. Biomaterials 2002;23(2):517-26.

[48] Kurtz SM, Ok M, M E, Edidin AA. Advances in the processing, sterilization, and crosslinking of ultra-high molecular weight polyethylene for total joint arthroplasty. Biomaterials 1999;20(18):1659-88.

[49] Kobayashi A, Freeman MA, Bonfield W, Kadoya Y, Yamac T, Al-Saffar N, et al. Number of polyethylene particles and osteolysis in total joint replacements. A quantitative study using a tissue-digestion method. J Bone Joint Surg Br 1997;79(5):844-8.

[50] Atkins GJ, Welldon KJ, Holding CA, Haynes DR, Howie DW, Findlay DM. The induction of a catabolic phenotype in human primary osteoblasts and osteocytes by polyethylene particles. Biomaterials 2009;30:3672-81.

[51] Atkins GJ, Haynes DR, Howie DW, Findlay DM. Role of polyethylene particles in peri-prosthetic osteolysis: A review. World journal of orthopedics 2011;2:93-101.

[52] Revell PA, Weightman B, Freeman MA, Roberts BV. The production and biology of polyethylene wear debris. Arch Orthop Trauma Surg 1978;30; 91(3):167-81.

[53] Looney RJ, Boyd A, Totterman S, Seo G, Tamez-Pena J, Campbell D, et al. Volumetric computerized tomography as a measurement of periprosthetic acetabular osteolysis and its correlation with wear. Arthritis Res 2002;4(1):59-63.

[54] Kim KJ, Kobayashi Y, Itoh T. Osteolysis model with continuous infusion of polyethylene particles. Clin Orthop Relat Res 1998;(352):46-52.

[55] Howie DW, Neale SD, Stamenkov R, McGee MA, Taylor DJ, Findlay DM. Progression of acetabular periprosthetic osteolytic lesions measured with computed tomography. J Bone Joint Surg Am 2007;89(8):1818-25.

[56] Oparaugo PC, Clarke IC, Malchau H, Herberts P. Correlation of wear debris-induced osteolysis and revision with volumetric wear-rates of polyethylene: a survey of 8 reports in the literature. Acta Orthop Scand 2001;72(1):22-8.

[57] Orishimo KF, Claus AM, Sychterz CJ, Engh CA. Relationship between polyethylene wear and osteolysis in hips with a second-generation porous-coated cementless cup after seven years of follow-up. J Bone Joint Surg Am 2003;85-A(6):1095-9.

[58] Sochart DH. Relationship of acetabular wear to osteolysis and loosening in total hip arthroplasty. Clin Orthop Relat Res 1999 (363)::135-50.

[59] Shanbhag AS, Jacobs JT, Glant TT, Gilbert Jl, Black J, Galante JO. Composition and morphology of wear debris in failed uncemented total hip replacement. J Bone Joint Surg Br 1994;76(1):60-7.

[60] Campbell P, Ma S, Yeom B, McKellop H, Schmalzried TP, Amstutz HC. Isolation of predominantly submicron-sized UHMWPE wear particles from periprosthetic tissues. J Biomed Mater Res 1995;29(1):127-31.

[61] Hirakawa K, Bauer TW, Stulberg BN, Wilde AH. Comparison and quantitation of wear debris of failed total hip and total knee arthroplasty. J Biomed Mater Res 1996;31(2):257-63.

[62] Willert HG, Bertram H, Buchhorn GH. Osteolysis in alloarthroplasty of the hip. The role of ultra-high molecular weight polyethylene wear particles. Clin Orthop Relat Res 1990;(258)::95-107.

[63] Migaud H, Putman S, Krantz N, Vasseur L, Girard J. Cementless metal-on-metal versus ceramic-on-polyethylene hip arthroplasty in patients less than fifty years of age: a comparative study with twelve to fourteen-year follow-up. J Bone Joint Surg Am 2011;93 Suppl 2:137-42.

[64] Migaud H, Jobin A, Chantelot C, Giraud F, Laffargue P, Duquennoy A. Cementless metal-on-metal hip arthroplasty in patients less than 50 years of age: comparison with a matched control group using ceramic-on-polyethylene after a minimum 5-year follow-up. J Arthroplasty 2004;19(8 Suppl 3):23-8.

[65] Horowitz SM, Gonzales JB. Effects of polyethylene on macrophages. J Orthop Res 1997;15(1):50-6.

[66] Grimaud E, Soubigou L, Couillaud S, Coipeau P, Moreau A, Passuti N, et al. Receptor activator of nuclear factor kappaB ligand (RANKL)/osteoprotegerin (OPG) ratio is increased in severe osteolysis. Am J Pathol 2003;163:2021-31.

[67] Wedemeyer C, Xu J, Neuerburg C, Landgraeber S, Malyar NM, von Knoch F, et al. Particle-induced osteolysis in three-dimensional micro-computed tomography. Calcif Tissue Int 2007;81:394-402.

[68] Tuan RS, Lee FY, Y TK, Wilkinson JM, Smith RL. What are the local and systemic biologic reactions and mediators to wear debris, and what host factors determine or modulate the biologic response to wear particles? J Am Acad Orthop Surg 2008;16 Suppl 1:S42-8.

[69] Rosen HN, Moses AC, Garber J, Iloputaife ID, Ross DS, Lee SL, et al. Serum CTX: a new marker of bone resorption that shows treatment effect more often than other markers because of low coefficient of variability and large changes with bisphosphonate therapy. Calcif Tissue Int 2000;66(2):100-3.

[70] Hernandez JL, Olmos JM, Romana G, Martinez J, Castillo J, Yezerska I, et al. Bone turnover markers in statin users: A population-based analysis from the Camargo Cohort Study. Maturitas 2013.

[71] Chailurkit L-o, Ongphiphadhanakul B, Piaseu N, Saetung S, Rajatanavin R. Biochemical Markers of Bone Turnover and Response of Bone Mineral Density to Intervention in Early Postmenopausal Women: An Experience in a Clinical Laboratory. Clinical Chemistry 2001;47:1083-8.

[72] Liu S, Virdi AS, Sena K, Hughes WF, Sumner DR. Bone turnover markers correlate with implant fixation in a rat model using LPS-doped particles to induced implant loosening. Journal of biomedical materials research Part A 2012;100:918-28.

[73] Gibson RJ, Bowen JM, Alvarez E, Finnie J, Keefe DM. Establishment of a single-dose irinotecan model of gastrointestinal mucositis. Chemotherapy 2007;53(5):360-9.

[74] Gibson RJ, Bowen JM, Keefe DM. Palifermin reduces diarrhea and increases survival following irinotecan treatment in tumor-bearing DA rats. Int J Cancer 2005;116(3):464-70.

[75] Gibson RJ, Bowen JM, Inglis MR, Cummins AG, Keefe DM. Irinotecan causes severe small intestinal damage, as well as colonic damage, in the rat with implanted breast cancer. J Gastroenterol Hepatol 2003;18(9):1095-100.

[76] Purdue PE, Koulouvaris P, Nestor BJ, Sculco TP. The central role of wear debris in periprosthetic osteolysis. HSS journal : the musculoskeletal journal of Hospital for Special Surgery 2006;2:102-13.

[77] Barrow AD, Raynal N, Andersen TL, Slatter DA, Bihan D, Pugh N, et al. OSCAR is a collagen receptor that costimulates osteoclastogenesis in DAP12-deficient humans and mice. 2011.

[78] Howie DW, Vernon-Roberts B, Oakeshott R, Manthey B. A rat model of resorption of bone at the cement-bone interface in the presence of polyethylene wear particles. J Bone Joint Surg Am 1988;70(2):257-63.

[79] Gelb H, Schumacher HR, Cuckler J, Ducheyne P, Baker DG. In vivo inflammatory response to polymethylmethacrylate particulate debris: effect of size, morphology, and surface area. J Orthop Res 1994;12(1):83-92.

[80] Boss JH, Shajrawi I, Soudry M, Mendes DG. Histological features of the interface membrane of failed isoelastic cementless prostheses. Int Orthop 1990;14(4):399-403.

[81] Yang SY, Ren W, Park Y, Sieving A, Hsu S, Nasser S, et al. Diverse cellular and apoptotic responses to variant shapes of UHMWPE particles in a murine model of inflammation. Biomaterials 2002;23(17):3535-43.

[82] Merkel KD, Erdmann JM, McHugh KP, Abu-Amer Y, Ross FP, Teitelbaum SL. Tumor necrosis factor-alpha mediates orthopedic implant osteolysis. Am J Pathol 1999;154:203-10.

[83] Schwarz EM, Benz EB, Lu AP, Goater JJ, Mollano AV, Rosier RN, et al. Quantitative small-animal surrogate to evaluate drug efficacy in preventing wear debris-induced osteolysis. J Orthop Res 2000;18(6):849-55.

[84] Landgraeber S, Jaeckel S, Loer F, Wedemeyer C, Hilken G, Canbay A, et al. Pan-caspase inhibition suppresses polyethylene particle-induced osteolysis. Apoptosis : an international journal on programmed cell death 2009;14:173-81.

[85] Tsutsumi R, C H, Cd B, St P, Sv B, H I, et al. Differential effects of biologic versus bisphosphonate inhibition of wear debris-induced osteolysis assessed by longitudinal micro-CT. 2008.

[86] Li Y, Zhang C, Zhou X, Wang H, Mao Y, Wang X. Parthenolide inhibits polyethylene particle-induced mouse calvarial osteolysis in vivo. The Journal of surgical research 2013.

[87] Wedemeyer C, Neuerburg C, Pfeiffer A, Heckelei A, von Knoch F, Hilken G, et al. Polyethylene particle-induced bone resorption in substance P-deficient mice. Calcif Tissue Int 2007;80(4):268-74.

[88] Wedemeyer C, Neuerburg C, Pfeiffer A, Heckelei A, Bylski D, von Knoch F, et al. Polyethylene particle-induced bone resorption in alpha-calcitonin gene-related peptide-deficient mice. J Bone Miner Res 2007;22:1011-9.

[89] Kong L, Yang C, Yu L, Smith W, Zhu S, Zhu J, et al. Pyrroloquinoline Quinine Inhibits RANKL-Mediated Expression of NFATc1 in Part via Suppression of c-Fos in Mouse Bone Marrow Cells and Inhibits Wear Particle-Induced Osteolysis in Mice. PloS one 2013;8:e61013.

[90] von Knoch M, Wedemeyer C, Pingsmann A, von Knoch F, Hilken G, Sprecher C, et al. The decrease of particle-induced osteolysis after a single dose of bisphosphonate. Biomaterials 2005;26:1803-8.

[91] von Knoch M, Sprecher C, Barden B, Saxler G, Löer F, M. W. [Size and shape of commercially available polyethylene particles for in-vitro and in-vivo-experiments]. Z Orthop Ihre Grenzgeb 2004;142(3):366-70.

[92] Catelas I, Huk OL, Petit A, Zukor DJ, Marchand R, Yahia L. Flow cytometric analysis of macrophage response to ceramic and polyethylene particles: effects of size, concentration, and composition. J Biomed Mater Res 1998;41(4):600-7.

[93] Ingham E, Fisher J. Biological reactions to wear debris in total joint replacement. Proc Inst Mech Eng H 2000;214(1):21-37.

[94] Revell PA. Tissue reactions to joint prostheses and the products of wear and corrosion. Curr Top Pathol 1982;71:73-101.

[95] Karin M, Greten FR. NF-kappaB: linking inflammation and immunity to cancer development and progression. Nat Rev Immunol 2005;5(10):749-59.

[96] Hitchins VM, Merritt K. Decontaminating particles exposed to bacterial endotoxin (LPS). J Biomed Mater Res 1999;46(3):434-7.

[97] Magalhaes PO, Lopes AM, Mazzola PG, Rangel-Yagui C, Penna TCV, Pessoa A, Jr. Methods of endotoxin removal from biological preparations: a review. J Pharm Pharm Sci 2007;10(3):388-404.

[98] Ogikubo Y, Norimatsu M, Noda K, Takahashi J, Inotsume M, Tsuchiya M, et al. Evaluation of the bacterial endotoxin test for quantification of endotoxin contamination of porcine vaccines. Biologicals 2004;32(2):88-93.

[99] Anspach FB. Endotoxin removal by affinity sorbents. J Biochem Biophys Methods 2001;49(1-3):665-81.

[100] Daneshian M, Guenther A, Wendel A, Hartung T, von Aulock S. In vitro pyrogen test for toxic or immunomodulatory drugs. J Immunol Methods 2006;313(1-2):169-75.

[101] Zhou X, Zhang P, Zhang C, Zhu Z. Promotion of bone formation by naringin in a titanium particle-induced diabetic murine calvarial osteolysis model. Journal of orthopaedic research : official publication of the Orthopaedic Research Society 2010;28:451-6.

[102] Poltorak A, He X, Smirnova I, Liu MY, Van Huffel C, Du X, et al. Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: mutations in Tlr4 gene. Science 1998;282(5396):2085-8.

[103] Zhou X, Zhang C, Wang X, An B, Zhang P, Zhu Z. Berberine inhibits lipopolysaccharide- and polyethylene particle-induced mouse calvarial osteolysis in vivo. The Journal of surgical research 2012;173:e47-52.

[104] Qin A, Cheng TS, Lin Z, Cao L, Chim SM, Pavlos NJ, et al. Prevention of wear particle-induced osteolysis by a novel V-ATPase inhibitor saliphenylhalamide through inhibition of osteoclast bone resorption. PloS one 2012;7:e34132.

[105] Kauther MD, Bachmann HS, Neuerburg L, Broecker-Preuss M, Hilken G, Grabellus F, et al. Calcitonin substitution in calcitonin deficiency reduces particle-induced osteolysis. BMC musculoskeletal disorders 2011;12:186.

[106] Zhang YH, Heulsmann A, Tondravi MM, Mukherjee A, Abu-Amer Y. Tumor necrosis factor-alpha (TNF) stimulates RANKL-induced osteoclastogenesis via coupling of TNF type 1 receptor and RANK signaling pathways. J Biol Chem 2001;276:563-8.

[107] Yip KH, Zheng MH, Feng HT, Steer JH, Joyce DA, Xu J. Sesquiterpene lactone parthenolide blocks lipopolysaccharide-induced osteolysis through the suppression of NF-kappaB activity. J Bone Miner Res 2004;19:1905-16.

[108] Kaar SG, Ragab AA, Kaye SJ, Kilic BA, Jinno T, Goldberg VM, et al. Rapid repair of titanium particle-induced osteolysis is dramatically reduced in aged mice. J Orthop Res 2001;19(2):171-8.

[109] Schmalzried TP, Harris WH. The Harris-Galante porous-coated acetabular component with screw fixation. Radiographic analysis of eighty-three primary hip replacements at a minimum of five years. J Bone Joint Surg Am 1992;74(8):1130-9.

[110] al Saffar N, Revell PA. Interleukin-1 production by activated macrophages surrounding loosened orthopaedic implants: a potential role in osteolysis. Br J Rheumatol 1994;33(4):309-16.

[111] Santavirta S, Hoikka V, Eskola A, Konttinen YT, Paavilainen T, Tallroth K. Aggressive granulomatous lesions in cementless total hip arthroplasty. J Bone Joint Surg Br 1990;72(6):980-4.

[112] Verborgt O, Gibson GJ, Schaffler MB. Loss of osteocyte integrity in association with microdamage and bone remodeling after fatigue in vivo. J Bone Miner Res 2000;15(1):60-7.

[113] Kurata K, Heino TJ, Higaki H, Vaananen HK. Bone marrow cell differentiation induced by mechanically damaged osteocytes in 3D gel-embedded culture. J Bone Miner Res 2006;21(4):616-25. Epub 2006 Apr 5.

[114] Teitelbaum SL. Bone resorption by osteoclasts. Science 2000;289:1504-8.

[115] Boyle WJ, Simonet WS, Lacey DL. Osteoclast differentiation and activation. Nature 2003;423:337-42.

[116] Chambers TJ. Regulation of the differentiation and function of osteoclasts. 2000.

[117] Parfitt AM. Bone and plasma calcium homeostasis. Bone 1987;8 Suppl 1:S1-8.

[118] Lerner UH. Osteoclast formation and resorption. Matrix Biology 2000;19:107.

[119] Manolagas SC. Birth and death of bone cells: basic regulatory mechanisms and implications for the pathogenesis and treatment of osteoporosis. Endocr Rev 2000;21:115-37.

[120] Suda T, Takahashi N, Udagawa N, Jimi E, Gillespie MT, Martin TJ. Modulation of osteoclast differentiation and function by the new members of the tumor necrosis factor receptor and ligand families. Endocr Rev 1999;20:345-57.

[121] Lane NE, Yao W, Nakamura MC, Humphrey MB, Kimmel D, Huang X, et al. Mice lacking the integrin beta5 subunit have accelerated osteoclast maturation and increased activity in the estrogen-deficient state. J Bone Miner Res 2005;20:58-66.

[122] Vaananen HK, Zhao H, Mulari M, Halleen JM. The cell biology of osteoclast function. J Cell Sci 2000;113 (Pt 3):377-81.

[123] Oguro I, Ozawa H. The histochemical localization of acid phosphatase activity in BMU. Journal of Bone and Mineral Metabolism 1988;6:44.

[124] Findlay DM, Haynes DR. Mechanisms of bone loss in rheumatoid arthritis. Mod Rheumatol 2005;15:232-40.

[125] Hirotani H, Tuohy NA, Woo JT, Stern PH, Clipstone NA. The calcineurin/nuclear factor of activated T cells signaling pathway regulates osteoclastogenesis in RAW264.7 cells. J Biol Chem 2004;279:13984-92.

[126] Rodan GA, Martin TJ. Therapeutic approaches to bone diseases. Science 2000;289:1508-14.

[127] Raisz LG. Physiology and pathophysiology of bone remodeling. Clin Chem 1999;45:1353-8.

[128] Vincent C, Kogawa M, Findlay DM, Atkins GJ. The generation of osteoclasts from RAW 264.7 precursors in defined, serum-free conditions. J Bone Miner Metab 2009;27(1):114-9.

[129] Lees RL, Sabharwal VK, Heersche JN. Resorptive state and cell size influence intracellular pH regulation in rabbit osteoclasts cultured on collagen-hydroxyapatite films. Bone 2001;28:187-94.

[130] Boyce BF, Yao Z, Xing L. Osteoclasts have multiple roles in bone in addition to bone resorption. Crit Rev Eukaryot Gene Expr 2009;19(3):171-80.

[131] Rissanen JP, Suominen MI, Peng Z, Halleen JM. Secreted tartrate-resistant acid phosphatase 5b is a Marker of osteoclast number in human osteoclast cultures and the rat ovariectomy model. Calcif Tissue Int 2008;82(2):108-15.

[132] Sabokbar A, Fujikawa Y, Neale S, Murray DW, Athanasou NA. Human arthroplasty derived macrophages differentiate into osteoclastic bone resorbing cells. Ann Rheum Dis 1997;56(7):414-20.

[133] Gallois A, Lachuer J, Yvert G, Wierinckx A, Brunet F, Rabourdin-Combe C, et al. Genome-wide expression analyses establish dendritic cells as a new osteoclast precursor able to generate bone-resorbing cells more efficiently than monocytes. J Bone Miner Res 2010;25(3):661-72.

[134] Suda T, Takahashi N, Martin TJ. Modulation of osteoclast differentiation. Endocr Rev 1992;13(1):66-80.

[135] Yamashita T, Asano K, Takahashi N, Akatsu T, Udagawa N, Sasaki T, et al. Cloning of an osteoblastic cell line involved in the formation of osteoclast-like cells. J Cell Physiol 1990;145:587-95.

[136] Miyazaki M, Fujikawa Y, Takita C, Tsumura H. Tacrolimus and cyclosporine A inhibit human osteoclast formation via targeting the calcineurin-dependent NFAT pathway and an activation pathway for c-Jun or MITF in rheumatoid arthritis. Clinical Rheumatology 2007;26:231.

[137] Collin-Osdoby P, Rothe L, Anderson F, Nelson M, Maloney W, Osdoby P. Receptor activator of NF-kappa B and osteoprotegerin expression by human microvascular endothelial cells, regulation by inflammatory cytokines, and role in human osteoclastogenesis. J Biol Chem 2001;276(23):20659-72.

[138] McHugh KP, Shen Z, Crotti TN, Flannery MR, Fajardo R, Bierbaum BE, et al. Role of cell-matrix interactions in osteoclast differentiation. Adv Exp Med Biol 2007;602:107-11.

[139] Teitelbaum SL, Ross FP. Genetic regulation of osteoclast development and function. Nat Rev Genet 2003;4:638-49.

[140] Hayman AR, Macary P, Lehner PJ, Cox TM. Tartrate-resistant acid phosphatase (Acp 5): identification in diverse human tissues and dendritic cells. J Histochem Cytochem 2001;49(6):675-84.

[141] Janckila AJ, Parthasarathy RN, Parthasarathy LK, Seelan RS, Hsueh YC, Rissanen JP, et al. Properties and expression of human tartrate-resistant acid phosphatase isoform 5a by monocyte-derived cells. J Leukoc Biol 2005;77(2):209-18.

[142] Burstone M. Histochemical demonstration of acid phosphatases with naphthol AS-phosphates. Journal of the National Cancer Institute 1958;21:523-39.

[143] Halleen JM, Tiitinen Sl, Ylipahkala H, Fagerlund KM, Vaananen HK. Tartrate-resistant acid phosphatase 5b (TRACP 5b) as a marker of bone resorption. Clin Lab 2006;52(9-10):499-509.

[144] Lam WK, Eastlund DT, Li CY, Yam LT. Biochemical properties of tartrate-resistant acid phosphatase in serum of adults and children. Clin Chem 1978;24(7):1105-8.

[145] Angel NZ, Walsh N, Forwood MR, Ostrowski MC, Cassady AI, Hume DA. Transgenic mice overexpressing tartrate-resistant acid phosphatase exhibit an increased rate of bone turnover. J Bone Miner Res 2000;15(1):103-10.

[146] Chu P, Chao TY, Lin YF, Janckila AJ, Yam LT. Correlation between histomorphometric parameters of bone resorption and serum type 5b tartrate-resistant acid phosphatase in uremic patients on maintenance hemodialysis. Am J Kidney Dis 2003;41(5):1052-9.

[147] Alatalo SL, Peng Z, Janckila AJ, Kaija H, Vihko P, Vaananen HK, et al. A novel immunoassay for the determination of tartrate-resistant acid phosphatase 5b from rat serum. J Bone Miner Res 2003;18(1):134-9.

[148] Drake FH, Dodds RA, James IE, Connor JR, Debouck C, Richardson S, et al. Cathepsin K, but not cathepsins B, L, or S, is abundantly expressed in human osteoclasts. J Biol Chem 1996;271(21):12511-6.

[149] Hou WS, Li Z, Gordon RE, Chan K, Klein MJ, Levy R, et al. Cathepsin k is a critical protease in synovial fibroblast-mediated collagen degradation. Am J Pathol 2001;159(6):2167-77.

[150] Sukhova GK, Shi GP, Simon DI, Chapman HA, Libby P. Expression of the elastolytic cathepsins S and K in human atheroma and regulation of their production in smooth muscle cells. J Clin Invest 1998;102(3):576-83.

[151] Corisdeo S, Gyda M, Zaidi M, Moonga BS, Troen BR. New insights into the regulation of cathepsin K gene expression by osteoprotegerin ligand. Biochem Biophys Res Commun 2001;285(2):335-9.

[152] Garnero P, Borel O, Byrjalsen I, Ferreras M, Drake FH, McQueney MS, et al. The collagenolytic activity of cathepsin K is unique among mammalian proteinases. J Biol Chem 1998;273(48):32347-52.

[153] Kafienah W, Bromme D, Buttle DJ, Croucher LJ, Hollander AP. Human cathepsin K cleaves native type I and II collagens at the N-terminal end of the triple helix. Biochem J 1998;331 (Pt 3):727-32.

[154] Johnson MR, Polymeropoulos MH, Vos HL, Ortiz de Luna RI, Francomano CA. A nonsense mutation in the cathepsin K gene observed in a family with pycnodysostosis. Genome Res 1996;6(11):1050-5.

[155] Ho N, Punturieri A, Wilkin D, Szabo J, Johnson M, Whaley J, et al. Mutations of CTSK result in pycnodysostosis via a reduction in cathepsin K protein. J Bone Miner Res 1999;14(10):1649-53.

[156] Gowen M, Lazner F, Dodds R, Kapadia R, Feild J, Tavaria M, et al. Cathepsin K knockout mice develop osteopetrosis due to a deficit in matrix degradation but not demineralization. J Bone Miner Res 1999;14(10):1654-63.

[157] Saftig P, Hunziker E, Wehmeyer O, Jones S, Boyde A, Rommerskirch W, et al. Impaired osteoclastic bone resorption leads to osteopetrosis in cathepsin-K-deficient mice. Proc Natl Acad Sci U S A 1998;95(23):13453-8.

[158] Xia L, Kilb J, Wex H, Li Z, Lipyansky A, Breuil V, et al. Localization of rat cathepsin K in osteoclasts and resorption pits: inhibition of bone resorption and cathepsin K-activity by peptidyl vinyl sulfones. Biol Chem 1999;380(6):679-87.

[159] Kim N, Takami M, Rho J, Josien R, Choi Y. A novel member of the leukocyte receptor complex regulates osteoclast differentiation. J Exp Med 2002;195(2):201-9.

[160] Davies J, Warwick J, Totty N, Philp R, Helfrich M, Horton M. The osteoclast functional antigen, implicated in the regulation of bone resorption, is biochemically related to the vitronectin receptor. J Cell Biol 1989;109(4 Pt 1):1817-26.

[161] Shinar DM, Schmidt A, Halperin D, Rodan GA, Weinreb M. Expression of alpha v and beta 3 integrin subunits in rat osteoclasts in situ. J Bone Miner Res 1993;8(4):403-14.

[162] Kitazawa S, Ross FP, McHugh K, Teitelbaum SL. Interleukin-4 induces expression of the integrin alpha v beta 3 via transactivation of the beta 3 gene. J Biol Chem 1995;270(8):4115-20.

[163] Zhu HJ, Ross FP, Cao X, Teitelbaum SL. Phorbol myristate acetate transactivates the avian beta 3 integrin gene and induces alpha v beta 3 integrin expression. J Cell Biochem 1996;61(3)::420-9.

[164] Horton MA, Taylor ML, Arnett TR, Helfrich MH. Arg-Gly-Asp (RGD) peptides and the anti-vitronectin receptor antibody 23C6 inhibit dentine resorption and cell spreading by osteoclasts. Exp Cell Res 1991;195(2):368-75.

[165] Nesbitt S, Nesbit A, Helfrich M, Horton M. Biochemical characterization of human osteoclast integrins. Osteoclasts express alpha v beta 3, alpha 2 beta 1, and alpha v beta 1 integrins. J Biol Chem 1993;268:16737-45.

[166] Nakamura I, Gailit J, Sasaki T. Osteoclast integrin alphaVbeta3 is present in the clear zone and contributes to cellular polarization. Cell Tissue Res 1996;286:507-15.

[167] Rao H, Lu G, Kajiya H, Garcia-Palacios V, Kurihara N, Anderson J, et al. Alpha9beta1: a novel osteoclast integrin that regulates osteoclast formation and function. J Bone Miner Res 2006;21:1657-65.

[168] McHugh KP, Hodivala-Dilke K, Zheng MH, Namba N, Lam J, Novack D, et al. Mice lacking beta3 integrins are osteosclerotic because of dysfunctional osteoclasts. J Clin Invest 2000;105(4):433-40.

[169] Grano M, Zigrino P, Colucci S, Zambonin G, Trusolino L, Serra M, et al. Adhesion properties and integrin expression of cultured human osteoclast-like cells. Exp Cell Res 1994;212(2):209-18.

[170] Crotti TN, O'Sullivan RP, Shen Z, Flannery MR, Fajardo RJ, Ross FP, et al. Bone matrix regulates osteoclast differentiation and annexin A8 gene expression. J Cell Physiol 2011;226:3413-21.

[171] Goebeler V, Poeter M, Zeuschner D, Gerke V, Rescher U. Annexin A8 regulates late endosome organization and function. Mol Biol Cell 2008;19(12):5267-78.

[172] Udagawa N, Takahashi N, Akatsu T, Tanaka H, Sasaki T, Nishihara T, et al. Origin of osteoclasts: mature monocytes and macrophages are capable of differentiating into osteoclasts under a suitable microenvironment prepared by bone marrow-derived stromal cells. Proc Natl Acad Sci U S A 1990;87:7260-4.

[173] Chambers TJ. Regulation of the differentiation and function of osteoclasts. J Pathol 2000;192:4-13.

[174] Hattersley G, Chambers TJ. Calcitonin Receptors as Markers for Osteoclastic Differentiation: Correlation between Generation of Bone-Resorptive Cells and Cells that Express Calcitonin Receptors in Mouse Bone Marrow Cultures. Endocrinology 1989;125:1606-12.

[175] Crotti TN, Flannery M, Walsh NC, Fleming JD, Goldring SR, McHugh KP. NFATc1 regulation of the human beta3 integrin promoter in osteoclast differentiation. Gene 2006;372:92-102.

[176] Crotti TN, Flannery M, Walsh NC, Fleming JD, Goldring SR, McHugh KP. NFATc1 directly induces the human β(3) integrin gene in osteoclast differentiation. Journal of musculoskeletal & neuronal interactions 2005;5:335-7.

[177] Roodman GD, Windle JJ. Paget disease of bone. J Clin Invest 2005;115:200-8.

[178] Yagi M, Miyamoto T, Sawatani Y, Iwamoto K, Hosogane N, Fujita N, et al. DC-STAMP is essential for cell-cell fusion in osteoclasts and foreign body giant cells. J Exp Med 2005;202:345-51.

[179] Bar-Shavit Z. The osteoclast: a multinucleated, hematopoietic-origin, bone-resorbing osteoimmune cell. J Cell Biochem 2007;102:1130-9.

[180] Boissy P, Saltel F, Bouniol C, Jurdic P, Machuca-Gayet I. Transcriptional activity of nuclei in multinucleated osteoclasts and its modulation by calcitonin. Endocrinology 2002;143(5):1913-21.

[181] Humphrey MB, Ogasawara K, Yao W, Spusta SC, Daws MR, Lane NE, et al. The signaling adapter protein DAP12 regulates multinucleation during osteoclast development. J Bone Miner Res 2004;19(2):224-34.

[182] Paloneva J, Mandelin J, Kiialainen A, Bohling T, Prudlo J, Hakola P, et al. DAP12/TREM2 deficiency results in impaired osteoclast differentiation and osteoporotic features. J Exp Med 2003;198:669-75.

[183] Lee SH, Rho J, Jeong D, Sul JY, Kim T, Kim N, et al. v-ATPase V0 subunit d2-deficient mice exhibit impaired osteoclast fusion and increased bone formation. Nat Med 2006;12(12):1403-9.

[184] Carano A, Teitelbaum SL, Konsek JD, Schlesinger PH, Blair HC. Bisphosphonates directly inhibit the bone resorption activity of isolated avian osteoclasts in vitro. J Clin Invest 1990;85:456-61.

[185] Vaananen K. Mechanism of osteoclast mediated bone resorption--rationale for the design of new therapeutics. Adv Drug Deliv Rev 2005;57:959-71.

[186] Zou W, Zhu T, Craft CS, Broekelmann TJ, Mecham RP, Teitelbaum SL. Cytoskeletal dysfunction dominates in DAP12-deficient osteoclasts. J Cell Sci 2010;123(Pt 17):2955-63.

[187] Reinholt FP, Hultenby K, Oldberg A, Heinegård D. Osteopontin--a possible anchor of osteoclasts to bone. Proceedings of the National Academy of Sciences of the United States of America 1990;87:4473-5.
[188] Takahashi N, Ejiri S, Yanagisawa S, Ozawa H. Regulation of osteoclast polarization. Odontology 2007;95:1-9.
[189] Abu-Amer Y, Erdmann J, Alexopoulou L, Kollias G, Ross FP, Teitelbaum SL. Tumor necrosis factor receptors types 1 and 2 differentially regulate osteoclastogenesis. J Biol Chem 2000;275(35):27307-10.
[190] Teitelbaum SL. The osteoclast and its unique cytoskeleton. Ann N Y Acad Sci 2011;1240:14-7.
[191] Blair HC, Teitelbaum SL, Ghiselli R, Gluck S. Osteoclastic bone resorption by a polarized vacuolar proton pump. Science 1989;245(4920):855-7.
[192] Mattsson JP, Schlesinger PH, Keeling DJ, Teitelbaum SL, Stone DK, Xie XS. Isolation and reconstitution of a vacuolar-type proton pump of osteoclast membranes. J Biol Chem 1994;269(40):24979-82.
[193] Gay CV, Mueller WJ. Carbonic anhydrase and osteoclasts: localization by labeled inhibitor autoradiography. Science 1974;183(4123):432-4.
[194] Schlesinger PH, Blair HC, Teitelbaum SL, Edwards JC. Characterization of the osteoclast ruffled border chloride channel and its role in bone resorption. J Biol Chem 1997;272(30):18636-43.
[195] Dorozhkin SV. Dissolution mechanism of calcium apatites in acids: A review of literature. World Journal of Methodology 2012;2:1-17.
[196] Teti A, Blair HC, Teitelbaum SL, Kahn AJ, Koziol C, Konsek J, et al. Cytoplasmic pH regulation and chloride/bicarbonate exchange in avian osteoclasts. Journal of Clinical Investigation 1989;83:227-33.
[197] Tezuka K, Nemoto K, Tezuka Y, Sato T, Ikeda Y, Kobori M, et al. Identification of matrix metalloproteinase 9 in rabbit osteoclasts. J Biol Chem 1994;269(21):15006-9.
[198] Delaissé J-M, Andersen TL, Engsig MT, Henriksen K, Troen T, Blavier L. Matrix metalloproteinases (MMP) and cathepsin K contribute differently to osteoclastic activities. Microscopy Research and Technique 2003;61:504-13.

[199] Zaidi M. "Calcium receptors" on eukaryotic cells with special reference to the osteoclast. Biosci Rep 1990;10(6):493-507.

[200] Pfeilschifter J, Wolf O, Naumann A, Minne HW, Mundy GR, Ziegler R. Chemotactic response of osteoblastlike cells to transforming growth factorβ. Journal of Bone and Mineral Research 1990;5:825-30.

[201] Kodama H, Nose M, Niida S, Yamasaki A. Essential role of macrophage colony-stimulating factor in the osteoclast differentiation supported by stromal cells. J Exp Med 1991;173:1291-4.

[202] Fujikawa Y, Sabokbar A, Neale SD, Itonaga I, Torisu T, Athanasou NA. The effect of macrophage-colony stimulating factor and other humoral factors (interleukin-1, -3, -6, and -11, tumor necrosis factor-alpha, and granulocyte macrophage-colony stimulating factor) on human osteoclast formation from circulating cells. Bone 2001;28(3):261-7.

[203] Lagasse E, Weissman IL. Enforced expression of Bcl-2 in monocytes rescues macrophages and partially reverses osteopetrosis in op/op mice. Cell 1997;89:1021-31.

[204] Yoshida H, Hayashi S, Kunisada T, Ogawa M, Nishikawa S, Okamura H, et al. The murine mutation osteopetrosis is in the coding region of the macrophage colony stimulating factor gene. Nature 1990;345(6274):442-4.

[205] Jimi E, Shuto T Fau - Koga T, Koga T. Macrophage colony-stimulating factor and interleukin-1 alpha maintain the survival of osteoclast-like cells. Endocrinology 1995;136(2):808-11.

[206] Miyamoto T, Ohneda O, Arai F, Iwamoto K, Okada S, Takagi K, et al. Bifurcation of osteoclasts and dendritic cells from common progenitors. Blood 2001;98:2544-54.

[207] Udagawa N. Mechanisms involved in bone resorption. Biogerontology 2002;3:79.

[208] Teitelbaum SL. Osteoclasts: what do they do and how do they do it? Am J Pathol 2007;170:427-35.

[209] Demer L, Tintut Y. The roles of lipid oxidation products and receptor activator of nuclear factor-kappaB signaling in atherosclerotic calcification. Circ Res 2011;108(12):1482-93.

[210] Takahashi N, Udagawa N, Suda T. A new member of tumor necrosis factor ligand family, ODF/OPGL/TRANCE/RANKL, regulates osteoclast differentiation and function. Biochem Biophys Res Commun 1999;256(3):449-55.

[211] Rolink AG, Nutt SL, Melchers F, Busslinger M. Long-term in vivo reconstitution of T-cell development by Pax5-deficient B-cell progenitors. Nature 1999;401(6753):603-6.
[212] Nutt SL, Heavey B, Rolink AG, Busslinger M. Commitment to the B-lymphoid lineage depends on the transcription factor Pax5. Nature 1999;401(6753):556-62.
[213] Kong YY, Yoshida H, Sarosi I, Tan HL, Timms E, Capparelli C, et al. OPGL is a key regulator of osteoclastogenesis, lymphocyte development and lymph-node organogenesis. Nature 1999;397:315-23.
[214] Simonet WS, Lacey DL, Dunstan CR. Osteoprotegerin: A novel secreted protein involved in the regulation of bone density. Cell 1997;89:309-19.
[215] Tsuda E, Goto M, Mochizuki S, Yano K, Kobayashi F, Morinaga T, et al. Isolation of a novel cytokine from human fibroblasts that specifically inhibits osteoclastogenesis. Biochem Biophys Res Commun 1997;234(1):137-42.
[216] Lacey DL, Timms E, Tan HL, Kelley MJ, Dunstan CR, Burgess T, et al. Osteoprotegerin ligand is a cytokine that regulates osteoclast differentiation and activation. Cell 1998;93:165-76.
[217] Quinn JM, Horwood NJ, Elliott J, Gillespie MT, Martin TJ. Fibroblastic stromal cells express receptor activator of NF-kappa B ligand and support osteoclast differentiation. J Bone Miner Res 2000;15:1459-66.
[218] Wong BR, Josien R, Lee SY, Vologodskaia M, Steinman RM, Choi Y. The TRAF family of signal transducers mediates NF-kappaB activation by the TRANCE receptor. J Biol Chem 1998;273:28355-9.
[219] Wong BR, Rho J, Arron J, Robinson E, Orlinick J, Chao M, et al. TRANCE is a novel ligand of the tumor necrosis factor receptor family that activates c-Jun N-terminal kinase in T cells. J Biol Chem 1997;272:25190-4.
[220] Nakashima T, Takayanagi H. Osteoimmunology: crosstalk between the immune and bone systems. J Clin Immunol 2009;29:555-67.
[221] Nakashima TaT, Hiroshi. Osteoclasts and the immune system. Journal of Bone and Mineral Metabolism 2009;27:519-29.
[222] Nakashima T, Kobayashi Y, Yamasaki S, Kawakami A, Eguchi K, Sasaki H, et al. Protein expression and functional difference of membrane-bound and soluble receptor

activator of NF-kappaB ligand: modulation of the expression by osteotropic factors and cytokines. Biochem Biophys Res Commun 2000;275:768-75.

