THE UNIVERSITY of ADELAIDE

A NEW ROLE FOR PEROXIDASES IN BONE REPAIR

Alexandra Jayne Shoubridge B.Sc. (Hons)

A Thesis submitted for the degree of Doctor of Philosophy

in

The Discipline of Surgery, School of Medicine

Faculty of Health Science

The University of Adelaide

July 2019

TABLE OF CONTENTS

“The world is full of wonders, but they become more wonderful, not less wonderful when Science looks at them.”

- Sir David Attenborough

DECLARATION

I, Alexandra Jayne Shoubridge, certify that this work contains no material which has been accepted for the award of any other degree or diploma in my name, in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously 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 work will, in the future, be used in a submission in my name, for any other degree or diploma in any university or other tertiary institution without the prior approval of the University of Adelaide and where applicable, any partner institution responsible for the joint-award of this degree.

I acknowledge that copyright of published works contained within this thesis resides with the copyright holder(s) of those works.

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 Search and also through web search engines, unless permission has been granted by the University to restrict access for a period of time.

ACKNOWLEDGEMENTS

Whenever I would ask someone who had completed their PhD what it was like and how they felt afterwards, I was always told the same thing, which is, “it is one of the toughest academic challenges they have faced, but it also the most rewarding.” Now, at the end of my PhD I can appreciate the views those people have. It has undoubtedly been the hardest three years of my life, both personally and academically but it has also been my greatest personal achievement. As someone whom success does not come easily to, I have found these last three years as an opportunity to prove my worth to the scientific community and very proud of myself. This would all have not been possible however, without all of the support from both colleagues and family who have helped bring out the best in me, both personally and as a researcher. Words alone cannot describe my appreciation for each person I am about to acknowledge but I hope you all understand how much I have valued your guidance and support.

First and foremost, I would like to thank my principal supervisor, Prof. Andreas Evdokiou, for giving me the opportunity to study within your laboratory group and for seeing potential in me before I began my Honours degree. Your encouragement and confidence in me throughout that year enabled me to have the self-confidence to undertake a PhD. I am very grateful to you for allowing me to continue on and complete a PhD in the Breast Cancer Research Unit and will always appreciate the support you provided throughout those years. Besides the course and laboratory work, I am also very appreciative of your support and feedback towards presentations. As someone who struggled with public-speaking I am now a very confident speaker and have always been thankful for your help in developing that critical personal skill.

I would also like to express my overwhelming gratitude to my co-supervisor, Prof. Peter Anderson who has supported me through both my Honours degree and now my PhD. I believe

I have been very fortunate in finding a supervisor and mentor like you, who has provided unwavering personal and academic support. Your outside views and expertise have added a great degree of depth to my work. The assistance you have provided has been paramount in broadening my ideas and skills. I have also deeply appreciated your ability to find the time to meet with me outside your constantly hectic schedule and provide advice for my personal development and career as a researcher. I will always be grateful.

Great appreciation and thanks also goes to my second co-supervisor, Dr. Bill Panagopoulos, whom I am greatly indebted to for jumping in as a key supervisor for the remainder of my PhD. Your knowledge and experience has supported me during the most critical point of my PhD and I am so thankful for you time. I hope we will always find time to catch up in the future and share a laugh but in the meantime, I wish you all the very best with your career and your family.

I am also very thankful to past and present laboratory members from the BCRU laboratory. Firstly, I would like to say thank you Aneta for being such a wonderful and amazing work-wife. I greatly miss our lunch trips to Salsa's and walks to see Possum but most of all, sharing our struggles and successes which always helped me to keep persisting. I am so thankful of the advice you gave me early in my studies, which is what led to my current position today. I am so proud of all you have accomplished and proud to call you a friend. Although we're both on our own different career paths now I am so happy in knowing that we will still be a part of each other's lives. Thank you also to other past members of the BCRU laboratory, Shelley and Bill. Also, to current members, Irene, Bee and Chris. Finally, many thanks go to everyone else at the Basil Hetzel Institute for their support, as well as The Hospital Research Foundation and The University of Adelaide. Without all of your support the past 3 years would have been an overwhelming challenge.

Next, I would like to thank my friends, especially my best friend Chloé, for providing moral support and many laughs during the toughest times during my PhD. Since we met on the first day of University you have always been there to boost my confidence and help me through the most demanding times of my studies. Thank you for allowing me to vent my frustrations and for giving me advice, you're a wonderful person and I am so grateful to have you as a friend.

I want to now thank the most important people in my life, my family. Especially to my Mum and Dad, I owe you so much. You have poured so much time and energy into my life and without your never-ending support I know I would not be where I am today. I cannot thank you enough for always supporting me and providing me with advice. I am proud and so very lucky to have the most wonderful and loving parents. To my brother, Andrew, I would like to thank you for always supporting me and for always being a great friend. I wish you all the best of luck for completing your PhD and as you move forward with your career. Finally, I owe so much to my dearest husband Tim. Words cannot describe how much I love and appreciate all the things you have done for me both before and during my PhD. I am certain that without your ability to bring out the best in me I would not have gotten through the last 3 years with composure. Your support and ability to always make me smile and laugh during the most mentally-demanding 3 years I have ever experienced is proof that to me, you truly are the dearest and most important person in my life.

ABSTRACT

When bone undergoes trauma or the architecture deteriorates, due to disease and is neglected or misdiagnosed, non-unions can occur, whereby bone does not heal correctly. As a consequence, patients experience pain, stiffness, loss of mobility and disability. In many cases this can result in an inability to perform normal duties in employment, which causes significant financial burden to the patient and economy. The repair of these large bone defects remains a significant challenge for orthopaedic surgeons. Bone grafting strategies have been developed to repair and restore bone function, however the demand for functional bone grafts is extremely high, with an estimated 2.2 million patients worldwide undergoing bone grafting procedures annually. Due to an aging population these numbers are expected to double by 2020, which will put further burden on health care costs worldwide. Autologous bone grafting remains the current standard to repair bone defects and fractures, however, this method of treatment has numerous surgical-associated morbidities and complication rates of up to 30%. Therefore, researchers are attempting to identify substitute grafting materials which possess the critical bone reparative characteristics required for successful healing. To date, a bone graft material which is comparable to autologous bone, with fewer associated morbidities is yet to be identified, thus, continued research is required to identify and develop new agents to promote and accelerate bone repair.

Agents which have been thoroughly investigated to enhance the bone repair process in combination with bone graft substitutes include the use of BMP-2. BMP-2 has proven to be successful due to its pro-osteogenic role whereby it promotes osteoblast functionality through the regulation of genes necessary for collagen biosynthesis and mineralisation of the extracellular matrix (ECM). Osteoblasts are one of the main cell types responsible for bone formation and bone repair. These cells are derived from the mesenchymal progenitor cell

population, along with endothelial cells and fibroblasts. Work published by our laboratory provides evidence that a group of enzymes with peroxidase activity, namely mammalian-derived myeloperoxidase (MPO) and eosinophil peroxidase (EPO) as well as plant-derived soybean peroxidase (SBP) stimulate the migration of fibroblastic cells and promote their ability to generate a functional ECM. In addition, we have presented evidence demonstrating the ability of these peroxidases, in promoting endothelial cell function and inhibiting osteoclastogenesis, suggesting a potential role for these enzymes in bone repair.

The work described in this thesis aims to provide evidence that mammalian and plant derived peroxidase enzymes including, MPO, EPO and SBP possess pro-osteogenic activities by influencing osteoblast functionality. Using physiologically relevant concentrations of peroxidases, this study showed that the enzymatic catalytic activities and substrate specificities of each of these enzymes which were shown to be different, resulted in differential responses in the context of osteoblast function. EPO and SBP demonstrated a well-conserved pro-osteogenic capacity to stimulate the biosynthesis of collagen I by primary human osteoblasts and promote mineralisation of the deposited ECM. In contrast, MPO, while it was able to promote ECM deposition, it failed to promote mineralisation and therefore unlikely to contribute to bone formation.

The ability of EPO and SBP to stimulate mineralisation by osteoblasts suggests that these enzymes may possess key properties for promoting bone repair. Of the two tested peroxidases however, SBP is more readily available and significantly cheaper than EPO, making it an attractive and realistic candidate for further pre-clinical assessment. Data presented in this thesis demonstrate for the first time the pro-osteogenic ability of SBP, in combination with a commercially available scaffold to significantly accelerate bone repair in an ovine critical-sized defect model. This was confirmed by quantitative micro-CT analysis. Histological assessment showed evidence of intramembranous bone formation and viable osteoblast and

osteocyte cell populations, indicative of bone repair and maturation. These results suggest that SBP may be beneficial as a therapeutic agent to accelerate localised repair of damaged bone.

The use of rodents over larger animals for different models of bone repair allows for high throughput analyses of multiple variables, such as dose and time. Using wildtype mice, we established a critical size defect model to validate SBP in this species, prior to investigating other models of bone repair. The doses of SBP investigated in this study demonstrated significant inhibition of bone formation with increased fibrous tissue present and an absence of bone remodelling indicators. The results presented in this thesis highlight the importance of further mechanistic investigation, to determine how SBP regulates the remodelling process and the necessity for optimisation before assessing the role of SBP in fracture healing.

In conclusion, our findings demonstrate for the first time that peroxidase enzymes likely regulate multiple cellular processes involved in new bone formation, including collagen I biosynthesis, bone matrix mineralisation and osteogenic regulation. Specifically, the plant derived peroxidase, SBP, displays significant pro-osteogenic potential by promoting intramembranous ossification. The studies presented in this thesis provide the first *in vivo* evidence for peroxidase enzymes as therapeutic agents with the potential to enhance bone repair and, identifies peroxidase inhibitors as a preventative target of pathological ossification.

PUBLICATIONS

Summary: Published Journal Articles: **2**; Papers in preparation **1**.

Peer Reviewed Journal Articles:

1. DeNichilo, MO., **Shoubridge, AJ**., Panagopoulos V., Liapis V., Zinonos I., Hay S., Atkins GJ., Findlay DM., and Evdokiou A., (2016). Peroxidase enzymes regulate collagen biosynthesis and matrix mineralization by cultured human osteoblasts. *Calcif Tissue Int. 98(3):294-305*.

2. Liapis V., Zysk A., DeNichilo MO., Zinonos I., Hay S., Panagopoulos V., **Shoubridge AJ**., Difelice C., Ponomarev V., Ingman W., Atkins GJ., Findlay DM., Zannettino ACW., and Evdokiou A., (2016). Anticancer efficacy of the hypoxia activated prodrug evofosfamide is enhanced in combination with proapoptotic receptor agonists against osteosarcoma. *Cancer Med. 6(9): 2164-2176.*

Articles in Progress:

1. **Shoubridge AJ**., Panagopoulos V., DeNichilo MO., Hay S., Anderson PJ., and Evdokiou A., (2018). The Effect of Soybean Peroxidase to Promote Bone Formation

CONFERENCE PRESENTATIONS

- **Alexandra J. Shoubridge**, Mark O. DeNichilo, Peter J. Anderson, Irene Zinonos, Vasilios Panagopoulos, Shelley Hay and Andreas Evdokiou. The role of Peroxidase Enzymes in Fracture Repair of Healthy and Osteoporotic Bone. ASMR SA Annual Scientific Meeting 2015. Adelaide, Australia. *Poster presentation*.

- **Alexandra J. Shoubridge**, Mark O. DeNichilo, Peter J. Anderson, Irene Zinonos, Vasilios Panagopoulos, Shelley Hay and Andreas Evdokiou. Peroxidases and their role in Promoting Bone Repair and Regeneration. Florey Postgraduate Research Conference 2015. Adelaide, Australia. *Poster presentation*.

- **Alexandra J. Shoubridge**, Mark O. DeNichilo, Peter J. Anderson, Irene Zinonos, Vasilios Panagopoulos, Shelley Hay and Andreas Evdokiou. Peroxidases and their role in Promoting Bone Repair and Regeneration. Clare Valley Bone Meeting 2016. Clare, Australia. *Oral and poster presentation*.

- **Alexandra J. Shoubridge**, Mark O. DeNichilo, Peter J. Anderson, Irene Zinonos, Vasilios Panagopoulos, Shelley Hay and Andreas Evdokiou. Peroxidases as a Novel Promoter of Bone Repair. ESA-SRB-ANZBMS Joint Scientific Meeting 2016. Gold Coast, Queensland. *Poster presentation*.

- **Alexandra J. Shoubridge**, Mark O. DeNichilo, Peter J. Anderson, John Field, Irene Zinonos, Vasilios Panagopoulos, Shelley Hay and Andreas Evdokiou. The Therapeutic Potential of Peroxidases in Promoting Bone Repair. ASMR SA Annual Scientific Meeting 2016. Adelaide, Australia. *Poster presentation*.

- **Alexandra J. Shoubridge**, Mark O. DeNichilo, Peter J. Anderson, John Field, Irene Zinonos, Vasilios Panagopoulos, Shelley Hay and Andreas Evdokiou. The Therapeutic Potential of Peroxidases in Promoting Bone Repair. Florey Postgraduate Research Conference 2016. Adelaide, Australia. *Poster presentation*.

- **Alexandra J. Shoubridge**, Mark O. DeNichilo, Peter J. Anderson, John Field, Irene Zinonos, Vasilios Panagopoulos, Shelley Hay and Andreas Evdokiou. Peroxidases and their role in Promoting Bone Repair and Regeneration. The Queen Elizabeth Hospital (TQEH) Research Day 2016. Adelaide, Australia. *Poster presentation*.

- **Alexandra J. Shoubridge**, Vasilios Panagopoulos, John Field, Siamak Saifzadeh, Roland Steck, Mark O. DeNichilo, Irene Zinonos, Shelley Hay, Peter J. Anderson & Andreas Evdokiou. Soybean Peroxidase Possesses Osteogenic Activity to Accelerate Bone Regeneration In Vivo. ASBMR Annual Meeting 2017. Denver, United States of America. *Poster presentation*.

- **Alexandra J. Shoubridge**, Vasilios Panagopoulos, John Field, Siamak Saifzadeh, Roland Steck, Mark O. DeNichilo, Irene Zinonos, Shelley Hay, Peter J. Anderson & Andreas Evdokiou. Soybean Peroxidase Possesses Osteogenic Activity to Accelerate Bone Regeneration In Vivo. The Queen Elizabeth Hospital Research Day 2017. Adelaide, Australia. *Poster presentation*.

PRIZES AWARDED

- Project with most commercial potential, awarded by Adelaide Research and Innovation. Florey Postgraduate Research Conference, Faculty of Health Science, University of Adelaide, 24th September 2015. National Wine Centre of Australia.

- Best Poster Presentation for the Florey Medical Research Foundation Prize. Florey Postgraduate Research Conference, Faculty of Health Science, University of Adelaide, 24th September 2015. National Wine Centre of Australia.

- Travel award for the ANZBMS Scientific Meeting. ESA-SRB-ANZBMS Joint Scientific Meeting, 21st August 2016. Gold Coast Convention Centre.

- AMS research travel award for the ASBMR 2017 Annual Meeting. Faculty of Health Science, University of Adelaide.

- The Hospital Research Foundation Research Travel Award for the ASBMR 2017 Annual Meeting. The hospital Research Foundation.

- Best Poster Presentation for The Queen Elizabeth Hospital Research Day 2017. TQEH Research Day, 20th October 2017. Basil Hetzel Institute.

- Faculty of Health Science Divisional Postgraduate Scholarship, University of Adelaide.

ABBREVIATIONS

3D	3 Dimensional
4-ABAH	4-Amino-Benzoic Acid Hydrazide
AA	Ascorbic Acid/Ascorbate
ALP	Alkaline Phosphatase
β-TCP	Beta-Tricalcium Phosphate
BCP	Biphasic Calcium Phosphate
BMP	Bone Morphogenetic Protein
BSA	Bovine Serum Albumin
BSP	Bone Sialoprotein
BV	Bone Volume
CCL	Chemokine (C-C motif) Ligand
cDNA	Complementary Deoxyribonucleic Acid
COL1A1	Alpha-1 Type I Collagen
CSD	Critical Size Defect
CXCL	Chemokine (C-X-C Motif) Ligand

CXCR	Chemokine (C-X-C Motif) Receptor
DMEM	Dulbecco's Modified Eagle Medium
DMOG	Dimethyloxalylglycine
ECM	Extracellular Matrix
EDTA	Ethylene-Diamine-Tetra Acetic Acid
ELISA	Enzyme-Linked Immunosorbent Assay
EPO	Eosinophil Peroxidase
FBS	Foetal Bovine Serum
FGF	Fibroblast Growth Factor
FRZB	Frizzled-Related Protein B
GAPDH	Glyceraldehyde-3-Phosphate Dehydrogenase
GDF-5	Growth Differentiation Factor-5
H&E	Hematoxylin and Eosin
H_2O_2	Hydrogen Peroxide
HA	Hydroxyapatite
HO	Heterotopic Ossification

HRP	Horseradish Peroxidase
HU	Hounsfield Units
IGF	Insulin-Like Growth Factor
IgG	Immunoglobulin-G
IL	Interleukin
LPO	Lactoperoxidase
M-CSF	Macrophage Colony-Stimulating Factor
MG	Mastergraft®
Micro-CT	Microcomputed Tomography
MMA	Methyl Methacrylate
MMP	Matrix Metalloproteinases
MPO	Myeloperoxidase
mRNA	Messenger Ribonucleic Acid
MSC	Mesenchymal Stem Cell
NADPH	Nicotinamide Adenine Dinucleotide Phosphate Hydrogen
NSAID	Nonsteroidal Anti-Inflammatory Drug

OCN	Osteocalcin
OPG	Osteoprotegerin
OSX	Osterix
PCR	Polymerase Chain Reaction
PEG	Polyethylene Glycol
PBS	Phosphate Buffered Saline
PDGF	Platelet-Derived Growth Factor
RANK	Receptor Activator of Nuclear Factor Kappa-B Receptor
RANKL	Receptor Activator of Nuclear Factor Kappa-B Ligand
RNA	Ribonucleic Acid
ROI	Region of Interest
ROS	Reactive Oxygen Species
RT-PCR	Real Time Polymerase Chain Reaction
RUNX2	Runt-Related Transcription Factor 2
SaOS-2	Sarcoma Osteogenic-2
SBP	Soybean Peroxidase
SDF-1	Stromal Cell-Derived Factor-1

SD	Standard Deviation
TGF- β	Transforming Growth Factor Beta
TNF-α	Tumour Necrosis Factor-Alpha
TRAP	Tartrate-Resistant Acid Phosphatase
TV	Total Volume
VEGF	Vascular Endothelial Growth Factor
VOI	Volume of Interest
WGFE	Whey Growth Factor Extract
WNT	Wingless-Type MMTV Integration Site

CHAPTER 1:

INTRODUCTION

BIOLOGY OF BONE

Bone is a highly specialised connective tissue, consisting of both organic and inorganic constituents and is responsible for providing locomotion, supporting and protecting soft tissues, maintaining calcium homeostasis and containing bone marrow for haematopoiesis (Datta, Ng, Walker, Tuck, & Varanasi, 2008; Robling, Castillo, & Turner, 2006). The inorganic components are responsible for providing compression, strength and stiffness, whereas the organic components provide tension properties. The composition of these components varies with species, age, sex, the specific bone and the presence of disease (Ontañón, Aparicio, Ginebra, & Planell, 2000). From a macroscopic viewpoint, bone appears non-homogenous, porous and anisotropic and can be defined between two types of bone tissue. The first type, trabecular or cancellous bone has a porosity of 50-95% and can be found in cuboidal and flat bones and at the ends of long bones (Figure. 1.1). The highly porous structure is interconnected and filled with bone marrow (R. B. Martin, Burr, & Sharkey, 1998). The other type of bone is termed cortical or compact bone and is 5-10% porous and contains a variety of different types of pores. Vascular pores are formed by Haversian canals that are aligned with the long axis and are connected by the transverse Volkmann canals, containing capillaries and nerves. Other pores are termed lacunae which are connected through the small canaliculi canals (Cowin, 1999). This network forms cylindrical structures termed osteons, or Haversian systems (Figure 1.2). Bone is a very unique tissue by its ability to grow, modify its shape, self-repair and continuously renews itself. The growth of bone mostly occurs during childhood, self-repair occurs only during fracture healing and its renewal or remodelling remains constant throughout our lifetime. The dynamic and highly complex process of bone formation and remodelling requires the coordination of osteoblasts and osteoclasts. These cells are influenced by growth factors, cytokines and hormones, which regulate their migration, attachment, proliferation, differentiation and activity.

Figure 1.1 *Cross-section of a bone showing both cortical and trabecular bone.* The cortical bone, which forms the cortex of most bones is very dense and contributes to approximately 80% of the weight of a human skeleton. Cancellous bone is highly vascular and exhibits greater surface area than cortical bone which allows for metabolic activity, such as the exchange of calcium (Netter, 1990).

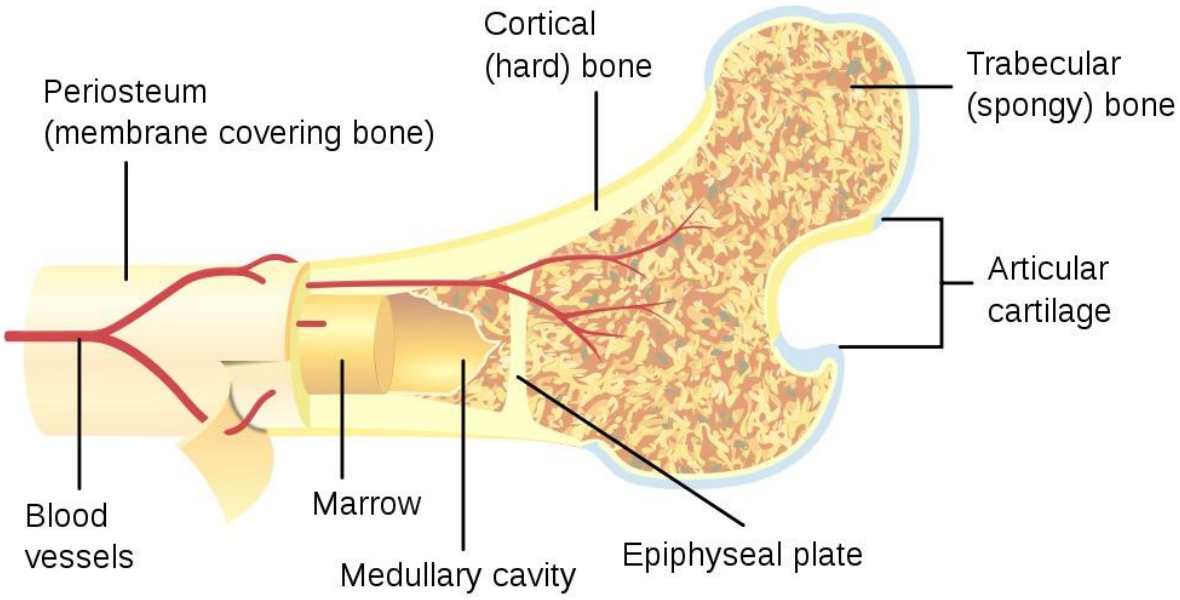
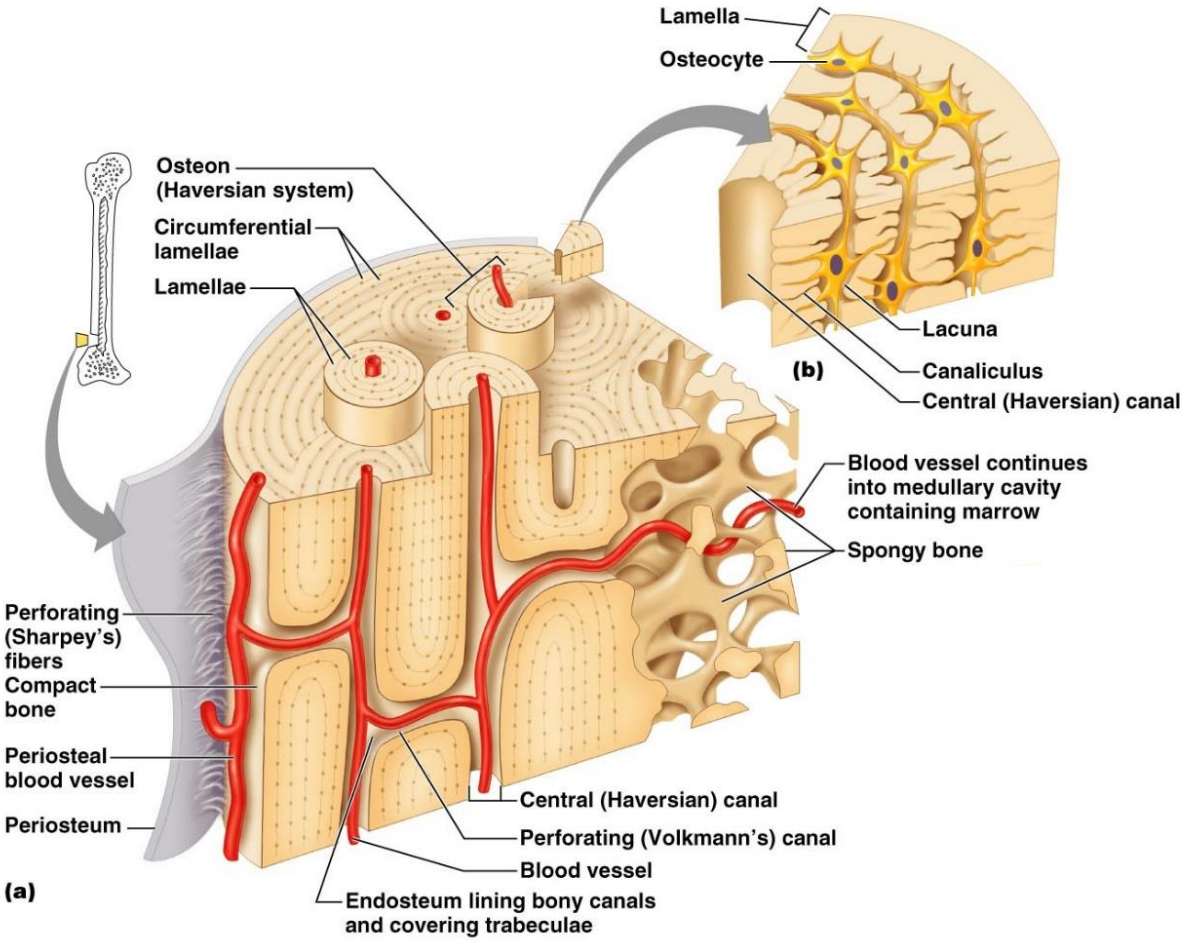

Periosteum
(membrane covering bone)
Cortical
(hard) bone
Trabecular
(spongy) bone
Articular
cartilage
Blood
vessels
Marrow
Medullary cavity
Epiphyseal plate

Figure 1.2 *Microscopical structure of cortical bone.* (a) 3D image of cortical bone showing the network of blood vessels and nerves interconnected within Haversian and Volkmann canals. The canals are a central point for individual osteons or harversian systems. (b) A cut section of a Harversian system shows the lamellae containing lacunae cavities, which house osteocytes and are connected by canaliculi. Figure adapted and modified from (Fridez, 1996).



Osteoblasts, osteoclasts and osteocytes

Bone contains two distinct cell types, the bone forming osteoblasts and the bone resorbing osteoclasts. The functional role between these two cell types are very intimate and continues throughout skeletal development and life. Cells from the osteoblastic lineage are responsible for the synthesis and secretion of molecules that in turn initiate and regulate osteoclast differentiation, which results in a tightly regulated process of bone resorption and formation (Teitelbaum, 2000). A third cell type, known as osteocytes plays a key role in this regulation of bone formation and remodelling by triggering the differentiation of osteoclasts (Matsuo & Irie, 2008).

Osteoblasts are cuboidal cells which represent 4-6% of the total bone cell population and their main role is the formation of new bone (Capulli, Paone, & Rucci, 2014). These cells are derived from mesenchymal stem cells (MSCs) and the commitment towards the osteoprogenitor lineage requires the timely expression and synthesis of specific osteogenic genes, including bone morphogenetic proteins (BMPs) and members of the Wnt pathway (Rawadi et al., 2003). Runt-Related Transcription Factor 2 (RUNX2) is critical in regulating osteoblast differentiation and has been shown to upregulate the expression of other key osteoblast-related genes, including alpha-1 type I collagen (COL1A1), alkaline phosphatase (ALP), bone sialoprotein (BSP) and osteocalcin (OCN) (Fakhry, Hamade, Badran, Buchet, & Magne, 2013). Once osteoblast progenitors express RUNX2 they begin the proliferative phase whereby the progenitors begin presenting ALP activity and become pre-osteoblasts (Capulli et al., 2014). Evidence of the formation of mature osteoblasts is observed by the increase in expression of Osterix (OSX) and secretion of bone matrix proteins, OCN, BSP and collagen type I.

The osteoclast is a multinucleated cell derived from mononuclear cells of the hematopoietic stem cell niche by the influence of several factors. These factors include,

macrophage colony-stimulating factor (M-CSF) which is secreted by osteoprogenitors and osteoblasts and receptor activator of nuclear factor kappa-B ligand (RANKL), secreted by osteoblasts, osteocytes and stromal cells. Combined, these factors regulate transcription factors and gene expression in osteoclasts (Karst, Gorny, Galvin, & Oursler, 2004). M-CSF is responsible for binding to osteoclast precursors, which leads to their proliferation and inhibits apoptosis. Whereas RANKL is critical in promoting osteoclastogenesis, by binding to its receptor RANK, in osteoclast precursors and a result induces osteoclast formation (Yavropoulou & Yovos, 2008).

Whilst the communication between osteoclasts and osteoblasts is critical, the most abundant and specialised bone cell type in mammalian bone is the osteocyte. Osteocytes are descendants of mesenchymal stem cells through osteoblast differentiation (Bonewald, 2011), that become encased within the bone matrix during bone formation. As osteoblasts transition to an osteocyte, the expression of various key regulatory markers changes, including reduction of ALP and increasing expression of osteocalcin (Mikuni- Takagaki, et al., 1995). The primary role of these cells is to maintain mineral homeostasis by detecting micro-fractures and micro-cracks in bone (Matsuo, Irie, 2008). During remodelling, healthy and dying osteocytes recruit osteoclasts at sites of micro-damage. Healthy osteocytes, not directly adjacent to a site of micro-damage will release anti-apoptotic molecules as a protective mechanism to prevent their own apoptosis. Osteocytes at the site of damage however, release pro-apoptotic molecules that contains RANKL to recruit osteoclasts directly to the site (Bonewald, 2011). In addition to maintaining bone maintenance, osteocytes also act as mechanosensors in the regulation of bone mass through hormonal and mechanical signalling. This includes signalling between osteocytes and the parathyroid, kidney, cardiac and skeletal muscle to regulate phosphate homeostasis and manage bone calcium levels (Dallas, Prideaux, & Bonewald, 2013).

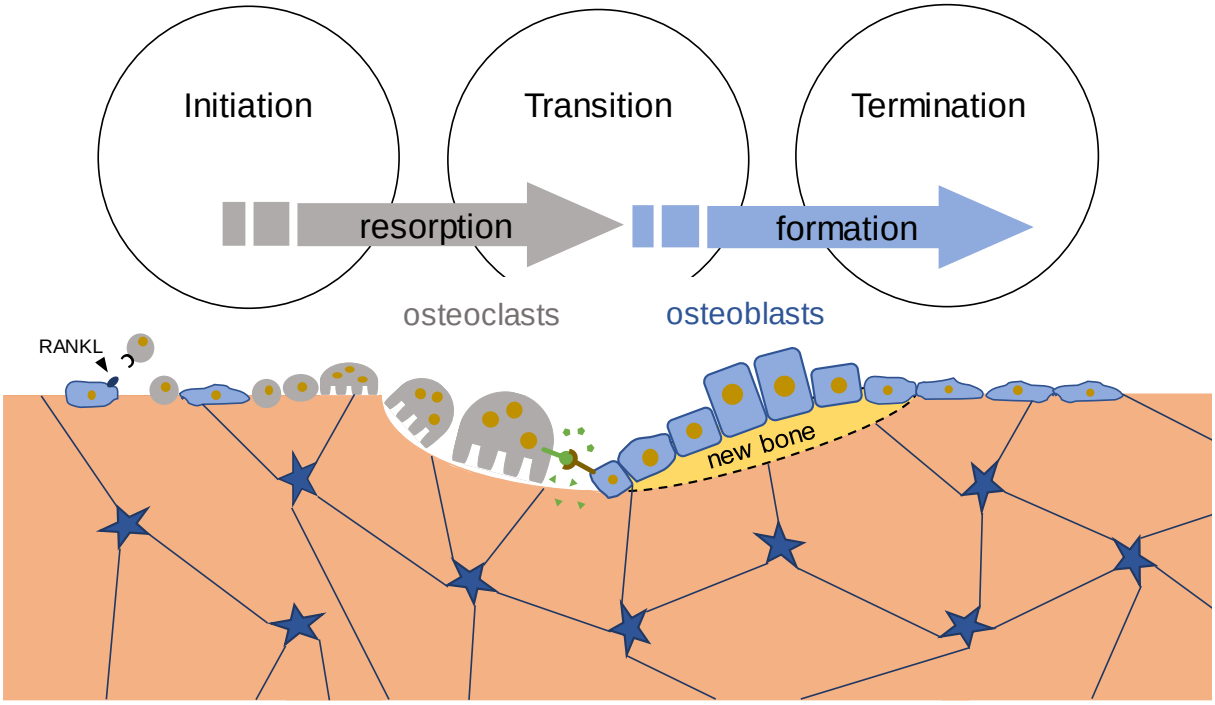
The maintenance and function of the skeletal system relies on a variety of cell types and factors, but predominantly the communication between osteoclasts and osteoblasts (T. J. Martin & Sims, 2005). However, whilst much of the maintenance is the responsibility of these two cell types, they rely on the specialised regulation and mechanosensory of osteocytes. Thus, a functional, highly communicative bone network, extending from the pre-osteoblast to the mature osteocyte and bone resorbing osteoclast is important for maintaining the integrity of bone as a tissue.

Bone remodelling

Bone is a dynamic organ that constantly undergoes remodelling, where old bone is continuously replaced by new bone. This cycle consists of three key phases, initiation of bone resorption, which is orchestrated by osteoclasts, transition from resorption to bone formation and then a long period of bone matrix formation mediated by osteoblasts, which then becomes mineralised (Sims & Gooi, 2008) (Figure 1.3). The initiation phase involves the recruitment and differentiation of osteoclast precursors to mature osteoclasts, which maintain bone resorption. This occurs by the expression of RANKL of bone lining cells which stimulates RANK on osteoclast precursors. In addition, nearby osteoblasts produce the decoy receptor osteoprotegerin (OPG), which effectively inhibits osteoclast formation and helps in regulating the extent of bone resorption (Bucay et al., 1998). As the bone matrix becomes resorbed, numerous factors are released which were embedded during the previous cycle of bone formation. This initiates the transition phase whereby the osteoclasts stimulate differentiation of osteoblast precursors through the release of various factors. These regulatory factors of bone formation include, insulin-like growth factors (IGF), fibroblast growth factors (FGF), transforming growth factor β (TGF-β) 1 and 2, BMPs and platelet-derived growth factors (PDGF) (Baylink, Finkelman, & Mohan, 1993; Linkhart, Mohan, & Baylink, 1996). The

amount of bone resorbed by the osteoclasts determines the concentration of factors released, thus ensuring bone formation remains proportional to the level of resorption. Once bone resorption has been completed the osteoclasts undergo apoptosis from released calcium and mononuclear phagocytes of haematopoietic origin or osteoblast-lineage cells complete the resorption process by forming a “reversal line” (Van Tran, Vignery, & Baron, 1982). The final termination phase, which is comprised of osteoblastic bone formation occurs at a much slower rate, taking approximately 3 months, compared to 3 weeks of bone resorption. The osteoblasts form new bone by communicating with one another via gap junctions as well as by releasing factors. Once bone formation is nearing completion and the secretion of osteoid begins to slow, osteoblasts begin differentiation into either bone lining cells, which remain on the surface or into osteocytes and become embedded into the bone matrix (Eriksen, 2010). Once bone formation has ceased and bone lining cells cover the bone surface, the matrix becomes mineralised through the signalling of osteocytes within the matrix. When the matrix is completely mineralised, it reaches a stable state until signalling cues the next cycle of remodelling. Through these intracellular communications bone can respond to various stimuli including, hormonal, mechanical and inflammatory changes. However, certain conditions can alter or impede the remodelling process which includes bone fractures, large osseous defects, and even systemic diseases such as osteoporosis. These forms of bone damage require bone formation to occur by alternate mechanisms.

Figure 1.3 *Three-phase model of bone remodelling.* Osteoclast (grey) and osteoblast (blue) lineage cells are shown on the bone surface (orange). Osteocytes (star-shaped) and the canaliculi (blue lines) are shown embedded in the bone. The initiation phase begins with the recruitment of haematopoietic precursors. Osteoblast linage cells express osteoclastogenic ligands such as RANKL to induce osteoclast differentiation. Once differentiated, the osteoclasts become multinucleated and begin to resorb old bone. The second phase, transition, represents the switch from bone resorption to formation, via coupling and diffusible factors and membrane bound molecules (green). The final termination phase involves the complete formation of resorbed bone by the osteoblasts. The mature osteoblasts eventually flatten and become bone lining cells over the new bone surface. Figure adapted from (Matsuo & Irie, 2008)


Initiation
Transition
Termination
resorption
formation
osteoclasts
osteoblasts
RANKL
new bone

Extracellular matrix

Bone is comprised of both inorganic mineral and organic matrix. Freshly synthesised osteoid prior to being mineralised, contains about 94% collagenous proteins, which are predominantly type I collagen. Other non-collagenous proteins of the organic matrix include osteocalcin, osteonectin, osteopontin, fibronectin, BSP, BMPs and other growth factors (Mizuno, Fujisawa, & Kuboki, 2000; Young, Kerr, Ibaraki, Heegaard, & Robey, 1992). These proteins are found embedded within the bone matrix and play crucial roles in signalling bone remodelling or influencing the mineralisation process (Delany et al., 2000; Ducy et al., 1996). As the organic matrix begins to be mineralised, the quantity of present mineral increases to 70% of its total content within 2 weeks. The final 30% of mineral is then slowly deposited over the next few months (Gunzburg & Szpalski, 2004). The inorganic component of bone consists mostly of phosphate and calcium, however other minerals are also abundant including, bicarbonate, sodium, potassium, citrate, magnesium and zinc (Downey & Siegel, 2006; Pereira, Rodrigues, Rodrigues, Oliveira, & Gama, 2017). The two key minerals calcium and phosphate form the major constituent of inorganic bone, called hydroxyapatite, which is represented by the chemical formula $Ca_{10}(PO_4)_6(OH)_2$. The collagenous and non-collagenous proteins combine to form a scaffold for the deposited hydroxyapatite crystals and this final association between the organic and inorganic components leads to the stiffness and strength of bone tissue (Datta et al., 2008). The calcified bone matrix provides both mechanical support and is essential in maintaining bone homeostasis. However, factors such as age, nutrition and disease can lead to varied concentrations of bone matrix proteins which can consequently contribute to increased bone fracture incidence (Tang, Zeenath, & Vashishth, 2007).

BONE DAMAGE

The highly adaptable nature of bone allows for efficient remodelling which prevents the occurrence of fractures. However, fracturing and bone damage can still arise, either by sudden loading which surpasses the bones strength, or by continuous activity of a lower loading which accumulates bone damage overtime. Once a bone fractures, the complex signalling between cells and stimuli leads to an automatic healing process to repair the bone. The healing process requires the coordination of several tissues which are directly influenced by the mechanical environment. However, in some cases, the mechanical and biological factors prevent the damaged bone from reforming properly and this results in either delayed union or non-union.

Mechanisms of bone repair

Trauma or deteriorating bone architecture, because of age or disease can reduce the bones structural integrity when combined with bone loading, leading to the creation of bone fractures. There are two distinct processes by which fractured bone can be repaired which are termed primary and secondary bone healing. Primary healing which includes contact healing, gap healing and direct union is characterised by union of the fractured bone. This form of healing can only occur in a mechanically stable environment, which can be supplied by using implants such as plates and screws (Einhorn, 1998). The haematoma that forms within the fracture gap initially after injury is removed by the contact between the fracture ends. Once removed, osteoclasts undergo a tunnelling resorptive response whereby they move from one side of the fracture to the other and form new harversian canals, by providing a pathway for penetrating blood vessels (McKibbin, 1978) (Figure 1.4A). Osteoblasts immediately follow the osteoclasts and form new osteons which bridge the fracture gap and the new bone begins to gain strength through calcification and remodelling, with the additional application of static and cyclic loading on the damaged bone (LaStayo, Winters, & Hardy, 2003).

Secondary or indirect fracture healing is the most common form of healing and is comprised of both intramembranous and endochondral bone repair. Intramembranous ossification is characterised by direct differentiation of MSCs into osteoblasts and occurs internally of the periosteum at the proximal and distal edges of the callus and forms a hard callus (Figure 1.4B). The bridging of the hard callus across the fracture gap provides the bone with the initial stabilisation and biomechanical function (Dimitriou, Tsiridis, & Giannoudis, 2005; Thompson, Miclau, Hu, & Helms, 2002). Endochondral ossification on the other hand, occurs externally to the periosteum, adjacent to the fracture site where the bone is much less stable. In addition, MSCs in endochondral bone differentiate into chondrocytes and deposit a cartilaginous matrix, which overtime is degraded and replaced by bone. Fracture repair requires several processes in order to successfully reinstate mechanical function and stability to the bone. This includes an acute inflammatory response, recruitment of MSCs, generation of a cartilaginous callus, revascularisation, mineralisation of the callus and final bone remodelling. Immediately following fracturing, a haematoma forms and consists of peripheral and intramedullary blood and bone marrow cells. The injury also initiates an inflammatory response and whilst it is known that chronic inflammation is detrimental to bone, the brief and highly regulated secretion of pro-inflammatories in the form of an acute response, is essential to the repair process. This acute response peaks within the first 24 hours of injury and is completed by 7 days post injury. Several pro-inflammatory factors are released at this time and include, chemokines, tumour necrosis factor-α (TNF-α) and interleukin-1 (IL-1), IL-6, IL-8, IL-11 and IL-18 (Gerstenfeld, Cullinane, Barnes, Graves, & Einhorn, 2003; Koch et al., 2001; Y.-C. Liu et al., 2014; Volpin et al., 2014). These factors are essential for the recruitment of inflammatory cells, differentiation of osteoblasts and osteoclasts and promote angiogenesis (Sfeir, Ho, Doll, Azari, & Hollinger, 2005; Yang et al., 2007). For the formation of new bone to repair the fracture, MSCs must be recruited from the surrounding soft tissue and bone marrow as well as

systemic circulation, followed by differentiation into osteogenic cells. The ability for MSCs to be recruited to sites of bone damage is regulated by stromal cell-derived factor-1 (SDF-1, CXCL12) and its receptor CXCR4, expressed on MSCs (Kitaori et al., 2009). Once MSCs are recruited, a molecular cascade leads to collagen type I and II matrix production and the upregulation of several signalling factors. The generation of an initial cartilaginous callus which later becomes mineralised, resorbed and replaced with bone is critical in the healing process. This endochondral formation occurs in between the fracture ends and forms a less stable soft tissue callus. During this process TGF-β and GDF-5, a close member of the BMP family promote chondrogenesis and endochondral ossification (T. J. Cho, Gerstenfeld, & Einhorn, 2002). At the same time, BMP-2, -5 and -6 induce cell proliferation and initiate intramembranous ossification at the periosteal sites. This generates a hard callus and once it has bridged the fracture gap it provides the site with a semi-stable structure for weight bearing (Marsell & Einhorn, 2009). Another critical component for successful fracture healing is the revascularisation of the fracture gap. This process firstly requires chondrocyte apoptosis and degradation of the cartilaginous matrix, to allow the ingrowth of blood vessels at the fracture site (Ai-Aql, Alagl, Graves, Gerstenfeld, & Einhorn, 2008). Vascular endothelial growth factor (VEGF), expressed in high levels by osteoblasts and chondrocytes, is critical in the regulation of vascular regeneration within the fracture gap, by promoting blood vessel invasion and transforming the cartilaginous matrix into a well-vascularised osseous tissue. Here, VEGF promotes both the proliferation of endothelial MSCs into a vascular network and angiogenesis (Keramaris, Calori, Nikolaou, Schemitsch, & Giannoudis, 2008). Once vascularisation has been established the cartilaginous callus is resorbed and replaced with a bony callus. This is brought about primarily by M-CSF, RANKL and OPG, which initiate resorption of the mineralised cartilage and recruit osteoblasts and osteoclasts to begin forming new bone (Kon et al., 2001). The Wnt-family is also involved in the differentiation of MSCs into osteoblasts and promote

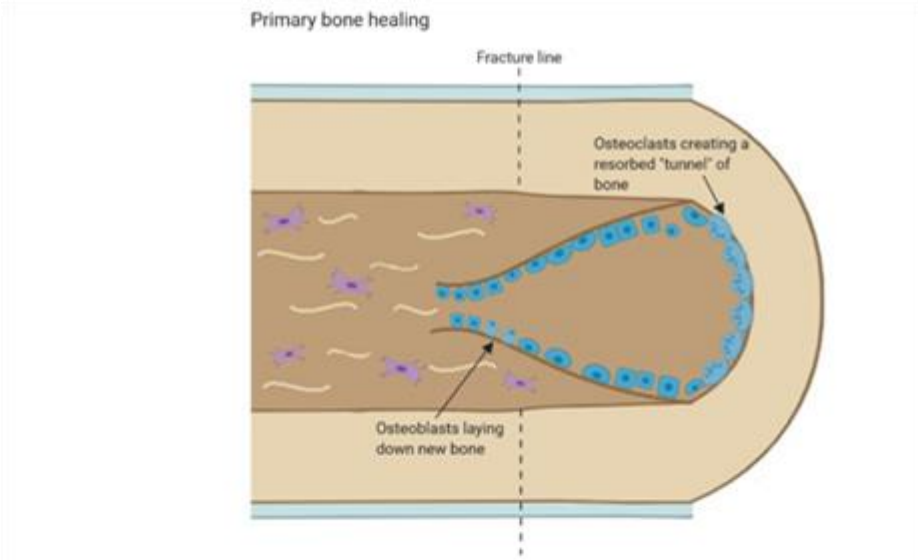
bone formation (Chen & Alman, 2009). Other molecules such as matrix metalloproteinases (MMPs) are also involved in endochondral ossification. This family of proteases is responsible for extracellular matrix (ECM) degradation and subsequent bone remodelling (Ortega, Behonick, & Werb, 2004). BSP is also observed near the resorptive front between the vascular bone and remaining cartilage where it acts as the nucleator for hydroxyapatite crystals (Scammell & Roach, 1996). As the hard callus forms, the calcified cartilage is replaced with woven bone and the callus within the fracture gap becomes increasingly stabilised. The final process to complete fracture healing is remodelling of the hard callus to normal bone. This involves the activation of a second resorptive phase whereby woven bone is resorbed and replaced with mature lamellar bone, which is orchestrated by IL-1, TNF-α and BMP-2 (Ai-Aql et al., 2008; Marsell & Einhorn, 2009). The process can take 3-4 weeks in humans, however, the completion of remodelling and a fully restored bone structure can take several years (Hall, 1963). In cases where mechanical stability is not created from bone remodelling or there is an inadequate blood supply, the development of fibrous non-union is likely to occur. This is also the case for larger fractures where bridging of the fracture ends is not successful.

Interestingly, the repair mechanisms of bone damage are not consistent between all bones of the skeleton. In long bones and limbs which are fractured and damaged, bone healing occurs via intramembranous and endochondral ossification. However, in flat bones such as calvarial and facial bones, the repair process occurs by intramembranous ossification alone, with no chondrogenic cells present (Alberius & Johnell, 1991). Furthermore, comparisons of bone formation between long and flat bones have revealed that the rate of healing in long bones occurs much more rapidly than flat bones. This is likely due to the absence of mechanical loading of the flat bones, which is a key physiological factor for regulating bone formation (Lim, Lee, Yun, Shin, & Park, 2013). These differences in healing rate between bones must be considered in cases where a scaffold material is required to aid the repair process. For instance,

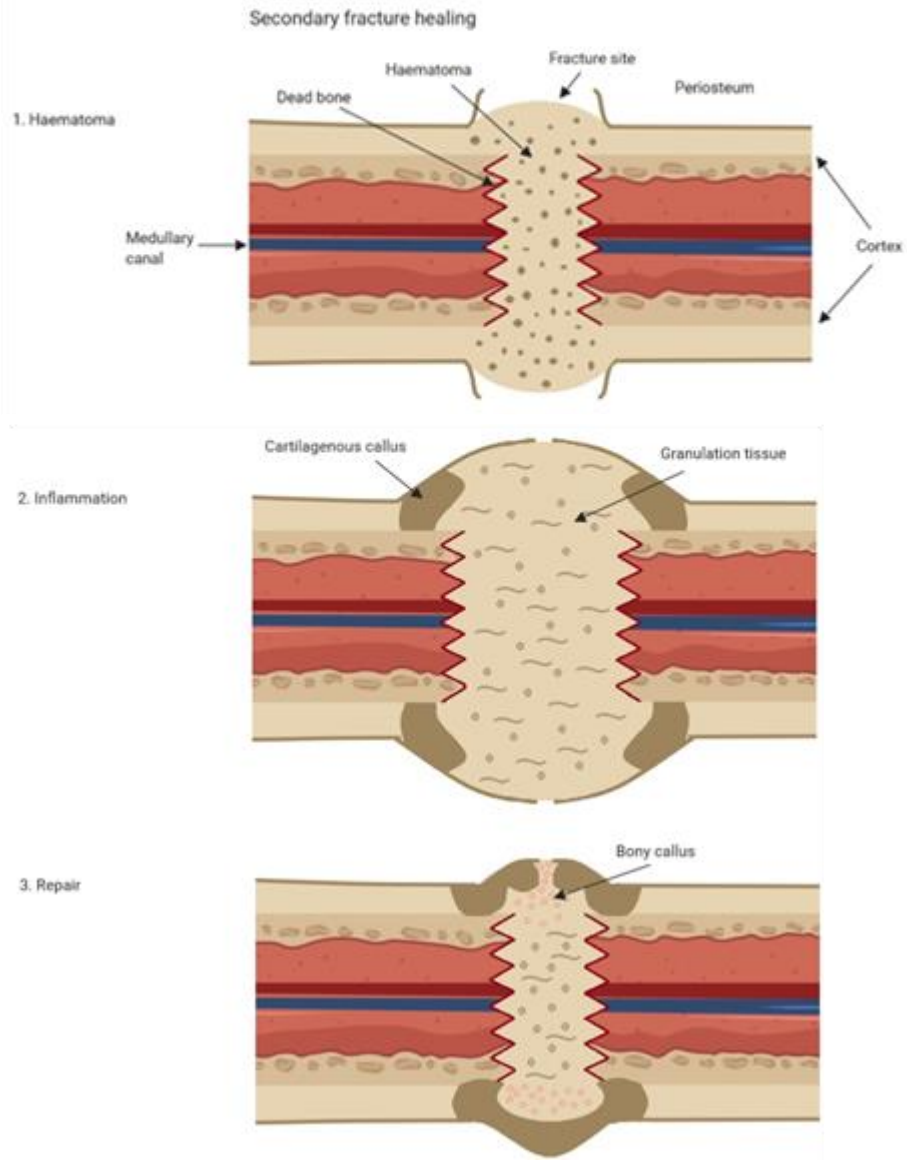
fast resorbing scaffolds would only be suitable in long bones to match the rate of new bone formation.

Figure 1.4 *Primary and secondary fracture healing.* Healing of a fracture is dependent on the stability of the fracture site. When bone is undergoing primary fracture healing, osteoclasts osteoclasts from the intact bone move across the fracture gap via tunnelling, resorptive mechanisms, to re-establish Harversian Canals. Along with the formation of new blood vessels, osteoblasts follow the tunnelling osteoclasts and produce new bone and fill the canals with osteons (A). Secondary fracture healing undergoes a series of phases in order to restore mechanical stability in addition to repairing the fracture. The three phases of fracture healing begin with the initial haematoma (B1). Bleeding from the bone and surrounding soft tissue results in a haematoma, providing some stability. The clotted site provides a framework for an inflammatory response (B2), leading to an influx of numerous cell types and release of various cytokines. Granulation tissue also begins to replace the haematoma and a cartilaginous callus begins to form. During the repair phase (B3), the external cartilaginous callus is converted into a bony callus, followed by bone remodelling. Figure adapted from (LaStayo, Winters, & Hardy, 2003).

