Investigating the effects of downstream parathyroid hormone (PTH) targets on bone strength and quality

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Abstract

The only currently approved anabolic therapy that increases bone mass and reduces fracture risk in patients with osteoporosis is parathyroid hormone (PTH). To investigate how PTH increases bone mass and improves bone strength, I have investigated the function of two gene products regulated by PTH in osteoblasts on bone structure and strength (gp130 and ephrinB2).

Gp130 deletion in late osteoblasts/osteocytes resulted in greater femoral dimensions indicating a role for osteocytic gp130 in maintaining bone width. Although the bending load required to fracture these bones was not altered, they showed a significant reduction in material strength which was associated with a greater proportion of disorganised woven bone suggesting that the increased bone width may have been a compensatory mechanism for poor bone quality. This indicated that gp130 in late osteoblasts/osteocytes maintains the material strength of the cortical bone matrix collagen production and the deposition of organised lamellar bone.

The role of another PTH downstream target, ephrinB2, in controlling bone strength and quality was investigated in both osteoblasts and osteocytes. Work conducted prior to my PhD showed that specific deletion of ephrinB2 within the entire osteoblast lineage in 12-week old female mice caused osteoblast apoptosis and delayed initiation of bone mineralisation, indicating a role for ephrinB2 in osteoblasts that promotes mineralisation by preventing apoptosis. During my PhD, I found that mice lacking ephrinB2 in the osteoblast lineage had slender bones which were more compliant when assessed by mechanical testing. Despite their greater bending ability, their bones were more fragile but they exhibited no change in bone composition measured by synchrotron-based Fourier transform infrared microscopy (sFTIRM). This suggested that the previously observed delay in the initiation of mineralisation caused by ephrinB2-deficient osteoblasts may have resulted in the fragile bone phenotype.

Since ephrinB2 is expressed throughout the entire osteoblast differentiation pathway, I next sought to determine the role of ephrinB2 in late osteoblasts/osteocytes in 12-week
old female mice. Mice lacking ephrinB2 in osteocytes showed an opposing strength phenotype compared to the earlier deletion of ephrinB2 mentioned above. Their bones were more brittle due to greater mineral deposition and parallel stretching/compression of the collagen fibres detected by sFTIRM, highlighting stage-specific roles of ephrinB2 during osteoblast differentiation that regulate bone size and material composition. Correlation analysis revealed that the relationships between collagen fibre alignment and bending strength and between mineral composition and material toughness that normally exist in control mice were lost in bones from mice lacking ephrinB2 in late osteoblasts/osteocytes. This demonstrated that ephrinB2 within late osteoblasts/osteocytes restrains mineral deposition to maintain bone strength.

I next investigated the trabecular bone structure in these mice and found that deletion of ephrinB2 in late osteoblasts/osteocytes resulted in greater trabecular bone containing enlarged osteoclast size observed in vivo suggesting impaired osteoclast function. This may also relate to greater carbonate content within the bone mineral which requires further investigation.

Finally, I assessed whether osteocytic ephrinB2 is required for the anabolic action of PTH. In contrast to impaired anabolic effect of PTH in mice lacking ephrinB2 within the entire osteoblast lineage, PTH treatment in mice lacking ephrinB2 in late osteoblasts/osteocytes did not significantly impair the anabolic action of PTH on trabecular and cortical bone formation. This led me to use the sFTIRM technique to analyse bone composition in vehicle and PTH-treated bones from control mice. This showed that bone deposited during PTH treatment undergoes a normal process of collagen maturation and mineral accrual.

In conclusion, downstream targets of PTH, gp130 and ephrinB2 play distinct roles in regulating bone size, strength and quality. This highlights that osteoblasts utilise these proteins during the formation of bone matrix and the initiation of its mineralisation. In contrast, osteocytes maintain the quality and composition of bone matrix and mineral by
regulating mineral accrual and collagen arrangement. Understanding how specific genes expressed in these cells can control bone strength, coupled with the sFTIRM technique developed during my PhD, can provide a new way for investigating different causes of bone fragility. Current diagnostic tests cannot fully predict risk of fracture and treatment options are not patient specific and are limited in their use. In the long term, this study may allow specific targeting of cell types to increase bone mass and improve bone quality which could lead to the development of different personalised treatments for bone fragility.
Declaration

This is to certify that:

i) This thesis comprises only my original work towards the degree Doctor of Philosophy except where indicated in the Preface,

ii) Due acknowledgement has been made in the text to all other material used,

iii) The thesis is fewer than 100,000 words in length, exclusive of tables and bibliography.

Christina Vrahnas
Preface

Pursuant to the regulations governing the degree of Doctor of Philosophy at the University of Melbourne, I hereby submit that:

I. This thesis contains no material that has been accepted for the award of any other degree or diploma in any university.

II. To the best of my knowledge and belief, this thesis contains no material previously published or written by another person, except where due reference has been made.

Chapter 1 of this thesis contains text from two review articles to which I contributed that have been published in peer-reviewed journals (Sims NA & Vrahnas C, Archives of Biochemistry and Biophysics, 561:22-28 (2014) and Vrahnas C & Sims NA, Current Molecular Biology Reports, 1:148-156 (2015). These manuscripts are included in Appendix 1 and Appendix 2.

Chapter 3 of this thesis contains findings and text that formed a part of a published peer-reviewed article that resulted from collaboration with others in the scientific community. Figure 3-1, Table 3-1, Figure 3-2, Figure 3-3, Figure 3-4 and Figure 3-5 were included in Johnson RW et al, Journal of Bone and Mineral Research 29(6):1492-1505 (2014). I produced these data and contributed to the writing of the manuscript. This manuscript is included in Appendix 3.

Chapter 4 of this thesis contains findings and text that formed a part of a published reviewed article that resulted from collaboration with others in the scientific community. Figure 4-1, Figure 4-2, Table 4-1, Figure 4-3, Figure 4-5, Table 4-2 were included in Tonna S et al, FASEB Journal 28(10): 4482-4496 (2014). I produced these data and contributed to the writing of the manuscript. This manuscript is included in Appendix 4.
Chapter 6 of this thesis contains findings that were published in a peer-reviewed journal and result from the collaboration with others in the scientific community. I declare that I am the primary author of the publication, having contributed >50% of the work presented. The declaration for thesis with publication forms for the paper have been submitted with this thesis, along with the signed co-author collaboration authorisation documents confirming collaborator contributions and that my contributions were >50%.

Contributions of all co-authors to this published work (Chapter 6) are as follows: Christina Vrahnas (60%), Thomas A Pearson (17.5%), Athena R. Brunt (2.5%), Mark R. Forwood (2.5%), Keith R. Bambery (2.5%), Mark J. Tobin (2.5%), T. John Martin (5%) and Natalie A. Sims (7.5%).

I would like to acknowledge the help of others in contributing to the work presented in this thesis:

Fluorescence-activated cell sorting was performed by Michael Thompson.

Roger Curtain (Bio21 Institute, Melbourne) provided assistance with obtaining images using the scanning backscattered electron microscope presented in Table 2-7.

Dr. Mark Tobin and Dr. Keith Bambery provided advice on collection and analysis of spectra presented in Figure 4-7 and Figure 5-10.

Dr. Eleftherios Paschalis and Dr. Cyril Petibios provided advice on performing sub peak analysis of spectra presented in Figure 5-11.

Dr. Cyril Petibios assisted with FTIR imaging and analysis presented in Figure 5-12.

Blessing Crimeen-Irwin analysed the backscattered electron microscopy images for osteocyte lacunae presented in Figure 5-13.
Ms Ingrid Poulton performed the TUNEL stain and Ms Blessing Crimeen-Irwin analysed osteocyte properties presented in Figure 5-14.

Associate Professor Mika Ikegame performed captured transmission electron microscopy images presented in Figure 5-15.
Publications

The following publications and prepared manuscripts are a result of work generated for this thesis:

Publication


In preparation
Acknowledgments

I would like to express my overwhelming appreciation to my supervisors, A/Prof Natalie Sims and Emeritus Prof Jack Martin. Thank you for the endless opportunities you have presented me throughout my PhD. They have been amazing, and at times challenging, but I could have never imagined we would achieve so much together when I first started my PhD. Natalie, your guidance, enthusiasm and positive attitude has been contagious. It was such an honour to be supervised and work alongside such an intelligent, happy, hard-working and successful woman! Thank you for allowing me to travel to so many international conferences, work in international labs and allow me to develop new and exciting techniques in the lab. Jack, your dedication to your students and research is so inspiring. Thank you for your help with my grammar and presentation skills! I really enjoyed your tough questions and advice. I hope I proved to you that my generation does have some good reading and writing skills. It has been an honour learning about bone biology from both of you and I am very grateful for your guidance and support.

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I would like to give a special thanks to some fellow students who have become close friends and have supported me throughout my PhD. Firstly, Leni Green, you were my best friend at SVI and couldn’t have been happier to share our PhD experience together. We have been through some challenging times together but your support, encouragement and friendship made my PhD so much more enjoyable! Shreya Bhattacharya-Mukherjee, you were my first friend at SVI and I can’t thank you enough for your amazing
friendship. You helped me through my PhD by giving me so much advice and bringing me back down to earth when I got too carried away with things. Niloufar Ansari, my friend who drinks too much beer! I am so glad you joined the Bone group because we could complain about our struggles with histomorphometry, micro-CT, genotyping, FACS etc. together. You are forever going out of your way to help me and others in the lab, I cannot be more grateful for your help and advice. I am so glad we have become such good friends! I hope I can help you like you have helped me in your final stages of your PhD. Toby Dite, thank you for always asking me to go for coffee. You have played a massive role in keeping me sane during my PhD. Our walks and chats in the park were the best stress relief and you constantly inspired me with your enthusiasm for good science! I cannot thank you enough for your advice, support and love which have made my PhD experience so much more special and enjoyable. Thank you for listening to my rants, helping me with my work and teaching me something new every day! I am also very grateful for the friendship and support from past and present students, thank you to: Ashleigh King, William Stanley, Julienne O’Rourke, Nick Scott, Vy Hoang, Matt O’Brien, Jasmina Markulić, Claudia Selck, Chacko Joseph, Alvin Ng and Ling Yeong Chia.

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<th>Description</th>
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<tbody>
<tr>
<td>αMEM</td>
<td>Alpha Modification of Eagle’s Medium</td>
</tr>
<tr>
<td>AC</td>
<td>Attenuation Coefficient</td>
</tr>
<tr>
<td>Alpl</td>
<td>Tissue non-specific Alkaline Phosphatase, Liver/Bone/Kidney</td>
</tr>
<tr>
<td>AP</td>
<td>Anteroposterior</td>
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<tr>
<td>β2m</td>
<td>Beta-2-Microglobulin</td>
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<tr>
<td>BaF₂</td>
<td>Barium Fluoride</td>
</tr>
<tr>
<td>BFR/BS</td>
<td>Bone Formation Rate/Bone Surface</td>
</tr>
<tr>
<td>Bglap</td>
<td>Osteocalcin</td>
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<tr>
<td>BMD</td>
<td>Bone Mineral Density</td>
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<tr>
<td>BMM</td>
<td>Bone Marrow Macrophages</td>
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<tr>
<td>BMU</td>
<td>Basic Multicellular Unit</td>
</tr>
<tr>
<td>BPO</td>
<td>Benzoyl peroxide</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine Serum Albumin</td>
</tr>
<tr>
<td>BV/TV</td>
<td>Bone Volume/Tissue Volume</td>
</tr>
<tr>
<td>C57BL/6</td>
<td>C57 black 6</td>
</tr>
<tr>
<td>Ca₅(PO₄)₃(OH)</td>
<td>Calcium Phosphate (hydroxyapatite mineral crystals)</td>
</tr>
<tr>
<td>CaCl₂</td>
<td>Calcium chloride</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary deoxyribonucleic acid</td>
</tr>
<tr>
<td>CNTF</td>
<td>Ciliary Neurotrophic Factor</td>
</tr>
<tr>
<td>CO₂</td>
<td>Carbon Dioxide</td>
</tr>
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<td>Cre recombinase</td>
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<td>Cardiotrophin-1</td>
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<td>CTan</td>
<td>CTAnalyser</td>
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<td>CTX-1</td>
<td>Carboxy-terminal collagen crosslinks</td>
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<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>DAB</td>
<td>3, 3’-Diaminobenzidine</td>
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<td>DBP</td>
<td>Dibutyl phthalate</td>
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<td>Double distilled H₂O</td>
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<td>DEPC</td>
<td>Diethylpyrocarbonate</td>
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<tr>
<td>DER</td>
<td>Di glycidyl ether of polypropylene glycol</td>
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<tr>
<td>dLS/BS</td>
<td>Double Labelled Surface/Bone Surface</td>
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<tr>
<td>DMAE</td>
<td>Dimethylaminoethanol</td>
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<td>DMEM</td>
<td>Dulbecco’s modified eagle medium</td>
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<td>DMF</td>
<td>N-Dimethyl formamide</td>
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<tr>
<td>dMMMA</td>
<td>Destabilised methylmethacrylate</td>
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<tr>
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<td>Dentin Matrix Protein 1</td>
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<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>DXA</td>
<td>Dual-energy X-ray absorptiometry</td>
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<tr>
<td>Ec.Pm</td>
<td>Endocortical Perimeter</td>
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<tr>
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<td>Ethylenediaminetetraacetic Acid</td>
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<tr>
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<td>EphrinB2</td>
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<tr>
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<tr>
<td>FACS</td>
<td>Fluorescence -activated cell sort</td>
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<td>Foetal Bovine Serum</td>
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<tr>
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<td>Floxed</td>
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<td>Fibroblast Growth Factor 23</td>
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<td>Flanked by LoxP</td>
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<td>Focal Plane Array</td>
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<tr>
<td>FTIRI</td>
<td>Fourier Transform Infrared Imaging</td>
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<tr>
<td>GFP</td>
<td>Green Fluorescence Protein</td>
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<tr>
<td>gp130</td>
<td>Glycoprotein-130</td>
</tr>
<tr>
<td>HCl</td>
<td>Hydrochloric Acid</td>
</tr>
<tr>
<td>HI</td>
<td>Heat inactivated</td>
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<tr>
<td>Hmbs</td>
<td>Hydroxymethylbilane Synthase</td>
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<tr>
<td>Hprt1</td>
<td>Hypoxanthine Phosphoribosyltransferase 1</td>
</tr>
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<td>Abbreviation</td>
<td>Description</td>
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<tr>
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</tr>
<tr>
<td>IDI</td>
<td>Indentation Distance Increase</td>
</tr>
<tr>
<td>IL-6</td>
<td>Interleukin-6</td>
</tr>
<tr>
<td>IL6st</td>
<td>Interleukin-6 signal transducer</td>
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<tr>
<td>IL-11</td>
<td>Interleukin-11</td>
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<tr>
<td>IMA</td>
<td>Integrated Morphometry Analysis</td>
</tr>
<tr>
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<td>Intraperitoneal</td>
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<td>Infrared</td>
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<td>M-CSF</td>
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<td>M.Ar</td>
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<tr>
<td>MAR</td>
<td>Mineral Apposition Rate</td>
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<td>Mineralising Surface/Bone Surface</td>
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<td>MSC</td>
<td>Mesenchymal Stem Cell</td>
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<td>Mepe</td>
<td>Matrix Extracellular Phosphoglycoprotein</td>
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<tr>
<td>MicroCT</td>
<td>Micro X-ray computed tomography</td>
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<td>ML</td>
<td>Mediolateral</td>
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<td>MLT</td>
<td>Mineralisation Lag Time</td>
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<tr>
<td>MMA</td>
<td>Methylmethacrylate</td>
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<tr>
<td>MMP</td>
<td>Matrix Metallopeptidase</td>
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<tr>
<td>MPMI</td>
<td>Mean Polar Moment of Inertisa</td>
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<td>Sodium Hydroxide</td>
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<tr>
<td>NSA</td>
<td>Nonenyl succinic anhydride</td>
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<tr>
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<td>Osteoclast Area</td>
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<tr>
<td>OI</td>
<td>Osteogenesis Imperfecta</td>
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<tr>
<td>OPG</td>
<td>Osteoprotegerin</td>
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<td>OPN</td>
<td>Osteopontin</td>
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<tr>
<td>OS/BS</td>
<td>Osteoid Surface/Bone Surface</td>
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OSM  Oncostatin M
Osmr  Oncostatin M receptor
O.Th  Osteoid Thickness
PBS  Phosphate Buffered Saline
PCR  Polymerase Chain Reaction
PFA  Paraformaldehyde
Ps  Periosteal
Ps.Pm  Periosteal Perimeter
PTH  Parathyroid Hormone
PTH1R  Parathyroid hormone receptor 1
PTHrP  Parathyroid Hormone-related Protein
qPCR  Quantitative-real time polymerase chain reaction
RANK  Receptor Activator of NF-κB
RANKL  Receptor Activator of NF-κB Ligand
RNA  Ribonucleic Acid
ROI  Region of Interest
RPI  Reference Point Indentation
rTDT  Terminal Deoxynucleotidyl Transferase, Recombinant, Enzyme
Runx2  Runt-related transcription factor 2
SDS  Sodium dodecyl sulphate
sEphB4  Soluble EphB4
sFTIRM  Synchrotron-based Fourier Transform Infrared Microscopy
SIBLING  Small Integrin Binding Ligand Interacting N-linked Glycoprotein
sLS/BS  Single Labelled Surface/Bone Surface
Sost  Sclerostin
Sp7  Osterix
Tb.N  Trabecular Number
Tb.Sp  Trabecular Separation
Tb.Th  Trabecular Thickness
TEM  Transmission Electron Microscopy
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<tr>
<td>TID</td>
<td>Total Indentation Distance</td>
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<tr>
<td>TMD</td>
<td>Tissue Mineral Density</td>
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<tr>
<td>Tnsfs11</td>
<td>Tumour Necrosis Factor (Ligand) Superfamily, Member 11</td>
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<tr>
<td>Tnfrsf11b</td>
<td>Tumour Necrosis Factor (Ligand) Superfamily, Member 11b</td>
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<tr>
<td>TRAP</td>
<td>Tartrate Resistance Acid Phosphatase</td>
</tr>
<tr>
<td>TUNEL</td>
<td>TdT-mediated dUTP Nick-End Labelling</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet</td>
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<tr>
<td>w/w</td>
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Aims and Hypotheses

CHAPTER 2: Materials and Methods

Animal models

Dmp1Cre.gp130 mice

OsxCre.EfnB2 mice

Dmp1Cre.EfnB2 mice

Dmp1Cre.DMP1-GFP.EfnB2 mice

Genotyping

Isolating tail DNA and genotyping of mouse strain

PTH treatment

Calcein administration in mice

Serum and tissue collection and storage

Micro-Computed tomography (micro-CT) 3D analysis of femora

Micro-CT analysis of femoral trabecular secondary spongiosa

Micro-CT analysis of femoral cortical bone

Tissue mineral density (TMD) measurements in the cortical region

Sample preparation for histomorphometry, TUNEL staining and synchrotron-based Fourier Transform Infrared Microscopy (sFTIRM)

Plastic section preparation

Preparation of destabilised methyl methacrylate (dMMA)

Dehydration, infiltration and embedding of bone samples

Cutting and polishing plastic sections

Staining of thin plastic sections

Toluidine Blue Stain

Xylenol Orange Stain
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CHAPTER 1: Introduction

1.1 Bone modelling and remodelling

The growth, repair and maintenance of the skeleton are controlled by the processes of bone modelling and remodelling. Bone modelling occurs during growth until adulthood and involves bone formation and resorption occurring in sequence at different sites resulting in a change in bone size and shape (5). In response to mechanical loading, bone modelling contributes to cortical expansion where bone is formed on the outer surface of bone (6).

Bone remodelling occurs asynchronously thereby renewing the skeleton throughout life. This is the fundamental process by which the skeleton changes in response to hormonal and mechanically-induced stresses (7). It is composed of repeated cycles of bone resorption by osteoclasts, followed by bone matrix production by osteoblasts, replacing old or damaged bone at the same location. When resorption and formation is balanced, bone size and shape remain the same (6). During remodelling, osteoclasts and osteoblasts carry out bone resorption and formation, respectively, in basic multicellular units (BMUs). Osteoclasts are large multinucleated bone resorbing cells derived from haematopoietic precursors (8, 9) and their activity and formation is regulated by a range of cell types, including osteoblasts that originate from the mesenchymal lineage. The BMU also contains other cells that contribute to remodelling such as T-cells, macrophages and osteocytes (10-12) however the signals from these cells converge on the osteoclast and the osteoblast. Osteoblast regulation of osteoclast formation is achieved by the expression of receptor activator of NFκB ligand (RANKL) by osteoblast lineage cells, which acts via its receptor, receptor activator of NFκB (RANK), on the cell surface of osteoclast progenitors. RANKL and macrophage-colony stimulating factor (M-CSF), also expressed by osteoblasts, are sufficient and necessary for osteoclast differentiation, function and survival. Osteoclast formation and attachment to bone initiates the dissolution of mineral and degradation of the collagen matrix, which is later filled by new bone matrix through the process of bone formation. The decoy receptor
inhibitor osteoprotegerin (OPG), also expressed by osteoblast lineage cells, can bind to RANK on osteoclast precursors to suppress osteoclastogenesis (13-15). By this mechanism, the same cell lineage both forms bone matrix and regulates osteoclast differentiation in response to paracrine and endocrine stimuli, including parathyroid hormone (PTH) (Section 1.4) (16).

Although the initial concept of remodelling focussed on the cells on the bone surface, we now understand that there are many other cellular contributors that regulate bone formation and resorption within the BMU. These include osteocytes, which are terminally differentiated osteoblast lineage cells that reside within the matrix as an interconnected network. Their network extends throughout the bone, responds to mechanical stimuli, regulates mineralisation (17) and communicates to osteoblasts, osteoclasts and other cell types in the marrow space (e.g., haemopoietic precursors, macrophages, T-cells, natural killer cells and adipocytes) (5).

For bone mass to remain constant, bone remodelling requires a strict balance between bone resorption and bone formation. Changes in this balance result in bone loss (net resorption) or bone gain (net formation), with a positive balance during growth, and a negative balance during aging (10). When this balance is negative, the resulting reduction in bone mass leads to compromised structural integrity of the skeleton and increased bone fragility (18). This literature review will discuss the roles of osteoblast- and osteocyte-derived paracrine, autocrine and endocrine factors, including ephrinB2 and the interleukin 6 (IL-6)/glycoprotein 130 (gp130) family of cytokines, their response to parathyroid hormone (PTH) and parathyroid hormone-related protein (PTHrP), at different stages of osteoblast differentiation to modify bone strength and quality.
1.2 Determinants of bone strength

Whole bone strength is defined as the maximal force a bone can tolerate before structural failure occurs (3) and depends on bone mass, cortical and trabecular microarchitecture, and intrinsic material properties (mineral and collagen) (19).

1.2.1 Bone mass and microarchitecture

Trabecular (cancellous) bone is mainly found at the metaphysis of long bones, in vertebrae, and at the inner parts of flat bones, and makes up 20% of the skeleton (Figure 1-1) (20). At the macroscopic level, trabecular bone is a spongy structure of interconnected trabecular plates and rods surrounding marrow-filled cavities. The remaining 80% of the skeleton is made up of cortical (compact) bone which is a dense and solid structure and is mainly found at the diaphysis of long bones (Figure 1-1). In humans, cortical bone comprises of concentric layers of bone surrounding central canals for blood supply, termed Haversian systems. Mouse cortical bone lack haversian systems (21) therefore humans, not mice, undergo remodelling of cortical bone which is carried out by osteoclasts and osteoblasts surrounding blood vessels (22).

Trabecular and cortical bone contribute to bone strength based on where they are predominantly found. The interconnecting trabeculae make up the majority of the vertebral bodies and so the porous sponge-like structure functions as a spring to absorb energy to maintain vertebral strength. Vertebral bodies therefore favour flexibility allowing them to deform more before cracking. In contrast, long bones such as femora or tibiae have a long diaphyseal shaft that consists of mainly cortical bone. Long bones favours stiffness over flexibility allowing long bones to tolerate the peak loads that vertebral bodies cannot withstand (18). For example, cortical perimeter defines the outer surface of the cortical bone, which is an important predictor of bone strength (Figure 1-1). An increase in perimeter or diameter of a hollow cylinder-shape, such as a femoral shaft, increases resistance to bending and torsional forces without increasing bone mass (3). The geometric contribution/spatial distribution of bone that resists bending and torsional loading is defined as the moment of inertia (23). For example, if two bones have the same cortical area, the bone with the large periosteal diameter has a larger moment of
inertia than a bone with a small periosteal diameter (24). This morphologic trait depends heavily on the outer bone diameter or periosteal expansion and to a lesser extent on changes in bone mass, cortical area or endosteal perimeter.

In addition to greater cortical perimeter, greater cortical thickness has been associated with higher bone strength and lower fracture risk in both human and rat (25, 26). During aging or menopause, the observed increase in bone perimeter or diameter has been argued to be a mechanism by which the skeleton compensates for the reduction in cortical and trabecular bone mass that occurs with age, thereby enabling the bone to maintain its strength somewhat (27-29). This highlights a way in which bone geometry adapts in response to compromised bone strength.

Age-related changes in bone include trabecular thinning and loss of connectivity, as well as cortical thinning and increased porosity (30). In conditions such as osteoporosis, the reduction in bone mass is associated with a disruption in trabecular connectivity and the conversion of normal plate-like trabeculae into thinner rod-like structures leading to increased bone fragility and fracture risk (31). A patient’s risk of fracture is currently assessed by areal bone mineral density (BMD) measurements by dual-energy X-ray absorptiometry (DXA) (32). BMD measures mass per unit area which considers the amount of mineral and the dimensions of the bone (33). However BMD only provides a measure of the density of a whole area of bone and cannot detect site-specific alterations in bone strength or quality, therefore incompletely describes fracture risk (34).
Figure 1-1 Trabecular and cortical bone within a stylised femur.

The proximal and distal ends of the femur, termed epiphyses, contain mostly trabecular bone. The trabeculae continue through to the metaphyses. Between these two regions is the epiphyseal (growth) plate which is the initial site of bone growth. The midshaft of the femur, diaphysis, contains mostly cortical bone and its cortical perimeter is the length of the outside surface (periosteum) of the diaphysis.
1.2.1.1 *Sex and age-specific effects on bone structure*

Male and female mammals, including humans, differ significantly in their rates of bone remodelling, their cortical dimensions and their bone strength. Briefly, after sexual maturity, male mice have higher trabecular bone mass (35, 36), a lower level of bone remodelling (35, 37), greater periosteal diameter and ultimate bone strength (38, 39) than females. For this reason, phenotypes of low bone remodelling are more readily detected in female mice, since the level of bone remodelling is already low in the male (40, 41), and phenotypes demonstrating high levels of bone remodelling and the effects of PTH injection on bone formation are more readily detected in male mice (42).

During aging, trabecular bones become thinner and cortical becomes more porous due to bone resorption on trabecular, endocortical and intracortical surfaces. Simultaneously, periosteal bone formation partially offsets the removal of bone on the inner surface (43). The net bone loss in men is less than in women, at least in part, because periosteal apposition is greater in men, leading to a higher proportion of women sustaining fractures than men (44, 45).