[223] Quinn JMW, Elliott J, Gillespie MT, Martin TJ. A Combination of Osteoclast Differentiation Factor and Macrophage-Colony Stimulating Factor Is Sufficient for both Human and Mouse Osteoclast Formation in Vitro. Endocrinology 1998;139:4424-7.

[224] Pettit AR, Ji H, von Stechow D, Muller R, Goldring SR, Choi Y, et al. TRANCE/RANKL knockout mice are protected from bone erosion in a serum transfer model of arthritis. Am J Pathol 2001;159:1689-99.

[225] Kong Y-Y, Feige U, Sarosi I, Bolon B, Tafuri A, Morony S, et al. Activated T cells regulate bone loss and joint destruction in adjuvant arthritis through osteoprotegerin ligand. Nature 1999;402:304-9.

[226] Anderson DM, Maraskovsky E, Billingsley WL, Dougall WC, Tometsko ME, Roux ER, et al. A homologue of the TNF receptor and its ligand enhance T-cell growth and dendritic-cell function. Nature 1997;390(6656):175-9.

[227] Feng X. RANKing intracellular signaling in osteoclasts. IUBMB Life 2005;57(6)::389-95.

[228] Wang Y, Grainger DW. siRNA Knock-Down of RANK Signaling to Control Osteoclast-Mediated Bone Resorption. Pharm Res 2010.

[229] Dougall WC, Glaccum M, Charrier K, Rohrbach K, Brasel K, De Smedt T, et al. RANK is essential for osteoclast and lymph node development. Genes Dev 1999;13(18):2412-24.

[230] Li J, Sarosi I, Yan X-Q, Morony S, Capparelli C, Tan H-L, et al. RANK is the intrinsic hematopoietic cell surface receptor that controls osteoclastogenesis and regulation of bone mass and calcium metabolism. Proceedings of the National Academy of Sciences 2000;97:1566-71.

[231] Yasuda H, Shima N, Nakagawa N. Identity of osteoclastogenesis inhibitory factor (OCIF) and osteoprotegerin (OPG): A mechanism by which OPG/OCIF inhibits osteoclastogenesis in vitro. Endocrinology 1998;139:1329-37.

[232] Haynes DR, Barg E, Crotti TN, Holding C, Weedon H, Atkins GJ, et al. Osteoprotegerin expression in synovial tissue from patients with rheumatoid arthritis,

spondyloarthropathies and osteoarthritis and normal controls. Rheumatology (Oxford) 2003;42:123-34.

[233] Crotti TN, Smith MD, Findlay DM, Zreiqat H, Ahern MJ, Weedon H, et al. Factors regulating osteoclast formation in human tissues adjacent to peri-implant bone loss: expression of receptor activator NFkappaB, RANK ligand and osteoprotegerin. Biomaterials 2004;25:565-73.

[234] Crotti T, Smith MD, Hirsch R, Soukoulis S, Weedon H, Capone M, et al. Receptor activator NF kappaB ligand (RANKL) and osteoprotegerin (OPG) protein expression in periodontitis. J Periodontal Res 2003;38:380-7.

[235] Oshita K, Yamaoka K, Udagawa N, Fukuyo S, Sonomoto K, Maeshima K, et al. Human mesenchymal stem cells inhibit osteoclastogenesis through osteoprotegerin production. Arthritis & Rheumatism 2011;63:1658-67.

[236] Yasuda H, Shima N, Nakagawa N. Osteoclast differentiation factor is a ligand for osteoprotegerin/osteoclastogenesis-inhibitory factor and is identical to TRANCE/RANKL. Proc Natl Acad Sci USA 1998;95:3597-602.

[237] Khosla S. Minireview: the OPG/RANKL/RANK system. Endocrinology 2001;142:5050-5.

[238] Bucay N, Sarosi L, Dunstan CR. Osteoprotegerin-deficient mice develop early onset osteoporosis and arterial calcification. Genes Dev 1998;12:1260-8.

[239] Stolina M, Dwyer D, Ominsky MS, Corbin T, Van G, Bolon B, et al. Continuous RANKL inhibition in osteoprotegerin transgenic mice and rats suppresses bone resorption without impairing lymphorganogenesis or functional immune responses. J Immunol 2007;179(11):7497-505.

[240] Asagiri M, Takayanagi H. The molecular understanding of osteoclast differentiation. Bone 2007;40:251.

[241] Gohda J, Akiyama T, Koga T, Takayanagi H, Tanaka S, Inoue J. RANK-mediated amplification of TRAF6 signaling leads to NFATc1 induction during osteoclastogenesis. EMBO J 2005;24(4):790-9.

[242] Takayanagi H, Kim S, Koga T, Nishina H, Isshiki M, Yoshida H, et al. Induction and activation of the transcription factor NFATc1 (NFAT2) integrate RANKL signaling in terminal differentiation of osteoclasts. Dev Cell 2002;3:889-901.

[243] Kim K, Kim JH, Lee JF-J, Hye-Mi, Jin HM, Lee SH, Fisher DE, et al. Nuclear factor of activated T cells c1 induces osteoclast-associated receptor gene expression during tumor necrosis factor-related activation-induced cytokine-mediated osteoclastogenesis. J Biol Chem 2005;280(42):35209-16.

[244] Matsuo K, Irie N. Osteoclast-osteoblast communication. Arch Biochem Biophys 2008;473:201-9.

[245] Grigoriadis AE, Wang ZQ, Cecchini MG, Hofstetter W, Felix R, Fleisch HA, et al. c-Fos: a key regulator of osteoclast-macrophage lineage determination and bone remodeling. Science 1994;266(5184):443-8.

[246] Kim Y, Sato K, Asagiri M, Morita I, Soma K, Takayanagi H. Contribution of nuclear factor of activated T cells c1 to the transcriptional control of immunoreceptor osteoclast-associated receptor but not triggering receptor expressed by myeloid cells-2 during osteoclastogenesis. J Biol Chem 2005;280:32905-13.

[247] Lomaga MA, Yeh WC, Sarosi I, Duncan GS, Furlonger C, Ho A, et al. TRAF6 deficiency results in osteopetrosis and defective interleukin-1, CD40, and LPS signaling. Genes Dev 1999;13(8):1015-24.

[248] Naito A, Azuma S, Tanaka S, Miyazaki T, Takaki S, Takatsu K, et al. Severe osteopetrosis, defective interleukin-1 signalling and lymph node organogenesis in TRAF6-deficient mice. Genes Cells 1999;4:353-62.

[249] Armstrong AP, Tometsko ME, Glaccum M, Sutherland CL, Cosman D, Dougall WC. A RANK/TRAF6-dependent signal transduction pathway is essential for osteoclast cytoskeletal organization and resorptive function. J Biol Chem 2002;277(46):44347-56.

[250] Lamothe B, Webster WK, Gopinathan A, Besse A, Campos AD, Darnay BG. TRAF6 ubiquitin ligase is essential for RANKL signaling and osteoclast differentiation. Biochemical and Biophysical Research Communications 2007;359:1044-9.

[251] Xu J, Wu HF, Ang ES, Yip K, Woloszyn M, Zheng MH, et al. NF-kappaB modulators in osteolytic bone diseases. Cytokine Growth Factor Rev 2009;20:7-17.

[252] Teitelbaum SL. Osteoclasts; culprits in inflammatory osteolysis. Arthritis Res Ther 2006;8(1):201.

[253] Soloviev A, Schwarz EM, Kuprash DV, Nedospasov SA, Puzas JE, Rosier RN, et al. The role of p105 protein in NFkappaB activation in ANA-1 murine macrophages following stimulation with titanium particles. J Orthop Res 2002;20(4):714-22.
[254] Barnes PJ, Karin M. Nuclear Factor-κB — A Pivotal Transcription Factor in Chronic Inflammatory Diseases. New England Journal of Medicine 1997;336:1066-71.
[255] Selvendiran K, Singh JPV, Krishnan KB, Sakthisekaran D. Cytoprotective effect of piperine against benzo[a]pyrene induced lung cancer with reference to lipid peroxidation and antioxidant system in Swiss albino mice. Fitoterapia 2003;74:109-15.
[256] Franzoso G, Carlson L, Xing L. Requirement for NF-kappaB in osteoclast and B-cell development. Genes Dev 1997;11:3482-96.
[257] Iotsova V, Caamano J, Loy J. Osteopetrosis in mice lacking NF-kappB1 and NF-kappB2. Nat Med 1997;3:1285-9.
[258] Yamamoto A, Miyazaki T, Kadono Y, Takayanagi H, Miura T, Nishina H, et al. Possible Involvement of IκB Kinase 2 and MKK7 in Osteoclastogenesis Induced by Receptor Activator of Nuclear Factor κB Ligand. Journal of Bone and Mineral Research 2002;17:612-21.
[259] Takatsuna H, Asagiri M, Kubota T, Oka K, Osada T, Sugiyama C, et al. Inhibition of RANKL-Induced Osteoclastogenesis by (−)-DHMEQ, a Novel NF-κB Inhibitor, Through Downregulation of NFATc1. Journal of Bone and Mineral Research 2005;20:653-62.
[260] Matsuo K, Galson DL, Zhao C, Peng L, Laplace C, Wang KZQ, et al. Nuclear Factor of Activated T-cells (NFAT) Rescues Osteoclastogenesis in Precursors Lacking c-Fos. Journal of Biological Chemistry 2004;279:26475-80.
[261] Luchin A, Suchting S, Merson T, Rosol TJ, Hume DA, Cassady AI, et al. Genetic and Physical Interactions betweenMicrophthalmia Transcription Factor and PU.1 Are Necessary for Osteoclast Gene Expression and Differentiation. Journal of Biological Chemistry 2001;276:36703-10.
[262] Crotti TN, Sharma SM, Fleming JD, Flannery MR, Ostrowski MC, Goldring SR, et al. PU.1 and NFATc1 mediate osteoclastic induction of the mouse beta3 integrin promoter. J Cell Physiol 2008;215:636-44.

[263] Ishida N, Hayashi K, Hoshijima M, Ogawa T, Koga S, Miyatake Y, et al. Large scale gene expression analysis of osteoclastogenesis in vitro and elucidation of NFAT2 as a key regulator. J Biol Chem 2002;277(43):41147-56.

[264] Asagiri M, Sato K, Usami T, Ochi S, Nishina H, Yoshida H, et al. Autoamplification of NFATc1 expression determines its essential role in bone homeostasis. J Exp Med 2005;202:1261-9.

[265] Koga T, Inui M, Inoue K, Kim S, Suematsu A, Kobayashi E, et al. Costimulatory signals mediated by the ITAM motif cooperate with RANKL for bone homeostasis. Nature 2004;428:758-63.

[266] Mocsai A, Humphrey MB, Van Ziffle JA, Hu Y, Burghardt A, Spusta SC, et al. The immunomodulatory adapter proteins DAP12 and Fc receptor gamma-chain (FcRgamma) regulate development of functional osteoclasts through the Syk tyrosine kinase. Proc Natl Acad Sci U S A 2004;101:6158-63.

[267] Humphrey MB, Lanier LL, Nakamura MC. Role of ITAM-containing adapter proteins and their receptors in the immune system and bone. Immunol Rev 2005;208:50-65.

[268] Barrow AD, Trowsdale J. You say ITAM and I say ITIM, let's call the whole thing off: the ambiguity of immunoreceptor signalling. European Journal of Immunology 2006;36:1646-53.

[269] Herman S, Muller RB, Kronke G, Zwerina J, Redlich K, Hueber AJ, et al. Induction of osteoclast-associated receptor, a key osteoclast costimulation molecule, in rheumatoid arthritis. Arthritis Rheum 2008;58:3041-50.

[270] Abram CL, Lowell CA. The Expanding Role for ITAM-Based Signaling Pathways in Immune Cells. Science Signaling 2007;2007:re2-re.

[271] Lanier LL, Bakker ABH. The ITAM-bearing transmembrane adaptor DAP12 in lymphoid and myeloid cell function. Immunology Today 2000;21:611-4.

[272] Takai T, Li M, Sylvestre D, Clynes R, Ravetch JV. FcR γ chain deletion results in pleiotrophic effector cell defects. Cell 1994;76:519-29.

[273] Takayanagi H. The role of NFAT in osteoclast formation. Ann N Y Acad Sci 2007;1116:227-37.

[274] Ishikawa S, Arase N, Suenaga T, Saita Y, Noda M, Kuriyama T, et al. Involvement of FcRgamma in signal transduction of osteoclast-associated receptor (OSCAR). Int Immunol 2004;16(7):1019-25.
[275] Lanier LL, Corliss B, Wu J, Phillips JH. Association of DAP12 with Activating CD94/NKG2C NK Cell Receptors. Immunity 1998;8:693-701.
[276] Bouchon A, Hernández-Munain C, Cella M, Colonna M. A Dap12-Mediated Pathway Regulates Expression of Cc Chemokine Receptor 7 and Maturation of Human Dendritic Cells. The Journal of Experimental Medicine 2001;194:1111-22.
[277] Daws MR, Lanier LL, Seaman WE, Ryan JC. Cloning and characterization of a novel mouse myeloid DAP12-associated receptor family. European Journal of Immunology 2001;31:783-91.
[278] McVicar DW, Taylor LS, Gosselin P, Willette-Brown J, Mikhael AI, Geahlen RL, et al. DAP12-mediated Signal Transduction in Natural Killer Cells: A DOMINANT ROLE FOR THE Syk PROTEIN-TYROSINE KINASE. Journal of Biological Chemistry 1998;273:32934-42.
[279] Faccio R, Zou W, Colaianni G, Teitelbaum SL, Patrick Ross F. High dose M-CSF partially rescues the Dap12−/− osteoclast phenotype. Journal of Cellular Biochemistry 2003;90:871-83.
[280] Zou W, Kitaura H, Reeve J, Long F, Tybulewicz VLJ, Shattil SJ, et al. Syk, c-Src, the αvβ3 integrin, and ITAM immunoreceptors, in concert, regulate osteoclastic bone resorption. The Journal of Cell Biology 2007;176:877-88.
[281] Fütterer K, Wong J, Grucza RA, Chan AC, Waksman G. Structural basis for syk tyrosine kinase ubiquity in signal transduction pathways revealed by the crystal structure of its regulatory SH2 domains bound to a dually phosphorylated ITAM peptide. Journal of molecular biology 1998;281:523-37.
[282] Pitcher LA, van Oers NS. T-cell receptor signal transmission: who gives an ITAM? Trends Immunol 2003;24(10):554-60.
[283] Blair HC, Robinson LJ, Zaidi M. Osteoclast signalling pathways. Biochem Biophys Res Commun 2005;328:728-38.
[284] Beals CR, Sheridan CM, Turck CW, Gardner P, Crabtree GR. Nuclear export of NF-ATc enhanced by glycogen synthase kinase-3. Science 1997;275:1930-4.

[285] Mao D, Epple H, Uthgenannt B, Novack DV, Faccio R. PLCγ2 regulates osteoclastogenesis via its interaction with ITAM proteins and GAB2. The Journal of Clinical Investigation 2006;116:2869-79.
[286] Rao A, Luo C, Hogan PG. TRANSCRIPTION FACTORS OF THE NFAT FAMILY:Regulation and Function. Annual Review of Immunology 1997;15:707-47.
[287] Crabtree GR, Olson EN. NFAT Signaling: Choreographing the Social Lives of Cells. Cell 2002;109:S67-S79.
[288] Paloneva J, Kestila M, Wu J, Salminen A, Bohling T, Ruotsalainen V, et al. Loss-of-function mutations in TYROBP (DAP12) result in a presenile dementia with bone cysts. Nat Genet 2000;25:357-61.
[289] Aoki N, Kimura S, Takiyama Y, Atsuta Y, Abe A, Sato K, et al. The Role of the DAP12 Signal in Mouse Myeloid Differentiation. The Journal of Immunology 2000;165:3790-6.
[290] Goronzy JJ, Henel G, Sawai H, Singh K, Lee EB, Pryshchep S, et al. Costimulatory Pathways in Rheumatoid Synovitis and T-Cell Senescence. Ann N Y Acad Sci 2005;1062:182-94.
[291] Tomasello E, Olcese L, Vély F, Geourgeon C, Bléry M, Moqrich A, et al. Gene Structure, Expression Pattern, and Biological Activity of Mouse Killer Cell Activating Receptor-associated Protein (KARAP)/DAP-12. Journal of Biological Chemistry 1998;273:34115-9.
[292] Chen X, Bai F, Sokol L, Zhou J, Ren A, Painter JS, et al. A critical role for DAP10 and DAP12 in CD8+ T cell–mediated tissue damage in large granular lymphocyte leukemia. Blood 2008;113:3226-34.
[293] Takai T, Inui M, Inoue K, Mori Y. [Regulation of osteoclast development by immunoglobulin-like receptors]. Nihon Rinsho 2005;63(9):1562-8.
[294] Helming L, Tomasello E, Kyriakides TR, Martinez FO, Takai T, Gordon S, et al. Essential Role of DAP12 Signaling in Macrophage Programming into a Fusion-Competent State. Science Signaling 2008;1:ra11-ra.
[295] Nakamagoe K, Shioya A, Yamaguchi T, Takahashi H, Koide R, Monzen T, et al. A Japanese Case with Nasu-Hakola Disease of DAP12 Gene Mutation Exhibiting Precuneus Hypoperfusion. Internal Medicine 2011;50:2839-44.

[296] Hemmi H, Idoyaga J, Suda K, Suda N, Kennedy K, Noda M, et al. A New Triggering Receptor Expressed on Myeloid Cells (Trem) Family Member, Trem-Like 4, Binds to Dead Cells and Is a DNAX Activation Protein 12-Linked Marker for Subsets of Mouse Macrophages and Dendritic Cells. The Journal of Immunology 2009;182:1278-86.
[297] Bouchon A, Dietrich J, Colonna M. Cutting Edge: Inflammatory Responses Can Be Triggered by TREM-1, a Novel Receptor Expressed on Neutrophils and Monocytes. The Journal of Immunology 2000;164:4991-5.
[298] Park-Min K-H, Ji J-D, Antoniv T, Reid AC, Silver RB, Humphrey MB, et al. IL-10 Suppresses Calcium-Mediated Costimulation of Receptor Activator NF-κB Signaling during Human Osteoclast Differentiation by Inhibiting TREM-2 Expression. The Journal of Immunology 2009;183:2444-55.
[299] Humphrey MB, Daws MR, Spusta SC, Niemi EC, Torchia JA, Lanier LL, et al. TREM2, a DAP12-Associated Receptor, Regulates Osteoclast Differentiation and Function. Journal of Bone and Mineral Research 2006;21:237-45.
[300] Kalliolias GD, Zhao B, Triantafyllopoulou A, Park-Min K-H, Ivashkiv LB. Interleukin-27 inhibits human osteoclastogenesis by abrogating RANKL-mediated induction of nuclear factor of activated T cells c1 and suppressing proximal RANK signaling. Arthritis & Rheumatism 2010;62:402-13.
[301] Roodman G. Mechanisms of abnormal bone turnover in Paget's disease. Bone 1999; vol. 24, no. 5 Suppl, May:pp. 39S-40S.
[302] Takegahara N, Takamatsu H, Toyofuku T, Tsujimura T, Okuno T, Yukawa K, et al. Plexin-A1 and its interaction with DAP12 in immune responses and bone homeostasis. Nat Cell Biol 2006;8:615-22.
[303] Quan DN, Cooper MD, Potter JL, Roberts MH, Cheng H, Jarvis GA. TREM-2 binds to lipooligosaccharides of Neisseria gonorrhoeae and is expressed on reproductive tract epithelial cells. Mucosal Immunol 2008;1:229-38.
[304] N'Diaye E-N, Branda CS, Branda SS, Nevarez L, Colonna M, Lowell C, et al. TREM-2 (triggering receptor expressed on myeloid cells 2) is a phagocytic receptor for bacteria. The Journal of Cell Biology 2009;184:215-23.

[305] Paloneva J, Autti T, Raininko R, Partanen J, Salonen O, Puranen M, et al. CNS manifestations of Nasu-Hakola disease: a frontal dementia with bone cysts. Neurology 2001;56:1552-8.

[306] Verloes A, Maquet P, Sadzot B, Vivario M, Thiry A, Franck G. Nasu-Hakola syndrome: polycystic lipomembranous osteodysplasia with sclerosing leucoencephalopathy and presenile dementia. Journal of Medical Genetics 1997;34:753-7.

[307] Kaifu T. Osteopetrosis and thalamic hypomyelinosis with synaptic degeneration in DAP12-deficient mice. J Clin Invest 2003;111:323-32.

[308] Hsieh CL, Koike M, Spusta SC, Niemi EC, Yenari M, Nakamura MC, et al. A role for TREM2 ligands in the phagocytosis of apoptotic neuronal cells by microglia. Journal of Neurochemistry 2009;109:1144-56.

[309] Prada I, Ongania GN, Buonsanti C, Panina-Bordignon P, Meldolesi J. Triggering receptor expressed in myeloid cells 2 (TREM2) trafficking in microglial cells: Continuous shuttling to and from the plasma membrane regulated by cell stimulation. Neuroscience 2006;140:1139-48.

[310] Athanasou NA, Quinn J. Immunophenotypic differences between osteoclasts and macrophage polykaryons: immunohistological distinction and implications for osteoclast ontogeny and function. Journal of Clinical Pathology 1990;43:997-1003.

[311] Maeda A, Kurosaki M, Kurosaki T. Paired Immunoglobulin-like Receptor (PIR)-A Is Involved in Activating Mast Cells through Its Association with Fc Receptor γ Chain. The Journal of Experimental Medicine 1998;188:991-5.

[312] Tenca C, Merlo A, Merck E, Bates EEM, Saverino D, Simone R, et al. CD85j (Leukocyte Ig-Like Receptor-1/Ig-Like Transcript 2) Inhibits Human Osteoclast-Associated Receptor-Mediated Activation of Human Dendritic Cells. The Journal of Immunology 2005;174:6757-63.

[313] Merck E, de Saint-Vis B, Scuiller M, Gaillard C, Caux C, Trinchieri G, et al. Fc receptor γ-chain activation via hOSCAR induces survival and maturation of dendritic cells and modulates Toll-like receptor responses. Blood 2005;105:3623-32.

[314] Merck E, Gaillard C, Gorman DM, Montero-Julian F, Durand I, Zurawski SM, et al. OSCAR is an FcRγ-associated receptor that is expressed by myeloid cells and is involved in antigen presentation and activation of human dendritic cells. Blood 2004;104:1386-95.

[315] Merck E, Gaillard C, Scuiller M, Scapini P, Cassatella MA, Trinchieri G, et al. Ligation of the FcRγ Chain-Associated Human Osteoclast-Associated Receptor Enhances the Proinflammatory Responses of Human Monocytes and Neutrophils. The Journal of Immunology 2006;176:3149-56.
[316] Goettsch C, Rauner M, Sinningen K, Helas S, Al-Fakhri N, Nemeth K, et al. The Osteoclast-Associated Receptor (OSCAR) Is a Novel Receptor Regulated by Oxidized Low-Density Lipoprotein in Human Endothelial Cells. Endocrinology 2011;152:4915-26.
[317] Kim N. Identification of Alternatively Spliced Forms of
human OSCAR in Osteoclasts. 2012.
[318] So H, Rho J, Jeong D, Park R, Fisher DE, Ostrowski MC, et al. Microphthalmia Transcription Factor and PU.1 Synergistically Induce the Leukocyte Receptor Osteoclast-associated Receptor Gene Expression. Journal of Biological Chemistry 2003;278:24209-16.
[319] Lee J, Kim K, Kim JH, Jin HM, Choi HK, Lee S-H, et al. Id helix-loop-helix proteins negatively regulate TRANCE-mediated osteoclast differentiation. Blood 2005;107:2686-93.
[320] Kim K, Kim JH, Lee J, Jin HM, Kook H, Kim KK, et al. MafB negatively regulates RANKL-mediated osteoclast differentiation. Blood 2006;109:3253-9.
[321] Matsumoto M, Mariathasan S, Nahm MH. Role of lymphotoxin and the type I TNF receptor in the formation of germinal centers. Science 1996;271:1289-91.
[322] Kim JH, Kim K, Jin HM, Youn BU, Song I, Choi HS, et al. Upstream stimulatory factors regulate OSCAR gene expression in RANKL-mediated osteoclast differentiation. Journal of molecular biology 2008;383:502-11.
[323] Malone JD, Teitelbaum SL, Griffin GL, Senior RM, Kahn AJ. Recruitment of osteoclast precursors by purified bone matrix constituents. The Journal of Cell Biology 1982;92:227-30.
[324] Komori T. Regulation of osteoblast differentiation by transcription factors. J Cell Biochem 2006;99:1233-9.
[325] Mackie EJ. Osteoblasts: novel roles in orchestration of skeletal architecture. The International Journal of Biochemistry & Cell Biology 2003;35:1301-5.

[326] Jilka RL, Weinstein RS, Bellido T, Roberson P, Parfitt AM, Manolagas SC. Increased bone formation by prevention of osteoblast apoptosis with parathyroid hormone. The Journal of Clinical Investigation 1999;104:439-46.

[327] Wright LM, Maloney W, Yu X, Kindle L, Collin-Osdoby P, Osdoby P. Stromal cell-derived factor-1 binding to its chemokine receptor CXCR4 on precursor cells promotes the chemotactic recruitment, development and survival of human osteoclasts. Bone 2005;36:840-53.

[328] Bonewald LF. Osteocytes as dynamic multifunctional cells. Ann N Y Acad Sci 2007;1116:281-90.

[329] Kamioka H, Honjo T, Takano-Yamamoto T. A three-dimensional distribution of osteocyte processes revealed by the combination of confocal laser scanning microscopy and differential interference contrast microscopy. Bone 2001;28:145-9.

[330] van Bezooijen RL, Roelen BA, Visser A, van der Wee-Pals L, de Wilt E, Karperien M, et al. Sclerostin is an osteocyte-expressed negative regulator of bone formation, but not a classical BMP antagonist. J Exp Med 2004;199:805-14.

[331] Poole KE, van Bezooijen RL, Loveridge N, Hamersma H, Papapoulos SE, Lowik CW, et al. Sclerostin is a delayed secreted product of osteocytes that inhibits bone formation. Faseb J 2005;19:1842-4.

[332] Poole AR, Kojima T, Yasuda T, Mwale F, Kobayashi M, Laverty S. Composition and structure of articular cartilage: a template for tissue repair. Clin Orthop Relat Res 2001;(391 Suppl):S26-33.

[333] Bentolila V, Boyce TM, Fyhrie DP, Drumb R, Skerry TM, Schaffler MB. Intracortical remodeling in adult rat long bones after fatigue loading. Bone 1998;23(3)::275-81.

[334] Noble BS, Peet N, Stevens HY, Brabbs A, Mosley JR, Reilly GC, et al. Mechanical loading: biphasic osteocyte survival and targeting of osteoclasts for bone destruction in rat cortical bone. American Journal of Physiology - Cell Physiology 2003;284:C934-C43.

[335] Gross TS, King KA, Rabaia NA, Pathare P, Srinivasan S. Upregulation of Osteopontin by Osteocytes Deprived of Mechanical Loading or Oxygen. Journal of Bone and Mineral Research 2005;20:250-6.

[336] Fujihara S, Yokozeki M, Oba Y, Higashibata Y, Nomura S, Moriyama K. Function and Regulation of Osteopontin in Response to Mechanical Stress. Journal of Bone and Mineral Research 2006;21:956-64.
[337] Terai K, Takano-Yamamoto T, Ohba Y, Hiura K, Sugimoto M, Sato M, et al. Role of Osteopontin in Bone Remodeling Caused by Mechanical Stress. Journal of Bone and Mineral Research 1999;14:839-49.
[338] Zhao S, Guo YY, Ding N, Yang LL, Zhang N. Changes in serum levels of soluble osteoclast-associated receptor in human rheumatoid arthritis. Chin Med J (Engl) 2011;124:3058-60.
[339] Xiong J, O'Brien CA. Osteocyte RANKL: new insights into the control of bone remodeling. J Bone Miner Res 2012 27(3):499-505.
[340] Kadoya Y, al-Saffar N, Kobayashi A, Revell PA. The expression of osteoclast markers on foreign body giant cells. Bone Miner 1994;27(2):85-96.
[341] Neale SD, Athanasou NA. Cytokine receptor profile of arthroplasty macrophages, foreign body giant cells and mature osteoclasts. Acta Orthop Scand 1999;70(5):452-8.
[342] Konttinen YT, Takagi M, Mandelin J, Lassus J, Salo J, Ainola MF, et al. Acid attack and cathepsin K in bone resorption around total hip replacement prosthesis. J Bone Miner Res 2001;16(10):1780-6.
[343] Clohisy JC, Harris WH. Primary hybrid total hip replacement, performed with insertion of the acetabular component without cement and a precoat femoral component with cement. An average ten-year follow-up study. J Bone Joint Surg Am 1999;81(2):247-55.
[344] Gruen TA, McNeice GM, Amstutz HC. "Modes of failure" of cemented stem-type femoral components: a radiographic analysis of loosening. Clin Orthop Relat Res 1979;(141):17-27.
[345] Kadoya Y, Revell PA, al-Saffar N, Kobayashi A, Scott G, Freeman MA. Bone formation and bone resorption in failed total joint arthroplasties: histomorphometric analysis with histochemical and immunohistochemical technique. J Orthop Res 1996;14(3):473-82.
[346] Greenfield EM, Bi Y, Ragab AA, Goldberg VM, Van De Motter RR. The role of osteoclast differentiation in aseptic loosening. J Orthop Res 2002;20(1):1-8.

[347] Chiba J, Maloney WJ, Inoue K, Rubash HE. Biochemical analyses of human macrophages activated by polyethylene particles retrieved from interface membranes after failed total hip arthroplasty. J Arthroplasty 2001;16(8 Suppl 1):101-5.
[348] Brooks RA, Sharpe JR, Wimhurst JA, Myer BJ, Dawes EN, Rushton N. The effects of the concentration of high-density polyethylene particles on the bone-implant interface. J Bone Joint Surg Br 2000;82(4):595-600.
[349] Crotti TN, Smith MD, Weedon H, Ahern MJ, Findlay DM, Kraan M, et al. Receptor activator NF-kappaB ligand (RANKL) expression in synovial tissue from patients with rheumatoid arthritis, spondyloarthropathy, osteoarthritis, and from normal patients: semiquantitative and quantitative analysis. Ann Rheum Dis 2002;61:1047-54.
[350] Haynes DR, Crotti TN, Loric M, Bain GI, Atkins GJ, Findlay DM. Osteoprotegerin and receptor activator of nuclear factor kappaB ligand (RANKL) regulate osteoclast formation by cells in the human rheumatoid arthritic joint. Rheumatology 2001;40:623-30.
[351] Lerner UH. Inflammation-induced Bone Remodeling in Periodontal Disease and the Influence of Post-menopausal Osteoporosis. Journal of Dental Research 2006;85:596-607.
[352] Schett G. Bone Formation Versus Bone Resorption in Ankylosing Spondylitis. In: López-Larrea C, Díaz-Peña R, editors. Molecular Mechanisms of Spondyloarthropathies: Springer New York; 2009. p. 114-21.
[353] Vanderborght A, Linsen L, Thewissen M, Geusens P, Raus J, Stinissen P. Osteoprotegerin and receptor activator of nuclear factor-kappaB ligand mRNA expression in patients with rheumatoid arthritis and healthy controls. The Journal of Rheumatology 2004;31:1483-90.
[354] Gehrke T, Sers C Fau - Morawietz L, Morawietz L Fau - Fernahl G, Fernahl G Fau - Neidel J, Neidel J Fau - Frommelt L, Frommelt L Fau - Krenn V, et al. Receptor activator of nuclear factor kappaB ligand is expressed in resident and inflammatory cells in aseptic and septic prosthesis loosening. Scand J Rheumatol 2003;32(5):287-94.
[355] Mandelin J, Li TF, Liljestrom M, Kroon ME, Hanemaaijer R, Santavirta S, et al. Imbalance of RANKL/RANK/OPG system in interface tissue in loosening of total hip replacement. J Bone Joint Surg Br 2003;85(8):1196-201.

[356] Wara-aswapati N, Surarit R, Chayasadom A, Boch JA, Pitiphat W. RANKL Upregulation Associated With Periodontitis and Porphyromonas gingivalis. Journal of Periodontology 2007;78:1062-9.
[357] Garlet GP, Cardoso CR, Campanelli AP, Martins W, Silva JS. Expression of suppressors of cytokine signaling in diseased periodontal tissues: a stop signal for disease progression? Journal of Periodontal Research 2006;41:580-4.
[358] Garlet GP, Cardoso CR, Silva TA, Ferreira BR, Ávila-Campos MJ, Cunha FQ, et al. Cytokine pattern determines the progression of experimental periodontal disease induced by Actinobacillus actinomycetemcomitans through the modulation of MMPs, RANKL, and their physiological inhibitors. Oral Microbiology and Immunology 2006;21:12-20.
[359] Lu HK, Chen YL, Chang HC, Li CL, Kuo MYP. Identification of the osteoprotegerin/receptor activator of nuclear factor-kappa B ligand system in gingival crevicular fluid and tissue of patients with chronic periodontitis. Journal of Periodontal Research 2006;41:354-60.
[360] Haynes DR, Crotti TN. Regulation of bone lysis in inflammatory diseases. Inflammopharmacology 2003;11:323-31.
[361] Redlich K, Hayer S, Ricci R, David JP, Tohidast-Akrad M, Kollias G, et al. Osteoclasts are essential for TNF-alpha-mediated joint destruction. J Clin Invest 2002;110:1419-27.
[362] Romas E, Sims NA, Hards DK, Lindsay M, Quinn JWM, Ryan PFJ, et al. Osteoprotegerin reduces osteoclast numbers and prevents bone erosion in collagen-induced arthritis. Am J Pathol 2002;161(4):1419-27.
[363] Cheng X, Kinosaki M, Takami M, Choi Y, Zhang H, Murali R. Disabling of Receptor Activator of Nuclear Factor-κB (RANK) Receptor Complex by Novel Osteoprotegerin-like Peptidomimetics Restores Bone Loss in Vivo. Journal of Biological Chemistry 2004;279:8269-77.
[364] Onyia JE, Galvin RJS, Ma YL, Halladay DL, Miles RR, Yang X, et al. Novel and Selective Small Molecule Stimulators of Osteoprotegerin Expression Inhibit Bone Resorption. Journal of Pharmacology and Experimental Therapeutics 2004;309:369-79.
[365] Han X, Kawai T, Taubman MA. Interference with immune-cell-mediated bone resorption in periodontal disease. Periodontology 2000 2007;45:76-94.

[366] Jin Q, Cirelli JA, Park CH, Sugai JV, Taba M, Kostenuik PJ, et al. RANKL Inhibition Through Osteoprotegerin Blocks Bone Loss in Experimental Periodontitis. Journal of Periodontology 2007;78:1300-8.
[367] Pioletti DP, Kottelat A. The influence of wear particles in the expression of osteoclastogenesis factors by osteoblasts. Biomaterials 2004;25:5803-8.
[368] Horiki M, Nakase T, Myoui A, Sugano N, Nishii T, Tomita T, et al. Localization of RANKL in osteolytic tissue around a loosened joint prosthesis. Journal of Bone and Mineral Metabolism 2004;22:346-51.
[369] Masui T, Sakano S, Hasegawa Y, Warashina H, Ishiguro N. Expression of inflammatory cytokines, RANKL and OPG induced by titanium, cobalt-chromium and polyethylene particles. Biomaterials 2005;26:1695-702.
[370] Kim KJ, Kotake S, Udagawa N, Ida H, Ishii M, Takei I, et al. Osteoprotegerin inhibits in vitro mouse osteoclast formation induced by joint fluid from failed total hip arthroplasty. Journal of Biomedical Materials Research 2001;58:393-400.
[371] von Knoch F, Heckelei A, Wedemeyer C, Saxler G, Hilken G, Brankamp J, et al. Suppression of polyethylene particle–induced osteolysis by exogenous osteoprotegerin. Journal of Biomedical Materials Research Part A 2005;75A:288-94.
[372] Childs LM, Paschalis EP, Xing L, Dougall WC, Anderson D, Boskey AL, et al. In Vivo RANK Signaling Blockade Using the Receptor Activator of NF-κB:Fc Effectively Prevents and Ameliorates Wear Debris-Induced Osteolysis via Osteoclast Depletion Without Inhibiting Osteogenesis. Journal of Bone and Mineral Research 2002;17:192-9.
[373] Nakashima Y, Sun DH, Trindade MC, Maloney WJ, Goodman SB, Schurman DJ, et al. Signaling pathways for tumor necrosis factor-alpha and interleukin-6 expression in human macrophages exposed to titanium-alloy particulate debris in vitro. J Bone Joint Surg Am 1999;81(5):603-15.
[374] Crotti TN, Dharmapatni AA, Alias E, Zannettino AC, Smith MD, Haynes DR. The immunoreceptor tyrosine-based activation motif (ITAM) -related factors are increased in synovial tissue and vasculature of rheumatoid arthritic joints. Arthritis research & therapy 2012;14:R245.