A


Primary bone healing
Fracture line
Osteoclasts creating a resorbed "tunnel" of bone
Osteoblasts laying down new bone

B


Secondary fracture healing
1. Haematoma
Haematoma
Fracture site
Dead bone
Periosteum
Medullary canal
Cortex
2. Inflammation
Cartilagenous callus
Granulation tissue
3. Repair
Bony callus

Limitations of bone regeneration and repair

Despite the characteristic regenerative capabilities that bone possesses, there are an array of conditions which negatively affect and impede the repair process. One of the main limiting factors of bone regeneration is the extent of damage, which beyond a certain degree or size will not naturally regenerate. Even in the case of smaller fractures, numerous factors can compromise the repair process, which include the patient's systemic status, nature of the injury, local host response and pharmacologic factors.

When considering a patient's systemic status there is an increased occurrence of fractures in women compared to men after the third decade of life (Hedlund & Lindgren, 1987). However, there is no specific data pertaining to gender and delayed healing. Whilst gender does not directly affect healing, aging and its associated health conditions can impact the repair process. Hormone deficiency, for example oestrogen, as a result of menopause in women is one of the most important factors in the pathogenesis of osteoporosis and as a consequence, significantly affects early fracture healing (Richelson, Wahner, Melton III, & Riggs, 1984). The link between hormone deficiency and delayed union have been further supported by replacement therapy studies, which have demonstrated that maintaining normal hormone levels is critical in ensuring bone healing (Felson et al., 1993).

Location of bone damage is another critical determinant of successful healing. It has been well reported that bone fractures can cause disruption of the bloody supply and consequently the bone marrow (Carano & Filvaroff, 2003). If the vascularisation is compromised, which supplies the osteogenic cells, then it is expected that disruption would impede the repair process (Gittens & Uludag, 2001). Another contributor to delayed or non-union is deep infection at the wound site. An infected non-union is defined as a persistent infection at the fracture site for a duration of 6-8 months (Meyer, Weiland, & Willenegger, 1975). In addition to resulting in delayed healing, infection at the fracture site also prevents

stable fixation which leads to mechanical instability (Friedrich & Klaue, 1977). Furthermore, the generation of a chronic inflammatory reaction in response to infection can also have deleterious effects on bone regeneration (Thomas & Puleo, 2011).

Within the host, the availability of collagen significantly influences callus formation and overall fracture healing. In a mechanically stable fracture, collagen type I is the major collagen type present, with collagens type II and V present within the trabeculae (Page, Hogg, & Ashhurst, 1986). Collagen type III is another crucial protein found at fracture sites and is expressed in early osteoblast differentiation. As the osteoblast matures and remodelling progresses, the level of collagen type III rapidly declines. However, in the case of a non-union it has been shown that type III collagen levels remain elevated and there is an apparent elongation of the osteoblasts early phase, resulting in fibrous tissue formation and consequently impaired bone repair (Lawton, Andrew, Marsh, Hoyland, & Freemont, 1997).

Finally, pharmacologic factors administered prior to fracturing or damaging bone can significantly impair the healing process. This includes systemic corticosteroid use which has been shown to inhibit bone repair, by reducing the synthesis of collagen type I and growth factors, as well as decreasing osteoblast activity (Waters et al., 2000). In addition, whilst chronic inflammatory responses can be detrimental to the repair process, the use of nonsteroidal anti-inflammatory drugs (NSAIDs) has been shown to have inhibitory effects on fracture healing by impeding blood flow to the fracture gap (Murnaghan, Li, & Marsh, 2006). It has also been shown that patients who smoke were 37% more likely to develop non-union and twice as likely to develop infection, which can have further detrimental effects on repair (Gaston & Simpson, 2007). With many reported factors that can influence non-union, the requirement to intervene to correctly repair the defect is essential. This can be done through orthopaedic surgery, by applying bone graft materials with or without fixations to bridge the defect or fracture and promote healing.

Types of non-union

A bone fracture is termed a non-union when the fracture ends fail to unite and requires additional treatment before successful healing can occur. Traditionally, non-unions are classified according to their radiographic appearance, as either hypervascular or avascular (Scott P Bruder, Fink, & Caplan, 1994). Hypervascular non-unions are associated with a sufficient blood supply with decreasing callus formation and appear as an elephant-foot or horse-shoe configuration (Panagiotis, 2005) (Figure 1.5). Additionally, oligotrophic non-unions have an adequate blood supply but minimal callus formation. The adequate blood supply present in these forms of non-union suggests the presence of a biological response and therefore the absence of fracture healing is due to insufficient mechanical stability. Alternatively, avascular non-unions have little or no callus formation due to an insufficient blood supply or bone forming cells (Phieffer & Goulet, 2006). These situations arise following bone loss or removal of the periosteum. Types of avascular non-unions include, severely displaced or comminuted fractures, defect non-unions, atrophic non-unions, that are comprised of thin fracture ends with excessive scar tissue and torsion wedge non-unions, that arise when the bone fragment has only healed at one end (Ebnezar & John, 2016; Panagiotis, 2005) (Figure 1.6). Avascular non-unions can arise not only from high-impact injuries but also following multiple failed procedures leading to significant bone loss (Alt et al., 2006). The treatment of non-unions involves the use of therapeutic interventions that improve the mechanical and/or biological environment at the fracture site. In cases where there is sufficient callus formation, such as hypervascular non-unions, the main cause of failure to heal is due to poor fracture stability. Once mechanical stability is achieved, calcification of the fibrous cartilage occurs, by the penetration of new blood vessels, allowing for bone remodelling and eventually bone union (Schenk, Müller, & Willenegger, 1968). Unlike hypervascular non-unions, avascular non-unions require both fracture stability and biological support. In such cases, the defects must be

filled with bone graft materials and supported by stable fixation. Currently, there are numerous methods of fixation to stabilise non-unions and a variety of bone graft materials that provide scaffolding, promote cellular differentiation and contain growth and differentiation factors which are necessary for successful fracture healing (Ring, Barrick, & Jupiter, 1997).

Figure 1.5 *Types of hypervascular non-unions.* Highly vascularised non-unions are defined by their radiographical appearance. The presence of a callus defines them as either, elephant-foot non-unions (A) or horse-shoe non-unions (B). Hypervascular non-unions with no observable callus are termed oligotrophic non-unions (C) (Ebnezar & John, 2016).

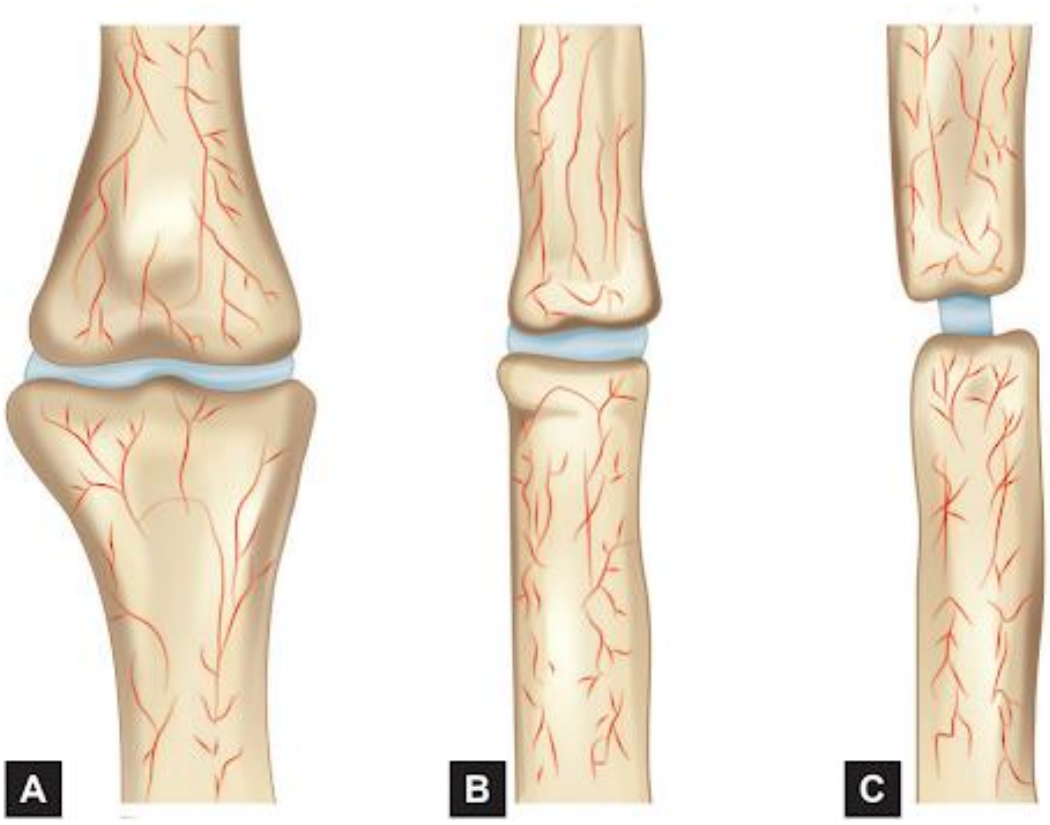
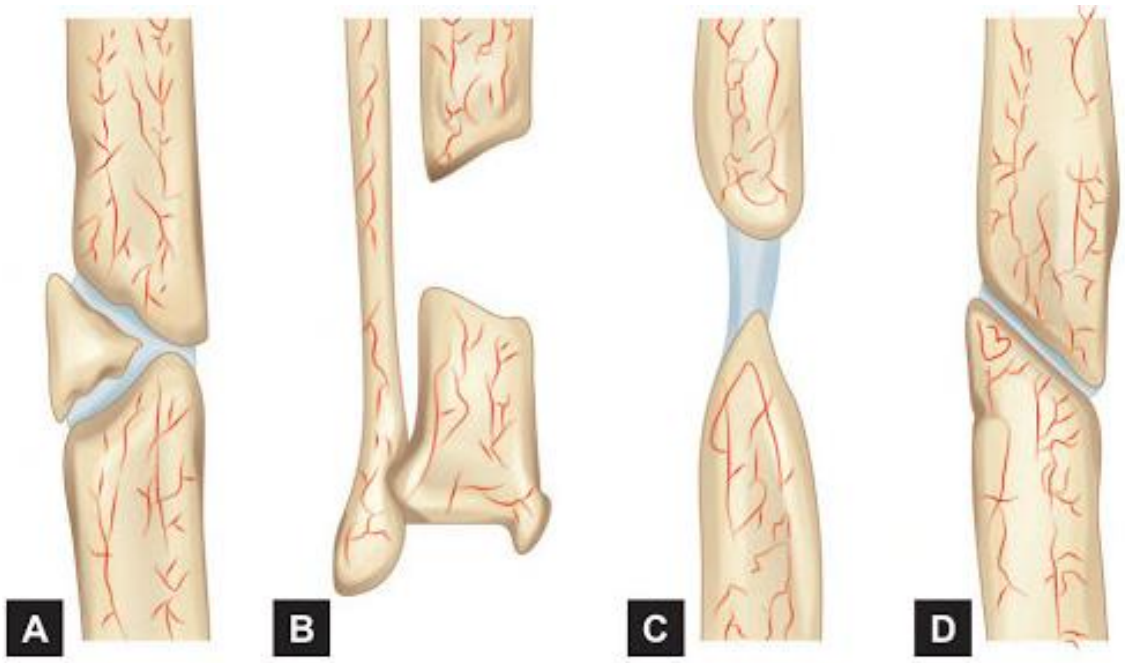

A
B
C

Figure 1.6 *Types of avascular non-unions.* Avascular non-unions are defined as bone fractures with little blood supply and are defined based on their appearance and size. Comminuted non-unions (A); Defect non-union (B); Atrophic non-union (C); Torsion wedge non-union (D) (Ebnezar & John, 2016).


A
B
C
D

BONE GRAFTING

The repair of large bony defects and non-unions that arise as a result of bone disease, trauma or tumour removal remains a significant challenge to orthopaedic surgeons. If bone damage is neglected, development of non-unions can occur, whereby the bone cannot repair itself correctly, which often leads to pain, stiffness of nearby joints and loss of mobility. Along with the associated morbidities, this disability often results in the patient being unable to resume normal duties in employment, which causes significant financial burden to the patient and the economy (Babhulkar, Pande, & Babhulkar, 2005; Bondurant, Cotler, Buckle, Miller-crotchett, & Browner, 1988; Kanakaris & Giannoudis, 2007). Therefore, in order to restore and stabilise the bone, bone grafting or the use of bone graft substitutes is required. To further complicate matters, the demand for functional bone grafts is becoming extremely high, with an estimated 2.2 million patients undergoing treatment for bone defects annually worldwide. This generates huge economic cost to health care and these numbers are expected to double by 2020, due to population ageing (B. Baroli, 2009; Giannoudis, Dinopoulos, & Tsiridis, 2005).

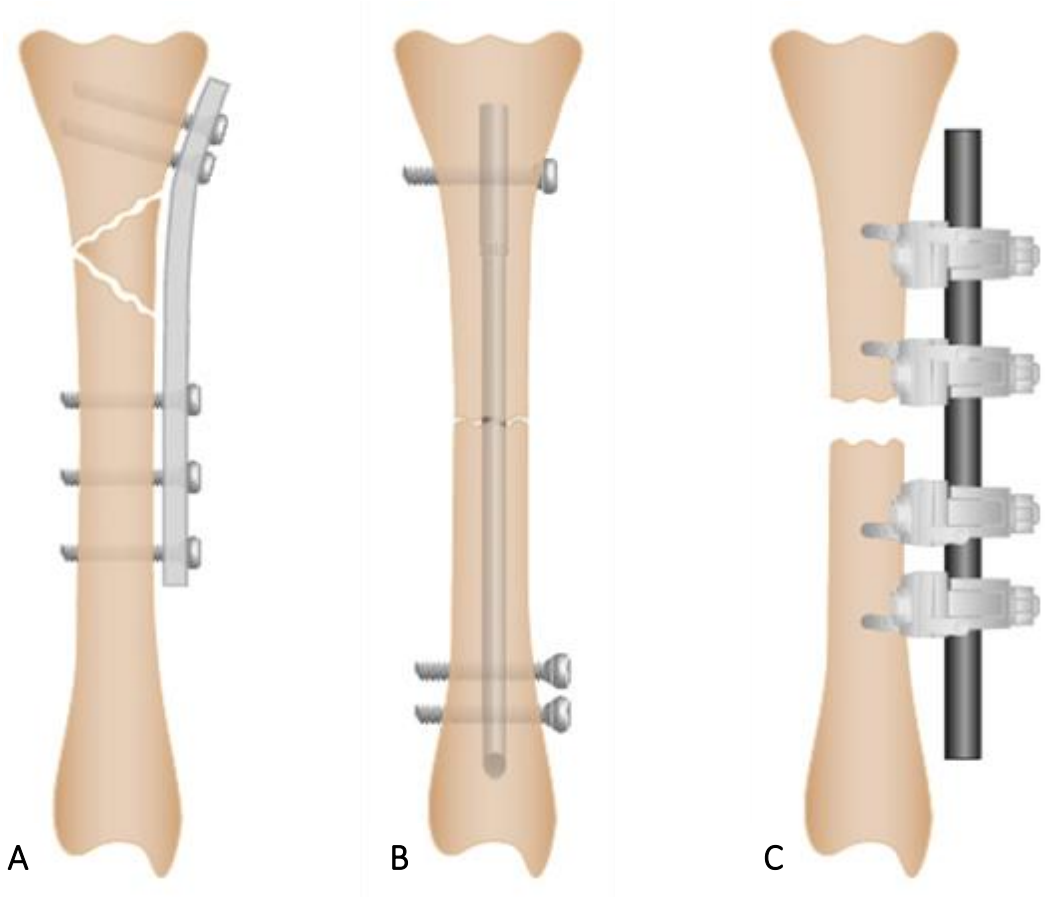
Fracture stabilisation

To repair large bone defects which are likely to undergo delayed or non-union, the mechanical and biological problems must both be addressed. Stabilisation of the bone is essential in ensuring the additional biological component can promote successful bone repair. Two forms of fixation are currently available, internal and external fixation (Figure 1.7). Plate fixation and intramedullary nailing are the most popular treatment options of internal fixation, by their ability to provide adequate stability without causing excessive rigidity (Rodriguez-Merchan & Forriol, 2004). Plate fixation can be used with or without bone grafts and is often accompanied by compression, which encourages ossification of the non-union. The disadvantages of using plates to repair non-unions is the increase in damage of the local

vasculature. Furthermore, the risk of infection is slightly higher compared to simple bone grafting techniques of uninfected tibia non-unions (Smith, 1974). Intramedullary nailing is the most common form of internal fixation and is the preferred treatment for most diaphyseal non-unions. Unlike plate fixation, reamed intramedullary nailing provides the advantage of reaching full function earlier, including weight-bearing and motion of adjacent joints. This is due to the effect of reaming, which increases the contact area between the nail and the rough surface of the medullary cavity. In addition, reamed intramedullary nailing possesses a broad range of applications, primarily for the treatment of uninfected non-unions. However, treating an infected, or previously infected non-union by nailing, has been associated with increased risk of infection (Phieffer & Goulet, 2006). Despite the association with infection, there have been reports of 100% successful union using reamed intramedullary nailing for treating tibia non-unions (Megas, Panagiotopoulos, Skriviliotakis, & Lambiris, 2001). In cases of non-unions with bony defects, it is recommended that the reamed nailing is supplemented with bone graft materials (Finkemeier & Chapman, 2002). For patients who present with delayed union and a previous infection, external fixation is the preferred method of stabilisation. External fixators present as either unilateral or circular fixation systems that provide several advantages including, wound access, distant stabilisation of the bone fragments and motion of adjacent joints which encourages patient mobility. Furthermore, external fixators cause less disruption to soft tissues and blood supply, which makes them beneficial for patients who present with open wounds or skin contusions (Fragomen & Rozbruch, 2007). Often, external fixation can successfully unite bone fractures, however in some patients the fixation can be ineffective and must be replaced. If the reoperation involves opening and exposing the non-union, it is recommended that bone grafting be done at the same time. Alternatively, if new fixation is performed without opening the non-union site it is recommended that bone grafting is delayed

(Rodriguez-Merchan & Forriol, 2004). In most cases, reoperation of an internal or external fixator requires the addition of a bone graft of graft substitute.

Figure 1.7 *Popular techniques of fixation for fractures.* Commonly used internal fixation strategies include, plate fixation (A) and intramedullary nailing (B). In place of internal fixation, an external fixator (C) can provide greater stabilisation, which is often required in more complex non-unions. Figure adapted from (Claes, 2017).


A
B
C

Osteogenic, osteoinductive and osteoconductive properties

Once mechanical stability has been achieved, the biological issues associated with large bone defects can be addressed, through the use of bone grafts. Three major characteristics of bone graft and graft substitutes that are essential to bonding, or osteointegration of the graft material to the host bone are osteogenesis, osteoinduction and osteoconduction. To successfully qualify as a bone graft, the material must possess at least one of these functions. Grafting materials harvested from donors may contain living osteoprogenitor cells which can survive transplantation and have the potential to differentiate into osteoblasts and osteocytes. The ability for grafting materials to contain these viable cells characterises the material as osteogenic (Cypher & Grossman, 1996). Bone graft and graft substitutes which stimulate proliferation of stem cells and the differentiation of stem cells to osteogenic cells are termed osteoinductive (Perry, 1999). Several growth factors are responsible for influencing this process and include BMPs, PDGFs, FGFs and other members of the TGF-β superfamily (Cypher & Grossman, 1996). Finally, the term osteoconductive refers to the graft material's ability to provide an interconnected structure which allows new cell migration and blood vessel formation, whilst also providing a framework for new cell attachment of osteoblasts and osteoprogenitors (Hak, 2007).

Autogenous and allogenic bone graft

Autogenous bone grafts which is bone tissue harvested from the patient is considered the current gold standard for reconstruction of large bone defects, as it possesses osteogenic, osteoinductive and osteoconductive properties. It also provides minimal immunogenic rejection and ample histocompatibility (Bauer & Muschler, 2000a; Homma, Zimmermann, & Hernigou, 2013; Perry, 1999; Samartzis, Shen, Goldberg, & An, 2005). The bone is often harvested from the iliac crest, but can also be taken from the distal femur, proximal tibia, fibula, ribs and radius,

before being implanted into the damaged site (Dimitriou, Mataliotakis, Angoules, Kanakaris, & Giannoudis, 2011). Although autologous bone is the best replacement material for a defect or fracture site, this method has a complication rate of up to 30%. Complications include, donor-site morbidity and pain from harvesting, extended hospitalisation and also surgical-associated morbidities such as, infection, inflammation and chronic pain (Ami R. Amini, Cato T. Laurencin, & Syam P. Nukavarapu, 2012; Banwart, Asher, & Hassanein, 1995). The quantity of bone that can be harvested is also very limited, which can pose a problem for larger defects (Jimi et al., 2012).

A common alternative to autogenous grafting is the use of allogenic bone, which accounts for approximately one-third of bone grafting procedures (T. Boyce, J. Edwards, & N. Scarborough, 1999). The use of allografts which are taken from donors or cadavers eliminates several patient morbidities and issues associated with limited supply. However, allografting also presents a number of hazards, including risk of disease transmission from the donor. The risk of HIV transmission is estimated to be 1 in 1.6 million and other viruses including hepatitis B and C have also been previously reported to be transmitted through allograft material (T. Boyce et al., 1999). Thorough screenings of donors and better sterilisation techniques such as tissue freezing and sterilisation by radiation have aided in minimising disease risk (Vangsness et al., 2003). However, these processing techniques eliminate any osteogenic capabilities and negatively affect the osteoinductive and osteoconductive potential of the graft material (Lane & Sandhu, 1987), which significantly decreases their biological capacity (Anderson et al., 1999; Oklund, Prolo, Gutierrez, & King, 1986).

Due to the above-mentioned limitations of natural bone grafts, a wide variety of synthetic bone graft substitutes have been developed. A summary of various bone graft and bone graft substitutes and their respective properties are described in Table 1. Many of these alternative graft substitutes utilise a range of materials, including natural and synthetic

polymers, ceramics and composites. Additionally, factor and cell-based strategies have been investigated, which can be used alone or in combination with other materials (Laurencin, Khan, & El-Amin, 2006). The development of a successful graft however, must possess the osteogenic, osteoinductive and osteoconductive characteristics, to ensure effective bone healing.

Table 1.1 Properties of bone graft and bone graft substitutes

Class	Description	Properties of action
Autograft	Used alone	• Osteogenic • Osteoinductive • Osteoconductive
Allograft	Used alone or in combination with other materials	• Osteoinductive • Osteoconductive
Ceramic based	Includes calcium phosphate, hydroxyapatite, used alone or in combination with other materials. e.g., Mastergraft®	• Osteoconductive
Collagen based	Biodegradable scaffold used alone or in combination with other materials. e.g., BioMend, CollaCote	• Osteoconductive
Factor based	Natural and recombinant growth factors used alone or in combination with other materials. e.g., BMPs	• Osteoinductive

Ceramic and collagen bone graft substitutes

Approximately 60% of bone graft substitutes currently available contain ceramics. Common commercially available forms include porous implants, non-porous implants and granular particles with pores, which all possess excellent osteoconductivity. The interest in using ceramics, specifically those comprised of calcium phosphates (CaP) is mostly because the primary inorganic component found in bone is calcium hydroxyapatite (HA), a subset of calcium phosphate (Zwingenberger et al., 2012). Whilst HA is naturally occurring in bone and has osteoconductive and osteointegrative properties, the high Ca/P ratio (1.67) and crystallinity causes a slow resorption rate (W. Wang & K. W. K. Yeung, 2017). As an alternative to HA, beta-tricalcium phosphate (β-TCP) use has been explored as it possesses a lower Ca/P ratio (1.5) which enables accelerated degradation and resorption (Torres, Tamimi, Alkhraisat, Prados-Frutos, & Lopez-Cabarcos, 2011). However, this results in significantly weaker mechanical properties (LeGeros, 2002). To address this issue, HA and β-TCP are combined to form biphasic calcium phosphate (BCP), where the resorption rate and mechanical properties can be controlled. A BCP ratio which has been rigorously tested and deemed suitable commercially is 15% HA:85% β-TCP, which is provided by several companies, including the Mastergraft® products provided by Medtronic (Khan & Lane, 2004).

Collagen type I is the main organic component of bone, accounting for 97% of protein found in bone (Glimcher & Lian, 1989). Collagen which serves as a template for biological bone, is one of the most commonly used scaffold materials, with a variety commercially available e.g., CollaCote™ and BioMend®. Type I collagen scaffolds are known to have good biocompatibility, biodegradability, osteoconductivity and weak antigenicity (W. Wang & K. W. K. Yeung, 2017). Furthermore, collagen scaffolds have been studied exhaustively and have been shown to support sustained release of a variety of osteoinductive factors, which makes them highly effective at repairing bone (Geiger, Li, & Friess, 2003; Winn, Uludag, & Hollinger,

1999). One drawback of collagen-based materials are the mechanical properties, which are very weak and so, these materials are often found in the form of composites such as collagen-HA (Kikuchi et al., 2004). Therefore, consideration of the bone type and mechanical properties are needed for graft substitutes. High weight-bearing bones, such as the femur, when fractured or presenting with a non-union require greater stiffness and structural stability. Whereas low weight-bearing applications such as the repair of cranial damage can benefit from materials that provide accurate aesthetic remodelling with reduced weight-bearing support. Although collagen and ceramic graft materials demonstrate high biocompatibility and osteoconductivity, new bone formation is often limited due to their poor osteoinductive capabilities (Yuan et al., 2002), therefore the addition of growth factors or cells is often required.

Bone morphogenetic proteins

Growth factors and proteins which reside in bone are responsible for the regulation of numerous cellular functions, including the production and resorption of bone. BMPs which are members of the TGF-β superfamily, are biologically active molecules with the ability to promote recruitment, proliferation, differentiation and migration of bone-forming cells (Lind, Eriksen, & Bünger, 1996). The capacity for BMPs, specifically BMP-2, 4 and 7 to bind to receptors on MSCs, osteoblasts and chondrocytes and promote their activity makes them potential osteoinductive agents for clinical use (Kloen et al., 2003). BMPs are often delivered to a surgical site by a variety of graft materials including allogenic bone and synthetic scaffolds. The osteogenic and osteoinductive potential of BMPs has been thoroughly proven in both preclinical and clinical studies with an analogous performance to autogenous bone (Boden, 2001; Friedlaender et al., 2001; Mussano, Ciccone, Ceccarelli, Baldi, & Bassi, 2007). Despite the potential of BMPs, only recombinant human BMP-2 and 7 have been approved for select use in Europe and the United States, where they have been used in spinal fusion surgery,

craniomaxillofacial surgeries and as a replacement for autogenous bone in tibial non-unions (Boden & Schimandle, 1995; Ristiniemi et al., 2007; Vaccaro et al., 2005). Although reports suggest multiple benefits for the use of BMPs in orthopaedic surgery, there are numerous complications to consider. In spinal surgery alone there have been reported complications of neurological impairment, bone resorption at the graft site and neck swelling (Benglis, Wang, & Levi, 2008; Wong, Kumar, Jatana, Ghiselli, & Wong, 2008). These reports, along with the significant high cost of using BMPs (Garrison et al., 2007) suggests the requirement to identify other factors possessing osteoinductive properties, that result in fewer adverse side effects and be of greater economic value.

PRE-CLINICAL MODELS

In order to biomechanically simulate humans as accurately as possible and assess the effects of experimental bone graft substitutes of defect and fracture repair, numerous animal models have been established. With the availability of numerous animal species and experimental approaches to test the capacity of graft materials, several factors need to be taken into consideration when determining the appropriate model for the tested material. Species of animal, age of the animal, type of defect, stability of the defect and anatomic location all have an influence on the quality and type of bone that forms within the defect site (Bosch, Melsen, & Vargervik, 1998). Therefore, researchers must ensure that the biological, biochemical and physiological characteristics of an animal species and the selected defect model will accurately replicate the repair mechanisms anticipated in humans.

Non-union models of bone repair

A critical prerequisite for any model used to test bone graft substitutes is to ensure that the empty defect heals more slowly than the experimental defect. The ideal scenario for any model is one by which the defect undergoes osseous union only with the addition of an applied bone graft substitute. A wound of this size is termed a critical size defect and was defined by Schmitz and Hollinger as the smallest intraosseous wound that would not heal by bone formation during the lifetime of the animal (Schmitz & Hollinger, 1986). As a result, the defect heals by fibrous connective tissue formation, not by new bone regeneration. It is hypothesised that this is due to the inability for cells to differentiate into osteoblasts and chondroblasts in the central region of the defect due to a lack of tissue factors (e.g., BMPs) to induce a rate of cellular differentiation. The reduced presence of these cells results in a failure to mineralise the matrix within the central region of the defect, leaving behind a dense fibrous connective tissue (Schmitz, Schwartz, Hollinger, & Boyan, 1990). Whilst the size of the defect is essential when

utilising this model, a number of other factors must also be considered which can influence healing of the defect, including animal species, animal age, anatomic location of the defect, bone structure and vascularisation and mechanical loading (Reichert et al., 2009; Schmitz & Hollinger, 1986).

Animal species as a testing model

Several animal species have been used to simulate the human *in-vivo* environment, physical conditions and comparability of bone graft substitutes, including rodents, rabbits, dogs, sheep, goats and pigs. When selecting a specific animal as the test model, certain factors must first be considered. Firstly, the animal model must clearly demonstrate comparable physiological and pathophysiological similarities to humans. Further selection criteria that must also be considered is cost of animal procurement, availability, tolerance to captivity and ease of housing (Pearce, Richards, Milz, Schneider, & Pearce, 2007). Additionally, it is important to consider the number of implants per animal, duration of the experiment and expected differences in biological response, before determining the most suitable animal species (Upman, 2006). Whilst there are numerous animal models available to test bone graft materials, as mentioned above, this thesis will focus primarily on rodents and sheep.

Rodents are a commonly used animal for bone-related research as they are small, easy to handle and relatively inexpensive to obtain and house, which is important to consider due to the long housing times required in orthopaedic research. For these reasons they are often considered the first *in-vivo* model for assessing the effectiveness of experimental bone graft substitutes (Gomes & Fernandes, 2011). Implantation of graft substitutes such as ceramics, collagen, BMPs and stem cells in various rat bone defect models have all successfully demonstrated osteoinductive and osteoconductive capabilities (Kirker-Head et al., 2007; Yoon,

Dhar, Chun, Gharibjanian, & Evans, 2007). This included the use of critically sized femoral and calvarial defects. It is also a common animal subject to study multiple aspects of bone regeneration, including fracture, defect repair, joint infection and osteoporosis (An & Freidman, 1998). Although mice are a much smaller rodent than rats, they have become increasingly popular as an animal model for skeletal research, specifically to screen potential novel substances for osteogenesis and chondrogenesis (An & Freidman, 1998). A highly useful model used in mice is the critical-size calvarial defect model, with a defect size > 2 mm considered a critical non-healing defect (Aalami et al., 2004; Cowan et al., 2004; Im et al., 2013; Levi et al., 2010). Whilst both rats and mice possess numerous advantages as pre-clinical animal models for investigating and assessing the potential of novel bone graft substitutes, there are limitations to consider. In particular, compared to larger animals such as sheep, rodents have much smaller long bones which can be difficult to use in fixation models and do not form haversian-type bone tissue, unlike larger animals and humans (Li et al., 2015; M Martiniaková, Grosskopf, Vondráková, Omelka, & Fabiš, 2005).

The use of small animals provides researchers with a significant amount of knowledge with regards to the effectiveness of tested bone graft substitutes. However, large animal models, such as sheep have been developed to verify the potential of novel materials, closer to a clinical setting. The advantages of using mature sheep is firstly, they possess a body weight similar to adult humans and the comparable dimensions of their long bones to humans allows for the use of human implants (Newman, Turner, & Wark, 1995). Secondly, the load bearing of the hind limbs and forces exerted is roughly half of what humans experience when walking (Taylor et al., 2006), which allows for comparisons of mechanical loading of implants in sheep to humans possible. Also, sheep have a rate of bone healing similar to humans (den Boer et al., 1999). This makes sheep a very useful model for femoral and tibial defect and fracture models. They have also been used as a model to assess the repair of mandibular, calvarial and metatarsal defects

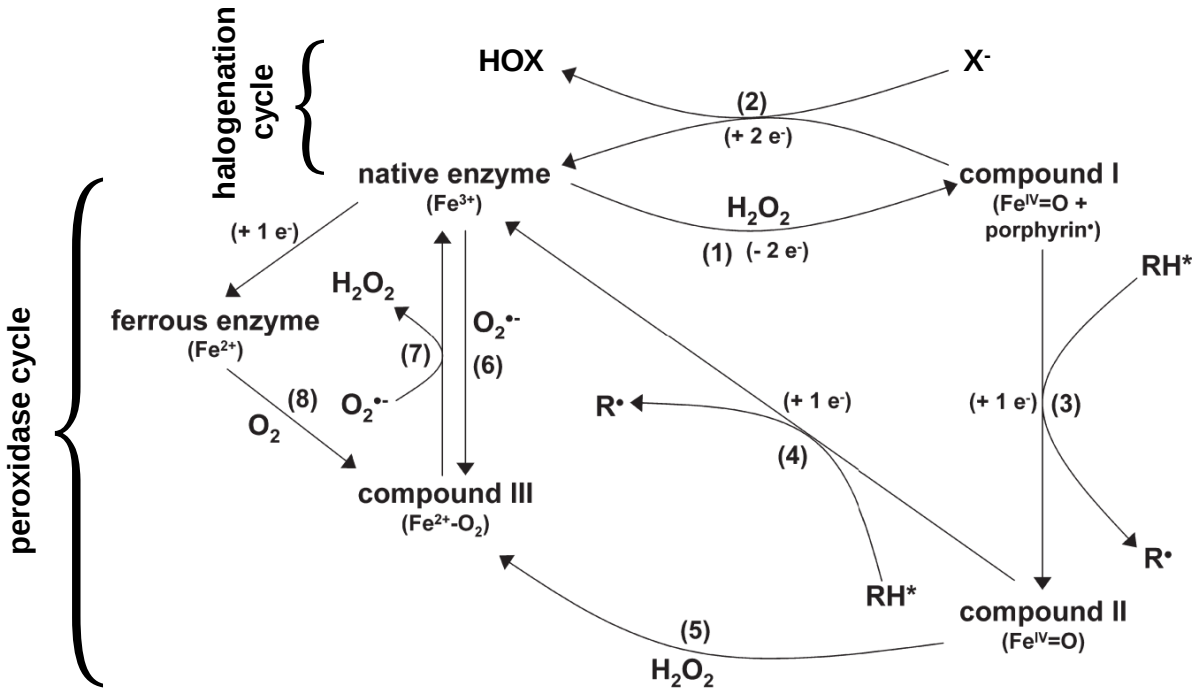
(Reichert et al., 2009). The creation of large segmental defects and critical sized defects are the most common models for sheep in orthopaedic research and a segmental defect size of 25-40 mm and cancellous defects >11 mm in diameter, do not heal spontaneously (Ehrnberg, De Pablos, Martinez-Lotti, Kreicbergs, & Nilsson, 1993; Malhotra, Pelletier, Yu, Christou, & Walsh, 2014; Moxham et al., 1996). Whilst sheep bones possess a similar macrostructure to humans, making them an ideal model, histologically, the bone structure of sheep is quite different. Small animals and humans are known to possess osteons or Harversian systems, that are the basic structural unit of compact bone (Pearce, Richards, Milz, Schneider, & Pearce, 2007). In larger animals such as sheep, plexiform bone predominates. Plexiform bone possesses a dense vascular canal network, which creates a "brick-wall" appearance and provides greater mechanical support than woven bone (Brits, Steyn, & Noelle, 2014). Furthermore, it has been reported that age can play a role in the extent of bone remodelling. Skeletally immature sheep have shown a similar bone density to humans, compared to mature sheep which have a significantly higher trabecular bone density and therefore greater bone strength than humans (Nafei, Danielsen, Linde, & Hvid, 2000). For these reasons it is critical to ensure that the age of sheep used within a study are consistent and considered when making comparisons between other studies. Although there is no perfect animal model that can simulate identical biological, physiological biochemical characteristics to humans, it is possible to obtain a great deal of pre-clinical information of a novel graft substitute by using the appropriate animal and model.

PEROXIDASE ENZYMES

Peroxidases are a haem-containing superfamily of ubiquitous and essential proteins found in all living organisms, including plants, microorganisms, fungi and animals (Hiraga, Sasaki, Ito, Ohashi, & Matsui, 2001). It is well-established that these enzymes possess antimicrobial action, through the formation of enzymatic reaction products. The term "haem" refers to the ferriprotoporphyrin IX prosthetic group which is an essential component of the enzymes active site and essential for reactivity with hydrogen peroxide (H_2O_2). In the enzymes native form, the oxidative status of iron within the haem group is in the ferric form of Fe^{3+}. In the presence of H_2O_2 peroxidases engage in several redox reactions which convert the enzyme into various catalytic intermediates (Figure 1.8). Firstly, the reaction is initiated by available H_2O_2 which results in two-electron oxidation, converting the enzyme from its native state of Fe^{3+} into Compound I (Fe^{4+}) and the H_2O_2 is reduced to H_2O. Compound I, which is a strong oxidant can be converted back to its ferric state by reacting with halide ions (X^-) ("halogenation cycle") (Davies, Hawkins, Pattison, & Rees, 2008). The conversion of the enzyme back to this state occurs via a two-electron reduction, oxidising the halide ions to the corresponding hypohalous acids (HOX). Additionally, Compound I can be reduced by one electron by oxidising organic and inorganic substrates (RH*), yielding a substrate free radical and Compound II, which is referred to as the oxy-ferryl (Fe^{4+}) haem intermediate ("peroxidase cycle"). In a second one-electron reduction step Compound II is reduced back to its resting ferric peroxidase and generates a free radical and water molecule. At the end of the cycle the peroxidase is returned to its original native state, to repeat the halogenation and peroxidase cycles through the initiation of available H_2O_2. Another intermediate, Compound III is usually formed when there is an excess of H_2O_2. This intermediate is also largely formed by the combination of superoxide, which is produced by the oxidation of H_2O_2, with the ferric enzyme (Fe^{2+}). Although, the superoxide could also be generated via a one-electron reduction from oxidised substrates to

molecular oxygen. In this state Compound III is not catalytically active, but the inactivation is reversible (Goodwin, Grover, & Aust, 1997).

Figure 1.8 *Catalytic cycle of peroxidases showing dual pathways of oxidation.* In the halogenation cycle the native enzyme is oxidised in the presence of H_2O_2 to form Compound I (1). In the presence of halides (X^-), Compound I is converted back its native state and, in the process, produces hypohalous acids (HOX) (2) (halogenation cycle). Alternatively, in absence of halides, Compound I can be converted to Compound II by one electron-donating substrates (RH*) (3) (peroxidase cycle). Compound III is formed in the presence of excess H_2O_2, which is an inactive form of the enzyme that slowly reverses back to its active native state. Figure adapted and modified from (Davies et al., 2008).


halogenation cycle
peroxidase cycle
HOX
X-
(2)
(+ 2 e-)
native enzyme
(Fe3+)
compound I
(FeIV=O + porphyrin•)
H2O2
(1) (- 2 e-)
(+ 1 e-)
RH*
H2O2
ferrous enzyme
(Fe2+)
O2•-
(7)
(6)
(8)
O2•-
O2
R•
(+ 1 e-)
(+ 1 e-)
(3)
(4)
compound III
(Fe2+-O2)
R•
RH*
compound II
(FeIV=O)
(5)
H2O2

Peroxidases and wound healing

There is sufficient evidence that peroxidases, specifically mammalian-derived are involved in inflammatory processes and therefore could have implications in wound healing. However, a discovery by Rayner and colleagues reported that compounds present in bovine milk whey growth factor extract (WGFE) have a direct effect on skin fibroblasts to increase tissue repair in normal and compromised models of wound healing (Rayner et al., 2000). The additional treatment with WGFE promoted cellular infiltration within the wound for both normal and steroid-treated rats compared to control (Figure 1.9), with a greater response to WGFE observed in the steroid-treated animals. It was first suggested that growth factors such as TGF-β were responsible for the enhanced wound response, however, the amount of growth factors that would be required to generate such a response were not present in WGFE. This led to the suggestion that another factor must be present to influence the difference in fibrogenic response. Using cell-based screening methods of fractionated WGFE, it was ascertained that bovine lactoperoxidase (LPO) was the protein responsible for generating the main functional component of WGFE. Additional research has demonstrated increased collagen production by dermal fibroblasts treated with mammalian peroxidases, myeloperoxidase (MPO) and eosinophil peroxidase (EPO) and plant-peroxidases soybean peroxidase (SBP) and horseradish peroxidase (HRP) (DeNichilo et al., 2015). The ability of SBP to promote a fibrogenic response in a full-thickness dermal porcine model further demonstrates the regenerative potential of these enzymes. Of significant relevance to this thesis, is their capacity to significantly increase the synthesis of a collagen-rich ECM which is essential for successful bone repair (Figure 1.10). Furthermore, the direct stimulation of fibroblasts to promote invasion, migration and collagen synthesis suggested that peroxidases may have a causative role on mesenchymal cellular function, in the context of repair. Studies have since demonstrated that MPO, EPO (Panagopoulos et al., 2015) and SBP (unpublished) can stimulate other mesenchymal lineage

cells, including endothelial cells, by acting as pro-angiogenic molecules capable of regulating vessel development (Figure 1.11). These findings suggest that peroxidases, both mammalian and plant-derived could have applications in tissue repair, through the regulation of mesenchymal lineage cells essential in wound healing and be utilised as therapeutic agents in regenerative medicine.

Figure 1.9 *Histology of a rat incisional wound model.* Photomicrographs show cross sections through day 5 incisional wounds from normal (A and B) and steroid-treated (C and D) rats that received WGFE (B and D) or collagen vehicle only (A and C) at time of wounding. Sections were stained with Masson's trichrome and photographed at ×250 magnification (Rayner et al., 2000).

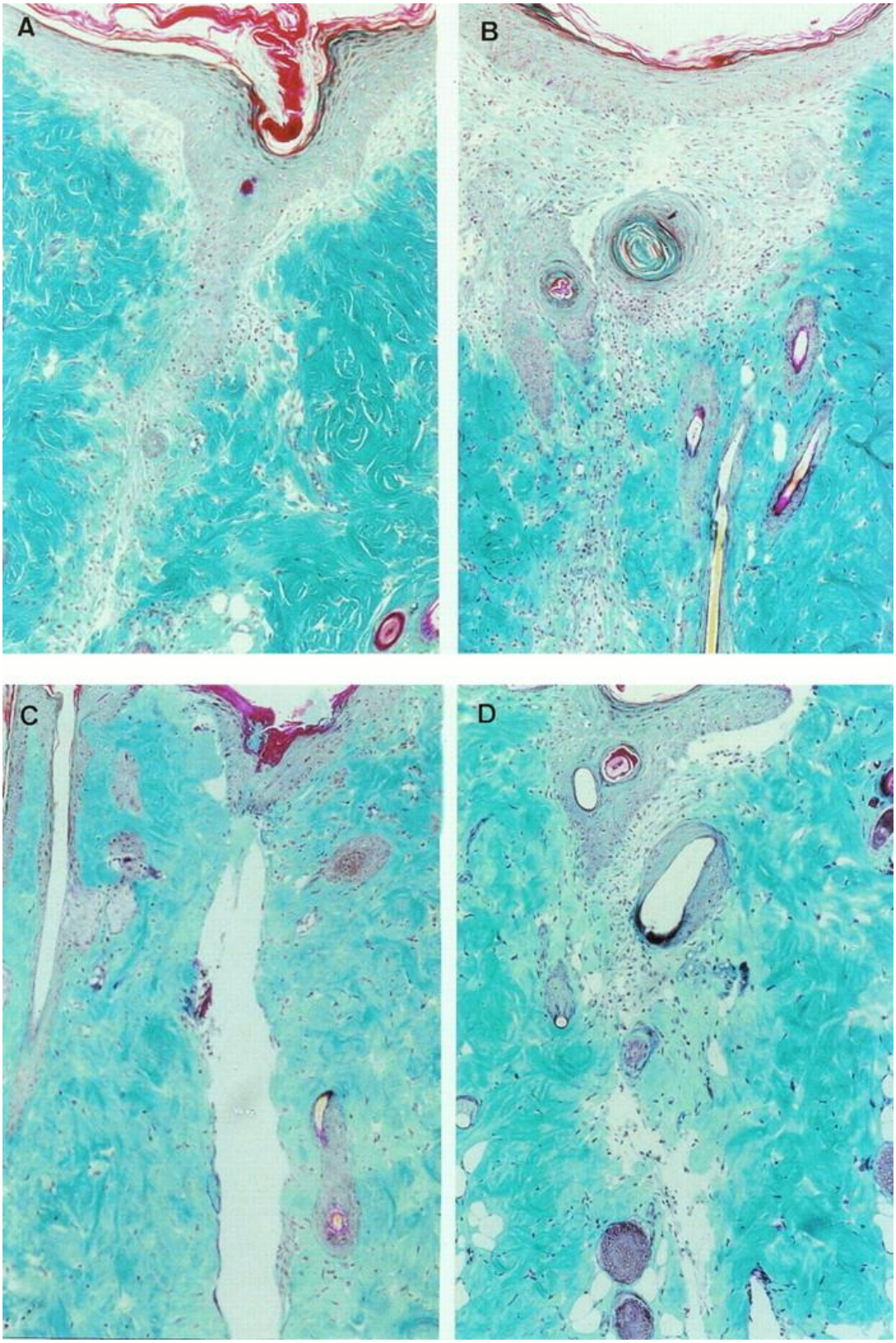

A
B
C
D

Figure 1.10 *Peroxidase enzymes stimulate potent tissue regeneration in a porcine excisional wound model.* A and B: The wounds with INTEGRA stapled in place. C and D: Representative low-power images of sections stained with Masson's trichrome. E and F: Representative higher-magnification images of boxed areas in panels C and D showing the control INTEGRA is poorly infiltrated with cells from the wound bed (E), whereas the INTEGRA treated with SBP (F) shows numerous fibroblasts and inflammatory cells interspersed with new extracellular matrix deposited between the INTEGRA fibres. Figure adapted and modified from (DeNichilo et al., 2015).

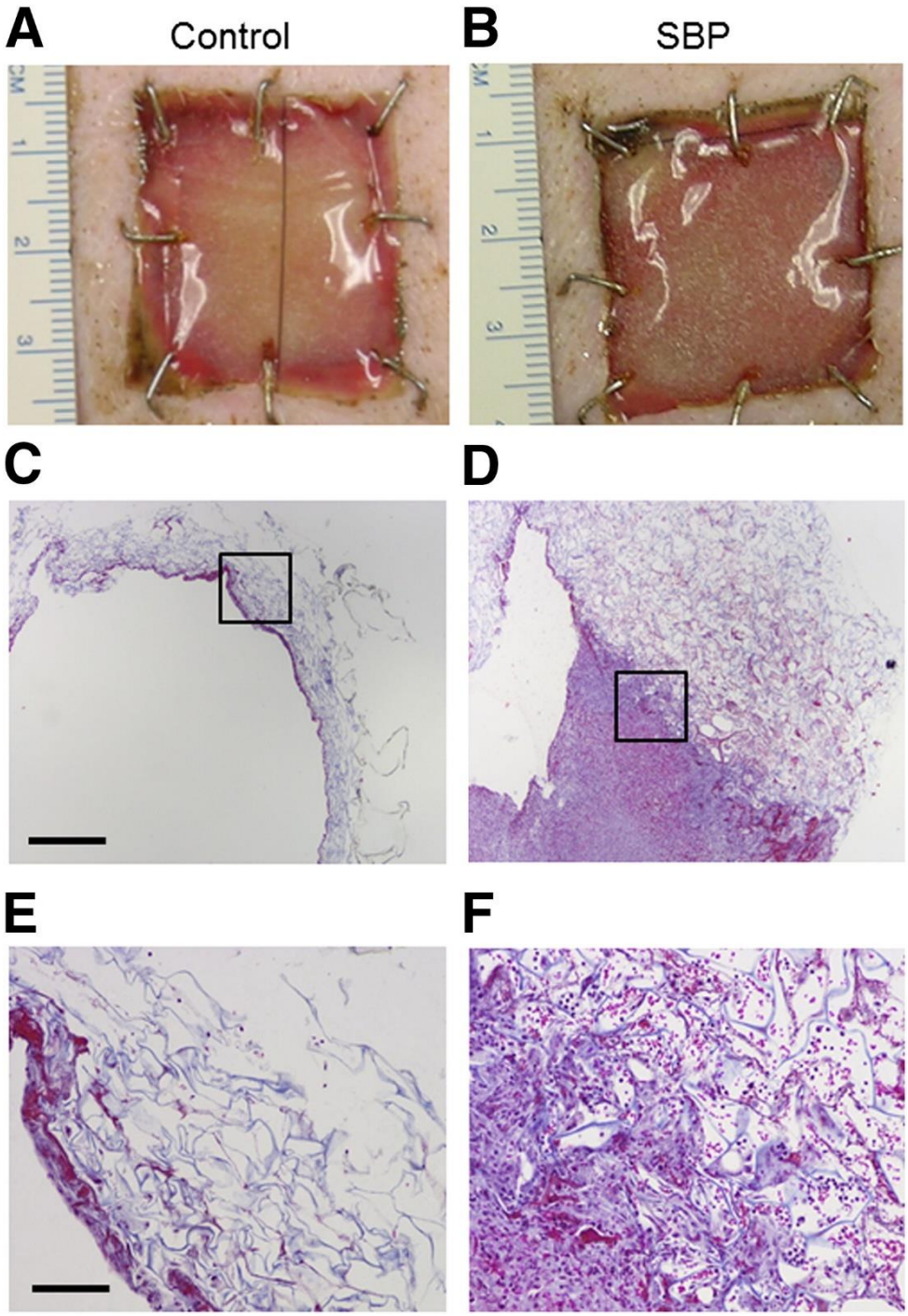
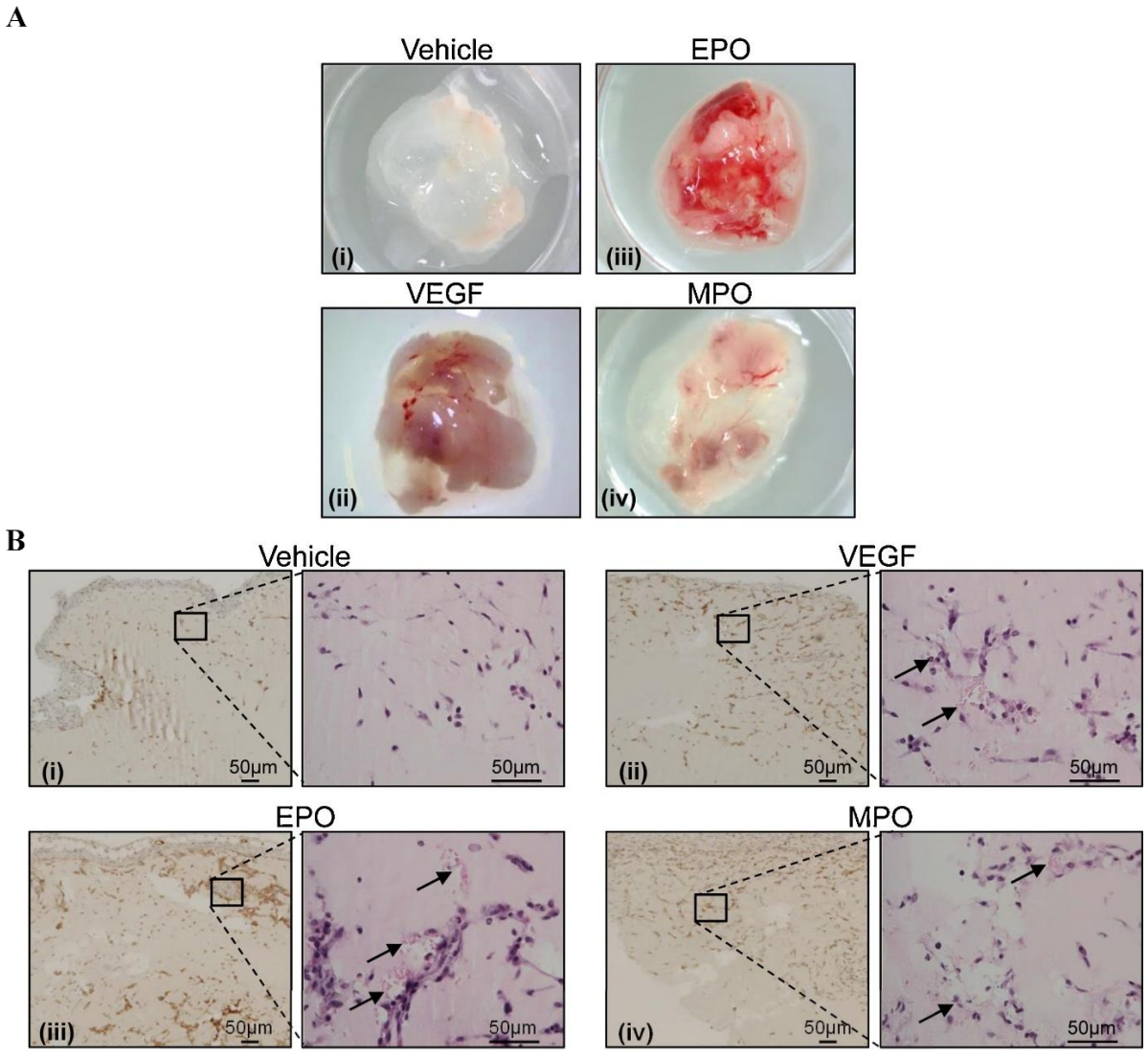

A
Control
B
SBP
C
D
E
F

Figure 1.11 *Peroxidases promote vascularisation of subcutaneously injected Matrigel in mice.* (A) Representative images of excised Matrigel plugs stimulated with (i) saline (vehicle), (ii) VEGF (100 ng/ml), (iii) EPO (5 μg) and (iv) MPO (5 μg), shows an increase in vasculature in the peroxidase treated Matrigel. (B) Representative images of CD31 (4×) and H&E (40×) immunostaining of (i) saline (vehicle), (ii) VEGF (100 ng/ml), (iii) EPO (5 μg), and (iv) MPO (5 μg), shows peroxidase induced infiltration of endothelial cells and an increase in red blood cells (arrows) within the Matrigel compared to saline and VEGF controls. Figure adapted and modified from (Panagopoulos et al., 2015).