1.2.2 Bone material properties

1.2.2.1 *Type I collagen*

The organic matrix is mainly made up of type 1 collagen (46) which is laid down by osteoblasts during bone formation and makes up 90% of the bone matrix (47). To a minor extent, bone material also consists of other non-collagenous proteins and proteoglycans (48). Type I collagen consists of two α1 and one α2 polypeptide chain assembled in a triple helix (49). It is first synthesised as procollagen and once secreted into the extracellular matrix, cleavage of N- and C- terminals of procollagen enables spontaneous self-assembly into collagen fibrils. Post-translational modifications stabilise the triple helical structure and allow the formation of intermolecular and interfibrillar crosslinks (50).
Abnormalities in collagen crosslinks, such as reduced stability, reduced collagen content or the formation of immature crosslinks have been associated with increased fracture risk (51-53). Additionally, a 50% decrease in mature collagen crosslinks was associated with reduced bending strength in rats with lathyrism (neurological disease) (54). Collagen fibre orientation is also an important predictor of bone tensile strength, defined as the resistance of a material to break under strain. This was evident in SAMP6 mice, where disorganized collagen fibrils and low overall collagen content resulted in weak and brittle bones (55, 56). However, collagen’s contribution to bone strength is very obvious in diseases such as osteogenesis imperfecta where abnormal collagen protein formation results in disorganised collagen polymer and matrix disruption leading to increased fracture risk (57). Changes in collagen content or collagen crosslinking can reduce the energy required to cause bone failure and therefore increase fracture risk (58). The energy required to cause bone failure defines the toughness of bone and indicates the resistance to fracture at the tissue level, independent of its size or geometry (23).

1.2.2.2 Hydroxyapatite mineral
The mineral laid down within the collagenous matrix is made up of crystals comprised of a modified hydroxyapatite [Ca₅(PO₄)₃OH]. Bone mineral crystals contain a variety of inclusions and substitutions which vary with age. The most prevalent substituent is carbonate which substitutes for hydroxyl and phosphate within the apatite surface and crystal lattice (60). This results in varying amounts of carbonate and hydrogen phosphate ions between samples and areas and a very small percentage of hydroxyl groups (59). X-ray diffraction analysis of iliac crest biopsies from patients aged 0-95 years (61) demonstrated that during the first 30 years, bone mineral crystal size and perfection increased and then decreased then slightly increased in the oldest individuals. In these same samples, carbonate substitution generally increased from 0-90 years of age. The increase in carbonate content with aging has also been reported in both Raman (62, 63) and infrared spectroscopy (64) studies.
The amount of mineral and size of mineral crystals can alter bone strength where bone containing a wider variety of hydroxyapatite mineral crystal sizes has been associated with the greater bone strength of younger experimental animals while older animals displayed mostly large mineral crystals (65). As mineral crystals grow between collagen fibrils, excessive growth can damage collagen fibres, reducing resistance to load, making bones more brittle (66). This reduction in heterogeneity and increased fragility with aging in humans (62) indicates that an optimal material might contain a broad distribution of crystal sizes.

In contrast to the collagen component which confers overall toughness of the bone tissue (67), the mineral component confers strength and stiffness to the tissue (68). Such material strength is a measure of resistance to permanent damage and is dependent on its mass and geometry. In general, the collagenous matrix provides bone’s ductility making it tougher than mineral while the mineral is stiff and brittle (69). This combination provides rigidity and resistance against fracture and changes in either component may affect bone strength.

1.3 Osteoblast differentiation, bone formation and mineralisation

1.3.2 Osteoblast differentiation

Osteoblasts are derived from mesenchymal stem cells (Figure 1-2) and two transcription factors are essential for their differentiation: Runt-related transcription factor 2 (Runx2), which is necessary for commitment of mesenchymal cells to the osteoblastic lineage (70, 71) and Osterix (Sp7), which acts downstream of Runx2 (72).

Mature osteoblasts deposit osteoid which in its mineralised form constitutes ~40% of bone (18) and is predominantly comprised of type I collagen (Col1a1) (Figure 1-2) (73).

During the process of bone formation, osteoblasts not only produce collagen matrix, but also control the mineralisation of that matrix by promoting the seeding of hydroxyapatite crystals and by propagating hydroxyapatite mineral into the collagen extracellular matrix
Tissue-nonspecific alkaline phosphatase (Alpl) plays a role in the bone matrix mineralisation process by generating inorganic phosphate required for hydroxyapatite crystal formation (76). Deactivating mutations in Alpl result in hypophosphatasia (77) characterised by poorly mineralised cartilage (rickets) and bones (osteomalacia), spontaneous fractures, and high levels of extracellular inorganic pyrophosphate concentrations (78). PTH receptor 1 (Pthr1) is also expressed on osteoblasts and promotes osteoblast differentiation and bone formation (Section 1.5) (79). During their post-proliferative stage, osteoblasts express osteocalcin (Bglap) which is maximally expressed during mineralisation in vivo (80) and in vitro (81). Osteocalcin-knockout mice were without bone defects at birth but by 6 months of age, they demonstrated increased cortical bone thickness, density and bone width. This was due to increased bone formation compared to controls indicating that osteocalcin functions by limiting bone matrix production (82).

When the production of osteoid matrix is complete, mature osteoblasts undergo one of three fates: (1) remain on the surface of bone as less metabolically active cells termed lining cells, (2) die by apoptosis or (3) become entrapped within the osteoid matrix and, as the osteoid is mineralised, they further differentiate to become osteocytes (Section 1.3.2.2) (83).

Different stages of osteoblast differentiation can regulate the activities of osteoclasts (5), and each other (84). This is particularly relevant for the initiation of the bone remodelling cycle, where both osteocytes and osteoprogenitors have been proposed to produce the RANKL required for osteoclastogenesis (5) indicating that osteoblast lineage cells have multiple functions, discussed in section 1.3.2.2.
1.3.2.2 Osteocytes

Osteocytes are terminally differentiated osteoblasts and constitute more than 95% of all cells in the bone (85). They become embedded within the bone matrix and exist in small cavities known as lacunae. Osteocytes develop long dendritic processes that form an intercellular communication network with each other and with cells on the bone surface, including osteoblasts and bone lining cells (86, 87). This extensive network coordinates local and systemic signals (88). These signals regulate bone formation and mineralisation (89, 90), phosphate metabolism (91, 92) and osteoclast differentiation via RANKL (93-96).

Mineralisation is regulated by a group of proteins, known as the SIBLING (small integrin- binding ligand N-linked glycoprotein) family, that directly influence bone mineralisation. These include dentin matrix protein 1 (Dmp1) (97) and matrix...
extracellular phosphoglycoprotein (Mepe) (98). Osteocytes are also known as the mechanosensors of bone because they detect and respond to mechanical stimulation by stimulating bone formation. Osteocytes from mechanically stimulated bones express lower levels of sclerostin (Sost) (99) which is an inhibitor of Wnt signalling, and therefore a negative regulator of bone formation (100).

Osteocytes also play an important role in phosphate metabolism via expression of Dmp1 (97) and fibroblast growth factor 23 (Fgf23) (101). Loss of Dmp1 in osteocytes results in a hypomineralised phenotype similar to hypophosphatemic rickets and osteomalacia, highlighting Dmp1 regulation of phosphate homeostasis (91). Fgf23 is also involved in hypophosphatemic diseases as demonstrated by severe growth retardation, short bones and reduced mineral content in Fgf23-null mice. Fgf23-null mice also displayed high serum phosphate with increased renal phosphate reabsorption indicating that FGF23 is essential for normal phosphate metabolism (102).

The Dmp1Cre mouse line has been used to study osteocytes using the 10kb Dmp1 promoter which drives the expression of Cre recombinase, allowing the deletion of genes specifically in Dmp1-expressing cells (103). While Dmp1 expression was originally thought to be specifically restricted to osteocytes and odontoblasts (104), Cre recombinase activity has also been detected in a subpopulation of surface osteoblasts (103) and in skeletal muscle (105). The 8kb region of the Dmp1 promoter has also been used to generate a transgenic mouse line with GFP expression directed to osteocytes in osteoid and bone as its expression is more restricted to osteocytes, but not osteoblasts compared to 10kb Dmp1Cre mouse (106). Subsequently, cells sorted for GFP from calvarial bones of neonatal DMP1-GFP mice have been used to examine osteocytic gene expression compared to osteoblasts (107, 108).

It has been proposed that osteocytes can support osteoclastogenesis and are a primary source of RANKL in vivo (93-96, 109, 110). A study conducted by Nakashima et al. (93) provided in vitro evidence that osteocytes express more RANKL and are more effective at supporting osteoclastogenesis than osteoblasts or bone marrow stromal cells. This
study and another which also reports RANKL expression in osteocytes and osteoblasts (93, 109) used cell preparations that would be contaminated by hematopoietic cells, including RANKL-expressing T-cells and natural killer (NK) cells, which also promote osteoclastogenesis (111, 112). The osteocytes from the Nakashima et al. study were isolated from transgenic mice (crossed between Dmp1Cre (103) and CAG-CAT-EGFP (113)) and sorted for GFP only. Thus, it is likely that cells defined as osteocytes were not only contaminated by haematopoietic lineage cells but also bone surface osteoblasts (103) that supported osteoclastogenesis. More recently, isolation of highly purified neonatal murine osteocytes (without the contamination of haematopoietic lineage and endothelial cells) (114) changed the interpretation of that earlier study. Highly purified neonatal murine osteocytes were generated by isolating GFP+ cells from DMP1-GFP mice and removing contaminating haematopoietic lineage and endothelial cells by fluorescence-activated cell sorting (FACS) (114). GFP+ osteocytes purified in this way demonstrated lower levels of RANKL (Tnfsf11) mRNA (approximately halved) than GFP negative cells (osteoblasts). Additionally, only binucleated tartrate-resistant acid phosphatase (TRAP) positive cells were formed when these haematopoietic-lineage depleted osteocytes were used as supportive cells for osteoclastogenesis. This suggested that highly purified osteocytes without contamination of haematopoietic cells cannot fully support osteoclastogenesis in vitro (114).

Osteocytes also control bone strength. This has been demonstrated in osteocyte-ablated mice which exhibit fragile bones with intracortical porosity and microfractures, osteoblast dysfunction and trabecular bone loss (115). Additionally, an overiectomized rat model showed that a greater number of empty lacunae were associated with reduced bone strength (116). Decreased osteocyte density in aging human bone was associated with a reduction in the bone’s ability to tolerate load and was also related with excessive mineralisation (117). Therefore, these studies implicate osteocytes as an important cell to maintain bone strength.
1.3.3 Bone formation and mineralisation

The process of bone formation involves the deposition of osteoid which can either be formed into woven or lamellar bone, which demonstrate differences in their organisation, osteocyte density and mechanical properties (118, 119). Firstly, woven bone is predominantly found in states of high bone remodelling rates where collagen fibres are rapidly deposited resulting in disorganised bundles containing large and numerous osteocytes, as well as delayed and irregular calcification (118). In contrast, lamellar bone has a more organised structure and collagen is arranged in thicker bundles than in woven bone. Collagen fibres within lamellar bone are oriented to maximise bone strength making it more mechanically competent (7). It is for this reason that woven bone is usually replaced by lamellar bone during bone remodelling (120).

After osteoid deposition, the mineralisation process involves the accumulation of hydroxyapatite crystals within the collagen fibrils of the bone matrix (Section 1.2.2) (18). The degree to which bone becomes mineralised is partially determined by the rate of bone remodelling (18, 121). Newly deposited organic matrix (osteoid) begins to mineralise after ~5-10 days (122), which is termed primary mineralisation. The rate of primary mineralisation can be measured in vivo using double tetracycline labels (123) which become incorporated into the mineralising bone and can be detected by its fluorescence. The process of secondary mineralisation occurs over a longer time period of time allowing for the slow and gradual maturation of mineral components, progressively increasing the mineral content of the bone matrix (122). This process occurs over months to years, including an increase in the number, size and perfection of crystals until maximum mineral content is reached (124, 125).

The size, density and alignment of hydroxyapatite crystals are different in lamellar and woven bone. Woven bone consists of crystals that are smaller in size compared to crystals in mature lamellar bone (119). However, crystal density was found to be greater within lamellar bone on the endosteal bone regions. In this region, crystals were aligned in the preferred orientation of the c-crystallographic (002) axis (parallel to the long axis direction of collagen fibrils) (126). In contrast, periosteal regions that contain mostly
woven bone showed less crystals aligned towards the c-axis orientation compared to the lamellar bone regions (126). The greater crystal density and c-axis aligned crystals in lamellar bone were proposed to be related to the greater elastic modulus and microhardness observed in the endosteal bone than in the periosteal region which contained more woven bone (127). The one similarity between lamellar and woven bone is the plate-like structure of hydroxyapatite crystals (128, 129).

Alterations in the level of mineralisation and the size of mineral crystals can influence bone strength. Compressive testing on human calcaneus bone samples showed greater elastic modulus and maximal strength due to increased bone mineralisation in mature bone compared to young human bone (130). Bone strength is also maintained by heterogeneity of hydroxyapatite crystal sizes (131, 132) where lower crystal heterogeneity is observed in aging human bone with impaired strength (62). The heterogeneity of mineralisation depends on a number of factors, including the rate at which bone remodelling occurs. For example, when the rate of bone remodelling is high, there may be insufficient time for the slow process of secondary mineralisation. This has been observed in cases of typical primary hyperparathyroidism where the degree of mineralisation is significantly lower than in control patients (122), and in patients with osteoporosis treated with PTH (133). In contrast, a very low rate of bone remodelling is associated with a low rate of bone matrix renewal; this allows accumulation of mineral, due to continuing secondary mineralization (134, 135). This has been demonstrated in postmenopausal osteoporotic women treated for 2-3 years with alendronate (134). A low bone turnover rate also results in an accumulation of damaged bone with reduced elastic properties facilitating microcrack proliferation and fracture occurrence (136, 137). Clinically, continued secondary mineralisation may be a cause of atypical fractures, such as those observed with bisphosphonate therapies that inhibit bone remodelling (138, 139). Therefore the rate of remodelling determines the amount of mineralisation that occurs to produce a properly mineralized bone structure that best combines stiffness and brittleness.
1.4 Anabolic parathyroid hormone (PTH) treatment and its effects on bone formation

Parathyroid hormone (PTH) is the only current clinically available pharmacological agent that increases bone mass and strength in patients with osteoporosis (140). PTH (1-34) is clinically administered as a daily subcutaneous injection (140) but its use is limited to 18 months because osteosarcoma was observed in a prolonged toxicity study in rats (141). The pharmacological effect of intermittent PTH treatment requires peak levels of circulating PTH to return to control levels within 3 hours (142). Bone biopsies from women pre- and post- treatment with daily PTH injections indicated that the main PTH effect was to increase remodelling, with some lesser effect on modelling (133, 143). The greater effect on bone remodelling occurs due to increases in the recruitment and activation of BMU’s where the balance of formation is favoured over resorption. Within the BMU, bone resorption takes approximately 3 weeks while bone formation takes 3-4 months, thus the increase in the number of BMU’s as well as a longer period of bone formation means an overall greater anabolic effect on bone. The anabolic effect of PTH is achieved by: promoting precursor differentiation (144), preventing mature osteoblast apoptosis (145), activating lining cells (144), and inhibiting sclerostin production by osteocytes (146, 147). However, concerns have been raised about lower mineral levels with PTH treatment associated with increased osteoid production (131, 148, 149).

In contrast to its anabolic actions, prolonged elevated levels of PTH stimulate osteoclast formation and bone resorption. This resorption effect is enhanced greatly with infusion of PTH over hours (150).

PTH-related protein (PTHrP) resembles PTH in its amino-terminal sequence (76), where 8 of the first 13 amino acids in the two peptides are identical. PTHrP and PTH both bind to and activate their common receptor, PTH type 1 receptor (PTH1R) (151). PTHrP’s importance in bone development has been shown through genetically altered mice lacking the PTHrP gene, which die at birth due to improper endochondral bone formation.
Additionally, mice heterozygous for PTHrP ablation had osteopenia that became evident after 3 months of age was reached due to reduced bone formation. This work and the recapitulation of that phenotype in osteoblast-specific PTHrP null mice, demonstrate the paracrine physiological role of PTHrP as a stimulator of bone formation, promoting recruitment and survival of osteoblasts.

1.5 Downstream targets of PTH and PTHrP

Osteoblasts are the key target cells for the anabolic action of both PTH and PTHrP on bone. PTH and PTHrP interact with PTH1R expressed on osteoblasts to promote osteoblast differentiation and stimulate bone formation. PTH also stimulates the expression of members of the IL-6 cytokine superfamily and ephrinB2, which are both expressed throughout osteoblast differentiation, as discussed below.

1.5.2 Glycoprotein 130 (gp130) is modified by PTH

Glycoprotein130 (gp130) is a signal transducer used by all interleukin-6 (IL-6) family cytokines. The most extensively studied members of this family are: IL-6, interleukin-11 (IL-11), leukaemia inhibitory factor (LIF), oncostatin M (OSM), ciliary neurotrophic factor (CNTF), and cardiotrophin-1 (CT-1). Each of these cytokines acts by forming a complex with gp130 to stimulate osteoblast development and differentiation, as well as osteoclast differentiation and activity. These cytokines have been shown to robustly stimulate osteoclast formation in vitro, an action mediated by their stimulation of RANKL expression by cultured osteoblasts. These findings led to the understanding that these cytokines are largely pro-osteoclastic. However, many of these same cytokines also stimulate bone formation. The anabolic action of IL-6...
cytokines appears to rely on two key mechanisms, firstly stimulating osteoblast commitment at the expense of adipogenesis (40, 173) while also inhibiting expression of the osteocyte-specific Wnt signalling antagonist sclerostin (40).

Both PTH and PTHrP stimulate the expression of gp130 by the osteoblast lineage (156) and rapidly promote the transcription of IL-6 family cytokines and receptors within the osteoblast lineage, including IL-6 (174), IL-11, OSM receptor and LIF (175). These IL-6 family cytokines share some actions and gene targets with PTH. For example, IL-6, IL-11, OSM, LIF and CT-1 promote osteoblast differentiation in vitro (176, 177) and OSM, LIF, and CT-1 stimulate bone formation in vivo (176-178). IL-11, LIF, OSM and CT-1 also, like PTH, inhibit osteocytic sclerostin expression (177, 179). Given the similar effects and up regulation of IL-6 family cytokines in osteoblasts by PTH, the requirement of gp130 signalling in osteocytes for the anabolic action of PTH was investigated in mice lacking gp130 in late osteoblasts and osteocytes directed by Dmp1Cre-mediated recombination (Dmp1Cre.gp130f/f) (180). The PTH-induced increase in bone formation in control mice was not observed in PTH-treated Dmp1Cre.gp130f/f mice (181). This impaired anabolic action of PTH was associated with 50% lower Pthr1 mRNA expression levels in Dmp1Cre.gp130f/f mice compared to controls. This indicated that osteocytic gp130 is required to maintain PTH receptor expression within the osteoblast lineage and for the stimulation of osteoblast differentiation that occurs in response to PTH (181).

Mice with global deletion of gp130 die at birth or earlier, depending on the genetic background, and exhibit abnormalities in cardiac and hematopoietic development (182). In addition, these gp130-deficient mice display less trabecular bone, lower osteoblast number and more osteoclasts compared to controls (183, 184), suggesting that gp130 cytokines stimulate osteoblast development while inhibiting bone resorption, a finding inconsistent with the roles described for these cytokines in vitro. However, since that mouse model could only be examined as an embryo, it is likely to reflect only the role of gp130 cytokines in bone development, not in remodelling.
In both mice lacking gp130 in the full osteoblast lineage (OsxCre.gp130<sup>f/f</sup>) and in late osteoblasts/osteocytes only (Dmp1Cre.gp130<sup>f/f</sup>), a low trabecular bone mass was observed compared to littermate controls and was attributed to a low level of bone formation at 12 and 26 weeks of age. Despite the known influence of IL-6 cytokines on osteoclastogenesis, there was no alteration in osteoclast number, surface or length in either mouse model. This led to the conclusion that increased RANKL production in the osteoblast lineage that occurs in response to IL-6 family cytokines does not play a key role in physiological bone growth and remodelling. Instead, the osteocytic pathway of action is most important for bone formation during bone remodelling since OsxCre.gp130<sup>f/f</sup> and Dmp1Cre.gp130<sup>f/f</sup> mice demonstrate essentially the same phenotype (180).

In contrast to the lower trabecular bone mass in 26-week old Dmp1Cre.gp130<sup>f/f</sup> mice; their femoral cortical dimensions were increased indicating an inhibitory role of gp130 in modelling periosteal bone. The striking difference between the cortical bone phenotype and trabecular bone phenotype in 26-week old Dmp1Cre.gp130<sup>f/f</sup> mice as well as the fact the greater femoral width was not observed in femora from 26-week old OsxCre.gp130<sup>f/f</sup> mice led us to focus on the cortical bone phenotype of 26-week old Dmp1Cre.gp130<sup>f/f</sup> mice. In this thesis, I investigated whether the increased periosteal growth in Dmp1Cre.gp130<sup>f/f</sup> mice altered their bone strength or quality. This is presented in Chapter 3.

1.5.3 EphrinB2 regulates osteoblast differentiation and bone strength

Eph receptors constitute the largest known family of membrane-bound tyrosine kinases and their ligands, Ephrins, are required for embryonic angiogenesis (185), and mediate inflammatory and neuropathic pain (186) and axon guidance (187). Complete deletion of ephrinB2 (188) or EphB4 (189) results in embryonic lethality before skeletal development, at days E11.5 and E10, respectively, due to disruption of early angiogenic remodelling. It was later found that reverse signalling through ephrinB2 was essential for this effect (190). Additionally, ephrinB2/EphB4 signalling was shown to mediate common interactions between arterial and venous endothelial cells and surrounding cells.
This indicated the importance of ephrinB2 and EphB4 in vascular development, network formation and vascular remodelling.

Two distinct features of membrane-bound Ephs and ephrins are: (1) their requirement for direct cell-to-cell interaction, and (2) their ability to generate bidirectional signals where forward signalling through the Eph receptor and reverse signalling through the ephrin ligand occur at the same time (192). Most ligands in this family can bind to more than one receptor and vice versa, with the exception being the receptor EphB4, which binds only to the ephrinB2 ligand (193).

In bone, ephrinB2 is expressed at all stages of osteoblast differentiation and in osteoclasts, while EphB4 is expressed in osteoblasts but not by osteoclasts (157, 173). It has been proposed that ephrinB2 expressed in osteoclasts and EphB4 expressed in osteoblasts can mediate communication between osteoblasts and osteoclasts (193), however ephrinB2/EphB4 signalling has been shown to be most important in osteoblast-osteoblast communication (157, 194, 195).

Based on in vitro data, it has been suggested that ephrinB2-expressing osteoclasts interact with EphB4-expressing osteoblasts to restrict osteoclast differentiation while stimulating bone formation (173). However this proposal has been controversial as contact between osteoclasts and osteoblasts although existing in cell culture conditions are rare in physiological conditions. For example, during bone remodelling at the BMU, osteoclasts and osteoblasts act on the same surface but at different times (196), while in bone modelling, they act on different surfaces. Additionally, osteoclast lineage-specific deletion of ephrinB2 presented no detectable bone phenotype (173, 195). While early data using a mouse that overexpressed EphB4 in the osteoblast lineage demonstrated a significant increase in osteoblast activity in female mice, suggesting that forward signalling may be most important (173), our analysis of these mice, backcrossed to C57BL/6, showed no difference in their bone mass or bone formation activity compared to littermate wild type males or females (197), suggesting that promoting EphB4 forward signalling may not stimulate bone formation in remodelling in all strains of mice.
PTH and PTHrP treatment also significantly increased the production of ephrinB2 in osteoblasts in vitro and in vivo, while other ephrin ligands or Eph receptors were not regulated (157). Specific blockade of ephrinB2/EphB4 signalling, both in vitro (157) and in vivo (194) impaired osteoblast function and the anabolic response to PTH in vivo. Therefore, the requirement of ephrinB2 signalling in osteoblasts for the anabolic action of PTH was investigated in mice lacking ephrinB2 within the osteoblast lineage, directed by OsxCre mediated recombination (OsxCre.EfnB2<sup>f/f</sup>). OsxCre.EfnB2<sup>f/f</sup> mice showed an impaired anabolic response to PTH with a 50% reduction in osteoblast differentiation induced by anabolic PTH treatment compared to controls (195), indicating that ephrinB2 signalling within the osteoblast lineage is required for the full anabolic action of PTH.

1.5.3.2 Impaired ephrinB2/EphB4 interaction affects osteoblast differentiation and bone mineralisation

The osteoblast lineage, including osteoblast precursors and osteocytes, expresses both ephrinB2 and EphB4 (157). In addition, extensive contact among these cells is required for bone formation (198-200). A key role for osteoblastic ephrinB2 signalling in bone formation was suggested by its strong and specific upregulation by parathyroid hormone (PTH) and its related paracrine protein PTHrP (201). The importance of the ephrinB2/EphB4 interaction within the osteoblast lineage was shown using a soluble extracellular domain of EphB4 (sEphB4) which binds to ephrinB2 to inhibit the binding of EphB4. sEphB4 inhibited late stage osteoblast differentiation and mineralisation in vitro (194, 201, 202), a finding that was reproduced in vivo, both in the presence and absence of PTH (194). In vivo, this inhibition of osteoblast differentiation resulted in an accumulation of early-stage osteoblasts, indicated by high mRNA levels of Runx2, Osx, Alpl, Col1a1 and Pthr1 both in the presence and absence of PTH (194). Even though more osteoblasts were present, treatment with sEphB4 did not increase bone formation rate in either vehicle or PTH-treated mice. This suggested that blocking the interaction of ephrinB2 with sEphB4 caused accumulation of partially differentiated osteoblasts with impaired ability to mineralise bone (194). These data suggested that ephrinB2/EphB4
mediated interactions within the osteoblast lineage are required for the progression of osteoblasts to full maturity, and for full expression of late osteoblast/osteocyte markers as well as subsequent bone mineralisation. Since sEphB4 blocks both forward and reverse signalling, the question of whether it is forward or reverse signalling that is most important during bone remodelling, and their relative roles in trabecular vs. cortical bone remained unresolved.