[375] Kim GS, Koh JM, Chang JS, Park BL, Kim LH, Park EK, et al. Association of the OSCAR promoter polymorphism with BMD in postmenopausal women. J Bone Miner Res 2005;20:1342-8.
[376] Ndongo-Thiam N, de Sallmard G, Kastrup J, Miossec P. Levels of soluble osteoclast-associated receptor (sOSCAR) in rheumatoid arthritis: link to disease severity and cardiovascular risk. Ann Rheum Dis 2014;73(6):1276-7.
[377] Takayanagi H. Inflammatory bone destruction and osteoimmunology. Journal of Periodontal Research 2005;40:287-93.
[378] Wu Y, Torchia J, Yao W, Lane NE, Lanier LL, Nakamura MC, et al. Bone microenvironment specific roles of ITAM adapter signaling during bone remodeling induced by acute
estrogen-deficiency. PloS One, 2007;2:no. 7, p. e586.
[379] Pal N, Quah B, Smith PN, Gladkis LL, Timmers H, Li RW. Nano-osteoimmunology as an important consideration in the design of future implants. Acta Biomaterialia 2011;7:2926-34.
[380] Sacomen D, Smith RL, Song Y, Fornasier V, Goodman SB. Effects of polyethylene particles on tissue surrounding knee arthroplasties in rabbits. Journal of Biomedical Materials Research 1998;43:123-30.
[381] Granchi D, Ciapetti G, Amato I, Pagani S, Cenni E, Savarino L, et al. The influence of alumina and ultra-high molecular weight polyethylene particles on osteoblast–osteoclast cooperation. Biomaterials 2004;25:4037-45.
[382] Lohmann CH, Dean DD, Bonewald LF, Schwartz Z, Boyan BD. Nitric Oxide and Prostaglandin E2 Production in Response to Ultra-High Molecular Weight Polyethylene Particles Depends on Osteoblast Maturation State. The Journal of Bone & Joint Surgery 2002;84:411-9.
[383] Kanaji A, Caicedo MS, Virdi AS, Sumner DR, Hallab NJ, Sena K. Co-Cr-Mo alloy particles induce tumor necrosis factor alpha production in MLO-Y4 osteocytes: a role for osteocytes in particle-induced inflammation. Bone 2009;45(3):528-33.
[384] Mulari MT, Qu Q, Harkonen PL, Vaananen HK. Osteoblast-like cells complete osteoclastic bone resorption and form new mineralized bone matrix in vitro. Calcif Tissue Int 2004;75(3):253-61.

[385] Rogers MJ, Gordon S, Benford HL, Coxon FP, Luckman SP, Monkkonen J, et al. Cellular and molecular mechanisms of action of bisphosphonates. Cancer 2000;88(12 Suppl):2961-78.
[386] Herman S, Kronke G, Schett G. Molecular mechanisms of inflammatory bone damage: emerging targets for therapy. Trends Mol Med 2008;14(6):245-53.
[387] D'Amelio P, Grimaldi A, Cristofaro MA, Ravazzoli M, Molinatti PA, Pescarmona GP, et al. Alendronate reduces osteoclast precursors in osteoporosis. Osteoporos Int 2009.
[388] Breuil V, Euller-Ziegler L. Bisphosphonate therapy in rheumatoid arthritis. Joint Bone Spine 2006;73(4):349-54.
[389] Bekker PJ, Holloway D, Nakanishi A, Arrighi M, Leese PT, Dunstan CR. The Effect of a Single Dose of Osteoprotegerin in Postmenopausal Women. Journal of Bone and Mineral Research 2001;16:348-60.
[390] Shanbhag AS, Hasselman CT, Rubash HE. The John Charnley Award. Inhibition of wear debris mediated osteolysis in a canine total hip arthroplasty model. Clin Orthop Relat Res 1997;(344):33-43.
[391] Millett PJ, Allen MJ, Bostrom MPG. Effects of Alendronate on Particle-Induced Osteolysis in a Rat Model. The Journal of Bone & Joint Surgery 2002;84:236-49.
[392] Iwase M, Kim KJ, Kobayashi Y, Itoh M, Itoh T. A novel bisphosphonate inhibits inflammatory bone resorption in a rat osteolysis model with continuous infusion of polyethylene particles. Journal of Orthopaedic Research 2002;20:499-505.
[393] O'Hara LJ, Nivbrant B, Rohrl S. 'Cross-linked polyethylene and bisphosphonate therapy for
osteolysis in total hip arthroplasty: a case report'. Journal of Orthopaedic Surgery (Hong Kong) 2004;12:pp. 114-21.
[394] Trevisan C, - NVF, Mattavelli M, Parra CG. Bisphosphonate treatment for osteolysis in total hip arthroplasty. A report of four cases. Clin Cases Miner Bone Metab 2013;10(1):61-4.
[395] Lewiecki EM. Treatment of osteoporosis with denosumab. Maturitas 2010.
[396] Vondracek SF. Managing osteoporosis in postmenopausal women. Am J Health Syst Pharm 2010;67:S9-19.
[397] Lewiecki EM. Treatment of osteoporosis with denosumab. Maturitas 2010;66:182-6.

[398] Looney RJ, Schwarz EM, Boyd A, O'Keefe RJ. Periprosthetic osteolysis: an immunologist's update. Current Opinion in Rheumatology 2006;18.

[399] Aramburu J, Yaffe MB, Lopez-Rodriguez C, Cantley LC, Hogan PG, Rao A. Affinity-driven peptide selection of an NFAT inhibitor more selective than cyclosporin A. Science 1999;285:2129-33.

[400] Fukunaga J, Yamaai T, Yamachika E, Ishiwari Y, Tsujigiwa H, Sawaki K, et al. Expression of osteoclast differentiation factor and osteoclastogenesis inhibitory factor in rat osteoporosis induced by immunosuppressant FK506. Bone 2004;34:425-31.

[401] Yu H, van Berkel TJ, Biessen EA. Therapeutic potential of VIVIT, a selective peptide inhibitor of nuclear factor of activated T cells, in cardiovascular disorders. Cardiovasc Drug Rev 2007;25:175-87.

[402] Miyazaki M, Fujikawa Y, Takita C, Tsumura H. Tacrolimus and cyclosporine A inhibit human osteoclast formation via targeting the calcineurin-dependent NFAT pathway and an activation pathway for c-Jun or MITF in rheumatoid arthritis. Clin Rheumatol 2007;26:231-9.

[403] Lee YH, Woo JH, Choi SJ, Ji JD, Bae SC, Song GG. Tacrolimus for the treatment of active rheumatoid arthritis: a systematic review and meta-analysis of randomized controlled trials. Scand J Rheumatol 2010;39:271-8.

[404] Varanasi SS, Datta HK. Characterisation of cytosolic FK506 binding protein 12 and its role in modulating expression of Cbfa1 and osterix in ROS 17/2.8 cells. Bone 2005;36:243-53.

[405] Yeo H, McDonald JM, Zayzafoon M. NFATc1: a novel anabolic therapeutic target for osteoporosis. Ann N Y Acad Sci 2006;1068:564-7.

[406] Aramburu J, Garcia-Cozar F, Raghavan A, Okamura H, Rao A, Hogan PG. Selective inhibition of NFAT activation by a peptide spanning the calcineurin targeting site of NFAT. Mol Cell 1998;1:627-37.

[407] Liu F, Zhu Z, Mao Y, Liu M, Tang T, Qiu S. Inhibition of titanium particle-induced osteoclastogenesis through inactivation of NFATc1 by VIVIT peptide. Biomaterials 2009;30:1756.

[408] Del Poeta M, Chen S-F, Von Hoff D, Dykstra CC, Wani MC, Manikumar G, et al. Comparison of In Vitro Activities of Camptothecin and Nitidine Derivatives against Fungal and Cancer Cells. Antimicrobial Agents and Chemotherapy 1999;43:2862-8.

[409] Addae-Mensah I, Muriuki G, Sofowora A. Structure and Anti-Hypertensive Properties of Nitidine Chloride from Fagara Species. Planta Med 1986;52:538-9.

[410] Liu L-M, Liu H-G, Luo D. Antitumor effect of nitidine chloride <EM>in vitro</EM> and <EM>in vivo</EM>. Chinese Journal of Pharmacology and Toxicology 2009;23:214-8.

[411] WANG B-L, LIU H-G, YANG B, QIN S-H. Anticancer activity of nitidine chloride from Zanthoxylum nitidum(Roxb.)DC.on multidrug resistant KBV200 cells in vitro. Chin J Pharmacol Toxicol 2007;21 (6).

[412] Zreiqat H, Crotti TN, Howlett CR, Capone M, Markovic B, Haynes DR. Prosthetic particles modify the expression of bone-related proteins by human osteoblastic cells in vitro. Biomaterials 2003;24:337-46.

[413] Shanmugam R, Kusumanchi P, Cheng L, Crooks P, Neelakantan S, Matthews W, et al. A water-soluble parthenolide analogue suppresses in vivo prostate cancer growth by targeting NFkappaB and generating reactive oxygen species. Prostate 2010.

[414] Natarajan K, Singh S, Burke TRJR, Grunberger D, Aggarwal BB. Caffeic acid phenethyl ester is a potent and specific inhibitor of activation of nuclear transcription factor NF-kappa B. Proc Natl Acad Sci U S A 1996; 93(17):9090–5.

[415] Marquez N, Sancho R, Macho A, Calzado MA, Fiebich BI, Munoz E. Caffeic acid phenethyl ester inhibits T-cell activation by targeting both nuclear factor of activated T-cells and NF-kappaB transcription factors. 2004.

[416] Ang ES, Pavlos NJ, Chai LY, Qi M, Cheng TS, Steer JH, et al. Caffeic acid phenethyl ester, an active component of honeybee propolis attenuates osteoclastogenesis and bone resorption via the suppression of RANKL-induced NF-kappaB and NFAT activity. J Cell Physiol 2009;221:642-9.

[417] Michaluart P, Masferrer JL, Carothers AM, Subbaramaiah K, Zweifel BS, Koboldt C, et al. Inhibitory effects of caffeic acid phenethyl ester on the activity and expression of cyclooxygenase-2 in human oral epithelial cells and in a rat model of inflammation. Cancer Res 1999;59:2347-52.

[418] Song JJ, Cho JG, Hwang SJ, Cho CG, Park SW, Chae SW. Inhibitory effect of caffeic acid phenethyl ester (CAPE) on LPS-induced inflammation of human middle ear epithelial cells. Acta oto-laryngologica 2008;128:1303-7.
[419] Fitzpatrick LR, Wang J, Le T. Caffeic acid phenethyl ester, an inhibitor of nuclear factor-kappaB, attenuates bacterial peptidoglycan polysaccharide-induced colitis in rats. J Pharmacol Exp Ther 2001;299(3):915-20.
[420] Duan W, Wang Q, Li F, Xiang C, Zhou L, Xu J, et al. Anti-catabolic effect of caffeic acid phenethyl ester, an active component of honeybee propolis on bone loss in ovariectomized mice: a micro-computed tomography study and histological analysis. Chin Med J (Engl) 2014;127(22):3932-6.

CHAPTER 2. Regulation of ITAM adaptor molecules and their receptors by inhibition of calcineurin-NFAT signalling during late stage osteoclast differentiation

M.S.F. Zawawi[1,2], A.A.S.S.K. Dharmapatni[2], M.D. Cantley[2], K.P. McHugh[3], D.R. Haynes[2], T.N. Crotti[2]

1. Universiti Sains Malaysia (USM), Malaysia.
2. Discipline of Anatomy and Pathology, School of Medical Sciences, University of Adelaide, Adelaide, SA 5005, Australia.
3. University of Florida, College of Dentistry, Fl, USA.

Biochemical and Biophysical Research Communications 2012; 427(2):404-9.

Chapter summary

Previous studies have shown FK506 and VIVIT inhibited osteoclast differentitation. However the mechanism is not fully understood. Chapter 2 investigated the effects of calcineurin/NFAT inhibitors FK506 and VIVIT on ITAM expression, late stage osteoclast markers and osteoclast resorption in PBMC-derived human osteoclast cultures *in vitro*. It was hypothesized that inhibition of NF-κB and NFATc1 modulates ITAM signalling and osteoclastogenesis in human osteoclast cultures *in vitro*.

Statement of Authorship

Title of Paper	Regulation of ITAM adaptor molecules and their receptors by inhibition of calcineurin-NFAT signalling during late stage osteoclast differentiation
Publication Status	Published
Publication Details	M.S.F. Zawawi, A.A.S.S.K. Dharmapatni, M.D. Cantley, K.P. McHugh, D.R. Haynes, T.N. Crotti. Biochemical and Biophysical Research Communications 2012; 427(2):404-9.

Statement of Authorship

NOTE:
Statements of authorship appear on pages 111-116 in the print copy of the thesis held in the University of Adelaide Library.

Zawawi, M.S.F., Dharmapatni, A.A.S.S.K., Cantley, M.D., McHugh, K.P., Haynes, D.R. & Crotti, T.N. (2012). Regulation of ITAM adaptor molecules and their receptors by inhibition of calcineurin- NFAT signalling during late stage osteoclast differentiation.
Biochemical and Biophysical Research Communications, v. 427 (2), pp. 404-409.

CHAPTER 3. Molecular mechanisms and therapeutic effects of NC100 on osteoclastic bone resorption and osteoporosis

Qian Liu[1, 2*], Tao Wang[1*], **Muhamad Syahrul Fitri Zawawi**[3, 4],Tania Crotti[3], An Qin[5], Zhen Lin[6], Jinbo Yuan[2], Huagang Liu[7], Minghao Zheng[8], Jiake Xu[1, 2], Jinmin Zhao[1]

1. Research Centre for Regenerative Medicine, the department of Orthopaedic Surgery, The First Affiliated Hospital of Guangxi Medical University, Guangxi 530021, China;
2. School of Pathology and Laboratory Medicine, The University of Western Australia, Crawley WA 6009, Australia;
3. Discipline of Anatomy and Pathology, School of Medical Sciences University of Adelaide, Adelaide SA 5005, Australia;
4. School of Medical Sciences, Universiti Sains Malaysia, Kelantan 16150, Malaysia;
5. Shanghai Key Laboratory of Orthopaedic Implant, Department of Orthopaedics, Shanghai Jiao Tong University School of Medicine, Ninth People's Hospital, Shanghai 200011, China;
6. 12A Genesta Crescent, Dalkeith, WA 6009, Australia;
7. Guangxi Medical University, Guangxi 530021, China;
8. Centre for Orthopaedic Research, School of Surgery, The University of Western Australia, Crawley WA 6009, Australia.

Chapter summary

NC100 exhibits anti-cancer or anti-inflammatory effects through its regulation of NF-κB. But its effects in bone-related studies have not been shown. This is the first study to investigate the effects of NC100 on osteoclastogenesis involving analyses of osteoclast formation and resorption activity on dentine, and mRNA expression of ITAM-related and late stage osteoclast markers, in BMM- and PBMC-derived osteoclast cultures *in vitro*. In addition we investigated the effects of NC100 in a murine model of osteoporosis.

Statement of Authorship

Title of Paper	Molecular mechanisms and therapeutic effects of NC100 on osteoclastic bone resorption and osteoporosis
Publication Status	In preparation
Publication Details	Qian Liu, Tao Wang, **Muhamad Syahrul Fitri Zawawi**,Tania Crotti, An Qin, Zhen Lin, Jinbo Yuan, Huagang Liu, Minghao Zheng, Jiake Xu, Jinmin Zhao Journal of Cellular Physiology 2014

Statement of Authorship

NOTE:
Statements of authorship appear on pages 124-134 in the print copy of the thesis held in the University of Adelaide Library.

Molecular mechanisms and therapeutic effects of NC100 on osteoclastic bone resorption and osteoporosis

Q Liu[1,2,*], T Wang[1,*], M S F Zawawi[3,4], T Crotti[3], A Qin[5], Z Lin[6], J B Yuan[2], H G Liu[7], M H Zheng[8], J Xu[1,2], J M Zhao[1,a]

[1]Research Centre for Regenerative Medicine, the department of Orthopaedic Surgery, The First Affiliated Hospital of Guangxi Medical University, Guangxi 530021, China;
[2]School of Pathology and Laboratory Medicine, The University of Western Australia, Crawley WA 6009, Australia;
[3]Discipline of Anatomy and Pathology, School of Medical Sciences University of Adelaide, Adelaide SA 5005, Australia;
[4]School of Medical Sciences, Universiti Sains Malaysia, Kelantan 16150, Malaysia;
[5]Shanghai Key Laboratory of Orthopaedic Implant, Department of Orthopaedics, Shanghai Jiao Tong University School of Medicine, Ninth People's Hospital, Shanghai 200011, China;
[6]12A Genesta Crescent, Dalkeith, WA 6009, Australia;
[7]Guangxi Medical University, Guangxi 530021, China;
[8]Centre for Orthopaedic Research, School of Surgery, The University of Western Australia, Crawley WA 6009, Australia.

[*]Equal contributors of the work.
[a]Corresponding author: Prof Jinmin Zhao, ZJinMin@hotmail.com

3.1.SUMMARY

BACKGROUND AND PURPOSE: Excessive osteoclast formation/function could result in osteoporotic diseases such as osteoporosis and Paget's disease. Many natural compounds have been shown to mitigate these osteoporotic diseases by regulating osteoclasts, while the effect of NC100 on this cell is unknown. Here, we aimed to investigate the effects of NC100, a bioactive alkaloid isolated from a folk medicine *Zanthoxylum nitidum (Roxb.)* on osteoclast formation and function, and to explore its underlining mechanisms.

EXPERIMENTAL APPROACH: Western blot, real-time PCR and luciferase report gene assays were used to explore the regulative effect of NC100 on osteoclastogenesis-related protein and gene expression. Ovariectomy (OVX) mouse model was established to determine the effects of NC100 on bone loss *in vivo* using micro-CT.

KEY RESULTS: Our results demonstrate that NC100 inhibited RANKL-mediated osteoclastogenesis, blocked NF-κB and NFATc1 signalling pathway, suppressed osteoclast-specific markers including calcitonin receptor, cathepsin K and TRAP *in vitro*. Interestingly, we found NC100 inhibited the expression of V-ATPase in osteoclast. Furthermore, micro-CT analysis showed that NC100 reversed the decrease BV/TV, Tb.Th, Tb.N and the increase of Tb.Sp in OVX mice.

CONCLUSIONS AND IMPLICATIONS: Overall, the data demonstrated that NC100 inhibits osteoclast formation and activity, and attenuates OVX-induced bone loss, by blocking NF-κB and NFATc1 signalling pathway. Therefore, NC100 has therapeutic potential for the treatment of osteoclast-related osteoporotic diseases.

Keywords: NC100, osteoclast, RANKL signalling pathway, bone resorption, osteoporosis

3.2.INTRODUCTION

Osteoclastic bone resorption outstripping bone synthesis results in various bone diseases, such as Paget's disease of the bone, bone metastatic diseases, osteoporosis, arthritis, aseptic bone loosening and non-union of fractures. RANKL signalling pathway has been proposed as a key therapeutic target in osteolytic bone diseases; including osteoporosis, Paget's diseases of bone, rheumatoid arthritis and cancer bone metastasis. Several approaches have been developed to inhibit RANKL function via the disruption of RANKL/RANK interactions. These include RANK-Fc (Feeley et al., 2006), Fc-OPG (Id Boufker et al., 2010; Romas et al., 2002), peptidomimetics (Aoki et al., 2006) and RANKL mutants (Cheng et al., 2009) which have been proposed for the treatment of osteolytic bone diseases. More importantly, anti-RANKL antibodies, Denosumab, a fully human monoclonal antibody to RANKL, have been found to increase bone density and reduce fracture risk.

RANKL associates with RANK to initiate a cascade of the intracellular signalling pathways that involve NF-κB, MAPK, NFAT, Akt, ionic calcium, and calcium/calmodulin-dependent kinase. The NF-κB signalling pathways play a key role in osteoclast formation (Franzoso et al., 1997; Iotsova et al., 1997; Xu et al., 2009). RANKL-induced activation of NFATc1 signalling pathway also represents a master switch for regulating terminal differentiation of osteoclasts (Miyazaki et al., 2000). Additionally, co-stimulatory pathway in osteoclasts, the immunoreceptor tyrosine-based activation motif (ITAM)-dependent pathway involving osteoclast-associated receptor (OSCAR), triggering receptor expressed in myeloid cells (TREM2), Fc receptor common gamma chain (FcRc) and DNAX-activating protein 12 kDa (DAP12), has also been shown to be important in regulating osteoclastogenesis via RANKL and NFAT (Takayanagi, 2007; Zawawi et al., 2012). In addition, antagonist on TRAF6, a signalling adaptor molecule has shown to be effective (Darnay et al., 2007). Therefore, targeting RANKL signalling pathway is a promising strategy for the treatment of osteoclast-related diseases.

NC100 is a benzophenanthridine alkaloid isolated from *Zanthoxylum Nitidum (Rutacease)* and *Fagara zanthoxyloide* (Cushman et al., 1984). Modern pharmacology studies have shown it has anti-tumor and anti-inflammation properties. NC100 inhibits breast cancer cells migration and invasion by suppressing MMP-9 and MMP-2 and

blocking c-Src/FAK signalling pathway (Pan et al., 2011). It also inhibits renal cancer cell metastasis by suppressing AKT and ERK signalling pathway (Fang et al., 2013; Fang et al., 2014). Interestingly, it was found that the anti-inflammatory activity of NC100 has suppressed LPS-induced TNF-α, IL-1β, and IL-6 expression by modulating MAPK and NF-κB pathway in RAW264.7 cells (Wang et al., 2012). However, the role of NC100 in osteoclastic bone resorption is unknown.

In order to understand the molecular mechanisms of NC100 on osteoclast formation, as well as its application in osteoclast-related diseases, we investigated the effect of NC100 on osteoclast both *in vitro* and *in vivo*. We hypothesize that NC100 inhibits osteoclast formation and bone resorption via the modulation of RANKL-induced signalling pathways. Therefore, in this study, we examined the effects of NC100 in RANKL-induced signalling pathways including NFAT, NF-κB and MAPK, important for osteoclast formation and function.

3.3.METHODS

3.3.1.Reagents

NC100 was purchased from National Institute for control of Pharmaceutical and Biological Products (Beijing, China) and dissolved to 100μM in DMSO. Murine monocyte cell line RAW264.7 cells were ordered from The American Type Culture Collection (Rockville, MD, USA). RAW264.7 C4 cells were transfected with a luciferase reporter gene 3κB-Luc-SV40 which contains three κB sites from the interferon gene. P3K cells were produced as previously described (Wang et al., 2003). Alpha modified of Eagles Medium (α-MEM) was purchased from Thermo, Electron (Sydney, Australia) and FBS was purchased from TRACE (Sydney, Australia). P3κB-Luc vector was obtained from Promega Corp. (USA) and pNFATc1-TA-Luc vector was obtained from BD Bioscience (MA, USA). The luciferase assay system was obtained from Promega (Sydney, Australia). M-CSF was obtained from R & D Systems (Minneapolis, USA). Recombinant GST-rRANKL protein was expressed and purified as previously described (Xu et al., 2000). For human peripheral blood mononuclear cell (PBMC), cells were cultured with recombinant human M-CSF (Chemicon, CA, USA), Minimum Essential Medium Alpha Medium (α-MEM)

(Invitrogen, Life Technologies, CA, USA) with 10% foetal calf serum (FCS) (Invitrogen, CA, USA), 1% 5µg/mL penicillin-50U/mL streptomycin (Gibco) and 1% 2mM L-glutamine (Invitrogen, CA, USA).

3.3.2.*In vitro* osteoclast cell culture

For bone marrow macrophage (BMM), osteoclast differentiation was modelled *in vitro* by isolating fresh bone marrow cells from C57BL6J mice. These cells were cultured with complete-MEM medium supplemented with 10 ng/ml of M-CSF. The cells were seeded onto a 96-well plate at 8×10^3 BMM cells per well with NC100, in the presence of RANKL (100 ng/ml) for 7 days. The medium was replaced every 2 days. Cells were then fixed with 4% paraformaldehyde (PFA) for 20 minutes at room temperature and washed thrice with 1×PBS. Tartrate resistant acid phosphatase (TRAP) staining was performed and osteoclast number was counted. TRAP-positive multinucleated cells with more than three nuclei were counted as osteoclasts.

For human PBMC-derived osteoclasts, PBMCs were isolated from whole blood buffy coats obtained from healthy donors from the Australian Red Cross Blood Service (Adelaide, South Australia) based on published methods (Cantley et al., 2011; Zawawi et al., 2012). PBMCs were washed with pre-warmed HANKs balanced salt solution (HBSS, 10mM Hepes, Sigma-Aldrich) and the monocytes isolated by Ficoll (Lymphoprep-SoFordium diatrizoate/polysucrose solution, Axix-Sheld, Oslo Norway) separation. Following final wash, the cell pellet was resuspended in complete medium (α-MEM with 10% FCS, 1% 5µg/mL penicillin-50U/mL streptomycin and 1% 2mM L-glutamine). Cells were resuspended in complete medium at $2x10^6$ cells/ml and seeded either into 12mm 48-well trays for mRNA expression analysis and onto sterilised 8mm whale tooth dentine in 96-well tray for pit resorption analysis. Cells were allowed to adhere for 24 hours at 37°C, 5% CO_2 in a humidified atmosphere, before non-adherent cells were removed and replaced with fresh complete medium containing 25ng/mL recombinant human M-CSF. The medium was replaced at days 1, 3 and 5. From day 7, cells were treated with 0.5µM or 1.0µM NC100. Control wells were treated with 0.01% DMSO. Osteoclasts were differentiated by addition of human recombinant RANKL (50ng/mL) (Chemicon, CA, USA) from day 7. Following this, the medium containing treatments was replaced at days

10 and 12. Doses were based on our preliminary data and results from Cell Proliferation Reagent WST-1 (Roche, Mannheim, Germany) from 3 donors after 48-hour treatment with NC100 (data not shown).

3.3.3.Reverse transcription (RT)-PCR

BMM cells were seeded into a 6-well plate at $1x10^5$ cells/well. Cells were cultured with RANKL in the presence or absence of 0.1 μM or 0.5 μM NC100 for 7 days. Total RNA was extracted using Trizolin accordance with the manufacturer's protocol. For reverse transcription, single-stranded cDNA was prepared from 2 μg of total RNA using reverse transcriptase with an oligo-dT primer. Each cDNA (1 μl) was subjected to PCR amplification using specific forward and reverse primers. 18sRNA was used as an internal control. The reactions were mixed and microcentrifuged before they were placed in a PCR thermal cycler set for initial denaturation at 94°C for 3 minutes. For the amplification of the primers, forward and reverse primers were used and PCR-amplified for 30 cycles (94°C, 40 seconds; 58°C, 40 seconds; and 72°C, 40 seconds). The final extension was performed at 72°C for 7 minutes, followed by 4°C indefinitely. Specific primers of the following genes were used for PCR amplification: 18sRNA (Forward: ACC ATA AAC GAT GCC GAC T; Reverse: TGT CAA TCC TGT CCG TGT C), 36B4 (Forward: TCA TTG TGG GAG CAG ACA; Reverse: TCC TCC GAC TCT TCC TTT), Calcitonin Receptor (Forward: TGG TTG AGG TTG TGC CCA; Reverse: CTC GTG GGT TTG CCT CAT C), Cathepsin K (Forward: GGG AGA AAA ACC TGA AGC; Reverse: ATT CTG GGG ACT CAG AGC), DC-STAMP (Forward: CTT GCA ACC TAA GGG CAA AG; Reverse: TCA ACA GCT CTG TCG TGA CC), V-ATPase d2 (Forward: GGA TCC GAA TTC ATG CTT GAG ACT GCA GAG; Reverse: GGT CTA GAT TAT AAA ATT GGA ATG TAG CT3), NFATc1 (mouseNFATc1) (Forward: CAA CGC CCT GAC CAC CGA TAG; Reverse: GGC TGC CTT CCG TCT CAT AGT), TRAP (Forward: TGT GGC CAT CTT TAT GCT; Reverse: GTC ATT TCT TTG GGG CTT).

3.3.4.Quantitative real-time polymerase chain reaction (PCR)

For BMM, real-time PCR was performed using a 7900HT Fast Real-Time PCR System (Applied Biosystems). Samples were loaded onto a 96-well plate and placed in the

AB17900HT. The thermocycler was set for initial denaturation at 95°C for 5 minutes; 40 cycles of 95°C for 15 seconds; 58°C for 20 seconds; 72°C for 20 seconds; and 72°C for 1 minute before the final extension. The cycle threshold value was determined, and the normalized fold expression of each gene in reference to the housekeeping gene 18sRNA was calculated using the Pfaffl equation on Microsoft® Excel 2003.

For PBMC-derived osteoclasts, RNA was isolated in duplicate at Day 3, 7 and 10 of the culture following addition of 300mL Trizol® (Invitrogen Corporation, Carlsbad, CA, USA) per well based on manufacturer's instructions and as previously described (Cantley et al., 2011; Zawawi et al., 2012). Samples were then DNase treated (Turbo DNA-free, Ambion Inc.). Complementary DNA (cDNA) was prepared by adding 1µg of total RNA with 1mM deoxyribonucleotide triphosphates (dNTP's, Bioline, London, UK) and 250ng random hexamer (Geneworks Pty. Ltd. Adelaide, Australia) and 200Units Superscript® III (Invitrogen) in a Rotor-Gene® 3000, software version 6.0.38 (Corbett Life Science, Mortlake, NSW, Australia) as per manufacturer's instructions.

The expression of the genes of interest was analyzed by quantitative RT-PCR. Amplification was performed using 1µL of the pre-diluted cDNA, 300nM of each forward and reverse primer, Platinum® SYBR® Green qPCR SuperMix-UDG (Invitrogen) and DEPC.H2O. PCR master mixes were made up for each gene of interest; human acidic ribosomal protein (hARP) (reference control gene) (Franssen et al., 2005), Cathepsin K, NFATc1, OSCAR, FcRγ, TREM2 and DAP12 (designed using Primer3Plus) (Alias et al., 2012; Zawawi et al., 2012). Each sample was prepared in triplicate, with a no RNA RT as a negative control. Samples were placed in the Rotor-GeneTM 3000 and analysed using Rotor-GeneTM Series 1.7 software for the PCR procedure. The endogenous reference gene hARP (Franssen et al., 2005) was used to normalize cycle threshold (Ct) data obtained from the genes investigated. For each sample, a value for Ct was determined, defined as the mean cycle at which the fluorescence curve reached an arbitrary threshold. The values for CathK, NFATc1, OSCAR, FcRγ, TREM2 and DAP12 were compared to those of hARP. The ΔCt for each sample was then calculated according to the formula Ct target gene - Ct hARP. The relative quantification was calculated in comparative Ct method, 2-ΔΔCt and plotted in the graphs (Livak et al., 2001; Zawawi et al., 2012).

3.3.5.NF-κB luciferase reporter gene assay

The transcriptional activity of nuclear factor kappa-light-chain-enhancer of activated B cells was examined by transfecting RAW264.7 cells with a luciferase reporter gene p-NF-κB-TA-Luc (Wang et al., 2003) at 1×10^5 cells/well in 24-well plates, and pre-treated with NC100 for 1 hour. After 8-hour stimulation with 100 ng/ml mouse RANKL, cells were immediately washed twice in cold 1×PBS and lysis buffer (100 µl) added to each well. Following harvest, RAW264.7 cell luciferase activity was measured using the Promega Luciferase Assay System according to the manufacturer's instructions (Promega, Sydney, Australia).

3.3.6.NFAT luciferase reporter gene assay

The transcriptional activity of nuclear factor of activated T-cells cytoplasmic 1 (NFATc1) was monitored using the NFATc1 luciferase reporter gene. Using the diethylaminoethyl-dextran method, RAW264.7 cells were temporarily transfected with 0.457 µg/µl of pNFATc1-TA-Luc vector in dulbecco's modified eagle medium (DMEM)/4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid medium. After transfection, cells were placed in a 24-well plate, cultured with complete DMEM for 18~24 hours and pre-treated with NC100 for 1 hour. Following stimulation with 100 ng/ml mouse RANKL for about 24~36 hours, cells were harvested and firefly luciferase activity was measured using the Promega Luciferase Assay System.

3.3.7.Bone resorption pit assay

For BMM-derived osteoclasts, BMM cells were isolated from the long bone of wild type mice and cultured in α-MEM supplemented with RANKL (100 ng/ml) and MCSF (10 ng/ml), in a 6-well plate for 3 days. Once mature osteoclasts formed, cells were trypsinized, pelleted and resuspended. Osteoclasts (1×10^3 cells/ml) were seeded onto ~0.75 mm thick bovine bone slices with complete α-MEM culture medium, RANKL (100 ng/ml) and MCSF (10 ng/ml) in a 96-well plate for 12 hours. NC100 was then added to the culture. Following 48 hours incubation at 37°C, the cells were fixed with 4% PFA and stained with the osteoclast marker TRAP. TRAP-positive cells with three or more nuclei were counted under a light microscope. The bone slices were gently brushed and sonicated

to remove the extraneous cells. The resorption pits were examined using a Philips XL30 scanning electron microscope and the percentage resorbed surface area quantified using the Scion Image software (Scion Cooperation, National Institutes of Health) (Yip et al., 2006).

3.3.8.Dentine resorption pit formation analysis

For PBMC-derived osteoclasts, after 10 days with RANKL, adherent cells were detached from dentine by addition of 0.1% v/v Trypsin in PBS. The dentine was then viewed under a scanning electron microscope (SEM) (Phillips XL20 SEM, Adelaide Microscopy) and three images from three different areas per dentine piece were taken at 150×magnification. The images were then traced and filled in Adobe Photoshop Elements 7 (2008). ImageJ (Version 1.36b, National Institutes of Health, USA) was used to calculate the area of bone resorption as a percentage of the total area.

3.3.9.Western blot assay

Freshly isolated BMM cells were cultured in a 6-well collagen-coated plate. BMMs were pre-treated with different doses of NC100 (0.5 μM or 1 μM) for 1 hour, then stimulated with RANKL (at 0, 5, 10, 20, 30 , 60 min or 0, 10, 20, 30, 60, 120 min) in a short-time western blot assay. BMMs were pre-treated with different doses of NC100 (0.5 μM or 1 μM) for 1 hour, then stimulated with RANKL (at 0, 4, 24, 72, 120, 168 hours) in a long-time western blot assay. Cells were then lysed for protein extraction. SDS-PAGE electrophoresis was performed. Following protein transfer to a nitrocellulose membrane, the membrane was blocked in a 5% dry skim milk with TBS-Tween solution for at least one hour. After washing with TBS-Tween, antibodies such as IKBα, D2, NFAT were used to detect RANKL-induced signal pathway. The immunoreactivity was visualized using Enhanced Chemiluminescence (ECL) reagents.

3.3.10.Animal Procedures

3.3.10.1.Animal housing and handling

Animals were housed in plastic cages with hay shavings in a temperature-regulated room (22 °C) under controlled lighting conditions (12 hours light and 12 hours dark). Food and water were provided ad libitum for mice. C57BL6J mice handling procedures were carried out in accordance with the protocols approved by The University of Western Australia Animal Ethics Committee and The Guangxi Medical University Ethics Committee (SCXK - (军) 2012-0004, Beijing, China).

3.3.10.2.Ovariectomy (OVX) animal model

Thirty female C57BL6J mice aged 6-8 weeks were randomly assigned into five groups of 6. The mice were anesthetized with 10% chloral hydrate solution. Small dorsal incisions were made, and each ovary, together with its capsule and part of the oviduct, was removed. A dissecting microscope was used to locate these organs. Each incision was closed by one stitch performed with a 5-0 synthetic absorbable suture. One week after surgery, mice were given an intraperitoneal (i.p.) injection of two doses of NC100 in DMSO (3 mg/kg for group 1; 6 mg/kg for group 2,) every 2 days for 6 weeks. The negative control group received 10% DMSO (mixed with sterile water) as a placebo. At 6 weeks post-treatment (7 weeks post-OVX), the mice were euthanized. The tibias were removed, fixed in 4% paraformaldehyde (PFA) for about 20-24 hours and radiologically analysed using micro-CT.

3.3.11.Micro-CT analysis

The tibias were then washed thrice with 1×PBS. Bones were scanned at 6.1 μm resolutions in a Skyscan 1174 microCT instrument (Skyscan, Aartselaar, Belgium). The source voltage used was 50 kV, the current was 800 μA, and the filter was 0.5 mm aluminum. Scans were reconstructed using NRecon software (cone beam reconstruction algorithm, Skyscan) with a constant global threshold. Reconstructed samples were then analyzed using CTAn software (Skyscan) with trabecular and cortical regions of interest determined with reference to the bottom of the growth plate. Trabecular bones were analyzed within a volume of slices spanning a 1 mm distance, starting 0.5 mm from the bottom of the growth plate. The trabecular regions of interest were outlined by interpolation of operator-drawn

regions exclusively representing trabecular bones. Regions of interest were binarized using constant threshold values and analyzed using CTAn software.

3.3.12.Statistical analysis

All data presented in this thesis are representative of one of three independent experiments, and results are presented as mean±SEM and resorption data were calculated as mean ± standard error (S.E) from 3 donors in the PBMC-derived osteoclasts. Statistical analyses included paired or unpaired Student's t-tests using Microsoft Office 2003. A *p*-value of <0.05 was set as statistically significant.

3.4.RESULTS

3.4.1.The effect of NC100 on osteoclastogenesis

Osteoclasts derived from BMM cell culture were stained for TRAP activity. Consistent with previous data, osteoclast formation decreased when treated with high concentration of NC100. The total number of multinucleated TRAP-positive cells was significantly lower in 0.03125 μM compared to 1 μM of NC100 (Figure 3.1 B). No osteoclast was observed in culture treated with 0.5 μM and 1.0 μM NC100 (Figure 3.1 C). These results suggest that NC100 dose-dependently inhibits RANKL-induced osteoclastogenesis in BMM cells.

3.4.2.The effect of NC100 on RANKL-induced expression of osteoclast genes

Expression of calcitonin receptor, cathepsin K and TRAP decreased in a dose-dependent manner during RANKL-induced osteoclastogenesis in the BMM-derived osteoclasts (Figure 3.2). Additionally, NC100 also suppressed osteoclast fusion (V-ATPase d2) and Cathepsin K at protein (Figure 3.2) and gene (Figure 3.3) levels. Together, these data show that NC100 suppresses osteoclast gene expression during RANKL-induced osteoclastogenesis.

In order to determine the temporal effects of drug treatments, mRNA expression of gene of interest was assessed in the PBMC-derived osteoclasts at days 3, 7 and 10. Figure 3.4 shows the data obtained after 10 days with 0.1μM and 1.0μM NC100 treatments. Cathepsin K expression, indicating the presence of mature osteoclasts (Shen et al., 2006),

was significantly reduced in response to NC100 treatments at day 10. NC100 treatments significantly reduced OSCAR, FcRγ, TREM2 and DAP12 expression at day 10 when compared to the controls. While NFATc1 expression was significantly reduced by 1.0μM NC100, this was not statistically significant in culture treated with 0.1μM NC100.

3.4.3.The effect of NC100 on osteoclastic bone resorption

NC100 significantly attenuated osteoclastic bone resorption area in murine BMM cell culture (Figure 3.5 A) and in human osteoclast culture derived from PBMCs (Figure 3.5 B). Noticeably, treatment with NC100 showed smaller and shallower resorption pits compared to untreated control. These data indicated that NC100 suppressed acquisition of osteoclast function, consistent with its effect on the suppression of osteoclastogenesis and gene expression.