A
Vehicle
EPO
(i)
(iii)
VEGF
MPO
(ii)
(iv)
B
Vehicle
VEGF
(i)
(ii)
50μm
EPO
MPO
(iii)
(iv)

Myeloperoxidase

MPO is a highly cationic, dimeric protein which consists of two monomer units of 73kDa each, joined by a cysteine disulphide bridge (Davies et al., 2008). They are located within neutrophils which provide the frontline of defence against invading microbes, by engulfing and ingesting foreign organisms. During granulocyte differentiation of promyelocytes and promyelomonocytes, MPO is synthesised throughout their maturation process in the bone marrow. The synthesis of MPO ceases after this stage of cell development and so circulating monocytes do not continue to synthesise this enzyme. During neutrophil activation and ingestion of a foreign body into a phagosomal compartment, azurophilic granules are secreted into the compartment, where MPO is subsequently released. Coinciding with the secretion of azurophilic granules, NADPH oxidase is assembled on the internal membrane surface and is responsible for providing a source of superoxide radicals and H_2O_2, which MPO subsequently transforms, into hypochlorous acid (Borregaard & Cowland, 1997).

Eosinophil peroxidase

EPO is a 70kDa cationic glycoprotein, which shares a sequence homology of 70% with MPO, however the catalytic behaviour and substrate specificities of these enzymes differ (Tahboub, Galijasevic, Diamond, & Abu-Soud, 2005). It is located within the cytoplasmic granules of human eosinophilic leukocytes and unlike MPO is much less active in Cl^- oxidation, but highly active with Br^-, I^- and SCN^- (Bozeman, Learn, & Thomas, 1990). Whilst neutrophils phagocytose their target and release MPO into the phagolysosomal compartment, the larger size of parasites targeted by eosinophils requires the exocytosis release of EPO onto the parasite surface. Eosinophils play an essential role in allergic reactions and a major part in host defence against parasites, fungi and bacteria by generating superoxide anions through an NADPH oxidase system. EPO is far more capable at generating large quantities of superoxide anions

and H_2O_2, with levels of these molecules estimated to be threefold to tenfold higher than those generated by MPO (Davies et al., 2008).

Mammalian peroxidases and fracture healing

In the occurrence of most fractures there is a certain amount of mechanical instability which leads to interfragmentary movement of the bone and consequently, secondary bone healing. This response requires a local inflammatory reaction by circulating inflammatory cells, including neutrophils, eosinophils and macrophages, which remain in the fracture region until regenerative cells invade the site (Prasad & Udupa, 1972). This inflammatory phase becomes initiated when the vasculature becomes disrupted and a haematoma forms around the fracture site, leading to the infiltration of these cells. Within 24 hours after injury there is a significant influx of neutrophils, which become the predominant leukocyte at the fracture haematoma (Glynne Andrew, Andrew, Freemont, & Marsh, 1994). Here, the inflammatory cells release pro-inflammatory factors that are essential in initiating the repair cascade. They carry out this role by having a chemotactic effect on other inflammatory cells, enhancing ECM synthesis, stimulating angiogenesis and recruiting fibrogenic cells to the fracture site (Dimitriou et al., 2005). The importance of the initial fracture haematoma and subsequent inflammatory phase has been demonstrated in animal models. Studies have shown that removal of the haematoma as well as repeated debridement of the haematoma, leads to delayed or non-union (Grundnes & Reikerås, 1993; Park, Silva, Bahk, McKellop, & Lieberman, 2002). MPO and EPO have not yet been directly implicated in the bone repair process beyond providing oxidative defence against bacteria and invading pathogens. However, the knowledge that these enzymes are released at sites of tissue injury and inflammation, suggests a potential role for these enzymes in the repair process. Furthermore, recent studies have demonstrated an association between eosinophilic inflammation and osteitis in patients diagnosed with chronic rhinosinusitis,

suggesting a link between an increased presence of eosinophils and pathological osteogenesis (Mehta, Campeau, Kita, & Hagan, 2008; Snidvongs et al., 2012). A link between inflammatory involvement and ectopic bone formation has also been reported in the soft tissue of patients following a traumatic injury, which is defined as heterotopic ossification (HO) (Evans et al., 2009). Whilst it is well established that inflammatory cells are a contributing factor to HO, the mechanisms and factors responsible are not yet defined (Convente, Wang, Pignolo, Kaplan, & Shore, 2015). These findings suggest that localised use of mammalian peroxidases MPO and EPO could potentiate the bone repair process.

Soybean peroxidase

SBP is a 37 kD glycoprotein expressed in the root, leaf and seed hull of *Glycine max* (soybean) (Gillikin & Graham, 1991). It belongs to the secretory plant peroxidase superfamily (class III), which play a role in the self defence system of plants, lignification (strengthening of the cell wall) and salt tolerance (Henriksen et al., 2001). Besides SBP, the most well-known member of the class III peroxidases in horseradish peroxidase (HRP), to which SBP shares 57% sequence homology (Welinder, Mauro, & Nørskov-Lauritsen, 1992). HRP has been identified as the most structurally similar protein to SBP, with key structural characteristics maintained between the two proteins. This includes the location of their four distinguishing disulphide bridges, a single tryptophan present and a prosthetic haem group (Welinder & Larsen, 2004). Whilst both HRP and SBP have been widely used for biosensing and diagnostic applications due to high stability, SBP has been recognised to be significantly more thermally stable, possess greater catalytic activity and is reportedly less susceptible to haem loss and permanent inactivation by hydrogen peroxide (McEldoon & Dordick, 1996; Wright & Nicell, 1999). The inactivation temperature of SBP is reportedly 90.5°C, compared to 81.5°C for HRP. Furthermore, SBP can maintain catalytic activity from a pH of 2.0, compared to HRP at pH 2.4

(Henriksen et al., 2001; McEldoon & Dordick, 1996). In addition, SBP is present in large amounts, readily available and inexpensive, which makes it an attractive candidate over HRP for large scale use (Gillikin & Graham, 1991). As expected, plant-derived peroxidases also differ from mammalian peroxidases in both size and binding of the haem group. Plant peroxidases, including SBP consist of ~ 300 amino acids and the haem-domain is not covalently bound, compared to mammalian peroxidases which are much larger, ranging from 576-738 amino acids and covalent bonding of the haem group (O'Brien, 2000; Obinger, 2006). Whilst mammalian peroxidases have been widely studied due to their greater physiological relevance, plant peroxidases have been tested for use in enzymatic crosslinking of hydrogels. These studies have demonstrated not only resistance to hydrogel degradation after subcutaneous injection but also *in vivo* non-cytotoxicity (Sofia, Singh, & Kaplan, 2002). The advantageous properties of SBP, particularly its stability, has given rise to numerous medical applications, such as a detecting agent in diagnostic toolkits and biosensing for various medical conditions (Hiraga et al., 2001). Although SBP's uses are still being fully elucidated, its superior functionality and availability makes it an attractive agent for further research.

Oxidative stress and bone maintenance

It is well-established that oxidative stress is involved in mineral tissue homeostasis and the production of reactive oxygen species (ROS) contributes by promoting bone resorption through the formation and activation of osteoclasts (Ha et al., 2004). Studies using bone marrow precursor cells have demonstrated that RANKL-induced osteoclastogenesis requires ROS production. Overexpression of glutathione peroxidase 1, the main antioxidant enzyme found in osteoclasts, has been shown to prevent RANKL-induced osteoclastogenesis (Lean, Jagger, Kirstein, Fuller, & Chambers, 2005; Lee et al., 2005). This suggests a key role for H_2O_2 in the formation of osteoclasts. In addition to promoting the formation of osteoclasts, H_2O_2 can induce

oxidative stress and inhibit osteoblast differentiation and reduce their lifespan. When mouse osteoblastic cells were incubated with H_2O_2, mineralisation levels decreased and osteogenic markers RUNX2, ALP and BSP decreased in expression (Arai, Shibata, Pugdee, Abiko, & Ogata, 2007). However, this can be overcome by the use of ROS inhibitors, which have been shown to restore osteoblast differentiation (A. L. Liu, Zhang, Zhu, Liao, & Liu, 2004). Whilst it is clear ROS production is present during normal bone remodelling, recent studies have shown increasing oxidative stress might be involved in the pathogenesis of some bone diseases. For example, postmenopausal women who are at greatest risk of developing osteoporosis, are found to have decreased catalase and glutathione peroxidase activity, which are critical for depleting H_2O_2 (Ozgocmen, Kaya, Fadillioglu, Aydogan, & Yilmaz, 2007). Furthermore, studies have shown diabetic patients who present with increased oxidative stress levels have an associated increase in fracture risk (Strotmeyer & Cauley, 2007). In addition to impairing these diseases, high levels of ROS can impede the fracture healing process, by inhibiting osteoblast formation. This increase in ROS cannot be depleted without the addition of antioxidant enzymatic activity. Thus, current data suggest antioxidants might be beneficial in bone health as a strategy to reduce bone loss and its associated morbidities and mortality.

AIMS AND SIGNIFICANCE OF THE PROJECT

The repair of large bony defects and non-unions that arise because of bone disease, trauma or tumour removal remains a significant challenge for orthopaedic surgeons. The occurrence of a non-union that cannot heal naturally is associated with several morbidities and can lead to disability and consequently financial burden on both the patient and economy. Furthermore, the number of patients requiring treatment is increasing due to the ageing population. To restore and stabilise the bone, bone grafting or the use of bone graft substitutes is required. Successful bone graft substitutes require three key characteristics, osteogenicity, osteoinduction and osteoconduction. Unfortunately, besides autologous grafting, which has several associated morbidities, researchers are yet to identify a graft substitute which possesses the three key characteristics required to successfully repair bone.

Our laboratory has been investigating a group of enzymes called peroxidases which may provide osteoinductive properties, to be used in combination with scaffold materials. Peroxidases are haem-containing enzymes whose functional involvement in organism health has mainly been limited to providing a mechanism for oxidative defence against invading bacteria and other pathogenic microorganisms. Work published by our laboratory provides evidence that mammalian peroxidases MPO and EPO, and plant-derived peroxidase SBP, stimulate the migration of fibroblastic cells and promote their ability to secrete collagen type I protein both *in vitro* and *in vivo* to generate a functional ECM (DeNichilo et al., 2015). In addition, we have presented further evidence demonstrating the ability of peroxidases to promote angiogenesis and inhibit osteoclastogenesis, suggesting a potential role for these enzymes in fracture repair.

Osteoblasts are the main cell type responsible for collagen type I biosynthesis during normal bone formation and fracture repair. It is well established that osteoblasts, endothelial cells and fibroblasts are derived from the same mesenchymal progenitor cell population. We

therefore hypothesise that peroxidase enzymes can influence osteoblast cellular processes to increase collagen biosynthesis and matrix mineralisation resulting in new bone formation. This novel concept raises the possibility that peroxidase enzymes may have therapeutic potential in regulating new bone formation in various clinical settings, including fracture repair and disease-related bone loss.

The hypothesis of this thesis is peroxidase enzymes have a causative role on osteoblast function, resulting in increased collagen ECM biosynthesis and mineralisation and as a result, possess the capacity to induce new bone formation at a locally targeted site.

The specific aims of this research project are:

Aim 1: investigate the effect of physiologically-relevant mammalian peroxidase enzymes on osteoblast cell functionality of collagenous ECM biosynthesis and deposition and mineralisation of the deposited matrix.

Aim 2: explore the ability of plant-derived peroxidase enzymes to promote osteoblast functionality and determine their effectiveness compared to mammalian peroxidases.

Aim 3: determine the osteoinductive potential of plant-derived peroxidase enzymes to accelerate bone formation *in vivo*, using a standardised animal model of bone repair.

CHAPTER 2:

PEROXIDASE ENZYMES REGULATE COLLAGEN BIOSYNTHESIS AND MATRIX MINERALISATION BY CULTURED HUMAN OSTEOBLASTS

Statement of Authorship

Title of Paper	Peroxidase Enzymes Regulate Collagen Biosynthesis and Matrix Mineralization by Cultured Human Osteoblasts
Publication Status	☑ Published ☐ Accepted for Publication ☐ Submitted for Publication ☐ Unpublished and Unsubmitted w ork w ritten in manuscript style
Publication Details	DeNichilo, M. O., Shoubridge, A. J., Panagopoulos, V., Liapis, V., Zysk, A., Zinonos, I., Evdokiou, A. (2016). Peroxidase Enzymes Regulate Collagen Biosynthesis and Matrix Mineralization by Cultured Human Osteoblasts. Calcified Tissue International, 98(3), 294-305. (IF=3.3)

Principal Author

Name of Principal Author	Mark DeNichilo		
Contribution to the Paper	Designed project, optimised and completed research experiments, performed data analysis and prepared manuscript.		
Signature		Date	19/10/2017

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate in include the publication in the thesis; and

iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

Name of Co-Author (Candidate)	Alexandra Shoubridge		
Contribution to the Paper	Completed *in vitro* experiments, performed data analysis, and contributed to the preparation of the manuscript. Specifically conducted experiments and provided data for figures 1a &b; 5a & b; 6.		
Overall percentage (%)	40%		
Signature		Date	01/11/2017

Name of Co-Author	Vasilios Panagopoulos		
Contribution to the Paper	Assisted in part with *in vitro* experiments, data interpretation and editing of manuscript.		
Signature		Date	01/11/2017

Name of Co-Author	Vasilios Liapis		
Contribution to the Paper	Assisted in part with *in vitro* experiments.		
Signature		Date	01/11/2017

Name of Co-Author	Aneta Zysk		
Contribution to the Paper	Assisted in part with *in vitro* experiments.		
Signature		Date	01/11/2017

Name of Co-Author	Irene Zinonos		
Contribution to the Paper	Assisted in part with *in vitro* experiments		
Signature		Date	01/11/2017

Name of Co-Author	Shelley Hay		
Contribution to the Paper	Assisted in part with *in vitro* experiments and data interpretation.		
Signature		Date	01/11/2017

Name of Co-Author	Gerald Atkins		
Contribution to the Paper	Assisted in data interpretation and manuscript evaluation.		
Signature		Date	01/11/2017

Name of Co-Author	David Findlay		
Contribution to the Paper	Assisted in data interpretation and manuscript evaluation.		
Signature		Date	01/11/2017

Name of Co-Author	Andreas Evdokiou		
Contribution to the Paper	Supervised development of work, assisted in data interpretation and manuscript evaluation.		
Signature		Date	01/11/2017

Calcif Tissue Int (2016) 98:294–305
DOI 10.1007/s00223-015-0090-6

ORIGINAL RESEARCH

Peroxidase Enzymes Regulate Collagen Biosynthesis and Matrix Mineralization by Cultured Human Osteoblasts

Mark O. DeNichilo[1,4] · Alexandra J. Shoubridge[1] · Vasilios Panagopoulos[1] · Vasilios Liapis[1] · Aneta Zysk[1] · Irene Zinonos[1] · Shelley Hay[1] · Gerald J. Atkins[2] · David M. Findlay[2] · Andreas Evdokiou[1,3]

Received: 28 May 2015 / Accepted: 20 November 2015 / Published online: 7 December 2015

Abstract The early recruitment of inflammatory cells to sites of bone fracture and trauma is a critical determinant in successful fracture healing. Released by infiltrating inflammatory cells, myeloperoxidase (MPO) and eosinophil peroxidase (EPO) are heme-containing enzymes, whose functional involvement in bone repair has mainly been studied in the context of providing a mechanism for oxidative defense against invading microorganisms. We report here novel findings that show peroxidase enzymes have the capacity to stimulate osteoblastic cells to secrete collagen I protein and generate a mineralized extracellular matrix in vitro. Mechanistic studies conducted using cultured osteoblasts show that peroxidase enzymes stimulate collagen biosynthesis at a post-translational level in a prolyl hydroxylase-dependent manner, which does not require ascorbic acid. Our studies demonstrate that osteoblasts rapidly bind and internalize both MPO and EPO, and the catalytic activity of these peroxidase enzymes is essential to support collagen I biosynthesis and subsequent release of collagen by osteoblasts. We show that EPO is capable of regulating osteogenic gene expression and matrix mineralization in culture, suggesting that peroxidase enzymes may play an important role not only in normal bone repair, but also in the progression of pathological states where infiltrating inflammatory cells are known to deposit peroxidases.

Keywords Peroxidase enzymes · Osteoblasts · Collagen biosynthesis · Matrix mineralization

✉ Mark O. DeNichilo
mark.denichilo@adelaide.edu.au

[1] Breast Cancer Research Unit, Discipline of Surgery, The University of Adelaide, Adelaide, Australia

[2] Discipline of Orthopaedics and Trauma, The University of Adelaide, Adelaide, Australia

[3] Centre for Personalized Cancer Medicine, The University of Adelaide, Adelaide, Australia

[4] TQEH, Basil Hetzel Research Institute, 28 Woodville Road, Woodville, SA 5011, Australia

Introduction

Fracture repair is a highly coordinated and complex process that involves the interplay of numerous cell types, growth factors, and extracellular matrix (ECM) components [1]. Recruited to the injury site from surrounding tissue, osteoblasts have a central role in new bone formation, where these cells are responsible for the synthesis and deposition of a collagen-rich ECM that is subsequently mineralized to form the hard bone structure. Circulating inflammatory cells, including neutrophils, eosinophils, and macrophages, are known to infiltrate the fracture site during the early phase of this well-orchestrated healing process [2–4], where they release pro-osteogenic factors locally that influence osteoblast functionality and the regenerative process [5, 6]. While disruption of this early inflammatory phase is known to severely impair the fracture healing outcome [7, 8], our understanding of the complex interplay between osteoblasts and the inflammatory cells of the immune system remains poorly defined.

Myeloperoxidase (MPO) and eosinophil peroxidase (EPO) are heme-containing enzymes that form part of the mammalian peroxidase family [9]. These enzymes are released at sites of tissue injury and disease by infiltrating inflammatory cells [10, 11], and until recently have been studied mainly in the context of providing oxidative defense against invading bacteria and other pathogenic

microorganisms [12]. Our laboratory has recently discovered new functional roles for peroxidase enzymes as regulators of fibroblast collagen ECM biosynthesis [13] and angiogenesis [14], offering new insight into the pro-fibrogenic mechanism by which inflammatory cells influence normal tissue regeneration and fibrotic disease progression. These important findings lead us to hypothesize that peroxidases may also regulate osteoblastic cellular processes that contribute to new bone formation, including collagen I biosynthesis and matrix mineralization.

We report here studies that characterize, for the first time, the ability of mammalian MPO and EPO to directly stimulate the secretion of collagen I by cultured human osteoblasts. We provide mechanistic data showing that peroxidase enzymes regulate collagen I biosynthesis at a post-translational level that is prolyl hydroxylase-dependent, but importantly, does not require ascorbic acid (AA), or transcriptional regulation of collagen I mRNA. Our studies demonstrate that cultured osteoblasts rapidly bind and internalize both MPO and EPO, and the catalytic activity of these peroxidase enzymes is essential to support collagen I biosynthesis and subsequent release of collagen from cells. We show that EPO is also capable of promoting osteoblast matrix mineralization in vitro, a response that is accompanied by the regulation of several key osteogenic effector genes, including bone morphogenetic protein (BMP)-2 and the non-canonical Wnt5a. Our findings that peroxidase enzymes can promote collagen I biosynthesis, osteogenic gene regulation, and matrix mineralization by cultured human osteoblasts suggest these enzymes may play an important role, not only in fracture repair, but also in the progression of pathological states where infiltrating inflammatory cells are known to deposit peroxidases.

Materials and Methods

Peroxidases

Native human eosinophil peroxidase (EPO) was obtained from Cell Sciences (Canton, MA) and Lee Biosolutions Inc. (St. Louis, MO). Recombinant human myeloperoxidase (rhMPO) was purchased from R&D Systems (Minneapolis, MN).

Osteoblast Cell Culture

Normal human bone-derived osteoblasts were isolated from intertrochanteric trabecular bone samples from female donors undergoing primary hip replacement surgery, as described previously [15]. Human osteoblasts were expanded in culture using Dulbecco's modified Eagle's medium (DMEM; high glucose with no AA), supplemented with 2 mmol/L glutamine, 100 IU/mL penicillin, 100 μg/mL streptomycin, 20 mmol/L HEPES, and 10 % fetal bovine serum (FBS; Invitrogen Life Technologies, Carlsbad, CA) in a 5 % CO_2-containing humidified atmosphere. These cells maintain an osteoblastic phenotype in culture and stain positive for alkaline phosphatase activity.

Collagen I Enzyme-Linked Immunosorbent Assay (ELISA)

To evaluate the effect of MPO and EPO on collagen I production, osteoblasts were seeded into 96-well plates (Nunc, Roskilde, Denmark) at a density of 1.2×10^4 cells per well and cultured for 5 days in DMEM/10 % FBS until reaching confluence. Cells were starved overnight in serum-free DMEM and then stimulated for an additional 72 h in serum-free DMEM containing either AA 2-phosphate at 100 μmol/L (Wako Chemical Industries, Osaka, Japan) as a positive control, or with the peroxidase proteins in the absence of AA supplementation. At the end of the 72-h treatment period, osteoblast-conditioned media were collected for measurement of secreted, soluble type I collagen by ELISA. Cell viability/growth was then assessed using the alamarBlue fluorescent dye assay (Invitrogen Life Technologies). Briefly, cells were incubated in a 10 % alamarBlue/phosphate-buffered saline (PBS) solution for 30 min at 37 °C and fluorescence measured at wavelengths of 530 nm excitation and 595 nm emission using a FLUOstar Optima plate reader (BMG Labtek Australia, Mornington, VIC).

The amount of soluble type I collagen in cell-conditioned medium was measured by a direct coat enzyme-linked immunosorbent assay method, using standard curves constructed from purified Type I human placental collagen (BD Biosciences Australia, North Ryde, NSW). Samples and standards (100 μL per well) were added to a 96-well Maxisorp plate (Nunc) and left at 4 °C overnight. The plate was then washed with PBS-Tween 0.05, 2.5 % bovine serum albumin (BSA)/PBS blocking solution added to each well and the plate incubated for 1 h at room temperature. The plate was then washed with PBS-Tween 0.05 % and primary antibody (0.25 μg/mL rabbit anti-human-collagen I polyclonal; Rockland Immunochemicals, Limerick, PA) in 5 % non-fat dairy milk added to each well for 3 h at room temperature. After washing, europium-tagged anti-rabbit secondary antibody (0.5 μg/mL in 1 % BSA/PBS; Perkin Elmer Life Sciences, Turku, Finland) was added for 1 h at room temperature. After a final wash, Enhancement Solution (Perkin Elmer Life Sciences) was added, and fluorescence was measured at excitation 355 nm and emission 620 nm using a FLUOstar Optima plate reader (BMG Labtek Australia). The collagen content of each

sample was determined from the standard curve (μg/mL), and then normalized to DMEM-only treated cells.

Immunofluorescence Staining

Primary human osteoblasts (1.2×10^4 cells per well) were seeded onto circular glass coverslip in DMEM supplemented with 2 mmol/L glutamine, 100 IU/mL penicillin, 100 μg/mL streptomycin, 20 mmol/L HEPES, and 10 % FBS (Invitrogen Life Technologies) and maintained in culture overnight. Cells were then treated with either 1 μg of MPO or EPO in serum-free DMEM for up to 3 h at 37 °C. After peroxidase exposure, indirect immunofluorescence staining was performed to detect cellular distribution of both MPO and EPO. In brief, cells were fixed with 4 % formaldehyde with 5 % sucrose in PBS for 10 min. Cells were permeated with 0.25 % Triton X-100 (Sigma-Aldrich) in PBS for 10 min to detect intracellular peroxidase uptake; non-specific binding sites were blocked by using 3 % BSA in PBS containing 0.1 % glycine and a 1:10 dilution of non-immune goat serum for 90 min. Cells were incubated for 1 h with the primary antibodies [1 μg/mL rabbit anti-human MPO from Dako Australia, North Sydney, NSW (A0398); 1:1000 dilution of mouse anti-human EPO from Merck Millipore Australia, Bayswater, NSW (MAB1087); 1 μg/mL non-immune rabbit IgG (Sigma-Aldrich); 1 μg/mL non-immune mouse IgG (Sigma-Aldrich)] in PBS containing BSA at 1 mg/mL and then counterstained with a 1:1000 dilution of Alexa Fluor 488-conjugated goat anti-mouse or goat anti-rabbit IgG (Cell Signaling Technologies, Beverly, MA) in PBS containing 1 mg/mL BSA. Coverslips were mounted on glass slides using Fluoromount (Sigma-Aldrich) and images captured using a fluorescence photomicroscope (Observer Z1; Carl Zeiss Microscopy, Jena, Germany).

RNA Isolation and Quantitative Real-Time PCR

For collagen I mRNA time-course studies, 6×10^4 osteoblasts were seeded into T25 culture flasks in DMEM/10 % FBS and maintained in culture for 5 days. On reaching confluence, cells were starved in serum-free DMEM overnight and then stimulated with a maximal dose of 10 ng/mL transforming growth factor (TGF)-β2, 100 μmol/L AA 2-phosphate, 1.56 μg/mL EPO, or 1.56 μg/mL recombinant human MPO in serum-free DMEM. To examine osteogenic gene expression, 6×10^4 osteoblasts were seeded into T25 culture flasks in DMEM/10 % FBS and maintained in culture for 5 days. On reaching confluence, cells were stimulated with either 5 μg/mL EPO or 5 μg/mL MPO for 12 days in osteogenic DMEM mineralization medium [DMEM supplemented with 5 % FBS, 100 μmol/L AA 2-phosphate (Wako Chemical Industries), 10^{-8} mol/L dexamethasone (Hospira Australia, Mulgrave, VIC), and 10 mmol/L β-glycerophosphate (Sigma-Aldrich)]. Total RNA was harvested using an RNeasy Mini Kit (Qiagen Australia, Chadstone, VIC) according to the manufacturer's instructions. RNA yield and purity were quantified by Nanodrop spectrophotometric measurement at 260 nm (Nanodrop Technologies, Thermo Fisher Scientific, Scoresby, VIC, Australia). Gene expression was examined using real-time PCR and normalized to the housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH). cDNA was synthesized by reverse transcription of 1 μg total RNA using random hexamer primers and SuperScript III Reverse Transcriptase (Invitrogen Life Technologies). Quantitative real-time RT-PCR was performed using SYBR Green Fluor qPCR mastermix (Qiagen Australia) in a CFX96 Real-Time System (BioRad, Hercules, CA). Each reaction volume of 25 μL contained cDNA templates, primer pairs, and SYBR Green mastermix. Amplification occurred after initial denaturation at 95 °C for 3 min, followed by 40 cycles at 95 °C for 15 s, 60 °C for 26 s, and 72 °C for 10 s. The primer combination used for human GAPDH, collagen I α1, BMP-2, BSP, Wnt5a, and FRZB is shown in Table 1.

In Vitro Mineralization

Primary human osteoblasts were seeded into 96-well plates (Nunc) at a density of 1.2×10^4 cells per well and cultured for 5 days in AA-free Dulbecco's minimum essential media containing 10 % fetal bovine serum/(10 % FBS/DMEM) at 37 °C and 5 %CO_2. Triplicate wells were stimulated with either MPO or EPO in osteogenic DMEM mineralization medium [DMEM supplemented with 5 % FBS, 100 μmol/L AA 2-phosphate (Wako Chemical Industries), 10^{-8} mol/L dexamethasone (Hospira Australia, Mulgrave, VIC), and 10 mmol/L β-glycerophosphate (Sigma-Aldrich)] to assist bone mineral formation. Cells were maintained in culture for up to 12 days, with fresh medium with or without EPO or MPO, changed every 4 days. To detect matrix mineralization, the Alizarin Red staining method was used. Cells were washed twice with PBS and then fixed with 10 % phosphate-buffered formalin for 15 min. The fixed cells were washed twice with distilled water and stained with 2 % Alizarin Red S solution (Sigma-Aldrich) for 5 min. The excess dye was removed by repeated washing with distilled water, and mineralized matrix stained by Alizarin Red photographed using a Nikon Eclipse 50i microscope attached to a DS-L2 control unit (Digital Sight, Nikon Europe, Amsterdam, The Netherlands) and a DS-Fi1 digital camera (Nikon Corporation, Tokyo, Japan). The extent of mineralization was

Table 1 qPCR primer sequences

Gene	Sense primer (5′–3′)	Anti-sense primer (5′–3′)
GAPDH	ACCCAGAAGACTGTGGATGG	TCAGTGAGCTTCCCGTTCAG
Col I α1	AGGGCTCCAACGAGATCGAGATCCG	TACAGGAAGCAGACAGGGCCAACGTCG
BMP-2	GCGGAAACGCCTTAAGTCCAGC	CGGGGGAGCCACAATCCA
BSP	GCATGCCTACTTTTATCCTCATTTAA	TCTTCTGAACTGTCATCTCCATTTTC
Wnt5a	TGCAGCCAACTGGCAGGACTT	TCGCGCCTTCTCCGATGT
FRZB	GCGGAAACGCCTTAAGTCCAGC	CGGGGGAGCCACAATCCA

quantitated by measuring calcium levels within the mineralized osteoblast monolayer after 12 days using the Arsenazo III assay (Thermo Fisher Scientific). Cells were washed twice in PBS, and 0.1 mL of 0.5 mol/L HCl added to each well and the mineral solubilized overnight at 4 °C. Following the manufacturer's instructions, 10 µL of sample was transferred to a clean 96-well plate, and 190 µL of Arsenazo III reagents was added. After 10 min of incubation at 37 °C, absorbance was measured at a wavelength of 650 nm using a FLUOstar Optima plate reader (BMG Labtek Australia). The calcium concentration (mmol/L) of each sample was determined from a $CaCl_2$ calibration curve.

Results

MPO and EPO Stimulate Cultured Osteoblasts to Secrete Type I Collagen

Type I collagen comprises approximately 80 % of the total protein present in bone, where it plays an important structural role in determining the biomechanical competence of bone. When stimulated for 72 h with either EPO or MPO in the absence of AA, cultured human osteoblasts demonstrated a dose-responsiveness to both mammalian peroxidases. Like AA, which was used as a control, the combined data from five adult donors showed the peroxidase enzymes increased the amount of soluble collagen I released into the medium approximately sixfold, with highly significant differences detected as low as 98 ng/mL (Fig. 1a). Assessment of cell viability following stimulation indicated that neither the peroxidases nor AA had a significant impact on the number of cultured osteoblasts over the 72-h stimulation period (Fig. 1b), indicating the increase in collagen I release is not due to an increase in cell number.

Peroxidase Catalytic Activity is Essential for Collagen I Production by Osteoblasts

The iron-containing heme catalytic domain is a highly conserved feature shared by both EPO and MPO. To

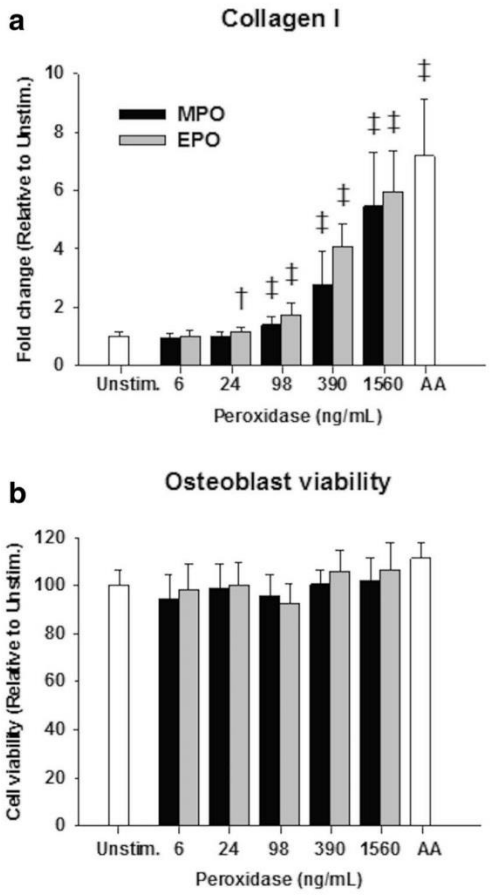


Fig. 1 Myeloperoxidase (MPO) and eosinophil peroxidase (EPO) promote collagen I release by cultured human osteoblasts. **a** ELISA detection of soluble collagen I in osteoblast-conditioned medium after 72 h stimulation with MPO and EPO at the doses indicated. Ascorbic acid 2-phosphate (AA) at 100 µmol/L served as the positive control, whereas cells treated with Dulbecco's modified Eagle's medium (DMEM) alone (Unstim.) served as the baseline control. The levels of collagen I are expressed as fold change and normalized so the average values of Unstim were set to 1. **b** Viability of cultured osteoblasts after 72 h stimulation as assessed using the alamarBlue dye assay. Cell viability was normalized so the average values of Unstim cells were set to 100 % relative to each peroxidase dose. The data are pooled from five experiments each conducted using cells derived from different individual donors. Data are the mean ± SD of fifteen determinations for Unstim, AA, and each peroxidase dose. $^{\dagger}P < 0.01$; $^{\ddagger}P < 0.001$

determine whether peroxidase catalytic activity had a role in promoting collagen I production, osteoblasts were stimulated with either EPO or MPO in the presence of 4 amino-benzoic acid hydrazide (4-ABAH), an irreversible inhibitor of mammalian peroxidase enzymatic activity [16]. A clear dose-dependent inhibition of collagen I release was observed over the 72-h time period, with maximal inhibition achieved at the highest dose of 250 µmol/L (Fig. 2a). In contrast, 4-ABAH over the same dose range had no impact on AA-induced collagen I release, confirming that 4-ABAH was inhibiting the peroxidase catalytic activity and not the cellular collagen biosynthetic pathway. Exposure of osteoblasts to increasing doses of 4-ABAH up to 250 µmol/L had no impact on cellular viability over the duration of the experimental period (Fig. 2b). These data confirm that the heme-containing catalytic domain plays an essential role in EPO and MPO-mediated collagen I release by osteoblasts.

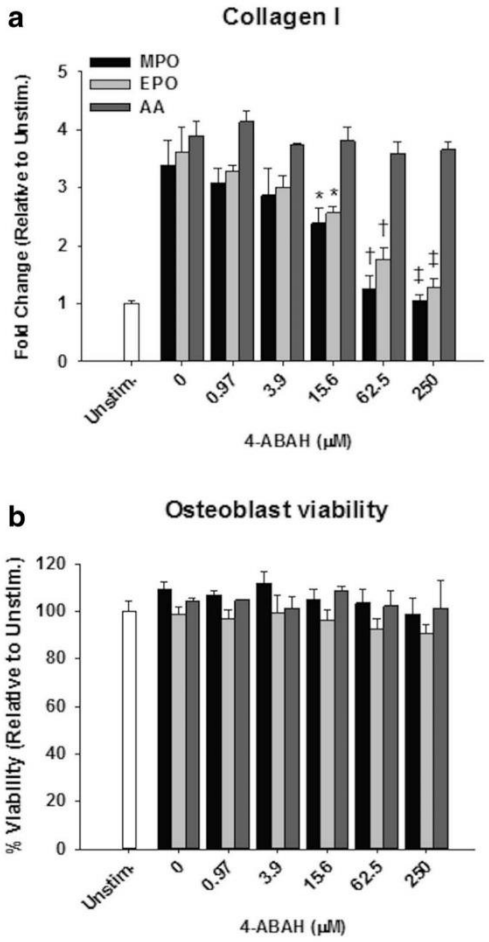


Fig. 2 Inhibition of peroxidase-induced collagen I release by 4 amino-benzoic acid hydrazide (4-ABAH). **a** ELISA detection of soluble collagen I levels in osteoblast-conditioned medium after cells were pre-treated for 30 min with 4-ABAH at the doses indicated, then stimulated by the addition of either 1.56 µg/mL myeloperoxidase (MPO), 1.56 µg/mL eosinophil peroxidase (EPO), or 100 µmol/L ascorbic acid 2-phosphate (AA) for a further 72 h. Soluble collagen I levels are expressed as fold change and normalized to the average values of cells treated with Dulbecco's modified Eagle's medium alone (Unstim.). **b** Viability of osteoblasts after 72 h treatment with 4-ABAH as assessed using the alamarBlue dye assay. Cell viability was normalized to the average values of cells treated with Dulbecco's modified Eagle's medium alone (Unstim.). Statistical significance was calculated by two-tailed Student's *t* test with the 4-ABAH-treated groups compared to the treatment group without 4-ABAH. Inhibition studies using 4-ABAH were independently performed three times. Data are the mean ± SD of triplicate determinations. $^{*}P < 0.05$; $^{\dagger}P < 0.01$; $^{\ddagger}P < 0.001$

Peroxidases Regulate Post-Translational Collagen Biosynthesis

To investigate the mechanism by which peroxidase enzymes regulate collagen I production, we compared the effect of EPO to the time-dependent secretion of collagen I stimulated by either AA or TGF-β2. In the absence of AA, EPO increased the levels of soluble collagen I detected in osteoblast-conditioned media by 12 h (Fig. 3a). The amount of collagen I stimulated by the peroxidase enzyme increased with time, reaching maximal levels by 48 h. The profile of collagen I release stimulated by EPO was comparable to that observed for AA, both in terms of magnitude and time dependency. In contrast, without AA supplementation, TGF-β2 failed to induce a significant increase in collagen I release over the same time frame (Fig. 3a). Quantitative real-time PCR analysis of collagen I α1 mRNA levels within these cells revealed that the robust increase in collagen I protein stimulated by EPO was not associated with a corresponding increase in collagen I mRNA levels (Fig. 3b). In contrast, TGF-β2 clearly induced a time-dependent increase in collagen I α1 mRNA, with a twofold increase detected at both the 24 and 48 h time points. AA failed to induce a significant increase in collagen Iα mRNA expression over the 48-h stimulation period, an observation that is consistent with previous reports in both human bone-derived osteoblasts and mouse MC3T3-E1 pre-osteoblasts treated with AA [17, 18].

AA is known to function as an enzymatic cofactor for prolyl hydroxylase to promote post-translational collagen biosynthesis [19–21]. As the AA and peroxidase-mediated collagen response profiles were very similar, further studies were conducted to establish whether peroxidase enzymes were also capable of regulating post-translational collagen I biosynthesis in a prolyl hydroxylase-dependent manner. Cultured osteoblasts were stimulated with either MPO or EPO in the presence of dimethyloxalylglycine (DMOG), a cell-permeable structural analogue of α-ketoglutarate,

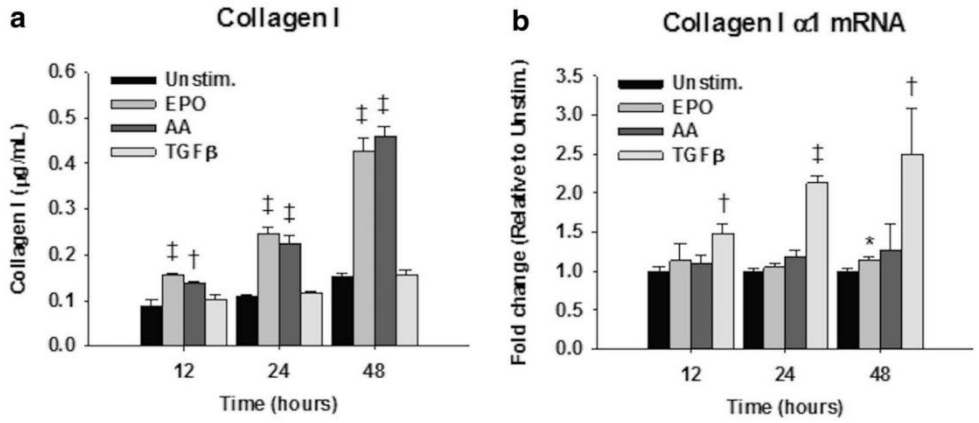


Fig. 3 Peroxidases induce collagen I release without regulating collagen I α1 mRNA. **a** ELISA detection of soluble collagen I levels in osteoblast-conditioned medium at the indicated time points upon stimulation with 1.56 μg/mL eosinophil peroxidase (EPO), 100 μmol/L ascorbic acid 2-phosphate (AA), or 10 ng/mL transforming growth factor (TGF)-β2. Cells treated with Dulbecco's modified Eagle's medium (DMEM) alone (Unstim.) served as the baseline control at each time point. The levels of soluble collagen I are expressed as μg/mL. **b** Quantitative real-time PCR analysis of the collagen I α1 mRNA expression (normalized to glyceraldehyde-3-phosphate dehydrogenase) in osteoblasts at the indicated time points upon stimulation with 1.56 μg/mL eosinophil peroxidase (EPO), 100 μmol/L ascorbic acid 2-phosphate (AA), or 10 ng/mL transforming growth factor (TGF)-β2. Cells treated with DMEM alone (Unstim.) served as the baseline control at each time point. The levels of collagen I α1 transcript are expressed as fold change and normalized so the average values of Unstim at each time point were set to 1. Statistical significance was calculated by two-tailed Student's *t* test, with the various treatment groups compared to the DMEM alone (Unstim) group at each time point. Each time-course experiment was independently performed three times. Data are the mean ± SD of triplicate determinations for each time point. $^{*}P < 0.05$; $^{\dagger}P < 0.01$; $^{\ddagger}P < 0.001$

which acts as a competitive inhibitor of prolyl hydroxylases [22]. As a positive control, cells stimulated with AA were also treated with DMOG to confirm that the release of collagen I was dependent upon prolyl hydroxylase-mediated hydroxylation. DMOG blocked the AA and peroxidase-induced release of collagen I from cultured osteoblasts in a dose-dependent manner, with complete inhibition occurring at 100 μmol/L (Fig. 4a). The inhibitory effect of DMOG was not associated with a loss in cell viability (Fig. 4b).

Eosinophil Peroxidase Promotes Osteoblast Matrix Mineralization In Vitro

To establish whether peroxidase enzymes could influence the matrix mineralization process in vitro, cultured osteoblasts were stimulated with either MPO or EPO in a dose range up to 12.5 μg/mL in the presence of DMEM mineralization medium containing AA 2-phosphate, dexamethasone, and *β*-glycerophosphate. After 12 days, cells were fixed and stained with Alizarin Red dye for the detection of calcium phosphate (mineral) deposition. Light microscopy imaging clearly shows that EPO stimulated a dose-dependent increase in mineral deposition by the osteoblasts (Fig. 5a), with Alizarin Red staining detected at EPO doses between 1.56 and 12.5 μg/mL. In contrast, MPO was not effective at promoting matrix mineralization over the same dose range (Fig. 5a). Quantitation of acid-extracted mineralized calcium using the Arsenazo III assay indicates that EPO induced a significant increase in mineralization at 1.56 μg/mL, with cells stimulated at the highest dose showing a greater than fivefold increase in mineral deposition compared to cells maintained under mineralization conditions without peroxidase enzymes (Fig. 5b). No increase in mineralized calcium was detected with MPO stimulation, consistent with the Alizarin Red staining.

EPO Regulates Osteogenic Gene Expression In Vitro

To establish whether peroxidase enzymes could regulate the expression of genes associated with osteoblast differentiation and matrix mineralization, primary human osteoblasts were grown to confluence and stimulated with either EPO or MPO under mineralizing conditions for 12 days. Quantitative real-time PCR analysis was performed to determine the effect peroxidases had on BMP-2, BSP, Wnt5a, and FRZB gene expression. BMP-2 plays a fundamental role in regulating the regenerative capacity of osteoblastic cells, and is possibly the most extensively investigated cytokine that enhances skeletal repair [23]. Relative to osteoblasts maintained in mineralization medium alone (Unstim), EPO stimulation resulted in a 14-fold increase in BMP-2 gene expression at a time when rapid mineralization of the cellular monolayer was occurring

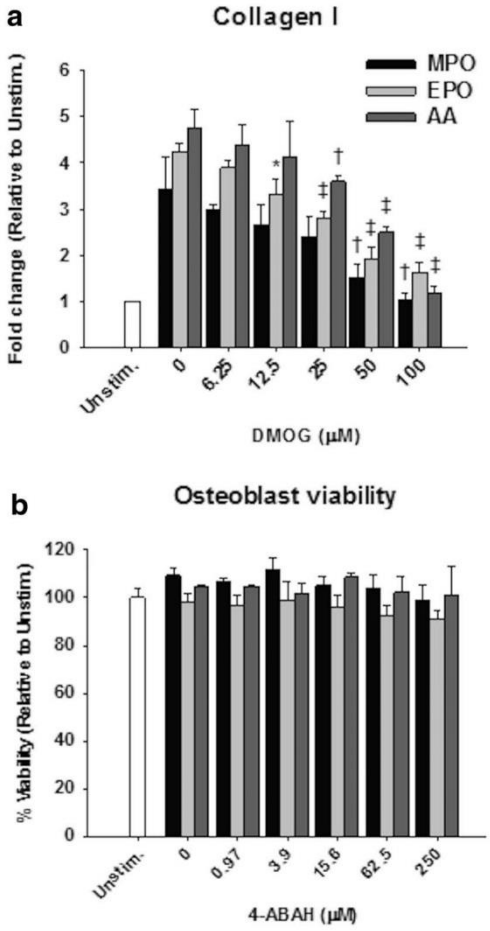


Fig. 4 Inhibition of peroxidase-induced collagen I release by the prolyl hydroxylase inhibitor dimethyloxalylglycine (DMOG). **a** ELISA detection of soluble collagen I levels in osteoblast-conditioned medium after cells were pre-treated for 30 min with DMOG at the doses indicated, then stimulated by the addition of either 1.56 μg/mL myeloperoxidase (MPO), 1.56 μg/mL eosinophil peroxidase (EPO), or 100 μmol/L ascorbic acid 2-phosphate (AA) for a further 72 h. Soluble collagen I levels are expressed as fold change and normalized to the average values of cells treated with Dulbecco's modified Eagle's medium (DMEM) alone (Unstim.). **b** Viability of osteoblasts after 72 h treatment with DMOG as assessed using the alamarBlue dye assay. Cell viability was normalized to the average values of cells treated with DMEM culture medium alone (Unstim.). Statistical significance was calculated by two-tailed Student's *t* test with the DMOG-treated groups compared to the treatment group without DMOG. Inhibition studies using DMOG were independently performed twice over the same dose range. Data are the mean ± SD of triplicate determinations. $^{*}P < 0.05$; $^{\dagger}P < 0.01$; $^{\ddagger}P < 0.001$

(Fig. 6). EPO was also found to induce the mRNA expression of BSP, an acidic non-collagenous bone ECM protein that facilitates mineralization by serving as a nucleation site for hydroxyapatite crystal formation [24]. Wnt5a-induced non-canonical signaling plays an essential role in skeletal development, and has been shown to promote osteoblast differentiation and mineralization [25, 26]. Our results show that EPO also induced a greater than twofold increase in Wnt5a expression, while at the same time down-regulating the expression of the secreted WNT inhibitor FRZB. In contrast to EPO, MPO stimulation of cultured osteoblasts had no effect on BMP-2, BSP, Wnt-5a, or FRZB gene expression (Fig. 6).

Cultured Human Osteoblasts Rapidly Bind and Internalize Peroxidase Enzymes

To determine whether exposure of cultured osteoblasts to MPO and EPO could promote cell surface binding and internalization of these enzymes, immunofluorescence localization studies were performed (Fig. 7). The images clearly show that osteoblasts bind and rapidly internalize peroxidase enzymes. Both MPO and EPO accumulate on the cell surface within minutes of peroxidase exposure (Fig. 7; Surface). After 3 h of peroxidase exposure, permeabilization of the cell surface membranes revealed intense intracellular MPO and EPO staining (Fig. 7; Intracellular). Both enzymes appear to localize to vesicle-like structures within the cell, with MPO adopting a distinct perinuclear distribution that is not evident with EPO staining. Probing peroxidase-treated cells with non-immune mouse or rabbit IgG confirms the specificity of the surface and intracellular EPO and MPO immunostaining (Fig. 7; Non-immune IgG staining).

Discussion

The studies reported here provide evidence for the first time that mammalian peroxidase enzymes have the capacity to regulate osteoblastic cell function in vitro, including collagen I biosynthesis, osteogenic gene expression, and matrix mineralization. These novel findings offer new insight into a potential mechanism, by which inflammatory cells are able to regulate osteogenesis at sites of fracture and pathological bone formation.

Collagen I biosynthesis by osteoblasts is a critical early-stage event required for the formation of new bone [27]. AA is known to function as an enzymatic cofactor for prolyl hydroxylase to promote post-translational collagen biosynthesis [19, 20]. AA increases cellular secretion of collagen proteins by promoting efficient hydroxylation of peptidyl proline, leading to the assembly of a stabilized procollagen triple helical structure. Without hydroxylation, unfolded procollagen is thermally unstable and is retained within the cell and degraded [20]. Our in vitro studies examining collagen I biosynthesis show that peroxidase enzymes elicit an overall response in cultured osteoblasts

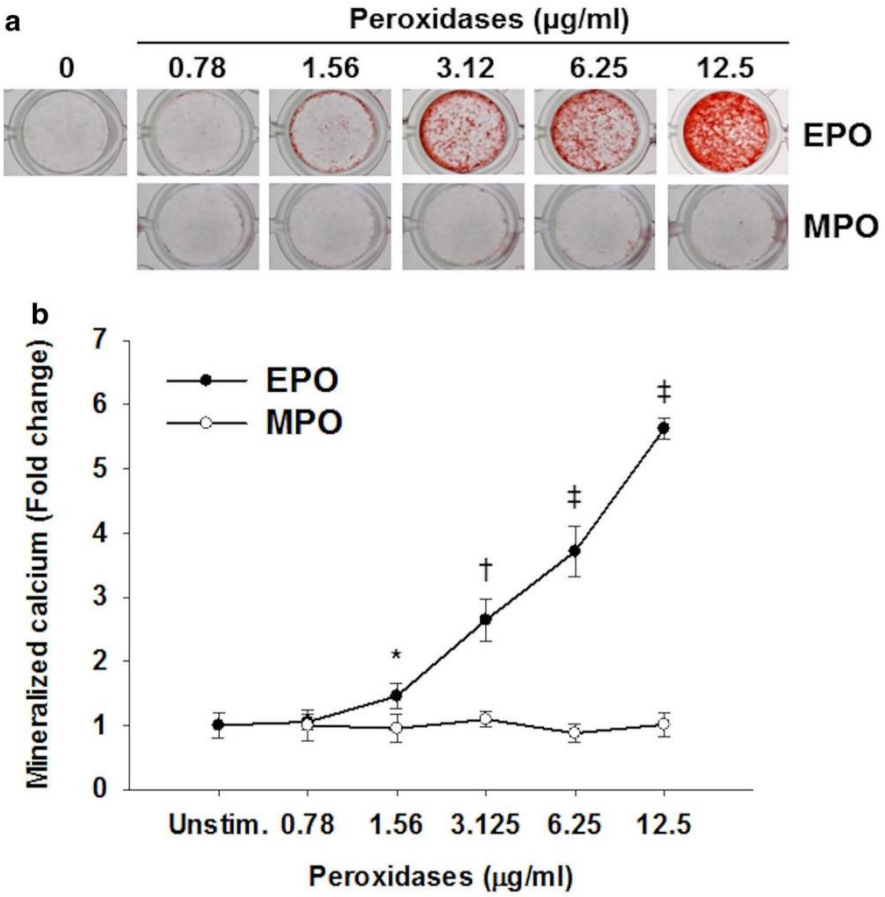


Fig. 5 Eosinophil peroxidase promotes osteoblast matrix mineralization in vitro. **a** Representative images of Alizarin Red stained cultured human osteoblasts stimulated with either EPO or MPO for 12 days at the various doses indicated. **b** Quantitation of mineralized calcium extracted from cultured osteoblast monolayers following stimulation with either EPO or MPO for 12 days at the doses indicated. Cells treated with mineralization medium alone (Unstim.) served as the baseline control. The levels of mineralized calcium are expressed as fold change and normalized so the average values of Unstim were set to 1. Statistical significance was calculated by two-tailed Student's *t* test with the various treatment groups compared to the mineralization medium alone (Unstim) group. Dose response experiments using osteoblasts were independently performed at least three times using cells derived from two different donors. Data are the mean ± SD of triplicate determinations for Unstim and each peroxidase dose. $^{*}P < 0.05$; $^{\dagger}P < 0.01$; $^{\ddagger}P < 0.001$ (Color figure online)

that is very similar to the effect we observed when stimulating these cells with AA. Exposure of osteoblasts to peroxidase proteins in the absence of AA supplementation stimulated a robust increase in soluble collagen I secretion without a corresponding increase in collagen I mRNA levels. Our data showing the inhibition of peroxidase-induced collagen I release by the prolyl hydroxylase inhibitor DMOG indicate that post-translational events are most likely to be affected by the peroxidases. Moreover, given that hydroxylation is an essential requirement for secretion of procollagen from the cell, we propose that peroxidase enzymes can also act at this point in the collagen I biosynthetic pathway to improve hydroxylation efficiency and the utilization of translated protein. We believe this important observation has previously been overlooked by others because AA is routinely added to osteoblast culture medium when examining collagen biosynthesis. When combined with AA, the stimulatory effects of peroxidase enzymes on osteoblast collagen release are masked (data not shown), suggesting both agents target the same regulatory point in collagen biosynthesis. These data obtained using cultured human osteoblasts are entirely consistent with our recently reported findings generated with primary fibroblasts derived from dermal, mammary, and prostate tissue [13], indicating the mechanism by which peroxidase enzymes regulate collagen I biosynthesis appears to be highly conserved across different mesenchymal-derived cell types.