This work led to the generation of a mouse model which genetically lacks ephrinB2 within the osteoblast lineage under the control of the Osx1Cre promoter (OsxCre.EfnB2\textsuperscript{f/f}). OsxCre.EfnB2\textsuperscript{f/f} mice displayed greater osteoblast numbers compared to controls but bone formation rate was not increased indicating that although their number is increased, ephrinB2-deficient osteoblasts have a defect in their mineralising ability (195). This was similar to the effects of sEphB4 treatment\textit{ in vivo} (194). The mineralisation defect was further confirmed by greater osteoid thickness in OsxCre.EfnB2\textsuperscript{f/f} mice accompanied by a reduced mineral apposition rate. This indicated that rather than osteoid production being increased, it was the progression of bone mineralisation that was delayed (195). Higher expression levels of early osteoblast markers (Runx2, Col1a1) and lower levels of late osteoblasts/osteocyte markers (Bglap, Sost) were observed in OsxCre.EfnB2\textsuperscript{f/f} mice compared to controls (195). Again this was consistent with the impaired late-stage osteoblast differentiation observed with sEphB4 treatment (194) highlighting that ephrinB2 reverse signalling was required for continued osteoblast differentiation. The impaired late-stage osteoblast differentiation in OsxCre.EfnB2\textsuperscript{f/f} mice was explained by a higher level of osteoblast and osteocyte apoptosis compared to controls (195). This demonstrated a key role of ephrinB2 reverse signalling within the osteoblast lineage in promoting late stage osteoblast differentiation and bone mineralisation by limiting osteoblast apoptosis. The unresolved question that remained for OsxCre.EfnB2\textsuperscript{f/f} mice was how their delayed bone mineralisation affected their bone strength and quality.
1.6 Aims and Hypotheses

While current therapies aim to reduce fracture risk by inhibiting bone loss or increasing bone mass, it is still not well understood how these therapies affect bone mineralisation and quality. I hypothesise that two factors stimulated by PTH (ephrinB2 and gp130) expressed in late osteoblasts/osteocytes play distinct roles in regulating bone formation and strength at a structural and material level. I have studied three mouse models with genetic deletion of gp130 and ephrinB2 in late osteoblasts/osteocytes to identify mechanisms by which bone remodelling maintains bone strength.

To this end, my specific aims are:

- To assess the effects of gp130 deletion in late osteoblasts/osteocytes on bone strength and quality (Dmp1Cre.gp130<sup>fl/fl</sup>)
- To examine the bone strength and composition of mice lacking ephrinB2 within the osteoblast lineage (OsxCre.EfnB2<sup>fl/fl</sup>)
- To determine whether ephrinB2 in late osteoblasts/osteocytes is required for normal bone metabolism (Dmp1Cre.EfnB2<sup>fl/fl</sup>)
- To determine whether ephrinB2 in late osteoblasts/osteocytes is required for the anabolic action of PTH on bone
CHAPTER 2: Materials and Methods

2.1 Animal models

All animals were housed in a 12 h light and dark cycle with food and water provided *ad libitum* at the St. Vincent’s BioResources Centre. For all experiments, matching Cre+ littermates were used as controls and both female and male mice were analysed for all lines. All animal work was approved by the St. Vincent’s Health Melbourne Animal Ethics Committee.

All mice used in experiments were produced from heterozygous breeders and littermate controls were used except for the PTH study (described in Chapter 5 and 6) where homozygous breeders were used.

2.1.1 Dmp1Cre.gp130 mice

For experiments reported in Chapter 3, *Il6st tm1Wme* (gp130-floxed) mice crossed onto a C57BL/6 background were obtained from Rodger McEver (Oklahoma Medical Research Foundation) (203). *Dmp1Cre* (Tg(Dmp1-Cre)1Jqfe) mice (containing the *DMP1* 10-kb promoter region) were obtained from Lynda Bonewald (University of Kansas, Kansas City, USA) (103) and crossed with gp130-floxed mice.

The gp130-floxed mouse targeted deletion of the transmembrane domain (exon 15) by flanking *loxP* sites. Cre expression resulted in ablation of intracellular gp130 signalling, as reported (204). Gene deletion was validated by a significant down regulation of the exon 15 domain of gp130 in mRNA from flushed bone samples (180). The *gp130-loxP* was detected using primers to distinguish 600bp band for gp130-floxed samples and a 400bp band for wild-type samples (See Section 2.1.4)

The *Dmp1Cre* transgene was detected according to the original report (205). Primers used to distinguish Cre+ (534 bp) and Cre- (no band) alleles are indicated in Table 2-3. The PCR protocol is indicated in
2.1.2 OsxCre.EfnB2 mice

Mice used in experiments outlined in Chapter 4 were interbred from Tg(Sp7-tTA,tetO-EGFP/cre)\textsuperscript{1Amc} (Osx1-GFP::Cre) mice obtained from Andrew MacMahon (Harvard University, Cambridge, MA, USA (205)) and Efnb2\textsuperscript{tm1And} (ephrinB2-flox) transgenic mice from David J. Anderson (Howard Hughes Medical Institute, California Institute of Technology, Pasadena, CA, USA (189)). Both had been backcrossed to C57Bl/6 mice for at least 5 generations respectively. The ephrinB2-flox mouse had a targeted deletion of the first coding exon of the intracellular signalling domain of ephrinB2 (185).

EphrinB2-\textit{loxP} was detected according to the original report (185). The primers used to distinguish intact (636 bp) and deleted (309 bp) ephrinB2-\textit{loxP} alleles are indicated in Table 2-3.

The Osx1-GFP::Cre allele was detected according to the original report (205). The primers used to distinguish Cre+ (450 bp) and Cre- (no band) alleles are indicated in Table 2-3. Deletion of ephrinB2 in osteoblasts was confirmed at the mRNA and protein level (195).

The PCR protocols for all alleles are indicated in
Table 2-2.

2.1.3 Dmp1Cre.EfnB2 mice

Mice used in experiments outlined in Chapter 5 were interbred from Dmp1Cre (Tg(Dmp1-Cre)1Jqfe) and Efnb2tm1And transgenic mice, each as described above.

Deleted ephrinB2-loxP was detected as described in section 2.1.2. The Dmp1Cre transgene was detected as described in section 2.1.1. Cre primers were used as an alternative to detect the presence of Cre when Dmp1Cre primers were difficult to observe to confirm recombination (Table 2-3).

2.1.3.1 Dmp1Cre.DMP1-GFP.EfnB2 mice

DMP1-GFP (Tg(Dmp1-Topaz)1Ikal/J) mice (106) were obtained from the colony of Dr Hong Zhou (ANZAC Research Institute, Sydney, Australia) and crossed with the Dmp1Cre.EfnB2 mouse line to obtain Dmp1Cre.DMP1-GFP-Tg.EfnB2+/+ and Dmp1Cre.DMP1-GFP-Tg.EfnB2+/− mice.

The DMP1-GFP transgene was detected according to the original report (106). The primers used to distinguish GFP + (550 bp) and GFP- (no band) alleles are indicated in Table 2-3. The PCR protocol is indicated in
2.1.4 Genotyping

2.1.4.1 Isolating tail DNA and genotyping of mouse strain

The genetic status of all mice was determined by semi-quantitative PCR. 600μl 50nM NaOH (Merck Pty. Ltd. Kilsyth, VIC, Australia) was added to each tail sample, which was denatured at 95°C for 20 minutes. 100μl Tris pH 8.0 (Merck KGaA, Darmstadt, Germany) was added; the samples were vortexed then centrifuged at 13,000 rpm for 5 minutes. 1μl of each sample was added to PCR mastermixes, as below (Table 2-1).

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume per sample</th>
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<tr>
<td>10μM primers (Forward + Reverse, see Table 2-3)</td>
<td>1 μl</td>
</tr>
<tr>
<td>5x My Taq Reaction Buffer</td>
<td>2μl</td>
</tr>
<tr>
<td>Taq Polymerase</td>
<td>0.1 μl</td>
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Each reaction was mixed gently and quickly spun to bring the contents to the bottom of the tube. Each sample was amplified by PCR (Biometra T3000 Thermocycler, Biometra GmbH, Germany) under cycling conditions described in

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<tbody>
<tr>
<td>dH₂O</td>
<td></td>
<td>5.9 µl</td>
</tr>
<tr>
<td>Tail DNA</td>
<td></td>
<td>1 µl</td>
</tr>
<tr>
<td>Final Volume</td>
<td></td>
<td>10 µl</td>
</tr>
</tbody>
</table>
Table 2-2 and the genotyping primers listed in Table 2-3.
Table 2-2 Cycling conditions for genotyping gene of interest

<table>
<thead>
<tr>
<th>Gene of interest</th>
<th>Temperature</th>
<th>Time</th>
<th>Number of cycles</th>
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<tbody>
<tr>
<td><strong>OsxCre</strong></td>
<td>94°C</td>
<td>1 minute</td>
<td>30</td>
</tr>
<tr>
<td></td>
<td>94°C</td>
<td>30 seconds</td>
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<tr>
<td></td>
<td>55°C</td>
<td>30 seconds</td>
<td></td>
</tr>
<tr>
<td></td>
<td>72°C</td>
<td>30 seconds</td>
<td></td>
</tr>
<tr>
<td></td>
<td>72°C</td>
<td>10 minutes</td>
<td></td>
</tr>
<tr>
<td></td>
<td>15°C</td>
<td>Pause</td>
<td></td>
</tr>
<tr>
<td><strong>Dmp1Cre</strong></td>
<td>94°C</td>
<td>5 minutes</td>
<td></td>
</tr>
<tr>
<td></td>
<td>94°C</td>
<td>30 seconds</td>
<td></td>
</tr>
<tr>
<td></td>
<td>60°C</td>
<td>30 seconds</td>
<td></td>
</tr>
<tr>
<td></td>
<td>72°C</td>
<td>30 seconds</td>
<td></td>
</tr>
<tr>
<td></td>
<td>72°C</td>
<td>5 minutes</td>
<td></td>
</tr>
<tr>
<td></td>
<td>15°C</td>
<td>Pause</td>
<td></td>
</tr>
<tr>
<td><strong>EfnB2&lt;sup&gt;x/w&lt;/sup&gt; or EfnB2&lt;sup&gt;y/y&lt;/sup&gt;</strong></td>
<td>95°C</td>
<td>4 minutes</td>
<td></td>
</tr>
<tr>
<td></td>
<td>95°C</td>
<td>30 seconds</td>
<td></td>
</tr>
<tr>
<td></td>
<td>60°C</td>
<td>30 seconds</td>
<td></td>
</tr>
<tr>
<td></td>
<td>72°C</td>
<td>3 minutes</td>
<td></td>
</tr>
<tr>
<td></td>
<td>72°C</td>
<td>10 minutes</td>
<td></td>
</tr>
<tr>
<td></td>
<td>15°C</td>
<td>Pause</td>
<td></td>
</tr>
<tr>
<td><strong>Cre</strong></td>
<td>94°C</td>
<td>10 minutes</td>
<td></td>
</tr>
<tr>
<td></td>
<td>94°C</td>
<td>20 seconds</td>
<td></td>
</tr>
<tr>
<td></td>
<td>55°C</td>
<td>30 seconds</td>
<td></td>
</tr>
<tr>
<td></td>
<td>68°C</td>
<td>30 seconds</td>
<td></td>
</tr>
<tr>
<td></td>
<td>72°C</td>
<td>5 minutes</td>
<td></td>
</tr>
<tr>
<td></td>
<td>12°C</td>
<td>Pause</td>
<td></td>
</tr>
<tr>
<td><strong>Dmp1-GFP</strong></td>
<td>94°C</td>
<td>5 minutes</td>
<td></td>
</tr>
<tr>
<td></td>
<td>94°C</td>
<td>30 seconds</td>
<td></td>
</tr>
<tr>
<td></td>
<td>62°C</td>
<td>30 seconds</td>
<td></td>
</tr>
<tr>
<td></td>
<td>72°C</td>
<td>45 seconds</td>
<td></td>
</tr>
<tr>
<td></td>
<td>72°C</td>
<td>5 minutes</td>
<td></td>
</tr>
<tr>
<td></td>
<td>15°C</td>
<td>Pause</td>
<td></td>
</tr>
</tbody>
</table>
Each sample, including a standard DNA ladder VI (Roche Pty. Ltd., Melbourne, VIC, Australia) was loaded into a 2% agarose gel containing 6µl of SYBR Safe (Invitrogen Australia Pty. Limited, Mulgrave, VIC, Australia) and run by electrophoresis at 120V for 40 minutes. The product bands (Table 2-3) were visualised under ultraviolet (UV) light.
Table 2-3 Genotyping primer sequences for mouse models used in this study

<table>
<thead>
<tr>
<th>Gene of Interest</th>
<th>Direction</th>
<th>Primer Sequence 5’-3’</th>
<th>Product size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>OsxCre (205)</strong></td>
<td>Forward</td>
<td>GCC AGG CAG GTG CCT GGA CAT</td>
<td>450</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>CTC TTC ATG AGG AGG ACC CT</td>
<td></td>
</tr>
<tr>
<td><strong>Dmp1Cre (103)</strong></td>
<td>Forward</td>
<td>CCC GCA GAA CCT GAA GAT G</td>
<td>534</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>GAC CCG GCA AAA CAG GTA G</td>
<td></td>
</tr>
<tr>
<td><strong>EfnB2^{f/f} (185)</strong></td>
<td>Forward</td>
<td>AAG TTA TAA GCT TCA ACG CGT CC</td>
<td>320</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>GAG CCC CAG GTT CTA GAA TAA CTT CG</td>
<td></td>
</tr>
<tr>
<td><strong>EfnB2^{w/w} (185)</strong></td>
<td>Forward</td>
<td>GCT GCC CGC GGC CGG TCC CAA CG</td>
<td>580</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>CCG TTA GTG GCA ACG TCC TCC GTT ATACG</td>
<td></td>
</tr>
<tr>
<td><strong>Cre (206)</strong></td>
<td>Forward</td>
<td>GAC CAG GTT CGT TCA CTC ATG G</td>
<td>200</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>AGG CTA AGT GCC TTC TCT ACA</td>
<td></td>
</tr>
<tr>
<td><strong>Dmp1-GFP (106)</strong></td>
<td>Forward</td>
<td>TCA TCT GCA CCA CCG GCA AGC</td>
<td>550</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>AGC AGG ACC ATG TGA TCG CGC</td>
<td></td>
</tr>
</tbody>
</table>
2.1.5 PTH treatment

8-week old $Dmp1Cre.EfnB2^{w/w}$ and $Dmp1Cre.EfnB2^{f/f}$ male mice were administered 50 µg/kg synthetic human PTH (1-34) (Bachem, Bubendorf, Switzerland) or Vehicle (2% heat inactivated (HI) mouse serum in saline (0.9% NaCl)) by intraperitoneal (IP) injection, 5 days a week for 4 weeks; this protocol has previously been reported to increase trabecular bone formation (175, 194). Mice were weighed daily and injection volumes were adjusted according to weight changes over the 4-week treatment period. Calcein was administered by IP injection 7 and 2 days before tissue collection as below.

2.1.6 Calcein administration in mice

Calcein (Sigma Chemical Co, USA) fluorochrome labels were administrered subcutaneously. The calcein label binds to calcium in circulation and becomes incorporated within the matrix upon initiation of mineralisation. These labels can be viewed under UV light and quantified as a measure of bone mineralising surface. Mice were administered calcein according to Table 2-4. The different time points that calcein injections were administered at was dependent on mice age. The level of remodelling slows down from 6 weeks to 26 weeks of age, thus a longer time period between labels is required to detect bone formation in older mice. Calcein was dissolved in 10mg/ml in saline (0.9% NaCl) containing 20mg/ml of sodium bicarbonate (207).

<table>
<thead>
<tr>
<th>Table 2-4 Calcein injection day protocol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (weeks)</td>
</tr>
<tr>
<td>6</td>
</tr>
<tr>
<td>12</td>
</tr>
<tr>
<td>26</td>
</tr>
</tbody>
</table>
2.1.7 Serum and tissue collection and storage

Mice from the Dmp1Cre.gp130, OsxCre.EfnB2 and Dmp1Cre.EfnB2 models were fasted for 16 hours prior to tissue collection. Immediately prior to collection, mice were anaesthetised with ketamine/xyaline. Terminal blood samples were collected by cardiac puncture exsanguination and stored on ice for less than 4 hours followed by centrifugation at 4000rpm for 4 minutes at 4°C.

While still anaesthetised, mice were killed by cervical dislocation. The following tissues were collected: 2 tibiae, 2 femora, vertebrae, and calvaria. Skin and muscle were removed from one femur and the distal epiphysis including the growth plate was transversely sliced off. The remaining long bone was held over a sterile 50ml tube, and flushed of bone marrow through the medullary cavity opening using a 10ml syringe filled with 1 x Phosphate buffered saline (PBS) until the PBS solution ran clear. Flushed femoral samples were snap-frozen in liquid nitrogen and stored in liquid nitrogen until RNA preparation (Section 2.4).

The remaining tissues were fixed in 4% paraformaldehyde (PFA) at 4°C overnight followed by storage in 70% ethanol until required for micro-CT (Section 2.2), histological analysis (Section 2.3.5) or mechanical testing (Section 2.6.1).

PTH-treated Dmp1Cre.EfnB2 mice were given their last PTH injection 1 hour before being killed and were collected the same way as described above.
2.2 Micro-Computed tomography (micro-CT) 3D analysis of femora

Femoral specimens were analysed by micro-CT using a Skyscan 1076 system (Bruker-microCT, Kontich, Belgium). During scanning, femora were wrapped in gauze and enclosed in a 5ml plastic tube filled with 70% ethanol. Images were acquired at 9 µm voxel resolution, with a 0.5 mm aluminium filter, 50 kV voltage and 100 µA current, 2600 ms exposure time, rotation 0.5 degrees, frame averaging =1 (180).

Image slices were reconstructed by NRecon (version 1.6.9.8) with the following settings: beam-hardening correction 30%, ring artefact correction 12, no smoothing, and no defect pixel masking. Reconstructed images were straightened by aligning the xyz crosshair in the centre of the femoral metaphysis in a sagittal view using DataViewer (version 1.4.4) and analyses were performed using CTAnalyser software (CTan) (version 1.11.8.0).

To determine trabecular and cortical bone regions of interest, femoral length was measured from micro-CT scans as the distance from the notch between the two condyles at the distal femoral end and the notch between the greater trochanter and the femoral neck (Table 2-5). Micro-CT analysis of trabecular and cortical bone parameters are shown in Table 2-5.
2.2.1 Micro-CT analysis of femoral trabecular secondary spongiosa

The femoral trabecular region of interest (ROI) was determined in the sagittal plane by identifying the base of the distal femoral growth plate and excluding the primary spongiosa (10% of the total femur length towards the femora midshaft). From that point, a further 15% of the total femur length was analysed as the secondary spongiosa trabecular ROI (Figure 2-1) (123).

2.2.2 Micro-CT analysis of femoral cortical bone

Cortical analysis was performed 30% above the distal end of the femur toward the femoral midshaft, also with an ROI of 15% of the length of the femur (Figure 2-1).
Figure 2-1 Trabecular and cortical regions of interest (ROI) for micro-CT analysis.

Refer to section 2.2.1 and section 2.2.2 for full description.
Analysis of bone structure was completed by adaptive thresholding (mean of minimum and maximum values) in CT Analyser. Thresholds for analysis were determined by performing automatic thresholding on 3 samples from each experimental group. The grayscale values obtained (0-255, where 0=black and 255=white) were averaged (Table 2-5).

**Table 2-5 Threshold values used for trabecular and cortical micro-CT analysis**

<table>
<thead>
<tr>
<th>Mouse Model</th>
<th>Threshold values</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Age</td>
</tr>
<tr>
<td><em>Dmp1Cre.EfnB2</em>&lt;sub&gt;w/w&lt;/sub&gt; and &lt;sub&gt;f/f&lt;/sub&gt;</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>12</td>
</tr>
<tr>
<td></td>
<td>26</td>
</tr>
<tr>
<td><em>Dmp1Cre.EfnB2</em> + PTH treatment</td>
<td>12</td>
</tr>
</tbody>
</table>
### Table 2-6 Derived parameters for trabecular and cortical bone micro-CT analysis

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Description</th>
<th>Units</th>
</tr>
</thead>
<tbody>
<tr>
<td>Femoral length</td>
<td>Defined by top and bottom landmarks (top = notch between the two condyles at the distal end of femur, bottom = notch between the greater trochanter and the femoral neck)</td>
<td>mm</td>
</tr>
<tr>
<td>Trabecular Bone Volume (BV/TV)</td>
<td>Percentage of bone volume to total tissue volume</td>
<td>%</td>
</tr>
<tr>
<td>Trabecular Thickness (Tb.Th)</td>
<td>The thickness of individual trabeculae structures</td>
<td>µm</td>
</tr>
<tr>
<td>Trabecular Number (Tb.N)</td>
<td>The inverse of the mean distance between the mid-axes of the trabecular structure</td>
<td>1/mm</td>
</tr>
<tr>
<td>Trabecular Separation (Tb. Sp)</td>
<td>The thickness of the spaces between the individual trabeculae structures</td>
<td>µm</td>
</tr>
<tr>
<td>Cortical Thickness (Ct.Th)</td>
<td>The thickness of the cortical bone</td>
<td>µm</td>
</tr>
<tr>
<td>Marrow Area (M.Ar)</td>
<td>The bone volume subtracted from the total volume which is then divided by the measured length of the cortical ROI (bone length x 15%)</td>
<td>µm²</td>
</tr>
<tr>
<td>Cortical Area (Ct.Ar)</td>
<td>Cortical bone volume divided by the measured length of the cortical ROI (bone length x 15%)</td>
<td>µm²</td>
</tr>
<tr>
<td>Endocortical Perimeter (Ec.Pm)</td>
<td>Tissue surface subtracted from bone surface, divided by the measure length of cortical ROI (bone length x 15%)</td>
<td>µm</td>
</tr>
<tr>
<td>Periosteal Perimeter (Ps.Pm)</td>
<td>Tissue surface divided by divided by the measure length of cortical ROI (bone length x 15%)</td>
<td>µm</td>
</tr>
<tr>
<td>Mean Polar Moment of Inertia (MPMI)</td>
<td>The resistance to rotation of a cross section about the central axis</td>
<td>mm⁴</td>
</tr>
<tr>
<td>Cortical tissue mineral density</td>
<td>The density measurement restricted to within the volume of calcified bone tissue</td>
<td>g/cm³</td>
</tr>
</tbody>
</table>
2.2.3 Tissue mineral density (TMD) measurements in the cortical region

Cortical tissue mineral density (Ct.TMD) was analysed in the cortical region of interest (ROI) using the built in TMD function CTAnalyser (CTAn) (version 1.12.0.0, Skyscan, Aartselaar, Belgium). TMD calibration was performed using two phantom rods with concentrations of calcium hydroxyapatite (Ca\(_5\)(PO\(_4\))\(_3\)(OH)) of 0.25 and 0.75g/cm\(^3\) that were 2mm in diameter to match the cross-sectional thickness of mouse bone. Rods were placed in a tube containing 70% ethanol to match \textit{ex vivo} scans and scanned under the same settings as the bone scan (Section 2.2). The same reconstruction parameters used for bone scans (Section 2.2) were applied to the calibration scan of the TMD rods. The two TMD rod scans were opened in CT-Analyser (CTAn) and a circle smaller than the phantom was selected as the region of interest. The attenuation coefficient values for both phantoms were determined using CTAn. The bone mineral density calibration was selected to calibrate TMD against attenuation. A density histogram was generated from the dataset of contents within the volume of interest to calculate the mean total attenuation coefficient values for the two calibration phantoms. The two attenuation values for the two BMD phantoms were applied to the calculation to calibrate BMD against attenuation coefficients (AC) based on the formula below;

\[
\text{BMD} = \frac{\text{AC} - 0.00681}{0.05404} \text{ g/cm}^3
\]

Each cortical bone ROI dataset was loaded to obtain the mean greyscale density which was calibrated to BMD.
2.3 Sample preparation for histomorphometry, TUNEL staining and synchrotron-based Fourier Transform Infrared Microscopy (sFTIRM)

2.3.1 Plastic section preparation

Tibiae were embedded in destabilised methylmethacrylate (dMMA) (208) to allow measurement of bone formation and the extent of osteoid deposition and to preserve cellular detail for histomorphometry (209-211).

2.3.1.1 Preparation of destabilised methyl methacrylate (dMMA)

500 mL of MMA was combined with 300 mL of 5% sodium hydroxide (NaOH) into a separating funnel to destabilise the MMA, shaken vigorously for 30 seconds then placed in a retort stand to settle for ~10 minutes. The solution separated and the heavier bottom NaOH layer was drained off. This was repeated three times and then MMA was washed three times by the same procedure using 500 mL of distilled water each time. The remaining dMMA was filtered through a Whatman paper funnel containing granular calcium chloride to remove water and stored with a small amount of calcium chloride to absorb excess water at 4°C until required.

2.3.1.2 Dehydration, infiltration and embedding of bone samples

After removal of extraneous muscle tissue, bone samples were dehydrated in graded acetones for at least 1 hour each (70%, 90%, 2 x 100%) at 4°C.

Bone samples were then infiltrated for 3 nights to 1 week at 4°C in infiltration solution containing 90% dMMA, 10% dibutylphthalate (DBP) and 0.05% benzoyl peroxide (BPO) (37).

During infiltration, polymerised MMA bases were made for each sample using 90% dMMA, 10% DBP and 4% BPO filtered through granular calcium chloride to ensure residual water was removed and polymerised overnight at 39°C.
Following infiltration, samples were embedded on top of the premade bases in 85% dMMA, 15% DBP and 4% BPO that was filtered through granular calcium chloride. 0.5 ml of this solution was pipetted onto polymerised 1ml bases and left at room temperature for ~ 30 minutes to soften the underlying base. Once soft, tibial samples were placed in the liquid resin and oriented so the fibula was pushed into the base and the anterior tibial crest pointed downwards. The fibulae were left intact. Femoral samples were oriented so the anterior side of the femur was pushed into the base and the femoral condyles were facing up. Vials were placed in a water bath and left at 37°C overnight to 3 days to polymerise.

For tibial samples, once the sample was totally encased in solid plastic, a top layer/holding base was added. The top layer contained 90% dMMA, 10% DBP and 5% BPO filtered through granular calcium chloride. ~7.5mL of this solution was added to each vial and polymerised dry at 37°C for 1-2 nights. When the top layer/holding base had completely set, embedded samples were removed from vials by breaking the glass vial with a hammer.

2.3.1.3 Cutting and polishing plastic sections

Thin sections

Embedded tibiae were ground down using a water-cooled Pheonix Beta grinder/polisher (Buehler, Illinois, USA) until the entire sagittal tibial face was showing. For static and dynamic histomorphometry, 5 µm longitudinal sections were then cut using a tungsten carbide blade (Dorn and Hard Microedge) on a Leica RM 2165 microtome (Leica Microsystems, Germany) under 70% ethanol to soften the plastic. Sections were carefully stretched onto slides coated with Fol’s chrom alum / gelatin solution, ensuring all folds were flattened out using a small drop of 90% ethanol. To coat slides, washed slides were coated in Fol’s solution (6 g of gelatin dissolved in 288 ml water at 60°C, plus 120 mL 95% ethanol and 4 g of chromium potassium sulphate in 20 mL) for 30 seconds and dried at room temperature overnight. Freshly cut sections were covered with a square of bagging plastic, clamped together with other sections and baked overnight in a 37°C incubator to allow the section to adhere to the slide.
For sFTIRM, 3µm longitudinal sections were cut as described above, but were not adhered to slides. Instead, sections were placed in between two pieces of filter paper and clamped between two glass slides to flatten and dry.