3.4.4.Effects of NC100 on RANKL-induced NF-κB activation and IκBα degradation

Data from RAW264.7 cells transfected with the 3κB-Luc-SV40 reporter gene demonstrated that NC100 suppressed RANKL-induced NF-κB activation at 0.5 μM and 1 μM in a dose-dependent fashion and the inhibition was statistically significant (Figure 3.6 A). To further evaluate whether NC100 was involved in NF-κB activation, we tested the effect of NC100 on IκBα degradation by RANKL stimulation in RAW264.7 cells and BMMs (Figure 3.6). The results showed that NC100 has little effect on RANKL-induced degradation of IκBα.

3.4.5.NC100 suppresses RANKL-induced NFAT activation and protein expression

NC100 suppressed RANKL-induced NFAT activation in a dose dependent manner (Figure 3.7 A). In addition, Figure 3.7 B indicated that NC100 inhibited osteoclast formation by suppressing RANKL-induced NFATc1 signalling pathway in a dose-dependent manner.

In the absence of NC100, the highest expression of both NFATc1 and D2 protein was found at 120 min. The NFATc1 and D2 expression was low at all time points in the presence of NC100 (Figure 3.7 C). Together these showed NC100 inhibits NFATc1 and D2 expression throughout RANKL-induced osteoclast-forming time course.

3.4.6.NC100 protects OVX-induced bone loss in mice

An ovariectomized mouse model (OVX) of postmenopausal bone loss was used to examine the effects of NC100 on osteolysis *in vivo* for 7 weeks. Quantitative micro-CT analysis of mouse tibias showed that treatment with NC100 inhibited the OVX-induced reduction of bone volume, trabecular thickness and trabecular number of trabecular bone (Figure 3.8). Furthermore, OVX-induced increase of trabecular separation was also reduced. Overall, it showed that NC100 inhibits OVX-induced bone loss in mice.

3.5.DISCUSSION

Current treatments for osteoporosis including bisphosphonates, selective estrogen receptor modulators (SERMs) and hormone treatment show negative effects (Lippuner, 2012; Putnam et al., 2007). Thus, development of new osteoporosis drug is urgent. Natural compounds such as mangiferin, naringin and NC100 regulate NF-κB and exhibit anti-cancer or anti-inflammatory activities (Del Poeta et al., 1999; Garcia et al., 2002; Prado et al., 2004; Sanchez et al., 2000). Naringin protects retinoic acid-induced osteoporosis (Wei et al., 2007). Furthermore, parthenolide, a natural product from feverfew, inhibits LPS-induced osteolysis and bone resorption (Yip et al., 2004). Recently, chemical composition of NC100 has been analyzed, but its pharmacological mechanism on bone and the role of NC100 in osteoclastogenesis are unknown. In this study, for the first time, we show NC100 suppresses gene expression of NFAT, NF-κB and osteoclast markers including calcitonin receptor, cathepsin K, TRAP and V-ATPase d2. These results suggest that NC100 targets osteoclasts and might have therapeutic potential for the treatment of osteoclast-related bone diseases including osteoporosis, Paget's disease and cancer metastasis to bone.

Osteoclastogenic factors including RANKL, TNF-α and interlukin-1 could contribute to osteoclastogenesis. A recent study has found hematopoietic precursors lacking RANKL, RANK or TRAF6 can become osteoclasts *in vitro* when co-stimulated with TNF-α and TGF-β, suggesting a RANKL-RANK-TRAF6 independent axis for osteoclastogenesis (Kim et al., 2005). The engagement of RANKL-RANK-TRAF6 will lead to activation of downstream signalling pathways including NF-κB, NFAT, and MAPK (Boyle et al., 2003). To date, there has been no report on the effects of NC100 on the RANKL-RANK-

TRAF6 signalling pathway. In this study, RAW264.7 cells, BMM cells and human PBMCs were used to define the direct role of NC100 on RANKL-induced activation of NF-κB, NFAT, and MAPK in osteoclastogenesis and we show NC100 inhibits RANKL-induced NFAT protein expression and activation.

NFAT has been demonstrated as a significant transcriptional factor in osteoclasts. NFATc1 induces the differentiation of osteoclast precursor cells into TRAP-positive multinucleated osteoclast-like cells (Ikeda et al., 2004). Calcineurin is the key mediator of NFAT signalling pathways. Cyclosporine A, FK506 and 11R-VIVIT peptide inhibit the activity of calcineurin. Cyclosporine A and FK506 suppress bone marrow-derived osteoclastogenesis through the induction of apoptosis (Igarashi et al., 2004) and RANKL-induced differentiation in RAW264.7 cells (Hirotani et al., 2004). Calcineurin-NFAT signalling has been inhibited by FK506 and 11R-VIVIT peptide in PBMC-derived osteoclast culture (Zawawi et al., 2012). It will be interesting to determine the effects of NC100 on NFAT/calcineurin pathways in great detail. While our preliminary result suggested that NC100 has little effect on calcineurin protein expression in a short time course (data not shown), we reported that NC100 has significantly reduced the expression of ITAM-related molecules OSCAR, TREM2, FcRγ and DAP12 at the upstream of NFAT/calcineurin pathway, during late stage osteoclast differentiation.

In addition to the role of NFAT signalling in osteoclasts, other studies have found that inhibition of RANKL-induced activation of NF-κB is also effective for the treatment of osteolysis (Xu et al., 2009). Over the past several years, our laboratory has identified several natural compounds that have potential effects on the inhibition of osteoclast formation, bone resorption and RANKL-induced NF-κB activation. These include parthenolide (Yip et al., 2004) and caffeic acid phenethyl ester (Ang et al., 2009). In this study, we have found that NC100 significantly modulates NF-κB activity. This effect could contribute to the inhibitory effect of NC100 in osteoclasts. Taken together, these data provide a mechanistic explanation for the inhibitory effects of NC100 on osteoclast formation and bone resorption.

To date, our knowledge regarding the role of NC100 in cell signalling is very limited. Only recently, NC100 has been found to inhibit phosphorylation of c-Src, FAK, MAPKs, and activation of RhoA, Rac1 and AP-1 transcriptional activity induced by PDGF in breast

cancer cells (Pan et al., 2011). However, we have found that NC100 has little effect on RANKL-induced C-Src phosphorylation. Other studies have shown that NC100 induces the activation of the caspase-dependent pathway in MG63, an osteosarcoma cell line (Ang et al., 2011). It remains to be determined whether NC100 could impact these signalling pathways, which also contribute to osteoclast formation and bone resorption.

More importantly, we have yet to define the molecular target of NC100 in osteoclasts. To this end, we have attempted to use protein binding and pull down assays combined with proteomics analysis to identify NC100 binding partners. Preliminary results indicate that NC100 binds to several mitochondrial-related proteins; including ATP5α ATP synthase α subunit, aldehyde dehydrogenase and NADH dehydrogenase iron-sulphur protein 2 (data not shown). However, further studies are required to confirm these initial findings. Dissecting the mechanism of action of NC100 at both molecular and cellular level in osteoclasts will provide very important information for its use in osteoporosis treatment.

The *in vitro* inhibition of osteoclastogenesis and bone resorption by NC100 also correlates to its *in vivo* effect of protecting osteolytic conditions in an OVX mouse model. These results suggest that NC100 might have therapeutic potential for the treatment of osteoclast-related bone diseases; including osteoporosis, Paget's disease of bone and cancer metastasis to bone, by targeting osteoclasts. However, the role of NC100 in osteoblast differentiation and mineralization is unknown. The maintenance of dynamic balancing of the bone involves both bone resorption by osteoclasts and bone synthesis by osteoblasts (Tomoyasu et al., 1998). The bone loss related diseases are not only associated with osteoclasts but also with osteoblasts. Thus, it will be interesting to test the effects of NC100 on the osteoblastic lineage in our future experiments. Similarly, since the effects of NC100 in other local bone cells are unknown, it remains to be seen how NC100 affects these cells; including osteocytes, and endothelial cells that might have contributed to the bone mineral density *in vivo* via bone remodelling processes.

Collectively, this study has shown that NC100 plays an essential role in RANKL-induced osteoclast differentiation and bone resorption. Defining the precise target of NC100 in signalling for osteoclast differentiation and function will require further investigation of the downstream RANKL-induced signalling pathways in osteoclasts.

Dissecting the mechanism of action of NC100 at the molecular and cellular level will provide very important information for their potential use in the treatment of osteoporosis.

3.6.ACKNOWLEDGEMENTS

The authors acknowledge the animal care and assistance by Guangxi Medical University Laboratory Animal Centre and expert Micro-CT by Centre for Microscopy, Characterisation and Analysis (CMCA) of The University of Western Australia. This work was supported by the health department of Guangxi Chinese medicine science and technology special grant (GZKZ-Z1104 and GZKZ10-011). This work was funded in part by National Health and Medical Research Council of Australia, Western Australia Medical & Health Research Infrastructure Fund, University of Western Australia Research Collaboration Awards, and a grant from the National Natural Science Foundation of China (NSFC, no. 81228013). Dr. Qian Liu is a visiting scholar to UWA sponsored by Guangxi Medical University.

3.7.REFERENCES

Alias E, Dharmapatni AS, Holding AC, Atkins GJ, Findlay DM, Howie DW, et al. (2012). Polyethylene particles stimulate expression of ITAM-related molecules in peri-implant tissues and when stimulating osteoclastogenesis in vitro. Acta Biomater 8(8): 3104-3112.

Ang E, Liu Q, Qi M, Liu HG, Yang X, Chen H, et al. (2011). Mangiferin attenuates osteoclastogenesis, bone resorption, and RANKL-induced activation of NF-kappaB and ERK. J Cell Biochem 112(1): 89-97.

Ang ES, Pavlos NJ, Chai LY, Qi M, Cheng TS, Steer JH, et al. (2009). Caffeic acid phenethyl ester, an active component of honeybee propolis attenuates osteoclastogenesis and bone resorption via the suppression of RANKL-induced NF-kappaB and NFAT activity. J Cell Physiol 221(3): 642-649.

Aoki K, Saito H, Itzstein C, Ishiguro M, Shibata T, Blanque R, et al. (2006). A TNF receptor loop peptide mimic blocks RANK ligand-induced signalling, bone resorption, and bone loss. J Clin Invest 116(6): 1525-1534.

Boyle WJ, Simonet WS, Lacey DL (2003). Osteoclast differentiation and activation. Nature 423(6937): 337-342.

Cantley MD, Fairlie DP, Bartold PM, Rainsford KD, Le GT, Lucke AJ, et al. (2011). Inhibitors of histone deacetylases in class I and class II suppress human osteoclasts in vitro. J Cell Physiol 226(12): 3233-3241.

Cheng T, Pavlos NJ, Wang C, Tan JW, Lin JM, Cornish J, et al. (2009). Mutations within the TNF-like core domain of RANKL impair osteoclast differentiation and activation. Mol Endocrinol 23(1): 35-46.

Cushman M, Mohan P, Smith EC (1984). Synthesis and biological activity of structural analogues of the anticancer benzophenanthridine alkaloid nitidine chloride. J Med Chem 27(4): 544-547.

Darnay BG, Besse A, Poblenz AT, Lamothe B, Jacoby JJ (2007). TRAFs in RANK signalling. Adv Exp Med Biol 597: 152-159.

Del Poeta M, Chen SF, Von Hoff D, Dykstra CC, Wani MC, Manikumar G, et al. (1999). Comparison of in vitro activities of camptothecin and nitidine derivatives against fungal and cancer cells. Antimicrob Agents Chemother 43(12): 2862-2868.

Fang Z, Tang Y, Jiao W, Xing Z, Guo Z, Wang W, et al. (2013). Nitidine chloride inhibits renal cancer cell metastasis via suppressing AKT signalling pathway. Food Chem Toxicol 60: 246-251.

Fang Z, Tang Y, Jiao W, Xing Z, Guo Z, Wang W, et al. (2014). Nitidine chloride induces apoptosis and inhibits tumor cell proliferation via suppressing ERK signalling pathway in renal cancer. Food Chem Toxicol 66: 210-216.

Feeley BT, Liu NQ, Conduah AH, Krenek L, Roth K, Dougall WC, et al. (2006). Mixed metastatic lung cancer lesions in bone are inhibited by noggin overexpression and Rank:Fc administration. J Bone Miner Res 21(10): 1571-1580.

Franssen ME, Zeeuwen PL, Vierwinden G, van de Kerkhof PC, Schalkwijk J, van Erp PE (2005). Phenotypical and functional differences in germinative subpopulations derived from normal and psoriatic epidermis. J Invest Dermatol 124(2): 373-383.

Franzoso G, Carlson L, Xing L, Poljak L, Shores EW, Brown KD, et al. (1997). Requirement for NF-kappaB in osteoclast and B-cell development. Genes Dev 11(24): 3482-3496.

Garcia D, Delgado R, Ubeira FM, Leiro J (2002). Modulation of rat macrophage function by the Mangifera indica L. extracts Vimang and mangiferin. Int Immunopharmacol 2(6): 797-806.

Hirotani H, Tuohy NA, Woo JT, Stern PH, Clipstone NA (2004). The calcineurin/nuclear factor of activated T cells signalling pathway regulates osteoclastogenesis in RAW264.7 cells. J Biol Chem 279(14): 13984-13992.

Id Boufker H, Lagneaux L, Najar M, Piccart M, Ghanem G, Body JJ, et al. (2010). The Src inhibitor dasatinib accelerates the differentiation of human bone marrow-derived mesenchymal stromal cells into osteoblasts. BMC Cancer 10: 298.

Igarashi K, Hirotani H, Woo JT, Stern PH (2004). Cyclosporine A and FK506 induce osteoclast apoptosis in mouse bone marrow cell cultures. Bone 35(1): 47-56.

Ikeda F, Nishimura R, Matsubara T, Tanaka S, Inoue J, Reddy SV, et al. (2004). Critical roles of c-Jun signalling in regulation of NFAT family and RANKL-regulated osteoclast differentiation. J Clin Invest 114(4): 475-484.

Iotsova V, Caamano J, Loy J, Yang Y, Lewin A, Bravo R (1997). Osteopetrosis in mice lacking NF-kappaB1 and NF-kappaB2. Nat Med 3(11): 1285-1289.

Kim N, Kadono Y, Takami M, Lee J, Lee SH, Okada F, et al. (2005). Osteoclast differentiation independent of the TRANCE-RANK-TRAF6 axis. J Exp Med 202(5): 589-595.

Lippuner K (2012). The future of osteoporosis treatment - a research update. Swiss Med Wkly 142: w13624.

Livak KJ, Schmittgen TD (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 25(4): 402-408.

Miyazaki T, Katagiri H, Kanegae Y, Takayanagi H, Sawada Y, Yamamoto A, et al. (2000). Reciprocal role of ERK and NF-kappaB pathways in survival and activation of osteoclasts. J Cell Biol 148(2): 333-342.

Pan X, Han H, Wang L, Yang L, Li R, Li Z, et al. (2011). Nitidine Chloride inhibits breast cancer cells migration and invasion by suppressing c-Src/FAK associated signalling pathway. Cancer Lett 313(2): 181-191.

Prado S, Michel S, Tillequin F, Koch M, Pfeiffer B, Pierre A, et al. (2004). Synthesis and cytotoxic activity of benzo[c][1,7] and [1,8]phenanthrolines analogues of nitidine and fagaronine. Bioorg Med Chem 12(14): 3943-3953.

Putnam SE, Scutt AM, Bicknell K, Priestley CM, Williamson EM (2007). Natural products as alternative treatments for metabolic bone disorders and for maintenance of bone health. Phytother Res 21(2): 99-112.

Romas E, Sims NA, Hards DK, Lindsay M, Quinn JW, Ryan PF, et al. (2002). Osteoprotegerin reduces osteoclast numbers and prevents bone erosion in collagen-induced arthritis. Am J Pathol 161(4): 1419-1427.

Sanchez GM, Re L, Giuliani A, Nunez-Selles AJ, Davison GP, Leon-Fernandez OS (2000). Protective effects of Mangifera indica L. extract, mangiferin and selected antioxidants against TPA-induced biomolecules oxidation and peritoneal macrophage activation in mice. Pharmacol Res 42(6): 565-573.

Shen Z, Crotti TN, McHugh KP, Matsuzaki K, Gravallese EM, Bierbaum BE, et al. (2006). The role played by cell-substrate interactions in the pathogenesis of osteoclast-mediated peri-implant osteolysis. Arthritis Res Ther 8(3): R70.

Takayanagi H (2007). The role of NFAT in osteoclast formation. Ann N Y Acad Sci 1116: 227-237.

Tomoyasu A, Goto M, Fujise N, Mochizuki S, Yasuda H, Morinaga T, et al. (1998). Characterization of monomeric and homodimeric forms of osteoclastogenesis inhibitory factor. Biochem Biophys Res Commun 245(2): 382-387.

Wang C, Steer JH, Joyce DA, Yip KH, Zheng MH, Xu J (2003). 12-O-tetradecanoylphorbol-13-acetate (TPA) inhibits osteoclastogenesis by suppressing RANKL-induced NF-kappaB activation. J Bone Miner Res 18(12): 2159-2168.

Wang Z, Jiang W, Zhang Z, Qian M, Du B (2012). Nitidine chloride inhibits LPS-induced inflammatory cytokines production via MAPK and NF-kappaB pathway in RAW 264.7 cells. J Ethnopharmacol 144(1): 145-150.

Wei M, Yang Z, Li P, Zhang Y, Sse WC (2007). Anti-osteoporosis activity of naringin in the retinoic acid-induced osteoporosis model. Am J Chin Med 35(4): 663-667.

Xu J, Tan JW, Huang L, Gao XH, Laird R, Liu D, et al. (2000). Cloning, sequencing, and functional characterization of the rat homologue of receptor activator of NF-kappaB ligand. J Bone Miner Res 15(11): 2178-2186.

Xu J, Wu HF, Ang ES, Yip K, Woloszyn M, Zheng MH, et al. (2009). NF-kappaB modulators in osteolytic bone diseases. Cytokine Growth Factor Rev 20(1): 7-17.

Yip KH, Feng H, Pavlos NJ, Zheng MH, Xu J (2006). p62 ubiquitin binding-associated domain mediated the receptor activator of nuclear factor-kappaB ligand-induced osteoclast formation: a new insight into the pathogenesis of Paget's disease of bone. Am J Pathol 169(2): 503-514.

Yip KH, Zheng MH, Feng HT, Steer JH, Joyce DA, Xu J (2004). Sesquiterpene lactone parthenolide blocks lipopolysaccharide-induced osteolysis through the suppression of NF-kappaB activity. J Bone Miner Res 19(11): 1905-1916.

Zawawi MS, Dharmapatni AA, Cantley MD, McHugh KP, Haynes DR, Crotti TN (2012). Regulation of ITAM adaptor molecules and their receptors by inhibition of calcineurin-NFAT signalling during late stage osteoclast differentiation. Biochem Biophys Res Commun 427(2): 404-409.

3.8.FIGURES

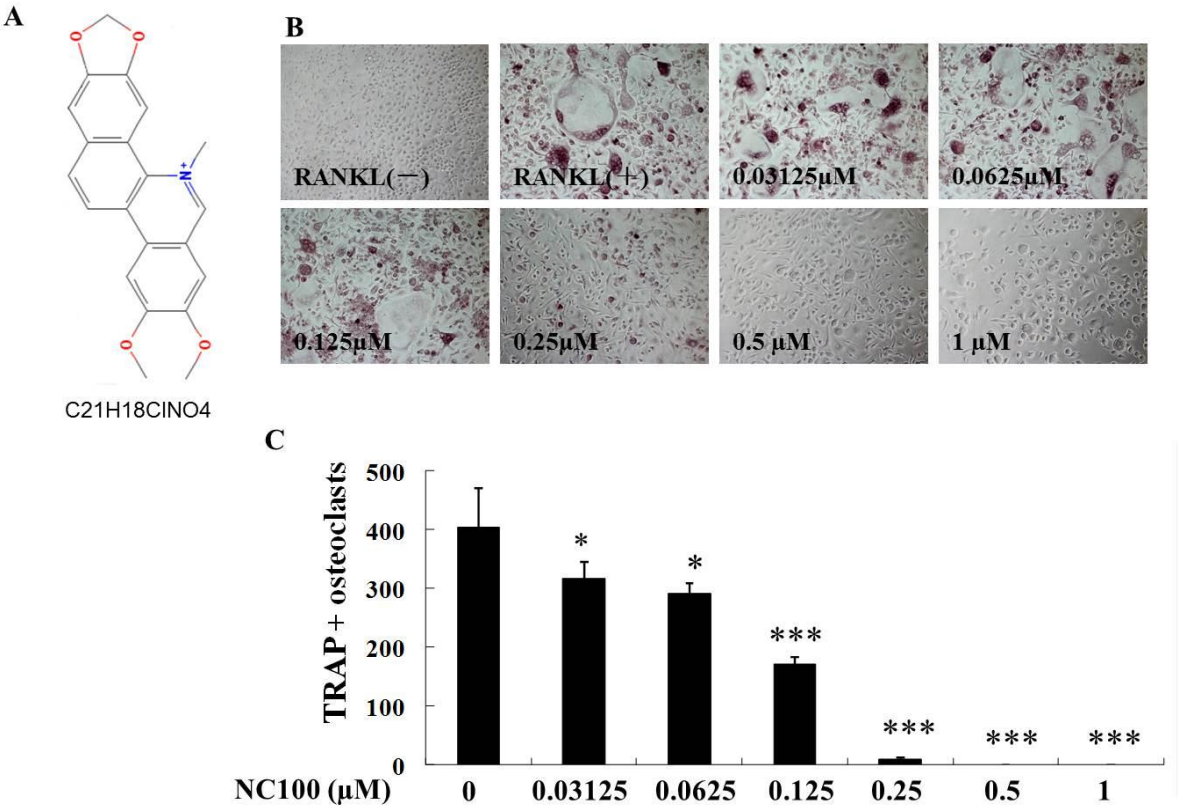

Fig. 3.1.NC100 inhibits RANKL-induced osteoclast formation.

A: Chemical structure of NC100 (PubChem substance ID 12013221). B: BMM cells were cultured in the presence of M-CSF and RANKL (100 ng/ml) with or without varying dose of NC100 for 5 days and stained for TRAP expression. Light microscope images depicting the dose-dependent effect of NC100 on RANKL-induced osteoclastogenesis. C: TRAP-positive multinuclear cells containing three or more nuclei were scored (*$p < 0.05$, ***$p < 0.001$ vs. control).

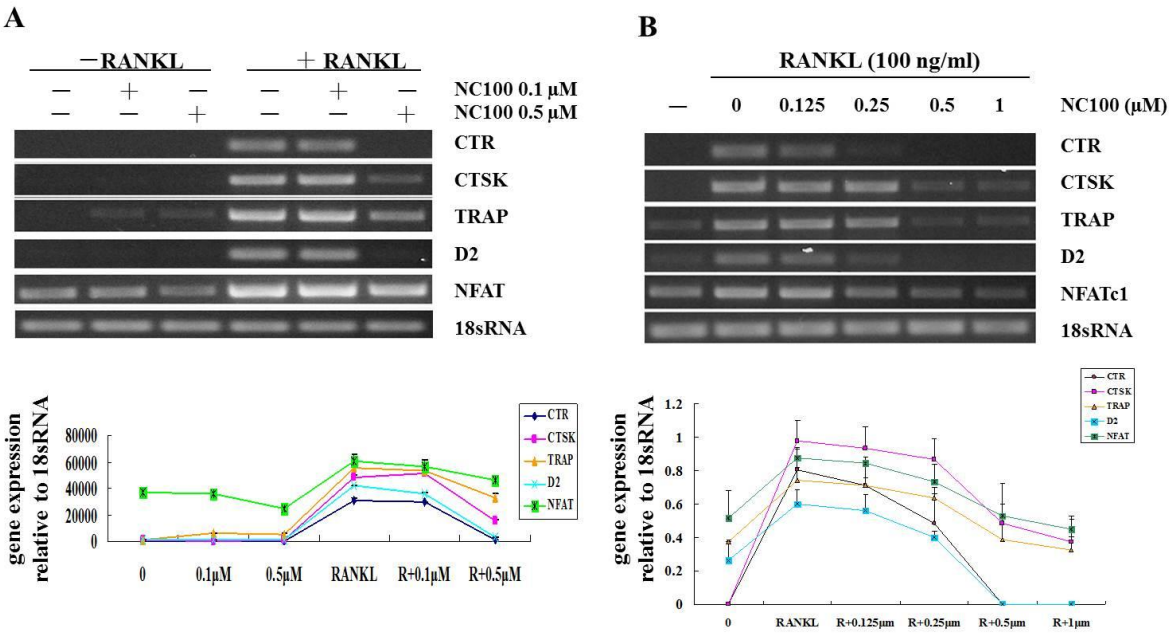

Fig. 3.2.Suppression of RANKL-induced gene expression by NC100.

A: BMMs were cultured with M-CSF and 100 ng/ml of RANKL in the presence or absence of 0.1 μM or 0.5 μM NC100 for 7 days. B: BMMs were seeded in the presence and absence of 100 ng/ml of RANKL with different dose of NC100 (0, 0.125, 0.25, 0.5 and 1 μM) for 7 days. The mRNA expression levels of the indicated genes were determined by RT-PCR.

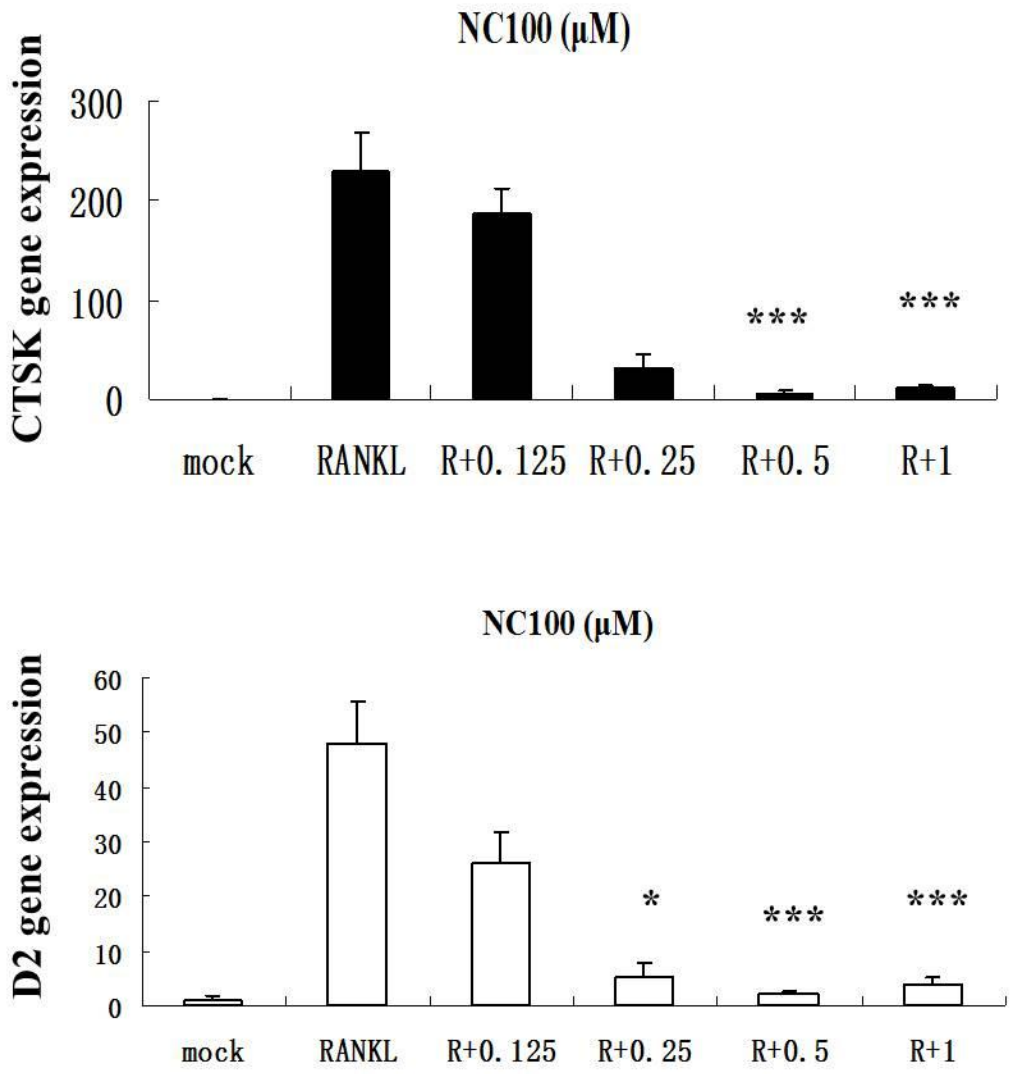

Fig. 3.3.NC100 dose-dependently affects RANKL-induced gene expression.

BMMs were seeded onto 6-well plates in the presence and absence of RANKL (100 ng/ml) with NC100 (0, 0.125 μM, 0.25 μM, 0.5 μM and 1 μM) for 7 days. The mRNA expression levels of the indicated genes were determined by quantitative real-time PCR. The relative amount of CTSK and V-ATPase d2 mRNA were treated with different doses of NC100 (*$p < 0.05$, ***$p < 0.001$ vs. control group).

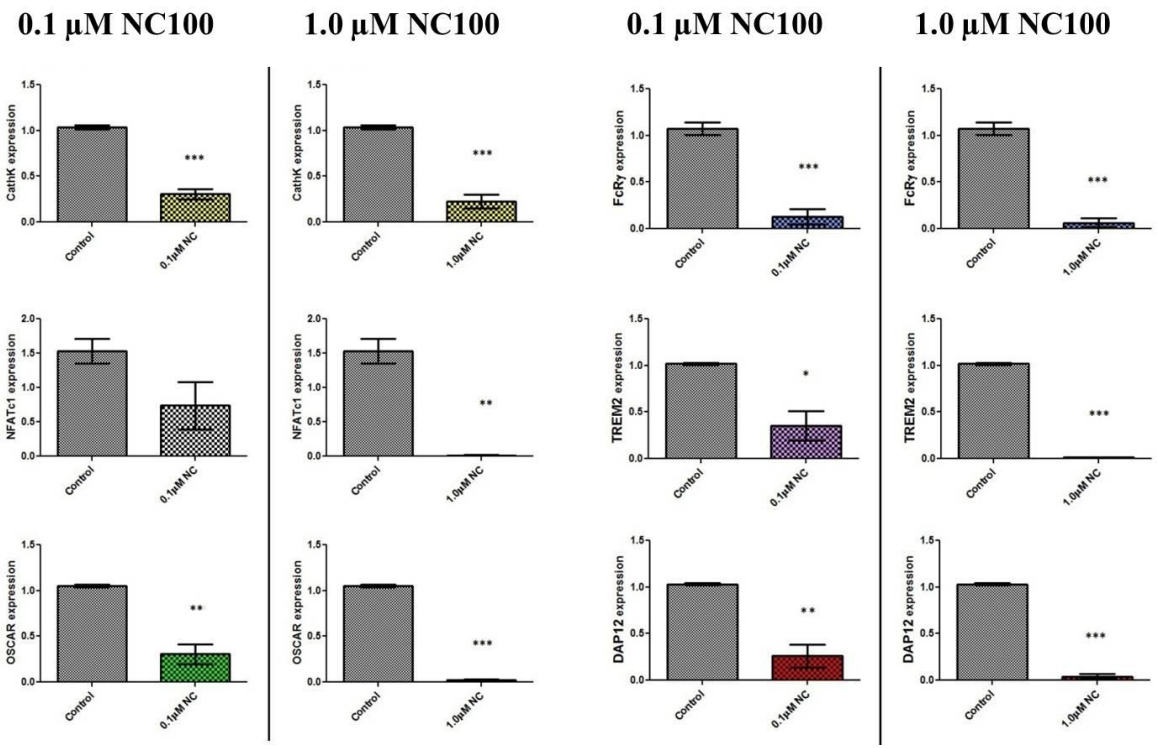

Fig. 3.4.Gene expression as assessed by quantitative real-time RT-PCR in 0.1 µM and 1.0 µM NC100 treatment, compared to control (no treatment).

The 2-ΔΔCt values of the expression of genes of interest relative to hARP as the reference gene are graphed and represented by mean±S.E (*p＜0.05, **p＜0.005, ***p＜0.001 vs. control).

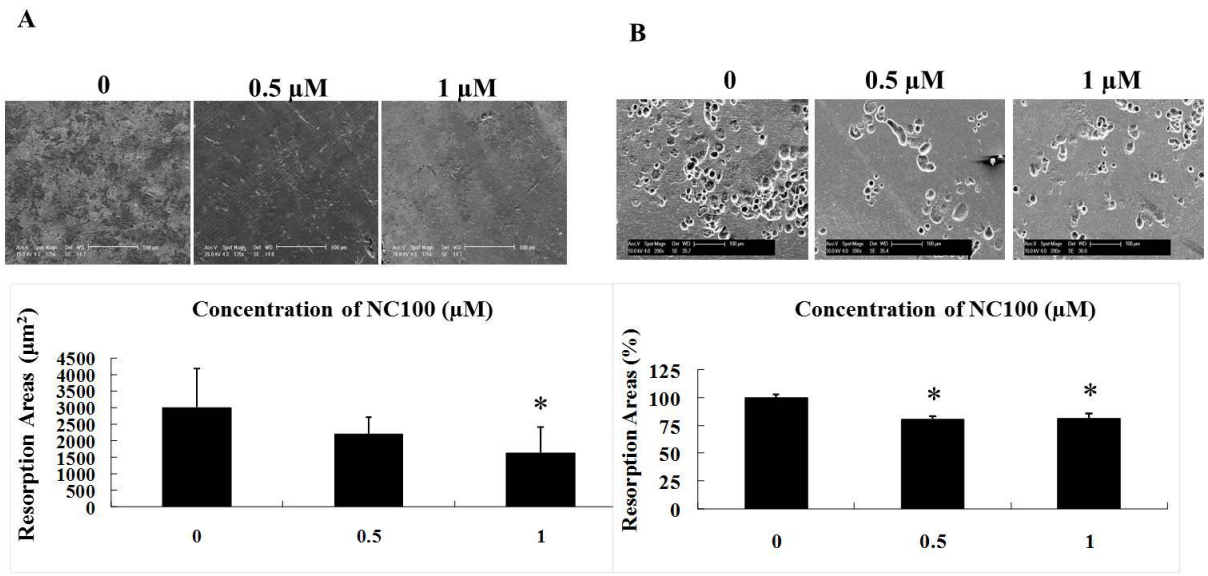

Fig. 3.5.NC100 reduces bone resorption.

A: Equal number of osteoclast-like cells derived from BMM cells was seeded onto bone slices and permitted to attach before exposure to NC100 at different doses (0, 0.5 and 1 μM) for 48 hours. B: Equal number of osteoclast-like cells derived from donor peripheral blood was seeded onto dentine bone slices and permitted to attach before exposure to NC100 at different doses (0, 0.5 and 1 μM) for 10 days. Representative scanning electron microscope images of bone resorption pits are shown. The summed areas of resorption pits were measured under scanning electron microscope and are presented graphically normalized with same numbers of osteoclasts (*p value＜0.05).

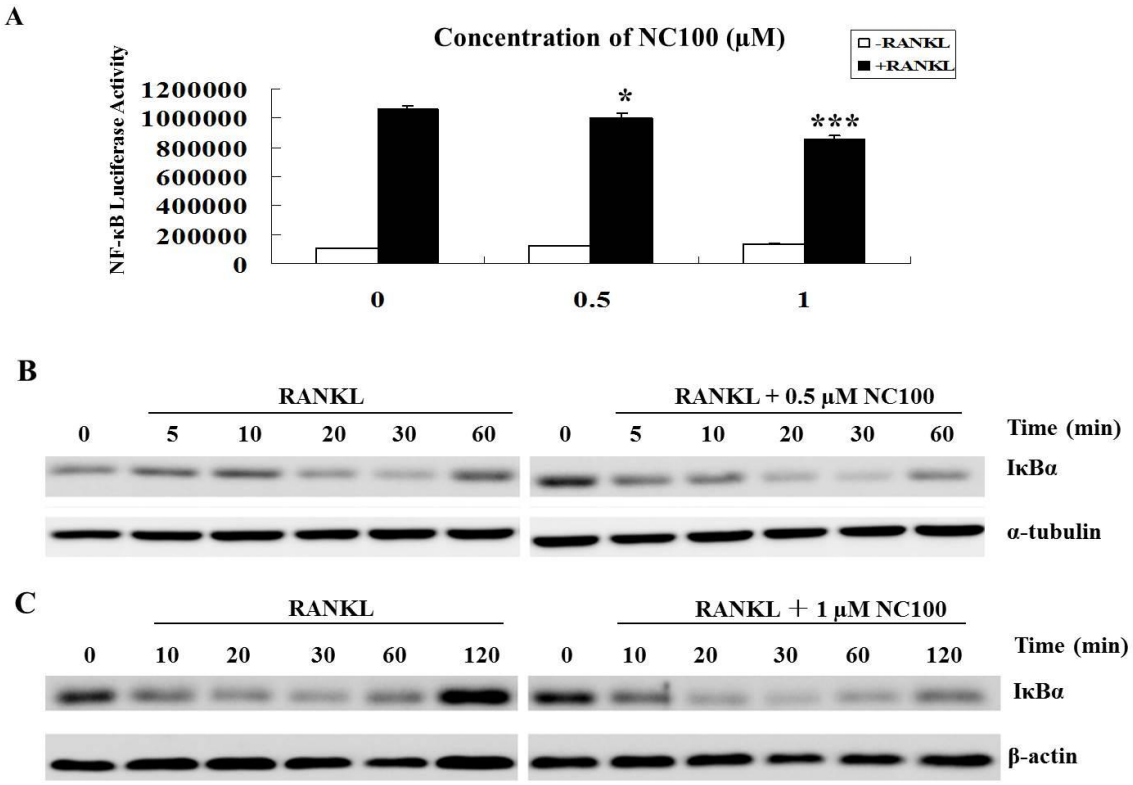

Fig. 3.6.The effect of NC100 on the degradation of of IκBα by RANKL.