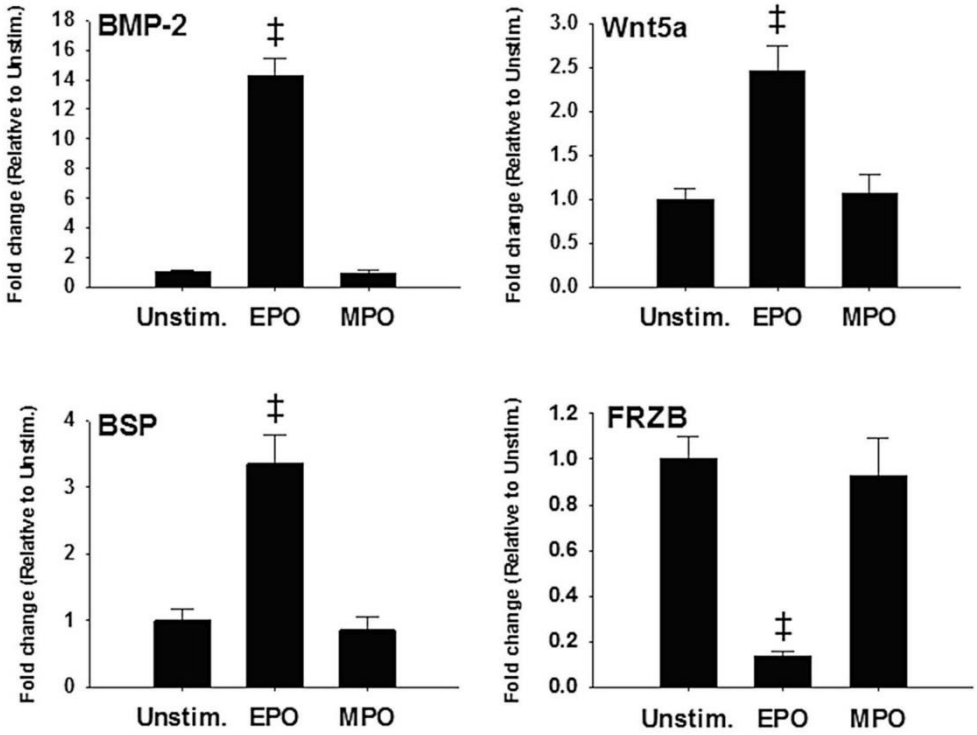


Fig. 6 Eosinophil peroxidase regulates osteogenic gene expression. Quantitative real-time PCR analysis of the BMP-2, BSP, Wnt5a, and FRZB mRNA expression (normalized to glyceraldehyde-3-phosphate dehydrogenase) in cultured osteoblasts following stimulation with either 5 μg/mL eosinophil peroxidase (EPO) or 5 μg/mL myeloperoxidase (MPO) for 12 days. Cells treated with DMEM mineralization medium alone (Unstim.) served as the baseline control. Transcript levels are expressed as fold change and normalized so the average values of Unstim were set to 1. Statistical significance was calculated by two-tailed Student's *t* test with the EPO and MPO treatment groups compared to the mineralization medium alone (Unstim) group. Data are the mean ± SD of triplicate determinations. $^{\ddagger}P < 0.001$

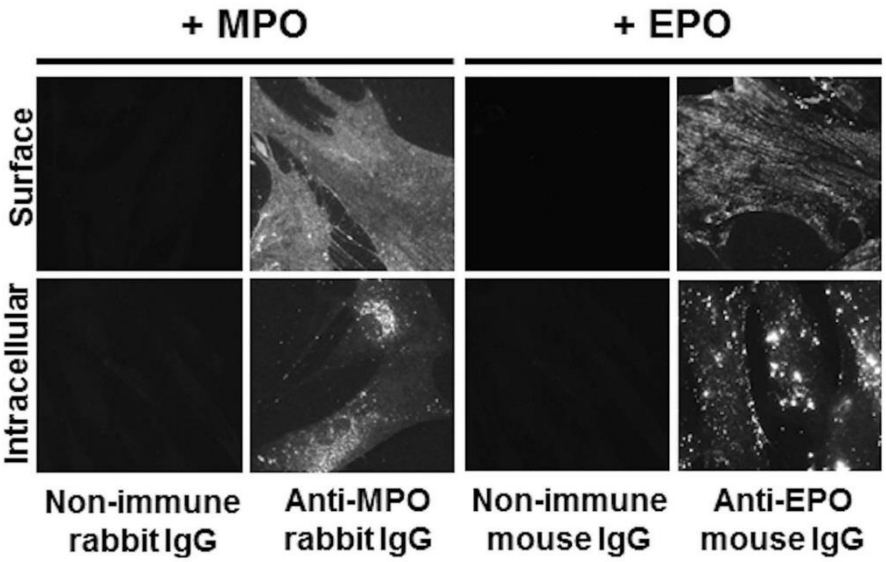


Fig. 7 Localization of peroxidase distribution by indirect immunofluorescence. Cultured human osteoblasts were exposed to 1 μg/mL myeloperoxidase (+MPO) or eosinophil peroxidase (+EPO) in serum-free Dulbecco's modified Eagle's medium for either 20 min (surface) or 3 h (intracellular). Cells were fixed, permeabilized (intracellular only), and stained for MPO and EPO localization, as described in "Materials and Methods" section. Original magnification: ×40 (Surface and Intracellular images)

Both MPO and EPO have been reported to bind to the surface of fibroblasts and endothelial cells in culture, triggering the intracellular uptake of these enzymes [13, 14, 28]. We have demonstrated by immunofluorescence localization studies that cultured osteoblasts can also rapidly bind and internalize both MPO and EPO. Once internalized, these catalytically active enzymes are able to consume hydrogen peroxide (H_2O_2) as an essential cofactor for the generation of multiple intracellular reactive oxidant species [9]. Cellular H_2O_2 is predominantly produced under inflammatory conditions through dismutation of superoxide that is formed in a reaction catalyzed by NADPH oxidase [29]. H_2O_2 is lipid soluble and can readily diffuse across cellular membranes and is known to oxidize the Fe^{2+} bound to the prolyl hydroxylase enzyme to Fe^{3+}, leading to inhibition of prolyl hydroxylase enzymatic activity and substrate hydroxylation [30]. Elevated H_2O_2 production has also been reported to deplete intracellular ascorbate levels, further dampening prolyl hydroxylase activity [31]. Given that prolyl hydroxylase plays an essential role in collagen biosynthesis, it is not surprising that supplementation of rabbit calvarial osteoblast cultures with exogenous H_2O_2 has previously been reported to suppress collagen I expression by these cells [32]. Moreover, 10-day chick embryo tibiae exposed to elevated H_2O_2 levels in an organ culture system showed a marked inhibition of collagen synthesis as measured by a decrease in ^{3}H-hydroxyproline incorporation into total collagen [33]. Importantly, our findings indicate the peroxidase heme-containing catalytic domain plays an essential role in MPO and EPO-mediated collagen biosynthesis in osteoblasts. One possible mechanism by which the peroxidase enzymes promote osteoblast collagen biosynthesis is by catalytically depleting intracellular H_2O_2 levels, thereby negating the inhibitory effect H_2O_2 has on prolyl hydroxylase enzymatic activity. It is therefore conceivable that peroxidase enzymes may play a dominant role in regulating osteoblast collagen biosynthesis during acute and chronic inflammatory conditions where H_2O_2 tissue levels are highly elevated, resulting in a depletion of cellular ascorbate availability.

In addition to stimulating collagen I biosynthesis, we have also demonstrated that peroxidases are capable of promoting osteoblast matrix mineralization in vitro. These mineralization studies were conducted in medium containing AA to promote the efficient cross-linking of soluble collagen into the ECM [21]. In the presence of AA, EPO was found to significantly promote matrix mineralization, while MPO failed to induce a mineralization response under the same culture conditions. These data suggest EPO has the capacity to influence the mineralization process beyond its ability to promote collagen biosynthesis. Concomitant with mineralization, EPO was also found to regulate the expression of several key osteogenic effector genes associated with osteoblast differentiation and matrix mineralization, including BMP-2, BSP, Wnt5a, and FRZB. BMP-2 has previously been reported to stimulate BSP and Wnt5a mRNA and protein levels in cultured osteoblasts [34, 35], raising the possibility that EPO indirectly regulates matrix mineralization via induction of BMP-2 which acts as an autocrine intermediate to regulate additional downstream effector genes including BSP and Wnt5a. As with mineralization, MPO also failed to regulate the expression of the osteogenic genes examined. Despite both MPO and EPO catalyzing reactions that use H_2O_2 as a cofactor, differences are observed regarding their substrate specificities, kinetics, and redox properties [36]. Depending on substrate availability within the cell, MPO and EPO will generate vastly different intracellular oxidant species which may account for difference in their ability to induce a mineralization response and regulate osteogenic gene expression [9].

While eosinophils are known to be recruited to sites of bone fracture in the immediate period following injury [2], the capacity of this inflammatory cell type to influence new bone formation has not been contemplated. Recent data, however, show that in patients suffering from chronic rhinosinusitis, a clear association exists between elevated mucosal eosinophilia and increased osteitic bone formation in the paranasal sinuses [37, 38], suggesting a causal link between eosinophil infiltration and pathological osteogenesis. Moreover, in an IL-5 overexpression transgenic mouse model where eosinophils comprise more than 60 % of circulating white blood cells, extensive eosinophilia and ectopic bone formation were observed in the spleen, together with increased cancellous bone growth in skeletal long bones compared to wild-type litter mates [39]. The authors of this study suggest that the release of latent TGF-β2 during the process of degranulation is one potential mechanism, by which activated eosinophils could stimulate osteoblasts to elicit new bone formation [39]. EPO is the most abundant secretory granule proteins released along with latent TGF-β during eosinophil degranulation [40, 41]. Our data showing eosinophil-derived EPO regulation of osteoblast collagen biosynthesis and matrix mineralization provide an alternative mechanistic explanation to account for the pro-osteogenic properties of eosinophils.

Heterotopic ossification (HO) is a debilitating condition characterized by the formation of ectopic bone in soft tissue such as muscle following traumatic injury. Inflammatory cell involvement is now recognized to be an important contributing factor in HO progression [42, 43], although the mechanism or identity of the specific factors released by these inflammatory cells at the injury site remains poorly defined. Based on our data showing that inflammatory cell-derived peroxidase enzymes can regulate

osteoblast functionality in vitro, consideration should be given to the potential role that peroxidase enzymes such as MPO and EPO may have in promoting ectopic bone formation in HO.

In conclusion, our novel in vitro findings suggest that the peroxidase enzymes MPO and EPO are likely to regulate multiple cellular processes involved in new bone formation, including osteoblast collagen I biosynthesis, osteogenic gene regulation, and bone matrix mineralization. Our data also highlight a potential mechanism to link pathological bone formation at sites where elevated tissue eosinophilia is a feature.

Acknowledgments This work was supported in part by The Hospital Research Foundation and the National Health and Medical Research Council (Career Development Fellowship/627015; Project Grant/1050694).

Compliance with Ethical Standards

Conflict of Interest Mark O. DeNichilo, Alexandra J. Shoubridge, Vasilios Panagopoulos, Vasilios Liapis, Aneta Zysk, Irene Zinonos, Shelley Hay, Gerald J. Atkins, David M. Findlay, and Andreas Evdokiou declare that they have no conflict of interest.

Human and Animal Rights and Informed Consent The use of all normal human donor-derived bone tissue was approved by the human ethics committees of the Royal Adelaide Hospital/University of Adelaide (Approval No. RAH130114). Human bone samples were obtained with informed written donor consent, as required and approved by the ethics committee.

References

1. Claes L, Recknagel S, Ignatius A (2012) Fracture healing under healthy and inflammatory conditions. Nat Rev Rheumatol 8:133–143
2. Prasad GC, Udupa KN (1972) Studies on ultrastructural patter of osteogenic cells during bone repair. Acta Orthop Scand 43:163–175
3. Andrew JG, Andrew SM, Freemont AJ, Marsh DR (1994) Inflammatory cells in normal human fracture healing. Acta Orthop Scand 65:462–466
4. Bastian O, Pillay J, Alblas L, Leenen L, Koenderman L, Blokhuis T (2011) Systemic inflammation and fracture healing. J Leukoc Biol 89:669–673
5. Dimitriou R, Tsiridis E, Giannoudis PV (2005) Current concepts of molecular aspects of bone healing. Injury 36:1392–1404
6. Einhorn TA, Gerstenfeld LC (2015) Fracture healing: mechanisms and interventions. Nat Rev Rheumatol 11:45–54
7. Grundnes O, Reikeras O (1993) The importance of the hematoma for fracture healing in rats. Acta Orthop Scand 64:340–342
8. Kolar P, Schmidt-Bleek K, Schell H et al (2010) The early fracture hematoma and its potential role in fracture healing. Tissue Eng Part B 16:427–434
9. Davies MJ, Hawkins CL, Pattison DI, Rees M (2008) Mammalian heme peroxidases: from molecular mechanisms to health implications. Antioxid Redox Signal 10:1199–1234
10. Van der Veen BS, de Winther MPJ, Heeringa P (2009) Myeloperoxidase: molecular mechanisms of action and their relevance to human health and disease. Antioxid Redox Signal 11:2899–2937
11. Acharya KR, Ackerman SJ (2014) Eosinophil granule proteins: form and function. J Biol Chem 289:17406–17415
12. Klebanoff SJ (2005) Myeloperoxidase: friend or foe. J Leukoc Biol 77:598–625
13. DeNichilo MO, Panagopoulos V, Rayner TE et al (2015) Peroxidase enzymes regulate collagen extracellular matrix biosynthesis. Am J Pathol 185:1372–1384
14. Panagopoulos V, Zinonos I, Leach DA et al (2015) Uncovering a new role for peroxidase enzymes as drivers of angiogenesis. Int J Biochem Cell Biol 68:128–138
15. Atkins GJ, Kostakis P, Pan B et al (2003) RANKL expression is related to the differentiation state of human osteoblasts. J Bone Miner Res 18:1088–1098
16. Kettle AJ, Gedye CA, Winterbourn CC (1997) Mechanism of inactivation of myeloperoxidase by 4-aminobenzoic acid hydrazide. Biochem J 321:503–508
17. Carinci F, Pezzetti F, Spina A et al (2005) Effect of Vitamin C on pre-osteoblast gene expression. Arch Oral Biol 50:481–496
18. Maehata Y, Takamizawa S, Ozawa S et al (2007) Type III collagen is essential for growth acceleration of human osteoblastic cells by ascorbic acid 2-phosphate, a long-acting vitamin C derivative. Matrix Biol 26:371–381
19. Kivirikko KI, Myllylä R, Pihlajaniemi T (1989) Protein hydroxylation: prolyl 4-hydroxylase, an enzyme with four cosubstrates and a multifunctional subunit. FASEB J 3:1609–1617
20. Myllyharju J, Kivirikko KI (2001) Collagens and collagen-related diseases. Ann Med 33:7–21
21. Canty EG, Kadler KE (2005) Procollagen trafficking, processing and fibrillogenesis. J Cell Sci 118:1341–1353
22. Baader E, Tschank G, Baringhaus K, Burghard H, Günzler V (1994) Inhibition of prolyl 4-hydroxylase by oxalyl amino acid derivatives in vitro, in isolated microsomes and in embryonic chicken tissues. Biochem J 300:525–530
23. Rosen V (2009) BMP2 signaling in bone development and repair. Cytokine Growth Factor Rev 20:475–480
24. Harris NL, Rattray KR, Tye CE et al (2000) Functional analysis of bone sialoprotein: identification of the hydroxyapatite-nucleating and cell-binding domains by recombinant peptide expression and site-directed mutagenesis. Bone 27:795–802
25. Yamaguchi TP, Bradley A, McMahon AP, Jones S (1999) A Wnt5a pathway underlies outgrowth of multiple structures in the vertebrate embryo. Development 126:1211–1223
26. Okamoto M, Udagawa N, Uehara S et al (2014) Noncanonical Wnt5a enhances Wnt/β-catenin signalling during osteoblastogenesis. Sci Rep 4:4493–4500
27. Cox G, Einhorn TA, Tzioupis C, Giannoudis PV (2010) Bone-turnover markers in fracture healing. J Bone Joint Surg 3:329–334
28. Baldus S, Eiserich JP, Mani A et al (2001) Endothelial transcytosis of myeloperoxidase confers specificity to vascular ECM proteins as targets of tyrosine nitration. J Clin Invest 108:1759–1770
29. Genestra M (2007) Oxyl radicals, redox-sensitive signalling cascades and antioxidants. Cell Signal 19:1807–1819
30. Gerald D, Berra E, Frapart YM et al (2004) JunD reduces tumor angiogenesis by protecting cells from oxidative stress. Cell 118:781–794
31. Page EL, Chan DA, Giaccia AJ et al (2008) Hypoxia-inducible factor-1α stabilization in nonhypoxic conditions: role of oxidation and intracellular ascorbate depletion. Mol Biol Cell 19:86–94
32. Bai XC, Lu D, Bai J et al (2004) Oxidative stress inhibits osteoblastic differentiation of bone cells by ERK and NF-kappaB. Biochem Biophys Res Commun 314:197–207

33. Ramp WK, Arnold RR, Russell JE, Yancey JM (1987) Hydrogen peroxide inhibits glucose metabolism and collagen synthesis in bone. J Periodontol 58:340–344
34. Lecanda F, Avioli LV, Cheng S (1997) Regulation of bone matrix protein expression and induction of differentiation of human osteoblasts and human bone marrow stromal cells by bone morphogenetic protein-2. J Cell Biochem 67:386–398
35. Robubi A, Berger C, Schmid M, Huber KR, Engel A, Krugluger W (2014) Gene expression profiles induced by growth factors in in vitro cultured osteoblasts. Bone Joint Res 3:236–240
36. Furtmüller PG, Zederbauer M, Jantschko W et al (2006) Active site structural and catalytic mechanisms of human peroxidases. Arch Biochem Biophys 445:199–213
37. Mehta V, Campeau NG, Kita H, Hagan JB (2008) Blood and sputum eosinophil levels in asthma and their relationships to sinus computed tomographic findings. Mayo Clin Proc 83:671–678
38. Snidvongs K, McLachlan R, Chin D et al (2012) Osteitic bone: a surrogate marker of eosinophilia in chronic rhinosinusitis. Rhinology 50:299–305
39. Macias MP, Fitzpatrick LA, Brenneise I, McGarry MP, Lee JJ, Lee NA (2001) Expression of IL-5 alters bone metabolism and induces ossification of the spleen in transgenic mice. J Clin Invest 107:949–959
40. Abu-Ghazaleh RI, Dunnette SL, Loegering DA et al (1992) Eosinophil granule proteins in peripheral blood granulocytes. J Leukoc Biol 52:611–618
41. Levi-Schaffer F, Garbuzenko E, Rubin A et al (1999) Human eosinophils regulate lung- and skin-derived fibroblast properties in vitro: a role for transforming growth factor β (TGF-β). Proc Natl Acad Sci USA 96:9660–9665
42. Evans KN, Forsberg JA, Potter BK et al (2012) Inflammatory cytokine and chemokine expression is associated with heterotopic ossification in high-energy penetrating war injuries. Orthop Trauma 26:204–212
43. Convente MR, Wang H, Pignolo RJ, Kaplan FS, Shore EM (2015) The immunological contribution to heterotopic ossification disorders. Curr Osteoporos Rep 13:116–124

CHAPTER 3:

SOYBEAN PEROXIDASE REGULATES COLLAGEN TYPE I BIOSYNTHESIS AND MATRIX MINERALISATION BY HUMAN OSTEOBLASTS

Statement of Authorship

Title of Paper	Soybean Peroxidase Regulates Collagen Type I Biosynthesis and Matrix Mineralisation by Human Osteoblasts
Publication Status	☐ Published ☐ Accepted for Publication ☐ Submitted for Publication ☑ Unpublished and Unsubmitted work written in manuscript style
Publication Details	

Principal Author

Name of Principal Author (Candidate)	Alexandra Shoubridge
Contribution to the Paper	Designed project, optimised and completed research experiments, performed data analysis and prepared manuscript.
Overall percentage (%)	75%
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.
Signature	Date 25/05/2018

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate in include the publication in the thesis; and

iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

Name of Co-Author (Candidate)	Vasilios Panagopoulos
Contribution to the Paper	Assisted in experimental design, data interpretation and manuscript revision.
Signature	Date 25/05/2018

Name of Co-Author	Mark DeNichilo
Contribution to the Paper	Completed *in vitro* experiments and assisted with project design.
Signature	Date 25/05/2018

Name of Co-Author	Shelley Hay		
Contribution to the Paper	Assisted in part with *in vitro* experiments.		
Signature		Date	25/05/2018

Name of Co-Author	Peter Anderson		
Contribution to the Paper	Supervised development of work, assisted in data interpretation and manuscript evaluation.		
Signature		Date	25/05/2018

Name of Co-Author	Andreas Evdokiou		
Contribution to the Paper	Supervised development of work, assisted in data interpretation and manuscript evaluation.		
Signature		Date	25/05/2018

Soybean Peroxidase Regulates Collagen Type I Biosynthesis and Matrix Mineralisation by Human Osteoblasts

Alexandra J. Shoubridge[1,*], Vasilios Panagopoulos[2], Mark O. DeNichilo[3], Shelley Hay[2]

Peter J. Anderson[4], Andreas Evdokiou[2,5]

[1]Musculoskeletal Biology Research Laboratory, School of Pharmacy and Medical Sciences, University of South Australia, Adelaide, Australia

[2]Breast Cancer Research Unit, Discipline of Surgery, University of Adelaide, Adelaide, Australia

[3]Centre for Cancer Biology, University of South Australia, Adelaide, Australia

[4]Australian Craniofacial Unit, Women's and Children's Hospital and Department of Paediatrics and Dentistry, University of Adelaide, Adelaide, Australia

[5]Centre for Personalised Cancer Medicine, University of Adelaide, Adelaide, Australia

*** Corresponding author.**

Ms. Alexandra J. Shoubridge

University of South Australia Cancer Research Institute

North Terrace, SA 5001, Australia

Tel.: +61 8 8302 7389

email: alex.shoubridge@unisa.edu.au

Keywords Soybean peroxidase, osteoblast, collagen biosynthesis, matrix mineralisation

ABSTRACT

Large bone defects and fractures remain a significant problem in regenerative medicine. Current treatment strategies which include autografting, allografting or the use of synthetic materials, promote bone repair but have numerous limitations including, poor osteoinductive effects. Growth factors such as bone morphogenetic proteins (BMPs) improve osteoinduction however, we are yet to identify one that is of greater economic value and with fewer adverse effects. Peroxidases are haem-containing enzymes which are normally released at sites of tissue injury and inflammation by infiltrating immune cells and until recently, have been studied mainly in the context of providing oxidative defence against invading pathogens. We have discovered new and previously unrecognised roles for haem peroxidases in extracellular matrix biosynthesis, angiogenesis and osteoclastogenesis, all of which play an important role in bone remodelling and repair. In this study, we used *in vitro* models to investigate the ability of the extremely cheap, stable and potent plant-derived soybean peroxidase, to regulate osteoblast function, which is necessary for normal bone repair. Here, we have demonstrated soybean peroxidase's pro-osteogenic role, by promoting collagen I biosynthesis and matrix mineralisation, which are both essential for the formation and maturation of a mineralised scaffold and subsequently, new bone. Mechanistically, we have also shown soybean peroxidase's ability to regulate osteogenic genes responsible for inflammation, extracellular matrix remodelling and ossification, which are necessary for normal bone healing. These findings show soybean peroxidase to be an effective regulator of osteoblast function and thus, could have numerous implications as a therapeutic agent in the context of bone repair.

INTRODUCTION

Large bone defects which occur as a result of trauma, tumour resection and fractures remain a significant problem in regenerative medicine. Autogenous bone graft transplantation is the current "gold standard" for the treatment of large bone defects. This form of transplantation consists of bone matrix, autologous cells and growth factors which provide a scaffold for new bone to form (Jimi et al., 2012). However, there are significant limitations associated with autografting including, pain from bone harvesting, longer rehabilitation and limited supply of grafting material (Homma et al., 2013). Despite alternatives such as allografts or synthetic graft materials, these treatment strategies are also limited by immunogenic or poor osteoinductive effects (Betz, 2002).

Recently, synthetic biomaterials which possess osteoconductive properties that promote the ingrowth of newly formed bone, have been widely investigated for use in bone regenerative medicine (Oryan, Alidadi, Moshiri, & Maffulli, 2014). For example, β-tricalcium phosphate (β-TCP) and synthetic hydroxyapatite (HA) are being developed due to their high biocompatibility and osteoconductive properties (Patlolla & Arinzeh, 2014). Despite their poor osteoinductive potential (e.g.; to signal stem cell differentiation), these calcium phosphate ceramics have proven to be effective carriers of osteoinductive agents such as bone morphogenetic proteins (BMPs), to accelerate bone formation (Ono, Gunji, Kaneko, Sait, & Kuboki, 1995). While BMPs play an important role in fracture repair, with the ability to promote recruitment, proliferation, differentiation and migration of bone-forming cells (Lind et al., 1996), their clinical use has resulted in a number of complications. These include higher revision rates, due to an increased inflammatory response accompanied by cyst-like bone and soft tissue swelling and ectopic bone formation (Zara et al., 2011). These reports, along with the significant high cost of using BMPs suggest the need to identify osteoinductive factors which result in fewer adverse side effects and be of greater economic value. A number of other agents have been

suggested to promote bone repair, such as those to enhance vascularisation (Curtin et al., 2015), but none have advanced to the clinic. Therefore, new therapeutic interventions for complex osseous defects and fractures are needed.

Peroxidases are a group of haem-containing enzymes found in animals, plants and micro-organisms (Hiraga et al., 2001). It is well-established that these enzymes share the same catalytic mechanism, by converting hydrogen peroxide and chloride ions into hypochlorous acid, which is one of the most reactive oxidants produced *in vivo* and is responsible for peroxidase anti-microbial actions (Aruoma & Halliwell, 1987). We have recently discovered new functional roles for peroxidase enzymes, including mammalian myeloperoxidase (MPO) and eosinophil peroxidase (EPO), plant-derived soybean peroxidase (SBP) and horseradish peroxidase (HRP). These roles include the ability to regulate fibroblast collagen extracellular matrix (ECM) biosynthesis (DeNichilo et al., 2015), drive angiogenesis (Panagopoulos et al., 2015) and inhibit osteoclastogenesis (Panagopoulos et al., 2017). Collectively, these findings suggest that peroxidases may have a causative role in tissue repair. In addition, we have recently discovered additional new roles for mammalian peroxidases in bone repair, by their ability to promote osteoblast collagen biosynthesis, osteogenic gene expression and bone matrix mineralisation (Mark O. DeNichilo et al., 2016). Despite the effectiveness demonstrated by mammalian peroxidases on osteoblast function *in vitro,* we have investigated the possible contribution of the plant-derived SBP in bone repair. SBP is one of the most biologically active peroxidase enzymes we have tested to-date, both *in vitro* and *in vivo* (DeNichilo et al., 2015). Furthermore, SBP is safe, highly stable and extremely cheap to manufacture in large quantities, making it a highly feasible and desirable candidate for clinical use.

In this report, we characterise for the first time, the ability of SBP to stimulate the release of collagen type I and promote matrix mineralisation by primary human osteoblasts. This is supported by mechanistic data that shows SBP regulates pro-osteogenic genes known

to be essential for bone regeneration. Our findings show SBP promotes osteoblast function *in vitro* and has the potential to play an important role as a therapeutic agent in bone repair.

MATERIALS AND METHODS

Ethics Statement

The use of all normal human donor-derived bone tissue was approved by the human ethics committee of the Royal Adelaide Hospital/University of Adelaide (Approval No. RAH 090101). Human bone samples were obtained with informed written donor consent, as required and approved by the ethics committee.

Osteoblast Cell Culture

Normal human bone-derived osteoblasts were isolated from intertrochanteric trabecular bone samples from four donors of both genders, age range 46-67 years, undergoing primary hip and knee replacement surgery, as described previously (Atkins et al., 2003). Human osteoblasts were expanded in culture using Dulbecco's Modified Eagle's Medium (DMEM; high glucose with no ascorbic acid; AA), supplemented with 2 mmol/L glutamine, 100 IU/mL penicillin, 100 µg/mL streptomycin, 20 mmol/L HEPES, and 10% foetal bovine serum (FBS; Invitrogen Life Technologies, Carlsbad, CA) in a 5% CO_2-containing humidified atmosphere. These cells maintain an osteoblastic phenotype in culture and stain positive for alkaline phosphatase activity.

Collagen I Enzyme-Linked Immunosorbent Assay (ELISA)

To evaluate the effect of SBP on collagen I production, osteoblasts were cultured and treated as previously described (Mark O. DeNichilo et al., 2016). Briefly, osteoblasts were cultured in 96-well plates for 5 days in DMEM/10% FBS until reaching confluence. Cells were starved overnight in serum-free DMEM and then stimulated for an additional 72 h in serum-free DMEM containing either AA 2-phosphate at 100 µmol/L (Wako Chemical Industries, Osaka, Japan) as a positive control, or with soybean peroxidase (SBP; Bio-Research Products, North

Liberty, IA) in the absence of AA supplementation. Osteoblast-conditioned media was then collected for measurement of secreted, soluble type I collagen by ELISA. Cell viability/growth was then assessed using the alamarBlue fluorescent dye assay (Invitrogen Life Technologies), according to manufacturer's instructions. Fluorescence was measured at wavelengths of 530 nm excitation and 595 nm emission using a FLUOstar Optima plate reader (BMG Labtek Australia, Mornington, VIC).

The amount of soluble type I collagen in cell-conditioned medium was measured by a direct coat enzyme-linked immunosorbent assay method, as previously described (Mark O. DeNichilo et al., 2016). Samples and standards were added to a 96-well Maxisorp plate (Nunc) and left at 4°C overnight. The plate was then washed with phosphate-buffered saline (PBS) with 0.05% Tween, 2.5% bovine serum albumin (BSA)/PBS blocking solution added to each well and the plate incubated for 1 h at room temperature. The plate was then washed and primary antibody (0.25 μg/mL rabbit anti-human-collagen I polyclonal; Rockland Immunochemicals, Limerick, PA) in 5% non-fat dairy milk added to each well for 3 h at room temperature. After washing, europium-tagged anti-rabbit secondary antibody (0.5 μg/mL in 1% BSA/PBS; Perkin Elmer Life Sciences, Turku, Finland) was added for 1h at room temperature. After a final wash, Enhancement Solution (Perkin Elmer Life Sciences) was added, and time-resolved fluorescence was measured at excitation 355 nm and emission 620 nm using a FLUOstar Optima plate reader (BMG Labtek Australia). The collagen content of each sample was determined from the standard curve (μg/mL), constructed from purified type I human placental collagen (BD Biosciences Australia, North Ryde, NSW) and then normalised to DMEM-only treated cells.

In Vitro Mineralisation

Normal human bone-derived osteoblasts were seeded into 96-well plates (Nunc) at a density of 1.2×10^4 cells per well and cultured for 5 days in AA-free 10% FBS/DMEM at 37°C and 5% CO_2. Triplicate wells were stimulated with SBP in osteogenic DMEM mineralisation medium [DMEM supplemented with 5% FBS, 100 μmol/L AA 2-phosphate (Wako Chemical Industries), 10^{-8} mol/L dexamethasone (Hospira Australia, Mulgrave, VIC), and 10 mmol/L β-glycerophosphate (Sigma-Aldrich)] to assist bone mineral formation. Cells were maintained in culture for 21 days, with fresh medium with or without SBP, changed every 7 days. To detect matrix mineralisation, the Alizarin Red staining method was used as previously described (Mark O. DeNichilo et al., 2016). Briefly, cells were washed with PBS and fixed with 10% phosphate-buffered formalin. The fixed cells were then washed twice with distilled water and stained with 2% Alizarin Red S solution (Sigma-Aldrich). The stained mineralised matrix was photographed using a Nikon Eclipse 50i microscope attached to a DS-L2 control unit (Digital Sight, Nikon Europe, Amsterdam, The Netherlands) and a DS-Fi1 digital camera (Nikon Corporation, Tokyo, Japan).

The extent of mineralisation was quantitated by eluting the Alizarin Red S dye with 10% (w/v) cetylpyridinium chloride (Sigma-Aldrich) in 10 mM phosphate buffer pH 7.0, for 10 mins to release remaining calcium-bound Alizarin Red S. Absorbance was measured at a wavelength of 570 nm using a FLUOstar Optima plate reader (BMG Labtek Australia).

Microarray

To evaluate the effects on known primary human osteoblast pro-osteogenic genes by SBP, a microarray was performed. Osteoblasts harvested from a single donor were seeded at a density of 6×10^4 into T25 culture flasks in DMEM/10 % FBS and maintained in culture for 5 days. On reaching confluence, cells were stimulated with or without 5 μg/mL SBP for 12 days in

osteogenic DMEM mineralisation medium [DMEM supplemented with 5 % FBS, 100 μmol/L AA 2-phosphate (Wako Chemical Industries), 10−8 mol/L dexamethasone (Hospira Australia, Mulgrave, VIC), and 10 mmol/L β-glycerophosphate (Sigma-Aldrich)]. Total RNA was harvested using an RNeasy Mini Kit (Qiagen Australia, Chadstone, VIC) according to the manufacturer's instructions. RNA yield and purity were quantified by Nanodrop spectrophotometric measurement at 260 nm (Nanodrop Technologies, Thermo Fisher Scientific, Scoresby, VIC, Australia). RNA was analysed using whole-genome Illumina sequencing (Australian Genome Research Facility, Parkville, VIC). The Illumina GenomeStudio software (Version 1.9.0) was used to extract and normalise the expression data for the mean intensity of the array. Genes that were statistically significant ($p = \leq 0.05$) and exceeded a pre-set threshold for significantly higher (≥1.5 fold change) or lower (≤1.5 fold change) expression compared to control were included for further analysis. Of the genes presented in the array, 836 genes met this prerequisite. Those genes which had the greatest difference in fold change and known key osteogenic genes were selected and classified according to associated gene ontology terms and participation in biological pathways. The genes were arranged in related groups using functional annotation tools and bioinformatics software provided by the open web-based Database for Annotation, Visualisation and Integrated Discovery (DAVID) Bioinformatics Resources 6.7 (Huang, Sherman, & Lempicki, 2009a, 2009b) (Table 1).

Quantitative Real-Time PCR (qRT-PCR)

To validate nominated genes from the microarray, qRT-PCR was performed. Custom TaqMan® Array Fast Plates (Thermo Fisher, Scoresby, VIC) were pre-spotted with TaqMan® Gene Expression Assay probes for BMP-2, BSP, WNT-5A, FRZ-B, CCL5, CXCL5, CXCL6, CXCL12, MMP1, MMP3, IL6 and IL8. GAPDH was used as the control house-keeping gene.

The assay numbers for each probe are defined in Table 2. Quantitative RT-PCR was performed using TaqMan® Fast Advanced Master Mix (Thermo Fisher, Scoresby, VIC), in a ViiA 7 Real-Time System (Applied Biosystems, Foster City, CA).

Data Analysis and Statistics

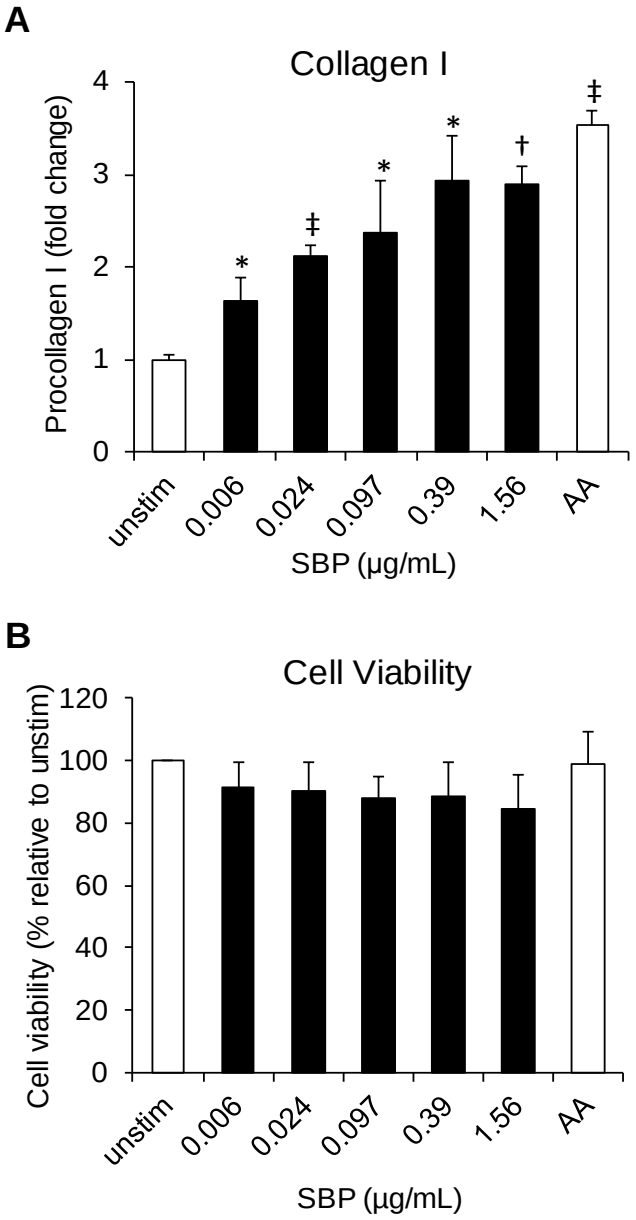
Data points derived from experiments are reported as the mean ± standard deviation (SD). Analysis of variance to determine significant difference between samples was performed using the paired Student's t-test.

RESULTS

SBP Stimulates Primary Human Osteoblasts to Release Type I Collagen

Collagen type I is the most abundant type of collagen and accounts for approximately 90% of the total collagen content of bone (Gay & Miller, 1978). When primary human osteoblasts were stimulated for 72 h with SBP, in the absence of AA we observed a dose-responsive increase in collagen type I release. Maximal doses of SBP resulted in a three-fold increase in soluble collagen type I release which was comparable to the amount measured in the presence of AA, which was used as the control (Fig. 1A). Significant increases in collagen secretion were observed even at the lowest dose of SBP of 6 ng/mL. Assessment of cell viability indicated that SBP and AA had no impact on osteoblast numbers (Fig. 1B), even up to 6.25µg/mL (data not shown). This confirmed that SBP is not toxic at the doses tested and importantly, shows the increase in collagen type I was not related to effects on cellular proliferation. Consistent with our previously published data of mammalian peroxidases, these results demonstrate that the plant-derived SBP can promote collagen type I secretion by osteoblasts in an ascorbic acid-independent manner (Mark O. DeNichilo et al., 2016).

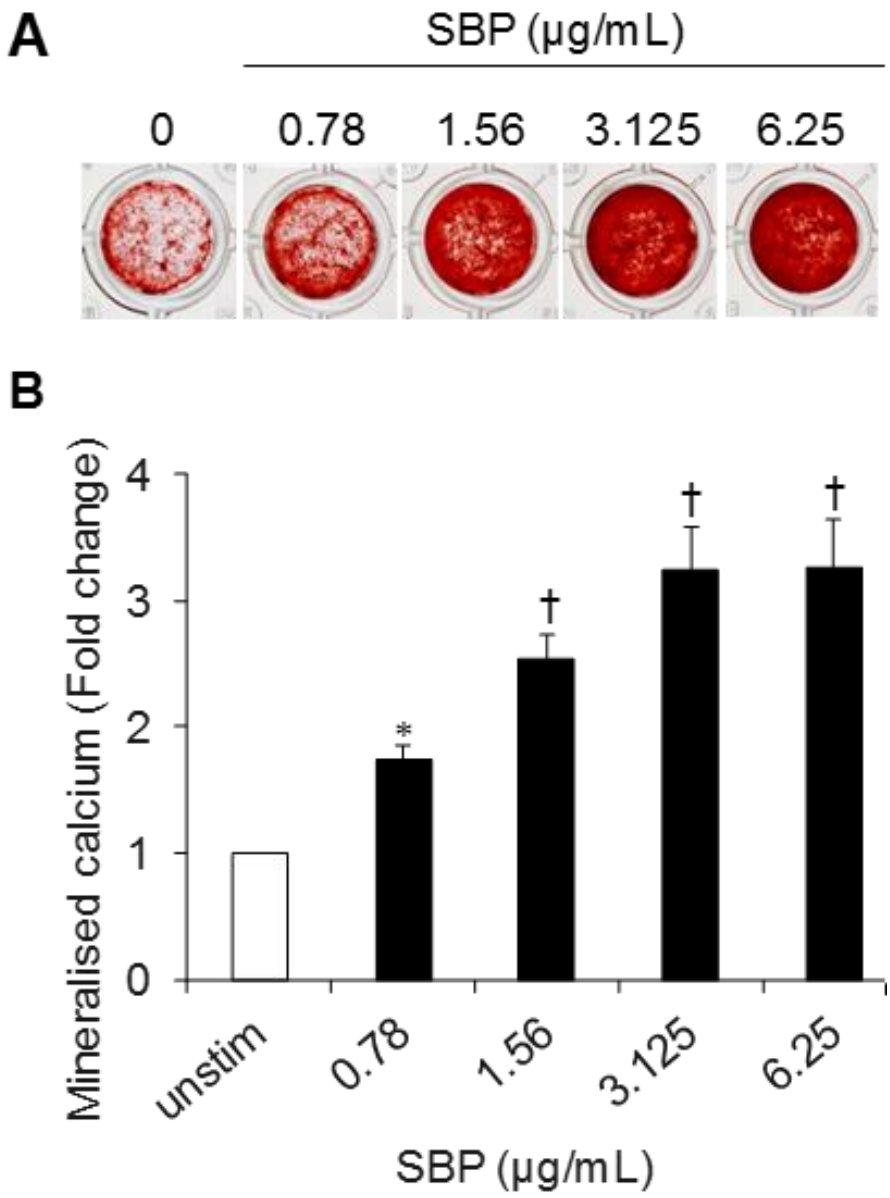
Figure 1. *Soybean peroxidase (SBP) promotes collagen I release by cultured human osteoblasts.* A ELISA detection of soluble collagen I in osteoblast-conditioned medium after 72h stimulation with SBP at the doses indicated. Ascorbic acid 2-phosphate (AA) at 100µmol/L served as the positive control, whereas cells treated with Dulbecco's modified Eagle's medium (DMEM) alone (unstim) served as the baseline control. The levels of collagen I are expressed as fold change and normalised so the average values of unstim were set to 1. The data are pooled from three experiments each conducted using cells derived from two donors. B Viability of cultured osteoblasts after 72h stimulation as assessed using the alamarBlue dye assay. Cell viability was normalised so the average values of unstim cells were set to 100% relative to each peroxidase dose. The data are pooled from three experiments each conducted using cells derived from three donors. Data are the mean±SD for unstim, AA, and each peroxidase dose. *$P < 0.05$; †$P < 0.01$; ‡$P < 0.001$


A
Collagen I
Procollagen I (fold change)
0
1
2
3
4
*
*
‡
*
†
‡
unstim
0.006
0.024
0.097
0.39
1.56
AA
SBP (µg/mL)
B
Cell Viability
Cell viability (% relative to unstim)
0
20
40
60
80
100
120
unstim
0.006
0.024
0.097
0.39
1.56
AA
SBP (µg/mL)

SBP Stimulates Primary Human Osteoblasts to Promote Matrix Mineralisation

We next examined the ability of SBP to promote the matrix mineralisation process of primary human osteoblasts. Light microscopy clearly shows SBP significantly increased mineral deposition of osteoblasts at each dose tested (Fig. 2A), as observed by the increased intensity of Alizarin Red staining from the lowest dose at 0.78 µg/mL. Quantification of the eluted Alizarin Red staining revealed that the addition of SBP resulted in a significant increase in mineralisation at 0.78 µg/mL (Fig. 2B), with cells stimulated by SBP at the highest dose of 6.25 µg/mL showing a three-fold increase in mineral deposition. This was compared to cells maintained under mineralising conditions without SBP. Together, these data suggest that SBP is promoting normal matrix mineralisation of stimulated primary human osteoblasts.

Figure 2. *Soybean peroxidase promotes osteoblast matrix mineralisation in vitro.* A Representative images of Alizarin Red stained cultured human osteoblasts stimulated with SBP for 21 days at the various doses indicated. B Quantitation of mineralised calcium extracted from cultured osteoblast monolayers following stimulation with SBP for 21 days at the doses indicated. Cells treated with mineralisation medium alone (unstim.) served as the baseline control. The levels of mineralised calcium are expressed as fold change and normalised so the average values of unstim were set to 1. Statistical significance was calculated by two-tailed Student's *t* test with the various doses of SBP compared to the mineralisation medium alone (unstim) group. Dose response experiments using osteoblasts were independently performed at least three times using cells derived from two different donors. Data are the mean ± SD of triplicate determinations for unstim and each peroxidase dose. *$P < 0.05$; †$P < 0.01$


A
SBP (µg/mL)
0
0.78
1.56
3.125
6.25
B
Mineralised calcium (Fold change)
0
1
2
3
4
*
†
†
†
unstim
0.78
1.56
3.125
6.25
SBP (µg/mL)

SBP Regulates Expression of Genes Involved in Osteoblast Function

We next sought to attribute the effects of SBP on mineralisation to levels of expression of genes associated with osteoblast differentiation and mineralisation. We performed a RNA microarray on SBP-treated osteoblasts which identified 6,554 genes to be differentially expressed. Those genes which had the greatest difference in fold change and known key genes involved in osteoblast function were selected and classified according to the molecular function of their related protein and their involvement in biological processes, using web-based classification programs as described in the Methods section. Based on that approach we present differentially expressed genes in six categories: genes involved in ossification, inflammatory and immune responses, cell migration, extracellular matrix and genes encoded in response to wounding (Table 1.). The expression of selected genes was confirmed by qRT-PCR. The data obtained with qRT-PCR confirmed the microarray data on gene expression for BMP-2, BSP, WNT-5A, FRZ-B, CCL5, CXCL5, CXCL6, CXCL12, MMP1, MMP3, IL-6 and IL-8 (Table 2.). Taken together, these results indicate the effects of peroxidases may be the culmination of differential regulation of effector genes that are key to osteogenesis and repair.

Table 1. Pathways of genes differentially expressed by soybean peroxidase-treated osteoblasts

Term	Count	Genes	Fold enrichment
GO:0001503~ossification	2	BSP, BMP2,	29.40
GO:0006954~inflammatory response	5	IL6, BMP2, IL8, CXCL6, CCL5	18.92
GO:0006955~immune response	6	IL6, IL8, CXCL5, CXCL6, CXCL12, CCL5	10.69
GO:0009611~response to wounding	5	IL6, BMP2, IL8, CXCL6, CCL5	11.60
GO:0016477~cell migration	4	IL6, IL8, CXCL12, CCL5	17.82
GO:0031012~extracellular matrix	3	WNT5A, MMP3, MMP1	12.34

Table 2: Soybean peroxidase regulates the expression of key osteoblast-related genes

Gene	Assay number	PCR product size (bp)	Fold change	SD	*p*-value
BMP-2	Hs00154192_m1	60	**4.87**	**0.93**	**0.001993**
BSP	Hs00173720_m1	95	**4.31**	**0.11**	**0.000001**
WNT-5A	Hs00998537_m1	61	**2.21**	**0.04**	**0.000012**
FRZ-B	Hs00173503_m1	108	**0.44**	**0.01**	**0.000012**
CCL5	Hs00982282_m1	70	**208.67**	**4.75**	**0.000001**
CXCL5	Hs01099660_g1	93	**461.44**	**31.25**	**0.000014**
CXCL6	Hs00605742_g1	125	**649.20**	**77.15**	**0.000130**
CXCL12	Hs03676656_mH	88	**5.11**	**0.40**	**0.000063**
MMP1	Hs00899658_m1	64	**26.08**	**0.98**	**0.000002**
MMP3	Hs00968305_m1	126	**403.35**	**17.89**	**0.000003**
IL6	Hs00174131_m1	95	**5.41**	**0.27**	**0.000011**
IL8	Hs00174103_m1	101	**147.57**	**9.46**	**0.000011**
GAPDH	Hs02786624_g1	157			

DISCUSSION

Collagen type I is the major extracellular matrix constituent of bone and its synthesis by osteoblasts is critical for normal bone formation (Cox, Einhorn, Tzioupis, & Giannoudis, 2010). It is well reported that AA, an enzymatic cofactor, is essential for the accumulation of a collagen-rich matrix which subsequently becomes mineralised (Franceschi & Iyer, 1992). The role of AA in ECM production has been well characterised, by its ability to promote hydroxylation of peptidyl-proline which results in the assembly of a stabilised triple-helical procollagen molecule. Without AA, the absence of hydroxylation results in unstable procollagen molecules which rapidly degrade within the cell (Myllyharju & Kivirikko, 2001). Importantly, *in vivo* studies have shown that AA deficiency results in delayed bone matrix mineralisation (Sugimoto et al., 1998), highlighting the link between collagen deposition and matrix mineralisation. We show here that the plant-derived SBP stimulates collagen I release by osteoblasts in the absence of AA supplementation. When considering the previously assumed, indispensable role of AA in ECM production and mineralisation, the ability of SBP to elicit a similar response in collagen synthesis in the absence of AA suggests a potential therapeutic role for this enzyme in regenerative medicine.

We demonstrate here that SBP promotes osteoblast matrix mineralisation. For these mineralisation studies, AA was maintained in the mineral media due to its essential role for normal cross-linking of soluble collagen and its presence in normal physiological conditions (Peterkofsky, 1991). In the presence of AA, we found SBP to significantly promote matrix mineralisation by human osteoblasts. When considering the limitations of current grafting strategies, particularly the healing time required for large bone defects, we believe SBP could be effective at accelerating the repair process as seen by the increase in mineralisation compared to AA alone. The ability of SBP to manipulate the mineralisation process beyond the initial

stage of collagen biosynthesis suggests that SBP possesses the necessary properties of an effective osteoinductive agent.