**Thick sections**

For production of 100µm thick transverse sections of the femur, femoral samples embedded in plastic were cut into rectangular prism-shaped blocks using a water-cooled Isomet Low Speed Saw and a Diamond Wafering Blade (Buehler, Lake Bluff, IL, USA). The distal end (femoral condyles) of the femur was ground down to the base of the growth plate and then 100 µm serial transverse sections were cut along the entire length of the femur, as described previously (212). To measure collagen fibre orientation by polarized light microscopy, unstained thick sections were mounted on glass slides and coverslipped with DePeX. For reflectance FTIR, thick sections were polished with 1200 grit carbide paper and cloths impregnated with diamond suspensions (particle sizes of 3, 1, 0.3 micron FiberMet Abrasive Discs (Buehler, Lake Bluff, IL, USA)) on the Pheonix Beta grinder/polisher (Buehler, Lake Bluff, IL, USA) for 30 seconds each. Sections were stored with filter paper clamped between two glass slides until analysis.

**2.3.2 Staining of thin plastic sections**

For all stains, sections were deplasticised in Cellosolve for 2 x 50 minutes before rehydration through a series of ethanol washes for 5 minutes each: 2 x 100%, 80% and 60% ethanol. Sections were placed in tap water for 5 minutes prior to staining.

**2.3.2.1 Toluidine Blue Stain**

**Toluidine Blue Buffer**

1.58 g Citric Acid, 0.75g disodium phosphate, 1 L dH₂O, pH to 3.7

**Toluidine Blue Stain**

2g Toluidine Blue O dissolved in 100 mL of toluidine blue buffer, filtered and adjusted pH to 3.7
Deplasticised and rehydrated sections were immersed in toluidine blue stain for 5 minutes, washed in two changes of toluidine blue buffer and then blotted on both sides to dry. Following staining, each slide was passed through the following solutions: Butanol (twice) 50% Butanol/50% Toluene (once), Toluene (twice) then coverslipped with DePex.

2.3.2.2 Xylenol Orange Stain
Deplasticised and rehydrated sections were immersed in Xylenol Orange stain (1% Xylenol Orange in 0.5M Tris (60.57g/L), pH 9) for 5 minutes. Sections were then blotted thoroughly on both sides to dry. Following staining, each slide was passed through the following solutions: Butanol (twice) 50% Butanol/50% Toluene (once), Toluene (twice) then coverslipped with DePex.

2.3.3 Paraffin embedding
Tibiae trimmed of any skin or muscle tissue were decalcified for 14 days in decalcification solution (150g ethylene-diamine-tetra-acetic acid (EDTA) and 5g of PFA dissolved in 1L DEPC-treated water pH 8.0) with gentle agitation at 4°C with a change of solution every three days. DEPC-water was prepared as follows: 1ml Diethyl pyrocarbonate (DEPC) was added to 1L Elix water and shaken vigorously. Solution was then incubated for 12 hours at room temperature, then autoclaved to ensure removal of all remaining traces of DEPC. Decalcified specimens were infiltrated in paraffin wax using the Shandon Excelsior™ ES Tissue Processor (Thermo Scientific) according to the following protocol in Table 2-6. Processed bone specimens were then embedded in paraffin (Paraplast, Leica Microsystems, St Louis, USA) using a Shandon Histocentre-2 embedding machine (Grale Scientific, Ringwood, Australia) and stored at 4°C prior to sectioning (176, 213).
Table 2-7 Paraffin embedding processing steps for Shandon Excelsior™ Tissue Processor

<table>
<thead>
<tr>
<th>Solution</th>
<th>Duration</th>
</tr>
</thead>
<tbody>
<tr>
<td>70% Ethanol</td>
<td>1 hour</td>
</tr>
<tr>
<td>85% Ethanol</td>
<td>1 hour</td>
</tr>
<tr>
<td>90% Ethanol</td>
<td>1 hour</td>
</tr>
<tr>
<td>100% Ethanol</td>
<td>1 hour (x3)</td>
</tr>
<tr>
<td>Xylene</td>
<td>1 hour (x3)</td>
</tr>
<tr>
<td>Wax</td>
<td>1.5 hours (x3)</td>
</tr>
</tbody>
</table>

2.3.3.1 Cutting paraffin sections

Paraffin-embedded samples were sectioned at 5μm using a Microm HM330 rotary microtome and Mx35 Premier+ microtome blade (Thermo Scientific). Sections were transferred to an illuminated flotation bath containing autoclaved water with a sprinkling of gelatin at 37°C. Sections were mounted onto SuperFrost®Plus slides and placed on a 37°C solid state control warming tray for at least 2 hours. Samples were transferred to a 37°C incubator overnight and stored at 4°C until required.

2.3.4 Staining of paraffin sections

2.3.4.1 TdT-mediated dUTP Nick-End Labelling (TUNEL) stain

TUNEL stain was performed on 5μm paraffin sections from 12-week old female Dmp1Cre.EfnB2 mice using the DeadEnd Colorimetric TUNEL system (Promega). Firstly, sections were baked for 2 hours at 55°C then for 2 days at 37°C before being dewaxed in histolene (Grale Scientific) (2x 5 minutes) and 30 seconds each in 100%, 90%, 80% and 70% ethanol. Slides were then washed in 0.85% NaCl for 5 minutes and PBS for 5 minutes. 30μl of a 20μl/ml Proteinase K solution was placed on each section for 10 minutes in a humidified chamber. Slides were then washed in PBS 3x 5 minutes, liquid tapped off and 30μl of DNase I (Ambion, Life Technologies) was added to a positive
control section to cause DNA fragmentation. Sections were then washed 3-4 times in double distilled H₂O (ddH₂O) (keeping DNase treated section in a separate jar) before being washed in PBS for 5 minutes. Liquid was removed and then 30µl/ml of Equilibrium buffer was added to section and incubated for 5-10 minutes in humidified chamber. Terminal Deoxynucleotidyl Transferase, Recombinant, Enzyme (rTdT) reaction mixes were prepared on ice (Table 2-8). Liquid was removed from the slides and 30µl of rTdT buffer solution mix was added, coverslipped with plastic coverslips and incubated in a humidified chamber for 60-90 minutes. 20x SSC was diluted 1:10 with ddH₂O and slides were immersed in separate jars for 15 minutes which terminated the reaction. Slides were washed separately in PBS using dH₂O for 3x 5 minutes. The endogenous peroxidases were blocked by immersing slides in 0.3% hydrogen peroxide solution in PBS for 5 minutes then washed in PBS for 3x 5 minutes (separate one for DNase treated). 30µl of Streptavidin HRP solution diluted 1:500 was added to sections and incubated for 30 minutes in a humidified chamber. Slides were washed again in PBS for 3x 5 minutes. A DAB kit (Dako) was used to make up a DAB solution consisting of 50µl of DAB Substrate 20X Buffer, 950µl dH₂O, 50µl of the DAB 20X Chromogen and 50µl Hydrogen Peroxide 20X. 30µl of this DAB solution was added to slides. Once stain developed, slides were rinsed several times in dH₂O and then counterstained with Mayer’s haematoxylin solution (Grale Scientific) for 30 seconds. Sections were then washed in Scott’s Tap water for 30 seconds, rehydrated in graded ethanols: 10 dips each in 70%, 90%, and 100% then coverslipped with DePex.

**Table 2-8 rTdT reaction mix components**

<table>
<thead>
<tr>
<th>Buffer component</th>
<th>Per reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Equilibration buffer</td>
<td>49 µl</td>
</tr>
<tr>
<td>Biotin Nucleotide mix</td>
<td>0.1 µl</td>
</tr>
<tr>
<td>rTdT Enzyme (dH₂O used for negative control)</td>
<td>0.1 µl</td>
</tr>
</tbody>
</table>
2.3.5 Static and dynamic histomorphometry of trabecular and cortical bone

2.3.5.1 Static Histomorphometry

Cellular parameters were measured in the secondary spongiosa of Toluidine blue stained tibial sections commencing 370 µm below the growth plate, in a 1110 µm² region of the proximal tibia, as previously described (181) (Osteomeasure; Osteometrics, Atlanta, GA, USA). Static histomorphometric parameters include: trabecular bone volume (BV/TV), trabecular thickness (Tb.Th), number (Tb.N) and separation (Tb.Sp), osteoid surface/bone surface (OS/BS), osteoid thickness (O.Th), osteoblast number/bone perimeter (ObN/B.Pm), osteoclast number/bone perimeter (OcN/B.Pm) and osteoclast area (Oc.Ar).

100 µm thick plastic cross sections were used to measure woven and lamellar bone proportions within cortical bone in the mid-diaphyseal region of femora. Polarized light was used to identify the birefringence of collagen fibres on thick transverse sections to demonstrate their orientation within the lamellar bone composite (214). Irregular fibre orientation was measured as woven bone and well-aligned fibre orientation was measured as lamellar bone. Woven and lamellar bone were reported as a percentage of total cortical bone.

2.3.5.2 Dynamic

Calcein labels (described in section 2.1.6) in the secondary spongiosa were measured in the same region as static histomorphometry measurements using a Leica fluorescence microscope. Dynamic histomorphometric parameters included: single-labelled surface/bone surface (sLS/BS), double-labelled surface/bone surface (dLS/BS), interlabelled width (Ir.L.Wi), mineralising surface/bone surface (MS/BS), mineral apposition rate (MAR) and bone formation rate/bone surface (BFR/BS).

Periosteal parameters were measured on the medial tibial midshaft (1500µm from the base of the growth plate in an 1110µm region) (181). These included: periosteal (Ps) single-labelled surface/bone surface (Ps.sLS/BS), double-labelled surface/bone surface (Ps.dLS/BS), mineralising surface/bone surface (Ps.MS/BS) calculated as double label +
single label/2, mineral apposition rate (Ps.MAR) and bone formation rate/bone surface (Ps.BFR/BS).

2.4 Assessment of bone mRNA by quantitative real-time PCR

2.4.1 RNA isolation from bone samples

Femora collected as described in section 2.1.7 were homogenised using two different instruments prior to RNA extraction. 12-week old female *Dmp1Cre.EfnB2* femora were homogenised using the Polytron (Brinkmann Instruments model PT 10/35, Westbury NY).

Bones were homogenised in 1mL of QIAzol Lysis Reagent at 4°C using a Polytron PTA 20S homogeniser at maximum speed for 30 seconds until no large pieces of bone remained. The probe of the homogenizer was soaked in 0.1M NaOH 1% sodium dodecyl sulphate (SDS) for 10 minutes, and then cleaned in purified Elix (de-ionised, pure water, Millipore) and Diethylpyrocarbonate (DEPC)-treated water four times each between specimens. The homogenates were stored on dry ice until all samples in that group were homogenized. Homogenates were then stored at -80°C.

After homogenization, the Qiagen RNeasy Lipid Tissue Mini Kit (Qiagen Sciences, Maryland, USA) was used to purify RNA from sample homogenates. This kit uses RNeasy spin columns for purifying up to 100 µg of RNA. Briefly, the homogenate was separated into aqueous and organic phases by centrifugation. The upper aqueous phase was extracted and ethanol was added to provide proper binding conditions when the sample was applied to the RNeasy spin column. Once centrifuged, the total RNA bound to the membrane, while all other contaminants were washed away. RNA was then eluted in 30-50 µl of RNase-free water.
2.4.2 Removal of genomic DNA

To remove contaminating genomic DNA, total RNA was treated with DNase using a Turbo DNA-free™ Kit (Ambion), which removes up to 50µg of DNA per ml of purified RNA. Reaction sizes used were 20µl. For a 10 µl sample, 1µl of 10X Turbo DNase buffer and 1µl Turbo DNase (2 U/µl) were added to the sample and incubated at 37°C for 30 minutes. Reactions were then inactivated with 0.1 volume of DNase Inactivation Reagent and incubated for 5 minutes at room temperature with gentle mixing at least 3 times. Tubes were centrifuged at 10,000 x g for 1.5 minutes at room temperature and the supernatant was transferred to a new microcentrifuge tube.

2.4.3 Reverse transcription polymerase chain reaction (RT-PCR)

2.4.3.1 Reverse transcriptase and complementary DNA (cDNA) synthesis

cDNA was synthesized from total RNA in 0.2ml thin-walled autoclaved microfuge tubes using the AffinityScript QPCR cDNA Synthesis Kit (Agilent Technologies, La Jolla, CA, USA). The components and PCR cycles were as follows:

Table 2-9 Synthesis of First strand cDNA using Reverse transcriptase

<table>
<thead>
<tr>
<th>Components</th>
<th>Number of samples (n=1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>First Strand Master Mix (µl)</td>
<td>10</td>
</tr>
<tr>
<td>Random Primers (0.1µg/µl)</td>
<td>3</td>
</tr>
<tr>
<td>Affinity Script RNase (µl)</td>
<td>1</td>
</tr>
<tr>
<td>Master Mix Total (µl)</td>
<td>14</td>
</tr>
<tr>
<td>RNA (2µg)</td>
<td>x</td>
</tr>
<tr>
<td>H₂O (µl)</td>
<td>6-x</td>
</tr>
<tr>
<td>Total (µl)</td>
<td>20</td>
</tr>
</tbody>
</table>

x represents the amount of RNA required per sample.
cDNA was generated by an Affinity reaction (Biometra T3000 Thermocycler, Biometra GmbH, Germany) using the following cycle:

**Table 2-10 cDNA synthesis Affinity reaction cycle**

<table>
<thead>
<tr>
<th>Temperature (Celsius)</th>
<th>Duration (minutes)</th>
</tr>
</thead>
<tbody>
<tr>
<td>25°</td>
<td>5</td>
</tr>
<tr>
<td>42°</td>
<td>15</td>
</tr>
<tr>
<td>95°</td>
<td>5</td>
</tr>
</tbody>
</table>

The cDNA was diluted (1 in 5) in RNase-free water and stored at -20°C until amplification by quantitative PCR (qPCR).

2.4.3.2 **Quantitative Polymerase Chain Reaction (qPCR)**

mRNA was analysed from synthesized cDNA using the Mx3000™ Multiplex Quantitative PCR System (Stratagene, USA). Each reaction contained the following:

**Table 2-11 Composition of quantitative PCR reaction**

<table>
<thead>
<tr>
<th>Components</th>
<th>Number of samples (n=1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>H₂O</td>
<td>2.25 µl</td>
</tr>
<tr>
<td>10 µM Forward Primer</td>
<td>0.4 µl</td>
</tr>
<tr>
<td>10 µM Reverse Primer</td>
<td>0.4 µl</td>
</tr>
<tr>
<td>2X Brilliant II SYBR Green QPCR Mastermix</td>
<td>5 µl</td>
</tr>
<tr>
<td><strong>Master mix Total</strong></td>
<td><strong>8.05 µl</strong></td>
</tr>
<tr>
<td>cDNA</td>
<td>2 µl</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>10.05 µl</strong></td>
</tr>
</tbody>
</table>
Each reaction was subject to the following PCR conditions:

**Table 2-12 SYBR Green Cycling Protocol**

<table>
<thead>
<tr>
<th>Cycle</th>
<th>Temperature</th>
<th>Duration</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>95°C</td>
<td>10 minutes</td>
</tr>
<tr>
<td>40</td>
<td>95°C</td>
<td>15 minutes</td>
</tr>
<tr>
<td></td>
<td>60°C</td>
<td>1 minute</td>
</tr>
<tr>
<td>1</td>
<td>95°C</td>
<td>1 minute</td>
</tr>
<tr>
<td></td>
<td>55°C</td>
<td>30 seconds</td>
</tr>
<tr>
<td></td>
<td>95°C</td>
<td>30 seconds</td>
</tr>
</tbody>
</table>

Data was analysed using Stratagene software MxPro and reported using linear ΔCT values normalised to the geometric average of hypoxanthine phosphoribosyltransferase 1 (*Hprt1*) and hydroxymethyl-bilane synthase (*Hmbs*) or *Hmbs* and beta-2-microglobulin (*B2m*). Primers used are listed in Table 2-13. mRNA expression levels of bones from GFP positive and negative osteocytes isolated from femora from *Dmp1Cre.EfnB2* mice were normalised against *B2m* and *Hmbs* (Section 5.3.1). mRNA expression levels of bones from 12-week old female *Dmp1Cre.EfnB2* mice were normalised against the geometric average of *Hprt1* and *Hmbs* (Section 5.3.4).
Table 2-13 List of primer sequences for qPCR used in this study

<table>
<thead>
<tr>
<th>Protein (Gene name)</th>
<th>Accession number</th>
<th>Forward sequence (5’-3’)</th>
<th>Reverse sequence (5’-3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alkaline Phosphatase (<em>Alpl</em>) (157)</td>
<td>NM_007431</td>
<td>AAA CCC AGA CAC AAG CAT TCC</td>
<td>TCC ACC AGC AAG AAG AAG CC</td>
</tr>
<tr>
<td>Hydroxymethylbilane Synthase (<em>Hmbs</em>) (180)</td>
<td>NM_013551</td>
<td>TCA TGT CCG GTA ACG GCG</td>
<td>CAC TCG AAT CAC CCT CAT CTT TG</td>
</tr>
<tr>
<td>Hypoxanthine phosphoribosyltransferase (<em>Hprt1</em>) (157)</td>
<td>J00423</td>
<td>TGA TTA GCG ATG ATG AAC CAG</td>
<td>AGA GGG CCA CAA TGT GAT G</td>
</tr>
<tr>
<td>Type I collagen alpha 1 (<em>Col1a1</em>) (215)</td>
<td>NM_007742.3</td>
<td>CTG GCG GTT CAG GTC CAA T</td>
<td>TTC CAG GCA ATC CAC GAG C</td>
</tr>
<tr>
<td>Type I collagen alpha 2 (<em>Col1a2</em>) (216)</td>
<td>NM_007743</td>
<td>AGT CGA TGG CTG CTC CAA AA</td>
<td>AGC ACC ACC AAT GTC CAG AG</td>
</tr>
<tr>
<td>Dentin Matrix Protein 1 (<em>Dmp1</em>) (157)</td>
<td>NM_016779</td>
<td>CCA GAG GGA CAG GCA AAT AG</td>
<td>CTG GAC TGT GTG GTG TCT GC</td>
</tr>
<tr>
<td>Matrix Extracellular Phosphoglycoprotein (<em>Mepe</em>) (114)</td>
<td>NM_053172</td>
<td>AGC AAA TGC CCA GAG ACT AAG CCC</td>
<td>TGA GGC CCT CTG GCG TCA TTC A</td>
</tr>
<tr>
<td>Osteocalcin (<em>Bglap</em>) (157)</td>
<td>NM_0138480</td>
<td>TCT CTC TGA CCT CAC AGA TCC</td>
<td>TAC CTT ATT GCC CTC CTG CTT G</td>
</tr>
<tr>
<td>Osteoprotegerin (<em>Tnfsf11b</em>)</td>
<td>NM_008764</td>
<td>CCT ACC TAA AAC AGC ACT GCA C</td>
<td>TAA CGC CCT TCC TCA CAC TC</td>
</tr>
<tr>
<td>Osterix (<em>Sp7</em>) (215)</td>
<td>AF184902</td>
<td>TAT GCT CCG ACC TCC TCA AC</td>
<td>AAT AAG ATT GGG AAG CAG AAA G</td>
</tr>
<tr>
<td>Receptor Activator of NFκB (<em>Tnfsf11</em>) (217)</td>
<td>NM_011613</td>
<td>TCC AGC TAT GAT GGA AGG CT</td>
<td>GTC CCA AGA GGA CAG AGT G</td>
</tr>
<tr>
<td>Runx-related transcription factor 2 (<em>Runx2</em>) (215)</td>
<td>AF010284</td>
<td>CTC CGC TGT TAT GAA AAA CC</td>
<td>TGA AAC TCT TGC CTC GTC C</td>
</tr>
<tr>
<td>Sclerostin (<em>Sost</em>) (114)</td>
<td>NM_024449</td>
<td>CCA CAG AGG TCA TCC CA</td>
<td>GAC ACA TCT TTG GCG TCA TAG</td>
</tr>
</tbody>
</table>
2.5 Fluorescence Activated Cell Sort (FACS) of green-fluorescent protein (GFP) positive osteocytes

6-8 week old \textit{Dmp1Cre.DMP1-GFP-Tg.EfnB2}^{w/w} and \textit{Dmp1Cre.DMP1-GFP-Tg.EfnB2}^{f/f} mice were culled and long bones (tibiae, femora, humeri and radii) and calvarie were collected. One \textit{Dmp1Cre-WT.DMP1-GFP-Tg.EfnB2}^{w/w} or \textit{Dmp1Cre-WT.DMP1-GFP-Tg.EfnB2}^{f/f} mouse was used as a negative control as no recombination would occur in the absence of \textit{Cre}-recombinase. The ends of all long bones were cut off using scissors and marrow was flushed out with PBS and discarded. Bones were placed in a 50ml Falcon tube containing 3ml of collagenase/dispase solution (45mg collagenase II (Worthington, 320 U/mg)/90mg of dispase (Gibco, 1.817 U/mg) dissolved in 45ml of \alpha\text{-MEM}/1% Penicillan-Steptomycin (PSA)) and disaggregated with scissors. Bone remnants were then placed in a 37°C water bath shaking for 5 minutes. The supernatant was discarded. Another 3ml of collagenase/dispase was added and incubated for 15 minutes in a 37°C water bath, shaking. The supernatant was aspirated and transferred into a 15ml tube, with an additional 3ml of \alpha\text{-MEM}/1% PSA/10% foetal bovine serum (FBS). 3ml fresh collagenase/dispase was added to the bone remnants, placed in the 37°C water bath, shaking for 15 minutes. This digestion procedure was repeated for a total of 7 times. All fractions were pooled and centrifuged for 5 minutes at 1600 rpm. The supernatant was discarded off and the remaining pellet was resuspended in 1ml of Tris-ure (Bioline) and stored at -80°C until further analysis.

FACS procedures were carried out by Mr Michael Thomson at Flow Cytometry Facility, St Vincent’s Institute. GFP+ and GFP– cells were sorted from \textit{Dmp1Cre.DMP1-GFP-Tg.EfnB2}^{w/w} and \textit{Dmp1Cre.DMP1-GFP-Tg.EfnB2}^{f/f} mice. After collection, samples were centrifuged, supernatant discarded and pellets resuspended in 1ml of Trisure (Bioline) and stored at -80°C until further analysis.
2.5.1 RNA extraction

Samples were thawed on ice and left at room temperature for 5 minutes to permit complete dissociation of nucleoprotein complexes. 0.2ml chloroform/1mL Trisure was added to each sample and mixed by vortexing. Samples were incubated for 15 minutes at room temperature before being centrifuged at 12,000 x g for 15 minutes at 4°C. Following centrifugation, the colourless upper aqueous phase was collected and transferred to a fresh tube. The RNA was precipitated from this by adding 2µl of glycogen (5mg/ml) and 0.5mL isopropyl alcohol/1mL Trisure. Samples were incubated for 20 minutes at room temperature then centrifuged at 12,000 x g for 10 minutes at 4°C. The RNA precipitate formed a gel-like pellet at the bottom of the tube and the supernatant was removed and discarded. The RNA precipitate was washed once with 1mL of 70% ethanol, vortexed and spun at 7,500 x g for 5 minutes at 4°C. The RNA pellet was air dried and then dissolved in 20µl of RNase-free water.

Samples were DNase treated as per section 2.4.2 and cDNA was made as per section 2.4.3.1.

2.6 Bone mechanical properties and material strength analysis

2.6.1 Mechanical Testing

Structural and material properties were analysed by 3-point bending on femora from 6, 12 and 26 week-old female and male Dmp1Cre.EfnB2 mice, 26 week-old female Dmp1Cre.gp130 mice and 12 week-old PTH-treated Dmp1Cre.EfnB2 male mice. Before bending tests were performed, the anteroposterior (AP) and mediolateral (ML) widths of femora were measured using digital callipers. The moment of inertia (mm⁴) was calculated from micro-CT scans from the cortical midshaft of the same femur (180).

Load was applied in the anteroposterior direction at the cortical midshaft midway between two supports that were 7.5 mm apart for 6-week old femora, 6.0 mm apart for
12-week old femora and 9.5 mm apart for 26-week old femora. Mechanical testing on femora from 6- and 26-week old male and female *Dmp1Cre.EfnB2* mice was conducted at the Department of Medicine, St. Vincent’s Hospital, with the help of Dr. Jonathon Gooi (The University of Melbourne). Femora were loaded to failure at 0.5mm/second using a Bose Biodynamic 5500 Test Instrument (Bose, DE, USA) and recorded using Wintest 7 software. Mechanical testing on femora from 26-week old *Dmp1Cre.gp130* mice, 12-week old male and female *Dmp1Cre.EfnB2* mice and PTH-treated 12-week old male *Dmp1Cre.EfnB2* mice were conducted at Griffith University with the help of Dr. Huynh Nguyen and Ms Athena Brunt. Load-displacement curves were recorded at a crosshead speed of 1.0 mm/second using an Instron 5564A dual column materials testing system, using Bluehill 2 software (Instron, Norwood MA, USA). Prior to testing, bones were kept moist in gauze swabs soaked in phosphate buffered saline (PBS).

Ultimate force (N), yield force (N), stiffness (N.mm-1), and energy to failure (mJ), deformation at failure (mm) and post-yield deformation (mm) were calculated from the load-deformation curves, as described previously (Figure 2-2) (3). Combining the geometric calculations and the biomechanical test results, the material properties of each bone were calculated as described by Schriefer et. al (1) to obtain yield strength (MPa); ultimate strength (MPa); elastic modulus (MPa), and toughness (MPa) (Figure 2- 3). Yield point was determined as the point in which the load-displacement curves became nonlinear (23). Mean load-deformation and stress-strain curves were generated by averaging all samples from each genotype.
Figure 2-2 Schematic force-deformation curve from 3-point bending. Image adapted from Schriever et al. (1). Structural properties such as ultimate force (peak of curve on y-axis), deformation at ultimate force (peak of curve on x-axis), yield point (the point at which the curve deviated from linear), stiffness (slope of linear portion of curve), energy absorbed to failure (area under the curve) and failure point (highest deformation point before failure) were derived from this graph.

Figure 2-3 Schematic stress-strain curve from 3-point bending tests. Image adapted from Turner and Burr (3). Material properties were derived from this graph such as the ultimate stress (peak of curve on y-axis), deformation at ultimate stress (peak of curve on x-axis), yield point (the point at which the curve deviated from linear), elastic modulus (slope of linear portion of curve), toughness (area under the curve) and failure point (highest deformation point before failure).
2.6.2 Reference Point Indentation

Local bone material properties at the femoral midshafts from 12-week old female *Dmp1Cre.EfnB2* and 26-week old female *Dmp1Cre.gp130* mice were examined by reference point indentation (RPI) using a Biodent with BP2 probe assembly (Biodent Hfc, Active Life Scientific Inc., Santa Barbara, CA, USA). The BP2 probe includes a 90 degree cono-spherical test probe with <5 µm radius point and a flat bevel reference probe with ~5 mm cannula length and friction <0.1 N A maximum indentation force of 2N was achieved by manually applying a specific load of 300 g (reference force) onto the femur midshaft. One indentation measurement involving a 2N force being applied for 10 repeated cycles was carried out on each bone (Table 2-14) (195).