A: Cells were treated with medium alone, RANKL (100 ng/ml), NC100 at 0.5 μM and 1 μM in the presence or absence of RANKL. RAW264.7 cells transfected with the 3kB-Luc-SV40 reporter gene were pre-incubated with varying doses of NC100. B: BMMs were pre-treated with different doses of NC100 for 1 hour, and then stimulated with RANKL for 30 minutes. Proteins from whole cell extracts were separated and transferred to membranes, which were blocked and probed with antibody to IκBα and β-actin. B and C: BMMs were pretreated with NC100 (0.5 μM or 1 μM) for 1 hour, and then stimulated with RANKL for the indicated times. Cell lysates were prepared and subjected to western blotting with IκBα antibody. The α-tubulin or β-actin blot is shown as loading control (*$p < 0.05$, ***$p < 0.001$ vs. control).

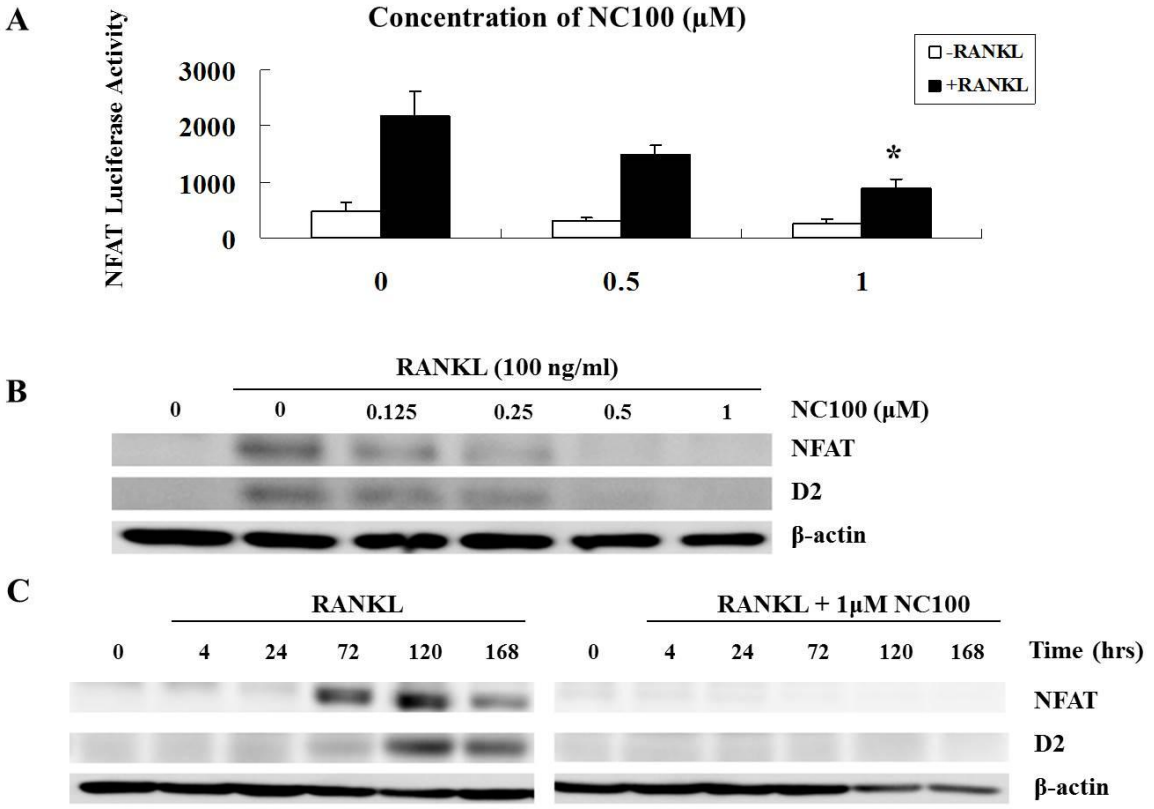

Fig. 3.7.NC100 inhibits RANKL-induced NFAT expression.

A: Cells were treated with medium alone, RANKL (100 ng/ml), NC100 at 0.5 μM and 1 μM in the presence or absence of RANKL. RAW264.7 cells were transiently transfected with pNFAT-TA-Luc vector. (*$p < 0.05$ vs. control) B: BMMs were incubated with different doses of NC100 for 1 hour and treated with 100 ng/ml RANKL for 7 days. C: BMMs were incubated with 1 μM NC100 for 1 hour and treated with RANKL for the indicated times. Quantitative Western Blot analysis of NFAT and D2 was performed, β-actin was used as a control.

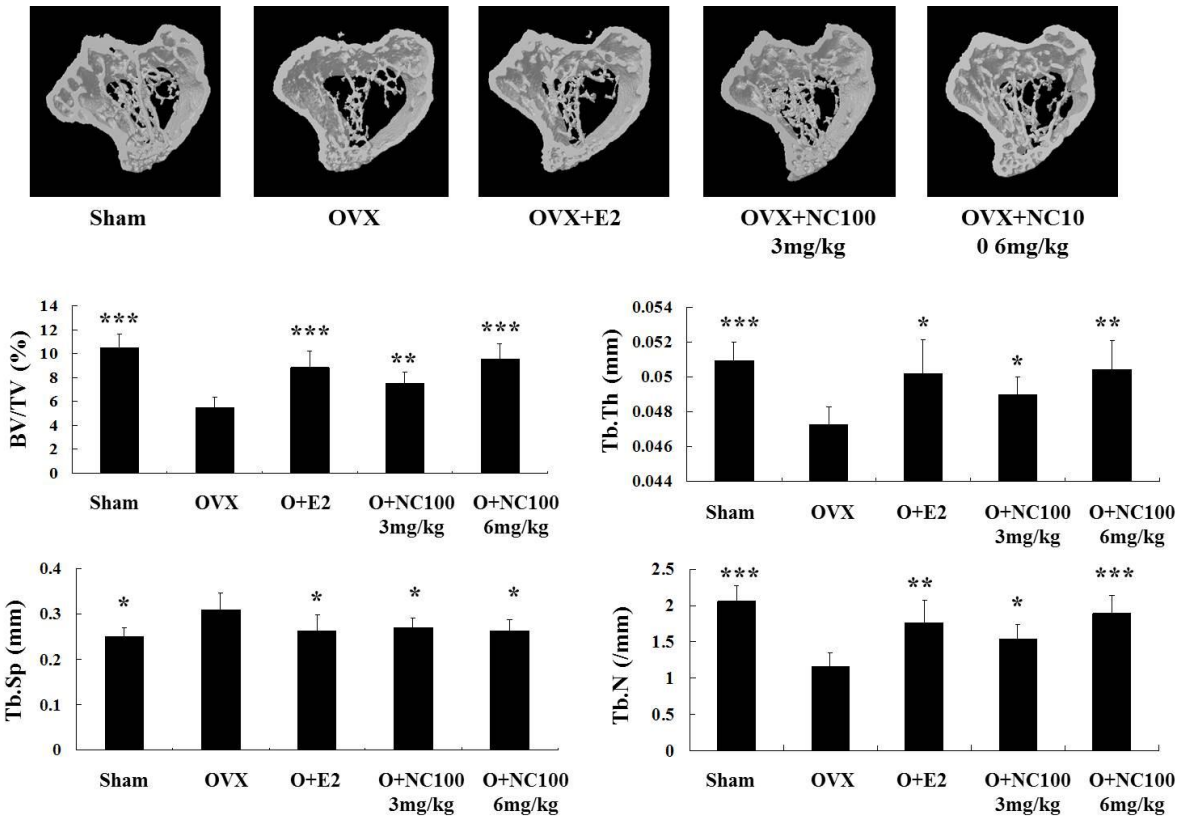

Fig. 3.8.NC100 prevented bone loss in OVX C57BL6J mice.

Trabecular bone microarchitecture by 3D micro-CT analysis in the proximal tibial metaphysis. Trabecular bone was analysed by micro-CT in sham-operated or ovariectomized mice after vehicle or NC100 treatment (3 and 6 mg/kg). Bone volume/tissue volume (BV/TV), trabecular space (Tb. Sp), trabecular thick (Tb. Th) and trabecular number (Tb. N). Values are means ± SE from 6 mice per group (*$p < 0.05$, **$p < 0.005$ and ***$p < 0.001$ vs. control).

CHAPTER 4. Parthenolide inhibits osteocyte apoptosis and osteoclastic bone surface resorption induced by polyethylene particles in a murine calvarial model of peri-implant osteolysis

Muhamad S F Zawawi,[1,2] Victor Marino,[3] Egon Perilli,[4] Melissa D Cantley,[1] Jiake Xu,[5] P. Edward Purdue,[6] Anak A S S K Dharmapatni,[1] David R Haynes,[1] Tania N Crotti[1]

1. School of Medical Sciences, The University of Adelaide, Australia.
2. Universiti Sains Malaysia, Malaysia.
3. School of Dentistry, The University of Adelaide, Australia.
4. School of Computer Science, Engineering and Mathematics, Flinders University, Australia.
5. School of Pathology and Laboratory Medicine, The University of Western Australia, Australia.
6. Hospital for Special Surgery, United States of America.

Chapter summary

In Chapter 4 the study aimed to investigate the effects of NF-κB inhibitor PAR on local and systemic bone resorption in a murine calvarial model of PE-induced peri-prosthetic osteolysis. We established a novel method to numerically analyse localised bone surface resorption from the μCT-derived images. Additionally the study determined the effects of PE particles and PAR treatment on the soluble OSCAR levels and osteocytes to better understand this pathology.

Statement of Authorship

Title of Paper	Parthenolide inhibits osteocyte apoptosis and osteoclastic bone surface resorption induced by polyethylene particles in a murine calvarial model of peri-implant osteolysis
Publication Status	Submitted (accepted April 2015 as "Parthenolide reduces empty lacunae and osteoclastic bone surface resorption induced by polyethylene particles in a murine calvarial model of peri-implant osteolysis").
Publication Details	**Muhamad S F Zawawi**,Victor Marino, Egon Perilli, Melissa D Cantley, Jiake Xu, P. Edward Purdue, Anak A S S K Dharmapatni, David R Haynes, Tania N Crotti Journal of Biomedical Materials Research Part A 2014

Statement of Authorship

NOTE:
Statements of authorship appear on pages 166-174 in the print copy of the thesis held in the University of Adelaide Library.

Parthenolide inhibits osteocyte apoptosis and osteoclastic bone surface resorption induced by polyethylene particles in a murine calvarial model of peri-implant osteolysis

Muhamad S F Zawawi,[1,2] Victor Marino,[3]Egon Perilli,[4] Melissa D Cantley,[1] Jiake Xu,[5] P. Edward Purdue,[6] Anak A S S K Dharmapatni,[1] David R Haynes,[1] Tania N Crotti[1]

[1]School of Medical Sciences, The University of Adelaide, SA, Australia; [2]Universiti Sains Malaysia, Malaysia; [3]School of Dentistry, The University of Adelaide, SA, Australia; [4]School of Computer Science, Engineering and Mathematics, Flinders University, SA, Australia; [5]School of Pathology and Laboratory Medicine, The University of Western Australia, WA, Australia; [6] Hospital for Special Surgery, NY, United States of America.

Corresponding author

Tania N. Crotti

Discipline of Anatomy and Pathology, School of Medical Sciences

The University of Adelaide, Adelaide, Australia, SA 5000

Email: tania.crotti@adelaide.edu.au

Telephone: +61 08 8313 5986

Fax: +61 08 8313 4408

Running title: Parthenolide suppresses calvarial surface resorption

4.1.ABSTRACT

The study aims to determine the effects of parthenolide (PAR) on bone volume (BV) and bone surface resorption (as assessed by live-animal μCT) and osteocyte death (as assessed by histology) on polyethylene (PE) particle-induced calvarial osteolysis in mice. Baseline μCT scans were conducted 7 days pre-implantation of 2×10^8 PE particles/mL over the calvariae (day 0). PAR at 1mg/kg/day was subcutaneously injected on days 0, 4, 7 and 10. At day 14, BV and surface resorption was analyzed with μCT. Calvarial tissue was processed for histomorphometric osteocyte evaluation. Serum was analyzed for type-1 carboxy-terminal collagen crosslinks (CTX-1) and osteoclast associated receptor (OSCAR) levels by ELISA. PE significantly decreased BV ($p=0.0368$), increased surface bone resorption area ($p=0.0022$) and increased osteocyte death ($p<0.0001$). Interestingly, PAR significantly reduced the resorption surface area in the PE-calvariae ($p=0.0022$), but it did not affect BV, serum CTX-1 or OSCAR levels. PAR also significantly reduced osteocyte death ($p<0.0001$). The ability of PAR to inhibit PE-induced surface bone erosion is important as it may better reflect the *in vivo* situation, where resorption occurs on the surface at the bone-implant interface and that may be related to the role of osteocytes in this pathology.

Keywords: Parthenolide; bone resorption; wear particles; calvarial model; osteolysis

4.2.INTRODUCTION

Total hip replacement (THR) is a highly successful procedure but these implants can fail prematurely for several reasons, of which the most common is osteolysis.[1] Prosthetic wear particles liberated from the implant surface over time are phagocytosed by macrophages. This leads to a chronic inflammatory response characterized by production of pro-inflammatory mediators, enhanced recruitment and activation of bone-resorbing osteoclasts[1] and suppression of bone formation by the osteoblasts resulting in bone loss.[2] Levels of receptor activator of nuclear factor κ B ligand (RANKL) relative to its inhibitor osteoprotegerin are increased in peri-implant tissues correlating with increased osteoclast resorption activity *ex vivo*.[3] RANKL binds to its receptor, RANK to activate critical osteoclast intracellular factors nuclear factor kappa B (NF-κB) and nuclear factor of activated T-cells, cytoplasmic calcineurin-dependent-1 (NFATc1). NFATc1 is induced by particles *in vitro* and NFATc1 is crucial to induction of genes required and crucial for osteoclast motility, morphology and activity.[4-6]

The effect of particles such as polyethylene (PE) particles on osteoblasts and osteocytes may also contribute to osteolysis. Osteocytes regulate osteoclast activity through several mechanisms, including microdamage to the bone and induction of osteocyte apoptosis.[7] In response to mechanical damage on the surface of the matrix, the osteocyte cellular network induces RANKL-mediated local osteoclastic formation and resorption *in vitro*.[8] It is possible the osteocyte response to wear particles can enhance surface bone resorption through similar mechanisms. PE particles promote osteoblast maturation to osteocytes and induce RANKL production by osteocytes.[2] Osteocytes undergo apoptosis and produce inflammatory cytokines in response to cobalt-chromium-molybdenum alloy (Co-Cr-Mo) particles *in vitro*.[9] Osteocyte apoptosis generates one or more signals that increase bone loss and direct it toward the area of bone containing the dead or dying cells.[7] This study determines the effects of PE particles on osteocyte apoptosis in a murine model of PE-induced osteolysis.

PE particles significantly induce osteolysis in a calvarial mouse model as shown histologically and by micro-computed tomography (μCT) with reduced bone volume (BV) assessed *ex vivo* at 7 days,[10] 12 days[11] and 14 days.[12] The current study extends this by investigating whether PE particle induced volumetric bone change overtime as well as

assessing surface bone resorption.

Parthenolide (PAR) is a Feverfew-derived natural product[10] that prevents NF-κB DNA binding in osteoclasts *in vitro*[13] and reduces lipopolysaccharide (LPS)-[13] and PE-induced[10] osteolysis in mice. The current study investigates whether PAR treatment reduces the PE particle-induced volumetric bone change over time in a murine calvarial model of osteolysis at day 14. Importantly the current study assesses the effects of PE particles and PAR treatment on surface bone resorption of the calvariae and osteocyte apoptosis.

In addition to RANKL-RANK signaling, the immunoreceptor tyrosine-based activation motif (ITAM)-dependent pathway provides co-stimulatory signals in the osteoclast to stimulate calcium signals that enhances NFATc1 expression via a positive feedback loop.[14] The ITAM factor osteoclast-associated receptor (OSCAR) contributes to the pathogenesis and severity of rheumatoid arthritis (RA) and peri-implant osteolysis.[4,15,16] Increased levels of membrane bound OSCAR is associated with peripheral blood monocytes, synovial tissue macrophages and the vasculature in RA synovial tissues.[15,16] OSCAR and NFATc1 protein expression is increased by PE in human peri-implant tissue and *in vitro* assays.[17] Additionally, calcineurin/NFAT inhibitors suppress OSCAR in osteoclast formation *in vitro*.[18] We propose soluble OSCAR in the serum and synovial fluid[15,16,19,20] may modulate osteoclast activity in the context of inflammatory induced osteolysis.

This study aims to investigate whether inhibition of NF-κB using PAR reduces localized osteolysis and modulates osteocyte death in a calvarial model of PE-induced bone loss 14 days after administration of PE particles. We further investigated the systemic effects of treatments on bone resorption marker serum type-1 carboxy-terminal collagen crosslinks (CTX-1), and OSCAR in the murine model and in human peri-implant osteolytic patients.

4.3.MATERIAL AND METHODS

4.3.1.Murine calvarial model of polyethylene particle-induced osteolysis

This murine model of PE particle-induced osteolysis was based on a model developed by Wedemeyer *et al.*[12] Twenty-four 6-8-week LPS-resistant[21] C3H/HEJ mice were randomly allocated to 4 groups of 6: Control (no disease and no treatment); PAR only (no disease but treated with PAR); PE only (disease and no treatment) and PE+PAR (disease with PAR treatment). Ethical approval was obtained from the Animal Ethics Committees of the University of Adelaide (M-2001-070) and SA Pathology (106/10) and complied with the National Health and Medical Research Council (Australia) Code of Practice for Animal Care in Research and Teaching (2014).

4.3.2.Polyethylene particle preparation

Commercially pure PE particles (UHMWPE, Ceridust VP 3610, Clariant, Gersthofen, Germany)[12] were washed in 100% ethanol for endotoxin removal[10] then washed in phosphate buffer solution (PBS) with 1% normal mouse serum (NMS).

4.3.3.Particle implantation and treatment

At day 0, mice were anaesthetized with 2% isoflurane anaesthetic in oxygen. Heads were shaved and a 2mm skin incision was made along the midline. The periosteum of the calvarium was lightly scratched and 30μL of PBS with 1% normal mouse serum (NMS) (Control and PAR only) or PE particles in PBS with 1% NMS at 2×10^8 particles/mL (PE+/-PAR) were placed onto the periosteum[11] and the incision was stapled.[22] At days 0, 4, 7 and 10, PAR only and PE+PAR mice were subcutaneously injected with PAR (Alexis Biochemicals; 350-258-M005' purity 99%) at 1mg/kg/day[10,13] in PBS with 0.04% dimethyl sulfoxide (DMSO). Control and PE only mice received PBS+0.04% DMSO. At day 14 post-surgery, mice were anaesthetized and blood was collected by cardiac puncture before cervical dislocation. *Ex vivo* μCT scans were performed and calvariae were processed for histology.

4.3.4.Micro-computed tomography (μCT) imaging and selection of the volume of interest

Baseline measurements were obtained 6-7 days prior to particle implantation (day 0), as previously described[23]. Mice were scanned using a live animal μCT system (Skyscan model 1076 high resolution *in vivo* scanner; Skyscan, Kontich, Belgium) with the following settings: 75kV voltage, 120mA current, 1.0mm aluminium filter,[23] field of view of 35mm×35mm, at 17μm isotropic pixel size and rotation step 0.5º.Each scan took 16 minutes. Following killing at day 14, an *ex vivo* μCT scan with the same settings was performed on the skinned heads.

The cross-section images were reconstructed using a filtered back-projection algorithm (NRecon software, V 1.12.04, Skyscan, Kontich, Belgium)[23] and saved as 8-bit greylevel files (.BMP format). For each skull, a stack of up to 1800 cross-sections was reconstructed, with an inter-slice distance of 1 pixel corresponding to a maximum reconstructed length of 16.2mm (Fig.4.1), recreating the full length of the skull. These were uniformly thresholded to segment the bone voxels as a solid and a 3D model of the skull was created (CT Analyser) and visualized (ParaView Software, V 3.1.2.0-RC2, New York, USA) (Fig. 4.1).

For the subsequent quantitative analysis of the segmented μCT images, a rectangular region of interest (ROI), 1.7mm length ×3.5mm width (200×400 pixel) was centered over the skull to include the area of PE implantation. The ROI, used over the stackof bone of 220 μCT images of each skull (corresponding to 2.0mm depth), formed a volume of interest (VOI). The VOI included the entire thickness of the skull and produced a rectangular slab in 3D (Fig. 4.1e-l) in which quantitative μCT analysis was performed.

4.3.5.Surface resorptivearea analysis via μCT

For each segmented VOI (rectangular slab) visualized in 3D, of the day 14 scans, two orthogonal images were taken (software Paraview). One image visualized the outer skull surface and one for the inner surface (Fig. 4.1e-l). Resorption areas that were visible as darker crater-like regions in these images were then traced manually using a tablet (Bamboo, Wacom Co. 2009) and areas of resorption filled (Adobe Photoshop Elements 7).[18] The area was quantified (pixel counting) using ImageJ analysis software (Version

1.36b, National Institutes of Health, USA).[18] The percentage area of resorption was calculated as a fraction of the total ROI area analyzed for the inner, outer and combined surfaces of the calvariae.

4.3.6.Volumetric bone loss analysis via µCT

For each VOI, the BV (mm^3) was calculated as the volume occupied by the voxels segmented as 'bone' using CTAn software.[24] For each animal, the change in BV (mm^3) overtime was determined as BV at day 14 minus BV at baseline.

4.3.7.Histomorphometric osteocyte evaluation of the calvarial tissue

Mouse heads were skinned and fixed in 10% PBS-buffered formalin for 48 hours. Following decalcification in a pH 7.4 10% dehydrate disodium salt (EDTA) solution over 8 weeks, heads were cut coronally into front and back regions and paraffin embedded. Sections of calvariae (5µm) were mounted on (3-Aminopropyltriethoxysilane 98% (APTS)-coated glass slides (Sigma-Aldrich) and stained with haemotoxylin and eosin (H&E) to screen for the depth at which particles were present within the calvarial tissue. Toluidine blue staining was performed to identify osteocytes with in the lacunae of calvarial tissues.[7] Slides were mounted with DPX Mountant for histology (Sigma-Aldrich) and imaged using the NanoZoomer Digital Pathology (NDP) (Hamamatsu Photonics K.K., Shizuoka Pref., Japan) at x40 magnifications. Within a 1.6x1.0mm box on the right and left sides of midline suture the percentage of dead osteocytes (empty osteocyte lacunae, E) over the total osteocyte number (total of E and normal-appearing nuclei, N)[7] was calculated. The two values from each mouse were averaged to represent each animal.

4.3.8.Serum analysis of a murine model of PE induced calvarial osteolysis

Blood collected at day 14 was allowed to clot in the Eppendorf tubes then centrifuged and serum was collected for analysis of levels of CTX-1 (RatLaps, Nordic Bioscience, Denmark) and OSCAR (Cusabio, Life Research, Australia), as per ELISA kit protocols.

4.3.9.Serum analysis of patients with peri-implant osteolysis

Serum was collected at the time of surgery from 10 patients with osteoarthritis (OA) receiving their primary hip implant or 19 patients undergoing revision for peri-implant osteolysis (metal-on-polyethylene total hips) at Hospital for Special Surgery following an IRB approved protocol. The osteolytic patients were aged 44-93 (mean=64.3) with time to revision 7-31 (mean=17) years. Informed consent was obtained from all participating patients. OSCAR levels were determined using a commercial ELISA kit (Cusabio CSB-EL017255HU).

4.3.10.Statistics and data presentation

All data was presented as mean ± standard error of the mean. The Kruskal–Wallis test was used for statistical significance among all groups (GraphPad Prism 6 for Windows,v6.0.0.289, 2012) followed by a Mann-Whitney on Control vs PAR only, Control vs PE only and PE only vs PE+PAR. Statistical significance was set as $p<0.05$.

4.4.RESULTS

4.4.1.Visual assessment of osteolysis via histology and μCT

H&E stained tissue showed the presence of fibrous granulomatous reaction and bone osteolysis in mice with PE (data not shown). In the PE-implanted group, at day 14 the 3D μCT images revealed the presence of craters (resorption regions) on the skull surface, adjacent to the location where the particles were placed (Fig. 4.1a-d). This was visible on both the outer (Fig. 4.1e-h) and inner surface (Fig.4.1i-l).

4.4.2.Quantitative assessment of bone surface resorptive area via μCT

The areas of surface bone resorption were quantified within the rectangular ROIs on the outer and inner surface (Fig. 4.1e-l). There was a statistically significant increase in the percentage of resorbed area for the outer, inner surfaces and combined surfaces in the PE group when compared to the control ($p= 0.0022$ for each) (Fig. 4.2c). PAR treatment significantly reduced the area resorbed over all examined surfaces ($p=0.0022$) in the PE

group compared with the PE untreated group, with a 67% decrease (from 0.53% to 0.17%) (Fig. 4.2c).

4.4.3.Quantitative changes in bone volume determined by µCT

By day 14, BV in the control group increased from baseline by 0.35mm^3 consistent with animal growth. BV in the PE group increased by only 0.09mm^3 indicating significant osteolysis induced by PE when compared to control ($p=0.0368$) (Fig. 4.2d). PAR treatment had no significant effect on BV when compared to control nor when comparing the PE groups.

4.4.4.Quantitative assessment of osteocyte death

Toluidine blue was used to stain mineralized tissue and identify lacunae with and without osteocytes (Fig. 4.3a-d). The percentage of empty osteocyte lacunae was significantly increased in animals given PE when compared to control ($p<0.0001$) (Fig. 4.3e). PAR treatment significantly reduced the percentage of osteocyte death when comparing the PE groups ($p<0.0001$). PAR treatment in the absence of particles increased osteocyte death ($p=0.0116$).

4.4.5.Murine serum levels of CTX-1 and soluble OSCAR

CTX-1 levels were measured as an indicator of systemic bone resorption. CTX-1 was significantly higher in the PE group ($p=0.0303$) and PAR treated group ($p=0.0411$) when compared to the control group (Fig. 4.4a). While CTX-1 levels were lower in the PAR treated PE group compared to the PE group, this was not statistically significant ($p=0.1429$).

Serum OSCAR levels (Fig. 4.4b) were assessed as a potential indicator of osteolytic activity.[16,19,20] OSCAR levels were significantly increased in mice with PE particles ($p=0.0286$) and decreased in PAR treated groups ($p=0.0286$) when compared to the control. PAR did not affect the OSCAR levels in the PE groups ($p=0.2286$).

4.4.6.Human serum levels of OSCAR in primary OA and revision patients

Serum levels of soluble OSCAR were measured in primary OA (n=10) and peri-implant osteolytic patients (n=19). OSCAR was not significantly different (p=0.6874) in the peri-implant (0.00-11.10pg/ml) compared to the OA patients (0.00-5.99pg/ml) (Fig. 4. 5).

4.5.DISCUSSION

Particle-induced osteolysis is a major cause of aseptic loosening and subsequent implant failure following total joint arthroplasty. Excessive osteoclast bone resorption induced by wear particles is largely responsible for peri-implant osteolysis and subsequent loosening.[1] Previous reports have shown osteocytes undergo apoptosis in response to wear particles[9] and fatigue-induced microdamage[7] resulting in bone containing the dead osteocytes being resorbed by osteoclasts, thereby contributing to osteolysis. The current study showed PE-induced osteolysis as measured by BV and surface resorption in a murine calvarial model of osteolysis. In addition PE caused osteocyte apoptosis, potentially contributing to osteolysis via this mechanism. The NF-κB inhibitor PAR abrogated PE-induced surface bone lysis but not BV.

This study is novel in that it involves *in vivo* μCT in order to calculate changes in bone volume overtime after 14 days administration of PE particles in a murine calvarial model of osteolysis, with each animal being its own control at baseline. At day 14 post-surgery BV in the PE group was significantly reduced when compared to control, consistent with the development of osteolysis at that time point as previously reported.[12]

PAR significantly inhibits NF-κB and osteoclastogenesis *in vitro,*[25] abrogates LPS-[13] and PE-induced[10] osteolysis in mice. In the current study, PAR was administered at 1mg/kg/3-day to PE implanted mice and PAR treatment did not significantly affect the BV after 14 days when compared to the baseline BV. In a previous study by Li *et al* mice receiving 1mg/kg/day PAR for 7-days post-surgery had a significantly reduced bone volume fraction (BV/TV) measurements at day 7.[10] Together these findings suggest PAR is able to prevent PE-induced osteolysis as assessed volumetrically at the earlier stage (day 7) rather than the late stage (day 14) of peri-implant osteolysis.

We contend that the surface erosions are highly important as these better reflect the *in vivo* situation as numerous studies have shown that resorption near implants occurs on the

bone surface at the interface[26] and at sites where there are high concentrations of PE wear particles present in the surface tissues.[27] This study includes extensive quantitative analysis of the area of macroscopically visible calvarial surface bone resorption using the μCT-derived images. PE significantly increased the percentage resorption surface area, whereas the PAR treatment significantly reduced this surface resorption. This suggests PAR treatment had a significant focal effect on reduction of the bone surface resorption adjacent to the implanted PE particles. Interestingly, osteolysis induced by PE particles resulted in both internal and external surface erosion, suggesting that the particles may also induce resorption at a distance. This is most likely due to mediators induced by PE particles stimulating osteoclast activity in the bone marrow. This may also occur within the bone and on the trabeculae.

While the analysis of the resorption bone surface is done over a surface area within a rectangular ROI over the skull, the bone volume (BV and BV/TV) measurements are done over the VOI, which encompasses the entire skull thickness, from the internal to external surface (3D slab). Importantly, our results indicate that at day 14, PAR significantly reduced surface bone resorption but not volume. However, until further work is carried out, it is difficult to know why at day 14 PAR affects only surface erosions but not volume. It is possible this is due to the different mechanisms of bone erosion occurring at the surface and within bone, and these have different sensitivities to PAR.

Osteocytes may also play a role in regulating peri-implant osteolysis via apoptosis signals[7,8] as they have been shown to be apoptotic in response to particles *in vitro*.[9] Consistent with this, the current study found PE particles significantly increased osteocyte death *in vivo*. Additionally, PE particles directly induce osteocytes to produce RANKL,[2] thus, significantly contributing to particle-induced osteolysis.[2] In the current study, PAR significantly reduced the numbers of empty lacune and this is consistent with surface osteolysis being regulated by osteocyte apoptosis. Since PAR acts by targeting NF-κB, it would be interesting to know if osteocyte death is regulated by NF-κB in osteocytes. A recent report found TNF-α enhanced sclerostin expression in an NF-κB-dependent manner in the osteocyte cell line, MLO-Y4, indicating NF-κB could have a role in osteocyte functions.[28] The possible mechanism by which PE induces NF-κB in osteocytes and thus induces osteolysis requires further studies.

Particles may also act distally by travelling and inducing inflammatory factors that exacerbate bone resorption elsewhere, as has been suggested previously.[29] As an indicator of systemic bone resorption CTX-1 levels were significantly increased in PE-given mice. Unexpectedly, in the absence of particles PAR increased CTX-1 levels.

Soluble OSCAR has been proposed as a potential regulator of osteoclast activity.[16,19] in this osteolysis. Decreased levels are present in active RA patients compared with healthy patients[16,19] and there is an inverse relationship between RA and the presence of erosions.[19] We found almost undetectable levels of serum OSCAR in patients with end-stage wear-induced osteolysis as well as in primary OA patients. This may be explained by limit of detection of the assay used. In our mouse model of osteolysis, OSCAR increased in the serum of mice with PE induced bone resorption, consistent with a recent finding that OSCAR was found at low levels in healthy individuals and high in active RA.[20] It is important to consider that in mice cell associated and possibly secreted OSCAR is limited to osteoclast cells[30] whereas in humans it is expressed and potentially released by multiple cells types[15] including dendritic cells,[31] osteoclasts[14] and endothelial cells.[32]

4.6.CONCLUSION

PE particles significantly induced osteocyte death, and bone loss as assessed by internal and external surface resorption and BV in a calvarial murine model of peri-implant osteolysis. At day 14, PAR treatment every 3 days resulted in a significant reduction in osteocyte death and PE particle-induced bone surface resorption but not bone volume change. The ability of PAR to inhibit surface erosion induced by PE particles is important as surface erosion may better reflect the *in vivo* situation where bone is resorbed on the surface at the bone-implant interface, and that may be related to the role of osteocytes in this pathology.

4.7.ACKNOWLEDGEMENTS

We thank Renee Orsmby for her contribution to the osteocyte analysis. The work has been supported by a New Appointment Grant, Faculty of Health Sciences, The University of Adelaide. The Malaysian government (USM) provided support for M.S.F.Z. The authors have no conflict of interest to declare.

4.8.REFERENCES

1. Purdue PE, Koulouvaris P, Nestor BJ, Sculco TP. The central role of wear debris in periprosthetic osteolysis. HSS J 2006;2(2):102-13.
2. Atkins GJ, Welldon KJ, Holding CA, Haynes DR, Howie DW, Findlay DM. The induction of a catabolic phenotype in human primary osteoblasts and osteocytes by polyethylene particles. Biomaterials 2009;30(22):3672-81.
3. Crotti TN, Smith MD, Findlay DM, Zreiqat H, Ahern MJ, Weedon H, Hatzinikolous G, Capone M, Holding C, Haynes DR. Factors regulating osteoclast formation in human tissues adjacent to peri-implant bone loss: expression of receptor activator NFkappaB, RANK ligand and osteoprotegerin. Biomaterials 2004;25(4):565-73.
4. Alias E, Dharmapatni ASSK, Holding AC, Atkins GJ, Findlay DM, Howie DW, Crotti TN, Haynes DR. Polyethylene particles stimulate expression of ITAM-related molecules in peri-implant tissues and when stimulating osteoclastogenesis in vitro. Acta Biomaterialia 2012;8(8):3104-12.
5. Crotti TN, Sharma SM, Fleming JD, Flannery MR, Ostrowski MC, Goldring SR, McHugh KP. PU.1 and NFATc1 mediate osteoclastic induction of the mouse beta3 integrin promoter. J Cell Physiol 2008;215(3):636-44.
6. Asagiri M, Takayanagi H. The molecular understanding of osteoclast differentiation. Bone 2007;40(2):251-64.
7. Verborgt O, Gibson GJ, Schaffler MB. Loss of osteocyte integrity in association with microdamage and bone remodeling after fatigue in vivo. J Bone Miner Res. 2000;15(1):60-7.
8. Kurata K, Heino TJ, Higaki H, Vaananen HK. Bone marrow cell differentiation induced by mechanically damaged osteocytes in 3D gel-embedded culture. J Bone Miner Res 2006;21(4):616-25. Epub 2006 Apr 5.
9. Kanaji A, Caicedo MS, Virdi AS, Sumner DR, Hallab NJ, Sena K. Co-Cr-Mo alloy particles induce tumor necrosis factor alpha production in MLO-Y4 osteocytes: a role for osteocytes in particle-induced inflammation. Bone. 2009;45(3):528-33.

10. Li Y, Zhang C, Zhou X, Wang H, Mao Y, Wang X. Parthenolide inhibits polyethylene particle-induced mouse calvarial osteolysis in vivo. J Surg Res 2013;187(1):176-81.
11. Landgraeber S, Jaeckel S, Loer F, Wedemeyer C, Hilken G, Canbay A, Totsch M, von Knoch M. Pan-caspase inhibition suppresses polyethylene particle-induced osteolysis. Apoptosis 2009;14(2):173-81.
12. Wedemeyer C, Xu J, Neuerburg C, Landgraeber S, Malyar NM, von Knoch F, Gosheger G, von Knoch M, Loer F, Saxler G. Particle-induced osteolysis in three-dimensional micro-computed tomography. Calcif Tissue Int 2007;81(5):394-402.
13. Yip KH, Zheng MH, Feng HT, Steer JH, Joyce DA, Xu J. Sesquiterpene lactone parthenolide blocks lipopolysaccharide-induced osteolysis through the suppression of NF-kappaB activity. J Bone Miner Res 2004;19(11):1905-16.
14. Kim N, Takami M, Rho J, Josien R, Choi Y. A novel member of the leukocyte receptor complex regulates osteoclast differentiation. J Exp Med. 2002;195(2):201-9.
15. Crotti TN, Dharmapatni AA, Alias E, Zannettino AC, Smith MD, Haynes DR. The immunoreceptor tyrosine-based activation motif (ITAM) -related factors are increased in synovial tissue and vasculature of rheumatoid arthritic joints. Arthritis Res Ther 2012;14(6):R245.
16. Herman S, Muller RB, Kronke G, Zwerina J, Redlich K, Hueber AJ, Gelse H, Neumann E, Muller-Ladner U, Schett G. Induction of osteoclast-associated receptor, a key osteoclast costimulation molecule, in rheumatoid arthritis. Arthritis Rheum 2008;58(10):3041-50.
17. Alias E, Dharmapatni AS, Holding AC, Atkins GJ, Findlay DM, Howie DW, Crotti TN, Haynes DR. Polyethylene particles stimulate expression of ITAM-related molecules in peri-implant tissues and when stimulating osteoclastogenesis in vitro. Acta biomaterialia 2012;8(8):3104-12.
18. Zawawi MSF, Dharmapatni AA, Cantley MD, McHugh KP, Haynes DR, Crotti TN. Regulation of ITAM adaptor molecules and their receptors by inhibition of calcineurin-NFAT signalling during late stage osteoclast differentiation. Biochem Biophysical Research Communication 2012;427(2):404-9.

19. Zhao S, Guo YY, Ding N, Yang LL, Zhang N. Changes in serum levels of soluble osteoclast-associated receptor in human rheumatoid arthritis. Chin Med J 2011;124(19):3058-60.
20. Ndongo-Thiam N, de Sallmard G, Kastrup J, Miossec P. Levels of soluble osteoclast-associated receptor (sOSCAR) in rheumatoid arthritis: link to disease severity and cardiovascular risk. Ann Rheum Dis. 2014;73(6):1276-7.
21. Poltorak A. Defective LPS Signaling in C3H/HeJ and C57BL/10ScCr Mice: Mutations in Tlr4 Gene. Science 1998;282(5396):2085-2088.
22. Merkel KD, Erdmann JM, McHugh KP, Abu-Amer Y, Ross FP, Teitelbaum SL. Tumor necrosis factor-alpha mediates orthopedic implant osteolysis. Am J Pathol 1999;154(1):203-10.
23. Cantley MD, Bartold PM, Marino V, Reid RC, Fairlie DP, Wyszynski RN, Zilm PS, Haynes DR. The use of live-animal micro-computed tomography to determine the effect of a novel phospholipase A2inhibitor on alveolar bone loss in anin vivomouse model of periodontitis. Journal of Periodontal Research 2009;44(3):317-322.
24. Perilli E, Le V, Ma B, Salmon P, Reynolds K, Fazzalari NL. Detecting early bone changes using in vivo micro-CT in ovariectomized, zoledronic acid-treated, and sham-operated rats. Osteoporos Int 2010;21(8):1371-82.
25. Xu J, Wu HF, Ang ES, Yip K, Woloszyn M, Zheng MH, Tan RX. NF-kappaB modulators in osteolytic bone diseases. Cytokine Growth Factor Rev 2009;20(1):7-17.
26. Schmalzried TP, Harris WH. The Harris-Galante porous-coated acetabular component with screw fixation. Radiographic analysis of eighty-three primary hip replacements at a minimum of five years. J Bone Joint Surg Am. 1992;74(8):1130-9.
27. Holding CA, Findlay DM, Stamenkov R, Neale SD, Lucas H, Dharmapatni AS, Callary SA, Shrestha KR, Atkins GJ, Howie DW and others. The correlation of RANK, RANKL and TNFalpha expression with bone loss volume and polyethylene wear debris around hip implants. Biomaterials 2006;27(30):5212-9.