In association with mineralisation, we found SBP to be an effective regulator of several genes involved in osteoblast function including, osteoblast differentiation, mineralisation and repair. Firstly, our *in vitro* studies exhibited significant up-regulation of BMP-2, WNT-5A and BSP. It was previously reported that BMP-2 regulates BSP and WNT-5A mRNA expression by cultured osteoblasts (Lecanda, Avioli, & Cheng, 1997; Robubi et al., 2014). SBP not only up-regulated these bone forming genes but down-regulated a known repressor of osteogenesis, FRZ-B related protein. FRZ-B acts as a competitive inhibitor of the WNT signalling pathway (S. W. Cho et al., 2008), which, like BMP-2 is a key modulator of bone formation (Takada, Kouzmenko, & Kato, 2009). These data suggest that SBP may indirectly regulate mineralisation via induction of BMP-2, which acts as an intermediate for the regulation of downstream effector genes. BMP-2 has been extensively tested both *in vitro* and *in vivo* as an agent for the repair of osseous defects both alone and in combination with other growth factors (Nakamura et al., 2005; Simmons, Alsberg, Hsiong, Kim, & Mooney, 2004; Street, Bao, Bunting, et al., 2002). Of these, vascular endothelial growth factor (VEGF) has been highly investigated by its ability to induce angiogenesis, act as an effective promoter of osteoblast differentiation and BMP-2 induced bone formation (Peng et al., 2005; Street, Bao, Bunting, et al., 2002). This is of particular relevance given our recently published findings which demonstrate the pro-angiogenic potential of peroxidases compared to VEGF (Panagopoulos et al., 2015).While BMP-2 appears to be the most potent osteoinductive agent identified to-date, several studies have shown osteoclastic activation and bone resorption in response to BMP-2 treatment (Seeherman, Li, Bouxsein, & Wozney, 2010; Toth et al., 2009), which may interfere with accelerated bone repair. This is further supported by a recent study which tested osteoprotegerin (OPG), a receptor activator of nuclear factor kappa-B ligand (RANKL) inhibitor which blocks

the differentiation and function of osteoclasts, increased bone repair of a mouse critical defect model in combination with locally delivered BMP-2 (Bougioukli et al., 2016). These reports are of significant interest, considering our recent findings which demonstrate the ability of peroxidases to inhibit RANKL-induced osteoclast formation and bone resorption (Panagopoulos et al., 2017). Therefore, our findings suggest that peroxidases may act as effective agents in regulating multiple aspects of the bone regenerative process.

In addition to the regulation of genes involved in osteoblast function by SBP, we observed significant up-regulation of numerous cytokines and chemokines including CCL5, CXCL5, CXCL6, CXCL12, IL-8, and IL-6, which are crucial in the recruitment and regulation of bone remodelling. For example, CCL5 when knocked out in mice, resulted in impaired bone formation and increased osteoclastogenesis (Wintges et al., 2013). Moreover, CCL5, was shown to promote osteoblast migration and survival (Yu, Huang, Collin-Osdoby, & Osdoby, 2004). The regulation of IL-8, CXCL5 and CXCL6, which are essential for endothelial cell proliferation and neutrophil attraction (Koch et al., 2001), further supports SBPs potential role in angiogenesis. The pro-inflammatory cytokine IL-6 was also upregulated by SBP, which has been shown to both enhance and inhibit osteoclastogenesis and more recently has been reported to be involved in promoting osteoclastogenesis by indirectly increasing RANKL expression by osteoblasts (Duplomb et al., 2008; Udagawa et al., 1995). However recent studies have found IL-6 may affect bone mass by influencing osteocyte communication towards osteoblasts (Bakker, Kulkarni, Klein-Nulend, & Lems, 2014). Despite the conflicting results it is evident IL-6 is necessary for bone remodelling and therefore essential in the repair process. Up-regulated CXCL12 is also substantially involved in bone formation and healing. Previous studies have shown that blocking CXCL12 or its receptor CXCR4, led to reduced osteoblastic differentiation markers, including their transcription factors (Zhu et al., 2007). Furthermore, in a mouse model, inactivation of CXCR4 resulted in reduced bone mass, decreased bone mineral

density and decreased expression of collagen type I (Zhu, Liang, Huang, Doty, & Boskey, 2011). Interestingly, increased collagen type I secretion in the presence of SBP may be responsible for the increase in MMP1 expression observed, due to its functional role in the degradation of collagen type I (Rao, Mohanam, Puppala, & Rao, 1999). Although MMP3 is not a collagenase like MMP1, it was reported previously to be an effective activator of proMMP1 (Rao et al., 1999). This investigation of the mechanistic relationship SBP has on osteoblast function was only conducted on a handful of key osteogenic genes, in order to better understand the role SBP has on mineralisation and repair. The limitation of only investigating a handful of key genes does provide preliminary evidence to suggest a causative role of SBP in the bone regenerative process by mechanisms involving the regulation of genes essential for bone repair.

In conclusion, our findings suggest SBP possesses a new pro-osteogenic role, to act as an osteoinductive agent, by promoting collagen I biosynthesis and matrix mineralisation. We have also presented the first evidence for potential mechanisms, demonstrating SBP's ability to regulate genes involved in osteoblast function that are responsible for inflammation, ECM remodelling and ossification, which are necessary for normal bone healing. However, further studies are required to fully elucidate the mechanisms by which SBP promotes osteoblast differentiation and activity. Although it remains to be seen how effective SBP will be *in vivo*, we believe that it has the potential to be used in a localised setting in combination with current, commercially available biomaterials and scaffolds. Continued research both *in vitro* and *in vivo* will further optimise SBP and ultimately determine its potential as an effective osteoinductive agent for bone repair.

ACKNOWLEDGEMENTS

This work was supported in part by The Hospital Research Foundation and the National Health and Medical Research Council (Career Development Fellowship/627015; Project Grant/1050694).

CONFLICT OF INTEREST

The authors declare that they have no competing interests.

REFERENCES

Aruoma, O. I., & Halliwell, B. (1987). Action of hypochlorous acid on the antioxidant protective enzymes superoxide dismutase, catalase and glutathione peroxidase. *Biochemical Journal, 248*(3), 973-976.

Atkins, G. J., Kostakis, P., Pan, B., Farrugia, A., Gronthos, S., Evdokiou, A., . . . Zannettino, A. C. (2003). RANKL expression is related to the differentiation state of human osteoblasts. *Journal of Bone and Mineral Research, 18*(6), 1088-1098.

Bakker, A., Kulkarni, R., Klein-Nulend, J., & Lems, W. (2014). IL-6 alters osteocyte signaling toward osteoblasts but not osteoclasts. *Journal of dental research, 93*(4), 394-399.

Betz, R. R. (2002). Limitations of autograft and allograft: new synthetic solutions. *Orthopedics, 25*(5), S561-S570.

Bougioukli, S., Jain, A., Sugiyama, O., Tinsley, B. A., Tang, A. H., Tan, M. H., . . . Lieberman, J. R. (2016). Combination therapy with BMP-2 and a systemic RANKL inhibitor enhances bone healing in a mouse critical-sized femoral defect. *Bone, 84*, 93-103.

Cho, S. W., Her, S. J., Sun, H. J., Choi, O. K., Yang, J.-Y., Kim, S. W., . . . Shin, C. S. (2008). Differential effects of secreted frizzled-related proteins (sFRPs) on osteoblastic differentiation of mouse mesenchymal cells and apoptosis of osteoblasts. *Biochemical and biophysical research communications, 367*(2), 399-405.

Cox, G., Einhorn, T., Tzioupis, C., & Giannoudis, P. (2010). Bone-turnover markers in fracture healing. *Bone & Joint Journal, 92*(3), 329-334.

Curtin, C. M., Tierney, E. G., McSorley, K., Cryan, S. A., Duffy, G. P., & O'brien, F. J. (2015). Combinatorial Gene therapy accelerates bone regeneration: non-viral dual delivery of VEGF and BMP2 in a collagen-nanohydroxyapatite scaffold. *Advanced healthcare materials, 4*(2), 223-227.

DeNichilo, M. O., Panagopoulos, V., Rayner, T. E., Borowicz, R. A., Greenwood, J. E., & Evdokiou, A. (2015). Peroxidase Enzymes Regulate Collagen Extracellular Matrix Biosynthesis. *The American Journal of Pathology, 185*(5), 1372-1384.

DeNichilo, M. O., Shoubridge, A. J., Panagopoulos, V., Liapis, V., Zysk, A., Zinonos, I., . . . Evdokiou, A. (2016). Peroxidase Enzymes Regulate Collagen Biosynthesis and Matrix Mineralization by Cultured Human Osteoblasts. *Calcified Tissue International, 98*(3), 294-305.

Duplomb, L., Baud'Huin, M., Charrier, C., Berreur, M., Trichet, V., Blanchard, F., & Heymann, D. (2008). Interleukin-6 inhibits receptor activator of nuclear factor κB ligand-induced osteoclastogenesis by diverting cells into the macrophage lineage: key role of serine727 phosphorylation of signal transducer and activator of transcription 3. *Endocrinology, 149*(7), 3688-3697.

Franceschi, R. T., & Iyer, B. S. (1992). Relationship between collagen synthesis and expression of the osteoblast phenotype in MC3T3-E1 cells. *Journal of Bone and Mineral Research, 7*(2), 235-246.

Gay, S., & Miller, E. J. (1978). *Collagen in the physiology and pathology of connective tissue*: Fischer.

Hiraga, S., Sasaki, K., Ito, H., Ohashi, Y., & Matsui, H. (2001). A large family of class III plant peroxidases. *Plant and Cell Physiology, 42*(5), 462-468.

Homma, Y., Zimmermann, G., & Hernigou, P. (2013). Cellular therapies for the treatment of non-union: the past, present and future. *Injury, 44*, S46-S49.

Huang, D. W., Sherman, B. T., & Lempicki, R. A. (2009a). Bioinformatics enrichment tools: paths toward the comprehensive functional analysis of large gene lists. *Nucleic acids research, 37*(1), 1-13.

Huang, D. W., Sherman, B. T., & Lempicki, R. A. (2009b). Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. *Nature protocols, 4*(1), 44-57.

Jimi, E., Hirata, S., Osawa, K., Terashita, M., Kitamura, C., & Fukushima, H. (2012). The current and future therapies of bone regeneration to repair bone defects. *International journal of dentistry, 2012*.

Koch, A. E., Volin, M. V., Woods, J. M., Kunkel, S. L., Connors, M. A., Harlow, L. A., . . . Strieter, R. M. (2001). Regulation of angiogenesis by the C-X-C chemokines interleukin-8 and epithelial neutrophil activating peptide 78 in the rheumatoid joint. *Arthritis & Rheumatology, 44*(1), 31-40.

Lecanda, F., Avioli, L. V., & Cheng, S. L. (1997). Regulation of bone matrix protein expression and induction of differentiation of human osteoblasts and human bone marrow stromal cells by bone morphogenetic protein-2. *Journal of cellular biochemistry, 67*(3), 386-398.

Lind, M., Eriksen, E., & Bünger, C. (1996). Bone morphogenetic protein-2 but not bone morphogenetic protein-4 and-6 stimulates chemotactic migration of human osteoblasts, human marrow osteoblasts, and U2-OS cells. *Bone, 18*(1), 53-57.

Myllyharju, J., & Kivirikko, K. I. (2001). Collagens and collagen-related diseases. *Annals of medicine, 33*(1), 7-21.

Nakamura, Y., Tensho, K., Nakaya, H., Nawata, M., Okabe, T., & Wakitani, S. (2005). Low dose fibroblast growth factor-2 (FGF-2) enhances bone morphogenetic protein-2 (BMP-2)-induced ectopic bone formation in mice. *Bone, 36*(3), 399-407.

Ono, I., Gunji, H., Kaneko, F., Sait, T., & Kuboki, Y. (1995). Efficacy of hydroxyapatite ceramic as a carrier for recombinant human bone morphogenetic protein. *Journal of Craniofacial Surgery, 6*(3), 238-244.

Oryan, A., Alidadi, S., Moshiri, A., & Maffulli, N. (2014). Bone regenerative medicine: classic options, novel strategies, and future directions. *Journal of orthopaedic surgery and research, 9*(1), 18.

Panagopoulos, V., Liapis, V., Zinonos, I., Hay, S., Leach, D. A., Ingman, W., . . . Zannettino, A. C. (2017). Peroxidase enzymes inhibit osteoclast differentiation and bone resorption. *Molecular and cellular endocrinology, 440*, 8-15.

Panagopoulos, V., Zinonos, I., Leach, D. A., Hay, S. J., Liapis, V., Zysk, A., . . . Evdokiou, A. (2015). Uncovering a new role for peroxidase enzymes as drivers of angiogenesis. *The international journal of biochemistry & cell biology, 68*, 128-138.

Patlolla, A., & Arinzeh, T. L. (2014). Evaluating apatite formation and osteogenic activity of electrospun composites for bone tissue engineering. *Biotechnology and bioengineering, 111*(5), 1000-1017.

Peng, H., Usas, A., Olshanski, A., Ho, A. M., Gearhart, B., Cooper, G. M., & Huard, J. (2005). VEGF Improves, Whereas sFlt1 Inhibits, BMP2-Induced Bone Formation and Bone Healing Through Modulation of Angiogenesis. *Journal of Bone and Mineral Research, 20*(11), 2017-2027.

Peterkofsky, B. (1991). Ascorbate requirement for hydroxylation and secretion of procollagen: relationship to inhibition of collagen synthesis in scurvy. *The American journal of clinical nutrition, 54*(6), 1135S-1140S.

Rao, C., Mohanam, S., Puppala, A., & Rao, J. S. (1999). Regulation of ProMMP-1 and ProMMP-3 activation by tissue factor pathway inhibitor-2/matrix-associated serine protease inhibitor. *Biochemical and biophysical research communications, 255*(1), 94-98.

Robubi, A., Berger, C., Schmid, M., Huber, K., Engel, A., & Krugluger, W. (2014). Gene expression profiles induced by growth factors in in vitro cultured osteoblasts. *Bone and Joint Research, 3*(7), 236-240.

Seeherman, H. J., Li, X. J., Bouxsein, M. L., & Wozney, J. M. (2010). rhBMP-2 induces transient bone resorption followed by bone formation in a nonhuman primate core-defect model. *The Journal of Bone & Joint Surgery, 92*(2), 411-426.

Simmons, C. A., Alsberg, E., Hsiong, S., Kim, W. J., & Mooney, D. J. (2004). Dual growth factor delivery and controlled scaffold degradation enhance in vivo bone formation by transplanted bone marrow stromal cells. *Bone, 35*(2), 562-569.

Street, J., Bao, M., Bunting, S., Peale, F. V., Ferrara, N., Steinmetz, H., . . . van Bruggen, N. (2002). Vascular endothelial growth factor stimulates bone repair by promoting angiogenesis and bone turnover. *Proceedings of the National Academy of Sciences, 99*(15), 9656-9661.

Takada, I., Kouzmenko, A. P., & Kato, S. (2009). Wnt and PPARγ signaling in osteoblastogenesis and adipogenesis. *Nature Reviews Rheumatology, 5*(8), 442-447.

Udagawa, N., Takahashi, N., Katagiri, T., Tamura, T., Wada, S., Findlay, D. M., . . . Kishimoto, T. (1995). Interleukin (IL)-6 induction of osteoclast differentiation depends on IL-6 receptors expressed on osteoblastic cells but not on osteoclast progenitors. *Journal of Experimental Medicine, 182*(5), 1461-1468.

Wintges, K., Beil, F. T., Albers, J., Jeschke, A., Schweizer, M., Claass, B., . . . Schinke, T. (2013). Impaired bone formation and increased osteoclastogenesis in mice lacking chemokine (C-C motif) ligand 5 (Ccl5). *Journal of Bone and Mineral Research, 28*(10), 2070-2080.

Yu, X., Huang, Y., Collin-Osdoby, P., & Osdoby, P. (2004). CCR1 chemokines promote the chemotactic recruitment, RANKL development, and motility of osteoclasts and are induced by inflammatory cytokines in osteoblasts. *Journal of Bone and Mineral Research, 19*(12), 2065-2077.

Zara, J. N., Siu, R. K., Zhang, X., Shen, J., Ngo, R., Lee, M., . . . Kwak, J. (2011). High doses of bone morphogenetic protein 2 induce structurally abnormal bone and inflammation in vivo. *Tissue Engineering Part A, 17*(9-10), 1389-1399.

Zhu, W., Boachie-Adjei, O., Rawlins, B. A., Frenkel, B., Boskey, A. L., Ivashkiv, L. B., & Blobel, C. P. (2007). A novel regulatory role for stromal-derived factor-1 signaling in bone morphogenic protein-2 osteogenic differentiation of mesenchymal C2C12 cells. *Journal of Biological Chemistry, 282*(26), 18676-18685.

Zhu, W., Liang, G., Huang, Z., Doty, S. B., & Boskey, A. L. (2011). Conditional inactivation of the CXCR4 receptor in osteoprecursors reduces postnatal bone formation due to impaired osteoblast development. *Journal of Biological Chemistry, 286*(30), 26794-26805.

CHAPTER 4:

THE EFFECT OF SOYBEAN PEROXIDASE TO PROMOTE BONE REPAIR: A PRELIMINARY STUDY

Statement of Authorship

Title of Paper	The Effect of Soybean Peroxidase to Promote Bone Repair: A Preliminary Study
Publication Status	☐ Published ☐ Accepted for Publication ☐ Submitted for Publication ☑ Unpublished and Unsubmitted work written in manuscript style
Publication Details	

Principal Author

Name of Principal Author (Candidate)	Alexandra Shoubridge
Contribution to the Paper	Designed project, participated in *in vivo* experiments, performed data analysis and prepared manuscript.
Overall percentage (%)	70%
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.
Signature	Date 10/07/2018

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);
ii. permission is granted for the candidate in include the publication in the thesis; and
iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

Name of Co-Author	Peter Anderson
Contribution to the Paper	Supervised development of work, assisted in data interpretation and manuscript evaluation.
Signature	Date 10/07/2018

Name of Co-Author	Vasilios Panagopoulos
Contribution to the Paper	Assisted in data interpretation, manuscript preparation and revision.
Signature	Date 10/07/2018

Name of Co-Author	Andreas Evdokiou		
Contribution to the Paper	Supervised development of work, assisted in data interpretation and manuscript evaluation.		
Signature		Date	10/07/2018

The Effect of Soybean Peroxidase to Promote Bone Repair: A Preliminary Study

Alexandra J. Shoubridge[1,*], Peter J. Anderson[2], Vasilios Panagopoulos[3], Andreas Evdokiou[3,4]

[1]Musculoskeletal Biology Research Laboratory, School of Pharmacy and Medical Sciences, University of South Australia, Adelaide, Australia

[2]Australian Craniofacial Unit, Women's and Children's Hospital and Department of Paediatrics and Dentistry, University of Adelaide, Adelaide, Australia

[3]Breast Cancer Research Unit, Discipline of Surgery, University of Adelaide, Adelaide, Australia

[4]Centre for Personalised Cancer Medicine, University of Adelaide, Adelaide, Australia

*** Corresponding author.**

Ms. Alexandra J. Shoubridge

University of South Australia Cancer Research Institute

North Terrace, SA 5001, Australia

Tel.: +61 8 8302 7389

email: alex.shoubridge@unisa.edu.au

Keywords: Soybean peroxidase, bone repair, BCP granules, drill-hole defect

ABSTRACT

Recently, the use of synthetic scaffolds to provide a three-dimensional platform for bone cells to integrate and deposit new bone for the treatment of bone defects has increased, in particular as carriers of osteoinductive factors. However, despite on-going research we are yet to identify an ideal factor that can provide controlled, accelerated regeneration of new bone without the limitations in current regenerative therapies. The aim of the present study was to investigate whether SBP possesses osteoinductive capabilities by accelerating bone repair when combined with commercially available biphasic calcium phosphate (BCP) granules. BCP granules were pre-treated with SBP and untreated BCP served as the control. The ability for SBP to promote bone repair beyond the capacity of BCP granules alone was evaluated by conducting microcomputed tomography and histological analyses after 4 weeks of implantation in the femoral condyles of 4 sheep, using a bilateral model. Micro-CT analysis revealed SBP-treated BCP significantly increased bone formation within the defects at 4 weeks compared to BCP alone. Histological assessment correlated with reconstructions from micro-CT data, showing new bone formation prominent at the defect margins and surrounding individual BCP granules. In addition, a significant presence of osteoblasts and embedded osteocytes were observed in all specimens, indicative of intramembranous ossification. Whilst this study only assessed a single, early time point, results showed that SBP may be beneficial to be used clinically as an osteoinductive agent to accelerate repair of large bone defects and fractures.

INTRODUCTION

Bone grafting is a common procedure in orthopaedic surgery to repair large osseous defects and fractures as a result of damaged, diseased or aged skeletal tissues. It is estimated that 2.2 million bone grafting procedures are performed annually world-wide and these figures are expected to double by 2020 due to population aging (Biancamaria Baroli, 2009; Giannoudis et al., 2005).

Clinically, autografting remains the current 'gold standard' grafting procedure for treating bone defects as it is histocompatible and non-immunogenic (Bauer & Muschler, 2000a). Autograft bone is also highly effective due to its three-dimensional scaffold structure, which provides osteoconduction and also by providing bone cells for osteogenesis and growth factors (ie. BMP-2) to augment osteoinduction (Finkemeier, 2002). However, the amount of autologous bone which can be harvested is very limited and there is the potential for donor site morbidity at the second surgical site and higher complication rates (Almaiman, Al-Bargi, & Manson, 2013; Finkemeier, 2002; Gamradt & Lieberman, 2003). Allografting is a common alternative to standard autografting as it can provide ample amounts of bone without the additional morbidities from harvesting, however it also possesses a number of limitations (Gazdag, Lane, Glaser, & Forster, 1995). These include loss of osteoinductive properties and cellular components due to processing of bones to avoid immunologic rejection, as well as the risk of infection and disease transmission (Bostrom & Seigerman, 2005; Greenwald et al., 2001). Due to these limitations the use of bone graft substitutes, often in the form of calcium phosphate ceramics is increasing, by their ability to eliminate many of the issues associated with the use of autologous and autograft bone (Fillingham & Jacobs, 2016). Of these, synthetic materials comprised of β-tricalcium phosphate (β-TCP) and hydroxyapatite (HA), termed biphasic calcium phosphate (BCP) have been studied extensively (Bouler, Pilet, Gauthier, & Verron, 2017) as the composition of these minerals is comparable to normal bone tissue (Kivrak & Taş, 1998). Although these materials demonstrate high biocompatibility and osteoconductive

properties, new bone formation is often limited due to their poor osteoinductive capabilities (Yuan et al., 2002). This limitation can be overcome however, by the incorporation of osteoinductive agents such as BMP-2 (Szpalski et al., 2012). Unfortunately, there are numerous concerns associated with the application of BMP-2, including local complications such as ectopic bone formation and significant costs attributed to the high doses administered (Tannoury & An, 2014). Therefore, researchers are currently investigating other growth factors and agents that possess osteoinductive properties which can effectively repair bone similarly to BMP-2, without producing comparable adverse effects.

Our laboratory has recently identified new functional roles for a group of haem-containing enzymes called peroxidases. These enzymes which are naturally occurring in many organisms such as animals, plants and microorganisms have been well reported to possess antimicrobial activity (Aruoma & Halliwell, 1987). However, we have recently discovered that these enzymes, specifically mammalian peroxidases myeloperoxidase (MPO) and eosinophil peroxidase (EPO) and additionally plant peroxidases horseradish peroxidase (HRP) and soybean peroxidase (SBP) possess the ability to regulate various processes critical for tissue repair. These include the ability to regulate fibroblast ECM collagen biosynthesis (DeNichilo et al., 2015), drive angiogenesis (Panagopoulos et al., 2015) and inhibit osteoclastogenesis (Panagopoulos et al., 2017). Additionally, in the context of bone repair we have presented evidence that our most potent peroxidases tested to date, EPO and SBP, significantly regulate osteoblast ECM collagen biosynthesis and matrix mineralisation *in vitro* (unpublished). These findings demonstrate a potential role for peroxidases to be used as osteoinductive agents for bone repair. Although EPO has proven to be highly effective in regulating osteoblast function, it is highly expensive and currently not available in large quantities, making the use of EPO impractical for clinical use. Therefore we have been focusing our research on plant-derived SBP. To date, SBP has proven to be one of the most biologically active peroxidases, both *in*

vitro and *in vivo* (DeNichilo et al., 2015), but importantly it is non-toxic, highly stable and very inexpensive to manufacture. These properties make it an attractive agent, with significant potential for translation. However, before we can consider their use clinically, we needed to assess the ability of SBP to repair bone *in vivo*.

Therefore, the objective of this preliminary study was to evaluate the osteoinductive capacity of SBP to accelerate bone regeneration when used in combination with a commercially available BCP. For this purpose SBP-soaked BCP granules (15:85 HA:β-TCP) were implanted in a critical-sized defect of the sheep femoral condyle. The use of a bilateral model allowed for individuals to serve as their own control, receiving ceramic granules alone or in combination with SBP. New bone formation was assessed 4 weeks post-surgery by histological and microcomputed tomography analyses. Our data demonstrate preliminary osteoinductive effects of SBP to improve the rate of bone regeneration in combination with BCP *in-vivo*.

MATERIALS AND METHODS

Ethics Statement

The study was approved by the Animal Ethics Committee of the University of Adelaide (M-2015-044A) to conduct this study at the Medical Engineering Research Facility, Chermside, QLD.

Animals

Four male castrated, wethers sheep aged 3-5, years weighing 56-69 kg (mean: 60; SD ± 5.9) were randomly assigned with even distribution, to receive the experimental treatment in either the left or right hind limb to eliminate bias. The opposing hind limb received the control treatment. Due to the comparatively low impact this procedure has on the animal, a bilateral model is well tolerated and allows for the control and experimental treatments to be conducted in each animal.

Experimental Procedure

The sheep were fasted for 12 h prior but had free access to water during this time. To alleviate post-operative pain, a fentanyl patch (2-3 µg/kg/hr) was applied 24 h prior to surgery and reapplied every 72 h for a minimum of 10 days. After pre-emptive sedation and analgesia with midazolam (0.2 mg/kg) and buprenorphine (0.005 mg/kg), animals were induced with Propofol, which was administered until effect (3-4 mg/kg). Animals were then intubated and administered Isoflurane in oxygen at 2%. A patient warmer was used during the procedure to keep the animal's body temperature stable and to prevent hypothermia. Cephalosporin (22 mg/kg) and Meloxicam (0.2 mg/kg) were administered pre-emptively and during the surgical procedure. Non-steroidal anti-inflammatory flunixin meglumine (1.1 mg/kg) was also administered.

Animals were positioned in dorsal recumbency and the operative site for each hind limb was prepared through sequential sterilising scrubs and draping.

Once marked, a 2-3 cm incision was made directly over the mid-medial aspect of the femoral condyle and underlying soft tissue removed to reveal the bone. A 12 mm diameter, 10 mm deep defect was then created with sequential use of differing size drill heads (8.5 mm, 10 mm and 12 mm). This method was used to avoid unnecessary thermal necrosis. The defect was then flushed with saline to remove any remaining bone fragments. Once the defect was created, commercially available Mastergraft® BCP [15% HA / 85% β-TCP] granules (Medtronic Sofamor Danek, Memphis, TN) were deposited into either condyle. In one defect, 1.25 cc BCP, which was pre-soaked for 15 mins in 5 mL soybean peroxidase (650 µg/mL) was added. The remaining defect received 1.25 cc BCP pre-soaked with 5 mL Ringer's lactate (saline) solution as the control. Once the soaked BCP granules were added, they were gently compacted into the defect to ensure consistent and even distribution. The wound was closed using 0-vicryl in a cruciate pattern for the deep fascial layer and continuous pattern for the subcutaneous layer. Metallic skin staples were then applied over the surface. Finally, a bandage with antimicrobial dressing was applied over the surgical site. Animals were given antibiotics and analgesics according to the standard operating protocols. Animals remained in a sling for 24 h before being moved to indoor housing for the entirety of the study. Sheep were monitored 3 times a day for the first 3 days followed by once per day for the remainder of the study. Bandages were removed 2-3 days post-surgery and surgical wounds assessed for signs of wound complication. The skin staples were removed 14 days post-surgery. The animals were sacrificed 4 weeks post-surgery and specimens were obtained by aseptic technique.

Microcomputed Tomography (Micro-CT) Imaging

Samples (n=4) in 70% ethanol were cut to size in order to fit within a 36.9 mm diameter sample tube with the drill hole defect in vertical orientation. The samples were scanned in a micro-CT scanner 40 (Scanco Medical, Bassersdorf, Switzerland) at an energy of 70 kVp and intensity of 114 µA with 200 µs integration time, resulting in an isotropic voxel size of 36 µm. The reconstructed scans were evaluated using Scanco µCT Evaluation Program (v6.5-3). A cylindrical volume of interest (VOI) with a diameter of 11 mm was defined for the analysis. The vertical evaluation length (cylinder height) was defined in a preview and consisted of the central, cylindrical section of the defect (approximately 100 slices or 3.6 mm), excluding the zones at the top and bottom ends of the defect to prevent "boundary effects". To identify newly formed bone throughout the VOI, and to reduce partial volume effect at the grain boundaries of the ceramic granules, a masking procedure was employed (Burghardt, Kazakia, Laib & Majumdar, 2008). First, using a high threshold of 5069 Hounsfield Units (HU, 872.6 mg HA/mm^3), with a gauss filter of σ=0.8 and a support of 1.0 was applied to segment the BCP granules within the defect. Next, the granules were enlarged by 2 voxels on all faces, and this enlarged granule volume was deducted from the VOI, leaving only the intra-granule space of the VOI. Newly formed bone was segmented from this space with a lower threshold of 2121 HU (356.3 mg HA/mm^3), a gauss filter of σ=1.8 and a support of 3.0. For the quantitative evaluation, the total cylindrical volume of interest (TV), the volume of the segmented newly formed bone (BV) and the average mineral density of the newly formed bone were calculated.

Histology

Femoral condyles were subjected to ethanol dehydration steps prior to being placed into a Methyl Methacrylate (MMA): Polyethylene Glycol 400 (PEG) solution (100% MMA: 10% PEG). Femurs were left in the MMA: PEG solution for 21 days, at which time polymerisation

was induced using a solution containing MMA: PEG: Perkadox (0.2%). Trimmed resin blocks were sectioned in the sagittal plane at 10 μm thickness. Sections were prepared for Toluidine blue staining. Images were taken using the NanoZoomer Digital Pathology (NDP-Hamamatsu, Hamamatsu City, Japan). Toluidine blue stained slides were used to visualise sites of new bone formation and the presence of osteoblasts and osteocytes. Sections were viewed using the associated software (NDP View Version 2.5; Hamamatsu, Hamamatsu City, Japan).

Statistical Analysis

Data points derived from experiments are reported as the mean ± standard deviation (SD). Analysis of variance to determine significant difference between samples was performed using the paired Student's t-test.

RESULTS

Surgery

Based on previous *in vitro* and *in vivo* studies (unpublished), we selected SBP at 650 µg/mL for *in vivo* testing in a standardised bilateral femoral defect model (Fig. 1). Saline and peroxidase-soaked BCP granules were prepared (Fig. 2) and implanted directly into the defect site (Fig. 3). No surgical complications or postoperative morbidities were detected in any of the sheep. The impact of this procedure on the animals' wellbeing, in particular, ambulation has been minimal with good use of both hind limbs throughout the study.

Figure 1. *Surgical procedure: an incision is created from the condylar notch to the proximal trochlear ridge (solid line).* The osteochondral defect (diameter 12mm, depth 10mm) is created in the medial aspect of both femoral condyles (bilateral model) and is controlled by a drill guide to a depth of 10mm (red circle).

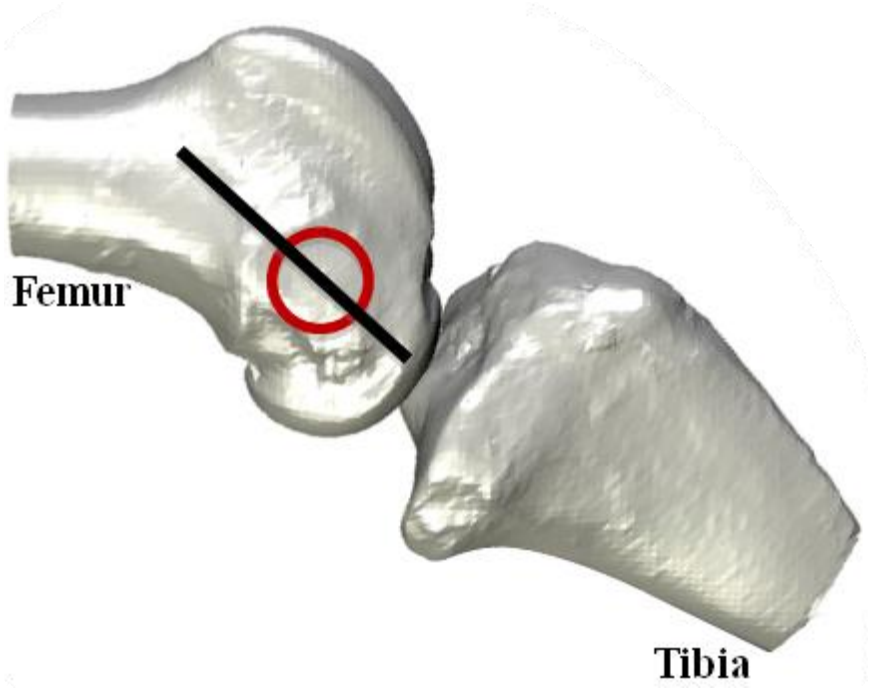

Femur
Tibia

Figure 2. *The SBP and control group treated BCP, shortly before implantation.*

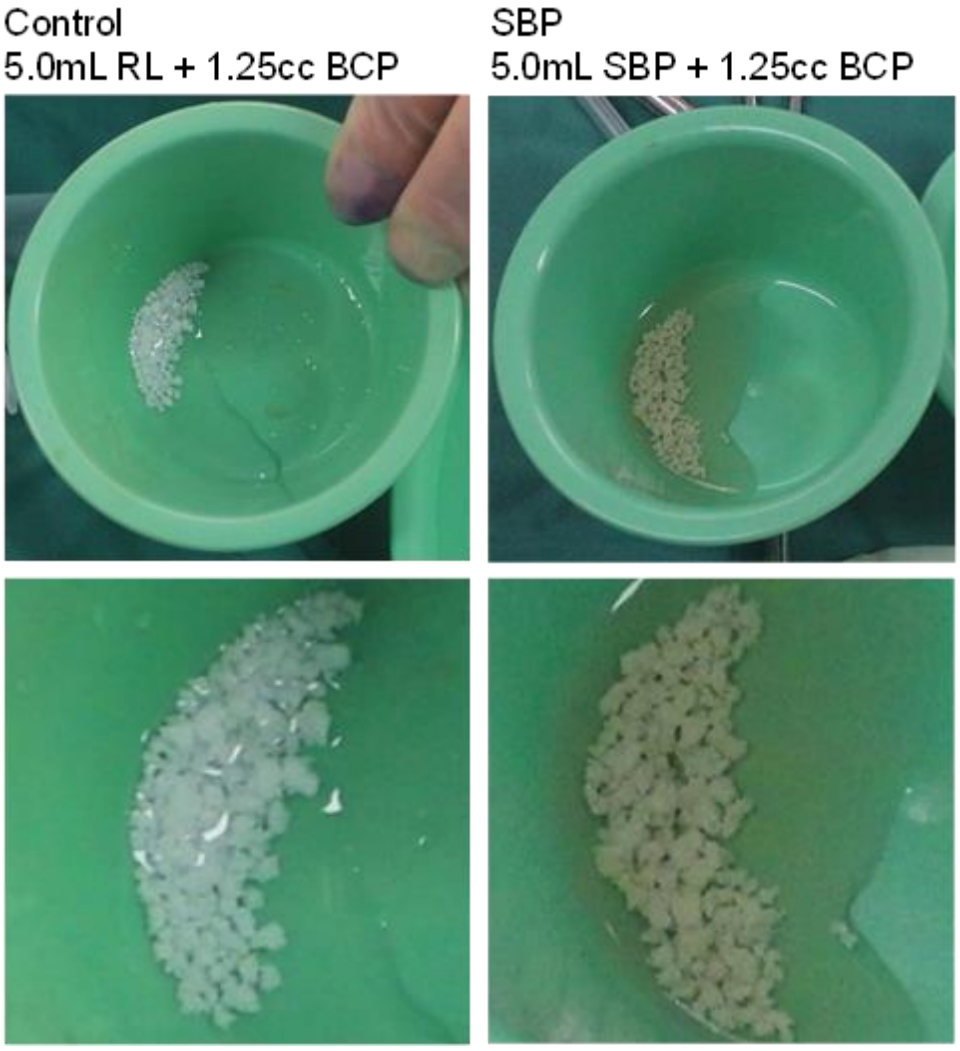
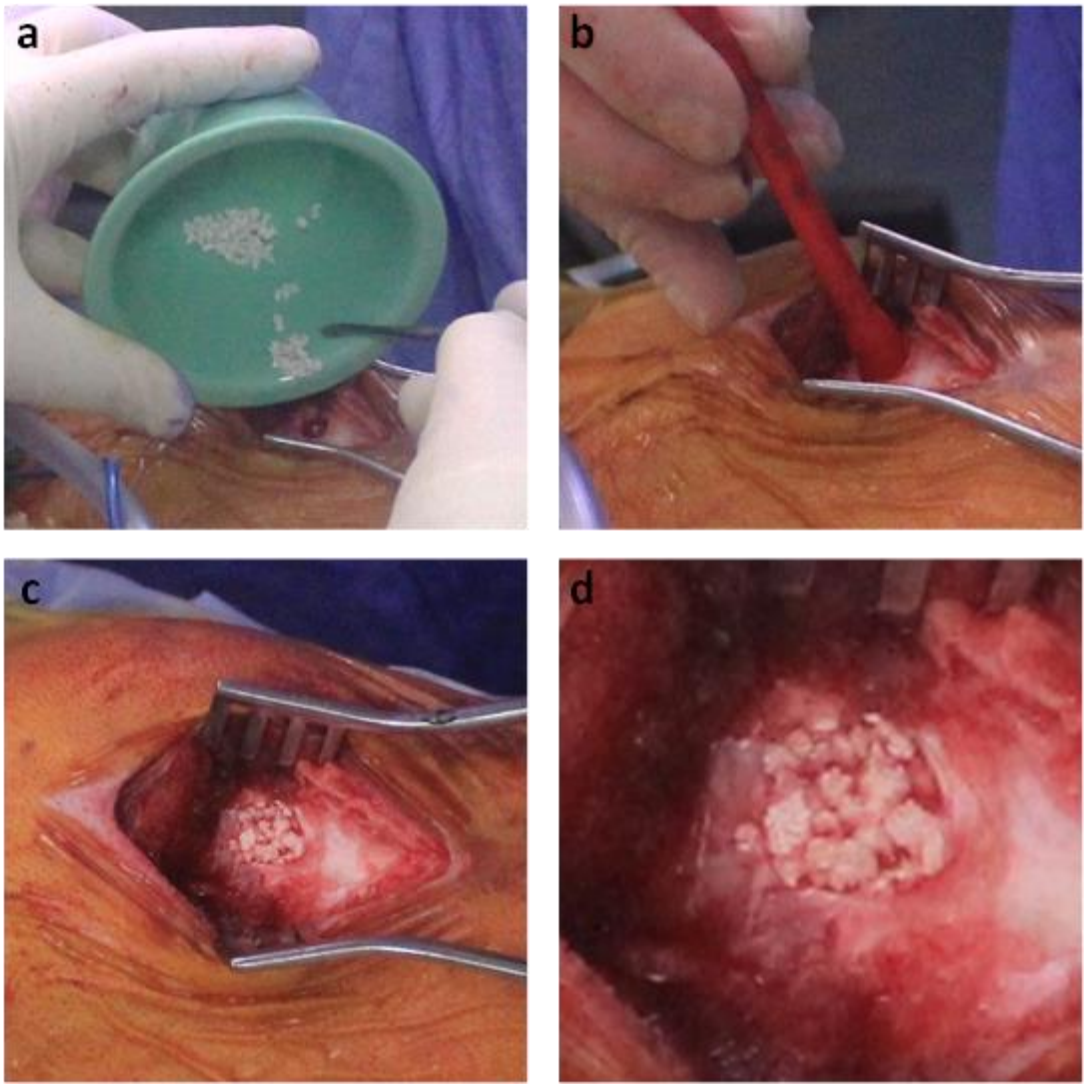

Control
5.0mL RL + 1.25cc BCP
SBP
5.0mL SBP + 1.25cc BCP

Figure 3. *Scaffold implantation (a), soft compaction into the defect (b) and after compaction ready for suturing (c) and (d).*


a
b
c
d

Micro-CT Evaluation

The effects of SBP in combination with biphasic ceramic granules on bone formation after 4 weeks of implantation were studied by micro-CT imaging. Representative three-dimensional reconstructions of the micro-CT images which were created in the central zone of the defect (Fig. 4) showed increased bone formation in the treatment hind limbs, compared to control (Fig. 5A). Quantitative analysis of the reconstructions depicted in Fig. 5A revealed a statistically significant increase in bone volume at the defect site in the SBP-treated limbs compared to control ($p = 0.04$) (Fig. 5B).

Figure 4. *Representative micro-CT image of reconstructed defect block used for quantification.* A cylindrical volume of interest (VOI) with a diameter of 11 mm and length of 3.6 mm, excluding the zones at the top and bottom ends of the defect to prevent "boundary effects".

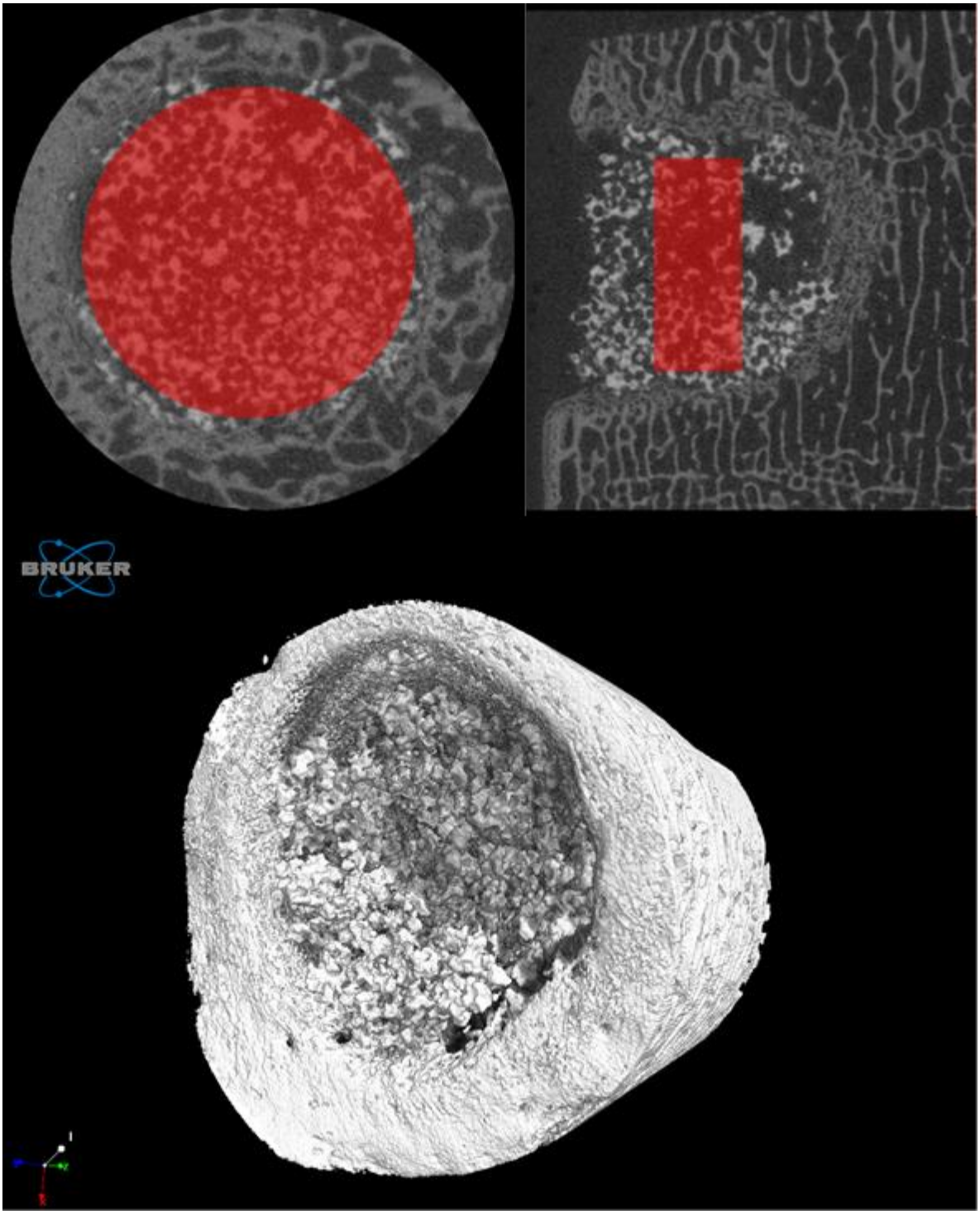
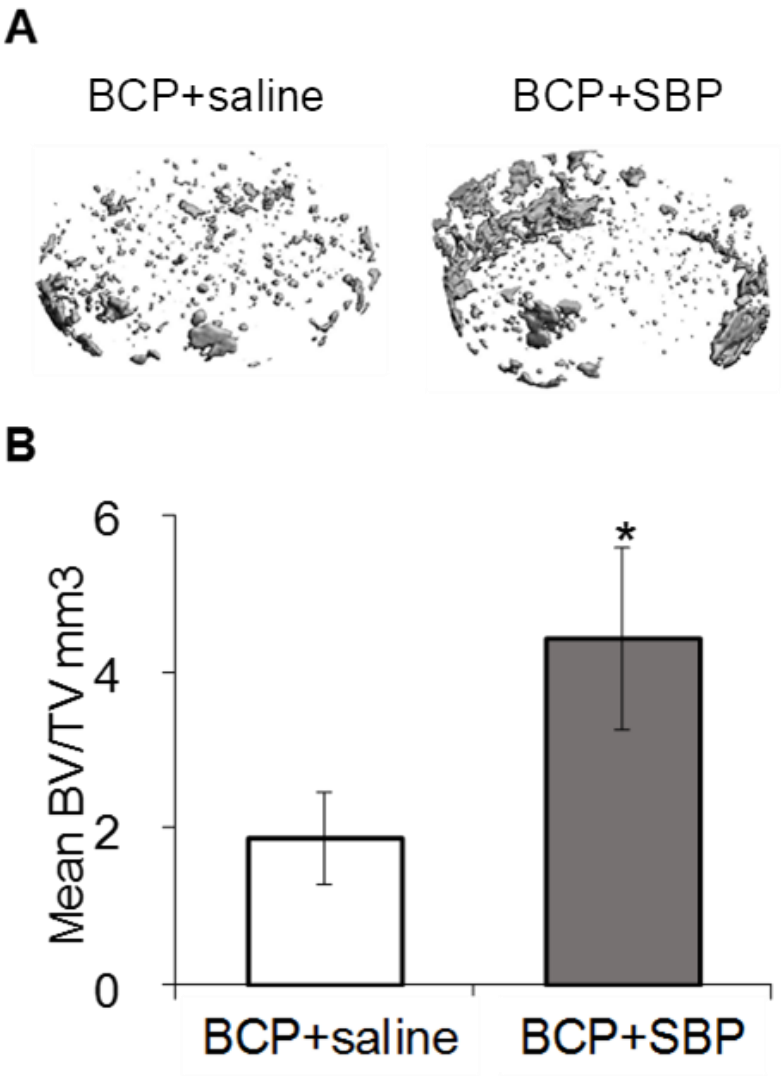

BRUKER

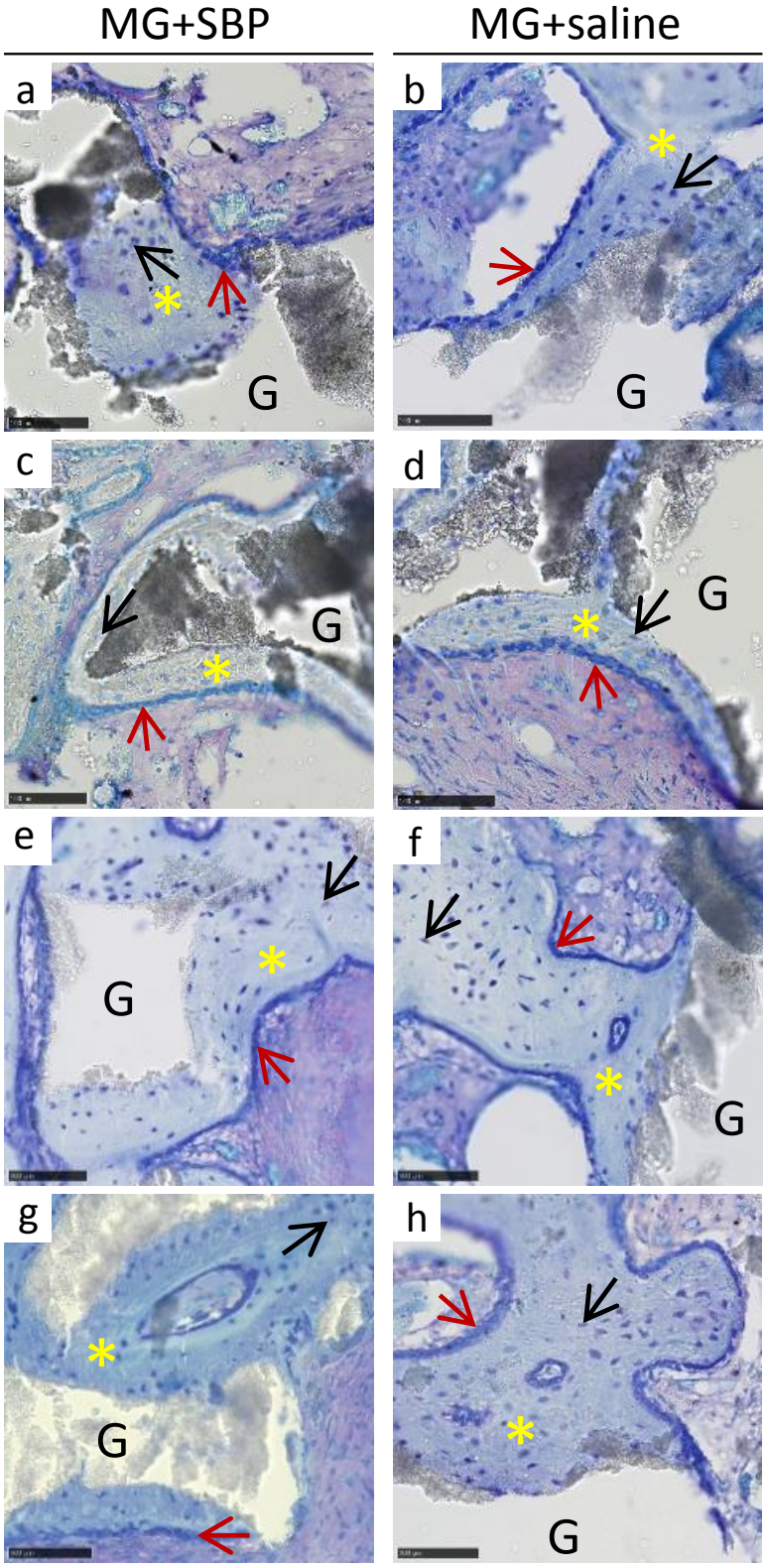
Figure 5. *Localised application of SBP-soaked BCP granules within a femoral defect increases bone formation.* Sheep received a 12mmx10mm defect within the femoral condyle of both hind limbs. Each defect was filled with saline or SBP pre-soaked granules. A: Representative micro-CT reconstructions. At 28 days the sheep were sacrificed, and micro-CT scanning of the bones was performed at the defect site. B**:** Quantitative analysis of the micro-CT data showed increased bone volume within the defects treated with BCP+SBP compared to BCP+saline (control). Analysis is presented as the mean bone volume (BV) to tissue volume (TV) of the SBP treatment compared to control. Data was analysed and reported as the mean ± standard deviation (SD). Analysis of variance to determine significant difference between samples was performed using the paired Student's *t*-test of four determinations for control and peroxidases. $^{*}P = 0.04$.