Table 2-14 Reference Point Indentation protocol for mouse femora

<table>
<thead>
<tr>
<th>Protocol: 2N Mouse Femur</th>
<th>Type Bone Probe 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Test Probe</td>
<td>90° cono-spherical with ≤5µm radius point</td>
</tr>
<tr>
<td>Reference Probe</td>
<td>Flat bevel with ~5mm cannula length</td>
</tr>
<tr>
<td>Fixture</td>
<td>Excised rodent bone fixture</td>
</tr>
<tr>
<td>Reference Force</td>
<td>260-310g</td>
</tr>
<tr>
<td>Indentation force</td>
<td>2N</td>
</tr>
<tr>
<td>Indentations per measurement (cycles)</td>
<td>10</td>
</tr>
<tr>
<td>Indentations per second (Hz)</td>
<td>2</td>
</tr>
</tbody>
</table>

To assure consistency between measurements RPI was carried out 6mm proximal to the femoral condyles on the posterior side, as data from my honours thesis showed a lower coefficient of variation was observed in this region compared to other cortical sites (Vrahnas C (2012) Influence of EphrinB2 Signalling on Bone Material Strength, Honours Thesis, La Trobe University).

Samples were kept partially hydrated throughout testing with phosphate buffered saline. Internal friction, defined as the force resisting motion between the test and reference
probe, was identified by the size and shape of the force-distance graph and kept at a constant of ≤0.3N to ensure that disruptions within the probe assembly would not affect results. Test measurements were taken pre- and post-experiment on polymerized methyl methacrylate (MMA) to ensure consistency. Data was discarded if graphs displayed high friction, or disruptions in the curves of the loading and unloading slopes.

The distance the probe travels into the bone (total indentation distance, TID) has been identified as a measure of resistance to fracture, indentation distance increase (IDI) is the indentation distance in the last cycle relative to the first, and has been correlated to bone tissue toughness (218).

2.7 Hydroxyproline assay

Hydroxyproline (4-hydroxyproline) is a non-proteinogenic amino acid formed by the post-translational hydroxylation of proline. Hydroxyproline is a major component of collagen, where it serves to stabilize the helical structure. Because hydroxyproline is largely restricted to collagen, the measurement of hydroxyproline levels in hydrolysed bone samples can be used as an indicator of collagen content.

Femoral samples were cleaned of muscle, flushed of marrow and cut with scissors at the mid-diaphysis. The distal half of the femur was weighed before and after dehydration in a 37°C incubator for one hour. Each sample was incubated overnight at 120°C in 1mL of 5M HCl per 20mg of bone. 50μl of each sample was used, and 6 serial dilutions of the hydroxyproline standard (1mg/mL) were used to generate a standard curve. 50μl of Chloramine T (Sigma-Aldrich) was added to each reaction and incubated at room temperature for 25 minutes. 500μl of Ehrlich’s Reagent (Dimethyl-amino-benzaldehyde (Sigma-Aldrich), n-propanol (99%, Sigma-Aldrich), perchloric acid (70%, AnalAR)) was added to each reaction and incubated at 65°C for 10 minutes (or longer if colour was not well developed). 100μl of each reaction was pipetted into a 96 well plate and absorbance measured using a POLARstar plate reader at 550nm and interpolated on the standard curve (219).
2.8 Bone mineralisation analysis by Fourier Transform Infrared Microscopy (FTIRM)

2.8.1 Sample preparation, scanning and spectral analysis

To investigate material composition of 12-week old female OsxCre.EfnB2 and Dmp1Cre.EfnB2 bones and PTH-treated male Dmp1Cre.EfnB2 control bones, sagittal sections were cut from tibiae embedded in methyl methacrylate (MMA) (Section 2.3.1).

FTIRM measurements of bone are usually performed in transmittance, where light is passed through the section, thus requiring preparation of 2-3µm thin sections. Sections were cut at 3µm to allow light to be transmitted through the section. Sections thicker than 3µm resulted in higher absorption peaks which increased the signal-to-noise ratio (220).

2.8.1.1 Synchrotron-based FTIRM (sFTIRM) transmission scanning protocol

For transmission scanning, 3µm sections were placed on 22mm diameter x 0.5mm polished barium fluoride (BaF₂) windows (Crystan Limited, UK). Spectra were collected in the mid-IR region from 750 to 3850 cm⁻¹ using a mercury cadmium telluride detector, at 8 cm⁻¹ and 128 scans/pixel spectral resolution in transmission mode using a Bruker Hyperion 2000 IR microscope coupled to a V80v FTIR spectrometer located at the IR Microspectroscopy beamline at the Australian Synchrotron. A matching background spectrum was collected through clear BaF₂. All data acquisition was undertaken with Bruker OPUS version 6.5 and data analysis completed with OPUS version 7.2.

For 12-week old female Dmp1Cre.EfnB2 mice and PTH-treated male controls, the microscope video camera was used to image the cortical diaphysis (1500 µm from the base of the growth plate), which was the same location used for histomorphometric measurements of periosteal mineral apposition (Figure 2-4B). (123). In both mouse models, sFTIRM mapping was performed with the synchrotron source, using a 15 x 15 µm aperture.
In my initial sFTIRM study, tibial samples from 12-week old female *OsxCre.EfnB2* and *Dmp1Cre.EfnB2* mice (Chapter 5) were imaged in 3 regions that commenced at the periosteal edge and progressed perpendicularly into the more mature bone of the cortex (Figure 2-4C). This analysis did not incorporate calcein labels on the periosteum. In my second sFTIRM study (Chapter 7), I utilized the epifluorescence accessory on the Hyperion microscope suitable for calcein visualisation (excitation/emission ~490nm/~515nm) to allow bone-age matched analysis in 12-week old PTH-treated male *Dmp1Cre.EfnB2* control mice. I made use of the double fluorescent calcein labels on the periosteal edge to mark bone ages equal to 2 days (region 1), 7 days (region 3), and an intermediate zone equally spaced between the two (region 2). Regions then progressed equidistant into the bone matrix (regions 4 – 6) (Figure 2-4D); based on the calcein labels, we termed these zones primary mineralization (regions 1-3) and secondary mineralization (regions 4-6) (Figure 2-4D). For each sample an additional MMA reference spectrum was collected from within the embedding material adjacent to the bone region assessed.
Figure 2-4 sFTIRM measurement regions on thin tibial sections.
(A) Representative micro-CT image of tibia indicating the depth at which tibial sections are taken from (white dotted line). (B) Transmitted visible light micrograph showing the region at which spectra were collected (1500 µm below the tibial growth plate on the medial side). Scale bar = 250 µm. (C) Representative fluorescence micrograph showing the 3 x 15 µm2 regions from where spectra were collected in 12 week-old female *OsxCre.EfnB2* and *Dmp1Cre.EfnB2* tibiae. Regions of analysis commenced at the periosteal edge and progressed a further 2 regions into the mature bone. Note: fluorescent labels were not used to mark bone ages in this study. Scale bar = 20 µm. (D) Representative fluorescence micrograph showing the 6 x 15 µm2 regions from where spectra were collected in 12 week-old PTH-treated male *Dmp1Cre.EfnB2* tibiae. Regions commenced at the periosteal edge on the calcein label indicating 2-day old mineralised bone (region 1), between the two labels (region 2), and on calcein label indicated 7-day old mineralised bone (region 3), then progressing into the more mature cortical bone (region 4-6). A methylmethacrylate (MMA) reading was taken in a blank area of section to subtract from bone spectra. Scale bar = 20 µm.
2.8.1.2 Transmission spectral analysis

Transmission spectra were analysed using OPUS version 7.2 by integrating the area of the following peaks: Phosphate (mineral) (916-1180-916 cm\(^{-1}\)), amide I (1588-1712 cm\(^{-1}\)), amide II (1600-1500 cm\(^{-1}\)) and carbonate (852-890 cm\(^{-1}\)) (Figure 2-5). Ratios of these integrated peak areas were calculated as follows: mineral:matrix ratio (221) (1180-916 cm\(^{-1}\)/588-1712 cm\(^{-1}\)), carbonate:mineral ratio (222) (1180-916 cm\(^{-1}\)/890-852 cm\(^{-1}\)) and amide I:II ratio (223) (1588-1712 cm\(^{-1}\)/1600-1500 cm\(^{-1}\)).

![Figure 2-5 A representative FTIR spectrum showing the main peaks analysed.](image)

Figure 2-5 A representative FTIR spectrum showing the main peaks analysed.
2.8.1.3  **Sub-peak analysis**

Absorbance spectra from 12-week old female *Dmp1Cre.EfnB2* mice were analysed for sub-peaks from the amide I, amide II and phosphate peaks by spectral curve fitting using Grams /AI (Version 9.2, Thermo Scientific, USA). Briefly, individual spectra were baseline corrected on either side of the amide I and II peaks for 1660 cm\(^{-1}\) and 1690 cm\(^{-1}\) sub-bands and on either side of the phosphate peak for 1030 cm\(^{-1}\), 1020 cm\(^{-1}\), 1127 cm\(^{-1}\) and 1096 cm\(^{-1}\) sub-bands (Figure 2-6). The second-derivative of each peak was used to estimate sub-peak positions that were then curve-fitted using 500 maximum iterations with an auto limit fix function. Sub-peak integrated areas were reported as collagen crosslinking (1660:1690 cm\(^{-1}\)) \(^{(2)}\) (Figure 2-6A) and crystallinity (1030/1020 cm\(^{-1}\)) \(^{(4)}\) (Figure 2-6B). Due to the high scanning resolution (8cm\(^{-1}\)), ± 5 wavenumbers were allowed to select for the peak position. The carbonate peak could not be performed because the peak was too small to allow spectral curve fitting. Advice for sub-peak analysis was provided by Dr. Eleftherios Paschalis (Ludwig Boltzmann Institute of Osteology, Vienna, Austria) and the sub-peak analysis was performed by Mr. Thomas A. Pearson.
Figure 2-6 Examples of spectral curve-fitting from amide I, amide II and phosphate peaks.
(A) Amide I and II peaks curve-fitted to obtain 1660 and 1690 cm$^{-1}$ sub peaks (collagen crosslinking) (2). (B) Phosphate peak curve-fitted to obtain 1030 and 1020 cm$^{-1}$ sub peaks (crystallinity) (4).
2.8.1.4  **FTIR imaging (FTIRI)**

In addition to our sFTIRM analysis, FTIR imaging (FTIRI) was applied to the same 3µm tibial sections from 12-week old female *Dmp1Cre.EfnB2* mice. Tissue sections were scanned in Dr. Cyril Petibois’ laboratory (University of Bordeaux) using a Hyperion 3000 spectral imaging system equipped with a Vertex-70 spectrometer (Bruker, Germany), a liquid-N\textsubscript{2} cooled focal plane array (FPA: 128x128 elements; 40x40 microns each) detector and a Globar source. The FPA detector was continuously maintained at liquid-N\textsubscript{2} temperature by an automated refilling system (Norhof LN2-cooling system #606; Maarssen, Netherland). The microscope and spectrometer were also continuously N\textsubscript{2}-purged and an insulation box protected the sample stage from ambient air. For all FTIR image acquisitions, a 15x magnification level and condenser were used. High-resolution FTIR images were obtained for microscopic analysis of tissue sections; 200 scans and an 8 cm\textsuperscript{-1} spectral resolution were used for image acquisitions (140 ms FPA detector exposure per scan; spectral range = 3800-900 cm\textsuperscript{-1}). The cortical diaphysis was imaged 1500µm from the base of the growth plate and images were taken using a 100 x 100µm aperture 100µm above and below the 1500 µm mid-point. Coupling the FTIR microscope with two different polarising filters allowed us to observe the molecular orientation of collagen fibres relative to the plane of the tissue section and determine collagen distribution. The 0° polarising filter was used to measure bonds “in plane” (orientation of bonds parallel to the bone section). The 90° polarising filter was used to measure bonds “out of plane” or the orientation of bonds perpendicular to the bone section. All FTIR images had an individual pixel dimension of 2.6x2.6 µm, thus at \(\sim \lambda/2\) for the mid-IR spectral interval. All infrared images were obtained in transmission mode. The images were obtained from sub-routines of the Opus 7.5 software (Bruker-Optics, France).
2.9 Backscatter scanning electron microscopy (BSEM)

To quantify osteocyte lacunae size and density, 100µm thick sections from the femoral midshaft, obtained as described in section 2.3.1.3, were scanned using a FEI Quanta FEG 200 backscattered scanning electron microscope (BSEM) from the Advanced Microscopy Facility in Bio21. Sections were placed on anodised aluminium SEM mounts (Proscitech, QLD, Australia) using double-sided sticky carbon tape (Proscitech, QLD, Australia) to provide conductivity and a uniform dark background. Carbon coating on sections was not used. With the assistance of Mr. Roger Curtain, the osteocyte lacunae were visualised on the femoral cross-sections and imaged on the anterior and posterior side of the femur at 500x magnification (Figure 2-7). Imaging was performed at low vacuum using water at a 9.8mm working distance. The BSE detector was a solid state backscatter.

Lacunae were quantified using MetaMorph (v7.8.3.0; Molecular Devices; Sunnyvale, CA, USA) by establishing an inclusive threshold for dark objects (0, 76) to distinguish bone from background. Integrated Morphometry Analysis (IMA) was applied with a 28-280 pixel filter for lacunae area, thus any objects below (eg. cracks) or above (eg. blood vessels) this range was excluded from measurements.

The threshold parameter included the light area of each image (cortical bone that does not include lacunae area) which was combined with total lacunar area to calculate the total bone area in pixels. The parameters measured included osteocyte lacunar size, the largest 20% of osteocyte lacunar size and osteocyte lacunar density which were obtained in pixels that were converted to µm using the conversion of 0.294695µm/pixel for an image captured at 500x magnification.
Figure 2-7 Representative backscattered scanning electron microscopic image of femoral thick section and regions of analysis.
2.10 Transmission electron microscopy (TEM)

TEM sample preparation, fixation, infiltration and embedding were conducted with the help of Ms. Liliana Tatarczuch from the Faculty of Veterinary Science, The University of Melbourne.

2.10.1 Sample preparation

Both tibiae were collected from 3 12-week old female $Dmp1\text{Cre.EfnB}2^{\text{w/w}}$ and 3 $Dmp1\text{Cre.EfnB}2^{\text{f/f}}$ mice. Tibiae were cut in half longitudinally with a scalpel and placed in TEM fixative (Section 2.10.1.1) for a minimum of 24 hours - 2 days.

2.10.1.1 TEM fixative (Karnovsky’s fixative)

A total volume of 150 ml TEM fixative was prepared by combining 60 ml of 4% paraformaldehyde (PFA), 60 ml of 0.2M sodium cacodylate buffer and 30ml of glutaraldehyde (yielding a final concentration of 2% PFA, 0.1M sodium cacodylate and 5% glutaraldehyde). Each sample was fixed in a volume of 20ml.

2.10.1.2 Decalcification

After two days, tibiae were decalcified for two weeks in 50 ml of 0.15M ethylenediaminetetraacetic acid (EDTA) and 0.1M sodium cacodylate. Solutions were changed twice a week. Samples were then washed (4x 30 minutes) in 10ml of 0.1M sodium cacodylate buffer and stored at 4°C overnight.

2.10.1.3 Washing and post-fixation

The next day, samples were washed (2x 15 minutes) in 0.1M sodium cacodylate. The samples were then post-fixed in 1% osmium tetroxide and 1.5% potassium ferrocyanide. After 2 – 5 hours, samples were washed (2x 5 minutes) and stored at 4°C in 0.1M sodium cacodylate.

2.10.1.4 Dehydration and infiltration

Samples were washed again (2x 15 minutes) in 0.1M sodium cacodylate and then washed (2x 15 minutes) with H$_2$O. Each sample proceeded through the following dehydration
steps: 70% acetone (2x 30 minutes), 95% acetone (2x 30 minutes), µ% acetone (4x 30 minutes). After dehydration, samples were put in an infiltration mixture of acetone and Spurr’s hard resin 1:1 overnight. Spurr’s resin was made up of a 20g ERL, 8g Di glycidyl ether of polypropylene glycol (DER) and 52g Nonenyl succinic anhydride (NSA) mixture where 0.8g Dimethylaminoethanol (DMAE) was added last. Resin was also used to make moulds for embedding and stored in a 60°C incubator overnight. The next day samples were changed to a 1:2 mixture of acetone and resin for infiltration during the day and then changed to pure resin to infiltrate overnight.

2.10.1.5 Infiltration and embedding

The pure resin was changed twice during the day every 2-3 hours. Samples were then embedded in the pre-filled moulds and stored in a 60°C incubator for 3 days to allow resin to cure.

Note: All steps during washing, post fixation, dehydration and infiltration were done on a rotator at room temperature.

2.10.2 Sectioning and scanning

TEM sectioning and scanning was conducted by Mika Ikegame, Okayama University, Japan. Ultrathin sections (~100 nm thickness) were obtained with an ultramicrotome (Ultracut E, Reichert-Jung (Leica microsystems, Wetzlar, Germany) were placed on collodion-coated copper grids. Sections were stained with uranyl acetate and lead citrate to enhance the contrast, as well as tannic acid to observe collagen fibres.

Scanning of sections were done on two different microscopes under H-7100 (Hitachi High-Technologies Corporation, Tokyo, Japan) using a CCD camera with TEM films.
2.11 Statistical analysis

Statistically significant differences were determined by an unpaired one-tailed t-test where only one comparison was being made. One-tailed t-test allowed for testing statistical significance when a significant change in one direction was observed. One-tailed t-test allowed for testing statistical significance when a significant change in one direction was observed. Two-way ANOVA followed by Fisher’s LSD post hoc test was used for sFTIRM analysis when comparing regions within one genotype or when comparing regions between genotypes (Chapters 4 and 5) and for comparing PTH effects between genotypes and genotype effects between sexes (Chapters 7). All data is presented as mean ± SEM. p<0.05 was considered statistically significant.

All statistical analyses were performed using GraphPad Prism 6.
CHAPTER 3: Bone quality and strength in mice lacking PTH target gene glycoprotein 130 (gp130)

3.1 Introduction

The amounts of mRNA for interleukin 6 (IL-6) family cytokine and receptor mRNA levels generated within the osteoblast lineage are rapidly increased by parathyroid hormone (PTH). These include Il6, Il11, oncostatin M receptor (Osmr), leukemia inhibitory factor (Lif), and cytokine receptor-like factor 1 (Crlf1) (175, 224). These cytokines and receptors all form signalling complexes with the co-receptor glycoprotein 130 (gp130) (158). Gp130 itself is also stimulated by PTH within the osteoblast lineage (156). Several cytokine-specific knockout mouse models have demonstrated necessary roles for each of these cytokines in the physiological regulation of longitudinal growth, periosteal expansion and trabecular structure (156, 176, 177, 215, 225-227). IL-6 family cytokines can act on osteoblasts at all stages of osteoblast differentiation, however the relevant stages at which they support bone formation and osteoclastogenesis are not established. To determine the stage-specific roles of gp130 in the osteoblast lineage during bone modelling and remodelling, our laboratory generated two mouse models with gp130 conditionally deleted either from the entire osteoblast lineage using the OsxCre transgene (Osx1Cre.gp130<sup>f/f</sup>) or in late osteoblasts/osteocytes using the Dmp1Cre transgene (Dmp1Cre.gp130<sup>f/f</sup>) (180). The two mouse models had similar phenotypes that included both a low trabecular bone formation rate and a low trabecular bone mass indicating that the key cell in which gp130 controls trabecular bone formation is the osteocyte (180).

In Dmp1Cre.gp130<sup>f/f</sup> mice, lower osteoblast number and reduced Sp7, Col1a1 and Bglap mRNA levels were found, indicating an important role for gp130 in osteocytes that promotes osteoblast differentiation (180). Despite their lower level of bone formation on trabecular surfaces, femora from 26-week old Dmp1Cre.gp130<sup>f/f</sup> mice demonstrated a different effect in their cortical bone structure (180). We observed greater periosteal
perimeter and cross-sectional moment of inertia in 26-week old \textit{Dmp1Cre.gp130^{w/w}} mice which led us to examine in the work of this chapter whether these structural differences affected bone strength and quality.

### 3.2 Specific Methods

Bone strength was measured using 3-point bending tests conducted on femora from male and female 26-week old \textit{Dmp1Cre.gp130^{w/w}} and \textit{Dmp1Cre.gp130^{f/f}} mice (Section 2.6). Prior to mechanical testing, anteroposterior width and mediolateral width were measured on the same bones on the femoral midshaft using digital callipers (Section 2.6.1). Following mechanical testing, all fragments of the broken femora were flushed of marrow and used for hydroxyproline assay in hydrolysed samples to measure collagen content (Section 2.7).

To assess collagen orientation, femora from 12-week old male \textit{Dmp1Cre.gp130} mice were embedded in plastic and thick 100µm sections were cut and assessed by polarized light microscopy and histomorphometry (Section 2.3.5.1).
3.3 Results

3.3.1 Cortical bone structure and strength in *Dmp1Cre.gp130* mice

Sex-specific differences were observed in 26 week old *Dmp1Cre* controls, where males demonstrated significantly wider bones in the mediolateral, but not anteroposterior direction compared to female *Dmp1Cre* controls (Figure 3-1A, B). Consistent with their greater periosteal perimeter (Ps.Pm) and mean polar moment of inertia (MPMI) (Figure 5A- from (180)), femora from 26 week-old female *Dmp1Cre.gp130<sup>f/f</sup>* mice displayed greater anteroposterior (AP) width compared to sex-matched *Dmp1Cre.gp130<sup>w/w</sup>* controls while femora from male *Dmp1Cre.gp130<sup>f/f</sup>* mice demonstrated greater anteroposterior and mediolateral widths (Figure 3-1A, B).

![Figure 3-1](image.png)

**Figure 3-1** Widths of femora from 26-week old female and male *Dmp1Cre.gp130<sup>w/w</sup>* (w/w) and *Dmp1Cre.gp130<sup>f/f</sup>* (f/f) mice.
(A) Anteroposterior and (B) mediolateral widths were measured for each bone by digital callipers at the femoral midshaft. Data are mean ± SEM, n = 5-8/group, *p<0.05, ***p<0.001 vs. *Dmp1Cre.gp130<sup>w/w</sup>* controls, ++ p<0.01 vs. female *Dmp1Cre.gp130<sup>w/w</sup>* controls.
Normal patterns of sex-specific changes in bone strength were observed. Femora from 26 week old male \textit{Dmp1Cre} controls showed significantly lower ultimate load and deformation, force at yield, post-yield deformation, stiffness and energy absorbed to failure compared to female \textit{Dmp1Cre} controls (Table 3-1). Normalisation for changes in bone geometry demonstrated significantly lower ultimate strain (Table 3-1), ultimate stress and toughness (Figure 3-2A, B) in femora from male \textit{Dmp1Cre} controls compared to female \textit{Dmp1Cre} controls.

Prior to normalisation for the observed changes in bone geometry, no difference in response to mechanical load was observed in femora from female or male \textit{Dmp1Cre.gp130}^{+/f} mice compared to \textit{Dmp1Cre.gp130}^{+/w} controls (Figure 3-3A). However, after normalisation for bone geometry, femora from \textit{Dmp1Cre.gp130}^{+/f} male mice appeared more compliant and withstood less stress than those from sex-matched \textit{Dmp1Cre} controls, as indicated by the average stress-strain curves (Figure 3-3B). Data analysis of the curves revealed that both female and male \textit{Dmp1Cre.gp130}^{+/f} femora demonstrated a significantly lower elastic modulus compared to controls (Figure 3-2C). In addition, deletion of gp130 in late osteoblasts/osteocytes in males resulted in a significantly greater mean polar moment of inertia compared to controls (Table 3-1). Ultimate and yield stress were significantly lower in male \textit{Dmp1Cre.gp130}^{+/f} femora compared to controls (Figure 3-2A, D). Toughness was not significantly affected by genotype in either sex (Figure 3-2B).
Table 3-1 Structural and material properties of femora from female and male *Dmp1Cre.gp130* 26-week old mice determined by 3-point bending tests.

<table>
<thead>
<tr>
<th></th>
<th>Female</th>
<th>Male</th>
</tr>
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<tbody>
<tr>
<td></td>
<td><em>Dmp1Cre.gp130</em> (n=5)</td>
<td><em>Dmp1Cre.gp130</em> (n=7)</td>
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<tr>
<td></td>
<td></td>
<td><em>Dmp1Cre.gp130</em> (n=8)</td>
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<td></td>
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<td><em>Dmp1Cre.gp130</em> (n=8)</td>
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<tr>
<td><strong>Structural properties</strong></td>
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<td></td>
</tr>
<tr>
<td>Ultimate force (N)</td>
<td>24.4 ± 1.3</td>
<td>19.1 ± 1.5*</td>
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<td></td>
<td>26.0 ± 2.2</td>
<td>19.0 ± 0.9</td>
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<tr>
<td>Ultimate deformation (mm)</td>
<td>0.22 ± 0.02</td>
<td>0.17 ± 0.02*</td>
</tr>
<tr>
<td></td>
<td>0.24 ± 0.03</td>
<td>0.16 ± 0.01</td>
</tr>
<tr>
<td>Moment of inertia (mm⁴)</td>
<td>0.67 ± 0.06</td>
<td>0.74 ± 0.09</td>
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<tr>
<td></td>
<td>0.83 ± 0.05</td>
<td>1.55 ± 0.17***</td>
</tr>
<tr>
<td>Force at Yield (N)</td>
<td>15.18 ± 0.56</td>
<td>12.72 ± 0.92*</td>
</tr>
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<td>15.36 ± 0.93</td>
<td>13.34 ± 0.82</td>
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<td>Deformation at Yield (µm)</td>
<td>110 ± 4</td>
<td>109 ± 6</td>
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<td></td>
<td>141 ± 18</td>
<td>112 ± 4</td>
</tr>
<tr>
<td>Post-yield deformation (µm)</td>
<td>108 ± 18</td>
<td>56 ± 11*</td>
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<tr>
<td></td>
<td>97 ± 20</td>
<td>52 ± 13</td>
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<tr>
<td>Stiffness (N/mm)</td>
<td>139 ± 3</td>
<td>118 ± 7*</td>
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<tr>
<td></td>
<td>116 ± 12</td>
<td>119 ± 5</td>
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<tr>
<td>Energy absorbed to failure (mJ)</td>
<td>2.83 ± 0.40</td>
<td>1.49 ± 0.27*</td>
</tr>
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<td>2.81 ± 0.45</td>
<td>1.48 ± 0.30</td>
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<td><strong>Material properties</strong></td>
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<tr>
<td>Ultimate strain (%)</td>
<td>4.97 ± 0.42</td>
<td>3.64 ± 0.32*</td>
</tr>
<tr>
<td></td>
<td>5.72 ± 0.57</td>
<td>4.24 ± 0.39</td>
</tr>
</tbody>
</table>

Data shown are mean ± SEM, where *p*<0.05 vs. female *Dmp1Cre.gp130* controls and *** *p*<0.001 vs. sex-matched *Dmp1Cre.gp130* controls.
Figure 3-2 Material properties of 26 week old w/w ($Dmp1Cre.gp130^{w/w}$) and f/f ($Dmp1Cre.gp130^{ff}$) femora determined by 3 point bending tests.