28. Baek K, Hwang HR, Park HJ, Kwon A, Qadir AS, Ko SH, Woo KM, Ryoo HM, Kim GS, Baek JH. TNF-alpha upregulates sclerostin expression in obese mice fed a high-fat diet. J Cell Physiol. 2014;229(5):640-50.
29. Tuan RS, Lee FY, Y TK, Wilkinson JM, Smith RL. What are the local and systemic biologic reactions and mediators to wear debris, and what host factors determine or modulate the biologic response to wear particles? The Journal of the American Academy of Orthopaedic Surgeons 2008;16 (1):S42-8.
30. Kim N, Takami M, Rho J, Josien R, Choi Y. A novel member of the leukocyte receptor complex regulates osteoclast differentiation. J Exp Med 2002;195(2):201-9.
31. Tenca C, Merlo A, Merck E, Bates EEM, Saverino D, Simone R, Zarcone D, Trinchieri G, Grossi CE, Ciccone E. CD85j (Leukocyte Ig-Like Receptor-1/Ig-Like Transcript 2) Inhibits Human Osteoclast-Associated Receptor-Mediated Activation of Human Dendritic Cells. The Journal of Immunology 2005;174(11):6757-6763.
32. Goettsch C, Rauner M, Sinningen K, Helas S, Al-Fakhri N, Nemeth K, Hamann C, Kopprasch S, Aikawa E, Bornstein SR and others. The Osteoclast-Associated Receptor (OSCAR) Is a Novel Receptor Regulated by Oxidized Low-Density Lipoprotein in Human Endothelial Cells. Endocrinology 2011;152(12):4915-4926.

4.9.FIGURE LEGENDS

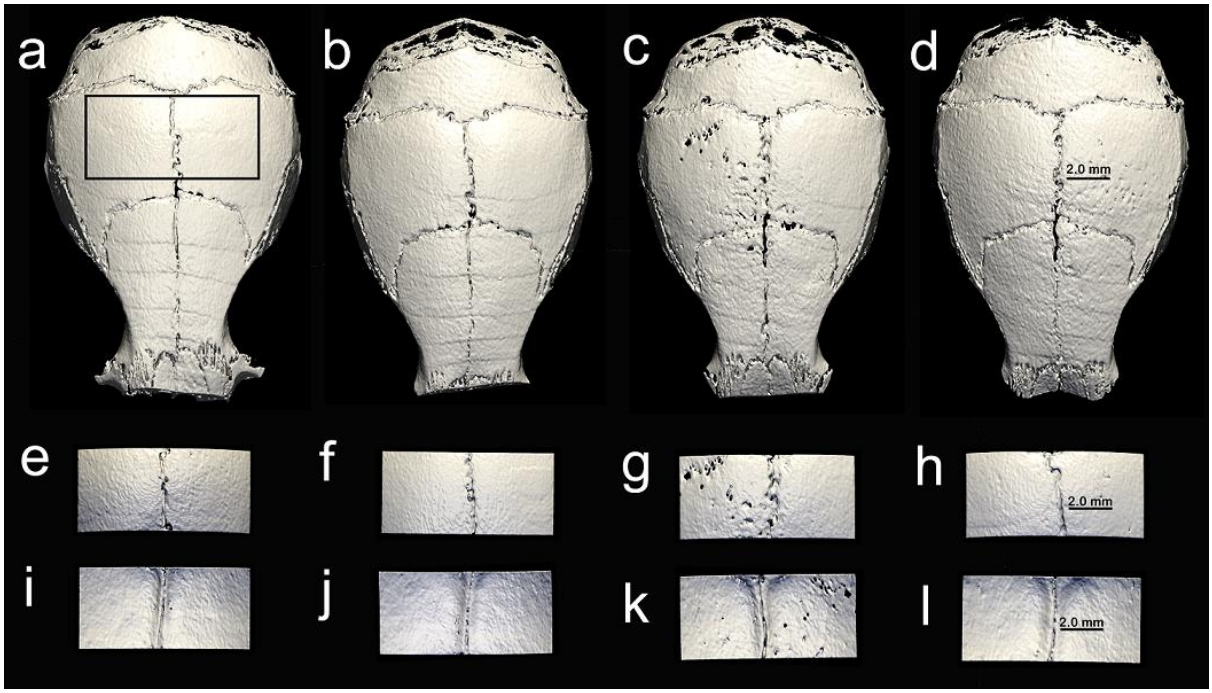

Figure 4.1

(a-d) Three-dimensional μCT images of mouse calvariae at day 14, (e-l) Rectangular ROI (1.7×3.5mm) selected for quantitative analysis over the calvariae, (e-h) view of the outer surface and (i-l) of the inner surface, (a, e, i) Control, (b, f, j) PAR only, (c, g, k) PE only, (d, h, l) PE+PAR. (a) Location of rectangular ROI depicted by black box. (c) The visible resorptive regions/craters on the surface of the calvariae of the PE group.

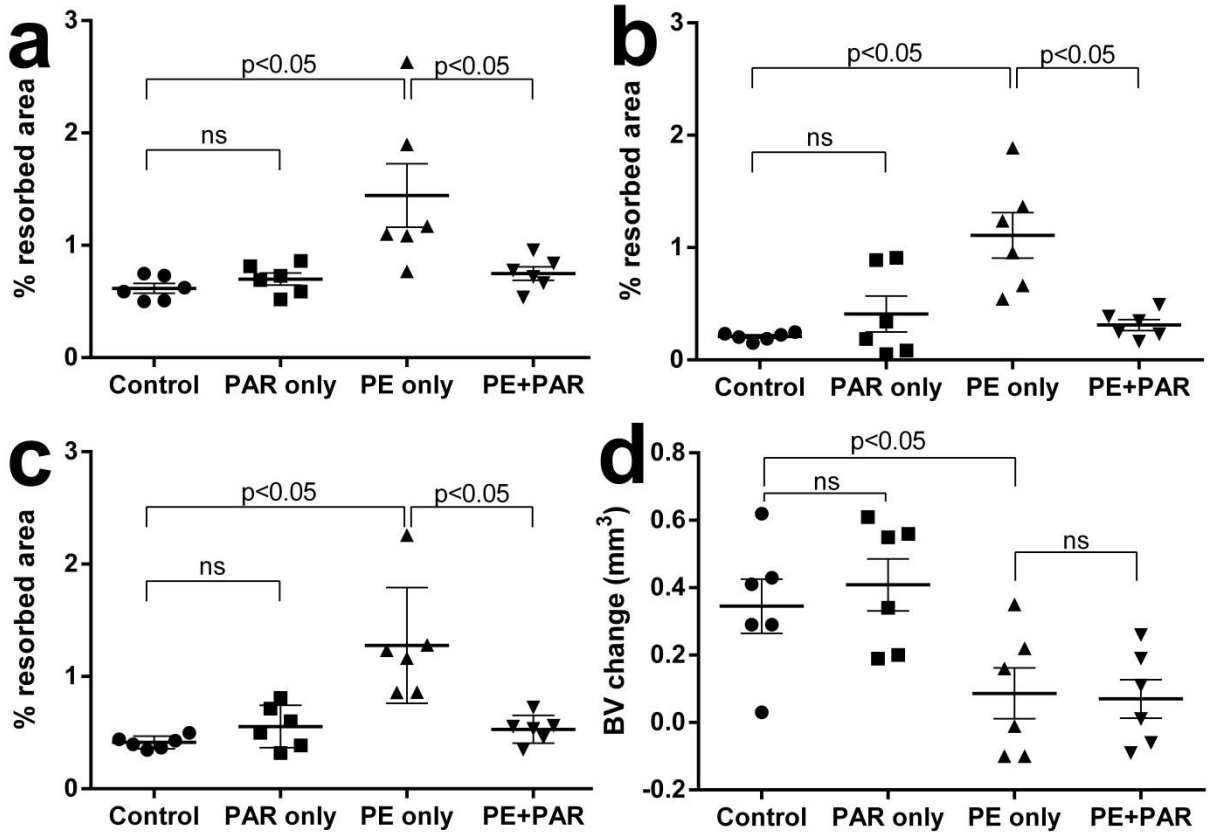

Figure 4.2

Quantitative μCT analysis within the rectangular ROI selected at day 14, percentage resorbed bone area for (a) the outer surface of the calvariae, (b) the inner surface of the calvariae, (c) combined outer and inner surfaces of the calvariae, (d) Change in Bone volume (BV) compared to baseline. The values are presented as mean ± S.E. Significance was considered as $p<0.05$; ns referred to not significant.

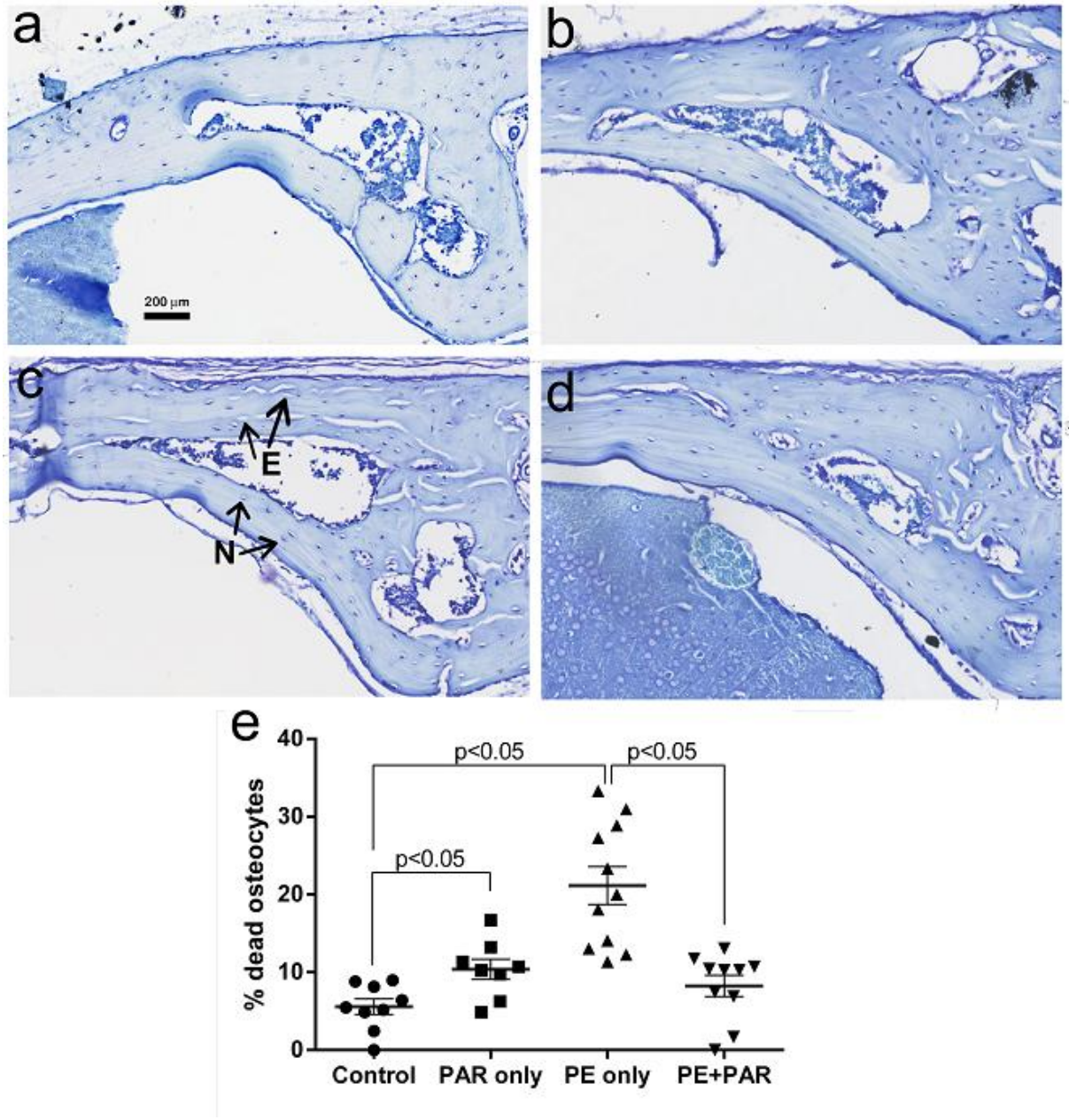

Figure 4.3

(a-d) Photomicrograph of toluidine blue-stained sections of murine calvarial tissue (left hand-side from midline suture), at 40× magnification. (a) Control, (b) PAR only, (c) PE only, (d) PE+PAR. (c) Empty osteocyte lacunae, E and normal-appearing nuclei, N. (e) The percentage of dead osteocyte in 1.6×1.0mm tissue section. The values are presented as mean ± S.E. Significance was set as $p<0.05$.

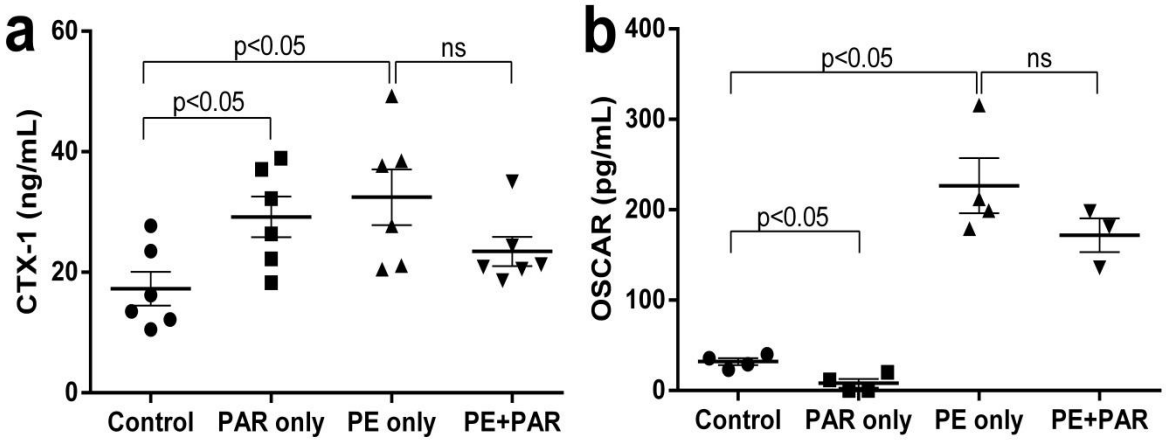

Figure 4.4

Biochemical analyses of the murine serum. CTX-1 concentration in a particle induced murine model of osteolysis. (b) Soluble OSCAR levels in a particle-induced murine model of osteolysis. The values are presented as mean ± S.E. Significance was considered as $p<0.05$; ns referred to not significant.

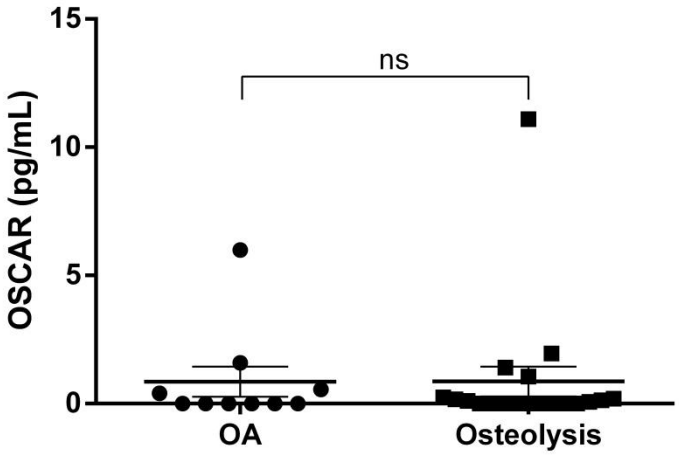

Figure 4.5

Serum levels of soluble OSCAR in human osteolysis. The values are presented as mean ± S.E. Significance was considered as $p<0.05$; ns referred to not significant.

CHAPTER 5. Caffeic acid phenethyl ester abrogates bone resorption in a murine calvarial model of polyethylene particle-induced osteolysis

M.S.F. Zawawi[a,b], E. Perilli[c], R.L. Stansborough[a], V. Marino[d], M.D. Cantley[a], J. Xu[e], A.A.S.S.K., Dharmapatni[a], D.R. Haynes[a] , R.J. Gibson[a], and T.N. Crotti[a]

a. School of Medical Sciences, The University of Adelaide, Australia.
b. School of Medical Sciences, Universiti Sains Malaysia (USM), Malaysia.
c. School of Computer Science, Engineering and Mathematics, Flinders University, Australia.
d. School of Dentistry, The University of Adelaide, Australia.
e. School of Pathology and Laboratory Medicine, The University of Western Australia, Australia.

Chapter summary

Chapter 4 showed the effects of NF-κB inhibition on bone resorption in a murine calvarial model of PE-induced peri-prosthetic osteolysis. Using this murine model, Chapter 5 investigated the effects of NF-κB and NFATc1 inhibitor CAPE on localised PE-induced calvarial bone resorption. The systemic effects of PE particles and CAPE treatment were assessed by analyses of serum CTX-1 levels, soluble OSCAR and GIT. It was hyposthesized inhibition of NF-κB and NFATc1 suppresses localized and systemic bone resorption in a murine calvarial model of particle-induced peri-prosthetic osteolysis.

Statement of Authorship

Title of Paper	Caffeic acid phenethyl ester abrogates bone resorption in a murine calvarial model of polyethylene particle-induced osteolysis
Publication Status	Submitted (accepted March 2015).
Publication Details	**M.S.F. Zawawi**, E. Perilli, R.L. Stansborough, V. Marino, M.D. Cantley, J. Xu, A.A.S.S.K., Dharmapatni, D.R. Haynes, R.J. Gibson, and T.N. Crotti Calcified Tissue International 2014

Statement of Authorship

NOTE:
Statements of authorship appear on pages 198-207 in the print copy of the thesis held in the University of Adelaide Library.

Caffeic acid phenethyl ester abrogates bone resorption in a murine calvarial model of polyethylene particle-induced osteolysis

M.S.F. Zawawi[a,b], E. Perilli[c], R.L. Stansborough[a], V. Marino[d], M.D. Cantley[a], J. Xu[e], A.A.S.S.K., Dharmapatni[a], D.R. Haynes[a] , R.J. Gibson[a], and T.N. Crotti[a]

[a]School of Medical Sciences, The University of Adelaide, SA, Australia.

[b]School of Medical Sciences, Universiti Sains Malaysia (USM), Malaysia.

[c]School of Computer Science, Engineering and Mathematics, Flinders University, SA, Australia.

[d]School of Dentistry, The University of Adelaide, SA, Australia.

[e]School of Pathology and Laboratory Medicine, The University of Western Australia, WA, Australia.

Corresponding author

Muhamad Syahrul Fitri Zawawi

Discipline of Anatomy and Pathology, School of Medical Sciences

The University of Adelaide, Adelaide, Australia, SA 5000

Email: muhamad.zawawi@adelaide.edu.au

Telephone: +61 08 8313 5986

Fax: +61 08 8313 4408

5.0.Abstract

Particle-induced bone loss by osteoclasts is a common cause of aseptic loosening around implants. The study investigates whether caffeic acid phenethyl ester (CAPE), a potent and specific inhibitor of NFATc1 and NF-κB, at a low dose reduces bone resorption in a murine calvarial model of polyethylene (PE) particle-induced osteolysis. The effects of particles and CAPE treatment on gastrointestinal tract (GIT) histopathology were also evaluated. Mice were scanned using *in vivo*-animal micro-computed tomography (μCT) as a baseline measurement. PE particles (2.82×10^9 particles/mL) were implanted over the calvariae on day 0. CAPE was administered subcutaneously (1mg/kg/day) at days 0, 4, 7 and 10. Mice were killed at day 14 and serum analysed for Type 1 carboxyterminal collagen crosslinks (CTX)-1 and Osteoclast Associated Receptor (OSCAR) levels. *Ex vivo* μCT scans were conducted to assess bone volume (BV) change and percentage area of calvarial surface resorbed. Calvarial and GIT tissue was processed for histopathology. By day 14, PE particles significantly induced calvarial bone loss compared to control animals as evidenced by resorptive areas adjacent to the implanted PE in three-dimensional μCT images, an increase in percentage of resorbed area (p=0.0022), reduction in BV (p=0.0012) and increased CTX-1 serum levels (p=0.0495). Serum OSCAR levels significantly increased in the PE implant group (p=0.0006). CAPE significantly inhibited PE particle-induced calvarial osteolysis as evidenced by a significant reduction in surface bone resorption (p=0.0012) and volumetric change (p=0.0154) compared to PE only but had no effect on systemic CTX-1**.** Neither particles nor CAPE had an effect on GIT histopathology.

Keywords: Osteolysis; CAPE; NF kappa B inhibitor; micro CT; CTX-1; OSCAR

5.1.Introduction

Particle-induced bone loss by osteoclasts is a common cause of aseptic loosening around implants. Macrophages phagocytose wear particles and the chronic inflammatory response [1] enhances differentiation and bone-resorbing activity of the osteoclasts mediated by receptor activator of nuclear factor κ B ligand (RANKL) [2, 3]. Our previous *in vivo* analyses demonstrated increased protein and mRNA expression of RANKL relative to its inhibitor osteoprotegerin in peri-implant tissues [3]. This correlated with increased resorptive activity confirming the role of osteoclasts in this osteolysis [3]. RANKL acts by binding to its receptor RANK and activating critical intracellular factors, nuclear factor kappa B (NF-κB) and nuclear factor of activated T-cells, cytoplasmic calcineurin-dependent-1 (NFATc1), to induce expression of key genes required for osteoclastogenesis [4, 5].

The local accumulation of wear particles induces local osteolysis [6] but particles have also been proposed to travel to other regions such as lymph nodes and induce distal bone resorption directly or indirectly via inflammatory factors that exacerbated bone resorption, elsewhere [7]. It is possible that distal inflammatory factors may affect other organs such as the gastrointestinal tract (GIT) as per other chronic inflammatory conditions such as rheumatoid arthritis (RA) and Crohn's disease.

NF-κB and calcineurin/NFAT signaling pathways play crucial roles in inflammatory pathological states involving increased osteoclast activity [8, 9]. Elevated activation of NF-κB has been reported in the inflamed joints of arthritic mice [10], lipopolysaccharide-induced bone destruction [11] and particle-induced peri-prosthetic osteolysis [12]. Suppression of NF-κB transcription *in vivo* results in decreased bone loss in chronically inflamed bone [13] and reduced tumor-mediated osteolysis [14]. Further to this, inhibition of calcineurin/NFAT signaling by Cyclosporin A reduces the severity of arthritis developed in a collagen antibody induced arthritis (CAIA) model in both the early and late phases of disease [15]. This is significant, as NFATc1 is induced by calcineurin and is crucial to both T cell activation and osteoclastogenesis.

Co-stimulatory pathways involving NFATc1 and NF-κB have been identified in osteoclasts [16-18]. The immunoreceptor tyrosine-based activation motif (ITAM)-dependent pathway stimulates calcium signals that activate the NFAT/calcineurin pathway

thus inducing NFATc1 [18]. The ITAM factor osteoclast-associated receptor (OSCAR) is induced via NFATc1 and on ligand interaction OSCAR signals via its adaptor protein Fc-receptor common gamma-chain (FcRγ) to further enhance ITAM signalling [17-20]. We have demonstrated increased expression of these factors adjacent to sites of bone loss in human peri-implant tissue and *in vitro* assays of PE particle-stimulated osteoclasts [21]. We have also shown calcineurin/NFAT inhibitors significantly reduced the expression of ITAM factors in human osteoclast differentiation *in vitro* [22]. OSCAR may contribute to the pathogenesis and severity of RA, peri-implant loosening and osteoporosis [21, 23-26]. Increased level of membrane bound OSCAR is associated with the peripheral blood monocytes and synovial tissue macrophages as well as the vasculature in RA synovial tissues [24, 26]. Conversely soluble OSCAR is lower in the serum of active RA patients compared with healthy individuals [24, 25]. Further to this, OSCAR inversely correlates with erosion in active RA [25]. We propose that soluble OSCAR acts as a negative regulator of osteoclast activity with serum levels reducing in the context of inflammatory induced osteolysis.

Caffeic acid phenethyl ester (CAPE) is a phenolic antioxidant derived from the propolis of honeybee hives, and acts as a potent and specific inhibitor of NFATc1 and NF-κB [27-29]. CAPE has been demonstrated to have anti-viral [27] and anti-inflammatory properties [28, 29]. Using *in vitro* osteoclast assays demonstrate that CAPE has been shown to act by inhibiting the RANKL-induced activation of NF-κB and p65 nuclear translocation resulting in abrogation of osteoclastogenesis and induction of apoptosis [28], supporting it as a potential candidate for the prevention or treatment of osteolytic and arthritic bone diseases. CAPE at high dose has been demonstrated to have anti-inflammatory properties in a rat air pouch model (10-100mg/ml) [30] and lipopolysaccharide (LPS)-induced inflammation of cultured human middle ear epithelial cells (HMEECs) (50-200μM) [31]. In a rat model of colitis, CAPE at 30mg/kg caused weight loss although it effectively reduced macroscopic colonic damage [32]. It has been demonstrated that CAPE at a low dose (0.5mg/kg) inhibits local bone loss in an ovariectomized (OVX) murine models of osteolysis (unpublished results from J Xu). It is therefore important to ascertain the effects of CAPE on locally induced bone loss and systemic effects on bone resorption and the GIT.

We have demonstrated inhibition of ITAM factors and resorptive activity with calcineurin-NFAT inhibitors in our human *in vitro* model of osteoclast formation [22]. We propose that NFATc1 and NF-κB inhibitors may suppress inflammation induced bone loss associated with particle-induced osteolysis. This investigates the effect of NFATc1 and NF-κB suppression by CAPE administered at low dose on PE-particle induced bone loss and systemic osteoclast activity in a murine calvarial model. Additionally we propose to investigate the systemic effects of PE wear particles and CAPE by assessing tissue histopathology in the GIT.

5.2.Materials and methods

5.2.1.Murine polyethylene particle-induced osteolysis model

Our murine model of PE particle-induced osteolysis was based on a model developed by Wedemeyer et al [6, 33, 34]. Twenty-two (22) 6-8-week LPS-resistant C3H/HEJ female mice were randomly divided into 3 groups: Control (no particles and no treatment, n=7), PE only (particle implantation, n=7) and PE+CAPE (particles and CAPE, n=8). This study was conducted in accordance with ethics approved by The University of Adelaide (M-2001-070) and SA Pathology (106/10) and complied with the National Health and Medical Research Council (Australia) Code of Practice for Animal Care in Research and Teaching (2014).

2.2.Preparation of polyethylene particles

Commercially pure polyethylene particles (UHMWPE, Ceridust VP 3610) were obtained from Clariant Company (Gersthofen, Germany). Greater than 35% of the particles were smaller than 1μm, with a mean particle size (given as equivalent circle diameter) of 1.75±1.43μm (range 0.05-11.6) [35-37]. Particles were washed in 100% ethanol for endotoxin decontamination, as previously described [38, 39], resuspended and washed in phosphate buffer solution (PBS) with 1% normal mouse serum (NMS) 6 times to eliminate all ethanol. The particles were stored in endotoxin free, sterile tubes.

5.2.3.Surgical procedure for PE implantation over murine calvariae

At day 0, mice were anaesthetized with 2% isoflurane anaesthetic in oxygen, heads shaved and a 2mm skin incision was made along the midline. The skin was retracted, the periosteum of the calvarium was lightly scratched and 30μL of PBS with 1% NMS (Control) or PE particles in PBS with 1% NMS at 2.82×10^9 particles/mL (PE only, and PE+CAPE) were placed onto the periosteum [40]. The incision was carefully stapled to ensure the particles remained in place and mice were allowed to recover [41]. At day 14 post-surgery mice were humanely killed and the calvaria were removed, as previously described [36, 42, 43].

5.2.4.Drug administration by subcutaneous injection

CAPE was purchased from Sigma–Aldrich (Sydney, Australia). To investigate the effect of CAPE on bone resorption, low dosage CAPE at 1mg/kg/day in PBS with 0.04% dimethyl sulfoxide (DMSO) (based on unpublished observations by J Xu) was administered to the mice in Group 3 at days 0, 4, 7 and 10 by subcutaneous injection over the shoulders, into the loose skin over the neck. Mice in Group 1 and 2 were subcutaneously injected with PBS with 0.04% DMSO vehicle control at the same time points.

5.2.5.Micro-computed tomography (μCT) imaging and selection of the volume of interest

Six-seven days prior to particle administration, mice were scanned *in vivo* using a live-animal micro-computed tomography (μCT) system (Skyscan model 1076, high resolution *in vivo* system, Skyscan, Bruker, Kontich, Belgium) to determine the bone volume (BV) levels at baseline. The scanning system settings were as follows: 48kV voltage, 110μA current, 0.5mm aluminium filter, field of view of 35mm×35mm, at 9μm isotropic pixel size and rotation step 0.8º. Mice were anaesthetized via an intraperitoneal injection (rat/mouse anaesthetic – 1ml xylazine, 2ml ketamine (100mg/ml), 17ml water in the injection (0.3ml for a 30g mouse)) for scanning, as previously described [44], with each scan taking an average of 24 minutes. Mice were then placed in a separate cage to allow recovery. On day 14 (completion of the study), mice were humanely killed and the heads

were skinned and an *ex vivo* μCT scan was performed with the same settings used during the *in vivo* scan.

The cross-section images were reconstructed using a filtered back-projection algorithm (NRecon software, V 1.12.04, Skyscan, Bruker, Kontich, Belgium) [44] and saved as 8-bit greylevel files (.BMP format). For each skull, a stack of up to 1800 cross-sections was reconstructed, with an inter-slice distance of 1 pixel corresponding to a maximum reconstructed height of 16.2mm (Figure 5.1), recreating the full length of the skull. Following uniform thresholding, which segmented the bone voxels as a solid, 3D models of the skulls were created (CT Analyser) and visualized (ParaView Software, V 3.1.2.0-RC2, New York, USA) (Figure 5.1).

For the subsequent quantitative analysis of the segmented μCT images, a rectangular region of interest (ROI) was selected, 4.35mm long (500 pixels), 5.22mm wide (600 pixels) and centred over the skull to include the area where the PE particles were implanted. This ROI was used over a stack of 220 μCT images of each skull (corresponding to 1.98 mm depth), forming a volume of interest (VOI) of size 4.35×5.22×1.98mm (length×width×depth) which included the entire thickness of the skull, producing a rectangular bone slab in 3D (Figure 5.1G-L). Over this VOI, quantitative μCT analysis was performed.

5.2.6.Percentage resorption area analysis via μCT

For the scans at day 14, for each segmented VOI (rectangular slab), 3D surface models were built (CT Analyser) and visualised in 3D (software Paraview). Two orthogonal screenshot images were taken, one 3D image visualizing the outer skull surface and one for the inner surface. Resorption areas, visible as darker crater-like regions in these images, had their boundaries contoured manually using a tablet (Bamboo, Wacom Co. 2009) and their areas of resorption filled (Adobe Photoshop Elements 7) [22]. The area of resorption was quantified (pixel counting) using ImageJ analysis software (Version 1.36b, National Institutes of Health, USA) [22]. The percentage area of resorption (%) was calculated as a fraction of the total area analyzed (the area of the ROI), for the inner and the outer surface of the calvariae separately. These values were averaged to represent the percentage of area resorbed for each animal.

5.2.7.Volumetric bone loss analysis via μCT

For each VOI, the BV (expressed in mm^3) was calculated as the volume occupied by the voxels segmented as 'bone' using CTAn software (Skyscan) [45]. For each animal, the change in BV (mm^3) over time was determined as BV at day 14 minus BV at baseline.

5.2.8.Biochemical analyses

Mice were deeply anaesthetized and blood was collected by cardiac puncture before cervical dislocation at the conclusion of the experiment. Following clotting and centrifugation, the serum was collected for analysis of bone resorption markers. Serum levels of CTX-1 and of OSCAR were measured by commercial ELISA (Nordic Bioscience, Denmark and Cusabio, Life Research, Australia, respectively), as per kit protocols.

5.2.9.Histologic evaluation by TRAP staining of the calvarial tissue

Following death, mouse heads were skinned and fixed in fixative 10% PBS-buffered formalin for 48 hours. Heads were decalcified in a pH 7.4 10% dehydrate disodium salt (EDTA) solution over 8 weeks. Decalcified heads were cut coronally into front and back regions and paraffin embedded. Five μm sections of calvariae were mounted on (3-Aminopropyltriethoxysilane 98% (APTS)-coated glass slides (Sigma-Aldrich) at the depth at which particles were detected morphologically within the calvarial tissue.

Tartrate-resistant acid phosphatase (TRAP) staining was performed to identify pre-osteoclasts/osteoclasts in the calvarial tissues [46, 47]. TRAP staining was performed using the napthol-based method [48]. The TRAP substrate was prepared according to previous methods [48, 49] and incubated with tissues at 37°C for 40 minutes. Tissues were counterstained in Harris' haematoxylin/lithium carbonate, cover-slipped using Aquatex (Merck) and viewed under a Nikon FXA Research light Microscope at ×10 magnification.

5.2.10.Histologic evaluation by H&E staining of the gastrointestinal tract (GIT)

The entire GIT was removed, contents were flushed with saline and small (1cm) sections of the jejunum and colon were fixed in 10% formalin for further analysis. Following

routine processing and embedding in paraffin wax, 4µm sections of jejunum and colon were cut using a rotary microtome. Sections were then stained using a standard Haematoxylin and Eosin Stain. Briefly, sections were dewaxed and re-hydrated through a graded series of ethanols before staining in haematoxylin for 2 minutes. Sections were differentiated in 1% hydrochloric acid and then counterstained with eosin for 2 minutes. After dehydration, sections were cleared in histolene, mounted and coverslipped [29, 50]. Analysis was conducted using a validated scoring system [50]. Briefly, the sections were analysed in a blinded fashion (RS) for identification of tissue structures and cell components including identification of apoptotic cells based on histopathology [51-53]. The number of apoptotic cells in the crypt and the crypt depth in the epithelium of jejunal and colonic mucosa, and the general structure of the jejunal and colonic mucosa including the presence of red blood cells, thickened blood vessels and crypt vesicles were analysed as per previous studies [53].

5.2.12.Statistics and data presentation

For all examined parameters data was presented as mean±standard error of the mean. To test for changes in BV between time points within each group, a Wilcoxon ranked signed test was used. To test for significant differences in percentage resorption area at day 14 and time-related changes in BV among the three groups, a Kruskal–Wallis test was used (GraphPad Prism 6 for Windows, Version 6.0.0.289, 2012), followed by a Mann-Whitney test on Control vs. PE only and PE only vs. PE+CAPE. Differences were deemed statistically significant for $p<0.05$.

5.3.Results

5.3.1.Visual assessment of osteolysis via histology and µCT

TRAP stained tissue (Figure 5.1A-F) showed the presence of fibrous granulomatous reaction accompanied by bone osteolysis in mice with PE, and higher number of TRAP positive cells were found in animals given PE compared to controls and CAPE-treated PE animals. PE particles in the tissues appeared bright when viewed under the polarized light (Figure 5.1D, F). In the PE implanted group, at day 14 the 3D µCT images (Figure 5.1G-I)

revealed the presence of craters reflecting resorptive regions on the skull surface, adjacent to the location where the particles were implanted. While there were resorption areas on the outer surface of the calvariae (Figure 5.1J-L), there were also craters visible on the inner surface of the calvariae (Figure 5.1M-O).

5.3.2.Quantitative assessment of bone resorption area via µCT

The outer and inner surfaces of the calvaria were assessed for resorbed regions (dark areas indicating bone loss) to indicate local direct effects of PE particles and CAPE treatment (Figure 5.2A, B). Compared to the control group, the PE implanted group exhibited significantly greater percentage resorption area at the outer ($p=0.0006$), inner ($p=0.0006$) and combined surfaces ($p=0.0022$) (Figure 5.2C). Compared to PE only group, the percentage resorption area was significantly reduced from 9.58% to 3.66%–in the PE+CAPE group at the outer ($p=0.0003$), inner ($p=0.0093$) and combined surfaces ($p=0.0012$) (Figure 5.2A-C respectively). Of note, the percentage of resorption area in the PE+CAPE group was not significantly different to the control ($p=0.0932$).

5.3.3.Quantitative changes in bone volume determined by µCT

As assessed by the Wilcoxon ranked signed test, the control ($p=0.0156$) and PE+CAPE ($p=0.0078$), showed significantly increased BV at the conclusion of the experiment compared to baseline, consistent with animal growth. The PE only group did not significantly increase in BV ($p=0.1094$). At day 14, the average increase in BV for the control group was $0.54\pm0.06 mm^3$. BV in the PE implanted group increased by only 0.15 ± 0.06 mm^3, which was significantly lower compared to the control ($p=0.0012$) and consistent with bone loss induced by PE particles (Figure 5.2D). In the PE+CAPE group, BV increased by $0.44\pm0.09 mm^3$ over time, which was significantly higher compared to the PE-only group ($p=0.0154$), and not significantly different from the control group ($p=0.3201$).

5.3.4.Murine serum levels of CTX-1

CTX-1 levels (Figure 5.3A) were measured as an indicator of systemic bone resorption. CTX-1 was significantly higher in the PE group ($p=0.0495$) when compared to the control

group. CAPE did not significantly (p=0.3916) alter PE induced CTX-1 levels when compared to the PE group.

5.3.5.Serum levels of soluble OSCAR in the PE calvarial model

Serum OSCAR levels (Figure 5.3B) were assessed as a potential regulator of osteolysis [24, 25]. Systemic OSCAR levels significantly increased in the PE implant group (p=0.0006) compared to the controls. CAPE did not affect the OSCAR levels in the PE-implanted group.