A
BCP+saline
BCP+SBP
B
Mean BV/TV mm3
6
4
2
0
*
BCP+saline
BCP+SBP

Histological Analysis

In conjunction with micro-CT evaluation, histological assessment of new bone formation in the presence of SBP compared to control was conducted. Toluidine blue staining of cross-sections through the cancellous bone and implant showed an observable increase in new bone formation at the defect margins (data not shown). However, this increase in new bone formation at the periphery was not uniform around the entire implant in both SBP-treated defects and control. Representative images (Fig. 7) of new bone formation for each sample was shown to occur around individual HA/β-TCP Mastergraft® (MG) BCP granules. However, no difference in the extent of new bone formation around individual BCP granules between SBP-treated and saline-treated control was observed. Despite no observable difference in bone formation around individual BCP granules, it is evident that SBP-treated granules did not impair normal bone formation processes. This can be deduced by the presence of osteoblasts and osteocytes adjacent to the treated granules in all animals tested (Fig. 7). Thus, observational analysis of histological specimens suggest that SBP-treated granules generated a comparable bone formation response to control.

Figure 7. *Toluidine blue staining demonstrates formation of new bone after localised treatment of SBP-soaked Mastergraft® granules at 28 days post-surgery.* Representative histological analysis of sheep femoral defects after 28 days treated with saline or SBP-soaked HA/β-TCP Mastergraft® (MG) BCP granules. Each individual animal has its own representative image for SBP and saline (a-b; c-d; e-f; g-h). Each representative image was taken near the defect margin where majority of new bone growth had occurred. Yellow asterisks indicate bone formation adjacent to BCP granules. Voids marked with a "G" indicate the location of MG granules. Cuboidal osteoblasts (dark blue) lining the new bone forming surface are indicated by red arrows and embedded osteocytes (pale, purple) are identified by black arrows.


MG+SBP
MG+saline
a
b
c
d
e
f
g
h
G

DISCUSSION

In this proof-of-concept study, the ability of SBP to accelerate bone repair in combination with commercially sourced BCP was assessed. The study successfully showed new bone formation after 4 weeks of BCP implantation, with increased healing observed in the bone defects treated with SBP.

The critical-sized femoral condyle defect model remains a suitable model for testing the effectiveness of osteoinductive factors. The decision to use sheep for testing SBP's osteoinductive capabilities was due to its reliability as a representative pre-clinical model to assess new bone formation (Apelt et al., 2004; Peters, Hines, Bachus, Craig, & Bloebaum, 2006; Theiss et al., 2005). It has been well reported that sheep possess a similar bone metabolism to humans and the ability to create larger defects in sheep to test therapies is of greater relevance than the use of rodents (N Patel et al., 2005).

The role that peroxidases have in regulating multiple cell types to promote tissue regeneration has been well-reported by our laboratory (DeNichilo et al., 2015; Panagopoulos et al., 2015). Of particular relevance to this study are our recent findings which have demonstrated that SBP is highly effective at stimulating primary osteoblasts to promote collagen biosynthesis and mineralisation of the deposited ECM (unpublished). The results presented in this current study, predominantly the significant increase in new bone formation as shown by micro-CT ($p = 0.04$), successfully validates this previously conducted *in vitro* data. These *in vitro* studies also presented early mechanistic evidence showing significant regulation of a number of key osteogenic genes. This further supports the rapid new bone deposition observed in this study and clearly demonstrates SBP's potent osteoinductive capabilities. In addition, the mechanistic data also demonstrates upregulation of a number of pro-inflammatory genes in the presence of SBP, including a number of MMP's and chemokines. Whilst the upregulation of these genes, which are known to be critical to generate an acute inflammatory response during early fracture

repair (Pape et al., 2010) would be beneficial, we must consider the potential risk of long-term inflammatory responses as a result of treatment with SBP, particularly in bone defects. Therefore, investigating late stage bone formation, specifically the integrity and strength of new bone after treatment with SBP is necessary for future studies.

From our *in vitro* studies and the broader research community, it is well established that peroxidases have a pro-inflammatory role, which could be beneficial for fracture healing. However, the significant increase in bone formation observed by this micro-CT at just four weeks post-surgery suggests a potential role for these enzymes not just for repair of non-unions, but also large bony defects. Furthermore, observational assessment of this preliminary study shows osteoblasts lining the deposited osteoid, with osteocytes entrapped within their lacunae. Signs of endochondral bone formation, such as the presence of chondrocytes or cartilage were absent. In regions of direct bone-implant contact at the defect margin, moderate bone ingrowth was observed. However, there were minimal regions of direct bone-implant contact at the defect margins (data not shown) which is potentially the cause of non-uniform bone formation at the periphery of the defects in all specimens. This observation correlates with the micro-CT reconstructions, where majority of new bone is at the defect margins, in a non-uniform distribution. The nature of the highly porous structure of the individual BCP granules created difficulty in ensuring the implant was uniform throughout the defect. Compaction of the granules once in the defect was conducted in an attempt to overcome this potential problem, however, to ensure granule pores remained intact, they were not compacted with significant force. It could be argued that an implant with direct bone-implant contact, such as a smooth press-fit implant would allow for greater uniformity in new bone formation at the defect margin. However, it has been reported that the addition of HA can eliminate the negative response of non-interference fit between bone and the implant (Søballe, Hansen, Brockstedt-Rasmussen, Pedersen, & Bünger, 1990). Taken together, this preliminary study has successfully

demonstrated an ability for SBP to promote new bone formation compared to BCP alone. In addition, the presence of SBP does not appear to generate a toxicity effect to the local cell population, where we see an abundance of osteoblasts and osteocytes, necessary for normal bone healing. Whilst this proof-of-concept study was successful, numerous limitations must be considered.

A limitation of this study was the number of animals used. When considering the literature, there is significant variability in sheep numbers when using the critical-sized femoral condyle defect model (Milano et al., 2010; Zscharnack et al., 2010). Whilst most studies range from 6-12 animals, using a bilateral model, some studies only use 4 animals per treatment. However, this study was conducted as a preliminary, proof-of-concept study and so no prior study power was determined. Despite this, a significant difference between the addition of SBP compared to BCP alone was observed. The positive result suggests future studies will not require significantly large numbers to obtain a conclusive result, however careful consideration will be needed due to the intent to optimise SBP for clinical use. An additional consideration for future *in vivo* studies is the use of non-steroidal anti-inflammatory drugs (NSAIDs). These drugs have been previously reported to impair bone healing however, the drug Meloxicam, used in this study reportedly has no effect of bone healing (Pountos, Georgouli, Calori, & Giannoudis, 2012). Furthermore, a decrease in healing was not observed in the SBP-treated group, suggesting that the NSAIDs used in this study did not inhibit the bone reparative effects of SBP. Future fracture studies where callus formation and inflammation are critical for successful healing should consider which NSAIDs are used and how this may affect the pro-inflammatory effects of peroxidases, including SBP. An aspect of this study which could also be considered a limitation is the single and early endpoint used to assess the effectiveness of SBP to accelerate the bone repair process. The single, short time point of 4 weeks was proposed to observe the early bone remodelling process and observe how SBP interacts with the local

bone environment. This early time point also enabled us to observe potential toxicity effects exhibited by the peroxidases at such a high concentration. Sampling across multiple time points at 2, 4, 6 and 12 weeks would have provided a more revealing image of the true bone formation and remodelling processes in the presence of SBP. A robust study of this nature would provide an understanding of where the agent is acting in the repair process and whether it provides any benefit in reducing healing time. However, with limited resources we were only afforded a single time point. An additional limitation to our analysis of the overall healing response was the limited histological data. Whilst observational assessment correlated with our micro-CT analysis, the use of double-labelled fluorochromes would enable us to directly measure the extent of new bone formation. Future studies will also be better equipped to conduct robust histomorphometrics with blinded analysis. A further limitation for this study is the absence of a positive control (ie. BMP-2). Future studies will require the comparison of SBP to the previous standard, BMP-2 to discern the clinical potential of SBP. Although this study does not allow us to compare to a positive control, the purpose of this study was to provide evidence that SBP can promote bone repair *in vivo*, when used in combination with an appropriate scaffold, which has been successfully demonstrated.

In conclusion, the present study provides a proof-of-concept role for peroxidases, by demonstrating that SBP, in combination with commercially available BCP could accelerate the repair of a critical-sized femoral condyle defect in sheep.

Furthermore, histological observations between SBP and control suggest that SBP is non-toxic to the local cell populations, which is a critical step when identifying new, experimental agents. The requirement for novel osteoinductive agents to replace BMP-2 in the clinic provides an opportunity to continue to explore the possible applications of SBP in repair. Based on the limitations drawn from this study, further studies with more robust analyses are required to fully elucidate the potential of SBP to accelerate the repair of damaged bone. This

includes testing SBP long-term, in large segmental defect and fracture models with comparisons to BMP-2, which will ultimately determine its clinical effectiveness.

ACKNOWLEDGEMENTS

This work was supported in part by The Hospital Research Foundation, Adelaide Research and Innovation Commercial Accelerator Scheme and the National Health and Medical Research Council (Career Development Fellowship/627015; Project Grant/1050694). The authors would like to acknowledge Dr. John Field for his technical expertise, Dr. Roland Steck and QUT MERF for their analytical and technical assistance and Ms. Rebecca Sawyer for her assistance in preparing specimens for histological evaluation.

CONFLICT OF INTEREST

The authors declare that they have no competing interests.

REFERENCES

Almaiman, M., Al-Bargi, H. H., & Manson, P. (2013). Complication of anterior iliac bone graft harvesting in 372 adult patients from may 2006 to may 2011 and a literature review. *Craniomaxillofacial Trauma and Reconstruction, 6*(04), 257-266.

Apelt, D., Theiss, F., El-Warrak, A. O., Zlinszky, K., Bettschart-Wolfisberger, R., Bohner, M., . . . von Rechenberg, B. (2004). In vivo behavior of three different injectable hydraulic calcium phosphate cements. *Biomaterials, 25*(7), 1439-1451.

Aruoma, O. I., & Halliwell, B. (1987). Action of hypochlorous acid on the antioxidant protective enzymes superoxide dismutase, catalase and glutathione peroxidase. *Biochemical Journal, 248*(3), 973-976.

Bauer, T. W., & Muschler, G. F. (2000a). Bone graft materials: an overview of the basic science. *Clinical orthopaedics and related research, 371*, 10-27.

Baroli, B. (2009). From natural bone grafts to tissue engineering therapeutics: Brainstorming on pharmaceutical formulative requirements and challenges. *Journal of Pharmaceutical Sciences, 98*(4), 1317-1375. doi: 10.1002/jps.21528

Bodde, E. W. H., Wolke, J. G. C., Kowalski, R. S. Z., & Jansen, J. A. (2007). Bone regeneration of porous β-tricalcium phosphate (Conduit™ TCP) and of biphasic calcium phosphate ceramic (Biosel®) in trabecular defects in sheep. *Journal of Biomedical Materials Research Part A, 82A*(3), 711-722.

Bostrom, M. P., & Seigerman, D. A. (2005). The clinical use of allografts, demineralized bone matrices, synthetic bone graft substitutes and osteoinductive growth factors: a survey study. *Hss Journal, 1*(1), 9-18.

Bouler, J. M., Pilet, P., Gauthier, O., & Verron, E. (2017). Biphasic calcium phosphate ceramics for bone reconstruction: A review of biological response. *Acta Biomaterialia, 53*, 1-12.

Burghardt, A. J., Kazakia, G. J., Laib, A., & Majumdar, S. (2008). Quantitative assessment of bone tissue mineralization with polychromatic micro-computed tomography. *Calcified tissue international, 83*(2), 129.

DeNichilo, M. O., Panagopoulos, V., Rayner, T. E., Borowicz, R. A., Greenwood, J. E., & Evdokiou, A. (2015). Peroxidase Enzymes Regulate Collagen Extracellular Matrix Biosynthesis. *The American Journal of Pathology, 185*(5), 1372-1384.

Fillingham, Y., & Jacobs, J. (2016). Bone grafts and their substitutes. *Bone Joint J, 98*(1 Supple A), 6-9.

Finkemeier, C. G. (2002). Bone-grafting and bone-graft substitutes. *JBJS, 84*(3), 454-464.

Gamradt, S. C., & Lieberman, J. R. (2003). Bone graft for revision hip arthroplasty: biology and future applications. *Clinical orthopaedics and related research, 417*, 183-194.

Gazdag, A. R., Lane, J. M., Glaser, D., & Forster, R. A. (1995). Alternatives to autogenous bone graft: efficacy and indications. *Journal of the American Academy of Orthopaedic Surgeons, 3*(1), 1-8.

Giannoudis, P. V., Dinopoulos, H., & Tsiridis, E. (2005). Bone substitutes: an update. *Injury, 36*(3), S20-S27.

Greenwald, A. S., Boden, S. D., Goldberg, V. M., Khan, Y., Laurencin, C. T., & Rosier, R. N. (2001). Bone-graft substitutes: facts, fictions, and applications. *JBJS, 83*(2_suppl_2), S98-103.

Kivrak, N., & Taş, A. C. (1998). Synthesis of calcium hydroxyapatite-tricalcium phosphate (HA-TCP) composite bioceramic powders and their sintering behavior. *Journal of the American Ceramic Society, 81*(9), 2245-2252.

Milano, G., Passino, E. S., Deriu, L., Careddu, G., Manunta, L., Manunta, A., . . . Fabbriciani, C. (2010). The effect of platelet rich plasma combined with microfractures on the treatment of chondral defects: an experimental study in a sheep model. *Osteoarthritis and Cartilage, 18*(7), 971-980.

Panagopoulos, V., Liapis, V., Zinonos, I., Hay, S., Leach, D. A., Ingman, W., . . . Zannettino, A. C. (2017). Peroxidase enzymes inhibit osteoclast differentiation and bone resorption. *Molecular and cellular endocrinology, 440*, 8-15.

Panagopoulos, V., Zinonos, I., Leach, D. A., Hay, S. J., Liapis, V., Zysk, A., . . . Evdokiou, A. (2015). Uncovering a new role for peroxidase enzymes as drivers of angiogenesis. *The international journal of biochemistry & cell biology, 68*, 128-138.

Pape, H.-C., Marcucio, R., Humphrey, C., Colnot, C., Knobe, M., & Harvey, E. J. (2010). Trauma-induced inflammation and fracture healing. *Journal of orthopaedic trauma, 24*(9), 522-525.

Patel, N., Brooks, R., Clarke, M., Lee, P., Rushton, N., Gibson, I. R., . . . Bonfield, W. (2005). In vivo assessment of hydroxyapatite and silicate-substituted hydroxyapatite granules using an ovine defect model. *Journal of Materials Science: Materials in Medicine, 16*(5), 429-440.

Peters, C. L., Hines, J. L., Bachus, K. N., Craig, M. A., & Bloebaum, R. D. (2006). Biological effects of calcium sulfate as a bone graft substitute in ovine metaphyseal defects. *Journal of Biomedical Materials Research Part A, 76A*(3), 456-462.

Pountos, I., Georgouli, T., Calori, G. M., & Giannoudis, P. V. (2012). Do Nonsteroidal Anti-Inflammatory Drugs Affect Bone Healing? A Critical Analysis. *The Scientific World Journal, 2012*, 606404.

Søballe, K., Hansen, E. S., Brockstedt-Rasmussen, H., Pedersen, C. M., & Bünger, C. (1990). Hydroxyapatite coating enhances fixation of porous coated implants: a comparison in dogs between press fit and noninterference fit. *Acta Orthopaedica Scandinavica, 61*(4), 299-306.

Szpalski, C., Nguyen, P. D., Vasiliu, C. E. C., Chesnoiu-Matei, I., Ricci, J. L., Clark, E., . . . Warren, S. M. (2012). Bony engineering using time-release porous scaffolds to provide sustained growth factor delivery. *Journal of Craniofacial Surgery, 23*(3), 638-644.

Tannoury, C. A., & An, H. S. (2014). Complications with the use of bone morphogenetic protein 2 (BMP-2) in spine surgery. *The Spine Journal, 14*(3), 552-559.

Theiss, F., Apelt, D., Brand, B., Kutter, A., Zlinszky, K., Bohner, M., . . . von Rechenberg, B. (2005). Biocompatibility and resorption of a brushite calcium phosphate cement. *Biomaterials, 26*(21), 4383-4394.

Yuan, H., Van Den Doel, M., Li, S., Van Blitterswijk, C., De Groot, K., & De Bruijn, J. (2002). A comparison of the osteoinductive potential of two calcium phosphate ceramics implanted intramuscularly in goats. *Journal of Materials Science: Materials in Medicine, 13*(12), 1271-1275.

CHAPTER 5:

SOYBEAN PEROXIDASE IN THE REPAIR OF CALVARIAL CRITICAL SIZE DEFECTS IN MICE

Statement of Authorship

Title of Paper	Soybean Peroxidase in the Repair of Calvarial Critical-Sized Defects in Mice
Publication Status	☐ Published ☐ Accepted for Publication ☐ Submitted for Publication ☑ Unpublished and Unsubmitted work written in manuscript style
Publication Details	

Principal Author

Name of Principal Author (Candidate)	Alexandra Shoubridge		
Contribution to the Paper	Designed project, performed *in vitro* and *in vivo* experiments, performed data analysis and prepared manuscript.		
Overall percentage (%)	75%		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
Signature		Date	10/07/2018

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);
ii. permission is granted for the candidate in include the publication in the thesis; and
iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

Name of Co-Author	Peter Anderson		
Contribution to the Paper	Supervised development of work, participated in *in vivo* experiments, assisted in experimental design and manuscript evaluation		
Signature		Date	10/07/2018

Name of Co-Author	Irene Zinonos		
Contribution to the Paper	Assisted in *in vivo* experiments and data analysis		
Signature		Date	10/07/2018

Name of Co-Author	Aneta Zysk		
Contribution to the Paper	Assisted in *in vivo* experiments and data analysis		
Signature		Date	10/07/2018

Name of Co-Author	Mark DeNichilo		
Contribution to the Paper	Supervised development of work, assisted in experimental design and data analysis		
Signature		Date	10/07/2018

Name of Co-Author	Vasilios Panagopoulos		
Contribution to the Paper	Assisted in data interpretation, manuscript preparation and revision		
Signature		Date	10/07/2018

Name of Co-Author	Andreas Evdokiou		
Contribution to the Paper	Supervised development of work, assisted in experimental design, data interpretation and manuscript evaluation		
Signature		Date	10/07/2018

Soybean Peroxidase in the Repair of Calvarial Critical Size Defects in Mice

Alexandra J. Shoubridge[1,*], Peter J. Anderson[2], Irene Zinonos[3], Aneta Zysk[3], Mark DeNichilo[4], Vasilios Panagopoulos[3], Andreas Evdokiou[3,5]

[1]Musculoskeletal Biology Research Laboratory, School of Pharmacy and Medical Sciences, University of South Australia, Adelaide, Australia

[2]Australian Craniofacial Unit, Women's and Children's Hospital and Department of Paediatrics and Dentistry, University of Adelaide, Adelaide, Australia

[3]Breast Cancer Research Unit, Discipline of Surgery, University of Adelaide, Adelaide, Australia

[4]Centre for Cancer Biology, University of South Australia, Adelaide, Australia

[5]Centre for Personalised Cancer Medicine, University of Adelaide, Adelaide, Australia

*** Corresponding author.**

Ms. Alexandra J. Shoubridge

University of South Australia Cancer Research Institute

North Terrace, SA 5001, Australia

Tel.: +61 8 8302 7389

email: alex.shoubridge@unisa.edu.au

Keywords: Soybean peroxidase, bone regeneration, critical size defect, calvarial

ABSTRACT

To date, the functional role of peroxidase enzymes in human health has mainly been limited to providing a mechanism for oxidative defence against invading bacteria and other pathogenic microorganisms. Our laboratory has recently discovered new and important roles for mammalian and plant-derived peroxidases that include, the ability to promote tissue regeneration by modulating the function of mesenchymal lineage cells. This suggests that they may be used to repair bone defects and fractures. We previously demonstrated that SBP has the capacity to enhance bone repair in an ovine critical size defect (CSD) model. Whilst we have successfully established the CSD model in sheep, future fracture models testing SBP will be determined firstly in rodents. Therefore, this study was undertaken to validate plant-derived soybean peroxidase (SBP) in a mouse CSD model, which has been used extensively to test new bone graft substitutes. Critical size (3 mm) calvarial defects were created in the right parietal bone of adult female Balb/C mice. Defects were either treated with a collagen scaffold alone, or a scaffold pre-loaded with SBP at three separate doses (50µg, 100µg & 200µg). At 8-weeks, implants were retrieved and evaluated by micro-CT and histological analysis. Contrarily to what was expected, micro-CT results showed inhibition of bone formation in the SBP-treated groups when compared to scaffold alone ($p < 0.01$). Histological analysis revealed that for all SBP-treated groups at 8-weeks, the majority of the defect contained dense and slightly vascularised fibrous tissue. Mature bone formation was observed in the scaffold alone group with many positive TRAP-stained osteoclasts present, which is indicative of bone remodelling. For the doses investigated, the results show that the addition of SBP compromised bone remodelling in this mouse CSD model. These results suggest that further investigation is required to determine how SBP regulates cells essential for the remodelling process and further optimisation is required before testing the osteoinductive potential of SBP in a fracture setting.

INTRODUCTION

The repair of large or complex bone defects and fractures that cannot repair correctly require the use of bone graft substitutes, in order to stabilise and restore bone integrity. To successfully qualify as a bone graft substitute the material should ideally possess two of the following characteristics, osteogenicity, osteoconductivity or osteoinductivity (Lane & Sandhu, 1987). The current standard surgical strategies to repair skeletal defects is to utilise autogenous or allogenic bone materials (Bauer & Muschler, 2000b; Boyce, Edwards & Scarborough, 1999). Although these current approaches are generally successful, there are a number of disadvantages. Autologous bone is often limited in availability and complications which can arise in 30% of cases have numerous associated morbidities (Amini, Laurencin, & Nukavarapu, 2012; Banwart et al., 1995; Jimi et al., 2012). Allogenic bone grafts overcome some of the disadvantages of autogenous bone, however the risk of disease transmission and decreased biological capacity compared to autografts makes them an unfavourable choice (Lane & Sandhu, 1987; Oklund et al., 1986). Thus, there is a requirement for alternative bone graft substitutes which possess or combine the aforementioned characteristics, needed to ensure effective bone healing.

Growth factors and proteins have been increasingly used and tested to act as osteoinductive agents for clinical use. Much focus has been on factors directly responsible for the regulation of numerous cellular functions, including the formation and resorption of bone. Bone morphogenetic proteins (BMPs), which are essential factors for promoting the activity of bone-forming cells, have been used extensively both pre-clinically and clinically and have demonstrated significant osteoinductive potential (Dragoo et al., 2003; Kloen et al., 2003; Mussano et al., 2007). However, there are now emerging reports of complications post-surgery and along with the significant high cost of using BMPs clinically, there is a need to identify safer and cheaper alternative agents (Flierl et al., 2013; Garrison et al., 2007).

Our laboratory has recently demonstrated that peroxidases, which are a ubiquitous group of haem-containing enzymes, known for their antimicrobial role during host defence (Hiraga et al., 2001) have implications in wound healing (Rayner et al., 2000). Previous *in vitro* studies have demonstrated that peroxidases are pro-angiogenic (Panagopoulos et al., 2015) and have the capacity to stimulate osteoblasts to increase collagen I biosynthesis and mineralise the surrounding extracellular matrix (ECM) (DeNichilo et al., 2016). *In vivo*, our laboratory has also demonstrated that plant-derived soybean peroxidase (SBP), which is an inexpensive and highly available peroxidase compared to previously tested peroxidases, stimulates a fibrogenic response and induces connective tissue regeneration in a porcine model (DeNichilo et al., 2015). Additionally, we have successfully employed a CSD model in the medial femoral condyles of sheep (unpublished), which has demonstrated that SBP successfully accelerates bone formation in a critical size sheep femoral defect. This study established the clinical potential of SBP in a femoral drill-hole defect model, however, we needed to further optimise and verify its effectiveness to repair bone fractures. Although sheep are an ideal model for testing bone repair therapies in a fracture setting, there are significant costs associated and ethical pressures, which must be taken into consideration when testing new treatments (Martini, Fini, Giavaresi, & Giardino, 2001). In comparison, the significantly lower cost of rodents allows for high throughput optimisation studies. This includes the ability to conduct longitudinal studies and assess multiple doses. Furthermore, the size of rodent bones allows for rapid analyses compared to sheep. Thus, the decision was made to use of rodents as the species for preliminary testing. We attempted to mimic the response observed in the sheep model by exploring a critical size defect (CSD) model in mice. CSDs are defects which cannot heal by bone formation throughout the lifetime of the animal, without the addition of an applied bone graft substitute (Schmitz & Hollinger, 1986).

In this study we hypothesised that delivery of SBP in combination with an osteoconductive, absorbable collagen scaffold, CollaCote™ would increase bony bridging and bone formation at 8 weeks, when compared with scaffold alone. To test this hypothesis, we implanted SBP-loaded scaffolds into a mouse cranial CSD of 3 mm in diameter and quantified new bone formation at 3 days and 8 weeks by longitudinal microCT analysis. Histological analysis was also performed at 8 weeks.

MATERIALS AND METHODS

Ethics Statement

The use of all normal human donor-derived bone tissue was approved by the human ethics committee of the Royal Adelaide Hospital/University of Adelaide (Approval No. RAH 090101). Human bone samples were obtained with informed written donor consent, as required and approved by the ethics committee. The animal study was approved by the Animal Ethics Committees of the University of Adelaide (M-2013-221A) and SA Pathology (8-15a).

Cell Culture

The osteosarcoma cell line SaOS-2 were kindly donated by TGR BioSciences (TGR BioSciences Pty Ltd, Adelaide, SA). SaOS-2 cells were expanded in culture using Dulbecco's Modified Eagle's Medium (DMEM; high glucose with no ascorbic acid; AA), supplemented with 2 mmol/L glutamine, 100 IU/mL penicillin, 100 µg/mL streptomycin, 20 mmol/L HEPES, and 10% foetal bovine serum (FBS; Invitrogen Life Technologies, Carlsbad, CA) in a 5% CO_2-containing humidified atmosphere. These cells maintain an osteoblastic phenotype in culture and stain positive for alkaline phosphatase activity.

Cell Viability

To determine the effects of SBP on cell viability, SaOS-2 cells were cultured in 96-well plates (Nunc, Roskilde, Denmark) at a density of 1.2×10^4 cells per well for 5 days in DMEM/10% FBS until reaching confluence. Cells were starved overnight in serum-free DMEM and then stimulated for an additional 72 h in serum-free DMEM containing either AA 2-phosphate at 100 µmol/L (Wako Chemical Industries, Osaka, Japan) as a positive control, or with soybean peroxidase (SBP; Bio-Research Products, North Liberty, IA) in the absence of AA supplementation. Cell viability/growth was then assessed using the alamarBlue fluorescent dye

assay (Invitrogen Life Technologies), according to manufacturer's instructions. Fluorescence was measured at wavelengths of 530 nm excitation and 595 nm emission using a FLUOstar Optima plate reader (BMG Labtek Australia, Mornington, VIC).

Animals

56 Female Balb/C mice at 8 weeks of age (Institute of Medical and Veterinary Services Division), weighing 13.6-23.6 g (mean: 19.9; SD ± 1.7) were acclimatised to the animal housing facility for a minimum period of 1 week before the commencement of experimentation. The general physical well-being and weight of animals were monitored continuously throughout the experiments. All mice were housed under pathogen-free conditions and all experimental procedures on animals were carried out with strict adherence to the rules and guidelines for the ethical use of animals in research and were approved by the Animal Ethics Committees of the University of Adelaide and the Institute of Medical and Veterinary Science, Adelaide, SA, Australia.

Scaffold Preparation

Scaffolds used in this study were collagen-based biodegradable scaffolds called CollaCote™ (Medtronic Australasia, Sydney, NSW). Before conducting the *in vivo* study, it was confirmed that this scaffold material could readily uptake SBP to the desired concentrations of 50µg, 100µg and 200µg. These doses were determined to be suitable based on previous *in vitro* studies and an *in vivo* pilot study. For *in vitro* assessment, the CollaCote™ material was cut as 3-mm diameter discs, using a manual biopsy puncture (Stiefel Laboratories, Melbourne, VIC). It was determined that each scaffold could hold 15 µl of solution. From this information, scaffolds were individually added to wells containing 30 µl of SBP at 3.33 mg/mL, 6.66 mg/mL and 13.33 mg/mL which was expected to result in the uptake of 50 µg, 100 µg and 200 µg total SBP

respectively. Scaffolds were maintained at room temperature for 24 h. Total concentration of SBP taken up by the scaffolds was quantified using the Nanodrop spectrophotometric measurement at 403 nm (Nanodrop Technologies, Thermo Fisher Scientific, Scoresby, VIC).

For *in vivo* preparation, scaffolds were cut as 3-mm diameter circles in a sterile setting and maintained under sterile conditions. 24 h before surgery and implantation, 14 scaffolds per treatment group were loaded individually into separate wells of a v-bottom 96-well plate. 30 µl of SBP at concentrations of 3.33 mg/mL, 6.66 mg/mL and 13.33 mg/mL were aliquoted onto each scaffold which would result in the final concentrations of SBP within each scaffold to be 50 µg, 100 µg and 200 µg. As a control, 30 µl of saline was also added to individual scaffolds. Scaffolds were loaded, sealed and kept at the IMVS Animal House at room temperature overnight, ready to be implanted the next day.

Mouse Calvarial Critical Size Defect Model

An established gold standard mouse calvarial size defect (CSD) model was used to test the ability of SBP-soaked collagen scaffolds to induce bone repair, as previously described with minor modifications (Aalami et al., 2004; Hollinger & Kleinschmidt, 1990). Briefly, each treatment group (n=14) which consisted of 8-week-old Balb/C female mice, were anaesthetised by 80 mg/kg Ketamine hydrochloride and 10mg/kg Xylazine via intraperitoneal (i.p) injection, followed by subcutaneous (s.c) injection of 0.1 mg/kg Buprenorphine analgesic. Once mice were non-responsive to paw pinching, hair on the scalp was clipped and disinfected with 70% ethanol before a C-shaped incision of the skin was made, over the right parietal bone. The pericranium was removed by blunt scraping and 3-mm calvarial defects were created in the right parietal bone with a biopsy puncture (Stiefel) with meticulous care taken to avoid damaging of underlying dura mater. Care was also taken to ensure the cranium did not dry out by applying phosphate buffered saline (PBS) as needed. Each parietal bone defect was filled

with a 3-mm-diameter CollaCote™ collagen scaffold (Zfx Australia Pty Ltd, Sydney, NSW), with the assigned treatment as mentioned above. The skin was sutured closed using resorbable sutures and mice were moved to a heat pad to recover before being transferred into cages. Mice were monitored twice a day for 5 days after surgery to ensure complete recovery.

In Vivo Live Microcomputed Tomography Imaging

Computed tomography (CT) images were obtained using a SkyScan-1076 *in-vivo* micro-CT scanner (Bruker-micro-CT, Kontich, Belgium) while the animals were anaesthetised via i.p injection, as previously described (Zinonos et al., 2009)**.** Briefly, the micro-CT Scanner was operated at 50 kV, 110 μA, rotation step of 0.8, 0.5-mm Al filter, scanning width of 35 mm, scanning resolution of 9 μm, and imaging time of 30 min. For *ex vivo* analysis, skulls were surgically resected before being inserted into the scanner. Micro-CT settings remained consistent to the *in vivo* scans. The cross-sections were reconstructed using a Skyscan reconstruction program (software NRecon, Bruker). Cross-section images were then realigned consistently between samples and the sagittal sections saved (software Data Viewer, Bruker). Files were then imported into CTAn software (Bruker) for three-dimensional analysis and three-dimensional image generation. All images were viewed and edited using CTVol (Bruker) visualisation software.

Ex Vivo Micro-CT Imaging

At 56 days post-surgery, mice were euthanised by CO_2 gas and cervical dislocation. Mice heads were then surgically excised and placed into labelled containers filled with 70% ethanol ready for *ex vivo* micro-CT. The micro-CT scanner and analysis was conducted as previously mentioned for *in vivo* imaging.

Histology

Skulls were fixed in 10% (v/v) buffered formalin (24 hours at 4°C), followed by 4 to 6 weeks of decalcification in 0.5 M EDTA–0.5% paraformaldehyde in PBS (pH 8.0) at 4°C. Complete decalcification was confirmed by radiography. The calvaria and underlying brain tissue was excised and then embedded in paraffin. 5 µm longitudinal sections were prepared and stained with hematoxylin and eosin (H&E). Additional sections were used for TRAP (Sigma-Aldrich) staining following the manufacture's protocol. Images were taken using the Olympus CKX41 (Olympus, Hachioji-shi, Tokyo) microscope.

Statistical Analysis

Data points derived from experiments are reported as the mean ± standard deviation (SD). Analysis of variance to determine significant difference between samples was performed using the paired Student's t-test.

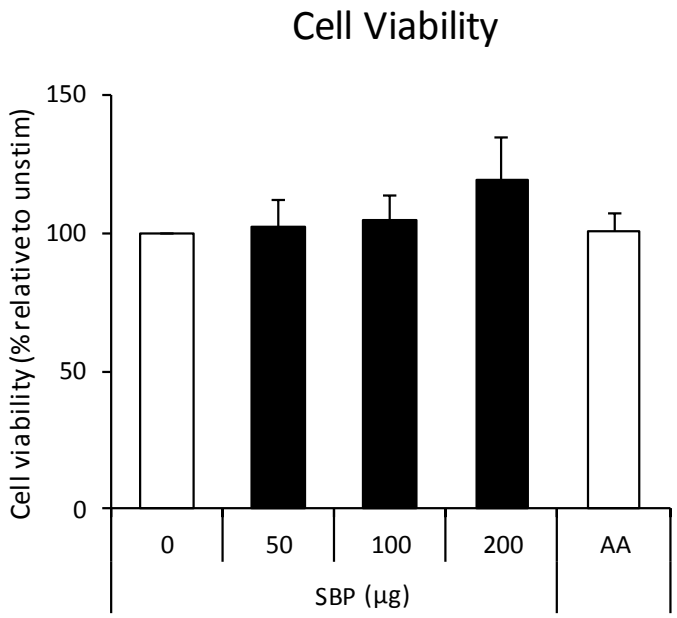
RESULTS

SBP promotes cell survival at high concentrations.

Assessment of cell viability after 72 h indicated that high dose SBP had no impact on SaOs-2 cells when compared to untreated cells (Fig. 1). This confirmed that SBP is not toxic at the high doses tested and without effect on cell proliferation. This is consistent with our previous *in vitro* studies, which demonstrated the non-toxic effects of SBP on primary human osteoblasts (unpublished).

Figure 1. *High dose SBP does not affect cell viability*. Viability of cultured osteosarcoma cell line SaOS-2 after 72h stimulation with SBP (µg), as assessed using the alamarBlue dye assay. Cell viability was normalised so the average values of untreated cells were set to 100% relative to each dose of SBP. Representative data are the mean ± SD of triplicate determinations, of three independent experiments.

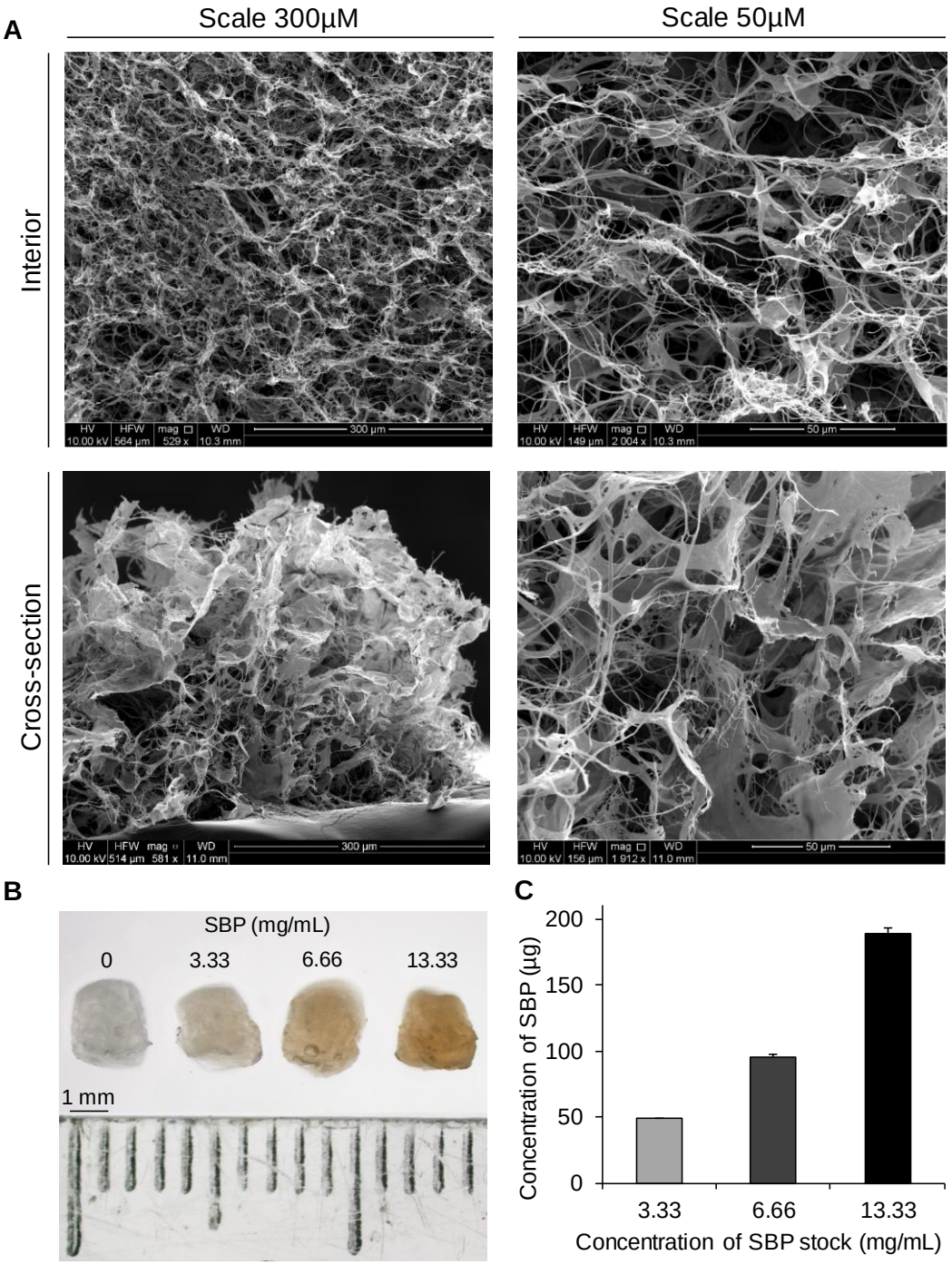
A


Cell Viability
Cell viability (% relative to unstim)
150
100
50
0
0
50
100
200
AA
SBP (µg)

CollaCote possesses favourable characteristics as a vessel to contain SBP.

Visualisation of CollaCote™ under SEM shows a highly porous, honeycomb-like structure within the material (Fig. 2A). Interior sections showed a uniform framework of Type I bovine collagen with voids ranging from approximately 10 µm to 100 µm. When the scaffold material was cut to 3-mm diameter discs and 15 µl of SBP added at increasing concentrations, we observed an ability of the CollaCote™ to retain the entire 15 µl solution by swelling in size and increasing in colour intensity with each increasing SBP concentration (Fig. 2B). Once we were confident that SBP could be absorbed by the material and retained, we assessed the ability of the material to take up desired concentrations of SBP for implantation into mice. We confirmed that the scaffolds, which hold 15 µl of solution, could successfully uptake our desired doses of SBP to be used *in vivo* of 50 µg, 100 µg and 200 µg by adding SBP at 3.33 mg/mL, 6.66 mg/mL and 13.33 mg/mL respectively, by Nanodrop quantification of remaining solution (Fig. 2C). From these *in vitro* experiments we were confident that CollaCote™ was a suitable material to contain SBP and to be utilised as an osteoconductive scaffold to assist SBP in accelerating the repair of critical size defects in mice.

Figure 2. *CollaCote readily uptakes desired concentrations of soybean peroxidase.* A: Representative SEM images of the CollaCote scaffold material. Images were taken of the interior and cross-sectional aspects of the material at low and high magnifications. B: Representative images of CollaCote scaffolds retaining SBP at increasing concentrations. 3-mm diameter scaffolds were cut and pre-soaked with SBP for 24 h at the doses indicated. C: Quantitation of SBP retained within the scaffolds after 24 h. 3-mm diameter scaffolds were soaked in SBP for 24 h at 3.33, 6.66 and 13.33 mg/mL. Experiments were independently performed two times and each reading was taken in duplicate. Data are the mean ± SD of triplicate determinations for each peroxidase dose.


A
Scale 300µM
Scale 50µM
Interior
Cross-section
300 µm
50 µm
B
SBP (mg/mL)
0
3.33
6.66
13.33
1 mm
C
Concentration of SBP (µg)
200
150
100
50
0
3.33
6.66
13.33
Concentration of SBP stock (mg/mL)

SBP-loaded CollaCote scaffolds inhibit bone remodelling in a Calvarial critical size defect model.

Previous *in vitro* studies conducted by our laboratory have shown SBP to be highly effective in promoting the production of procollagen I and generating a mineralised ECM by primary human osteoblasts (unpublished). Furthermore, we have demonstrated that SBP has the capacity to increase the deposition of new bone at 4 weeks, in an ovine defect model. To further optimise and validate the effectiveness of SBP in bone and fracture repair using rodents as an extremely cheap and high throughput species, we needed to establish the gold-standard CSD model in mice. Representative micro-CT images via 3D reconstruction at day 3 post-surgery demonstrate consistent creation of the defects across each tested group including control (Fig. 3A). 3D modelling and quantitative analysis of the defects containing saline-treated CollaCote™ at day 56 confirmed the osteoconductive ability of the scaffold material. Bone formation presented as total bone volume (BV), showed an approximately 50% increase in BV at day 56 compared to baseline ($p < 0.05$) (Fig. 3B). In support of the micro-CT data, histological evaluation confirms the presence of new bone formation, by CollaCote™ alone, as indicated by the formation of bone marrow pockets, angiogenesis and new bone surfaces (Fig. 4). Furthermore, TRAP staining provided further evidence of new bone formation and remodelling due to the number of positive-stained osteoclasts lining the new bone surfaces. In contrast, SBP-loaded scaffolds significantly inhibited bone formation compared to control and demonstrated a BV comparable to baseline ($p > 0.05$). In addition, Histological evaluation of the maximal dose of SBP administered (200µg) showed significant reduction in bone formation and remodelling when compared to the saline control (Fig. 4). H&E staining shows a lack of newly formed bone and bone marrow pockets within the defect. A large amount of irregular collagen fibres can be observed within the defect surrounding small blood vessels. Finally, a number of positive TRAP-stained osteoclasts can be observed, however they are only located

along the original bone surfaces. These findings demonstrate the complexity of bone formation and remodelling and furthermore, the numerous potential roles SBP has on the regulation of cellular function *in vivo*.

Figure 3. *SBP combined with CollaCote inhibits bone regeneration within a calvarial critical size defect.* Mice (n=14) received a 3-mm defect within the right parietal bone of the calvarium. Each defect was filled with saline or SBP-soaked collagen scaffold (CollaCote). A: Representative micro-CT reconstructions. At day 3 live micro-CT scanning of the calvarium of each mouse was conducted to establish a baseline. At day 56 mice were sacrificed and ex vivo micro-CT scanning of the defect site was performed. B: Quantitative analysis of the micro-CT data showed increased bone volume within the defects treated with CollaCote+saline (control) compared to CollaCote+SBP at each dose. Analysis is presented as the mean bone volume (BV) of each SBP dose compared to control. Data was analysed using paired Student's t-test and are the mean ± SD of fourteen determinations for control and each SBP dose. *$P<0.01$.

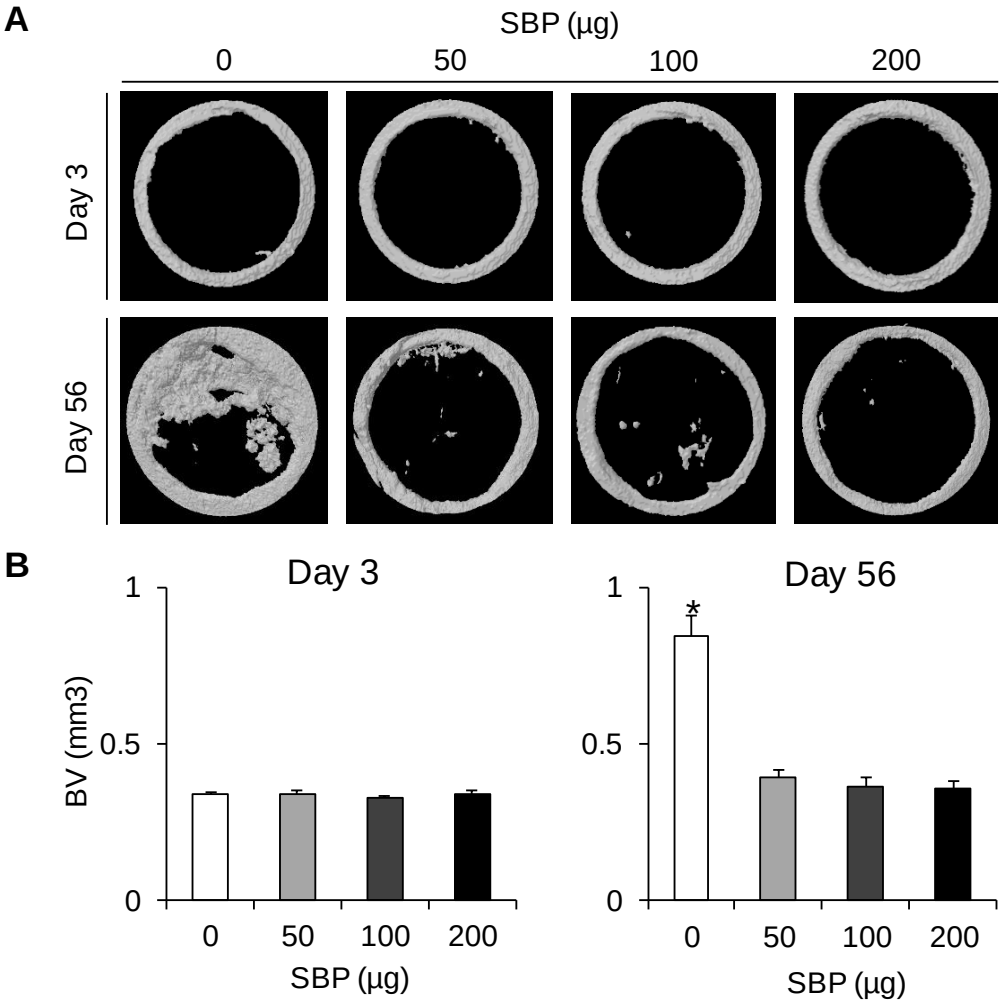
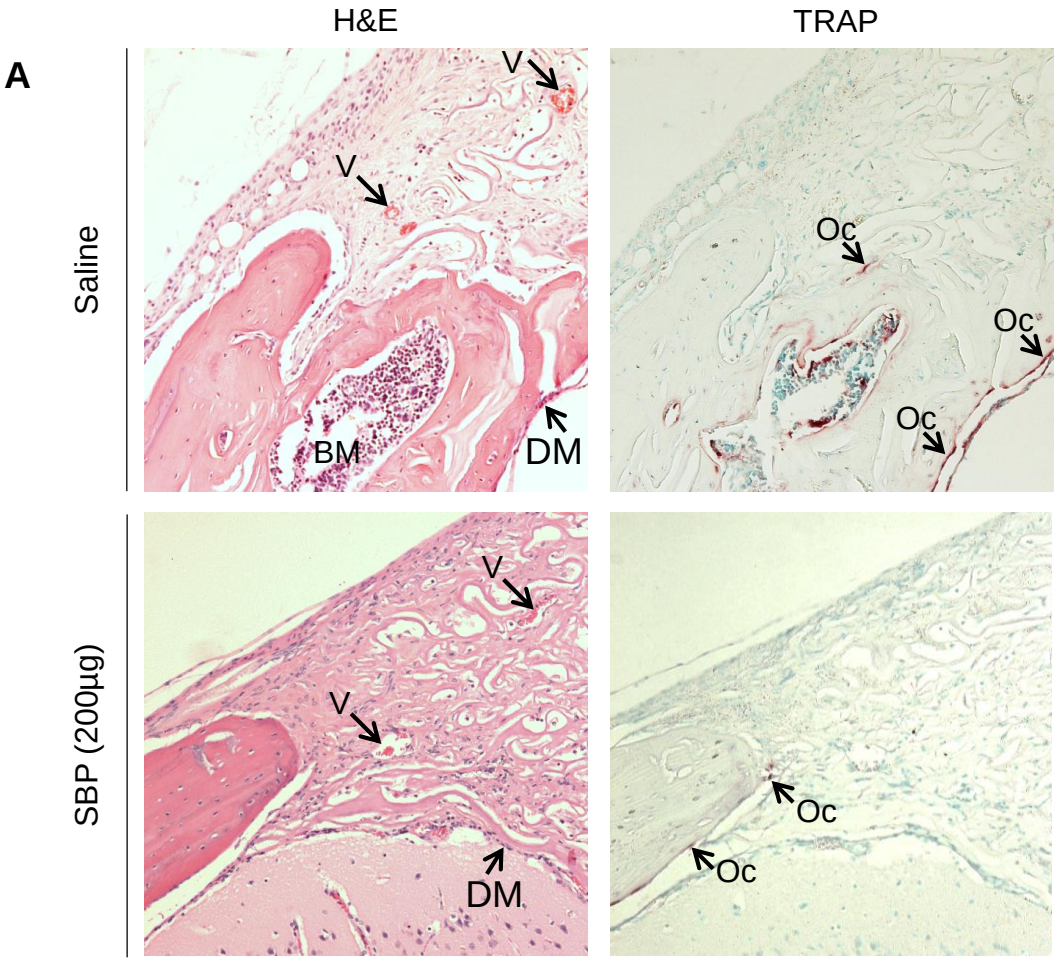

A
SBP (µg)
0
50
100
200
Day 3
Day 56
B
Day 3
Day 56
BV (mm3)
1
0.5
0
*
0
50
100
200
SBP (µg)

Figure 4. *Reduced detection of TRAP-positive osteoclasts and bone formation in the presence of SBP-loaded scaffolds.* A: Representative images of histological analysis using H&E and TRAP to visually compare between the calvarial CSD treated with SBP (200µg) and control. V: blood vessel; DM: dura mater; BM: bone marrow; Oc: osteoclast.


A
H&E
TRAP
Saline
SBP (200µg)
V
V
BM
DM
Oc
Oc
Oc
V
V
DM
Oc
Oc

DISCUSSION

The purpose of this study was to establish a cheaper and high throughput model of bone repair by using rodents, which can be used to further optimise SBP as an osteoinductive agent. Therefore, the objective of this study was to evaluate the efficacy of SBP in combination with an osteoconductive scaffold to accelerate repair of a 3 mm critical size bone defect in mice. Based on our previously successful ovine model (unpublished), we hypothesised that the delivery of SBP, which would act as an osteoinductive agent with an osteoconductive scaffold, would result in a synergistic response to increase bone formation within the CSD compared to the scaffold alone.

We have previously demonstrated in an ovine CSD model, that SBP is an effective agent at accelerating bone formation in combination with an osteoconductive scaffold. However, whilst the CSD model is an established model for assessing the regenerative potential of bone graft substitutes (Hollinger & Kleinschmidt, 1990), the mechanism of repair within these cancellous bone defects is predominantly via direct intramembranous ossification (Pobloth et al., 2016; Uebersax, 2008). This mechanism of repair differs to the combination of endochondral and intramembranous ossification observed in fracture models and so, new bone graft substitutes need to be verified in different models which replicate these mechanisms. We therefore aim to assess the potential of SBP to enhance repair of a fracture model initially in rodents, before progressing to sheep. However, the purpose of this study was to firstly establish SBP in a rodent model, by using a CSD model in the mouse calvaria. It was hypothesised that the calvarial CSD would reproduce what was observed in the sheep drill-hole CSD, due to the comparable healing mechanisms of intramembranous ossification (Alberius & Johnell, 1991).