(A) Ultimate stress, (B) Toughness, (C) Elastic modulus and (D) Yield stress. Data shown are mean + SEM, n = 5-8/group, +p<0.05, ++p<0.01 vs. female $Dmp1Cre.gp130^{w/w}$ controls, **p<0.01, ***p<0.001 vs. sex-matched $Dmp1Cre.gp130^{w/w}$ controls.
Figure 3-3 Average Load-Deformation and Stress-Strain curve of 26-week old female and male Dmp1Cre.gp130 femora generated from 3-point bending tests. Each dot point represents the average load, deformation, stress and strain for the noted sample group. Error bars were excluded to highlight the shape of curves. Data shown are mean, n = 5-8/group.
3.3.2 Collagen fibre organisation in male *Dmp1Cre.gp130*<sup>ff</sup> femora

Cortical bone from *Dmp1Cre.gp130*<sup>ff</sup> mice showed a significantly higher proportion of disorganized woven bone in the femoral metaphysis compared to lamellar bone matrix in the proximal femur compared to controls (Figure 3-4). This can be observed in the representative images (Figure 3-5).

![Graph showing proportion of lamellar and woven bone](image)

**Figure 3-4** Proportion of lamellar and woven bone in femora from 12-week old male *Dmp1Cre.gp130* mice assessed by polarised light microscopy.

Percentage bone area from thick transverse sections collected approximately 500µm from the base of the femoral condyles of the distal femur. Data shown are mean + SEM, n = 8-9/group, **p<0.01 vs. *Dmp1Cre.gp130*<sup>w/w</sup> controls.
Figure 3-5 Representative images of the proportion of lamellar vs. woven bone in 12-week old male (A) Dmp1Cre.gp130<sup>w/w</sup> and (B) Dmp1Cre.gp130<sup>ff</sup> femora assessed by polarised light microscopy.

White arrows indicate woven bone, grey arrows indicate lamellar bone. Scale bars represent 100 microns.
Despite a greater proportion of woven bone, potentially indicating a less mature bone matrix, hydrolysates of femora from 26-week old \textit{Dmp1Cre.gp130}\textsuperscript{ff} mice did not reveal any significant alteration in hydroxyproline content in female or male \textit{Dmp1Cre.gp130}\textsuperscript{ff} femora, compared to their respective controls (Figure 3-6).

\textbf{Figure 3-6} Hydroxyproline levels in hydrolysates of female and male 26-week old \textit{Dmp1Cre.gp130} femora.
Data shown are mean + SEM, n= 5-8/group.
3.4 Discussion

This study demonstrated that deletion of gp130 in late osteoblasts/osteocytes (Dmp1Cre.gp130f/f) resulted in femora with reduced material strength and a greater proportion of woven bone. This indicated that gp130 in late osteoblasts/osteocytes maintains the material strength of the cortical bone matrix and modifies collagen organisation. These mice also exhibited greater femoral width, which may have occurred to compensate for the material defect.

The impairment in bone quality in 26-week old Dmp1Cre.gp130f/f mice compared to controls suggests that cortical bone strength is regulated by gp130 signalling in late osteoblasts/osteocytes. Greater anteroposterior and mediolateral width, as well as greater mean polar moment of inertia, of the femoral diaphysis were observed in 26-week old male Dmp1Cre.gp130f/f mice compared to controls. The greater femoral dimension was confirmed by significantly greater periosteal perimeter and marrow area, a phenotype also observed at 12 weeks of age. However, no detectable changes in periosteal mineral apposition rate or periosteal bone formation rate were found (180), suggesting that the periosteal expansion in these bones has occurred slowly over a long period of time. Bone length remained unchanged (180) suggesting that this role of gp130 in maintaining bone size is specific to control of bone width.

Bones from 26-week old male Dmp1Cre.gp130f/f mice withstood less stress compared to controls when normalised for changes in bone size indicating a defect in material strength. This material defect was demonstrated by a greater proportion of woven bone. Woven bone, which is laid down rapidly, displays a random orientation of collagen fibres (228) while lamellar bone is laid down more slowly, creating a defined thick ordered structure. The loose structure and random orientation of woven bone suggests that it is mechanically weaker than lamellar bone (126, 229). This has been shown in regions that contain mostly woven bone which were associated with lower elasticity and lower resistance to penetration (microhardness) (127). A higher proportion of abnormal woven bone observed in patients with Paget’s disease resulted in increased bone fragility despite increased bone density and size (230). This confirms that greater proportion of woven
bone in male Dmp1Cre.gp130\textsuperscript{f/f} femora may be at least one cause of their greater bone fragility. Despite their reduction in material strength, the bending load required to cause fracture in Dmp1Cre.gp130\textsuperscript{f/f} mice remained unchanged, indicating that the greater diaphyseal dimensions may have retained fracture resistance and mechanical strength. The Mov13 mouse, which also shows a greater proportion of woven bone, is a model of mild osteogenesis imperfecta (OI) model with reduced type I collagen production (231). Mov13 mice, like the Dmp1Cre.gp130\textsuperscript{f/f} mice, display a greater proportion of woven bone which is compensated for by increasing periosteal apposition leading to an increase in mechanical integrity (232). This similar structural adaptation confirms that the increase in bone width in Dmp1Cre.gp130\textsuperscript{f/f} mice is a mechanism to compensate for poor material integrity.

As no significant change in tissue mineral density assessed by micro-CT (Supporting figure 6A from (180)) or periosteal mineral apposition rate was detected, the poor material strength in Dmp1Cre.gp130\textsuperscript{f/f} mice was like due to a defect in collagen deposition rather than altered mineralisation. This was further supported by a lower Col1a1:Col1a2 mRNA ratio compared to controls (180). Type I collagen consists of two α1 and one α2 polypeptide chains assembled in a triple helix (49) and the ratio of Col1a1:Col1a2 normally exists in a 2:1 ratio at the protein level (233). The altered ratio was caused by significantly lower Col1a1 mRNA expression level without any change in Col1a2 mRNA levels suggesting the dysfunctional synthesis of the triple helical structure of collagen (180).

Alteration in the distribution of mineral may also contribute to the poor material strength of Dmp1Cre.gp130\textsuperscript{f/f} mice. Even though tissue mineral density assessment by micro-CT may not be a sensitive technique to detect any subtle changes that may have occurred in these mice (234), the lower osteocalcin mRNA levels detected in Dmp1Cre.gp130\textsuperscript{f/f} mice than in controls and the fact that osteocalcin-knockout mice at 6 months of age exhibit lower mineral:matrix and carbonate:mineral ratios than controls (235) suggests that Dmp1Cre.gp130\textsuperscript{f/f} mice may also have altered mineralisation. We plan to examine whether the greater proportion of woven bone in Dmp1Cre.gp130\textsuperscript{f/f} is associated with
changes in mineral or matrix composition by synchrotron-based Fourier Transform Infrared Microscopy (sFTIRM).

The reduced ability of bone material to return to its original shape when an applied stress is removed in female $Dmp1:\text{Cre}.gp130^{ff}$ mice was shown by their reduced elastic modulus, normalised for bone size, compared to their gp130-replete controls. Consistent with a more minor change, female $Dmp1:\text{Cre}.gp130^{ff}$ mice showed a compensatory increase in bone width only in the anteroposterior direction. The more minor change in material strength in female $Dmp1:\text{Cre}.gp130^{ff}$ mice may relate to the baseline sex-difference in bone structure, that female mice have more slender femora than males. Previous work has demonstrated that slender bones have a higher degree of mineralisation and tissue mineral density to compensate for their smaller bone size (236); we also observed significantly higher tissue mineral density of in female mice (both $Dmp1:\text{Cre}.gp130^{ff}$ and control) compared to males (180). The higher mineral content in the female bones may therefore provide some protection against the effect of gp130 deletion on bone stiffness.

Additional expected effects of sex differences on bone size and strength were observed in control mice. The smaller bone width in female compared to male controls mentioned above is consistent with studies that show greater bone width in male bones due to greater periosteal growth, while females demonstrate greater bone loss on the endosteal surface (38, 39). The greater outward (periosteal) distribution in males confers greater mechanical strength compared to females (38, 237-239) and contributes to lower fracture rates in elderly men compared to women (237, 240-242). These differences in periosteal apposition and endosteal resorption between males and females could also account for the greater cortical expansion in male $Dmp1:\text{Cre}.gp130^{ff}$ mice compared to $Dmp1:\text{Cre}.gp130^{ff}$ female mice as their baseline cortical width would have been greater in male controls compared to female controls. Although wider bones are less susceptible to damage, slender bones can compensate for their smaller bone size by increasing their tissue mineral density (236). While increases in mineralisation are suggested to result in a more brittle, damageable material that would cause increased fragility (243), the greater
mineralisation in female controls increases their bone stiffness. The ultimate load and work required to cause fracture were also greater in female controls, making them ultimately stronger than male controls. Therefore, female mice compensate for their slender bone by increasing mineralisation to improve their bone strength.

Earlier deletion of gp130 in osteoblasts (OsxCre.gp130<sup>−/−</sup>) also resulted in bones with a larger periosteal circumference in male mice. This confirms the above finding in the Dmp1Cre.gp130<sup>−/−</sup> mice.

It should be noted that Dmp1Cre expression is not exclusive to osteocytes (as discussed in section 1.3.2.2) and Cre-mediated recombination in muscle may also contribute to the increased bone width observed in 26-week old male Dmp1Cre.gp130<sup>−/−</sup> mice. However, since these observations were made in both Dmp1Cre.gp130<sup>−/−</sup> mice and in 12-week old male OsxCre.gp130<sup>−/−</sup> mice, and in 12- and 26- week old female OsxCre.gp130<sup>−/−</sup> mice (<sup>180</sup>), changes in the muscle seems unlikely to be a major contributor.

Overall, this work demonstrates that in addition to the role of gp130 signalling in osteocytes to stimulate trabecular bone formation, gp130 in osteocytes also maintains bone quality in adult cortical bone.
CHAPTER 4: Bone quality and strength in mice lacking PTH target gene EphrinB2 within the osteoblast lineage

4.1 Introduction

EphrinB2 is stably expressed throughout all stages of osteoblast differentiation and is significantly upregulated by PTH treatment (157). The interaction of ephrinB2 with its receptor EphB4 is required as a checkpoint through which the differentiating osteoblast must pass for continued differentiation in vitro (157) and in vivo (194). Systemic ephrinB2:EphB4 inhibition in vivo caused an increased number of osteoblasts and greater osteoid production without any change to the bone mineralisation rate. Additionally, mRNA levels of early osteoblast markers were upregulated with no change in late stage osteoblast markers (194). This accumulation of matrix-producing osteoblasts with reduced ability to fully differentiate and initiate mineralisation suggested that the ephrinB2:EphB4 interaction was not required for osteoblasts to produce osteoid matrix but is required for osteoblasts to pass into later stages of differentiation that promote osteoid mineralisation.

Work conducted prior to my PhD showed that deletion of ephrinB2 in the osteoblast lineage directed by OsxCre (OsxCre.EfnB2<sup>−/−</sup>) resulted in greater osteoblast numbers and osteoid thickness than OsxCre.EfnB2<sup>+/+</sup> controls in female mice at 12 weeks of age. This suggested that greater number of osteoblasts from OsxCre.EfnB2<sup>−/−</sup> mice still produce a normal amount of matrix (195). However, bone formation rate was reduced by more than half in these mice, causing a doubling of the mineralisation lag time (MLT), which is the time between osteoid deposition and mineralisation (244). Additionally, cortical tissue mineral density assessed by micro-CT remained unchanged in OsxCre.EfnB2<sup>−/−</sup> mice compared to controls (Figure 3F from (195)). This suggested that ephrinB2-deficient osteoblasts have impaired mineralising ability whereby the osteoid deposition stage was normal but the initiation of the mineralisation process was delayed.

The delayed bone mineralisation in female OsxCre.EfnB2<sup>−/−</sup> mice was associated with a higher level of osteoblast and osteocyte apoptosis detected by TUNEL staining and
transmission electron microscopy (TEM). OsxCre.EfnB2/ff osteoblasts also demonstrated increased sensitivity to proapoptotic agents (195). In contrast to the several mouse models that have high levels of osteoblast apoptosis with lower osteoblast numbers on trabecular bone (155, 245-247), female OsxCre.EfnB2+/f mice displayed significantly greater numbers of osteoblasts as identified histologically (195). These cells could not be distinguished as apoptotic cells by normal histomorphometry at the light microscope level, and were counted as normal active osteoblasts. This may have accounted for the higher osteoblast number detected in female OsxCre.EfnB2+/f mice. Since osteoblasts in the late stages of differentiation, including osteocytes, regulate the maturation and mineralisation of osteoid, apoptosis before osteoblasts reach this stage could explain that delayed mineralisation. Osteoblast and osteocyte apoptosis is a critical determinant of bone strength. This has been shown in mice with a blockade of glucocorticoid action by overexpression of the 11β-hydroxysteroid dehydrogenase type 2 (11β-HSD2) enzyme under the control of the osteocalcin promoter. Glucocorticoid treatment in wild-type mice increased osteoblast apoptosis but this was prevented in transgenic mice, an effect that preserved bone strength. This indicated that glucocorticoid-induced loss of bone strength was in part due to increased death of osteocytes (248). Greater osteoblast and osteocyte apoptosis due to unloading on the skeleton by hind limb suspension (247) have also led to reduced vertebral compressive strength. This suggested that without the anti-apoptotic role of ephrinB2, osteoblasts undergo apoptosis rather than differentiating into the fully mature stages to promote osteoid mineralisation. In this chapter, I assessed whether the subsequent delay in mineralisation in OsxCre.EfnB2+/f mice compromised bone strength and quality.
4.2 Specific Methods

Bone strength was measured by 3-point bending tests of femora from 12 week old female and male OsxCre.EfnB2\textsuperscript{w/w} and OsxCre.EfnB2\textsuperscript{f/f} mice. Prior to mechanical testing, anteroposterior (AP) and mediolateral (ML) widths were measured at the femoral midshaft using digital callipers (Section 2.6.1).

Following mechanical testing, broken femora from female and male OsxCre.EfnB2\textsuperscript{w/w} and OsxCre.EfnB2\textsuperscript{f/f} mice were flushed of marrow and hydrolysed for a hydroxyproline assay to measure collagen content, as described in Section 2.7.

To measure local bone material properties, reference point indentation (RPI) was performed on the femoral cortical midshaft of 12-week old female and male OsxCre.EfnB2 mice, as described in Section 2.6.2.

To assess collagen orientation, femora from 6-week old female OsxCre.EfnB2 mice were embedded in plastic and thick 100µm sections were cut and assessed by polarized light microscopy as described in Section 2.3.5.

To examine bone composition, 3 µm sagittal sections of methyl methacrylate (MMA)-embedded tibiae from 12-week old female OsxCre.EfnB2 mice were analysed by sFTIRM, as described in Section 2.8.
4.3 Results

4.3.1 Cortical bone structure and strength in OsxCre.EfnB2 mice

12-week old male OsxCre controls displayed wider femora at the midshaft in both the anteroposterior and mediolateral directions compared to female OsxCre controls (Figure 4-1A, B). Female OsxCre.EfnB2^ff^ femora demonstrated significantly lower mediolateral width compared to sex-matched controls, but this was not significantly different in the anteroposterior direction (Figure 4-1B). Bone width of male OsxCre.EfnB2^ff^ femora was not significantly different compared to sex-matched OsxCre controls.

Figure 4-1 Cortical width of femora from 12 week old female and male OsxCre.EfnB2 femora.

(A) Anteroposterior and (B) Mediolateral width was measured for each bone by digital callipers at the femoral midshaft. Data shown are mean + SEM, n = 8-11/group. ++p<0.01, +++p<0.001 vs. female OsxCre.EfnB2^w/w^ controls, *p<0.05 vs. sex-matched OsxCre.EfnB2^w/w^ controls.
Sex-specific differences were observed in the mechanical properties of OsxCre control mice. 3-point bending tests demonstrated that while with the same force was required to fracture the femora from 12-week old male and female OsxCre controls (Figure 4-2A), male control bones were able to deform more before breaking compared to female OsxCre controls (Figure 4-2B). While stiffness was not different between sexes (Figure 4-2C), bones from male OsxCre controls absorbed more energy before breaking (Figure 4-2D) and had a greater moment of inertia (Table 4-1). Sex-specific differences in material strength were also observed. In 12-week old male OsxCre controls maximum stress was significantly lower (Figure 4-3A) while ultimate strain was significantly greater (Figure 4-3B) than female controls. The yield points for stress and strain demonstrated a similar pattern (Table 4-1). Elastic modulus was significantly lower in male OsxCre controls compared to female controls (Figure 4-3C), but work required to cause fracture remained unchanged (Figure 4-3D).

The ephrinB2 deficient genotype was also associated with differences in bone strength. Femora from 12-week old female OsxCre.EfnB2\(^{\text{f/f}}\) mice reached a greater ultimate deformation before failure (Figure 4-2B) and showed lower bone stiffness (Figure 4-2C) compared to sex-matched OsxCre.EfnB2\(^{\text{w/w}}\) controls. While the force at yield point was lower, post-yield deformation and toughness were both significantly greater in femora from female OsxCre.EfnB2\(^{\text{f/f}}\) mice compared to sex-matched controls (Table 4-1, Figure 4-3D). Moment of inertia was also significantly lower in female OsxCre.EfnB2\(^{\text{f/f}}\) femora compared to controls (Table 4-1). No significant differences were observed in bone strength of male OsxCre.EfnB2\(^{\text{f/f}}\) femora compared to their sex-matched controls measured by 3 point bending.

The average load-deformation curve highlights the greater deformation in bones of female OsxCre.EfnB2\(^{\text{f/f}}\) mice compared to controls (Figure 4-4A). After normalisation for bone geometry, femora from OsxCre.EfnB2\(^{\text{f/f}}\) female mice demonstrated greater bone toughness than those from sex-matched Dmp1Cre controls, as indicated by the area under the average stress-strain curves (Figure 4-4Figure 3-3B).
Figure 4-2 Structural properties of 12-week old female and male OsxCre.EfnB2 femora assessed by 3-point bending tests.
(A) Ultimate force, (B) Ultimate deformation, (C) Stiffness and (D) Energy absorbed to failure. Data shown are mean ± SEM, n = 8-10/group, *p<0.05 vs. female OsxCre.EfnB2<sup>w/w</sup> controls, +p<0.05 vs. sex-matched OsxCre.EfnB2<sup>w/w</sup> controls.
Table 4-1 Structural and material properties of 12-week old female and male OsxCre.EfnB2 femora assessed by 3-point bending tests.

<table>
<thead>
<tr>
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<th>Female</th>
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<th>Male</th>
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<tbody>
<tr>
<td></td>
<td>OsxCre.EfnB2&lt;sup&gt;w/w&lt;/sup&gt; (n=8)</td>
<td>OsxCre.EfnB2&lt;sup&gt;f/f&lt;/sup&gt; (n=10)</td>
<td>OsxCre.EfnB2&lt;sup&gt;w/w&lt;/sup&gt; (n=9)</td>
<td>OsxCre.EfnB2&lt;sup&gt;f/f&lt;/sup&gt; (n=10)</td>
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<tr>
<td><strong>Structural properties</strong></td>
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<tr>
<td>Moment of inertia (mm&lt;sup&gt;4&lt;/sup&gt;)</td>
<td>0.30 ± 0.03</td>
<td>0.22 ± 0.02&lt;sup&gt;*&lt;/sup&gt;</td>
<td>0.63 ± 0.08&lt;sup&gt;**&lt;/sup&gt;</td>
<td>0.48 ± 0.07</td>
</tr>
<tr>
<td>Force at Yield (N)</td>
<td>13.01 ± 10.00</td>
<td>10.60 ± 0.50&lt;sup&gt;*&lt;/sup&gt;</td>
<td>14.62 ± 1.42</td>
<td>12.03 ± 0.67</td>
</tr>
<tr>
<td>Deformation at Yield (µm)</td>
<td>116.38 ± 4.21</td>
<td>118.30 ± 5.89</td>
<td>139.33 ± 8.83&lt;sup&gt;*&lt;/sup&gt;</td>
<td>130.00 ± 7.07</td>
</tr>
<tr>
<td>Post-yield Deformation (µm)</td>
<td>104.78 ± 16.59</td>
<td>150.43 ± 18.81&lt;sup&gt;*&lt;/sup&gt;</td>
<td>144.24 ± 19.75</td>
<td>177.92 ± 19.95</td>
</tr>
<tr>
<td><strong>Material properties</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yield Stress (MPa)</td>
<td>41.05 ± 3.45</td>
<td>44.06 ± 3.78</td>
<td>25.49 ± 3.26&lt;sup&gt;**&lt;/sup&gt;</td>
<td>26.33 ± 2.78</td>
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<tr>
<td>Yield Strain (mm/mm)</td>
<td>2.33 ± 0.12</td>
<td>2.27 ± 0.13</td>
<td>3.06 ± 0.20&lt;sup&gt;**&lt;/sup&gt;</td>
<td>2.71 ± 0.15</td>
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Data shown are mean ± SEM, where + p<0.05, ++ p<0.01 vs. female OsxCre.EfnB2<sup>w/w</sup> controls and * p<0.05 vs. sex-matched OsxCre.EfnB2<sup>w/w</sup> controls.
Figure 4-3 Material properties of 12-week old female and male OsxCre.EfnB2 femora assessed by 3-point bending tests.

(A) Ultimate stress, (B) Ultimate strain, (C) Elastic modulus and (D) Toughness. Data shown are mean + SEM, n = 8-10/group, ++ p<0.01, +++ p<0.001 vs. female OsxCre.EfnB2w/w controls, *p<0.05 vs. sex-matched OsxCre.EfnB2w/w controls.
Figure 4-4 Load-Deformation and Stress-Strain curve of female and male *OsxCre.EfnB2* femora generated from 3-point bending tests.

Each dot point represents the average load, deformation, stress and strain for the noted sample group. Error bars were excluded to highlight the shape of curves. Data shown are mean, n =11/group.
In control mice, reference point indentation showed sex differences in material properties at the periosteum. The total distance travelled by the test probe into the cortex relative to the reference probe (total indentation distance) was significantly less in the femoral midshaft of 12-week old male controls compared to female controls (Figure 4-5A). The distance travelled in the first indentation cycle was also significantly less in male compared to female controls (Table 4-2). This was associated with a greater average loading and unloading slope (Figure 4-5B, C), as well as the unloading slope in the first indentation cycle (Table 4-2) in males compared to females. Other parameters such as the creep indentation distance, which measures the distance travelled by the test probe when held at maximum force in the first indentation cycle, were significantly smaller in male controls compared to female controls. Additionally, male control femora dissipated less energy than females (Table 4-2).

Although no difference was observed in material strength of bones from male OsxCre.EfnB2^{flo} femora, they showed significantly greater total indentation distance (Figure 4-5A) and significantly lower average loading and unloading slopes (Figure 4-5B, C) than their sex-matched controls. Female OsxCre.EfnB2^{flo} mice did not show any differences in any material properties measured by reference point indentation compared to controls.
Figure 4-5 Material properties of 12-week old female and male OsxCre.EfnB2 femora measured by Reference Point Indentation.

(A) Total indentation distance, (B) Average loading slope and (C) Average unloading slope. Data shown are mean + SEM, n= 8-10/group, +p<0.05, ++p<0.01 vs. female OsxCre.EfnB2<sup>w/w</sup> controls, *p<0.05 vs. sex-matched OsxCre.EfnB2<sup>w/w</sup> controls.
Table 4-2 Additional parameters measured by Reference Point Indentation on femora from 12-week old female and male OsxCre.EfnB2.

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<tr>
<td></td>
<td><em>OsxCre.EfnB2&lt;sup&gt;w/w&lt;/sup&gt;</em></td>
<td><em>OsxCre.EfnB2&lt;sup&gt;+/+&lt;/sup&gt;</em></td>
</tr>
<tr>
<td>Indentation Distance - 1st cycle (µm)</td>
<td>21.70 ± 0.28 (n=8)</td>
<td>22.94 ± 0.71 (n=10)</td>
</tr>
<tr>
<td>Indentation Distance Increase (µm)</td>
<td>3.23 ± 0.18</td>
<td>3.26 ± 0.10</td>
</tr>
<tr>
<td>Creep Indentation Distance - 1st cycle (µm)</td>
<td>2.08 ± 0.05</td>
<td>2.12 ± 0.05</td>
</tr>
<tr>
<td>Average Creep Indentation Distance (µm)</td>
<td>1.0 ± 0.00</td>
<td>1.0 ± 0.00</td>
</tr>
<tr>
<td>Average Energy Dissipated (µJ)</td>
<td>2.37 ± 0.17</td>
<td>2.47 ± 0.11</td>
</tr>
<tr>
<td>Unloading slope - 1st cycle (N/µm)</td>
<td>0.322 ± 0.008</td>
<td>0.320 ± 0.011</td>
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Data shown are mean ± SEM, where + p<0.05, ++ p<0.01 vs. female *OsxCre.EfnB2<sup>+/+</sup>* controls.
4.3.2 Collagen content in female and male OsxCre.EfnB2^{ff} femora

To investigate whether the greater compliance in femora from 12-week old OsxCre.EfnB2^{ff} mice was associated with altered collagen content, a hydroxyproline assay was conducted on hydrolysates of bone. Hydroxyproline is a major component of collagen and stabilizes the helical structure, therefore hydroxyproline levels can be used as an indicator of collagen content. 12-week old female OsxCre.EfnB2^{ff} femora hydrolysates showed significantly greater hydroxyproline concentration, thus collagen content, compared to controls. Male OsxCre.EfnB2^{ff} femora hydrolysates did not demonstrate any significant changes (Figure 4-6).

Figure 4-6 Hydroxyproline levels in female and male 12 week old OsxCre.EfnB2 femora.

Data shown are mean + SEM, n= 10-12/group, *p<0.05 vs. sex-matched OsxCre.EfnB2^{ww} controls
4.3.3 Cortical bone composition of 12-week old female OsxCre.EfnB2 tibiae assessed by synchrotron-based Fourier Transform Infrared Microscopy (sFTIRM)

To examine whether impaired bone strength in 12-week old female OsxCre.EfnB2\textsuperscript{f/f} mice was due to altered bone composition, region-based sFTIRM analysis was performed from the periosteal edge through to mature regions of the tibia. No significant change in mineral or matrix composition was observed in OsxCre.EfnB2\textsuperscript{f/f} mice compared to their controls (Figure 4-7A-C). However, as proof of principle of the validity of this method to measure the process of bone mineralisation, I observed that femora of OsxCre.EfnB2\textsuperscript{w/w} controls demonstrated significantly greater mineral incorporation (mineral:matrix ratio) in the most mature regions of bone (region 3) compared to the most newly formed region of bone (region 1) (Figure 4-7A) but this was not observed in femora of OsxCre.EfnB2\textsuperscript{f/f} mice. The amount of carbonate incorporated within the hydroxyapatite mineral crystal lattice (carbonate:mineral ratio), which represents mineral composition, did not change throughout the maturing bone in control mice (Figure 4-7B). The molecular orientation of the collagen (amide I:II ratio), showed no significant differences within the maturing bone of female OsxCre.EfnB2\textsuperscript{w/w} controls (Figure 4-7C).
Figure 4-7 Analysis of maturing bone regions in 12-week old female OsxCre.EfnB2 tibiae assessed by sFTIRM.
(A) Mineral:matrix ratio, (B) Carbonate:mineral ratio and (C) Amide I:II ratio. Ratios were calculated from integrated areas of the phosphate (mineral), matrix (amide I) and amide II peaks. Data shown are mean + SEM, n = 7/group, *p<0.05 vs. Region 1 of same genotype.
4.4 Discussion

Deletion of ephrinB2 directed by OsxCre (OsxCre.EfnB2\(^{−/−}\)) in female mice resulted in femora that were more slender and compliant than controls. This was associated with delayed initiation of mineralisation and a higher level of osteoblast and osteocyte apoptosis (195). This suggested that ephrinB2-deficient osteoblasts undergo apoptosis rather than differentiating into the fully mature stages required to promote osteoid mineralisation; this subsequent delay in mineralisation may be an explanation for the reduction in bone stiffness.