5.3.6.Gastrointestinal changes

The histopathology of the jejunum (Figure 5.4A-C) and colon (Figure 5.5A-C) was not significantly different between groups. No significant difference was detected between the three groups in jejunum (Figure 5.4D-E); crypt apoptosis p=0.9773, crypt depth p=0.2707, and colon (Figure 5.5D-E); crypt apoptosis p=0.1430, crypt depth p=0.4559.

5.4.Discussion

PE particles are one of the most frequent and biologically relevant sources of wear debris in clinical practice [54]. Additionally, they are a major driver of aseptic bone loss resulting in osteolysis [55] and implant failure. In the current study, PE particles significantly induced osteolysis in a mouse calvarial model, consistent with previous findings [6, 33, 34, 38, 40, 56]. In addition to the analysis of BV to investigate volumetric osteolysis [6], the current study extended previous analysis of this model by extensive analysis of the area of macroscopically visible calvarial surface bone resorption using the µCT-derived 3D images. This enabled quantification of the effects of PE and CAPE on the bone surface in these regions of macroscopically visible calvarial surface bone resorption using the µCT-derived 3D images. This study investigates the effects of PE at 2.82×10^9 particles/mL on bone by quantifying BV using live µCT. Baseline scans of the same animals are used for time-related comparisons, reducing the number of animals required. The current study found that PE particle implantation significantly reduced BV, and significantly increased percentage of resorptive area both on the outer and inner surfaces of the calvariae, when compared to the control. Levels of CTX-1 were significantly increased suggesting particles

perhaps travelled systemically and induced bone resorption elsewhere directly or indirectly via induction of cytokines [7]. This finding supported the reports that wear debris such as PE particles can enhance the pronounced osteoclastogenesis and subsequent osteolysis [57] locally [6] and systemically [7].

Natural compounds that inhibit NF-κB and NFAT have been shown to inhibit osteoclast activity *in vitro* and bone loss in LPS [11] and OVX (unpublished results from J Xu) murine models of osteolysis. Studies have shown that a propilis, CAPE, inhibited both the DNA-binding and transcriptional activity of NFAT [29] and NF-κB [27, 29] *in vitro*. CAPE blocked the induction of NFATc1 and c-Fos following RANKL stimulation [58]. CAPE inhibited NF-κB-dependent transcriptional activity without affecting the degradation of the cytoplasmic NF-κB inhibitory protein, Iκβα [29]. CAPE has also been shown to significantly inhibit the RANKL-induced osteoclast formation in mouse calvariae *in vivo* and the M-CSF and RANKL-induced osteoclast differentiation in dose-dependent manner *in vitro* mouse bone marrow-derived macrophages (BMMs) [58]. Further to this when CAPE was used to inhibit NF-□B and NFAT activation in RANKL-induced osteoclasts *in vitro*, osteoclast differentiation and resorption was impeded [59]. These findings indicate that CAPE is a potential therapeutic agent to inhibit osteoclast bone resorption and osteolysis and thus could potentially be used to prevent aseptic loosening.

Crucially in the current experiment, CAPE has been shown for the first time to significantly inhibit murine calvarial osteolysis induced by PE particles. CAPE significantly reduced the localized surface resorption area on the outer and inner surfaces of the calvariae in the PE+CAPE group, as well as significantly reduced the volumetric bone loss, restoring the levels of percentage resorptive area and BV change of the control group. This suggests the effectiveness of CAPE to prevent bone resorption medicated by PE particles likely due to its modulatory properties to abrogate osteoclastogenesis via NFATc1 and NF-□B signaling. Of note, CAPE did not reduce the systemic resorption induced in PE group as reflected by the serum levels of CTX-1.

Conflicting reports exist as to the levels of OSCAR in erosive disease such as RA. Decreased soluble OSCAR levels have been reported in active RA patients compared with healthy patients [24, 25]. Further to this, Zhao demonstrated that there is an inverse

relationship between RA and the presence of erosions, and hence soluble OSCAR has been proposed to be a potential regulator of osteoclast activity [25]. However a more recent finding found low levels in healthy individuals and high in active RA [60]. Our findings are consistent with this more recent report that in our murine model of osteolysis, OSCAR was increased in the serum of mice with PE-induced bone resorption. Of note, it is important to consider that in mice cell associated and possibly secreted OSCAR is limited to osteoclasts [61], whereas in humans it is expressed and potentially released by multiple cells types including dendritic cells, osteoclast and endothelial cells in the human [26, 62].

Additionally, in this study the effects of PE particles and CAPE treatment on the GIT were investigated. Although PE particles may travel elsewhere in the body and induce bone resorption systemically [7], this study found no effect on the gastrointestinal tract. Literature has suggested that CAPE at high dose may cause systemic toxicity, in particular reduced body weight as evidenced in a rat model of colitis [32]. CAPE has also been reported to induce apoptotic cell death in a dose-dependent fashion on a glucocorticoid-sensitive and –resistant cell line of lymphoid origin, increased leukocyte apoptosis and lead to a marked reduction in exudate leukocyte, neutrophil and monocyte concentrations at the inflammatory site in a rat model of carrageenan-induced subcutaneous inflammation [63]. Therefore it was a critical finding that that low doses of CAPE did not adversely affect the GIT.

5.5.Conclusion

PE particles significantly induced bone loss as assessed by increased bone surface resorption and local volumetric bone loss in a calvarial murine model of PE induced osteolysis. Additionally, although PE affected systemic bone resorption as indicated by CTX-1 levels, it did not affect GIT histopathology. Importantly an NF-κB and NFAT inhibitor, CAPE at low dose significantly reduced PE-induced surface bone resorption and local volumetric bone loss in the calvarial animal model. Low dose CAPE in the PE treated mice had inhibitory effect on bone resorption but did not adversely affect the GIT histopathology.

5.6.Acknowledgement

The work has been supported by a New Appointment Grant, Faculty of Health Sciences, The University of Adelaide. The Malaysian government (USM) provided support for M.S.F.Z.

5.7.References

1. Purdue PE, Koulouvaris P, Nestor BJ, Sculco TP (2006) The central role of wear debris in periprosthetic osteolysis. HSS journal : the musculoskeletal journal of Hospital for Special Surgery 2:102-113
2. Haynes DR, Crotti TN, Potter AE, Loric M, Atkins GJ, Howie DW, Findlay DM (2001) The osteoclastogenic molecules RANKL and RANK are associated with periprosthetic osteolysis. J Bone Joint Surg Br 83:902-911
3. Crotti TN, Smith MD, Findlay DM, Zreiqat H, Ahern MJ, Weedon H, Hatzinikolous G, Capone M, Holding C, Haynes DR (2004) Factors regulating osteoclast formation in human tissues adjacent to peri-implant bone loss: expression of receptor activator NFkappaB, RANK ligand and osteoprotegerin. Biomaterials 25:565-573
4. Crotti TN, Sharma SM, Fleming JD, Flannery MR, Ostrowski MC, Goldring SR, McHugh KP (2008) PU.1 and NFATc1 mediate osteoclastic induction of the mouse beta3 integrin promoter. J Cell Physiol 215:636-644
5. Crotti TN, Flannery M, Walsh NC, Fleming JD, Goldring SR, McHugh KP (2006) NFATc1 regulation of the human beta3 integrin promoter in osteoclast differentiation. Gene 372:92-102
6. Wedemeyer C, Xu J, Neuerburg C, Landgraeber S, Malyar NM, von Knoch F, Gosheger G, von Knoch M, Loer F, Saxler G (2007) Particle-induced osteolysis in three-dimensional micro-computed tomography. Calcif Tissue Int 81:394-402
7. Tuan RS, Lee FY, Y TK, Wilkinson JM, Smith RL (2008) What are the local and systemic biologic reactions and mediators to wear debris, and what host factors determine or modulate the biologic response to wear particles? J Am Acad Orthop Surg 16 Suppl 1:S42-48

8. Xu J, Wu HF, Ang ES, Yip K, Woloszyn M, Zheng MH, Tan RX (2009) NF-kappaB modulators in osteolytic bone diseases. Cytokine Growth Factor Rev 20:7-17
9. Jimi E, Ghosh S (2005) Role of nuclear factor-kappaB in the immune system and bone. Immunol Rev 208:80-87
10. Clohisy JC, Roy BC, Biondo C, Frazier E, Willis D, Teitelbaum SL, Abu-Amer Y (2003) Direct inhibition of NF-kappa B blocks bone erosion associated with inflammatory arthritis. J Immunol 171:5547-5553
11. Yip KH, Zheng MH, Feng HT, Steer JH, Joyce DA, Xu J (2004) Sesquiterpene lactone parthenolide blocks lipopolysaccharide-induced osteolysis through the suppression of NF-kappaB activity. J Bone Miner Res 19:1905-1916
12. Schwarz EM, Benz EB, Lu AP, Goater JJ, Mollano AV, Rosier RN, Puzas JE, Okeefe RJ (2000) Quantitative small-animal surrogate to evaluate drug efficacy in preventing wear debris-induced osteolysis. J Orthop Res. 18(6):849-855
13. Jimi E, Aoki K, Saito H, D'Acquisto F, May MJ, Nakamura I, Sudo T, Kojima T, Okamoto F, Fukushima H, Okabe K, Ohya K, Ghosh S (2004) Selective inhibition of NF-kappa B blocks osteoclastogenesis and prevents inflammatory bone destruction in vivo. Nat Med 10:617-624
14. Gordon AH, O'Keefe RJ, Schwarz EM, Rosier RN, Puzas JE (2005) Nuclear factor-kappaB-dependent mechanisms in breast cancer cells regulate tumor burden and osteolysis in bone. Cancer Res 65:3209-3217
15. Mitamura M, Nakano N, Yonekawa T, Shan L, Kaise T, Kobayashi T, Yamashita K, Kikkawa H, Kinoshita M (2007) T cells are involved in the development of arthritis induced by anti-type II collagen antibody. International immunopharmacology 7:1360-1368
16. Koga T, Inui M, Inoue K, Kim S, Suematsu A, Kobayashi E, Iwata T, Ohnishi H, Matozaki T, Kodama T, Taniguchi T, Takayanagi H, Takai T (2004) Costimulatory signals mediated by the ITAM motif cooperate with RANKL for bone homeostasis. Nature 428:758-763
17. Kim Y, Sato K, Asagiri M, Morita I, Soma K, Takayanagi H (2005) Contribution of nuclear factor of activated T cells c1 to the transcriptional control of

immunoreceptor osteoclast-associated receptor but not triggering receptor expressed by myeloid cells-2 during osteoclastogenesis. J Biol Chem 280:32905-32913

18. Kim K, Kim JH, Lee JF-J, Hye-Mi, Jin HM, Lee SH, Fisher DE, Kook H, Kim KK, Choi Y, Kim N (2005) Nuclear factor of activated T cells c1 induces osteoclast-associated receptor gene expression during tumor necrosis factor-related activation-induced cytokine-mediated osteoclastogenesis. J Biol Chem. 280(42):35209-35216
19. Ishikawa S, Arase N, Suenaga T, Saita Y, Noda M, Kuriyama T, Arase H, Saito T (2004) Involvement of FcRgamma in signal transduction of osteoclast-associated receptor (OSCAR). Int Immunol. 16(7):1019-1025
20. Takayanagi H (2007) The role of NFAT in osteoclast formation. Ann N Y Acad Sci. 1116:227-237.
21. Alias E, Dharmapatni ASSK, Holding AC, Atkins GJ, Findlay DM, Howie DW, Crotti TN, Haynes DR (2012) Polyethylene particles stimulate expression of ITAM-related molecules in peri-implant tissues and when stimulating osteoclastogenesis in vitro. Acta Biomaterialia
22. Zawawi MSF, Dharmapatni AA, Cantley MD, McHugh KP, Haynes DR, Crotti TN (2012) Regulation of ITAM adaptor molecules and their receptors by inhibition of calcineurin-NFAT signalling during late stage osteoclast differentiation. Biochem Biophysical Research Communication 427(2):404-409
23. Kim GS, Koh JM, Chang JS, Park BL, Kim LH, Park EK, Kim SY, Shin HD (2005) Association of the OSCAR promoter polymorphism with BMD in postmenopausal women. J Bone Miner Res 20:1342-1348
24. Herman S, Muller RB, Kronke G, Zwerina J, Redlich K, Hueber AJ, Gelse H, Neumann E, Muller-Ladner U, Schett G (2008) Induction of osteoclast-associated receptor, a key osteoclast costimulation molecule, in rheumatoid arthritis. Arthritis Rheum 58:3041-3050
25. Zhao S, Guo YY, Ding N, Yang LL, Zhang N (2011) Changes in serum levels of soluble osteoclast-associated receptor in human rheumatoid arthritis. Chin Med J (Engl) 124:3058-3060

26. Crotti TN, Dharmapatni AA, Alias E, Zannettino AC, Smith MD, Haynes DR (2012) The immunoreceptor tyrosine-based activation motif (ITAM) -related factors are increased in synovial tissue and vasculature of rheumatoid arthritic joints. Arthritis research & therapy 14:R245
27. Natarajan K, Singh S, Burke TRJR, Grunberger D, Aggarwal BB (1996) Caffeic acid phenethyl ester is a potent and specific inhibitor of activation of nuclear transcription factor NF-kappa B. Proc Natl Acad Sci U S A. 93(17):9090–9095
28. Ang ES, Pavlos NJ, Chai LY, Qi M, Cheng TS, Steer JH, Joyce DA, Zheng MH, Xu J (2009) Caffeic acid phenethyl ester, an active component of honeybee propolis attenuates osteoclastogenesis and bone resorption via the suppression of RANKL-induced NF-kappaB and NFAT activity. J Cell Physiol 221:642-649
29. Marquez N, Sancho R, Macho A, Calzado MA, Fiebich BL, Munoz E (2004) Caffeic acid phenethyl ester inhibits T-cell activation by targeting both nuclear factor of activated T-cells and NF-kappaB transcription factors. The Journal of pharmacology and experimental therapeutics 308:993-1001
30. Michaluart P, Masferrer JL, Carothers AM, Subbaramaiah K, Zweifel BS, Koboldt C, Mestre JR, Grunberger D, Sacks PG, Tanabe T, Dannenberg AJ (1999) Inhibitory effects of caffeic acid phenethyl ester on the activity and expression of cyclooxygenase-2 in human oral epithelial cells and in a rat model of inflammation. Cancer Res 59:2347-2352
31. Song JJ, Cho JG, Hwang SJ, Cho CG, Park SW, Chae SW (2008) Inhibitory effect of caffeic acid phenethyl ester (CAPE) on LPS-induced inflammation of human middle ear epithelial cells. Acta oto-laryngologica 128:1303-1307
32. Fitzpatrick LR, Wang J, Le T (2001) Caffeic acid phenethyl ester, an inhibitor of nuclear factor-kappaB, attenuates bacterial peptidoglycan polysaccharide-induced colitis in rats. J Pharmacol Exp Ther. 299(3):915-920.
33. Wedemeyer C, Neuerburg C, Pfeiffer A, Heckelei A, von Knoch F, Hilken G, Brankamp J, Henschke F, von Knoch M, Loer F, Saxler G (2007) Polyethylene particle-induced bone resorption in substance P-deficient mice. Calcif Tissue Int. 80(4):268-274

34. Wedemeyer C, Neuerburg C, Pfeiffer A, Heckelei A, Bylski D, von Knoch F, Schinke T, Hilken G, Gosheger G, von Knoch M, Loer F, Saxler G (2007) Polyethylene particle-induced bone resorption in alpha-calcitonin gene-related peptide-deficient mice. J Bone Miner Res 22:1011-1019
35. Kauther MD, Bachmann HS, Neuerburg L, Broecker-Preuss M, Hilken G, Grabellus F, Koehler G, von Knoch M, Wedemeyer C (2011) Calcitonin substitution in calcitonin deficiency reduces particle-induced osteolysis. BMC musculoskeletal disorders 12:186
36. von Knoch M, Sprecher C, Barden B, Saxler G, Löer F, M. W (2004) [Size and shape of commercially available polyethylene particles for in-vitro and in-vivo-experiments]. Z Orthop Ihre Grenzgeb. 142(3):366-370
37. Wedemeyer C, Neuerburg C, Pfeiffer A, Heckelei A, von Knoch F, Hilken G, Brankamp J, Henschke F, von Knoch M, Loer F, Saxler G (2007) Polyethylene particle-induced bone resorption in substance P-deficient mice. Calcif Tissue Int 80:268-274
38. Li Y, Zhang C, Zhou X, Wang H, Mao Y, Wang X (2013) Parthenolide inhibits polyethylene particle-induced mouse calvarial osteolysis in vivo. The Journal of surgical research
39. Zhou X, Zhang P, Zhang C, Zhu Z (2010) Promotion of bone formation by naringin in a titanium particle-induced diabetic murine calvarial osteolysis model. J Orthop Res 28:451-456
40. Landgraeber S, Jaeckel S, Loer F, Wedemeyer C, Hilken G, Canbay A, Totsch M, von Knoch M (2009) Pan-caspase inhibition suppresses polyethylene particle-induced osteolysis. Apoptosis : an international journal on programmed cell death 14:173-181
41. Merkel KD, Erdmann JM, McHugh KP, Abu-Amer Y, Ross FP, Teitelbaum SL (1999) Tumor necrosis factor-alpha mediates orthopedic implant osteolysis. Am J Pathol 154:203-210
42. von Knoch M, Wedemeyer C, Pingsmann A, von Knoch F, Hilken G, Sprecher C, Henschke F, Barden B, Loer F (2005) The decrease of particle-induced osteolysis after a single dose of bisphosphonate. Biomaterials 26:1803-1808

43. Zhou X, Zhang C, Wang X, An B, Zhang P, Zhu Z (2012) Berberine inhibits lipopolysaccharide- and polyethylene particle-induced mouse calvarial osteolysis in vivo. The Journal of surgical research 173:e47-52
44. Cantley MD, Bartold PM, Marino V, Reid RC, Fairlie DP, Wyszynski RN, Zilm PS, Haynes DR (2009) The use of live-animal micro-computed tomography to determine the effect of a novel phospholipase A2inhibitor on alveolar bone loss in anin vivomouse model of periodontitis. Journal of Periodontal Research 44:317-322
45. Perilli E, Le V, Ma B, Salmon P, Reynolds K, Fazzalari NL (2010) Detecting early bone changes using in vivo micro-CT in ovariectomized, zoledronic acid-treated, and sham-operated rats. Osteoporos Int 21:1371-1382
46. Gravallese E, Harada Y, Wang J, Gorn A, Thornhill T, Goldring S (1998) Identification of cell types responsible for bone resorption in rheumatoid arthritis and juvenile rheumatoid arthritis. The American journal of pathology 152:943-952
47. Shen Z, Crotti TN, McHugh KP, Matsuzaki K, Gravallese EM, Bierbaum BE, Goldring SR (2006) The role played by cell-substrate interactions in the pathogenesis of osteoclast-mediated peri-implant osteolysis. Arthritis Res Ther 8:R70
48. Burstone M (1958) Histochemical demonstration of acid phosphatases with naphthol AS-phosphates. Journal of the National Cancer Institute 21:523-539
49. Angel N, Walsh N, Forwood M, Ostrowski M, Cassady A, Hume D (2000) Transgenic mice overexpressing tartrate-resistant acid phosphatase exhibit an increased rate of bone turnover. Journal of bone and mineral research : the official journal of the American Society for Bone and Mineral Research 15:103-110
50. Logan RM, Gibson RJ, Bowen JM, Stringer AM, Sonis ST, Keefe DM (2008) Characterisation of mucosal changes in the alimentary tract following administration of irinotecan: implications for the pathobiology of mucositis. Cancer Chemother Pharmacol. 62(1):33-41
51. Gibson RJ, Bowen JM, Alvarez E, Finnie J, Keefe DM (2007) Establishment of a single-dose irinotecan model of gastrointestinal mucositis. Chemotherapy. 53(5):360-369

52. Gibson RJ, Bowen JM, Keefe DM (2005) Palifermin reduces diarrhea and increases survival following irinotecan treatment in tumor-bearing DA rats. Int J Cancer. 116(3):464-470
53. Gibson RJ, Bowen JM, Inglis MR, Cummins AG, Keefe DM (2003) Irinotecan causes severe small intestinal damage, as well as colonic damage, in the rat with implanted breast cancer. J Gastroenterol Hepatol 18(9):1095-1100
54. Kurtz SM, Ok M, M E, Edidin AA (1999) Advances in the processing, sterilization, and crosslinking of ultra-high molecular weight polyethylene for total joint arthroplasty. Biomaterials 20(18):1659-1688
55. Atkins GJ, Haynes DR, Howie DW, Findlay DM (2011) Role of polyethylene particles in peri-prosthetic osteolysis: A review. World journal of orthopedics 2:93-101
56. Nich C, Rao AJ, Valladares RD, Li C, Christman JE, Antonios JK, Yao Z, Zwingenberger S, Petite H, Hamadouche M, Goodman SB (2012) Role of direct estrogen receptor signaling in wear particle-induced osteolysis. Biomaterials
57. Goldring SR, Schiller AL, Roelke M, Rourke CM, O'Neil DA, Harris WH (1983) The synovial-like membrane at the bone-cement interface in loose total hip replacements and its proposed role in bone lysis. J Bone Joint Surg Am 65(5):575-584
58. Ha J, Choi HS, Lee Y, Lee ZH, Kim HH (2009) Caffeic acid phenethyl ester inhibits osteoclastogenesis by suppressing NF kappaB and downregulating NFATc1 and c-Fos. International immunopharmacology 9:774-780
59. Ang E, Pavlos NJ, Rea SL, Qi M, Chai T, Walsh JP, Ratajczak T, Zheng MH, Xu J (2009) Proteasome inhibitors impair RANKL-induced NF-kappaB activity in osteoclast-like cells via disruption of p62, TRAF6, CYLD, and IkappaBalpha signaling cascades. J Cell Physiol 220:450-459
60. Ndongo-Thiam N, de Sallmard G, Kastrup J, Miossec P (2014) Levels of soluble osteoclast-associated receptor (sOSCAR) in rheumatoid arthritis: link to disease severity and cardiovascular risk. Ann Rheum Dis. 73(6):1276-1277

61. Kim N, Takami M, Rho J, Josien R, Choi Y (2002) A novel member of the leukocyte receptor complex regulates osteoclast differentiation. J Exp Med 195:201-209
62. Nemeth K, Schoppet M, Al-Fakhri N, Helas S, Jessberger R, Hofbauer LC, Goettsch C (2011) The role of osteoclast-associated receptor in osteoimmunology. J Immunol. 186(1):13-18
63. Orban Z, Mitsiades N, Burke TRJ, Tsokos M, Chrousos GP (2000) Caffeic acid phenethyl ester induces leukocyte apoptosis, modulates nuclear factor-kappa B and suppresses acute inflammation. Neuroimmunomodulation. 7(2):99-105

5.8.Figure legends

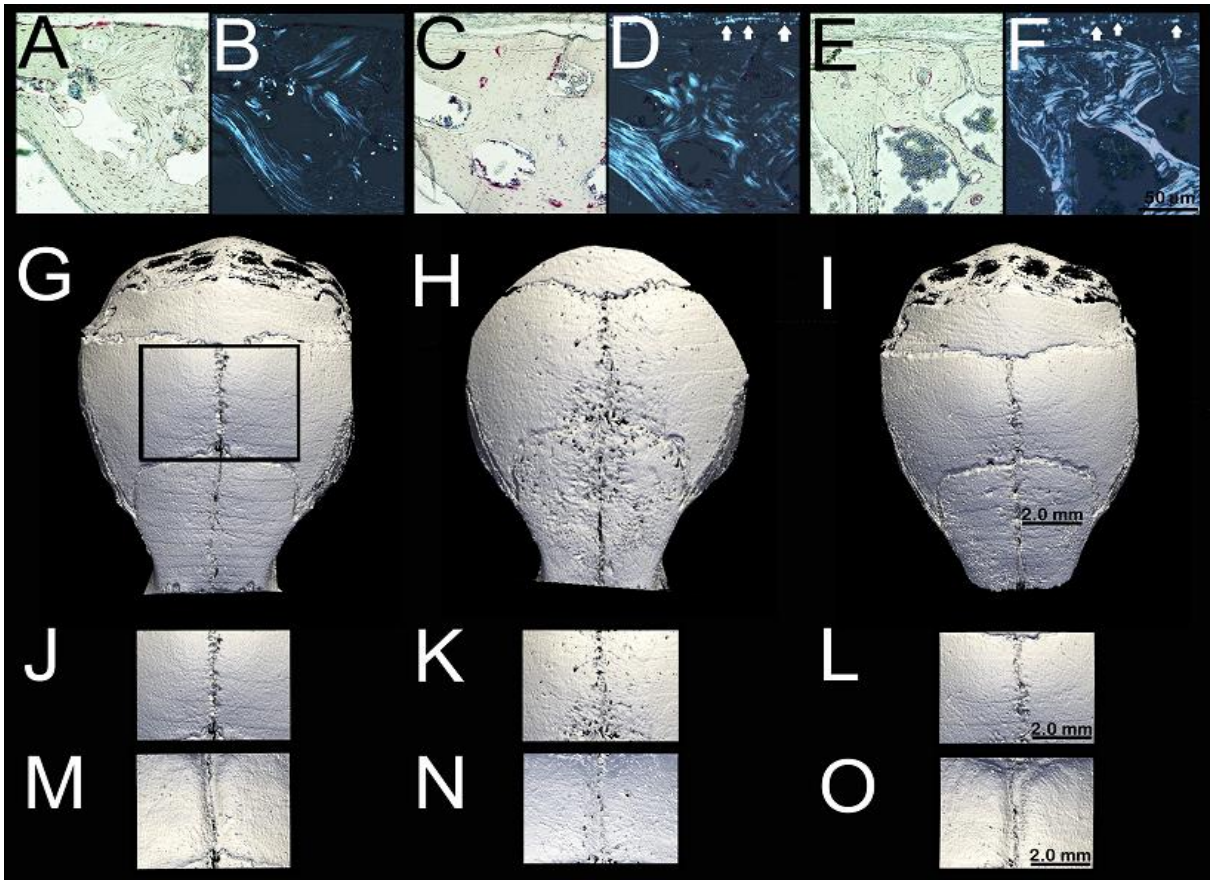

Figure 5.1

(A, B, G, J, M) Control. (C, D, H, K, N) PE only. (E, F, I, L, O) PE+CAPE. (A-F) Calvarial tissue sections stained with TRAP, with the periosteal surface found at the top of the image, and the midsagittal sutures or their remnants are located on the right hand-side of images. Magnification: ×100. (A, C, E) TRAP+ve cells stained red. (B, D F) Tissues viewed under polarized light, and the PE particles appeared bright as shown by arrows. (G-I) Three-dimensional μCT images of mouse calvariae at day 14. (G) Location of ROI (length×width, 4.35×5.22mm) depicted by black box. (J-O) Rectangular ROI images (length×width, 4.35×5.22mm) selected for quantitative analysis over the calvariae. (J-L) View of the outer surface and (M-O) of the inner surface of the ROI. (H) The resorption area on the surface of the calvariae of the PE implanted group are visible whilst (I) in the CAPE-treated PE group have been abrogated.

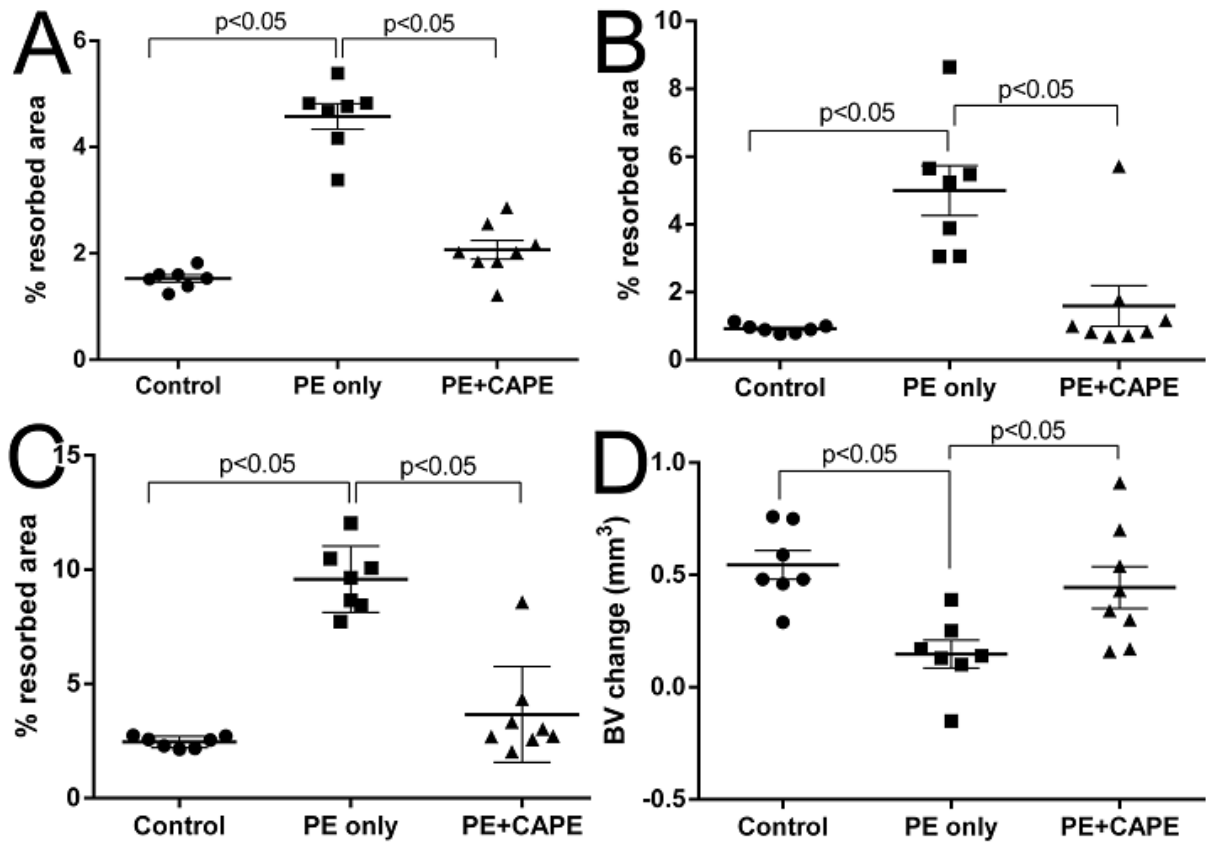

Figure 5.2

Quantitative μCT analysis within the rectangular ROI selected (see Figure 5.1) at day 14, percentage resorption bone area for (A) the outer surface of the calvariae, (B) the inner surface of the calvariae, (C) combined outer and inner surfaces of the calvariae, (D) Change in bone volume (BV) compared to baseline. The values are presented as mean±S.E. Significance was considered as $p<0.05$.

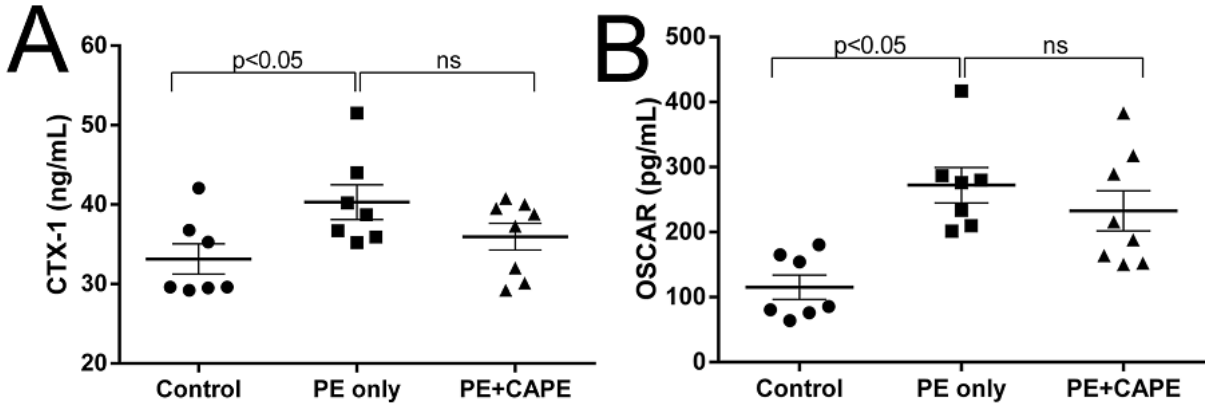

Figure 5.3

Biochemical analyses of the murine serum in a particle induced murine model of osteolysis. (A) CTX-1 concentration as an indicator of systemic bone resorption. (B) Soluble OSCAR levels as a potential modulator of osteoclast activity. The values are presented as mean±S.E. Significance was considered as $p<0.05$.

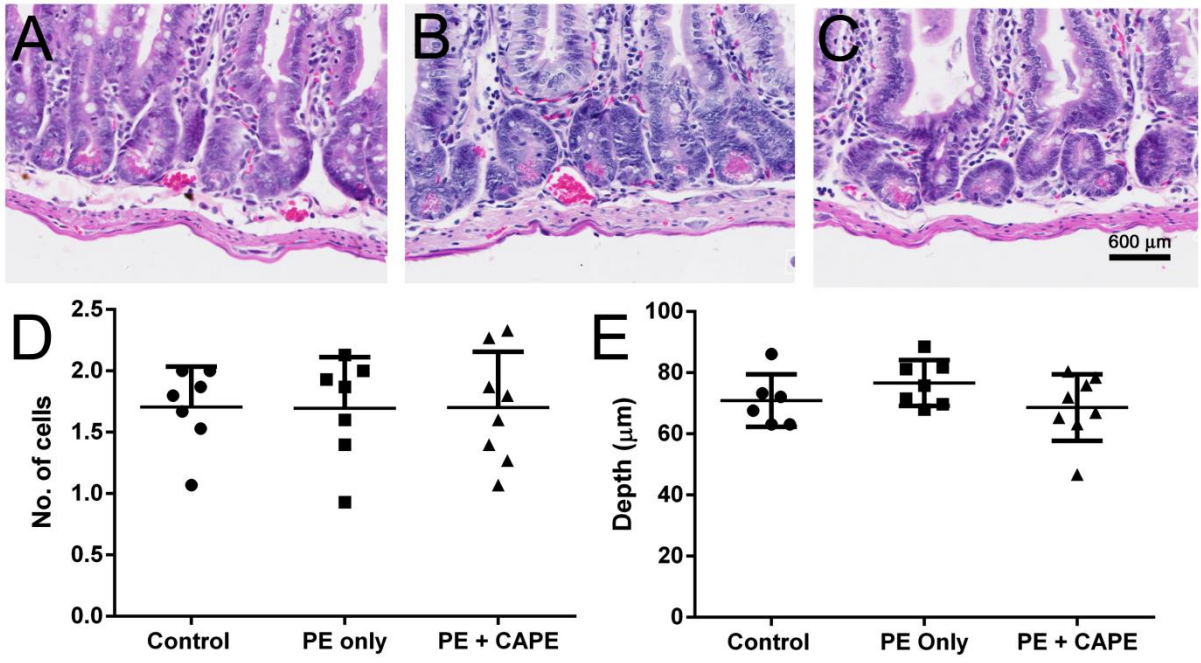

Figure 5.4

Morphological analysis of the jejunum by H&E staining. Magnification ×300. (A) Control. (B) PE only. (C) PE+CAPE (D) Number of jejunul crypt apoptosis. (E) Jejunul crypt depth.

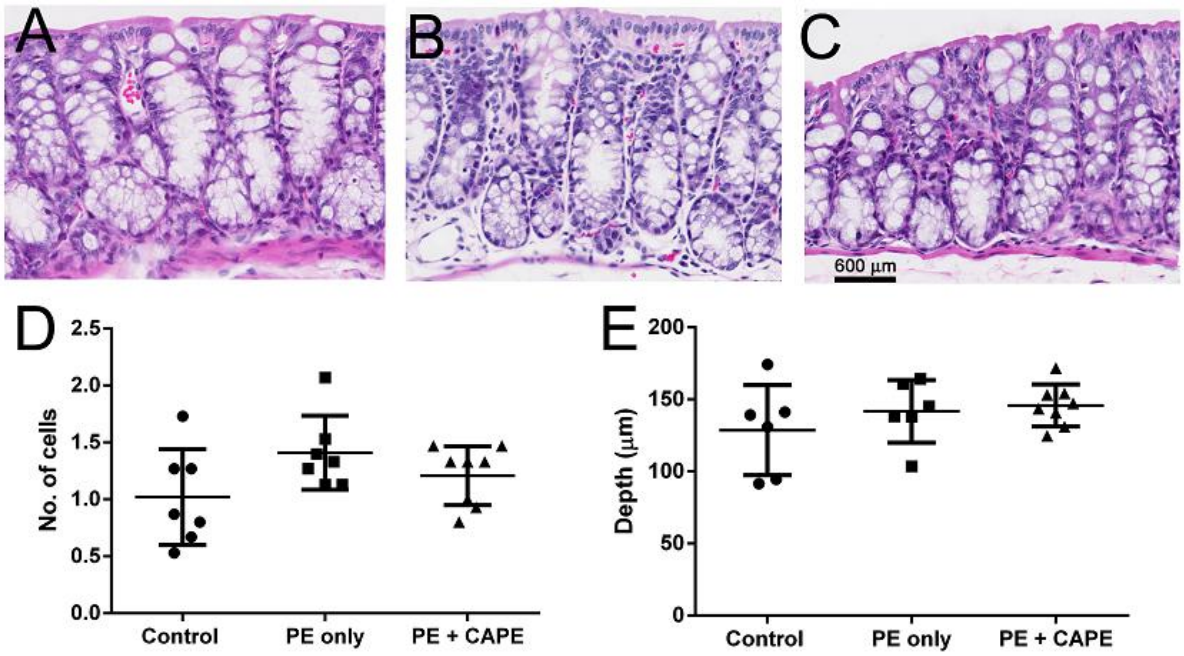

Figure 5.5

Morphological analysis of the colon by H&E staining. Magnification ×300. (A) Control. (B) PE only. (C) PE+CAPE. (D) Number of jejunul crypt apoptosis. (E) Jejunul crypt depth.