Due to this understanding, we anticipated similar outcomes in the context of bone formation using the mouse CSD model. In contrast, micro-CT and histological analysis showed exactly the opposite effect, where SBP inhibited the response. Comparisons of bone formation

between long and flat bones have previously shown that the rate of healing in long bones occurs much more rapidly and the absence of mechanical loading within the calvaria can impede the healing process (Lim et al., 2013). Whilst this was initially overlooked in our study design, the presence of new bone within the scaffold alone group in this study clearly demonstrates that the model itself may not be the problem. We are certain the collagen scaffold itself is osteoconductive and responsible for the partial healing of the defect, as previous experiments conducted in our group revealed that empty defects were generally filled with fibrous tissue within the 8-week timespan and absent of new bone. The 8-week timespan was selected based on previous studies that have shown the critical time of healing is between the 4^{th} and 8^{th} week post-surgery, with minimal healing beyond the 8-week time point (Cooper et al., 2010).

The use of carriers for sustained release of growth factors is necessary for delivering bone-inducing agents. In addition, they act as osteoconductive scaffolds to provide structural support for the infiltrating bone-remodelling cells (Wang & Yeung, 2017). Type I collagen scaffolds are an ideal candidate for the delivery of growth factors, as type I collagen is a major component of bone (Glimcher & Lian, 1989). Collagen sponges have been extensively tested and have demonstrated successful release of growth factors, including BMP-2 (Friess, Uludag, Foskett, Biron, & Sargeant, 1999; Schmidmaier, Schwabe, Strobel, & Wildemann, 2008). In this study, CollaCote firstly proved to be a suitable scaffold by its ability to take-up desired concentrations of SBP. Secondly, it appeared to be nearly completely resorbed by 8-weeks. Interestingly, in the SBP-treated defects, there is a significant amount of collagen within the defect. It is likely that the dense collagenous tissue is unresorbed CollaCote, due to the absence of remodelling.

The successful partial healing in the control group and the intact dura mater observed in each treatment group and animal, which is critical for normal bone repair (Cooper et al., 2010), confirms the model's success. It also strongly suggests that SBP is responsible for the

inhibition of bone formation within the CSD. Recent unpublished evidence by our laboratory has demonstrated that SBP can effectively promote osteoblast function by increasing collagen biosynthesis and matrix mineralisation. With comparable results also observed in the presence of mammalian peroxidases (DeNichilo et al., 2016). However, we have also recently demonstrated that both plant and mammalian peroxidases can inhibit osteoclastogenesis *in vitro* (Panagopoulos et al., 2017). Whilst we have investigated both osteoclasts and osteoblasts in the context of peroxidase treatment, we have not yet investigated the effect these enzymes have in a dynamic co-culture system. Since bone remodelling and repair is a dynamic process which requires communication between both cell types (Sims & Gooi, 2008), it is possible the doses of SBP used disrupted this cellular communication and consequently the remodelling process.

It is also a possibility that the absence of healing was partially related to the model itself. From the literature it is clear that peroxidases are released at sites of tissue injury by inflammatory cells, including neutrophils, macrophages and eosinophils (Acharya & Ackerman, 2014; Van der Veen, de Winther, & Heeringa, 2009). Furthermore, we have since presented *in vitro* evidence that plant-derived SBP promotes the expression of pro-inflammatory markers, expressed by osteoblasts necessary for recruitment and remodelling, (unpublished). This suggests that peroxidases may play a greater role at sites of inflammation. This is of relevance to the absence of healing observed in this study, as controlled surgeries created in the calvaria, such as the CSD model, would not be expected to generate the same inflammatory response generated in the long bones, where the previous CSD model in sheep was conducted (unpublished). It is therefore a possibility that for peroxidases to work effectively as regenerative agents, a significant inflammatory response may be required. Inflammation does not solely provide a reason for the inhibitory effect observed in this study. However, since during normal fracture repair, eosinophils, neutrophils and macrophages are recruited to the fracture site (Andrew, Andrew, Freemont, & Marsh, 1994) where they release

peroxidases the fracture setting likely represents the best therapeutic approach for SBP in bone repair. It is also well reported that peroxidases, particularly SBP, are highly stable under a range of temperatures and pH (Henriksen et al., 2001; McEldoon & Dordick, 1996) which suggests that it is unlikely SBP degraded once at the defect site.

Several limitations need to be considered when interpreting the data presented here. Firstly, the absence of a positive control such as BMP-2 would have provided valuable information regarding the effectiveness of SBP compared to the current clinical agent. Although SBP impeded the repair process, future experiments should include BMP-2 to provide more information as to the reliability of this model. A further limitation of this study was the lack of *in vitro* evidence to determine the suitable dose of SBP to be used. To address this, three increasing doses of SBP were used, however the range of doses were broad and for future experiments smaller doses should be considered. In addition, studies should have been conducted to assess the release of SBP from the scaffold material, to determine the rate and concentration of release. Furthermore, whilst these doses of SBP were tested in the SaOS-2 osteosarcoma cell line, which demonstrated a nontoxic effect of these cells, validation of toxicity effects at high doses should be tested using primary human osteoblasts. Finally, we have evidence of SBP's ability to promote osteoblast function and inhibit osteoclastogenesis, which suggests a role for this enzyme in bone repair when optimised. However, further *in vitro* studies are essential to understand the inhibitory effect observed in this study and should be conducted before pursuing the fracture model.

Taken together, the results of this present study demonstrate an inhibition of bone formation following delivery of SBP within the bone defects. Despite the results of this study, the model was conducted successfully and CollaCote™ proved to be a suitable osteoconductive scaffold, by partially healing the bone defects. Moreover, SBP does not cause cytotoxicity, which could have been a cause for inhibition of bone formation. However, this should be further

validated using primary human osteoblasts. Based on previous *in vitro* studies and the successful sheep CSD model the potential for SBP to act as an osteoinductive agent should not be disregarded, despite the results of this study. Therefore, future studies using rodents as a cheap and high-throughput model should investigate lower range doses of SBP. The link peroxidases have with inflammation also suggests that the fracture setting would be a more appropriate model to explore, compared to the calvarial CSD model where inflammation is limited. Whilst there is still a considerable amount of research to be done, the requirement for novel osteoinductive agents is clear, due to the current complications associated with the use of BMP-2. Therefore, further research is essential, to fully elucidate the mechanistic role of SBP in promoting osteoblast function and confirm whether SBP can be used as a cheap, stable and effective osteoinductive agent in fracture healing or various settings of bone damage.

ACKNOWLEDGEMENTS

This work was supported in part by The Hospital Research Foundation and the National Health and Medical Research Council (Career Development Fellowship/627015; Project Grant/1050694).

CONFLICT OF INTEREST

The authors declare that they have no competing interests.

REFERENCES

Acharya, K. R., & Ackerman, S. J. (2014). Eosinophil granule proteins: form and function. *Journal of Biological Chemistry, 289*(25), 17406-17415.

Alberius, P., & Johnell, O. (1991). Repair of intra-membranous bone fractures and defects in rats: Immunolocalization of bone and cartilage proteins and proteoglycans. *Journal of Cranio-Maxillofacial Surgery, 19*(1), 15-20.

Amini, A. R., Laurencin, C. T., & Nukavarapu, S. P. (2012). Bone Tissue Engineering: Recent Advances and Challenges. *Critical reviews in biomedical engineering, 40*(5), 363-408.

Andrew, J. G., Andrew, S. M., Freemont, A. J., & Marsh, D. R. (1994). Inflammatory cells in normal human fracture healing. *Acta Orthop Scand, 65*(4), 462-466.

Banwart, J. C., Asher, M. A., & Hassanein, R. S. (1995). Iliac crest bone graft harvest donor site morbidity. A statistical evaluation. *Spine, 20*(9), 1055-1060.

Bauer, T. W., & Muschler, G. F. (2000a). Bone graft materials: an overview of the basic science. *Clinical orthopaedics and related research, 371*, 10-27.

Boyce, T., Edwards, J., & Scarborough, N. (1999). Allograft bone: the influence of processing on safety and performance. *Orthopedic Clinics, 30*(4), 571-581.

Cooper, G. M., Mooney, M. P., Gosain, A. K., Campbell, P. G., Losee, J. E., & Huard, J. (2010). Testing the "critical-size" in calvarial bone defects: revisiting the concept of a critical-sized defect (CSD). *Plastic and reconstructive surgery, 125*(6), 1685.

DeNichilo, M. O., Panagopoulos, V., Rayner, T. E., Borowicz, R. A., Greenwood, J. E., & Evdokiou, A. (2015). Peroxidase Enzymes Regulate Collagen Extracellular Matrix Biosynthesis. *The American Journal of Pathology, 185*(5), 1372-1384.

DeNichilo, M. O., Shoubridge, A. J., Panagopoulos, V., Liapis, V., Zysk, A., Zinonos, I., . . . Evdokiou, A. (2016). Peroxidase Enzymes Regulate Collagen Biosynthesis and Matrix Mineralization by Cultured Human Osteoblasts. *Calcified Tissue International, 98*(3), 294-305.

Dragoo, J. L., Choi, J. Y., Lieberman, J. R., Huang, J., Zuk, P. A., Zhang, J., . . . Benhaim, P. (2003). Bone induction by BMP-2 transduced stem cells derived from human fat. *Journal of orthopaedic research, 21*(4), 622-629.

Flierl, M. A., Smith, W. R., Mauffrey, C., Irgit, K., Williams, A. E., Ross, E., . . . Stahel, P. F. (2013). Outcomes and complication rates of different bone grafting modalities in long bone fracture nonunions: a retrospective cohort study in 182 patients. *Journal of orthopaedic surgery and research, 8*(1), 33.

Friess, W., Uludag, H., Foskett, S., Biron, R., & Sargeant, C. (1999). Characterization of absorbable collagen sponges as rhBMP-2 carriers. *International journal of pharmaceutics, 187*(1), 91-99.

Garrison, K. R., Donell, S., Ryder, J., Shemilt, I., Mugford, M., Harvey, I., & Song, F. (2007). Clinical effectiveness and cost-effectiveness of bone morphogenetic proteins in the non-healing of fractures and spinal fusion: a systematic review.

Glimcher, M. J., & Lian, J. B. (1989). *The chemistry and biology of mineralized tissues: proceedings of the Third International Conference on the Chemistry and Biology of Mineralized Tissues, held in Chatham, Massachusetts on October 16-21, 1988* (Vol. 1): CRC Press.

Henriksen, A., Mirza, O., Indiani, C., Teilum, K., Smulevich, G., Welinder, K. G., & Gajhede, M. (2001). Structure of soybean seed coat peroxidase: A plant peroxidase with unusual stability and haem-apoprotein interactions. *Protein Science, 10*(1), 108-115.

Hiraga, S., Sasaki, K., Ito, H., Ohashi, Y., & Matsui, H. (2001). A large family of class III plant peroxidases. *Plant and Cell Physiology, 42*(5), 462-468.

Hollinger, J. O., & Kleinschmidt, J. C. (1990). The critical size defect as an experimental model to test bone repair materials. *Journal of Craniofacial Surgery, 1*(1), 60-68.

Jimi, E., Hirata, S., Osawa, K., Terashita, M., Kitamura, C., & Fukushima, H. (2012). The current and future therapies of bone regeneration to repair bone defects. *International journal of dentistry, 2012*.

Kloen, P., Di Paola, M., Borens, O., Richmond, J., Perino, G., Helfet, D., & Goumans, M. (2003). BMP signaling components are expressed in human fracture callus. *Bone, 33*(3), 362-371.

Lane, J. M., & Sandhu, H. (1987). Current approaches to experimental bone grafting. *The Orthopedic clinics of North America, 18*(2), 213-225.

Lim, J., Lee, J., Yun, H.-S., Shin, H.-I., & Park, E. K. (2013). Comparison of bone regeneration rate in flat and long bone defects: calvarial and tibial bone. *Tissue Engineering and Regenerative Medicine, 10*(6), 336-340.

Martini, L., Fini, M., Giavaresi, G., & Giardino, R. (2001). Sheep model in orthopedic research: a literature review. *Comparative medicine, 51*(4), 292-299.

McEldoon, J. P., & Dordick, J. S. (1996). Unusual thermal stability of soybean peroxidase. *Biotechnology progress, 12*(4), 555-558.

Mussano, F., Ciccone, G., Ceccarelli, M., Baldi, I., & Bassi, F. (2007). Bone morphogenetic proteins and bone defects: a systematic review. *Spine, 32*(7), 824-830.

Oklund, S. A., Prolo, D. J., Gutierrez, R. V., & King, S. E. (1986). Quantitative comparisons of healing in cranial fresh autografts, frozen autografts and processed autografts, and allografts in canine skull defects. *Clinical orthopaedics and related research*(205), 269-291.

Panagopoulos, V., Liapis, V., Zinonos, I., Hay, S., Leach, D. A., Ingman, W., . . . Zannettino, A. C. (2017). Peroxidase enzymes inhibit osteoclast differentiation and bone resorption. *Molecular and cellular endocrinology, 440*, 8-15.

Panagopoulos, V., Zinonos, I., Leach, D. A., Hay, S. J., Liapis, V., Zysk, A., . . . Evdokiou, A. (2015). Uncovering a new role for peroxidase enzymes as drivers of angiogenesis. *The international journal of biochemistry & cell biology, 68*, 128-138.

Rayner, T. E., Cowin, A. J., Robertson, J. G., Cooter, R. D., Harries, R. C., Regester, G. O., . . . Belford, D. A. (2000). Mitogenic whey extract stimulates wound repair activity in vitro and promotes healing of rat incisional wounds. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology, 278*(6), R1651-R1660.

Schmidmaier, G., Schwabe, P., Strobel, C., & Wildemann, B. (2008). Carrier systems and application of growth factors in orthopaedics. *Injury, 39*, S37-S43.

Schmitz, J. P., & Hollinger, J. O. (1986). The critical size defect as an experimental model for craniomandibulofacial nonunions. *Clinical orthopaedics and related research*(205), 299-308.

Sims, N. A., & Gooi, J. H. (2008). *Bone remodeling: Multiple cellular interactions required for coupling of bone formation and resorption.* Paper presented at the Seminars in cell & developmental biology.

Van der Veen, B. S., de Winther, M. P., & Heeringa, P. (2009). Myeloperoxidase: molecular mechanisms of action and their relevance to human health and disease. *Antioxidants & redox signaling, 11*(11), 2899-2937.

Wang, W., & Yeung, K. W. K. (2017). Bone grafts and biomaterials substitutes for bone defect repair: A review. *Bioactive Materials, 2*(4), 224-247.

Zinonos, I., Labrinidis, A., Lee, M., Liapis, V., Hay, S., Ponomarev, V., . . . Evdokiou, A. (2009). Apomab, a fully human agonistic antibody to DR5, exhibits potent antitumor activity against primary and metastatic breast cancer. *Mol Cancer Ther, 8*(10), 2969-2980.

CHAPTER 6:

DISCUSSION

The work reported in this thesis provides new contributions to knowledge on the ever-expanding function of peroxidase enzymes and their role in health and disease. The data presented shows for the first time the potential of these enzymes to be used as therapeutic agents to promote bone repair. Specifically, mammalian and plant-derived peroxidase enzymes EPO and SBP respectively, potently stimulate ECM biosynthesis and mineralisation of the bone matrix through regulation of osteoblast activity. In view of these findings, the link between inflammatory cells and heterotopic ossification (HO) identifies a potential role for peroxidase inhibitors in reducing unwanted pathological bone formation.

Despite current treatment strategies, including autologous grafting for bone repair and regeneration, researchers are yet to identify an agent that possesses the key characteristics of an optimal bone graft material. To date, autologous grafting remains the only material that provides osteoconductive, osteoinductive and osteogenic capabilities. Tissue engineering is considered an alternative grafting strategy due to the reduction in patient morbidities which commonly arise from autologous and allografting materials. This strategy comprises of biodegradable scaffold materials which are combined with inductive biomaterials, cells or growth factors to promote the repair process. Whilst a substantial amount of research has centred on the use of factors that promote osteogenic responses, there is great interest in investigating the importance of an inflammatory cell presence to improve healing (Bastian, Koenderman, Alblas, Leenen, & Blokhuis, 2016; Spiller et al., 2014). The initial acute inflammatory phase is critical for bone healing because it acts as the primary source of cytokines, chemokines and growth factors to the damaged site. However, the mechanisms by which these signalling cascades initiate bone repair are unclear. What is clear is the crosstalk between inflammatory cells and cells related to bone healing and remodelling is necessary, but also highly complex (Mountziaris, Spicer, Kasper, & Mikos, 2011). The importance of the inflammatory response during healing suggests that locally released factors may be involved in

influencing the regenerative process. Our laboratory has recently established that peroxidase enzymes, including, MPO and EPO, released by inflammatory cells that amass at sites of infection or trauma within the skeleton, act as regulators of fibroblast collagen ECM biosynthesis, angiogenesis and osteoclastogenesis. These studies provide insight into the potential role inflammatory cells and peroxidases play in normal tissue regeneration and fibrotic disease. Therefore, this thesis explored the role of peroxidases in promoting osteoblast functionality and bone repair. The findings presented in this thesis, identifies for the first time new and previously unrecognised roles of mammalian and plant-derived peroxidase enzymes as regulators of osteoblast functionality. To my knowledge this is the first report that describes a functional role for peroxidase enzymes in promoting osteoblast function and bone repair.

The first aim of this project was to assess the effect of mammalian peroxidases MPO and EPO on osteoblast functionality *in vitro* and to establish the role these enzymes have in bone formation. When osteoblasts were treated with MPO or EPO in the absence of AA, we observed a significant increase in collagen I biosynthesis without a corresponding increase in collagen I mRNA levels. Inhibition of collagen I secretion by peroxidase-treated osteoblasts in the presence of prolyl hydroxylase inhibitor DMOG, also indicated that post-translational modifications were likely affected by the peroxidases. Importantly, the work presented here also demonstrates that MPO and EPO can be bound and internalised by osteoblasts. This supports previous studies demonstrating their ability to bind and be internalised by members of the MSC family, including fibroblasts and endothelial cells. In the presence of AA, EPO alone significantly promoted matrix mineralisation and regulated mRNA expression of osteogenic genes. These findings, together with recent data linking eosinophilia and ectopic bone formation (Macias et al., 2001; Snidvongs et al., 2012), collectively suggests a mechanistic explanation for the pro-osteogenic properties of eosinophils and a role for EPO in bone repair. Unlike EPO, MPO failed to promote mineralisation and did not regulate the expression of

osteogenic genes examined. Despite both MPO and EPO sharing a 70% structural homology, the catalytic behaviour, substrate specificities and kinetics of these enzymes differ significantly. Furthermore, MPO and EPO generate different intracellular oxidant species (Borregaard & Cowland, 1997; Bozeman, Learn, & Thomas, 1990), which could be responsible for the difference in their ability to promote mineralisation and regulate osteogenic gene expression. A recent review has highlighted the importance of MPO released by neutrophils and macrophages in the progression of heterotopic ossification (HO) (Kraft et al., 2016). They hypothesised that neutrophils may be involved in the early stages of HO as neutrophils are a major source of prostaglandin E2 (PGE2). PGE2 has been the target of clinical utility including fracture healing and osteoporosis due to its diverse actions on inflammation, bone healing and bone formation (Li, Thompson, & Paralkar, 2007). The authors reported that the role of neutrophils in HO is due to enhanced prostaglandin E2 (PGE2) released from these cells, which leads to a dose-dependent increase in BMP-2 and osteoblast differentiation. This increase in PGE2 is reportedly due to a MPO-dependent mechanism, whereby MPO transforms prostaglandins to inactive products and consequently alters their activity at an inflammatory site (Paredes & Weiss, 1982). Whilst we did not observe a response in mineralisation by MPO-treated osteoblasts in this thesis, the association of inflammatory MPO and bone formation will need to be further explored, including its role in HO. The presence of eosinophils and neutrophils in inflammatory diseases collectively highlights a potential mechanistic link for MPO and EPO in diseases of chronic inflammation. These findings present an opportunity to explore the use of peroxidase inhibitors as a therapeutic strategy, using models of osteitis and ectopic bone formation. Importantly, *in vitro* data to address this aim, demonstrates the capacity of EPO to regulate osteoblast activity, including mineralisation and osteogenic expression, which are critical processes of bone repair. By fully elucidating the molecular mechanism(s) by which

peroxidases regulate osteoblast function we may identify multiple therapeutic strategies where peroxidases may be used to regulate bone formation.

An important goal for bone repair strategies is to identify potent agents that possess controlled osteoinductive and/or osteogenic capabilities. The intent for these agents is to be utilised with osteoconductive materials, to provide surgeons with an effective synthetic bone graft substitute. Osteoconductive scaffolds are currently available for clinical use, however the identification of effective osteoinductive and osteogenic agents is still required. A critical aspect for a successful clinical agent, aside from its bone repair capabilities is the cost, due to the unavoidable expense of orthopaedic surgery. Furthermore, agents need to demonstrate potency and safety to ensure effective repair with minimal adverse effects. We have now demonstrated that the mammalian peroxidase EPO, regulates osteoblast functionality and promotes the cellular processes required for bone formation, including collagen I biosynthesis and matrix mineralisation. However, EPO is an impractical candidate due to the prohibitively expensive and large-scale manufacturing costs. Conversely, the plant-derived peroxidase SBP, is inexpensive, commercially available in large quantities and has demonstrated potency in promoting collagen I section by fibroblasts and drive angiogenesis. The highly stable nature of SBP and its lack of toxicity in previous studies, attest to the safety of such an agent for clinical utility (DeNichilo et al., 2015; Hiraga, Sasaki, Ito, Ohashi, & Matsui, 2001; Panagopoulos et al., 2015).

Therefore, the second aim of this project was to investigate the ability of plant-derived peroxidase SBP, an inexpensive and nontoxic factor, to promote osteoblast functionality as a potential therapeutic candidate. *In vitro* experiments presented in this thesis demonstrated that SBP stimulated collagen I release by osteoblasts, promoted mineralisation of the deposited ECM and concomitantly regulated mRNA expression of numerous key osteogenic genes. SBP, like EPO demonstrated an ability to promote collagen I release by up to 3-fold compared to the

positive control, AA (Ch. 3, Fig. 1). In addition, osteoblasts stimulated with SBP under mineralising conditions showed up to a 3-fold increase in ECM mineralisation (Ch. 3, Fig 2), similarly to what was observed in the presence of EPO (Ch. 2, Fig. 5). Whilst EPO demonstrated greater potency at maximal doses of up to 6-fold, under the same mineralising conditions, the effectiveness of SBP, combined with its numerous benefits, including low cost, availability and stability suggests this enzyme could be an effective and economic therapeutic agent. The greatest variability between EPO and SBP was the mRNA expression level of BMP-2. EPO showed ~14-fold increase in expression (Ch. 2, Fig. 6) compared to a near 5-fold increase observed in the presence of SBP (Ch. 3, Table 2). The comparable mRNA expression of downstream genes regulated in the presence of SBP and EPO, including WNT-5A and BSP, suggests that a minimal increase in BMP-2 expression is evidently sufficient to promote expression of downstream targets. Therefore, the reduced BMP-2 expression of SBP-treated osteoblasts is still sufficient to significantly promote mineralisation. Although EPO has demonstrated greater potency than SBP, the effectiveness of SBP, combined with its numerous benefits compared to EPO, including low cost, availability, stability and safety make it a much greater therapeutic candidate in both its effectiveness and economic use. The work conducted here addresses a critical aspect of this thesis which was to identify a cheaper yet comparable peroxidase to EPO, in the context of promoting osteoblast functionality. Moreover, when compared to the complications and high cost associated with BMP-2, SBP may be considered an attractive option. Finally, although being plant-derived, SBP possessed the capacity to promote expression of pro-inflammatory and ECM remodelling genes, which are critical for successful bone repair. This suggests that SBP can influence human osteoblast function beyond normal levels and could be considered for clinical use in the context of promoting bone formation.

The third aim of this study was to assess whether localised delivery of SBP, using commercially available scaffold materials could enhance bone repair at a locally targeted site. For this study, biphasic granules comprised of HA/β-TCP were pre-treated with SBP and dispensed into bilateral femoral condyle defects in adult sheep. Whilst it could be argued that a fracture model would provide information of greater value, the simplistic approach for a novel agent was to assess its effectiveness in a critical size defect (CSD) model. The results of this preliminary study demonstrated that while HA/β-TCP granules are an effective osteoconductive material, SBP significantly increased intramembranous bony healing beyond the scaffold alone at 4 weeks, as shown by micro-CT analysis. Toluidine Blue staining of bone defects revealed no distinct difference in local cell populations between SBP-treated and control groups. Taken together, SBP enhanced the biphasic granules' ability to promote bone formation, with no observable toxic effects at the localised region. Since bone regeneration can take up to 12 weeks in sheep, long-term studies will be required to assess the continuing effects of these enzymes *in vivo* and confirm their non-toxic effects, as this is a critical determinant for the clinical use of novel agents.

However, the prohibitively expensive nature of the sheep model together with the number of sheep needed to provide statistical power made it unfeasible to pursue at this stage, thus, we explored validating SBP in a high throughput mouse model. This would allow us to explore fracture models in rodents and the use of various scaffold-materials and multiple end points with greater efficiency. CSD mouse models are commonly established in the calvaria of the skull, specifically the parietal bones (Aalami et al., 2004; Cowan et al., 2004). Whilst the long bones, which was the site of the ovine model, differ anatomically to the calvaria and can heal more rapidly due to the additional benefit of mechanical loading, the structure of trabecular bone in the ends of long bones, is comparable to flat bones including the calvaria (R. B. Martin, Burr, & Sharkey, 1998). Furthermore, our laboratory has previously established the calvarial

CSD model successfully in mice. Therefore, the CSD model in the calvaria was utilised as a high-throughput model to assess the bone regenerative potential of SBP, instead of a long bone defect or fracture model. However, in this study, inhibition of bone formation was observed in SBP treated defects (Ch. 5, Fig. 3) with a significant fibrous tissue presence and an absence of remodelling indicators, including osteoclasts (Ch. 5, Fig. 4). The mouse CSD model is well-established in the literature and was successfully reproduced by our laboratory as shown by the successful partial healing when using an osteoconductive scaffold alone as the control. It can therefore be concluded that the inhibition of bone formation observed in the presence of SBP, was not a result of failure to reproduce the CSD model, but likely due to the dose of SBP used. The inhibition of bone repair and absence of osteoclast-positive TRAP staining of the SBP treated defect sites suggests that the high concentrations tested, were impairing numerous bone repair and remodelling processes. For successful bone healing to occur there must be constant communication between sufficient population numbers of osteoclasts and osteoblasts (T. J. Martin & Sims, 2005). It is therefore plausible based on these studies and the absence of osteoclasts at the defect site, the concentrations of SBP utilised in this study significantly inhibited osteoclast formation, which resulted in disruption of cellular communication to repair the bony defects. However, given that our recent findings indicate that peroxidases possess a pro-fibrogenic and angiogenic response both *in vitro* and *in vivo* (DeNichilo et al., 2015; Panagopoulos et al., 2015), combined with data presented in this thesis, at optimal doses, peroxidases can promote tissue repair. Therefore, further studies which involve testing a broad range of peroxidase concentrations and determining the underlying mechanism(s) by which peroxidases regulate osteoblast and osteoclast function are needed. Future *in vivo* studies must involve careful consideration of dosage to prevent inhibitory responses from occurring.

Taken together, the significant findings observed in the sheep model presents a proof-of-concept role for SBP as a therapeutic agent to promote bone repair. Furthermore, it replicates

the pro-osteogenic role observed *in vitro*. The results obtained in the mouse model clearly present the issues of optimisation in pre-clinical models and whilst the result may be concerning for the future of SBP in the context of bone repair, the evidence presented in the physiologically comparable sheep model was positive. Further studies and more robust *ex vivo* analyses are needed to ensure that SBP is an effective agent pre-clinically and optimisation within the high throughput rodent model will be beneficial for establishing future models, including fracture models. Fracture models are of interest due to the relationship inflammation has on successful fracture healing and the known pro-inflammatory role of peroxidases. Thorough studies using multiple models and species, including future sheep studies to validate rodent models are necessary to fully elucidate the therapeutic potential of peroxidases in bone repair.

The findings in this thesis not only demonstrate a new functional role for peroxidases in promoting osteoblast function, but also provide the foundations for future studies where optimisation of these enzymes could be beneficial as novel therapeutic agents to enhance bone repair. In addition, the potential link that peroxidases have in pathological ossification provides us with the opportunity to explore the use of selective peroxidase inhibitors to manage the progression of ectopic bone formation.

FUTURE DIRECTIONS

The limitations of current bone graft substitutes are a major challenge for orthopaedic surgeons for managing complex bone defects and fractures. Due to the limitations of gold-standard autologous grafting and severe adverse effects associated with alternative bone graft substitutes, such as BMP-2, there is a need to identify alternative osteoinductive agents. The current study suggests that peroxidases, whether plant or mammalian-derived such as SBP and EPO, have the capacity to act as osteoinductive agents by their ability to promote osteoblast function. Furthermore, this thesis has presented evidence that SBP has the ability to promote bone formation *in vivo*. These preliminary studies identify a potential new range of osteoinductive agents for aiding bone repair. However, the work presented in this thesis also presents conflicting results for peroxidase enzymes in promoting bone repair, as seen in chapter 5, which shows significant inhibition of murine calvaria bone repair, when treated with high concentrations of SBP. Whilst one could argue the data presented in chapter 4, demonstrating the ability of SBP to increase bone repair of an ovine defect model is conducted in an animal of greater physiological relevance to humans, it is evident that further studies and robust analyses need to be performed to optimise the concentration of SBP to investigate bone healing. Well-established mouse models of fracture healing that also offer high throughput analysis in drug development exist and are likely more amenable to testing the clinical utility of SBP. Rodents are a useful animal model for testing therapies, due to being inexpensive compared to large animals and in this context a much more relevant model of bone repair. Once a range of optimal doses of SBP has been identified using the CSD model, more complex models of bone repair can be explored. This includes the fracture repair model of the long bones, which involves a greater complexity of remodelling mechanisms, a significantly greater inflammatory response and other factors such as mechanical loading. These studies will improve our understanding of

the osteoinductive potential peroxidases have and optimisation of this enzyme could lead to the development of a clinically effective agent to improve the rate of bone healing in patients.

The work described in this thesis identifies a previously unrecognised role for peroxidase enzymes on osteoblast functionality, associated with increased expression of osteogenic genes, including autocrine intermediate BMP-2. It is well established that autocrine BMP-2 production is essential for activating downstream targets in osteoblasts, necessary for osteoblast differentiation and bone formation (Phimphilai, Zhao, Boules, Roca, & Franceschi, 2006). Further findings presented by this thesis indicate that peroxidases are rapidly bound and internalised by primary osteoblasts and can promote mineralisation of these cells *in vitro*. Collectively, these findings suggest that one mechanism by which peroxidases increase mineralisation is by promoting endogenous BMP-2 production. Future studies investigating the role peroxidases have on BMP-2 production and therefore osteoblast function will be vital in their development as a therapeutic agent. The use of small molecule BMP inhibitors will be vital for investigating the mechanistic link between peroxidases and BMP signalling. A recently described BMP inhibitor called K02288, has demonstrated successful inhibition of stimulated Smad1/5/8 phosphorylation, without affecting TGF-β signalling (Sanvitale et al., 2013). This particular BMP inhibitor has also shown greater potency and selectivity than other small molecule BMP inhibitors, including Compound C and LDN-193189 (Vogt, Traynor, & Sapkota, 2011). By blocking BMP-2 signalling with the use of an inhibitor we will be able to determine if EPO and SBP directly upregulate BMP-2 production or by an independent mechanism.

An alternative mechanism by which peroxidases promote osteoblast activity is through depletion of ROS. ROS, including H2O2 are generated during normal cellular metabolism and at high levels can push cells into a state of oxidative stress, which is detrimental to cellular function. Recent findings have shown that oxidative stress leads to inhibition of bone cell

differentiation of pre-osteoblastic cells such as MC3T3-E1, primary rabbit osteoblasts and bone marrow stromal cells (Mody, Parhami, Sarafian, & Demer, 2001). The precise mechanism by which oxidative stress-induced inhibition of osteoblast differentiation occurs is still unclear. However, there is sufficient evidence that activation of both ERK and NF-KB are essential for the oxidative-stress induced inhibition of both alkaline phosphatase activity and collagen type I expression (Bai et al., 2004). It is well-established that peroxidases utilise H2O2 as a substrate to generate ROS. Based on this knowledge and our data, which demonstrates rapid binding and internalisation of peroxidases by cultured osteoblasts (DeNichilo et al., 2016) raises the distinct possibility that peroxidases can regulate intracellular H2O2. Therefore, we hypothesise that a potential mechanism by which peroxidases regulate osteoblast function in the context of osteogenesis is by depleting intracellular H2O2 levels. To assess this potential mechanism, *in vitro* studies will be conducted to determine the intracellular ROS levels of osteoblasts after peroxidase treatment. This will be assessed by measuring oxidative stress of osteoblastic cell cultures, using 2,7 dichlorofluorescein diacetate (DCF), a cell permeable dye which becomes fluorescent once reacted with intracellular ROS, such as H2O2 (Mody et al., 2001). We anticipate that these studies will collectively shed light on the mechanism(s) by which peroxidases regulate osteoblast function. These findings will be necessary to provide further understanding of how peroxidases function *in vivo*, which will enable us to further optimise this enzyme for pre-clinical and potentially in the future, clinical use.

Whilst there are several options available to manage and treat pathological ossification, including heterotopic ossification (HO), such as nonsteroidal anti-inflammatory medications (NSAIDs), bisphosphonates, radiation and physical therapy, there is a lack of clinical improvement for non-surgical interventions (Ranganathan et al., 2015). Operative intervention is currently the most successful treatment option, however severe complications can occur and depending on the extent of surgery required, disability can arise as a result of surgery. Though

the mechanistic processes involved in the progression of HO are not clearly defined, which has made targeted treatment of HO difficult, evidence suggests a mechanistic link between the local inflammatory response and HO development (Convente, Wang, Pignolo, Kaplan, & Shore, 2015). Specifically, enhanced recruitment of wound healing fibroblasts and a shift from normal soft tissue repair towards ectopic bone formation. Furthermore, it is hypothesised that treating HO at the inflammatory stage, prior to the formation of osseous tissue would be the ideal treatment strategy. However, the only current form of treatment in this context is the use of broad immunosuppressive drugs and whilst these drugs have shown success in inhibiting HO formation, therapies which target specific immune cells, including macrophages and neutrophils could be of greater benefit (Kraft et al., 2016). In addition to HO, the potential link between eosinophil infiltration and ectopic bone formation in chronic rhinosinusitis patients suggests a potential treatment target for this disease and others where increased eosinophilia is observed. The work presented in this thesis suggests that peroxidase enzymes, MPO and EPO are involved in multiple cellular processes critical to bone formation, which suggests a potential link for these enzymes in pathological ossification. Furthermore, it identifies a potential new avenue of targeted therapy for pathological ossification, where inflammation plays a critical role in the progression of these diseases. Small molecule peroxidase inhibitors have been developed over the years which have demonstrated their effectiveness with minimal detrimental effects (Kubin, 2011; Winterbourn, Kettle, & Hampton, 2016). These inhibitors have been designed specifically for inflammatory diseases such as chronic asthma and chronic sinusitis. Having now established a potential link for mammalian-derived peroxidases in pathological ossification, MPO and EPO inhibitors should be further investigated for their targeted anti-inflammatory therapy and subsequent prevention of ectopic bone formation. This would require the use of small animal models which mimic acquired HO, such as BMP-4-overexpressing mice (Kan & Kessler, 2010). These studies would not only enhance our understanding of the role

peroxidases have in inflammation but also the effect inflammation has on the progression of pathological ossification. Furthermore, by conducting studies utilising peroxidase inhibitors, we may increase our understanding of the mechanisms involved between peroxidases and osteogenesis, which subsequently may provide further knowledge of the role these enzymes have as therapeutic agents for bone repair.

Collectively, these future directions will not only provide valuable pre-clinical information that will help determine the therapeutic capacity of peroxidases in enhancing bone repair, compared to current osteoinductive agents, but also allow us to develop therapies which target MPO and EPO selectively against pathological ossification, thereby potentially inhibiting ectopic bone formation.

CONCLUSION

The findings presented in this thesis provide novel insights into the role of peroxidase enzymes in bone repair. MPO, EPO and SBP were proven to exhibit potent effects in inducing formation of the extracellular matrix, which is critical for ensuring successful bony healing. EPO and SBP were also recognised to be effective promoters of osteoblast function by regulating osteogenic gene expression and matrix mineralisation. The physiological relevance of EPO in promoting osteoblast function and its release from eosinophils at sites of inflammation suggests a potential mechanistic link for EPO in pathological bone formation at sites of tissue eosinophilia. Importantly, SBP has demonstrated the ability to enhance bone repair at a locally targeted site. The discovery that plant-derived SBP, like EPO, can promote osteoblast function, whilst also effectively enhance bone repair *in vivo*, gives rise to the idea of utilising novel non-mammalian-derived, cheaper agents which are easier to manufacture for promoting tissue regeneration.

In conclusion, data presented in this thesis demonstrates that peroxidase enzymes possess the capacity to regulate multiple cellular processes by osteoblasts, including collagen I biosynthesis, matrix mineralisation and regulate numerous pro-osteogenic genes. This thesis has also demonstrated the potential role SBP could have in a clinical setting by its ability to significantly enhance localised bone formation in a physiologically relevant animal model. Collectively, the findings of this thesis provide new evidence for peroxidase enzymes as novel osteoinductive agents likely to be used locally, in combination with bone graft substitutes to enhance bone repair.

BIBLIOGRAPHY

Aalami, O. O., Nacamuli, R. P., Lenton, K. A., Cowan, C. M., Fang, T. D., Fong, K. D., . . . Longaker, M. T. (2004). Applications of a mouse model of calvarial healing: differences in regenerative abilities of juveniles and adults. *Plastic and reconstructive surgery, 114*(3), 713-720.

Abu- Ghazaleh, R. I., Dunnette, S. L., Loegering, D. A., Checked, J. L., Kita, H., Thomas, L. L., & Gleich, G. J. (1992). Eosinophil granule proteins in peripheral blood granulocytes. *Journal of leukocyte biology, 52*(6), 611-618.

Acharya, K. R., & Ackerman, S. J. (2014). Eosinophil granule proteins: form and function. *Journal of Biological Chemistry, 289*(25), 17406-17415.

Ai-Aql, Z., Alagl, A. S., Graves, D. T., Gerstenfeld, L. C., & Einhorn, T. A. (2008). Molecular mechanisms controlling bone formation during fracture healing and distraction osteogenesis. *Journal of dental research, 87*(2), 107-118.

Alberius, P., & Johnell, O. (1991). Repair of intra-membranous bone fractures and defects in rats: Immunolocalization of bone and cartilage proteins and proteoglycans. *Journal of Cranio-Maxillofacial Surgery, 19*(1), 15-20.

Almaiman, M., Al-Bargi, H. H., & Manson, P. (2013). Complication of anterior iliac bone graft harvesting in 372 adult patients from may 2006 to may 2011 and a literature review. *Craniomaxillofacial Trauma and Reconstruction, 6*(04), 257-266.

Alt, V., Leung, K.-S., Kempf, I., Haarman, H., Taglang, G., Seidel, H., & Schnettler, R. (2006). *Practice of intramedullary locked nails: New developments in techniques and applications* (Vol. 3): Springer Science & Business Media.

Amini, A. R., Laurencin, C. T., & Nukavarapu, S. P. (2012). Bone Tissue Engineering: Recent Advances and Challenges. *Critical reviews in biomedical engineering, 40*(5), 363-408.

An, Y. H., & Freidman, R. J. (1998). *Animal models in orthopaedic research*: CRC Press.

Anderson, M. L., Dhert, W. J., de Bruijn, J. D., Dalmeijer, R. A., Leenders, H., van Blitterswijk, C. A., & Verbout, A. J. (1999). Critical size defect in the goat's os ilium: A model to evaluate bone grafts and substitutes. *Clinical Orthopaedics and Related Research®, 364*, 231-239.

Andrew, J. G., Andrew, S. M., Freemont, A. J., & Marsh, D. R. (1994). Inflammatory cells in normal human fracture healing. *Acta Orthop Scand, 65*(4), 462-466.

Apelt, D., Theiss, F., El-Warrak, A. O., Zlinszky, K., Bettschart-Wolfisberger, R., Bohner, M., . . . von Rechenberg, B. (2004). In vivo behavior of three different injectable hydraulic calcium phosphate cements. *Biomaterials, 25*(7), 1439-1451.

Arai, M., Shibata, Y., Pugdee, K., Abiko, Y., & Ogata, Y. (2007). Effects of reactive oxygen species (ROS) on antioxidant system and osteoblastic differentiation in MC3T3-E1 cells. *IUBMB life, 59*(1), 27-33.

Arinzeh, T. L., Peter, S. J., Archambault, M. P., Van Den Bos, C., Gordon, S., Kraus, K., . . . Kadiyala, S. (2003). Allogeneic mesenchymal stem cells regenerate bone in a critical-sized canine segmental defect. *JBJS, 85*(10), 1927-1935.

Aruoma, O. I., & Halliwell, B. (1987). Action of hypochlorous acid on the antioxidant protective enzymes superoxide dismutase, catalase and glutathione peroxidase. *Biochemical Journal, 248*(3), 973-976.

Atkins, G. J., Kostakis, P., Pan, B., Farrugia, A., Gronthos, S., Evdokiou, A., . . . Zannettino, A. C. (2003). RANKL expression is related to the differentiation state of human osteoblasts. *Journal of Bone and Mineral Research, 18*(6), 1088-1098.

Baader, E., Tschank, G., Baringhaus, K. H., Burghard, H., & Günzler, V. (1994). Inhibition of prolyl 4-hydroxylase by oxalyl amino acid derivatives in vitro, in isolated microsomes and in embryonic chicken tissues. *Biochemical Journal, 300*(2), 525-530.

Babhulkar, S., Pande, K., & Babhulkar, S. (2005). Nonunion of the diaphysis of long bones. *Clinical orthopaedics and related research, 431*, 50-56.

Bai, X.-c., Lu, D., Bai, J., Zheng, H., Ke, Z.-y., Li, X.-m., & Luo, S.-q. (2004). Oxidative stress inhibits osteoblastic differentiation of bone cells by ERK and NF-κB. *Biochemical and Biophysical Research Communications, 314*(1), 197-207.

Bakker, A., Kulkarni, R., Klein-Nulend, J., & Lems, W. (2014). IL-6 alters osteocyte signaling toward osteoblasts but not osteoclasts. *Journal of dental research, 93*(4), 394-399.

Baldus, S., Eiserich, J. P., Mani, A., Castro, L., Figueroa, M., Chumley, P., ... & Brennan, M. L. (2001). Endothelial transcytosis of myeloperoxidase confers specificity to vascular ECM proteins as targets of tyrosine nitration. *The Journal of clinical investigation, 108*(12), 1759-1770.

Banwart, J. C., Asher, M. A., & Hassanein, R. S. (1995). Iliac crest bone graft harvest donor site morbidity. A statistical evaluation. *Spine, 20*(9), 1055-1060.

Baroli, B. (2009). From natural bone grafts to tissue engineering therapeutics: Brainstorming on pharmaceutical formulative requirements and challenges. *Journal of Pharmaceutical Sciences, 98*(4), 1317-1375. doi: 10.1002/jps.21528

Bastian, O. W., Koenderman, L., Alblas, J., Leenen, L. P. H., & Blokhuis, T. J. (2016). Neutrophils contribute to fracture healing by synthesizing fibronectin+ extracellular matrix rapidly after injury. *Clinical Immunology, 164*, 78-84.

Bauer, T. W., & Muschler, G. F. (2000a). Bone graft materials: an overview of the basic science. *Clinical orthopaedics and related research, 371*, 10-27.

Baylink, D. J., Finkelman, R. D., & Mohan, S. (1993). Growth factors to stimulate bone formation. *Journal of Bone and Mineral Research, 8*(S2).

Benglis, D., Wang, M. Y., & Levi, A. D. (2008). A comprehensive review of the safety profile of bone morphogenetic protein in spine surgery. *Operative Neurosurgery, 62*(suppl_5), ONS423-ONS431.

Betz, R. R. (2002). Limitations of autograft and allograft: new synthetic solutions. *Orthopedics, 25*(5), S561-S570.

Bochi, G. V., Torbitz, V. D., de Campos, L. P., Sangoi, M. B., Fernandes, N. F., Gomes, P., . . . Moresco, R. N. (2016). In Vitro Oxidation of Collagen Promotes the Formation of Advanced Oxidation Protein Products and the Activation of Human Neutrophils. *Inflammation, 39*(2), 916-927.

Bodde, E. W. H., Wolke, J. G. C., Kowalski, R. S. Z., & Jansen, J. A. (2007). Bone regeneration of porous β-tricalcium phosphate (Conduit™ TCP) and of biphasic calcium phosphate ceramic (Biosel®) in trabecular defects in sheep. *Journal of Biomedical Materials Research Part A, 82A*(3), 711-722.

Boden, S. D. (2001). Clinical application of the BMPs. *JOURNAL OF BONE AND JOINT SURGERY-AMERICAN VOLUME-, 83*(2), 161-161.

Boden, S. D., & Schimandle, J. H. (1995). Biologic enhancement of spinal fusion. *Spine, 20*(24 Suppl), 113S-123S.

Bondurant, F. J., Cotler, H. B., Buckle, R., Miller-crotchett, P., & Browner, B. D. (1988). The medical and economic impact of severely injured lower extremities. *Journal of Trauma and Acute Care Surgery, 28*(8), 1270-1273.

Bonewald, L. F. (2011). The amazing osteocyte. *Journal of bone and mineral research, 26*(2), 229-238.

Borregaard, N., & Cowland, J. B. (1997). Granules of the human neutrophilic polymorphonuclear leukocyte. *Blood, 89*(10), 3503-3521.

Bosch, C., Melsen, B., & Vargervik, K. (1998). Importance of the critical-size bone defect in testing bone-regenerating materials. *The Journal of craniofacial surgery, 9*(4), 310-316.

Bostrom, M. P., & Seigerman, D. A. (2005). The clinical use of allografts, demineralized bone matrices, synthetic bone graft substitutes and osteoinductive growth factors: a survey study. *Hss Journal, 1*(1), 9-18.

Bougioukli, S., Jain, A., Sugiyama, O., Tinsley, B. A., Tang, A. H., Tan, M. H., . . . Lieberman, J. R. (2016). Combination therapy with BMP-2 and a systemic RANKL inhibitor enhances bone healing in a mouse critical-sized femoral defect. *Bone, 84*, 93-103.

Bouler, J. M., Pilet, P., Gauthier, O., & Verron, E. (2017). Biphasic calcium phosphate ceramics for bone reconstruction: A review of biological response. *Acta Biomaterialia, 53*, 1-12.

Boyce, T., Edwards, J., & Scarborough, N. (1999). Allograft bone: the influence of processing on safety and performance. *Orthopedic Clinics, 30*(4), 571-581.

Bozeman, P. M., Learn, D. B., & Thomas, E. L. (1990). Assay of the human leukocyte enzymes myeloperoxidase and eosinophil peroxidase. *Journal of Immunological Methods, 126*(1), 125-133.

Brits, D., Steyn, M., & Noelle, E. (2014). A histomorphological analysis of human and non-human femora. *International journal of legal medicine, 128*(2), 369-377.

Bruder, S. P., Fink, D. J., & Caplan, A. I. (1994). Mesenchymal stem cells in bone development, bone repair, and skeletal regenaration therapy. *Journal of cellular biochemistry, 56*(3), 283-294.

Bruder, S. P., Kurth, A. A., Shea, M., Hayes, W. C., Jaiswal, N., & Kadiyala, S. (1998). Bone regeneration by implantation of purified, culture-expanded human mesenchymal stem cells. *Journal of Orthopaedic Research, 16*(2), 155-162.

Bucay, N., Sarosi, I., Dunstan, C. R., Morony, S., Tarpley, J., Capparelli, C., . . . Lacey, D. L. (1998). Osteoprotegerin-deficient mice develop early onset osteoporosis and arterial calcification. *Genes & development, 12*(9), 1260-1268.

Burghardt, A. J., Kazakia, G. J., Laib, A., & Majumdar, S. (2008). Quantitative assessment of bone tissue mineralization with polychromatic micro-computed tomography. *Calcified tissue international, 83*(2), 129.

Canty, E. G., & Kadler, K. E. (2005). Procollagen trafficking, processing and fibrillogenesis. *Journal of cell science, 118*(7), 1341-1353.

Capulli, M., Paone, R., & Rucci, N. (2014). Osteoblast and osteocyte: games without frontiers. *Archives of biochemistry and biophysics, 561*, 3-12.

Carano, R. A., & Filvaroff, E. H. (2003). Angiogenesis and bone repair. *Drug discovery today, 8*(21), 980-989.

Carinci, F., Pezzetti, F., Spina, A. M., Palmieri, A., Laino, G., De Rosa, A., ... & Piattelli, A. (2005). Effect of Vitamin C on pre-osteoblast gene expression. *Archives of oral biology, 50*(5), 481-496.

Chen, Y., & Alman, B. A. (2009). Wnt pathway, an essential role in bone regeneration. *Journal of cellular biochemistry, 106*(3), 353-362.

Cho, S. W., Her, S. J., Sun, H. J., Choi, O. K., Yang, J.-Y., Kim, S. W., . . . Shin, C. S. (2008). Differential effects of secreted frizzled-related proteins (sFRPs) on osteoblastic differentiation of mouse mesenchymal cells and apoptosis of osteoblasts. *Biochemical and biophysical research communications, 367*(2), 399-405.

Cho, T. J., Gerstenfeld, L. C., & Einhorn, T. A. (2002). Differential temporal expression of members of the transforming growth factor β superfamily during murine fracture healing. *Journal of Bone and Mineral Research, 17*(3), 513-520.

Claes, L. (2017). Mechanobiology of fracture healing part 1: Principles. *Der Unfallchirurg, 120*(1), 14-22.

Convente, M. R., Wang, H., Pignolo, R. J., Kaplan, F. S., & Shore, E. M. (2015). The immunological contribution to heterotopic ossification disorders. *Current osteoporosis reports, 13*(2), 116-124.

Cooper, G. M., Mooney, M. P., Gosain, A. K., Campbell, P. G., Losee, J. E., & Huard, J. (2010). Testing the "critical-size" in calvarial bone defects: revisiting the concept of a critical-sized defect (CSD). *Plastic and reconstructive surgery, 125*(6), 1685.

Cowan, C. M., Shi, Y.-Y., Aalami, O. O., Chou, Y.-F., Mari, C., Thomas, R., . . . Longaker, M. T. (2004). Adipose-derived adult stromal cells heal critical-size mouse calvarial defects. *Nature biotechnology, 22*(5), 560.

Cowin, S. C. (1999). Bone poroelasticity. *Journal of biomechanics, 32*(3), 217-238.

Cox, G., Einhorn, T., Tzioupis, C., & Giannoudis, P. (2010). Bone-turnover markers in fracture healing. *Bone & Joint Journal, 92*(3), 329-334.

Curtin, C. M., Tierney, E. G., McSorley, K., Cryan, S. A., Duffy, G. P., & O'brien, F. J. (2015). Combinatorial Gene therapy accelerates bone regeneration: non-viral dual delivery of VEGF and BMP2 in a collagen-nanohydroxyapatite scaffold. *Advanced healthcare materials, 4*(2), 223-227.

Cypher, T. J., & Grossman, J. P. (1996). Biological principles of bone graft healing. *The Journal of foot and ankle surgery, 35*(5), 413-417.

Dallas, S. L., Prideaux, M., & Bonewald, L. F. (2013). The osteocyte: an endocrine cell… and more. *Endocrine reviews, 34*(5), 658-690.

Datta, H., Ng, W., Walker, J., Tuck, S., & Varanasi, S. (2008). The cell biology of bone metabolism. *Journal of clinical pathology, 61*(5), 577-587.

Davies, M. J., Hawkins, C. L., Pattison, D. I., & Rees, M. D. (2008). Mammalian heme peroxidases: from molecular mechanisms to health implications. *Antioxidants & redox signaling, 10*(7), 1199-1234.

Delany, A., Amling, M., Priemel, M., Howe, C., Baron, R., & Canalis, E. (2000). Osteopenia and decreased bone formation in osteonectin-deficient mice. *The Journal of clinical investigation, 105*(7), 915-923.

den Boer, F. C., Patka, P., Bakker, F. C., Wippermann, B. W., van Lingen, A., Vink, G. Q., . . . Haarman, H. J. T. M. (1999). New segmental long bone defect model in sheep: Quantitative analysis of healing with dual energy X-ray absorptiometry. *Journal of orthopaedic research, 17*(5), 654-660.

DeNichilo, M. O., Panagopoulos, V., Rayner, T. E., Borowicz, R. A., Greenwood, J. E., & Evdokiou, A. (2015). Peroxidase Enzymes Regulate Collagen Extracellular Matrix Biosynthesis. *The American Journal of Pathology, 185*(5), 1372-1384.