The femora from 12-week old female OsxCre.EfnB2\(^{−/−}\) mice displayed both greater compliance and a greater ultimate force withstood before fracturing. This might be considered a positive effect on bone strength. However, despite their greater bending ability, the force that caused permanent damage (yield force) to femoral from 12-week old female OsxCre.EfnB2\(^{−/−}\) mice was lower than that of controls. Their greater flexibility may be attributed to their delayed initiation of mineralisation, demonstrated by the thickened osteoid and lower rate of bone mineralisation). These are two hallmarks of osteomalacia (249), a pathological condition characterised by weak, ductile bones due to vitamin D deficiency (221). Two mouse models of osteomalacia have been described. Dmp1 knockout mice exhibit excessive osteoid, shorter and wider bones and thinner cortical walls (97), and their ulnae produced greater strains compared to controls indicating greater elasticity (250) when subjected to mechanical loading. The Hyp mouse, a model of X-linked hypophosphatemia, also displayed excessive osteoid, significantly shorter bones and reduced cortical thickness, significantly greater angular deformation and reduced torsional stiffness (251). Although these phenotypes were much more severe than the OsxCre.EfnB2\(^{−/−}\) mice, the similarities shared between these three models suggest that deletion of ephrinB2 within the osteoblast lineage causes mild osteomalacia.

sFTIRM analysis did not reveal any changes in bone composition in OsxCre.EfnB2\(^{−/−}\) mice compared to controls. In control animals, an increase in mineral:matrix ratio was observed from the periosteal edge through to the maturing bone. This increase in mineral:matrix ratio was not observed in OsxCre.EfnB2\(^{−/−}\) mice. This is suggestive of a slower progression of mineralisation in OsxCre.EfnB2\(^{−/−}\) mice, but the difference in effect was not statistically significant between genotypes by two-way ANOVA and would require a larger sample size to determine whether this effect is real. OsxCre.EfnB2\(^{−/−}\) mice did not show any changes in the
carbonate:mineral or amide I:II ratio compared to controls indicating that mineral and matrix composition is normal once the mineralisation process commences.

The increase in mineral:matrix ratio in control animals was the first observation of mineral accumulation in a mouse model by sFTIRM. Our sFTIRM analysis focused on the peristeal surface at the cortical midshaft which is a location where bone formation occurs without prior resorption (252), thus with increasing maturity we expected to observe increased mineral incorporation into the matrix. A similar increase in mineral:matrix ratio has previously been observed with increasing tissue age in rabbit, rat, and baboon cortical bone (63, 253, 254) and within human osteonal bone (255, 256). In addition, carbonate:mineral ratio and amide I:II ratio remained unchanged during matrix maturation in control bones. An increase in the carbonate:mineral ratio has previously been shown to increase with increasing tissue age on the periosteal edge of rat cortical bone from 4-day old tissue to 15-day old tissue (63). Our first region of analysis includes these two measurement regions of tissue age and for this reason may not be able to observe a similar increase in carbonate:mineral ratio on the peristeal edge. In contrast, osteonal bone showed a reduction in the carbonate:mineral ratio with increasing distance away from the osteonal centre (254, 256). This may be due to osteons undergoing remodelling while our analysis is on a surface where only bone formation occurs. There have been no reports on the amide I:II ratio in bone with increasing tissue age.

Greater collagen content in OsxCre.EfnB2/0 mice was demonstrated by higher hydroxyproline levels than in controls. This suggested that the mineral:matrix ratio which remained unchanged in OsxCre.EfnB2/0 mice may be due to greater the collagen content contributing to more matrix compared to controls. An alteration in collagen content is also suggested by the unusually distended rough endoplasmic reticulum detected by transmission electron microscopy in OsxCre.EfnB2/0 osteoblasts (195), since previously described mouse models with defective collagen secretion (257-259) have similar osteoblastic intracellular morphology.

In contrast to female mice, bone size and strength remained unchanged in 12-week old male OsxCre.EfnB2/0 mice compared to male controls. However male OsxCre.EfnB2/0 mice showed a greater total indentation distance and lower unloading and loading slopes by reference point indentation than controls. Total indentation distance and unloading slopes are
indicators of material stiffness (260). This suggests that rather than any compromise in whole bone strength, the local intrinsic material properties of the periosteum are impaired in male OsxCre.EfnB2\textsuperscript{f/f} bones.

The sex-difference in strength phenotypes suggests that ephrinB2 may have a sex-specific role in regulating bone size and strength at a whole tissue level and may be more important during higher states of bone remodelling, such as in female mice. There have been no reports of estrogen effects on ephrinB2 and EphB4 expression in bone, however ephrinB2 and EphB4 expression levels in the mammary gland are stimulated in overiectomized mice by estradiol injection (261). If this same principle applied to ephrinB2 expression in osteoblasts, higher estrogen levels in 12-week old females compared to males may suggest lower ephrinB2 expression in females compared to males. Deletion of ephrinB2 in female Dmp1Cre.EfnB2\textsuperscript{f/f} mice may further reduce the expression of ephrinB2 but it is unlikely that this reduction in ephrinB2 would result in such a significant female-only phenotype. In order to investigate sex-specific effects of ephrinB2, future experiments may include treating osteoblasts with estradiol and analysing ephrinB2 expression levels or examining whether estradiol treatment enhances apoptosis since ephrinB2 has an anti-apoptotic role in osteoblasts.

Sex-specific differences in bone size and strength were also observed in control mice in this study. At 12 weeks of age, male OsxCre controls had significantly wider bones in both the anteroposterior and mediolateral directions, compared to female controls. This is subtly different from the Dmp1Cre.gp130 mouse model (Chapter 3) where male bones were wider only in the mediolateral direction. The structural and material strength parameters assessed by 3-point bending also showed differences between these two models. Male OsxCre controls were capable of deforming more than females before fracturing while male Dmp1Cre.gp130 controls required less force to fracture compared to female controls. These differences between the two studies might be explained by the difference in ages of these mice (Dmp1Cre.gp130 mice were 26 weeks old while OsxCre.gp130 mice were 12 weeks old). It could also be due to effects of the Cre transgene on the skeleton. The OsxCre transgene has been reported to cause delayed cortical bone growth at 6 weeks of age compared to wild-type controls, which is overcome by 12 weeks of age (212). Although we did not observe any significant alteration in bone structure or turnover at 12 weeks of age in male OsxCre mice compared to wild-type mice (195) we cannot exclude this possibility.
In conclusion, this chapter shows that ephrinB2 in the osteoblast lineage maintains the elasticity of cortical bone in female mice, likely by allowing osteoblasts to reach mature stages that initiate the process of mineralisation.
CHAPTER 5: Bone quality and strength in mice lacking ephrinB2 in late osteoblasts/osteocytes

5.1 Introduction

EphrinB2 is expressed throughout osteoblast differentiation (157) and its interaction with its receptor EphB4 has previously been shown to be required as a checkpoint for continued osteoblast differentiation (194). Specific deletion of ephrinB2 throughout the osteoblast lineage (OsxCre.EfnB2<sup>f/f</sup>) impaired osteoblast differentiation, stimulated osteoblast apoptosis and delayed initiation of bone mineralisation, indicating a role for ephrinB2 in osteoblasts that promotes mineralisation by allowing late stages of osteoblast differentiation to occur (195). In chapter 4, I showed that the delayed mineralisation in OsxCre.EfnB2<sup>f/f</sup> mice is associated with more slender femora and greater flexibility. Since ephrinB2 is also expressed beyond the checkpoint where it is required for continued osteoblast differentiation (i.e. in osteocytes), I sought to determine the role of ephrinB2 in these more mature cells by generating a mouse model lacking ephrinB2 specifically in late osteoblasts/osteocytes (Dmp1Cre.EfnB2<sup>f/f</sup>). In this chapter, I describe the cortical structure, strength and bone composition of femora from Dmp1Cre.EfnB2<sup>f/f</sup> mice during growth and compare it to the OsxCre.EfnB2<sup>f/f</sup> mouse model to determine the stage-specific roles of ephrinB2 during osteoblast differentiation. This chapter also describes the trabecular bone structure of Dmp1Cre.EfnB2<sup>f/f</sup> mice.
5.2 Specific Methods

Micro-CT analysis of cortical and trabecular bone was performed on femora from 6, 12 and 26 week old female and male *Dmp1Cre.EfnB2<sup>+</sup>w/w* and *Dmp1Cre.EfnB2<sup>−/−</sup>* mice, as described in Section 2.2. 12- and 26-week old female and male *Dmp1Cre.EfnB2<sup>+</sup>w/w* and *Dmp1Cre.EfnB2<sup>−/−</sup>* tibiae were further analysed by trabecular histomorphometry in the secondary spongiosa as described in section 2.3.5.1.

Femora were also subjected to 3-point bending tests to determine the structural and material property changes due to ephrinB2 deletion in late osteoblasts/osteocytes, as described in Section 2.6.1. Anteroposterior and mediolateral widths were measured from the midshaft slice from micro-CT scans of the cortical region.

Since a strength phenotype was observed at 12 weeks of age in female *Dmp1Cre.EfnB2<sup>−/−</sup>* mice, further analysis was carried out at this age and sex. Reference point indentation was performed as described in Section 2.6.2. mRNA expression levels were assessed as described in section 2.4. Dynamic histomorphometric analysis was performed as described in section 2.3.5.2. To assess collagen fibre orientation, femora were embedded in plastic and thick 100µm sections were cut, coverslipped and assessed by polarized light microscopy as described in section 2.3.5.1. 3 µm sagittal sections of methyl methacrylate (MMA)-embedded tibiae were analysed by sFTIRM, as described in section 2.8.1.1. The same sections were analysed by FTIR imaging, at the University of Bordeaux, France, with the help of Dr. Cyril Petibois, as described in section 2.8.1.4. 100µm thick sections taken in series from the femoral cortical midshaft were measured by backscattered electron microscopy (BSEM) for analysis of osteocyte lacunae parameters assessed by Ms Blessing Crimeen-Irwin (Section 2.9). Immunohistochemistry for TUNEL staining was performed by Ms Ingrid Poulton (Section 2.3.4.1). Transmission electron microscopy analysis was performed ultra-thin sections of tibial cortical bone by Dr. Mika Ikegame (Okayama University, Japan) (Section 2.10).
5.3 Results

5.3.1 EphrinB2 knockdown in late osteoblasts/osteocytes

Deletion of ephrinB2 in late osteoblasts/osteocytes was confirmed in fluorescence-activated cell-sorted (FACS) green fluorescent protein (GFP) positive (+) and GFP negative (-) cells isolated from control $Dmp1Cre.DMP1-GFP.EfnB2^{w/w}$ and $Dmp1Cre.DMP1-GFP.EfnB2^{f/f}$ mice. $EfnB2$ mRNA was undetectable in GFP+ cells isolated from $Dmp1Cre.EfnB2^{f/f}$ mice but was detected in GFP+ cells isolated from control mice, confirming ephrinB2 knockdown. $EfnB2$ knockdown in GFP+ cells was further supported by similar $EfnB2$ mRNA expression levels as controls in GFP- cells isolated from $Dmp1Cre.EfnB2^{f/f}$ mice (Figure 5-1).

![Figure 5-1](image)

Figure 5-1 $EfnB2$ mRNA levels from FACS-sorted GFP+ and GFP- cells isolated from marrow-flushed femora of $Dmp1Cre.DMP1-GFP.EfnB2^{w/w}$ (w/w) and $Dmp1Cre.EfnB2^{f/f}$ (f/f) mice.

Data shown are mean ± SEM, n = 2 repeated experiments, each including 6 mice mRNA expression levels of $EfnB2$ is shown as the geometric average of Beta-2-microglobulin ($B2m$) and Hydroxymethylbilane Synthase ($Hmbs$) housekeeping genes.
5.3.2  Cortical bone structure and strength in Dmp1Cre.EfnB2 mice

Normal patterns of age-related bone growth were observed in female and male control mice. Femoral length increased significantly in Dmp1Cre control mice with increasing age (Table 5-1). This was accompanied by significant thickening of the cortical bone (Table 5-1), increasing cortical area and increasing mean polar moment of inertia and from 6 to 12 weeks of age in both sexes (Figure 5-2A, B). At 26 weeks of age, the cortical area in female controls remained higher, but without any change to marrow area or endosteal or periosteal perimeters, while male controls did not show any changes between 12 and 26 weeks of age (Table 5-1). Femora of male controls were significantly wider in the mediolateral direction at 26 weeks of age (Figure 5-2D) however mean polar moment of inertia (MPMI) (Figure 5-2B), marrow area and periosteal perimeter were all significantly lower at 26 weeks of age compared to 12 weeks of age (Table 5-1). Female controls showed no changes in MPMI or mediolateral width from 12 to 26 weeks of age (Figure 5-2B, D). Anteroposterior cortical width remained unchanged in controls of both sexes between 6 and 26 weeks of age (Figure 5-2C).

Femoral cortical structure was mildly altered in female and male Dmp1Cre.EfnB2ff mice compared to their sex-matched controls. 6-week old female and male Dmp1Cre.EfnB2ff mice showed no change in cortical structure. At 12 weeks of age, female Dmp1Cre.EfnB2ff mice had thinner cortices (Table 5-1) but all other cortical parameters including moment of inertia were unchanged compared to controls. At 26 weeks of age, female Dmp1Cre.EfnB2ff femora showed a smaller mediolateral width compared to controls (Figure 5-2D). 12-week old male Dmp1Cre.EfnB2ff mice had normal cortical thickness (Table 5-1), but transiently smaller mediolateral than controls (Figure 5-2D); this recovered by 26 weeks of age. 26-week old male Dmp1Cre.EfnB2ff mice showed significantly greater MPMI, anteroposterior width (Figure 5-2B, C), marrow area and periosteal perimeter than controls (Table 5-1).
Table 5-1 Cortical parameters measured by micro-CT in the femoral midshaft of 6, 12 and 26-week old female and male Dmp1Cre.EfnB2^{w/w} (w/w) and Dmp1Cre.EfnB2^{ff} (f/f) mice

<table>
<thead>
<tr>
<th></th>
<th>6 week old</th>
<th>12 week old</th>
<th>26 week old</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Female</td>
<td>Male</td>
<td>Female</td>
</tr>
<tr>
<td></td>
<td>w/w (n=8)</td>
<td>f/f (n=8)</td>
<td>w/w (n=8)</td>
</tr>
<tr>
<td>Femoral length (mm)</td>
<td>12.25 ± 0.15</td>
<td>12.52 ± 0.13</td>
<td>12.53 ± 0.12</td>
</tr>
<tr>
<td>Cortical Thickness (μm)</td>
<td>159.09 ± 3.62</td>
<td>167.65 ± 3.45</td>
<td>190.63 ± 6.65***</td>
</tr>
<tr>
<td>Marrow Area (μm²)</td>
<td>1.08 ± 0.04</td>
<td>1.11 ± 0.04</td>
<td>1.13 ± 0.04</td>
</tr>
<tr>
<td>Periosteal perimeter (μm)</td>
<td>6.97 ± 0.14</td>
<td>7.28 ± 0.15</td>
<td>7.53 ± 0.17*</td>
</tr>
<tr>
<td>Endocortical perimeter (μm)</td>
<td>2.94 ± 0.05</td>
<td>2.88 ± 0.17</td>
<td>3.22 ± 0.09**</td>
</tr>
</tbody>
</table>

Data shown are mean ± SEM, n = 6-12/group

Sex effect:  + p<0.05, ++ p<0.01, +++p<0.001 vs. female Dmp1Cre.EfnB2^{w/w} controls

Age effect: ° p<0.05, °° p<0.01, °°° p<0.001 vs. sex-matched Dmp1Cre.EfnB2^{w/w} controls at earlier time points

Genotype effect: *p<0.05 vs. sex-matched Dmp1Cre.EfnB2^{w/w} controls at same age
Figure 5-2 Cortical structural parameters by micro-CT in Dmp1Cre.EfnB2 femora. (A) Cortical Area, (B) Mean polar moment of inertia, (C) Anteroposterior width and (D) Mediolateral width from the femoral midshaft of female and male Dmp1Cre.EfnB2 mice at 6, 12, and 26 weeks of age. Data shown are mean + SEM, n = 6-12/group, + p<0.05, ++ p<0.01, +++ p<0.001 vs. female Dmp1Cre.EfnB2<sup>+/+</sup> controls, *p<0.05, **p<0.01 vs. sex-matched Dmp1Cre.EfnB2<sup>+/+</sup> controls, ° p<0.05, °° p<0.01, °°° p<0.001 vs. previous age group of sex-matched Dmp1Cre.EfnB2<sup>+/+</sup> controls.
In control femora, sex-specific differences were also observed in mechanical properties. At 6 weeks of age, male control femora had significantly greater moment of inertia compared to female controls (Table 5-2). Ultimate and yield stress were significantly lower in male controls compared to female controls (Table 5-2).

Femora from 6-week old female Dmp1Cre.EfnB2\textsuperscript{ff} mice showed no significant difference in bone strength compared to controls (Table 5-2). Femora from 6-week old male Dmp1Cre.EfnB2\textsuperscript{ff} mice demonstrated significantly lower force at yield and failure force compared to controls (Table 5-2), but no other parameters were significantly altered.

As observed at 6 weeks, 12-week old male control femora had significantly greater moment of inertia compared to female controls (Table 5-3), confirming the micro-CT measurement. Ultimate force (Figure 5-3A) and bone stiffness (Figure 5-3C) were also significantly greater in male controls compared to female controls. Ultimate and yield stress were also significantly lower in male controls compared to female controls at 12 weeks of age (Table 5-3).

At 12 weeks of age, femora from female Dmp1Cre.EfnB2\textsuperscript{ff} mice broke at a significantly lower ultimate force (Figure 5-3A) with less deformation (Figure 5-3B) compared to controls of the same age and sex. This was associated with significantly lower yield force, yield deformation, failure force and post-yield deformation (Table 5-3). Fifty percent less energy was required to break female Dmp1Cre.EfnB2\textsuperscript{ff} femora compared to controls (Figure 5-3D). 12-week old female Dmp1Cre.EfnB2\textsuperscript{ff} femora also demonstrated significantly lower ultimate stress, strain and bone toughness compared to controls (Figure 5-4A-C). This was associated with significantly lower yield and failure force, as well as post-yield strain (Table 5-3).

The dramatic strength phenotype in female Dmp1Cre.EfnB2\textsuperscript{ff} mice can be observed by the average load-deformation curve which highlights that femora from Dmp1Cre.EfnB2\textsuperscript{ff} mice are unable to withstand as much force and deform as much before breaking compared controls (Figure 5-5A). After normalisation for bone geometry, the average
stress-strain curves showed that femora from Dmp1Cre.EfnB2^ff female mice demonstrated a similar profile of impaired material properties (Figure 5-5B).

No significant differences were observed in the structural or material properties of male Dmp1Cre.EfnB2^ff femora compared to controls at 12 weeks of age (Figure 5-3, Figure 5-4 and Table 5-3).
Table 5-2 Structural and material properties of 6-week old female and male Dmp1Cre.EfnB2 femora determined by 3-point bending tests.

<table>
<thead>
<tr>
<th></th>
<th>Female</th>
<th></th>
<th>Male</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(n=8)</td>
<td>(n=8)</td>
<td>(n=8)</td>
<td>(n=5)</td>
</tr>
<tr>
<td><strong>Structural properties</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Moment of Inertia (mm⁴)</td>
<td>0.334 ± 0.031</td>
<td>0.380 ± 0.030</td>
<td>0.489 ± 0.063</td>
<td>0.398 ± 0.0473</td>
</tr>
<tr>
<td>Ultimate Force (N)</td>
<td>10.36 ± 0.60</td>
<td>11.08 ± 0.30</td>
<td>12.13 ± 0.91</td>
<td>10.17 ± 0.74</td>
</tr>
<tr>
<td>Ultimate Deformation (µm)</td>
<td>0.78 ± 0.48</td>
<td>0.79 ± 0.84</td>
<td>0.79 ± 0.63</td>
<td>0.82 ± 0.80</td>
</tr>
<tr>
<td>Stiffness (N/mm)</td>
<td>30.46 ± 2.22</td>
<td>37.52 ± 3.85</td>
<td>37.34 ± 3.81</td>
<td>37.80 ± 4.27</td>
</tr>
<tr>
<td>Energy absorbed to failure (mJ)</td>
<td>5.4 ± 0.4</td>
<td>6 ± 0.7</td>
<td>6.51 ± 0.5</td>
<td>6.1 ± 0.9</td>
</tr>
<tr>
<td>Force at Yield (N)</td>
<td>8.85 ± 0.43</td>
<td>10.20 ± 0.41</td>
<td>10.61 ± 1.03</td>
<td>8.31 ± 0.76*</td>
</tr>
<tr>
<td>Deformation at Yield (µm)</td>
<td>419 ± 50</td>
<td>402 ± 41</td>
<td>376 ± 47</td>
<td>255 ± 16</td>
</tr>
<tr>
<td>Post-yield Deformation (µm)</td>
<td>356 ± 53</td>
<td>387 ± 71</td>
<td>414 ± 45</td>
<td>569 ± 94</td>
</tr>
<tr>
<td>Failure Force (N)</td>
<td>8.88 ± 0.82</td>
<td>9.25 ± 0.48</td>
<td>10.11 ± 0.99</td>
<td>7.02 ± 0.65*</td>
</tr>
<tr>
<td><strong>Material properties</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ultimate Stress (MPa)</td>
<td>37.80 ± 2.37</td>
<td>35.74 ± 1.91</td>
<td>31.42 ± 1.52</td>
<td>29.95 ± 2.21</td>
</tr>
<tr>
<td>Ultimate Strain (%)</td>
<td>10.38 ± 0.70</td>
<td>10.77 ± 1.28</td>
<td>10.89 ± 0.92</td>
<td>10.80 ± 1.33</td>
</tr>
<tr>
<td>Elastic Modulus (MPa)</td>
<td>887 ± 149</td>
<td>886 ± 92</td>
<td>749 ± 129</td>
<td>874 ± 129</td>
</tr>
<tr>
<td>Toughness (ml/mm³)</td>
<td>2.60 ± 0.18</td>
<td>2.52 ± 0.24</td>
<td>2.35 ± 0.19</td>
<td>2.28 ± 0.20</td>
</tr>
<tr>
<td>Yield Stress (MPa)</td>
<td>34.18 ± 2.05</td>
<td>32.67 ± 1.29</td>
<td>27.11 ± 1.05++</td>
<td>24.61 ± 2.62</td>
</tr>
<tr>
<td>Yield Strain (%)</td>
<td>5.66 ± 0.736</td>
<td>5.46 ± 0.59</td>
<td>5.25 ± 0.72</td>
<td>3.32 ± 0.22</td>
</tr>
<tr>
<td>Post-yield Strain (%)</td>
<td>4.72 ± 0.70</td>
<td>5.31 ± 1.00</td>
<td>5.64 ± 0.53</td>
<td>6.18 ± 0.73</td>
</tr>
<tr>
<td>Failure Stress (MPa)</td>
<td>31.89 ± 2.33</td>
<td>30.23 ± 2.88</td>
<td>25.94 ± 1.68</td>
<td>20.74 ± 2.14</td>
</tr>
</tbody>
</table>

Data shown are mean ± SEM, n = 5-8/group.

Sex effect: +p<0.05, ++p<0.01 vs. female Dmp1Cre.EfnB2+/− controls
Genotype effect: *p<0.05 vs. sex-matched Dmp1Cre.EfnB2+/− controls at same age.
Figure 5-3 Structural properties of 12-week old female and male *Dmp1Cre.EfnB2* femora assessed by 3-point bending tests.

(A) Ultimate force, (B) Ultimate deformation, (C) Stiffness and (D) Energy absorbed to failure of female and male *Dmp1Cre.EfnB2* mice at 12 weeks of age. Data shown are mean ± SEM, n = 10-12/group, + p<0.05 vs. female *Dmp1Cre.EfnB2*/*w/w* controls, *p<0.05, ***p<0.001 vs. sex-matched *Dmp1Cre.EfnB2*/*w/w* controls.
Figure 5-4 Material properties of 12-week old female and male Dmp1Cre.EfnB2 femora assessed by 3-point bending.

(A) Ultimate stress, (B) Ultimate strain, (C) Elastic modulus and (D) Toughness of Dmp1Cre.EfnB2 mice at 12 weeks of age. Data shown are mean + SEM, n = 10-12/group, ++ p<0.01, +++ p<0.001 vs. female Dmp1Cre.EfnB2<sup>w/w</sup> controls, * p<0.05, ** p<0.01, ***p<0.001 vs. sex-matched Dmp1Cre.EfnB2<sup>w/w</sup> controls.
Table 5-3 Additional parameters measured by 3-point bending tests on 12-week old female and male \textit{Dmp1Cre.\textit{EfnB2}} femora.

<table>
<thead>
<tr>
<th></th>
<th>Female</th>
<th>Male</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>\textit{Dmp1Cre.\textit{EfnB2}}\textsubscript{w/w} (n=12)</td>
<td>\textit{Dmp1Cre.\textit{EfnB2}}\textsubscript{+/+} (n=10)</td>
</tr>
<tr>
<td><strong>Structural properties</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Moment of inertia (mm(^4))</td>
<td>0.460 ± 0.044</td>
<td>0.426 ± 0.020</td>
</tr>
<tr>
<td>Force at Yield (N)</td>
<td>14.98 ± 0.63</td>
<td>10.78 ± 0.92**</td>
</tr>
<tr>
<td>Deformation at Yield (µm)</td>
<td>176.27 ± 13.95</td>
<td>115.78 ± 6.84**</td>
</tr>
<tr>
<td>Post-yield Deformation (µm)</td>
<td>264.11 ± 22.69</td>
<td>177.83 ± 27.47*</td>
</tr>
<tr>
<td>Failure Force (N)</td>
<td>13.74 ± 0.91</td>
<td>9.13 ± 1.02*</td>
</tr>
<tr>
<td><strong>Material properties</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yield Stress (MPa)</td>
<td>31.96 ± 2.12</td>
<td>26.64 ± 4.09*</td>
</tr>
<tr>
<td>Yield Strain (%)</td>
<td>3.62 ± 0.28</td>
<td>2.24 ± 0.13**</td>
</tr>
<tr>
<td>Post-yield Strain (%)</td>
<td>5.42 ± 0.46</td>
<td>3.74 ± 0.58*</td>
</tr>
<tr>
<td>Failure Stress (MPa)</td>
<td>29.26 ± 2.41</td>
<td>20.90 ± 1.93*</td>
</tr>
</tbody>
</table>

Data shown are mean ± SEM, n = 10-12/group
Sex effect: +++p<0.001 vs. female \textit{Dmp1Cre.\textit{EfnB2}}\textsubscript{w/w} controls
Genotype effect: *p<0.05, **p<0.01 vs. sex-matched \textit{Dmp1Cre.\textit{EfnB2}}\textsubscript{w/w} controls.
Figure 5-5 Average Load-Deformation and Stress-Strain curve of female and male Dmp1Cre.EfnB2 femora generated from 3-point bending tests.