CHAPTER 6: Discussion

6.1.Discussion

Particle-induced osteolysis is a major cause of aseptic loosening and subsequent implant failure following total joint arthroplasty. Following phagocytosis of wear debris macrophages and FBGCs release the osteoclastic pro-inflammatory mediators, including RANKL, TNFα and IL-1 [1-4], that increase recruitment of osteoclast precursor cells into tissues around the prostheses and enhance osteoclast differentiation [5-7]. Thus chronic inflammation within the tissues [8-12] induces excessive osteoclast bone resorption which is largely responsible for particle-induced peri-prosthetic osteolysis and subsequent loosening of the prosthesis [13]. It has been shown that particles regulate the expression of osteoclast-associated genes including NFATc1 [14], NF-κB [15], RANKL and OPG [8, 9, 16], as well as ITAM-related molecules [17]. Hence, investigation of factors regulating osteoclastogenesis and their regulation by wear particles will help us better understand the pathology of particle-induced peri-prosthetic osteolysis.

As shown in normal osteoclastogenesis [18-20] and particle-induced peri-prosthetic osteolysis [17], NFATc1 and NF-κB play key roles to enhance RANKL-mediated osteoclast formation and differentiation [17-20]. In addition to this, the ITAM-dependent pathway provides co-stimulatory signals that stimulate calcium signals to activate the calcineurin [21], thereby enhancing NFATc1 expression via a positive feedback loop [19, 20]. Supporting this concept, calcineurin-NFAT inhibitors including FK506 [18, 22] and VIVIT [23, 24] have been demonstrated to suppress osteoclastogenesis during pathological bone loss. Consistent with previous publications, the present study (Chapter 2) confirmed that FK506 [25] and VIVIT [26] inhibit RANKL-induced osteoclast differentiation, as assessed by number of multinucleated cells expressing TRAP, and osteoclast resorption activity *in vitro*. To date NC100 has not been studied in regard to its effects on osteoclastogenesis but its anti-cancer or anti-inflammatory activities are well established through its regulation of NF-κB [27-30]. Here, for the first time the inhibitory effect of NC100 on osteoclast formation and resorption activity in normal human PBMC- and BMM-derived osteoclast cultures *in vitro* is demonstrated (Chapter 3).

The effects of FK506 or VIVIT on osteoclast-associated gene expression during osteoclastogenesis have been shown *in vitro* [25] and *in vivo* [26]. In this study (Chapter

2), we found FK506 significantly reduced both CathK and NFATc1 expression in RANKL-induced osteoclast cultures. This is consistent with the previous reports demonstrating a reduction in NFATc1 expression by FK506 [25]. VIVIT has been shown to inhibit CathK and NFATc1 expression in a murine model of Ti particleinduced osteoclast formation [26]. However, VIVIT in this study (Chapter 2) significantly reduced CathK but not NFATc1 expression in normal PBMC-derived human osteoclast development. In addition to the inhibition of osteoclast formation and resorption *in vitro* (Chapter 3), NC100 suppressed osteoclast marker genes, CathK and NFATc1. This indicates NC100 takes its effect through the regulation of NFATc1 and NF-κB *in vitro*. NC100 also inhibited murine OVX-induced bone loss *in vivo* through NF-κB and NFATc1 inhibition (Chapter 3).

Collectively, various studies [18, 25, 26], including those presented here (Chapters 2 and 3), demonstrate that inhibition of NFATc1 plays an essential role in RANKL-induced osteoclast differentiation and resorption. In addition to regulating NFATc1 signalling in osteoclasts, other studies [31, 32] and ours (Chapter 3) found that inhibition of RANKL-induced NF-κB activation also inhibits osteolysis. Over the past few years studies have investigated several natural compounds that have inhibitory effects on osteoclast formation and bone resorption through suppression of RANKL-induced NF-κB activation [31, 33-38]. These include two natural inhibitors being investigated in this thesis: PAR (Chapter 4) which inhibited LPS-induced osteolysis in mice through NF-κB suppression [35] and CAPE (Chapter 5) which suppressed osteoclastogenesis *in vitro* [36, 39]. CAPE also inhibited OVX-induced bone loss in a murine model through NF-κB and NFATc1 inhibition [39].

In the context of particle-induced peri-prosthetic osteolysis, PE wear rate has been shown to correlate with enhanced peri-prosthetic osteolysis and subsequent implant failure [10, 40-44]. A murine calvarial model has been developed to investigate the osteoclast-associated cellular responses to the exposure to PE particles in this thesis, similar to that used by others [45-50]. The findings presented here were the first μCT studies to calculate volumetric changes overtime after administration of PE particles in a murine calvarial model. The use of live animal μCT allows us to use each animal as its own control thus reducing number of animals required and increasing the accuracy of measurement of bone

loss (Chapters 4 and 5). The bone volumetric measurements (BV and BV/TV) were determined over the VOI which encompasses the entire skull thickness, from the internal to external surface (3D slab) [51]. In the current study, PE particles at day 14 post-surgery significantly induced osteolysis when compared to the control (Chapters 4 and 5), consistent with numerous previous analyses of the *ex vivo* μCT scans at final day of experiments [46, 48-50, 52, 53].

Previously, PAR and CAPE have been shown to inhibit NF-κB [31, 35] and both NF-κB and NFATc1 [36, 54-57], respectively, and both were shown to suppress osteoclastogenesis and osteoclast resorption *in vivo*. This demonstrates the therapeutic potential of PAR and CAPE for the treatment of disease where excessive osteoclast bone resorption occurs, such as aseptic loosening. In our murine calvarial model of PE-induced peri-prosthetic osteolysis, PAR has been shown to not significantly affecting BV after 14 days when compared to the baseline (Chapter 4). This contradicted a previous finding which showed PE-given mice receiving daily treatment of 1mg/kg PAR for 7 days post-surgery had a significant reduction in bone volume fraction (BV/TV) measurements at day 7 [53]. However, the findings suggest PAR is able to prevent volumetric PE-induced osteolysis at the earlier stage (day 7) rather than the late stage (day 14) of peri-prosthetic osteolysis. Interestingly CAPE significantly reduced the volumetric bone loss at day 14 when compared to the baseline. This was consistent with the fact that CAPE inhibited osteoclast differentiation *in vitro* [36] and osteoporotic bone loss in mice [57].

Many studies including ours (Chapters 4 and 5) have shown volumetric effects of PE particles on bone, but there is no report quantifying the bone surface resorption. We contend that the surface erosions are highly important as these better reflect the *in vivo* situation in human as numerous studies have shown that resorption near implants occurs on the bone surface at the interface [58] and at sites with high concentrations of PE wear particles in the soft tissues [10]. Our studies included extensive quantitative analysis of the calvarial surface bone resorption using the μCT-derived images of the region of interest of the skull (Chapters 4 and 5). This enabled measurement of significantly increased resorptive area both on the outer and inner surfaces of the calvariae when compared to the control (Chapters 4 and 5). The osteolysis induced of both internal and external surface erosion suggests that the particles can induce resorption at a distance. This is most likely

due to diffusion of mediators induced by PE particles stimulating osteoclast activity into the bone marrow. This may also occur within the bone and on the trabeculae.

Importantly, NF-κB inhibition by PAR (Chapter 4) and NF-κB and NFATc1 inhibition by CAPE (Chapter 5) treatment significantly reduced this surface resorption on the outer and inner surfaces of the calvariae in PE-given mice. This suggests that PAR and CAPE can focally reduce bone surface resorption adjacent to the implanted PE particles (Chapters 4 and 5). Further work is necessary to identify why at day 14 PAR affects only PE-induced calvarial surface erosions but not volume (Chapter 4). It is possible this is due to the different mechanisms of bone erosion occurring at the surface and within bone, and these have different sensitivities to PAR. This thesis showed for the first time that CAPE significantly reduce the localized surface resorption area on the outer and inner surfaces of the calvariae in the PE-given mice (Chapter 5). Together with the significant reduction in the volumetric bone loss, the ability of CAPE to restore normal levels of surface resorptive area and BV may be due to the fact that CAPE can prevent PE-induced bone resorption via both NFATc1 and NF-κB signalling (Chapter 5).

There are multiple reports of particulate wear debris in the serum, urine and end-organs in patients [59-64]. Thus, it is important to investigate whether PE particles can affect bone resorption systemically. CTX-1 levels, a good indicator of systemic bone loss [65], were significantly increased in PE-given mice (Chapters 4 and 5) suggesting particles either directly or indirectly induce bone loss possibly via the movement of particles or cytokines [66]. Our findings in Chapters 4 and 5 supported previous reports that wear debris can enhance osteoclastogenesis and subsequent osteolysis [67] locally [50] and systemically [66]. Notably while we found PAR and CAPE treatment reduced CTX-1 levels, the differences were not significant when compared to mice not given PE (Chapters 4 and 5).

Although PE particles appeared to increase systemic osteolysis (Chapters 4 and 5), we found no significant systemic effects of PE particles on the colon and jejunum of GIT (Chapter 5). Previous findings have shown CAPE at a high dose caused systemic toxicity and reduced body weight in a rat model of colitis [68]. CAPE also can induce apoptotic cell death in a dose-dependent fashion significantly reducing leukocyte, neutrophil and monocyte concentrations at the inflammatory site in a rat model of carrageenan-induced subcutaneous inflammation [69]. Therefore, it was important to determine the effects of

CAPE during treatment and it was found that it did not adversely affect the GIT (Chapter 5). An unexpected result was that, in the absence of particles, PAR increased CTX-1 levels (Chapter 4), suggesting CAPE can affect normal bone metabolism.

Consistent with previous findings [22, 25], ITAM related molecules are strongly expressed during the late stages of osteoclastogenesis *in vitro* (Chapters 2 and 3). The expression of ITAM adaptor molecules, FcRγ and DAP12 was significantly reduced by FK506, whereas, VIVIT significantly reduced FcRγ but not DAP12 (Chapter 2). To our knowledge, NFATc1 has not been shown to directly induce DAP12 and this is consistent with the lack of inhibition with the more specifically acting inhibitor VIVIT. In a study using murine BMM-derived osteoclasts OSCAR, but not TREM2 expression, was inhibited by FK506 [22]. Importantly, our studies demonstrated that both TREM and OSCAR were inhibited by FK506 in the late stage of human PBMC-derived osteoclast formation (Chapter 2). Interestingly, we noted that VIVIT suppressed OSCAR but not TREM2 expression (Chapter 2). This independent regulation of OSCAR and TREM2 was consistent with promoter studies demonstrating direct induction of OSCAR, but not TREM2, by NFATc1 [22]. This may be because VIVIT inhibits calcineurin-mediated dephosphorylation of NFATs without affecting calcineurin phosphatase signalling and non-NFAT mediated signalling. In contrast, FK506 acts further upstream, more broadly affecting both calcineurin phosphatase signalling and non-NFAT mediated signalling [23, 70]. In this study (Chapter 3) for the first time we reported that NC100 significantly reduced the expression of OSCAR, TREM2, FcRγ and DAP12 during late stage osteoclast differentiation.

Soluble OSCAR has been proposed as a regulator of osteoclast activity in osteolysis in RA [71, 72]. It has been suggested that there is an inverse relationship in RA with the presence of erosions [72] corresponding to lower levels of soluble OSCAR in active RA patients compared with healthy patients [71, 72]. We found almost undetectable levels of serum OSCAR in patients with end-stage wear-induced osteolysis as well as in primary osteoarthritis patients (Chapter 4). Importantly, in our mouse model of osteolysis, higher levels of soluble OSCAR were found in the serum of mice with PE-induced bone resorption when compared to control (Chapters 4 and 5). Our findings were consistent with a recent finding showing higher levels of OSCAR in active RA patients compared to

healthy individuals [73]. In mice, OSCAR was only associated with osteoclasts [19], whereas, in humans OSCAR can be expressed by multiple cells types [74, 75] including dendritic cells [76], osteoclasts [19] and endothelial cells [77]. Together these data strengthen the idea that modulation of osteoclastogenesis through inhibition of NF-κB/NFATc1 regulate ITAM-associated molecule OSCAR in osteolysis (Chapters 4 and 5) further supporting our *in vitro* findings in normal human osteoclast cultures (Chapters 2 and 3).

It has been suggested that osteocytes may also contribute to the development of particle-induced osteolysis. Osteocytes may regulate peri-prosthetic osteolysis via apoptotic signals [78, 79]. As osteocytes have been shown to undergo apoptosis in response to wear particles *in vitro* [80] as well as surface fatigue-induced microdamage [78], resulting in bone containing the dead osteocytes being resorbed by osteoclasts. Consistent with this, the current study found PE particles significantly increased osteocyte death as assessed by empty lacunae *in vivo* (Chapter 4). Additionally, PE particles can directly induce osteocytes to produce RANKL, thus directly stimulating particle-induced osteolysis [81]. This may explain the significant resorption observed on the bone surface around the location of the PE particles as well as the bone volumetric changes (Chapters 4 and 5). A recent report found TNF-α enhanced sclerostin expression in an NF-κB-dependent manner in the osteocyte cell line, MLO-Y4, indicating NF-κB could have a role in osteocyte functions [82]. It is possible PE particles induce NF-κB in osteocytes, thus inducing osteolysis, and this merits further study.

In Chapter 4 it was shown that PAR significantly reduced numbers of dead osteocytes and surface osteolysis indicating that these may be related as discussed above. Likewise, PAR treatment resulted in a significant reduction in PE-induced bone surface resorption as assessed by internal and external surface resorption but not bone volume resorption (Chapter 4). The ability of PAR to inhibit surface erosion induced by PE particles is important as surface erosion may better reflect the *in vivo* situation where bone is resorbed on the surface at the bone-implant interface. This may be related to the role of osteocytes in this pathology discussed above. CAPE treatment significantly reduced both PE-induced bone surface resorption and reduction in bone volume (Chapter 5). It would be valuable to further investigate the mechanisms behind the different effects of PAR and CAPE

treatments. In particular, whether osteocyte death is regulated by NF-κB and if inhibition of NF-κB/NFATc1 by CAPE could also result in reduced osteocyte death similar to inhibition of NF-κB by PAR. Overall, the data presented in this thesis demonstrates osteocytes are likely to have an important role in PE-induced osteolysis.

As more specific inhibitors of NF-κB and NFATc1 are developed, more studies will be carried out on the effects of this type of regulation of osteoclastogenesis and bone resorption. This is likely to identify a novel therapeutic approach to combat inflammation-mediated bone loss including particle-induced peri-prosthetic osteolysis.

6.2.References

[1] Baumann B, Rader CP, Seufert J, Noth U, Rolf O, Eulert J. Effects of polyethylene and TiAlV wear particles on expression of RANK, RANKL and OPG mRNA. Acta Orthop Scand 2004;75:295-302.

[2] Rader CP, Sterner T, Jakob F, Schutze N, Eulert J. Cytokine response of human macrophage-like cells after contact with polyethylene and pure titanium particles. J Arthroplasty 1999;14(7):840-8.

[3] Ishiguro N, Kojima T, Ito T, Saga S, Anma H, Kurokouchi K, et al. Macrophage activation and migration in interface tissue around loosening total hip arthroplasty components. Journal of Biomedical Materials Research 1997;35:399-406.

[4] Matthews JB, Green TR, Stone MH, Wroblewski BM, Fisher J, Ingham E. Comparison of the response of three human monocytic cell lines to challenge with polyethylene particles of known size and dose. J Mater Sci Mater Med 2001;12(3):249-58.

[5] Kadoya Y, Revell PA, al-Saffar N, Kobayashi A, Scott G, Freeman MA. Bone formation and bone resorption in failed total joint arthroplasties: histomorphometric analysis with histochemical and immunohistochemical technique. J Orthop Res 1996;14(3):473-82.

[6] Eftekhar NS, Doty SB, Johnston AD, Parisien MV. Prosthetic synovitis. Hip 1985:169-83.

[7] Greenfield EM, Bi Y, Ragab AA, Goldberg VM, Van De Motter RR. The role of osteoclast differentiation in aseptic loosening. J Orthop Res 2002;20(1):1-8.

[8] Haynes DR, Crotti TN, Loric M, Bain GI, Atkins GJ, Findlay DM. Osteoprotegerin and receptor activator of nuclear factor kappaB ligand (RANKL) regulate osteoclast formation by cells in the human rheumatoid arthritic joint. Rheumatology 2001;40:623-30.
[9] Haynes DR, Crotti TN, Potter AE, Loric M, Atkins GJ, Howie DW, et al. The osteoclastogenic molecules RANKL and RANK are associated with periprosthetic osteolysis. J Bone Joint Surg Br 2001;83:902-11.
[10] Holding CA, Findlay DM, Stamenkov R, Neale SD, Lucas H, Dharmapatni AS, et al. The correlation of RANK, RANKL and TNFalpha expression with bone loss volume and polyethylene wear debris around hip implants. Biomaterials 2006;27:5212-9.
[11] Shen Z, Crotti TN, McHugh KP, Matsuzaki K, Gravallese EM, Bierbaum BE, et al. The role played by cell-substrate interactions in the pathogenesis of osteoclast-mediated peri-implant osteolysis. Arthritis Res Ther 2006;8:R70.
[12] Howie DW. Tissue response in relation to type of wear particles around failed hip arthroplasties. J Arthroplasty 1990;5(4):337-48.
[13] Purdue PE, Koulouvaris P, Nestor BJ, Sculco TP. The central role of wear debris in periprosthetic osteolysis. HSS journal : the musculoskeletal journal of Hospital for Special Surgery 2006;2:102-13.
[14] Haynes DR, Crotti TN, Zreiqat H. Regulation of osteoclast activity in peri-implant tissues. Biomaterials 2004;25:4877-85.
[15] Nakashima Y, Sun DH, Trindade MC, Maloney WJ, Goodman SB, Schurman DJ, et al. Signaling pathways for tumor necrosis factor-alpha and interleukin-6 expression in human macrophages exposed to titanium-alloy particulate debris in vitro. J Bone Joint Surg Am 1999;81(5):603-15.
[16] Crotti TN, Smith MD, Findlay DM, Zreiqat H, Ahern MJ, Weedon H, et al. Factors regulating osteoclast formation in human tissues adjacent to peri-implant bone loss: expression of receptor activator NFkappaB, RANK ligand and osteoprotegerin. Biomaterials 2004;25:565-73.
[17] Alias E, Dharmapatni ASSK, Holding AC, Atkins GJ, Findlay DM, Howie DW, et al. Polyethylene particles stimulate expression of ITAM-related molecules in peri-implant tissues and when stimulating osteoclastogenesis in vitro. Acta Biomaterialia 2012;8(8):3104-12.

[18] Hirotani H, Tuohy NA, Woo JT, Stern PH, Clipstone NA. The calcineurin/nuclear factor of activated T cells signaling pathway regulates osteoclastogenesis in RAW264.7 cells. J Biol Chem 2004;279:13984-92.

[19] Kim N, Takami M, Rho J, Josien R, Choi Y. A novel member of the leukocyte receptor complex regulates osteoclast differentiation. J Exp Med 2002;195(2):201-9.

[20] Kim K, Kim JH, Lee JF-J, Hye-Mi, Jin HM, Lee SH, Fisher DE, et al. Nuclear factor of activated T cells c1 induces osteoclast-associated receptor gene expression during tumor necrosis factor-related activation-induced cytokine-mediated osteoclastogenesis. J Biol Chem 2005;280(42):35209-16.

[21] Blair HC, Robinson LJ, Zaidi M. Osteoclast signalling pathways. Biochem Biophys Res Commun 2005;328:728-38.

[22] Kim Y, Sato K, Asagiri M, Morita I, Soma K, Takayanagi H. Contribution of nuclear factor of activated T cells c1 to the transcriptional control of immunoreceptor osteoclast-associated receptor but not triggering receptor expressed by myeloid cells-2 during osteoclastogenesis. J Biol Chem 2005;280:32905-13.

[23] Aramburu J, Yaffe MB, Lopez-Rodriguez C, Cantley LC, Hogan PG, Rao A. Affinity-driven peptide selection of an NFAT inhibitor more selective than cyclosporin A. Science 1999;285:2129-33.

[24] Crotti TN, Flannery M, Walsh NC, Fleming JD, Goldring SR, McHugh KP. NFATc1 regulation of the human beta3 integrin promoter in osteoclast differentiation. Gene 2006;372:92-102.

[25] Miyazaki M, Fujikawa Y, Takita C, Tsumura H. Tacrolimus and cyclosporine A inhibit human osteoclast formation via targeting the calcineurin-dependent NFAT pathway and an activation pathway for c-Jun or MITF in rheumatoid arthritis. Clin Rheumatol 2007;26:231-9.

[26] Liu F, Zhu Z, Mao Y, Liu M, Tang T, Qiu S. Inhibition of titanium particle-induced osteoclastogenesis through inactivation of NFATc1 by VIVIT peptide. Biomaterials 2009;30:1756-62.

[27] Del Poeta M, Chen S-F, Von Hoff D, Dykstra CC, Wani MC, Manikumar G, et al. Comparison of In Vitro Activities of Camptothecin and Nitidine Derivatives against Fungal and Cancer Cells. Antimicrobial Agents and Chemotherapy 1999;43:2862-8.

[28] García D, Delgado R, Ubeira FM, Leiro J. Modulation of rat macrophage function by the Mangifera indica L. extracts Vimang and mangiferin. International immunopharmacology 2002;2:797-806.
[29] Prado S, Michel S, Tillequin F, Koch M, Pfeiffer B, Pierré A, et al. Synthesis and cytotoxic activity of benzo[c][1,7] and [1,8]phenanthrolines analogues of nitidine and fagaronine. Bioorganic & Medicinal Chemistry 2004;12:3943-53.
[30] Sánchez GM, Re L, Giuliani A, Núñez-Sellés AJ, Davison GP, León-Fernández OS. Protective effects of Mangifera indica L. extract, mangiferin and selected antioxidants against TPA-induced biomolecules oxidation and peritoneal macrophage activation in mice. Pharmacological Research 2000;42:565-73.
[31] Xu J, Wu HF, Ang ES, Yip K, Woloszyn M, Zheng MH, et al. NF-kappaB modulators in osteolytic bone diseases. Cytokine Growth Factor Rev 2009;20:7-17.
[32] Lin TH, Tamaki Y, Pajarinen J, Waters HA, Woo DK, Yao Z, et al. Chronic inflammation in biomaterial-induced periprosthetic osteolysis: NF-kappaB as a therapeutic target. Acta Biomater 2014;10(1):1-10.
[33] Zou W, Amcheslavsky A, Takeshita S, Drissi H, Bar-Shavit Z. TNF-α expression is transcriptionally regulated by RANK ligand. Journal of Cellular Physiology 2005;202:371-8.
[34] Bharti AC, Takada Y, Aggarwal BB. Curcumin (Diferuloylmethane) Inhibits Receptor Activator of NF-κB Ligand-Induced NF-κB Activation in Osteoclast Precursors and Suppresses Osteoclastogenesis. The Journal of Immunology 2004;172:5940-7.
[35] Yip KH, Zheng MH, Feng HT, Steer JH, Joyce DA, Xu J. Sesquiterpene lactone parthenolide blocks lipopolysaccharide-induced osteolysis through the suppression of NF-kappaB activity. J Bone Miner Res 2004;19:1905-16.
[36] Ang ES, Pavlos NJ, Chai LY, Qi M, Cheng TS, Steer JH, et al. Caffeic acid phenethyl ester, an active component of honeybee propolis attenuates osteoclastogenesis and bone resorption via the suppression of RANKL-induced NF-kappaB and NFAT activity. J Cell Physiol 2009;221:642-9.
[37] Ichikawa H, Takada Y, Shishodia S, Jayaprakasam B, Nair MG, Aggarwal BB. Withanolides potentiate apoptosis, inhibit invasion, and abolish osteoclastogenesis through

suppression of nuclear factor-κB (NF-κB) activation and NF-κB–regulated gene expression. Molecular Cancer Therapeutics 2006;5:1434-45.

[38] Shishodia S, Gutierrez AM, Lotan R, Aggarwal BB. N-(4-Hydroxyphenyl)Retinamide Inhibits Invasion, Suppresses Osteoclastogenesis, and Potentiates Apoptosis through Down-regulation of IκBα Kinase and Nuclear Factor-κB–Regulated Gene Products. Cancer Research 2005;65:9555-65.

[39] Wu X, Li Z, Yang Z, Zheng C, Jing J, Chen Y, et al. Caffeic acid 3,4-dihydroxy-phenethyl ester suppresses receptor activator of NF-kappaB ligand-induced osteoclastogenesis and prevents ovariectomy-induced bone loss through inhibition of mitogen-activated protein kinase/activator protein 1 and Ca2+-nuclear factor of activated T-cells cytoplasmic 1 signaling pathways. J Bone Miner Res 2012;27:1298-308.

[40] Looney RJ, Boyd A, Totterman S, Seo G, Tamez-Pena J, Campbell D, et al. Volumetric computerized tomography as a measurement of periprosthetic acetabular osteolysis and its correlation with wear. Arthritis Res 2002;4(1):59-63.

[41] Orishimo KF, Claus AM, Sychterz CJ, Engh CA. Relationship between polyethylene wear and osteolysis in hips with a second-generation porous-coated cementless cup after seven years of follow-up. J Bone Joint Surg Am 2003;85-A(6):1095-9.

[42] Wilkinson JM, Hamer AJ, Stockley I, Eastell R. Polyethylene wear rate and osteolysis: Critical threshold versus continuous dose-response relationship. Journal of Orthopaedic Research 2005;23:520-5.

[43] Kurtz SM, Ok M, M E, Edidin AA. Advances in the processing, sterilization, and crosslinking of ultra-high molecular weight polyethylene for total joint arthroplasty. Biomaterials 1999;20(18):1659-88.

[44] Atkins GJ, Haynes DR, Howie DW, Findlay DM. Role of polyethylene particles in peri-prosthetic osteolysis: A review. World journal of orthopedics 2011;2:93-101.

[45] Kauther MD, Bachmann HS, Neuerburg L, Broecker-Preuss M, Hilken G, Grabellus F, et al. Calcitonin substitution in calcitonin deficiency reduces particle-induced osteolysis. BMC musculoskeletal disorders 2011;12:186.

[46] Landgraeber S, Jaeckel S, Loer F, Wedemeyer C, Hilken G, Canbay A, et al. Pan-caspase inhibition suppresses polyethylene particle-induced osteolysis. Apoptosis : an international journal on programmed cell death 2009;14:173-81.

[47] von Knoch M, Wedemeyer C, Pingsmann A, von Knoch F, Hilken G, Sprecher C, et al. The decrease of particle-induced osteolysis after a single dose of bisphosphonate. Biomaterials 2005;26:1803-8.

[48] Wedemeyer C, Neuerburg C, Pfeiffer A, Heckelei A, Bylski D, von Knoch F, et al. Polyethylene particle-induced bone resorption in alpha-calcitonin gene-related peptide-deficient mice. J Bone Miner Res 2007;22:1011-9.

[49] Wedemeyer C, Neuerburg C, Pfeiffer A, Heckelei A, von Knoch F, Hilken G, et al. Polyethylene particle-induced bone resorption in substance P-deficient mice. Calcif Tissue Int 2007;80(4):268-74.

[50] Wedemeyer C, Xu J, Neuerburg C, Landgraeber S, Malyar NM, von Knoch F, et al. Particle-induced osteolysis in three-dimensional micro-computed tomography. Calcif Tissue Int 2007;81:394-402.

[51] Perilli E, Briggs AM, Kantor S, Codrington J, Wark JD, Parkinson IH, et al. Failure strength of human vertebrae: prediction using bone mineral density measured by DXA and bone volume by micro-CT. Bone 2012;50:1416-25.

[52] Nich C, Rao AJ, Valladares RD, Li C, Christman JE, Antonios JK, et al. Role of direct estrogen receptor signaling in wear particle-induced osteolysis. Biomaterials 2012.

[53] Li Y, Zhang C, Zhou X, Wang H, Mao Y, Wang X. Parthenolide inhibits polyethylene particle-induced mouse calvarial osteolysis in vivo. The Journal of surgical research 2013.

[54] Marquez N, Sancho R, Macho A, Calzado MA, Fiebich BL, Munoz E. Caffeic acid phenethyl ester inhibits T-cell activation by targeting both nuclear factor of activated T-cells and NF-kappaB transcription factors. The Journal of pharmacology and experimental therapeutics 2004;308:993-1001.

[55] Natarajan K, Singh S, Burke TRJR, Grunberger D, Aggarwal BB. Caffeic acid phenethyl ester is a potent and specific inhibitor of activation of nuclear transcription factor NF-kappa B. Proc Natl Acad Sci U S A 1996; 93(17):9090–5.

[56] Ha J, Choi HS, Lee Y, Lee ZH, Kim HH. Caffeic acid phenethyl ester inhibits osteoclastogenesis by suppressing NF kappaB and downregulating NFATc1 and c-Fos. International immunopharmacology 2009;9:774-80.

[57] Duan W, Wang Q, Li F, Xiang C, Zhou L, Xu J, et al. Anti-catabolic effect of caffeic acid phenethyl ester, an active component of honeybee propolis on bone loss in ovariectomized mice: a micro-computed tomography study and histological analysis. Chin Med J (Engl) 2014;127(22):3932-6.
[58] Schmalzried TP, Harris WH. The Harris-Galante porous-coated acetabular component with screw fixation. Radiographic analysis of eighty-three primary hip replacements at a minimum of five years. J Bone Joint Surg Am 1992;74(8):1130-9.
[59] Bozic KJ, Ries MD. Wear and Osteolysis in Total Hip Arthroplasty. Seminars in Arthroplasty 2005;16:142-52.
[60] Jacobs JJ, Skipor AK, Black J, Urban Rm, Galante JO. Release and excretion of metal in patients who have a total hip-replacement component made of titanium-base alloy. The Journal of Bone & Joint Surgery 1991;73:1475-86.
[61] Urban RM, Jacobs JJ, Tomlinson MJ, Gavrilovic J, Black J, Peoc'h M. Dissemination of wear particles to the liver, spleen, and abdominal lymph nodes of patients with hip or knee replacement. J Bone Joint Surg Am 2000;82(4):457-76.
[62] Urban RM, Tomlinson MJ, Hall DJ, Jacobs JJ. Accumulation in liver and spleen of metal particles generated at nonbearing surfaces in hip arthroplasty. The Journal of Arthroplasty 2004;19:94-101.
[63] Jacobs JJ, Skipor AK, Patterson LM, Hallab NJ, Paprosky WG, Black J, et al. Metal Release in Patients Who Have Had a Primary Total Hip Arthroplasty. A Prospective, Controlled, Longitudinal Study*. The Journal of Bone & Joint Surgery 1998;80:1447-58.
[64] Case CP, Langkamer VG, James C, Palmer MR, Kemp AJ, Heap PF, et al. Widespread dissemination of metal debris from implants. Journal of Bone & Joint Surgery, British Volume 1994;76-B:701-12.
[65] Rosen HN, Moses AC, Garber J, Iloputaife ID, Ross DS, Lee SL, et al. Serum CTX: a new marker of bone resorption that shows treatment effect more often than other markers because of low coefficient of variability and large changes with bisphosphonate therapy. Calcif Tissue Int 2000;66(2):100-3.
[66] Tuan RS, Lee FY, Y TK, Wilkinson JM, Smith RL. What are the local and systemic biologic reactions and mediators to wear debris, and what host factors determine or

modulate the biologic response to wear particles? J Am Acad Orthop Surg 2008;16 Suppl 1:S42-8.

[67] Goldring SR, Schiller AL, Roelke M, Rourke CM, O'Neil DA, Harris WH. The synovial-like membrane at the bone-cement interface in loose total hip replacements and its proposed role in bone lysis. J Bone Joint Surg Am 1983;65(5):575-84.

[68] Fitzpatrick LR, Wang J, Le T. Caffeic acid phenethyl ester, an inhibitor of nuclear factor-kappaB, attenuates bacterial peptidoglycan polysaccharide-induced colitis in rats. J Pharmacol Exp Ther 2001;299(3):915-20.

[69] Orban Z, Mitsiades N, Burke TRJ, Tsokos M, Chrousos GP. Caffeic acid phenethyl ester induces leukocyte apoptosis, modulates nuclear factor-kappa B and suppresses acute inflammation. Neuroimmunomodulation 2000;7(2):99-105.

[70] Yu H, van Berkel TJ, Biessen EA. Therapeutic potential of VIVIT, a selective peptide inhibitor of nuclear factor of activated T cells, in cardiovascular disorders. Cardiovasc Drug Rev 2007;25:175-87.

[71] Herman S, Muller RB, Kronke G, Zwerina J, Redlich K, Hueber AJ, et al. Induction of osteoclast-associated receptor, a key osteoclast costimulation molecule, in rheumatoid arthritis. Arthritis Rheum 2008;58:3041-50.

[72] Zhao S, Guo YY, Ding N, Yang LL, Zhang N. Changes in serum levels of soluble osteoclast-associated receptor in human rheumatoid arthritis. Chin Med J (Engl) 2011;124:3058-60.

[73] Ndongo-Thiam N, de Sallmard G, Kastrup J, Miossec P. Levels of soluble osteoclast-associated receptor (sOSCAR) in rheumatoid arthritis: link to disease severity and cardiovascular risk. Ann Rheum Dis 2014;73(6):1276-7.

[74] Nemeth K, Schoppet M, Al-Fakhri N, Helas S, Jessberger R, Hofbauer LC, et al. The role of osteoclast-associated receptor in osteoimmunology. J Immunol 2011;186(1):13-8.

[75] Crotti TN, Dharmapatni AA, Alias E, Zannettino AC, Smith MD, Haynes DR. The immunoreceptor tyrosine-based activation motif (ITAM) -related factors are increased in synovial tissue and vasculature of rheumatoid arthritic joints. Arthritis research & therapy 2012;14:R245.

[76] Tenca C, Merlo A, Merck E, Bates EEM, Saverino D, Simone R, et al. CD85j (Leukocyte Ig-Like Receptor-1/Ig-Like Transcript 2) Inhibits Human Osteoclast-

Associated Receptor-Mediated Activation of Human Dendritic Cells. The Journal of Immunology 2005;174:6757-63.

[77] Goettsch C, Rauner M, Sinningen K, Helas S, Al-Fakhri N, Nemeth K, et al. The Osteoclast-Associated Receptor (OSCAR) Is a Novel Receptor Regulated by Oxidized Low-Density Lipoprotein in Human Endothelial Cells. Endocrinology 2011;152:4915-26.

[78] Verborgt O, Gibson GJ, Schaffler MB. Loss of osteocyte integrity in association with microdamage and bone remodeling after fatigue in vivo. J Bone Miner Res 2000;15(1):60-7.

[79] Kurata K, Heino TJ, Higaki H, Vaananen HK. Bone marrow cell differentiation induced by mechanically damaged osteocytes in 3D gel-embedded culture. J Bone Miner Res 2006;21(4):616-25. Epub 2006 Apr 5.

[80] Kanaji A, Caicedo MS, Virdi AS, Sumner DR, Hallab NJ, Sena K. Co-Cr-Mo alloy particles induce tumor necrosis factor alpha production in MLO-Y4 osteocytes: a role for osteocytes in particle-induced inflammation. Bone 2009;45(3):528-33.

[81] Atkins GJ, Welldon KJ, Holding CA, Haynes DR, Howie DW, Findlay DM. The induction of a catabolic phenotype in human primary osteoblasts and osteocytes by polyethylene particles. Biomaterials 2009;30:3672-81.

[82] Baek K, Hwang HR, Park HJ, Kwon A, Qadir AS, Ko SH, et al. TNF-alpha upregulates sclerostin expression in obese mice fed a high-fat diet. J Cell Physiol 2014;229(5):640-50.

CHAPTER 7: Conclusion and future directions

7.1.Conclusion

The effects of disrupting NFATc1 and NF-κB signalling and the role of ITAM-related factors in osteoclastogenesis and bone resorption are only just becoming understood. Overall the findings of this thesis demonstrate there is a crucial interaction between NFATc1, NF-κB and ITAM molecules in osteoclastogenesis and bone resorption *in vitro* and *in vivo*. NF-κB or NF-κB/NFATc1 inhibition can regulate *in vivo* volumetric and surface particle-induced osteolysis as well as one of the ITAM-related molecules, OSCAR in murine particle-induced peri-prosthetic osteolysis. In addition to the effects of particles and inhibitors of NF-κB or NF-κB/NFATc1 on osteoclasts, the study shows that osteocytes are affected. As we are beginning to understand the important role osteocytes play in regulating bone metabolism, the contribution of osteocytes to the progression of osteolysis is worth investigating further. Understanding this complex interaction of bone cells may offer novel ways in inhibiting osteolysis seen in many bone loss pathologies particularly peri-prosthetic osteolysis.

7.2.Future directions

These following future directions may be useful to better understand the mechanisms involved in the studies in this thesis:

1. In Chapter 1 we have discovered the effects of NFATc1 and NF-κB inhibition by FK506, VIVIT and NC100 on osteoclast formation and activity. In addition, gene expression of osteoclast-associated factors and ITAM-related molecules in normal human PBMC-derived osteoclast cultures *in vitro* was investigated. This indicates that it would be valuable to repeat similar experiments in the presence of PE particles *in vitro.* This would allow us to understand the effects of PE particles on osteoclastogenesis as well as ITAM factors in an *in vitro* model that better reflects that occurring *in vivo*.
2. Additionally it would be important to determine the effects of FK506, VIVIT and NC100 in a murine calvarial model of PE-induced peri-prosthetic osteolysis as these have not been reported yet.

3. Studies similar to that carried out in Chapters 4 and 5 to study the gene and protein expression of NFATc1, NF-κB, and ITAM-associated molecules in the tissues would allow us understand the effects of PE particles and inhibitor treatments *in vivo* .
4. We have shown the effects of PAR and CAPE in a murine PE-induced calvarial osteolysis in Chapters 4 and 5. In order to further understand the effectiveness of PAR and CAPE to suppress osteolysis, it would be interesting to determine the effects of PAR and/or CAPE on other inflammatory driven disease models, such as, collagen antibody-induced arthritic (CAIA) mouse model, already established in our research group.
5. Due to the time limitations only the effects of NF-κB inhibition by PAR on osteocytes could be investigated (Chapter 4). It would be worthwhile to extend this study to determine whether NF-κB and NFATc1 inhibition by CAPE (Chapter 5) also affected osteocytes in this disease model.
6. To date there has been only limited *in vitro* research on the effects of PAR on osteoclastogenesis. It would be useful to investigate the effects of PAR more extensively, particularly, on gene expression of osteoclast-associated factors and ITAM-related molecules similar to the approach in Chapters 2 and 3.

The results of this thesis have demonstrated the potential of NF-κB and NFATc1 inhibition to suppress inflammation-mediated osteolysis particularly in PE-induced peri-prosthetic osteolysis. Although our knowledge on the mechanisms of action is limited, the data presented here and the work of others illustrate that the effects of NF-κB and NFATc1 inhibition may lead to effective treatments of human bone loss pathologies.