DeNichilo, M. O., Shoubridge, A. J., Panagopoulos, V., Liapis, V., Zysk, A., Zinonos, I., . . . Evdokiou, A. (2016). Peroxidase Enzymes Regulate Collagen Biosynthesis and Matrix Mineralization by Cultured Human Osteoblasts. *Calcified Tissue International, 98*(3), 294-305.

Dimitriou, R., Mataliotakis, G. I., Angoules, A. G., Kanakaris, N. K., & Giannoudis, P. V. (2011). Complications following autologous bone graft harvesting from the iliac crest and using the RIA: a systematic review. *Injury, 42*, S3-S15.

Dimitriou, R., Tsiridis, E., & Giannoudis, P. V. (2005). Current concepts of molecular aspects of bone healing. *Injury, 36*(12), 1392-1404.

Downey, P. A., & Siegel, M. I. (2006). Bone biology and the clinical implications for osteoporosis. *Physical therapy, 86*(1), 77-91.

Dragoo, J. L., Choi, J. Y., Lieberman, J. R., Huang, J., Zuk, P. A., Zhang, J., . . . Benhaim, P. (2003). Bone induction by BMP-2 transduced stem cells derived from human fat. *Journal of orthopaedic research, 21*(4), 622-629.

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

Duplomb, L., Baud'Huin, M., Charrier, C., Berreur, M., Trichet, V., Blanchard, F., & Heymann, D. (2008). Interleukin-6 inhibits receptor activator of nuclear factor κB ligand-induced osteoclastogenesis by diverting cells into the macrophage lineage: key role of serine727 phosphorylation of signal transducer and activator of transcription 3. *Endocrinology, 149*(7), 3688-3697.

Ebnezar, J., & John, R. (2016). *Textbook of Orthopedics*: JP Medical Ltd.

Ehrnberg, A., De Pablos, J., Martinez-Lotti, G., Kreicbergs, A., & Nilsson, O. (1993). Comparison of demineralized allogeneic bone matrix grafting (the Urist procedure) and the Ilizarov procedure in large diaphyseal defects in sheep. *Journal of Orthopaedic Research, 11*(3), 438-447.

Einhorn, T. A. (1998). The cell and molecular biology of fracture healing. *Clinical Orthopaedics and Related Research, 355*, S7-S21.

Eriksen, E. F. (2010). Cellular mechanisms of bone remodeling. *Reviews in Endocrine and Metabolic Disorders, 11*(4), 219-227.

Evans, K., Forsberg, J. A., Hawksworth, J. S., Tadaki, D. K., Brown, T. S., Dunne, J. R., & Elster, E. A. (2009). 224. Inflammatory Cytokine and Chemokine Expression is Associated With Heterotopic Ossification in High-Energy Penetrating War Injuries. *Journal of Surgical Research, 151*(2), 272.

Evans, K. N., Forsberg, J. A., Potter, B. K., Hawksworth, J. S., Brown, T. S., Andersen, R., ... & Elster, E. A. (2012). Inflammatory cytokine and chemokine expression is associated with heterotopic ossification in high-energy penetrating war injuries. *Journal of orthopaedic trauma, 26*(11), e204-e213.

Fakhry, M., Hamade, E., Badran, B., Buchet, R., & Magne, D. (2013). Molecular mechanisms of mesenchymal stem cell differentiation towards osteoblasts. *World journal of stem cells, 5*(4), 136.

Felson, D. T., Zhang, Y., Hannan, M. T., Kiel, D. P., Wilson, P., & Anderson, J. J. (1993). The effect of postmenopausal estrogen therapy on bone density in elderly women. *New England Journal of Medicine, 329*(16), 1141-1146.

Fillingham, Y., & Jacobs, J. (2016). Bone grafts and their substitutes. *Bone Joint J, 98*(1 Supple A), 6-9.

Finkemeier, C. G. (2002). Bone-grafting and bone-graft substitutes. *JBJS, 84*(3), 454-464.

Finkemeier, C. G., & Chapman, M. W. (2002). Treatment of femoral diaphyseal nonunions. *Clinical Orthopaedics and Related Research, 398*, 223-234.

Flierl, M. A., Smith, W. R., Mauffrey, C., Irgit, K., Williams, A. E., Ross, E., . . . Stahel, P. F. (2013). Outcomes and complication rates of different bone grafting modalities in long bone fracture nonunions: a retrospective cohort study in 182 patients. *Journal of orthopaedic surgery and research, 8*(1), 33.

Fragomen, A. T., & Rozbruch, S. R. (2007). The mechanics of external fixation. *HSS Journal, 3*(1), 13-29.

Franceschi, R. T., & Iyer, B. S. (1992). Relationship between collagen synthesis and expression of the osteoblast phenotype in MC3T3-E1 cells. *Journal of Bone and Mineral Research, 7*(2), 235-246.

Freeman, T. A., Parvizi, J., Della Valle, C. J., & Steinbeck, M. J. (2009). Reactive oxygen and nitrogen species induce protein and DNA modifications driving arthrofibrosis following total knee arthroplasty. *Fibrogenesis & Tissue Repair, 2*(1), 5.

Fridez, P. (1996). Modélisation de l'adaptation osseuse externe. *Physics Department, EPFL, Lausanne*.

Friedlaender, G. E., Perry, C. R., Cole, J. D., Cook, S. D., Cierny, G., Muschler, G. F., . . . Yin, S. (2001). Osteogenic protein-1 (bone morphogenetic protein-7) in the treatment of tibial nonunions: a prospective, randomized clinical trial comparing rhOP-1 with fresh bone autograft. *The Journal of bone and joint surgery. American volume, 83*(Pt 2), S151.

Friedrich, B., & Klaue, P. (1977). Mechanical stability and post-traumatic osteitis: an experimental evaluation of the relation between infection of bone and internal fixation. *Injury, 9*(1), 23-29.

Friess, W., Uludag, H., Foskett, S., Biron, R., & Sargeant, C. (1999). Characterization of absorbable collagen sponges as rhBMP-2 carriers. *International journal of pharmaceutics, 187*(1), 91-99.

Furtmüller, P. G., Zederbauer, M., Jantschko, W., Helm, J., Bogner, M., Jakopitsch, C., & Obinger, C. (2006). Active site structure and catalytic mechanisms of human peroxidases. *Archives of biochemistry and biophysics, 445*(2), 199-213.

Gamradt, S. C., & Lieberman, J. R. (2003). Bone graft for revision hip arthroplasty: biology and future applications. *Clinical orthopaedics and related research, 417*, 183-194.

Garrison, K. R., Donell, S., Ryder, J., Shemilt, I., Mugford, M., Harvey, I., & Song, F. (2007). Clinical effectiveness and cost-effectiveness of bone morphogenetic proteins in the non-healing of fractures and spinal fusion: a systematic review.

Gaston, M., & Simpson, A. (2007). Inhibition of fracture healing. *Bone & Joint Journal, 89*(12), 1553-1560.

Gay, S., & Miller, E. J. (1978). *Collagen in the physiology and pathology of connective tissue*: Fischer.

Gazdag, A. R., Lane, J. M., Glaser, D., & Forster, R. A. (1995). Alternatives to autogenous bone graft: efficacy and indications. *Journal of the American Academy of Orthopaedic Surgeons, 3*(1), 1-8.

Geiger, M., Li, R., & Friess, W. (2003). Collagen sponges for bone regeneration with rhBMP-2. *Advanced drug delivery reviews, 55*(12), 1613-1629.

Genestra, M. (2007). Oxyl radicals, redox-sensitive signalling cascades and antioxidants. *Cellular signalling, 19*(9), 1807-1819.

Gerald, D., Berra, E., Frapart, Y. M., Chan, D. A., Giaccia, A. J., Mansuy, D., ... & Mechta-Grigoriou, F. (2004). JunD reduces tumor angiogenesis by protecting cells from oxidative stress. *Cell, 118*(6), 781-794.

Gerstenfeld, L. C., Cullinane, D. M., Barnes, G. L., Graves, D. T., & Einhorn, T. A. (2003). Fracture healing as a post-natal developmental process: Molecular, spatial, and temporal aspects of its regulation. *Journal of cellular biochemistry, 88*(5), 873-884.

Giannoudis, P. V., Dinopoulos, H., & Tsiridis, E. (2005). Bone substitutes: an update. *Injury, 36*(3), S20-S27.

Gillikin, J. W., & Graham, J. S. (1991). Purification and developmental analysis of the major anionic peroxidase from the seed coat of Glycine max. *Plant physiology, 96*(1), 214-220.

Gittens, S. A., & Uludag, H. (2001). Growth factor delivery for bone tissue engineering. *Journal of drug targeting, 9*(6), 407-429.

Glimcher, M. J., & Lian, J. B. (1989). *The chemistry and biology of mineralized tissues: proceedings of the Third International Conference on the Chemistry and Biology of Mineralized Tissues, held in Chatham, Massachusetts on October 16-21, 1988* (Vol. 1): CRC Press.

Glynne Andrew, J., Andrew, S. M., Freemont, A. J., & Marsh, D. R. (1994). Inflammatory cells in normal human fracture healing. *Acta Orthopaedica Scandinavica, 65*(4), 462-466.

Gomes, P., & Fernandes, M. (2011). Rodent models in bone-related research: the relevance of calvarial defects in the assessment of bone regeneration strategies. *Laboratory animals, 45*(1), 14-24.

Goodwin, D. C., Grover, T. A., & Aust, S. D. (1997). Roles of efficient substrates in enhancement of peroxidase-catalyzed oxidations. *Biochemistry, 36*(1), 139-147.

Greenwald, A. S., Boden, S. D., Goldberg, V. M., Khan, Y., Laurencin, C. T., & Rosier, R. N. (2001). Bone-graft substitutes: facts, fictions, and applications. *JBJS, 83*(2_suppl_2), S98-103.

Grigoriadis, A. E., Heersche, J., & Aubin, J. E. (1988). Differentiation of muscle, fat, cartilage, and bone from progenitor cells present in a bone-derived clonal cell population: effect of dexamethasone. *The Journal of cell biology, 106*(6), 2139-2151.

Grundnes, O., & Reikerås, O. (1993). The importance of the hematoma for fracture healing in rats. *Acta Orthopaedica Scandinavica, 64*(3), 340-342.

Gunzburg, R., & Szpalski, M. (2004). Biology of Bone Grafting: Autograft and Allograft. *The Lumbar Spine*, 247.

Ha, H., Kwak, H. B., Lee, S. W., Jin, H. M., Kim, H.-M., Kim, H.-H., & Lee, Z. H. (2004). Reactive oxygen species mediate RANK signaling in osteoclasts. *Experimental cell research, 301*(2), 119-127.

Hak, D. J. (2007). The use of osteoconductive bone graft substitutes in orthopaedic trauma. *Journal of the American Academy of Orthopaedic Surgeons, 15*(9), 525-536.

Hall, M. C. (1963). Fractures. *Canadian Medical Association Journal, 89*(6), 255.

Harris, N. L., Rattray, K. R., Tye, C. E., Underhill, T. M., Somerman, M. J., D'errico, J. A., ... & Goldberg, H. A. (2000). Functional analysis of bone sialoprotein: identification of the hydroxyapatite-nucleating and cell-binding domains by recombinant peptide expression and site-directed mutagenesis. *Bone*, *27*(6), 795-802.

Haynesworth, S. E., Reuben, D., & Caplan, A. I. (1998). Cell-based tissue engineering therapies: the influence of whole body physiology. *Advanced drug delivery reviews, 33*(1-2), 3-14.

Hedlund, R., & Lindgren, U. (1987). Trauma type, age, and gender as determinants of hip fracture. *Journal of Orthopaedic Research, 5*(2), 242-246.

Henriksen, A., Mirza, O., Indiani, C., Teilum, K., Smulevich, G., Welinder, K. G., & Gajhede, M. (2001). Structure of soybean seed coat peroxidase: A plant peroxidase with unusual stability and haem-apoprotein interactions. *Protein Science, 10*(1), 108-115.

Hiraga, S., Sasaki, K., Ito, H., Ohashi, Y., & Matsui, H. (2001). A large family of class III plant peroxidases. *Plant and Cell Physiology*, *42*(5), 462-468.

Hollinger, J. O., & Kleinschmidt, J. C. (1990). The critical size defect as an experimental model to test bone repair materials. *Journal of Craniofacial Surgery*, *1*(1), 60-68.

Homma, Y., Zimmermann, G., & Hernigou, P. (2013). Cellular therapies for the treatment of non-union: the past, present and future. *Injury*, *44*, S46-S49.

Huang, D. W., Sherman, B. T., & Lempicki, R. A. (2009a). Bioinformatics enrichment tools: paths toward the comprehensive functional analysis of large gene lists. *Nucleic acids research, 37*(1), 1-13.

Huang, D. W., Sherman, B. T., & Lempicki, R. A. (2009b). Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. *Nature protocols*, *4*(1), 44-57.

Im, J.-Y., Min, W.-K., You, C., Kim, H.-O., Jin, H.-K., & Bae, J.-s. (2013). Bone regeneration of mouse critical-sized calvarial defects with human mesenchymal stem cells in scaffold. *Laboratory animal research, 29*(4), 196-203.

Jimi, E., Hirata, S., Osawa, K., Terashita, M., Kitamura, C., & Fukushima, H. (2012). The current and future therapies of bone regeneration to repair bone defects. *International journal of dentistry, 2012*.

Kan, L., & Kessler, J. A. (2010). Animal models of typical heterotopic ossification. *BioMed Research International, 2011*.

Kanakaris, N. K., & Giannoudis, P. V. (2007). The health economics of the treatment of long-bone non-unions. *Injury, 38*, S77-S84.

Karst, M., Gorny, G., Galvin, R. J. S., & Oursler, M. J. (2004). Roles of stromal cell RANKL, OPG, and M-CSF expression in biphasic TGF-β regulation of osteoclast differentiation. *Journal of cellular physiology, 200*(1), 99-106.

Keramaris, N., Calori, G., Nikolaou, V., Schemitsch, E., & Giannoudis, P. (2008). Fracture vascularity and bone healing: a systematic review of the role of VEGF. *Injury, 39*, S45-S57.

Kettle, A. J., Gedye, C. A., & Winterbourn, C. C. (1997). Mechanism of inactivation of myeloperoxidase by 4-aminobenzoic acid hydrazide. *Biochemical Journal, 321*(2), 503-508.

Khan, S. N., & Lane, J. M. (2004). The use of recombinant human bone morphogenetic protein-2 (rhBMP-2) in orthopaedic applications. *Expert opinion on biological therapy, 4*(5), 741-748.

Kikuchi, M., Matsumoto, H. N., Yamada, T., Koyama, Y., Takakuda, K., & Tanaka, J. (2004). Glutaraldehyde cross-linked hydroxyapatite/collagen self-organized nanocomposites. *Biomaterials, 25*(1), 63-69.

Kirker-Head, C., Karageorgiou, V., Hofmann, S., Fajardo, R., Betz, O., Merkle, H., . . . Abrahamsen, L. (2007). BMP-silk composite matrices heal critically sized femoral defects. *Bone, 41*(2), 247-255.

Kitaori, T., Ito, H., Schwarz, E. M., Tsutsumi, R., Yoshitomi, H., Oishi, S., . . . Nakamura, T. (2009). Stromal cell–derived factor 1/CXCR4 signaling is critical for the recruitment of mesenchymal stem cells to the fracture site during skeletal repair in a mouse model. *Arthritis & Rheumatology, 60*(3), 813-823.

Kivirikko, K. I., Myllylä, R., & Pihlajaniemi, T. (1989). Protein hydroxylation: prolyl 4-hydroxylase, an enzyme with four cosubstrates and a multifunctional subunit. *The FASEB Journal, 3*(5), 1609-1617.

Kivrak, N., & Taş, A. C. (1998). Synthesis of calcium hydroxyapatite-tricalcium phosphate (HA-TCP) composite bioceramic powders and their sintering behavior. *Journal of the American Ceramic Society, 81*(9), 2245-2252.

Klebanoff, S. J. (2005). Myeloperoxidase: friend and foe. *Journal of leukocyte biology, 77*(5), 598-625.

Kloen, P., Di Paola, M., Borens, O., Richmond, J., Perino, G., Helfet, D., & Goumans, M. (2003). BMP signaling components are expressed in human fracture callus. *Bone, 33*(3), 362-371.

Koch, A. E., Volin, M. V., Woods, J. M., Kunkel, S. L., Connors, M. A., Harlow, L. A., . . . Strieter, R. M. (2001). Regulation of angiogenesis by the C-X-C chemokines interleukin-8 and epithelial neutrophil activating peptide 78 in the rheumatoid joint. *Arthritis & Rheumatology, 44*(1), 31-40.

Kolar, P., Schmidt-Bleek, K., Schell, H., Gaber, T., Toben, D., Schmidmaier, G., ... & Duda, G. N. (2010). The early fracture hematoma and its potential role in fracture healing. *Tissue Engineering Part B: Reviews, 16*(4), 427-434.

Kon, T., Cho, T. J., Aizawa, T., Yamazaki, M., Nooh, N., Graves, D., . . . Einhorn, T. A. (2001). Expression of Osteoprotegerin, Receptor Activator of NF-κB Ligand (Osteoprotegerin Ligand) and Related Proinflammatory Cytokines During Fracture Healing. *Journal of Bone and Mineral Research, 16*(6), 1004-1014.

Kraft, C. T., Agarwal, S., Ranganathan, K., Wong, V. W., Loder, S., Li, J., . . . Levi, B. (2016). Trauma induced heterotopic bone formation and the role of the immune system: a review. *The journal of trauma and acute care surgery, 80*(1), 156.

Kubin, A., Furtmuller, P., Wolber, G. & Schuster, D. (2011). Compounds for use in the treatment of diseases. Google Patents.

Lane, J. M., & Sandhu, H. (1987). Current approaches to experimental bone grafting. *The Orthopedic clinics of North America, 18*(2), 213-225.

LaStayo, P. C., Winters, K. M., & Hardy, M. (2003). Fracture healing: Bone healing, fracture management, and current concepts related to the hand. *Journal of Hand Therapy, 16*(2), 81-93.

Laurencin, C., Khan, Y., & El-Amin, S. F. (2006). Bone graft substitutes. *Expert review of medical devices, 3*(1), 49-57.

Lawton, D., Andrew, J., Marsh, D., Hoyland, J., & Freemont, A. (1997). Mature osteoblasts in human non-union fractures express collagen type III. *Molecular Pathology, 50*(4), 194.

Lean, J. M., Jagger, C. J., Kirstein, B., Fuller, K., & Chambers, T. J. (2005). Hydrogen peroxide is essential for estrogen-deficiency bone loss and osteoclast formation. *Endocrinology, 146*(2), 728-735.

Lecanda, F., Avioli, L. V., & Cheng, S. L. (1997). Regulation of bone matrix protein expression and induction of differentiation of human osteoblasts and human bone marrow stromal cells by bone morphogenetic protein-2. *Journal of cellular biochemistry, 67*(3), 386-398.

Lee, N. K., Choi, Y. G., Baik, J. Y., Han, S. Y., Jeong, D.-w., Bae, Y. S., . . . Lee, S. Y. (2005). A crucial role for reactive oxygen species in RANKL-induced osteoclast differentiation. *Blood, 106*(3), 852-859.

LeGeros, R. Z. (2002). Properties of osteoconductive biomaterials: calcium phosphates. *Clinical orthopaedics and related research, 395*, 81-98.

Levi, B., James, A. W., Nelson, E. R., Vistnes, D., Wu, B., Lee, M., . . . Longaker, M. T. (2010). Human adipose derived stromal cells heal critical size mouse calvarial defects. *PloS one, 5*(6), e11177.

Levi-Schaffer, F., Garbuzenko, E., Rubin, A., Reich, R., Pickholz, D., Gillery, P., ... & Maquart, F. A. X. (1999). Human eosinophils regulate human lung-and skin-derived fibroblast properties in vitro: a role for transforming growth factor β (TGF-β). *Proceedings of the National Academy of Sciences, 96*(17), 9660-9665.

Li, Y., Chen, S.-K., Li, L., Qin, L., Wang, X.-L., & Lai, Y.-X. (2015). Bone defect animal models for testing efficacy of bone substitute biomaterials. *Journal of Orthopaedic Translation, 3*(3), 95-104.

Lim, J., Lee, J., Yun, H.-S., Shin, H.-I., & Park, E. K. (2013). Comparison of bone regeneration rate in flat and long bone defects: calvarial and tibial bone. *Tissue Engineering and Regenerative Medicine, 10*(6), 336-340.

Lind, M., Eriksen, E., & Bünger, C. (1996). Bone morphogenetic protein-2 but not bone morphogenetic protein-4 and-6 stimulates chemotactic migration of human osteoblasts, human marrow osteoblasts, and U2-OS cells. *Bone, 18*(1), 53-57.

Linkhart, T. A., Mohan, S., & Baylink, D. J. (1996). Growth factors for bone growth and repair: IGF, TGFβ and BMP. *Bone, 19*(1, Supplement 1), S1-S12.

Liu, A. L., Zhang, Z.-M., Zhu, B.-F., Liao, Z.-H., & Liu, Z. (2004). Metallothionein protects bone marrow stromal cells against hydrogen peroxide-induced inhibition of osteoblastic differentiation. *Cell biology international, 28*(12), 905-911.

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

Macias, M. P., Fitzpatrick, L. A., Brenneise, I., McGarry, M. P., Lee, J. J., & Lee, N. A. (2001). Expression of IL-5 alters bone metabolism and induces ossification of the spleen in transgenic mice. *The Journal of clinical investigation, 107*(8), 949-959.

Maehata, Y., Takamizawa, S., Ozawa, S., Izukuri, K., Kato, Y., Sato, S., ... & Hata, R. I. (2007). Type III collagen is essential for growth acceleration of human osteoblastic cells by ascorbic acid 2-phosphate, a long-acting vitamin C derivative. *Matrix Biology, 26*(5), 371-381.

Mehta, V., Campeau, N. G., Kita, H., & Hagan, J. B. (2008, June). Blood and sputum eosinophil levels in asthma and their relationship to sinus computed tomographic findings. In *Mayo Clinic Proceedings* (Vol. 83, No. 6, pp. 671-678). Elsevier.

Malhotra, A., Pelletier, M. H., Yu, Y., Christou, C., & Walsh, W. R. (2014). A sheep model for cancellous bone healing. *Frontiers in surgery, 1*, 37.

Marsell, R., & Einhorn, T. A. (2009). The role of endogenous bone morphogenetic proteins in normal skeletal repair. *Injury, 40*, S4-S7.

Martin, R. B., Burr, D. B., & Sharkey, N. A. (1998). *Skeletal tissue mechanics* (Vol. 190): Springer.

Martin, T. J., & Sims, N. A. (2005). Osteoclast-derived activity in the coupling of bone formation to resorption. *Trends in molecular medicine, 11*(2), 76-81.

Martini, L., Fini, M., Giavaresi, G., & Giardino, R. (2001). Sheep model in orthopedic research: a literature review. *Comparative medicine, 51*(4), 292-299.

Martiniaková, M., Grosskopf, B., Vondráková, M., Omelka, R., & Fabiš, M. (2005). Observation of the microstructure of rat cortical bone tissue.

Matsuo, K., & Irie, N. (2008). Osteoclast–osteoblast communication. *Archives of biochemistry and biophysics, 473*(2), 201-209.

McEldoon, J. P., & Dordick, J. S. (1996). Unusual thermal stability of soybean peroxidase. *Biotechnology progress, 12*(4), 555-558.

McKibbin, B. (1978). The biology of fracture healing in long bones. *J Bone Joint Surg Br, 60-b*(2), 150-162.

Megas, P., Panagiotopoulos, E., Skriviliotakis, S., & Lambiris, E. (2001). Intramedullary nailing in the treatment of aseptic tibial nonunion. *Injury, 32*(3), 233-239.

Mehta, V., Campeau, N. G., Kita, H., & Hagan, J. B. (2008). *Blood and sputum eosinophil levels in asthma and their relationship to sinus computed tomographic findings.* Paper presented at the Mayo Clinic Proceedings.

Meyer, S., Weiland, A., & Willenegger, H. (1975). The treatment of infected non-union of fractures of long bones. Study of sixty-four cases with a five to twenty-one-year follow-up. *The Journal of bone and joint surgery. American volume, 57*(6), 836-842.

Mikuni- Takagaki, Y., Kakai, Y., Satoyoshi, M., Kawano, E., Suzuki, Y., Kawase, T., & Saito, S. (1995). Matrix mineralization and the differentiation of osteocyte- like cells in culture. Journal of Bone and Mineral Research, 10(2), 231-242.

Milano, G., Passino, E. S., Deriu, L., Careddu, G., Manunta, L., Manunta, A., . . . Fabbriciani, C. (2010). The effect of platelet rich plasma combined with microfractures on the treatment of chondral defects: an experimental study in a sheep model. *Osteoarthritis and Cartilage, 18*(7), 971-980.

Mizuno, M., Fujisawa, R., & Kuboki, Y. (2000). Type I collagen-induced osteoblastic differentiation of bone-marrow cells mediated by collagen-α2β1 integrin interaction. *Journal of cellular physiology, 184*(2), 207-213.

Mody, N., Parhami, F., Sarafian, T. A., & Demer, L. L. (2001). Oxidative stress modulates osteoblastic differentiation of vascular and bone cells. *Free Radical Biology and Medicine, 31*(4), 509-519. doi: http://dx.doi.org/10.1016/S0891-5849(01)00610-4

Mountziaris, P. M., Spicer, P. P., Kasper, F. K., & Mikos, A. G. (2011). Harnessing and modulating inflammation in strategies for bone regeneration. *Tissue Engineering Part B: Reviews, 17*(6), 393-402.

Moxham, J. P., Kibblewhite, D. J., Dvorak, M., Perey, B., Tencer, A. F., Bruce, A. G., & Strong, D. M. (1996). TGF-beta 1 forms functionally normal bone in a segmental sheep tibial diaphyseal defect. *J Otolaryngol, 25*(6), 388-392.

Murnaghan, M., Li, G., & Marsh, D. R. (2006). Nonsteroidal anti-inflammatory drug-induced fracture nonunion: an inhibition of angiogenesis? *JBJS, 88*, 140-147.

Mussano, F., Ciccone, G., Ceccarelli, M., Baldi, I., & Bassi, F. (2007). Bone morphogenetic proteins and bone defects: a systematic review. *Spine, 32*(7), 824-830.

Myllyharju, J., & Kivirikko, K. I. (2001). Collagens and collagen-related diseases. *Annals of medicine, 33*(1), 7-21.

Nafei, A., Danielsen, C., Linde, F., & Hvid, I. (2000). Properties of growing trabecular ovine bone: Part I: mechanical and physical properties. *Bone & Joint Journal, 82*(6), 910-920.

Nakamura, Y., Tensho, K., Nakaya, H., Nawata, M., Okabe, T., & Wakitani, S. (2005). Low dose fibroblast growth factor-2 (FGF-2) enhances bone morphogenetic protein-2 (BMP-2)-induced ectopic bone formation in mice. *Bone, 36*(3), 399-407.

Netter, F. H. (1990). *Musculoskeletal system: anatomy, physiology, and metabolic disorders*: CIBA Medical Education Division.

Newman, E., Turner, A., & Wark, J. (1995). The potential of sheep for the study of osteopenia: current status and comparison with other animal models. *Bone, 16*(4), S277-S284.

O'Brien, P. J. (2000). Peroxidases. *Chemico-biological interactions, 129*(1-2), 113-139.

Obinger, C. (2006). Chemistry and biology of human peroxidases: Academic Press.

Okamoto, M., Udagawa, N., Uehara, S., Maeda, K., Yamashita, T., Nakamichi, Y., ... & Kobayashi, Y. (2014). Noncanonical Wnt5a enhances Wnt/β-catenin signaling during osteoblastogenesis. *Scientific reports, 4*, srep04493.

Oklund, S. A., Prolo, D. J., Gutierrez, R. V., & King, S. E. (1986). Quantitative comparisons of healing in cranial fresh autografts, frozen autografts and processed autografts, and allografts in canine skull defects. *Clinical orthopaedics and related research*(205), 269-291.

Ono, I., Gunji, H., Kaneko, F., Sait, T., & Kuboki, Y. (1995). Efficacy of hydroxyapatite ceramic as a carrier for recombinant human bone morphogenetic protein. *Journal of Craniofacial Surgery, 6*(3), 238-244.

Ontañón, M., Aparicio, C., Ginebra, M., & Planell, J. (2000). Structure and mechanical properties of cortical bone *Pergamon Materials Series* (Vol. 4, pp. 33-71): Elsevier.

Ortega, N., Behonick, D. J., & Werb, Z. (2004). Matrix remodeling during endochondral ossification. *Trends in cell biology, 14*(2), 86-93.

Oryan, A., Alidadi, S., Moshiri, A., & Maffulli, N. (2014). Bone regenerative medicine: classic options, novel strategies, and future directions. *Journal of orthopaedic surgery and research, 9*(1), 18.

Ozgocmen, S., Kaya, H., Fadillioglu, E., Aydogan, R., & Yilmaz, Z. (2007). Role of antioxidant systems, lipid peroxidation, and nitric oxide in postmenopausal osteoporosis. *Molecular and cellular biochemistry, 295*(1-2), 45-52.

Pagé, E. L., Chan, D. A., Giaccia, A. J., Levine, M., & Richard, D. E. (2008). Hypoxia-inducible factor-1α stabilization in nonhypoxic conditions: role of oxidation and intracellular ascorbate depletion. *Molecular biology of the cell, 19*(1), 86-94.

Page, M., Hogg, J., & Ashhurst, D. E. (1986). The effects of mechanical stability on the macromolecules of the connective tissue matrices produced during fracture healing. I. The collagens. *The Histochemical Journal, 18*(5), 251-265.

Panagiotis, M. (2005). Classification of non-union. *Injury, 36*(4, Supplement), S30-S37.

Panagopoulos, V., Liapis, V., Zinonos, I., Hay, S., Leach, D. A., Ingman, W., . . . Zannettino, A. C. (2017). Peroxidase enzymes inhibit osteoclast differentiation and bone resorption. *Molecular and cellular endocrinology, 440*, 8-15.

Panagopoulos, V., Zinonos, I., Leach, D. A., Hay, S. J., Liapis, V., Zysk, A., . . . Evdokiou, A. (2015). Uncovering a new role for peroxidase enzymes as drivers of angiogenesis. *The international journal of biochemistry & cell biology, 68*, 128-138.

Pape, H.-C., Marcucio, R., Humphrey, C., Colnot, C., Knobe, M., & Harvey, E. J. (2010). Trauma-induced inflammation and fracture healing. *Journal of orthopaedic trauma, 24*(9), 522-525.

Paredes, J., & Weiss, S. (1982). Human neutrophils transform prostaglandins by a myeloperoxidase-dependent mechanism. *Journal of Biological Chemistry, 257*(6), 2738-2740.

Park, S.-H., Silva, M., Bahk, W.-J., McKellop, H., & Lieberman, J. R. (2002). Effect of repeated irrigation and debridement on fracture healing in an animal model. *Journal of Orthopaedic Research, 20*(6), 1197-1204.

Patel, N., Brooks, R., Clarke, M., Lee, P., Rushton, N., Gibson, I. R., . . . Bonfield, W. (2005). In vivo assessment of hydroxyapatite and silicate-substituted hydroxyapatite granules using an ovine defect model. *Journal of Materials Science: Materials in Medicine, 16*(5), 429-440.

Patlolla, A., & Arinzeh, T. L. (2014). Evaluating apatite formation and osteogenic activity of electrospun composites for bone tissue engineering. *Biotechnology and bioengineering, 111*(5), 1000-1017.

Pearce, A., Richards, R., Milz, S., Schneider, E., & Pearce, S. (2007). Animal models for implant biomaterial research in bone: a review. *Eur Cell Mater, 13*(1), 1-10.

Peng, H., Usas, A., Olshanski, A., Ho, A. M., Gearhart, B., Cooper, G. M., & Huard, J. (2005). VEGF Improves, Whereas sFlt1 Inhibits, BMP2-Induced Bone Formation and Bone

Healing Through Modulation of Angiogenesis. *Journal of Bone and Mineral Research, 20*(11), 2017-2027.

Pereira, I., Rodrigues, C., Rodrigues, A., Oliveira, M., & Gama, M. (2017). Injectable hydrogels as a delivery system for bone regeneration *Bioinspired Materials for Medical Applications* (pp. 241-271): Elsevier.

Perry, C. R. (1999). Bone repair techniques, bone graft, and bone graft substitutes. *Clinical orthopaedics and related research, 360*, 71-86.

Peterkofsky, B. (1991). Ascorbate requirement for hydroxylation and secretion of procollagen: relationship to inhibition of collagen synthesis in scurvy. *The American journal of clinical nutrition, 54*(6), 1135S-1140S.

Peters, C. L., Hines, J. L., Bachus, K. N., Craig, M. A., & Bloebaum, R. D. (2006). Biological effects of calcium sulfate as a bone graft substitute in ovine metaphyseal defects. *Journal of Biomedical Materials Research Part A, 76A*(3), 456-462.

Phieffer, L. S., & Goulet, J. A. (2006). Delayed unions of the tibia. *JBJS, 88*(1), 205-216.

Phimphilai, M., Zhao, Z., Boules, H., Roca, H., & Franceschi, R. T. (2006). BMP signaling is required for RUNX2-dependent induction of the osteoblast phenotype. *J Bone Miner Res, 21*(4), 637-646.

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

Pobloth, A.-M., Johnson, K. A., Schell, H., Kolarczik, N., Wulsten, D., Duda, G. N., & Schmidt-Bleek, K. (2016). Establishment of a preclinical ovine screening model for the investigation of bone tissue engineering strategies in cancellous and cortical bone defects. *BMC Musculoskeletal Disorders, 17*(1), 111.

Pountos, I., Georgouli, T., Calori, G. M., & Giannoudis, P. V. (2012). Do Nonsteroidal Anti-Inflammatory Drugs Affect Bone Healing? A Critical Analysis. *The Scientific World Journal, 2012*, 606404.

Prasad, G., & Udupa, K. (1972). Studies on ultrastructural pattern of osteogenic cells during bone repair. *Acta Orthopaedica Scandinavica, 43*(3), 163-175.

Ramp, W. K., Arnold, R. R., Russell, J. E., & Yancey, J. M. (1987). Hydrogen peroxide inhibits glucose metabolism and collagen synthesis in bone. *Journal of periodontology, 58*(5), 340-344.

Ranganathan, K., Loder, S., Agarwal, S., Wong, V. W., Forsberg, J., Davis, T. A., . . . Levi, B. (2015). Heterotopic ossification: basic-science principles and clinical correlates. *JBJS, 97*(13), 1101-1111.

Rao, C., Mohanam, S., Puppala, A., & Rao, J. S. (1999). Regulation of ProMMP-1 and ProMMP-3 activation by tissue factor pathway inhibitor-2/matrix-associated serine protease inhibitor. *Biochemical and biophysical research communications, 255*(1), 94-98.

Rayner, T. E., Cowin, A. J., Robertson, J. G., Cooter, R. D., Harries, R. C., Regester, G. O., . . . Belford, D. A. (2000). Mitogenic whey extract stimulates wound repair activity in vitro and promotes healing of rat incisional wounds. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology, 278*(6), R1651-R1660.

Rawadi, G., Vayssière, B., Dunn, F., Baron, R., & Roman-Roman, S. (2003). BMP-2 controls alkaline phosphatase expression and osteoblast mineralization by a Wnt autocrine loop. Journal of Bone and Mineral Research, 18(10), 1842-1853.

Reichert, J. C., Saifzadeh, S., Wullschleger, M. E., Epari, D. R., Schütz, M. A., Duda, G. N., . . . Hutmacher, D. W. (2009). The challenge of establishing preclinical models for segmental bone defect research. *Biomaterials, 30*(12), 2149-2163.

Richelson, L. S., Wahner, H. W., Melton III, L., & Riggs, B. L. (1984). Relative contributions of aging and estrogen deficiency to postmenopausal bone loss. *New England Journal of Medicine, 311*(20), 1273-1275.

Ring, D., Barrick, W. T., & Jupiter, J. B. (1997). Recalcitrant nonunion. *Clinical orthopaedics and related research, 340*, 181-189.

Ristiniemi, J., Flinkkilä, T., Hyvönen, P., Lakovaara, M., Pakarinen, H., & Jalovaara, P. (2007). RhBMP-7 accelerates the healing in distal tibial fractures treated by external fixation. *Bone & Joint Journal, 89*(2), 265-272.

Robling, A. G., Castillo, A. B., & Turner, C. H. (2006). Biomechanical and molecular regulation of bone remodeling. *Annu. Rev. Biomed. Eng., 8*, 455-498.

Robubi, A., Berger, C., Schmid, M., Huber, K., Engel, A., & Krugluger, W. (2014). Gene expression profiles induced by growth factors in in vitro cultured osteoblasts. *Bone and Joint Research, 3*(7), 236-240.

Rodriguez-Merchan, E. C., & Forriol, F. (2004). Nonunion: general principles and experimental data. *Clinical orthopaedics and related research, 419*, 4-12.

Rosen, V. (2009). BMP2 signaling in bone development and repair. *Cytokine & growth factor reviews, 20*(5-6), 475-480.

Ruiz-Gaspa, S., Nogues, X., Enjuanes, A., Monllau, J. C., Blanch, J., Carreras, R., . . . Díez-Perez, A. (2007). Simvastatin and atorvastatin enhance gene expression of collagen type 1 and osteocalcin in primary human osteoblasts and MG-63 cultures. *Journal of cellular biochemistry, 101*(6), 1430-1438.

Samartzis, D., Shen, F. H., Goldberg, E. J., & An, H. S. (2005). Is autograft the gold standard in achieving radiographic fusion in one-level anterior cervical discectomy and fusion with rigid anterior plate fixation? *Spine, 30*(15), 1756-1761.

Sanvitale, C. E., Kerr, G., Chaikuad, A., Ramel, M.-C., Mohedas, A. H., Reichert, S., . . . Paul, B. Y. (2013). A new class of small molecule inhibitor of BMP signaling. *PloS one, 8*(4), e62721.

Scammell, B. E., & Roach, H. I. (1996). A new role for the chondrocyte in fracture repair: endochondral ossification includes direct bone formation by former chondrocytes. *Journal of Bone and Mineral Research, 11*(6), 737-745.

Schenk, R., Müller, J., & Willenegger, H. (1968). Experimental histological contribution to the development and treatment of pseudarthrosis. *Hefte zur Unfallheilkunde, 94*, 15-24.

Schmidmaier, G., Schwabe, P., Strobel, C., & Wildemann, B. (2008). Carrier systems and application of growth factors in orthopaedics. *Injury, 39*, S37-S43.

Schmitz, J. P., & Hollinger, J. O. (1986). The critical size defect as an experimental model for craniomandibulofacial nonunions. *Clinical orthopaedics and related research*(205), 299-308.

Schmitz, J. P., Schwartz, Z., Hollinger, J. O., & Boyan, B. D. (1990). Characterization of rat calvarial nonunion defects. *Cells Tissues Organs, 138*(3), 185-192.

Seeherman, H. J., Li, X. J., Bouxsein, M. L., & Wozney, J. M. (2010). rhBMP-2 induces transient bone resorption followed by bone formation in a nonhuman primate core-defect model. *The Journal of Bone & Joint Surgery, 92*(2), 411-426.

Sempuku, T., Ohgushia, H., Okumur, M., & Tamai, S. (1996). Osteogenic potential of allogeneic rat marrow cells in porous hydroxyapatite ceramics: a histological study. *Journal of Orthopaedic Research, 14*(6), 907-913.

Sfeir, C., Ho, L., Doll, B. A., Azari, K., & Hollinger, J. O. (2005). Fracture repair *Bone regeneration and repair* (pp. 21-44): Springer.

Simmons, C. A., Alsberg, E., Hsiong, S., Kim, W. J., & Mooney, D. J. (2004). Dual growth factor delivery and controlled scaffold degradation enhance in vivo bone formation by transplanted bone marrow stromal cells. *Bone, 35*(2), 562-569.

Sims, N. A., & Gooi, J. H. (2008). *Bone remodeling: Multiple cellular interactions required for coupling of bone formation and resorption.* Paper presented at the Seminars in cell & developmental biology.

Smith, J. (1974). Results of Early and Delayed Internal Fixation for Tibial Shaff Fractures: A Review of 470 Fractures. *J Bone Joint Surg Br, 56*(3), 469-477.

Snidvongs, K., McLachlan, R., Chin, D., Pratt, E., Sacks, R., Earls, P., & Harvey, R. J. (2012). Osteitic bone: a surrogate marker of eosinophilia in chronic rhinosinusitis. *Rhinology, 50*(3), 299-305.

Søballe, K., Hansen, E. S., Brockstedt-Rasmussen, H., Pedersen, C. M., & Bünger, C. (1990). Hydroxyapatite coating enhances fixation of porous coated implants: a comparison in dogs between press fit and noninterference fit. *Acta Orthopaedica Scandinavica, 61*(4), 299-306.

Sofia, S. J., Singh, A., & Kaplan, D. L. (2002). Peroxidase-catalyzed crosslinking of functionalized polyaspartic acid polymers. *Journal of Macromolecular Science, Part A, 39*(10), 1151-1181.

Spiller, K. L., Anfang, R. R., Spiller, K. J., Ng, J., Nakazawa, K. R., Daulton, J. W., & Vunjak-Novakovic, G. (2014). The role of macrophage phenotype in vascularization of tissue engineering scaffolds. *Biomaterials, 35*(15), 4477-4488.

Street, J., Bao, M., Bunting, S., Peale, F. V., Ferrara, N., Steinmetz, H., . . . van Bruggen, N. (2002). Vascular endothelial growth factor stimulates bone repair by promoting angiogenesis and bone turnover. *Proceedings of the National Academy of Sciences, 99*(15), 9656-9661.

Strotmeyer, E. S., & Cauley, J. A. (2007). Diabetes mellitus, bone mineral density, and fracture risk. *Current Opinion in Endocrinology, Diabetes and Obesity, 14*(6), 429-435.

Sugimoto, M., Hirota, S., Sato, M., Kawahata, H., Tsukamoto, I., Yasui, N., . . . Nomura, S. (1998). Impaired Expression of Noncollagenous Bone Matrix Protein mRNAs During Fracture Healing in Ascorbic Acid–Deficient Rats. *Journal of Bone and Mineral Research, 13*(2), 271-278.

Szpalski, C., Nguyen, P. D., Vasiliu, C. E. C., Chesnoiu-Matei, I., Ricci, J. L., Clark, E., . . . Warren, S. M. (2012). Bony engineering using time-release porous scaffolds to provide sustained growth factor delivery. *Journal of Craniofacial Surgery, 23*(3), 638-644.

Tahboub, Y. R., Galijasevic, S., Diamond, M. P., & Abu-Soud, H. M. (2005). Thiocyanate modulates the catalytic activity of mammalian peroxidases. *Journal of Biological Chemistry, 280*(28), 26129-26136.

Takada, I., Kouzmenko, A. P., & Kato, S. (2009). Wnt and PPARγ signaling in osteoblastogenesis and adipogenesis. *Nature Reviews Rheumatology, 5*(8), 442-447.

Tang, S., Zeenath, U., & Vashishth, D. (2007). Effects of non-enzymatic glycation on cancellous bone fragility. *Bone, 40*(4), 1144-1151.

Tannoury, C. A., & An, H. S. (2014). Complications with the use of bone morphogenetic protein 2 (BMP-2) in spine surgery. *The Spine Journal, 14*(3), 552-559.

Taylor, W. R., Ehrig, R. M., Heller, M. O., Schell, H., Seebeck, P., & Duda, G. N. (2006). Tibio-femoral joint contact forces in sheep. *Journal of biomechanics, 39*(5), 791-798.

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

Theiss, F., Apelt, D., Brand, B., Kutter, A., Zlinszky, K., Bohner, M., . . . von Rechenberg, B. (2005). Biocompatibility and resorption of a brushite calcium phosphate cement. *Biomaterials, 26*(21), 4383-4394.

Thomas, M., & Puleo, D. (2011). Infection, inflammation, and bone regeneration: a paradoxical relationship. *Journal of dental research, 90*(9), 1052-1061.

Thompson, Z., Miclau, T., Hu, D., & Helms, J. A. (2002). A model for intramembranous ossification during fracture healing. *Journal of Orthopaedic Research, 20*(5), 1091-1098.

Torres, J., Tamimi, F., Alkhraisat, M., Prados-Frutos, J. C., & Lopez-Cabarcos, E. (2011). Bone substitutes *Implant dentistry-the most promising discipline of dentistry*: InTech.

Toth, J. M., Boden, S. D., Burkus, J. K., Badura, J. M., Peckham, S. M., & McKay, W. F. (2009). Short-term osteoclastic activity induced by locally high concentrations of recombinant human bone morphogenetic protein–2 in a cancellous bone environment. *Spine, 34*(6), 539-550.

Udagawa, N., Takahashi, N., Katagiri, T., Tamura, T., Wada, S., Findlay, D. M., . . . Kishimoto, T. (1995). Interleukin (IL)-6 induction of osteoclast differentiation depends on IL-6 receptors expressed on osteoblastic cells but not on osteoclast progenitors. *Journal of Experimental Medicine, 182*(5), 1461-1468.

Uebersax, L. (2008). *Silk fibroin scaffolding for growth factor delivery in tissue repair*. ETH Zurich.

Upman, P. J. (2006). ISO 10993-6: Test for local effects after implantation. *BONEZone, 5*(1), 50-52.

Vaccaro, A. R., Anderson, D. G., Patel, T., Fischgrund, J., Truumees, E., Herkowitz, H. N., . . . Wetzel, T. (2005). Comparison of OP-1 Putty (rhBMP-7) to iliac crest autograft for posterolateral lumbar arthrodesis: a minimum 2-year follow-up pilot study. *Spine, 30*(24), 2709-2716.

Van der Veen, B. S., de Winther, M. P., & Heeringa, P. (2009). Myeloperoxidase: molecular mechanisms of action and their relevance to human health and disease. *Antioxidants & redox signaling, 11*(11), 2899-2937.

Van Tran, P., Vignery, A., & Baron, R. (1982). An electron-microscopic study of the bone-remodeling sequence in the rat. *Cell and tissue research, 225*(2), 283-292.

Vangsness, C. T., Garcia, I. A., Mills, C. R., Kainer, M. A., Roberts, M. R., & Moore, T. M. (2003). Allograft transplantation in the knee: tissue regulation, procurement, processing, and sterilization. *The American Journal of Sports Medicine, 31*(3), 474-481.

Vogt, J., Traynor, R., & Sapkota, G. P. (2011). The specificities of small molecule inhibitors of the TGFss and BMP pathways. *Cellular signalling, 23*(11), 1831-1842.

Volpin, G., Cohen, M., Assaf, M., Meir, T., Katz, R., & Pollack, S. (2014). Cytokine levels (IL-4, IL-6, IL-8 and TGFβ) as potential biomarkers of systemic inflammatory response in trauma patients. *International orthopaedics, 38*(6), 1303-1309.

Wang, W., & Yeung, K. W. K. (2017). Bone grafts and biomaterials substitutes for bone defect repair: A review. *Bioactive Materials, 2*(4), 224-247.

Waters, R. V., Gamradt, S. C., Asnis, P., Vickery, B. H., Avnur, Z., Hill, E., & Bostrom, M. (2000). Systemic corticosteroids inhibit bone healing in a rabbit ulnar osteotomy model. *Acta Orthopaedica Scandinavica, 71*(3), 316-321.

Welinder, K. G., & Larsen, Y. B. (2004). Covalent structure of soybean seed coat peroxidase. *Biochimica et Biophysica Acta (BBA)-Proteins and Proteomics, 1698*(1), 121-126.

Welinder, K. G., Mauro, J. M., & Nørskov-Lauritsen, L. (1992). Structure of plant and fungal peroxidases: Portland Press Limited.

Winn, S. R., Uludag, H., & Hollinger, J. O. (1999). Carrier systems for bone morphogenetic proteins. *Clinical orthopaedics and related research, 367*, S95-S106.

Winterbourn, C. C., Kettle, A. J., & Hampton, M. B. (2016). Reactive Oxygen Species and Neutrophil Function. *Annual Review of Biochemistry, 85*(1), 765-792.

Wintges, K., Beil, F. T., Albers, J., Jeschke, A., Schweizer, M., Claass, B., . . . Schinke, T. (2013). Impaired bone formation and increased osteoclastogenesis in mice lacking chemokine (C-C motif) ligand 5 (Ccl5). *Journal of Bone and Mineral Research, 28*(10), 2070-2080.

Wong, D. A., Kumar, A., Jatana, S., Ghiselli, G., & Wong, K. (2008). Neurologic impairment from ectopic bone in the lumbar canal: a potential complication of off-label PLIF/TLIF use of bone morphogenetic protein-2 (BMP-2). *The Spine Journal, 8*(6), 1011-1018.

Wright, H., & Nicell, J. A. (1999). Characterization of soybean peroxidase for the treatment of aqueous phenols. *Bioresource Technology, 70*(1), 69-79.

Yamaguchi, T. P., Bradley, A., McMahon, A. P., & Jones, S. (1999). A Wnt5a pathway underlies outgrowth of multiple structures in the vertebrate embryo. *Development, 126*(6), 1211-1223.

Yang, X., Ricciardi, B. F., Hernandez-Soria, A., Shi, Y., Camacho, N. P., & Bostrom, M. P. (2007). Callus mineralization and maturation are delayed during fracture healing in interleukin-6 knockout mice. *Bone, 41*(6), 928-936.

Yavropoulou, M., & Yovos, J. (2008). Osteoclastogenesis--current knowledge and future perspectives. *J Musculoskelet Neuronal Interact, 8*(3), 204-216.

Yoon, E., Dhar, S., Chun, D. E., Gharibjanian, N. A., & Evans, G. R. (2007). In vivo osteogenic potential of human adipose-derived stem cells/poly lactide-co-glycolic acid constructs for bone regeneration in a rat critical-sized calvarial defect model. *Tissue engineering, 13*(3), 619-627.

Young, M. F., Kerr, J. M., Ibaraki, K., Heegaard, A.-M., & Robey, P. G. (1992). Structure, expression, and regulation of the major noncollagenous matrix proteins of bone. *Clinical orthopaedics and related research*(281), 275-294.

Yu, X., Huang, Y., Collin-Osdoby, P., & Osdoby, P. (2004). CCR1 chemokines promote the chemotactic recruitment, RANKL development, and motility of osteoclasts and are induced by inflammatory cytokines in osteoblasts. *Journal of Bone and Mineral Research, 19*(12), 2065-2077.

Yuan, H., Van Den Doel, M., Li, S., Van Blitterswijk, C., De Groot, K., & De Bruijn, J. (2002). A comparison of the osteoinductive potential of two calcium phosphate ceramics implanted intramuscularly in goats. *Journal of Materials Science: Materials in Medicine, 13*(12), 1271-1275.

Zara, J. N., Siu, R. K., Zhang, X., Shen, J., Ngo, R., Lee, M., . . . Kwak, J. (2011). High doses of bone morphogenetic protein 2 induce structurally abnormal bone and inflammation in vivo. *Tissue Engineering Part A, 17*(9-10), 1389-1399.

Zhu, W., Boachie-Adjei, O., Rawlins, B. A., Frenkel, B., Boskey, A. L., Ivashkiv, L. B., & Blobel, C. P. (2007). A novel regulatory role for stromal-derived factor-1 signaling in bone morphogenic protein-2 osteogenic differentiation of mesenchymal C2C12 cells. *Journal of Biological Chemistry, 282*(26), 18676-18685.

Zhu, W., Liang, G., Huang, Z., Doty, S. B., & Boskey, A. L. (2011). Conditional inactivation of the CXCR4 receptor in osteoprecursors reduces postnatal bone formation due to impaired osteoblast development. *Journal of Biological Chemistry, 286*(30), 26794-26805.

Zinonos, I., Labrinidis, A., Lee, M., Liapis, V., Hay, S., Ponomarev, V., . . . Evdokiou, A. (2009). Apomab, a fully human agonistic antibody to DR5, exhibits potent antitumor activity against primary and metastatic breast cancer. *Mol Cancer Ther, 8*(10), 2969-2980.

Zscharnack, M., Hepp, P., Richter, R., Aigner, T., Schulz, R., Somerson, J., . . . Marquass, B. (2010). Repair of Chronic Osteochondral Defects Using Predifferentiated Mesenchymal Stem Cells in an Ovine Model. *The American Journal of Sports Medicine, 38*(9), 1857-1869.

Zwingenberger, S., Nich, C., Valladares, R. D., Yao, Z., Stiehler, M., & Goodman, S. B. (2012). Recommendations and considerations for the use of biologics in orthopedic surgery. *BioDrugs, 26*(4), 245-256.