Each dot point represents the average load, deformation, stress and strain for the noted sample group. Error bars were excluded to highlight the shape of curves. Data shown are mean, n = 10-12/group.
In contrast to 6- and 12-week old male Dmp1Cre.EfnB2\textsuperscript{w/w} controls, femora from 26-week old male controls had lower bone stiffness than female controls (Table 5-4). Their elastic modulus, toughness, yield, ultimate and failure stress were also significantly lower than female controls (Table 5-4).

Although 26-week old female Dmp1Cre.EfnB2\textsuperscript{f/f} femora showed few changes in mechanical strength compared to their age- and sex-matched Dmp1Cre controls, yield stress and ultimate stress were significantly elevated (Table 5-4). Male Dmp1Cre.EfnB2\textsuperscript{f/f} femora showed greater moment of inertia compared to age- and sex-matched controls, but no other indicators of bone strength were modified (Table 5-4).
Table 5-4 Structural and material properties of femora from 26-week old female and male *Dmp1Cre.EfnB2* mice determined by 3-point bending tests

<table>
<thead>
<tr>
<th></th>
<th>Female</th>
<th>Male</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(n=8)</td>
<td>(n=8)</td>
</tr>
<tr>
<td></td>
<td>(n=7)</td>
<td>(n=8)</td>
</tr>
<tr>
<td>Structural properties</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Moment of Inertia (mm⁴)</td>
<td>0.53 ± 0.03</td>
<td>0.46 ± 0.04</td>
</tr>
<tr>
<td>Ultimate force (N)</td>
<td>18.63 ± 0.85</td>
<td>18.10 ± 0.70</td>
</tr>
<tr>
<td>Ultimate deformation (mm)</td>
<td>0.47 ± 0.03</td>
<td>0.43 ± 0.03</td>
</tr>
<tr>
<td>Stiffness (N/mm)</td>
<td>72.70 ± 6.15</td>
<td>64.30 ± 5.69</td>
</tr>
<tr>
<td>Energy absorbed to failure (MJ)</td>
<td>4.81 ± 0.385</td>
<td>4.19 ± 0.45</td>
</tr>
<tr>
<td>Force at Yield (N)</td>
<td>16.58 ± 0.63</td>
<td>17.98 ± 0.29</td>
</tr>
<tr>
<td>Deformation at Yield (μm)</td>
<td>338 ± 51</td>
<td>308 ± 40</td>
</tr>
<tr>
<td>Post-yield Deformation (μm)</td>
<td>130 ± 35</td>
<td>125 ± 34</td>
</tr>
<tr>
<td>Failure Force (N)</td>
<td>17.98 ± 1.10</td>
<td>17.35 ± 0.67</td>
</tr>
<tr>
<td>Material properties</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ultimate Stress (MPa)</td>
<td>53.41 ± 2.27</td>
<td>60.34 ± 2.90 *</td>
</tr>
<tr>
<td>Ultimate Strain (%)</td>
<td>3.96 ± 0.32</td>
<td>3.62 ± 0.28</td>
</tr>
<tr>
<td>Elastic Modulus (MPa)</td>
<td>2470 ± 222</td>
<td>2457 ± 184</td>
</tr>
<tr>
<td>Toughness (MJ/mm³)</td>
<td>1.16 ± 0.09</td>
<td>1.14 ± 0.08</td>
</tr>
<tr>
<td>Yield Stress (MPa)</td>
<td>47.87 ± 2.42</td>
<td>58.52 ± 5.21 *</td>
</tr>
<tr>
<td>Yield Strain (%)</td>
<td>2.85 ± 0.42</td>
<td>2.57 ± 0.34</td>
</tr>
<tr>
<td>Post Yield Strain (%)</td>
<td>1.11 ± 0.31</td>
<td>1.05 ± 0.29</td>
</tr>
<tr>
<td>Failure Stress (MPa)</td>
<td>51.60 ± 3.04</td>
<td>58.19 ± 3.47</td>
</tr>
</tbody>
</table>

Data shown are mean ± SEM, n = 7-8/group
Sex effect: + p<0.05, ++ p<0.01, +++ p<0.001 vs. female *Dmp1Cre.EfnB2*+/+ controls
Genotype effect: * p<0.05, ** p<0.01 vs. sex-matched *Dmp1Cre.EfnB2*+/+ controls
5.3.2.1 Material properties measured at the microindentation level in Dmp1Cre.EfnB2 femora

At 12 weeks of age, control mice exhibited no sex difference in periosteal material properties assessed by reference point indentation. 12-week old female Dmp1Cre.EfnB2^{f/f} femora had a significantly greater indentation distance increase compared to controls (Table 5-5), but no other parameter measured by reference point indentation was altered in female Dmp1Cre.EfnB2^{f/f} femora compared to controls (Table 5-5). Male Dmp1Cre.EfnB2^{f/f} femora showed no significant alterations in parameters measured by reference point indentation (Table 5-5).
Table 5-5 Reference Point Indentation parameters measured on the cortical midshaft of femora from 12-week old female and male *Dmp1Cre.EfnB2* mice

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Female Dmp1Cre.EfnB2&lt;sup&gt;w/w&lt;/sup&gt; (n=12)</th>
<th>Male Dmp1Cre.EfnB2&lt;sup&gt;+/+&lt;/sup&gt; (n=11)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Indentation Distance - 1st cycle (μm)</td>
<td>19.43 ± 0.44</td>
<td>19.75 ± 0.36</td>
</tr>
<tr>
<td>Indentation Distance Increase (μm)</td>
<td>2.48 ± 0.06</td>
<td>2.80 ± 0.10 **</td>
</tr>
<tr>
<td>Total Indentation Distance Increase (μm)</td>
<td>20.77 ± 0.45</td>
<td>21.18 ± 0.38</td>
</tr>
<tr>
<td>Average Loading Slope (N/μm)</td>
<td>0.31 ± 0.01</td>
<td>0.29 ± 0.01</td>
</tr>
<tr>
<td>Unloading slope - 1st cycle (N/μm)</td>
<td>0.39 ± 0.02</td>
<td>0.37 ± 0.02</td>
</tr>
<tr>
<td>Average Unloading slope (N/μm)</td>
<td>0.41 ± 0.02</td>
<td>0.38 ± 0.02</td>
</tr>
<tr>
<td>Creep Indentation Distance - 1st cycle (μm)</td>
<td>1.57 ± 0.09</td>
<td>1.65 ± 0.10</td>
</tr>
<tr>
<td>Average Creep Indentation Distance (μm)</td>
<td>0.58 ± 0.10</td>
<td>0.62 ± 0.11</td>
</tr>
<tr>
<td>Average Energy Dissipated (μJ)</td>
<td>2.47 ± 0.16</td>
<td>2.60 ± 0.18</td>
</tr>
</tbody>
</table>

Data shown are mean ± SEM, n= 11-12/group, **p<0.01 vs. sex-matched *Dmp1Cre.EfnB2<sup>w/w</sup>* controls
Since the most dramatic alteration in bone strength was detected in femora of 12-week old female Dmp1Cre.EfnB2^{ff} mice, and this was the same age and sex at which bone strength was modified in OssCre.EfnB2^{ff} mice, I focused on determining the underlying defect in the bones of female 12-week old Dmp1Cre.EfnB2^{ff} mice for the rest of this chapter.

5.3.3 Cortical Tissue Mineral Density assessed by micro-CT

No significant difference in cortical tissue mineral density was detected at the femoral midshaft in 12-week old female Dmp1Cre.EfnB2^{ff} mice compared to controls (Figure 5-6).

Figure 5-6 Cortical tissue mineral density measured at the femoral midshaft from 12-week old female Dmp1Cre.EfnB2 mice assessed by micro-CT.
Data shown are mean + SEM, n=12/group

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5.3.4 mRNA levels of osteoblast and osteocyte markers

mRNA levels of early and late osteoblast markers were not significantly different in marrow-flushed femora from 12-week old female *Dmp1Cre.EfnB2*/*f/f* mice compared to controls.

Table 5-6 Relative expression of osteoblast, osteoclast and osteocyte mRNA measured in RNA extracted from femora of 12-week old female *Dmp1Cre.EfnB2* mice.

<table>
<thead>
<tr>
<th>Gene</th>
<th><em>Dmp1Cre.EfnB2</em>/<em>w/w</em> (n=12)</th>
<th><em>Dmp1Cre.EfnB2</em>/<em>f/f</em> (n=9)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Runx2</em></td>
<td>1.41 ± 0.145</td>
<td>1.43 ± 0.16</td>
</tr>
<tr>
<td><em>Osx</em></td>
<td>2.26 ± 0.47</td>
<td>1.48 ± 0.16</td>
</tr>
<tr>
<td><em>Alpl</em></td>
<td>4.16 ± 0.85</td>
<td>2.55 ± 0.32</td>
</tr>
<tr>
<td><em>Col1a1</em></td>
<td>456.77 ± 89.92</td>
<td>274.79 ± 61.85</td>
</tr>
<tr>
<td><em>Col1a2</em></td>
<td>799.56 ± 182.89</td>
<td>519.33 ± 108.51</td>
</tr>
<tr>
<td><em>Tnfsf11</em></td>
<td>0.11 ± 0.02</td>
<td>0.13 ± 0.02</td>
</tr>
<tr>
<td><em>Tnfrsf11b</em></td>
<td>0.22 ± 0.07</td>
<td>0.15 ± 0.04</td>
</tr>
<tr>
<td><em>Dmp1</em></td>
<td>1.47 ± 0.25</td>
<td>1.15 ± 0.23</td>
</tr>
<tr>
<td><em>Bglap</em></td>
<td>62.12 ± 13.48</td>
<td>37.02 ± 8.09</td>
</tr>
<tr>
<td><em>Mepe</em></td>
<td>0.02 ± 0.00</td>
<td>0.01 ± 0.00</td>
</tr>
<tr>
<td><em>Sost</em></td>
<td>2.00 ± 0.52</td>
<td>1.43 ± 0.34</td>
</tr>
</tbody>
</table>

Data shown are mean ± SEM, n = 9-12/group. Expression of genes are shown relative to the geometric average of Hypoxanthine Phosphoribosyltransferase 1 (*Hprt1*) and Hydroxymethylbilane Synthase (*Hmbs*) housekeeping genes.
5.3.5 Cortical bone mineralisation rate measured by histomorphometry

In contrast to the thinner cortical bone in femora from 12-week old \textit{Dmp1Cre.EfnB2}^{\textit{f/f}} mice, dynamic histomorphometric analysis detected no significant change in tibial cortical thickness compared to controls (Figure 5-7A). Periosteal single (Ps.sLS/BS) and double labelled (Ps.dLS/BS) surfaces, periosteal mineralising surface (Ps.MS/BS), periosteal mineral apposition rate (Ps.MAR) and periosteal bone formation rate (Ps.BFR/BS) were not significantly different at the tibial midshaft in female \textit{Dmp1Cre.EfnB2}^{\textit{f/f}} mice compared to controls (Figure 5-7B, Table 5-7).

![Figure 5-7](image)

Figure 5-7 Dynamic histomorphometric parameters of tibiae from 12-week old female \textit{Dmp1Cre.EfnB2} mice.
(A) Cortical Thickness, (B) Periosteal bone formation rate/Bone surface from tibiae of 12-week old \textit{Dmp1Cre.EfnB2} mice. Data shown are mean + SEM, n = 7-10/group.
Table 5-7 Other dynamic histomorphometric parameters of tibial cortical bone from 12-week old female *Dmp1Cre.EfnB2* mice

<table>
<thead>
<tr>
<th></th>
<th><em>Dmp1Cre.EfnB2</em> w/w (n=7)</th>
<th><em>Dmp1Cre.EfnB2</em> f/f (n=10)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Periosteal Single labelled surface/Bone Surface (%)</td>
<td>8.48 ± 7.96</td>
<td>18.94 ± 9.84</td>
</tr>
<tr>
<td>Periosteal Double labelled surface /Bone Surface (%)</td>
<td>84.73 ± 10.53</td>
<td>67.47 ± 10.76</td>
</tr>
<tr>
<td>Periosteal Mineralising Surface/Bone Surface (%)</td>
<td>88.9 ± 7.60</td>
<td>76.9 ± 6.93</td>
</tr>
<tr>
<td>Periosteal Mineral Apposition Rate (µm/day)</td>
<td>0.98 ± 0.12</td>
<td>1.07 ± 0.10</td>
</tr>
<tr>
<td>Periosteal Bone Formation Rate/Bone Surface (µm/µm²/day)</td>
<td>90.50 ± 15.65</td>
<td>88.60 ± 11.71</td>
</tr>
</tbody>
</table>

5.3.6 Collagen fibre organisation in female *Dmp1Cre.EfnB2* f/f femora

Collagen fibre organisation was assessed to determine whether alterations in the percentage of woven or lamellar bone were associated with impaired bone strength. Cortical bone from 12-week old *Dmp1Cre.EfnB2* f/f mice showed no significant difference in the proportion of woven bone compared to lamellar bone matrix in the midshaft of the femur compared to controls (Figure 5-8).

![Figure 5-8](image)

**Figure 5-8** Proportion of lamellar and woven bone in 12-week old female *Dmp1Cre.EfnB2* w/w (w/w) and *Dmp1Cre.EfnB2* f/f (f/f) mice assessed by polarised light microscopy.

Percentage bone area from thick transverse sections collected at the midshaft of the femur. Data shown are mean ± SEM, n = 7-9/group.
5.3.7 Cortical bone composition of female Dmp1Cre.EfnB2 mice assessed by synchrotron-based Fourier Transform Infrared Microscopy (sFTIRM)

Cortical bone composition was assessed in tibiae from 12-week old female Dmp1Cre.EfnB2\textsuperscript{f/f} mice at a site 1500 µm below the tibial growth plate on the medial side. Three 15µm\textsuperscript{2} regions were analysed commencing from the periosteal edge through to the mature bone by sFTIRM, as described in Section 2.8.1.1.

Averaged spectra from all regions and all samples indicated that the phosphate (mineral) and carbonate peak were greater in Dmp1Cre.EfnB2\textsuperscript{f/f} mice compared to Dmp1Cre.EfnB2\textsuperscript{w/w} controls. Spectra from Dmp1Cre.EfnB2\textsuperscript{f/f} mice also showed a lower amide I peak but a higher amide II peak compared to controls (Figure 5-9).

Quantified sFTIRM indices demonstrated a significant increase in mineral:matrix ratio from the periosteal edge (region 1) to the more mature regions (2 and 3) in Dmp1Cre control bones, as observed in OsxCre controls (Section 4.3.3). Carbonate:mineral ratio remained unchanged with increasing bone maturity in Dmp1Cre controls (Figure 5-10B), but there was a significant reduction in the amide I:II ratio with increasing bone maturity (Figure 5-10C).

Like the controls, Dmp1Cre.EfnB2\textsuperscript{f/f} mice also showed an increase in mineral:matrix with increasing bone maturity. In addition, they showed significantly greater mineral:matrix compared to controls, particularly in the two regions of most newly formed bone at the periosteal edge, (Figure 5-10A). Additionally, there was significantly more carbonate within the matrix in the two more mature regions of Dmp1Cre.EfnB2\textsuperscript{f/f} bone compared to controls, and the level of carbonate increased with increasing bone maturity (Figure 5-10B), an effect not observed in control mice. The amide I:II ratio in Dmp1Cre.EfnB2\textsuperscript{f/f} bones was notably lower than that of controls, significant in the two regions of most newly formed bone (Figure 5-10C).
Figure 5-9 Averaged absorbance spectra from 12-week old female $Dmp1Cre.EfnB2^{w/w}$ (blue) and $Dmp1Cre.EfnB2^{ff}$ (red) mice. All spectra from regions 1-3 were averaged per genotype. The main peaks analysed were amide I (1712-1588 cm$^{-1}$) (orange), amide II (1600-1500 cm$^{-1}$) (blue), phosphate (1180-916 cm$^{-1}$) and carbonate (890-852 cm$^{-1}$). n= 13/group
Figure 5-10 Analysis of maturing bone regions in tibiae from 12-week old female *Dmp1Cre.EfnB2* mice assessed by sFTIRM.

Ratios were calculated from integrated areas of the phosphate (mineral), matrix (amide I) and amide II peaks. (A) Mineral:matrix ratio, (B) Carbonate:mineral ratio and (C) Amide I:II ratio. Data shown are mean ± SEM, n = 13/group, *p<0.05, **p<0.01, ***p<0.001 vs. Region 1 of same genotype, + p<0.05, ++ p<0.01 vs. same region from *Dmp1Cre.EfnB2* controls.
5.3.7.1 Sub peak analysis

The integrated area ratio of the 1660:1690 sub peaks, which indicate collagen crosslinks (representative of collagen secondary structure) (2), showed no differences with increasing maturity in female control or *Dmp1Cre.EfnB2^{f/f}* specimens. *Dmp1Cre.EfnB2^{f/f}* bones demonstrated a significantly higher 1660:1690 ratio in the intermediate region (region 2) of bone compared to controls (Figure 5-11A). Crystallinity, indicated by the integrated area of the 1030:1020 sub-bands, was significantly greater in the most mature bone region compared to the intermediate region in *Dmp1Cre.EfnB2^{f/f}* mice. However, *Dmp1Cre.EfnB2^{f/f}* bones showed no significant differences in crystallinity compared to controls (Figure 5-11B).

![Figure 5-11](image)

**Figure 5-11 Integrated area of the sub peaks from the amide I, amide II and phosphate peaks obtained from 12-week old female *Dmp1Cre.EfnB2* bones.**

(A) Collagen crosslinking and (B) Crystallinity. Data shown are mean + SEM, n = 13/group, \( \phi \) p<0.05 vs. region 2 of same genotype.
5.3.7.3 *Investigating collagen alignment and distribution using FTIR with 0° and 90° polarising filters*

Collagen fibre alignment and distribution was examined using a 0° polarising filter (views bonds aligned parallel to the tissue section) and a 90° polarising filter (views bonds aligned perpendicular to the tissue section). FTIR images were taken 1500 µm from the base of the growth plate with an additional 100 µm region on the top and bottom of this location.

Under the 0° polarising filter, tibiae from 12-week old *Dmp1Cre.EfnB2*<sup>+/−</sup> control mice showed an amplification of amide I signal but not amide II at the cortical midshaft (Figure 5-12A). This indicates that a greater proportion of amide I bonds are aligned in the same plane as the bone tissue section. Consistent with this observation, the 90° polarising filter raised the amide II signal relative to amide I in control tibiae (Figure 5-12B). This indicates that a greater proportion of amide II than amide I bonds are aligned perpendicular to the plane of the tissue section. Both polarising filters showed higher levels of amide I bonds on the endosteal and periosteal cortical sides of a central region which contained less amide I bonds. Additionally, the 90° polarising filter showed higher levels of amide II bonds on both the endosteal and periosteal cortical surfaces (indicated by the asterisks in Figure 5-12B).

As observed in control tibiae, the 0° polarising filter amplified the amide I signal in *Dmp1Cre.EfnB2*<sup>+/−</sup> bones, confirming similar orientation of the collagen fibres relative to the plane of the tissue section. The amide II signal was also amplified in *Dmp1Cre.EfnB2*<sup>+/−</sup> bones relative to the amide II signals from control tibiae. This was particularly noticeable on the endosteal side (Figure 5-12C). Additionally, amide II bonds were amplified under the 90° polarising filter in *Dmp1Cre.EfnB2*<sup>+/−</sup> bones relative to amide I bonds (Figure 5-12D). In contrast to controls, amide I bonds were less visible, and did not show the distinctive pattern of control amide I bonds either side of the central core of trabecular bone. Instead, they showed a more heterogeneous pattern through the
tibial midshaft cortex and amide II bonds were more visible on the endosteal side of the tibiae (Figure 5-12D).
Figure 5-12 Representative FTIR images of amide I and II bonds in the cortical mid diaphysis of tibiae from 12-week old female Dmp1Cre.EfnB2\textsuperscript{w/w} and Dmp1Cre.EfnB2\textsuperscript{f/f} mice under 0° and 90 polarising filters. These are representative images from 13 samples per group. The relative intensity scale represents the proportion of bonds aligned in a parallel (0°) or perpendicular (90°) direction to the plane of the tissue section. Amide I and II bonds analysed using the 0° (A,C) and 90° (B,D) polarising filter in Dmp1Cre.EfnB2\textsuperscript{w/w} and Dmp1Cre.EfnB2\textsuperscript{f/f} tibiae.
5.3.8 Osteocyte lacunae properties assessed by Backscattered Scanning Electron Microscopy (BSEM)

BSEM revealed that osteocyte lacunar size within the cortical femoral midshaft was unchanged in 12-week old female Dmp1Cre.EfnB2<sup>f/f</sup> femora compared to controls (Figure 5-13A). The size of the top 20% largest osteocyte lacunae, a parameter previously reported (262), was also unchanged in 12-week old female Dmp1Cre.EfnB2<sup>f/f</sup> femora compared to controls (Figure 5-13B). However, osteocyte lacunar density was significantly greater in Dmp1Cre.EfnB2<sup>f/f</sup> femora compared to controls (Figure 5-13C).
Figure 5-13 Osteocyte lacunae parameters of 12-week old female $Dmp1Cre.EfnB2^{w/w}$ (w/w) and $Dmp1Cre.EfnB2^{f/f}$ (f/f) mice measured at the cortical femoral midshaft by Backscattered electron microscopy.

(A) Osteocyte lacunar size, (B) Largest 20% osteocyte lacunar size, (C) Osteocyte lacunar density. Data shown are mean ± SEM, $n = 10-12$ / group. *$p<0.05$ vs. $Dmp1Cre.EfnB2^{w/w}$ controls.
5.3.9  Apoptotic osteocytes measured by TUNEL stain in \textit{Dmp1Cre.EfnB2} tibiae

12-week old female \textit{Dmp1Cre.EfnB2}^{ff} tibiae demonstrated no significant differences in the percentage of TUNEL positive (+) and TUNEL negative (-) osteocytes within the tibial cortical midshaft compared to controls. Similarly, the percentage of empty lacunae was not different in \textit{Dmp1Cre.EfnB2}^{ff} mice compared to controls (Figure 5-14).

Figure 5-14 Percentage of TUNEL stained osteocytes and empty lacunae from the tibial midshaft in 12-week old female \textit{Dmp1Cre.EfnB2} mice. Measurements were carried out by Blessing Crimeen-Irwin.
5.3.10 Osteocyte morphology in *Dmp1Cre.EfnB2* tibiae assessed by Transmission Electron Microscopy (TEM)

Tibiae from 12-week old female *Dmp1Cre.EfnB2* control mice demonstrated normal osteocyte lacunae morphology (Figure 5-15A,B). However, *Dmp1Cre.EfnB2* tibiae showed many degenerating osteocytes with condensed cytoplasm and amorphous matrix with some empty space within the osteocyte lacunae under the light microscope (Figure 5-15C). This abnormal morphology of *Dmp1Cre.EfnB2* osteocytes was confirmed by TEM, with the lacunae appearing to contain large vacuoles (Figure 5-15D). This is in contrast to the osteoblast and osteocyte apoptosis observed in *OsxCre.EfnB2* mice by TEM.
Figure 5-15 Representative light microscope and transmission electron microscope images of 12-week old female *Dmp1Cre.EfnB2* tibiae.

Light microscope images from (A) *Dmp1Cre.EfnB2*<sup>w/w</sup> and (C) *Dmp1Cre.EfnB2*<sup>f/f</sup> osteocyte lacunae within cortical bone. Intact osteocytes (yellow arrows). TEM images of (B) *Dmp1Cre.EfnB2*<sup>w/w</sup> and (D) *Dmp1Cre.EfnB2*<sup>f/f</sup> osteocytes embedded within cortical bone. Degenerating osteocytes with condensed cytoplasm and empty space (purple arrows) and degenerating osteocytes with condensed cytoplasm and amorphous matrix (black arrows). Images were taken by Dr. Mika Ikegame.
5.3.11 The role of ephrinB2 in late osteoblasts/osteocytes in trabecular bone structure

The expected age-related differences in trabecular bone structure were detected by micro-CT in control mice. Trabecular bone volume (BV/TV), and trabecular number (Tb.N) were reduced with age in male and female \textit{Dmp1Cre.EfnB2\textsuperscript{w/w}} control mice (Figure 5-16A, C) with an inverse relationship observed for trabecular separation (Tb.Sp) (Figure 5-16D). Trabecular thickness (Tb.Th) was significantly lower at 12 and 26 weeks of age compared to 6 weeks of age in females, but not males (Figure 5-16B). Sex differences in trabecular bone structure were observed at all ages with male \textit{Dmp1Cre.EfnB2\textsuperscript{w/w}} controls having significantly greater BV/TV, Tb.Th., Tb.N and lower Tb.Sp than females.

There was no significant trabecular phenotype in male \textit{Dmp1Cre.EfnB2\textsuperscript{f/f}} femora at any age assessed. Deletion of ephrinB2 in late osteoblasts/osteocytes resulted in mildly altered trabecular bone structure at all ages in female mice. 6-week old female \textit{Dmp1Cre.EfnB2\textsuperscript{f/f}} mice showed significantly lower BV/TV and Tb.N accompanied by significantly higher Tb.Sp compared to age- and sex-matched controls (Figure 5-16A, C, and D). A mild reversal of that phenotype was observed in 12 and 26 week old female \textit{Dmp1Cre.EfnB2\textsuperscript{f/f}} mice compared to controls.
Figure 5-16 Micro-CT analysis of trabecular bone structure in 6, 12 and 26-week old female and male Dmp1Cre.EfnB2 femora. 

(A) Trabecular bone volume, (B) Trabecular thickness, (C) Trabecular number and (D) Trabecular separation. Data shown are Mean + SEM, n= 6-12/group, *p<0.05, **p<0.01 vs. sex-matched Dmp1Cre.ephrinB2<sup>W/W</sup> controls, ++p<0.01, +++p<0.001 vs. female Dmp1Cre.ephrinB2<sup>W/W</sup> controls, °p<0.05, °°p<0.01, °°°p<0.001 vs. previous age group of sex-matched Dmp1Cre.EfnB2<sup>W/W</sup> controls.
Histomorphometric analysis of tibiae from 12-week old female *Dmp1Cre.EfnB2*/*f/f* mice confirmed the reduced Tb.Sp at 12 weeks of age, in addition BV/TV was significantly greater than controls. This was accompanied by significantly greater Tb.Th and Tb.N than controls (Figure 5-17A-D). While there was no change in osteoblast parameters (Figure 5-18A-C), osteoclast surface, average area per osteoclast and osteoclast length were all significantly greater in 12-week old female *Dmp1Cre.EfnB2*/*f/f* mice compared to controls (Figure 5-19B, D).

**Figure 5-17** Trabecular structural parameters measured by static histomorphometry of *Dmp1Cre.EfnB2*/*w/w* (w/w) and *Dmp1Cre.EfnB2*/*f/f* (f/f) mice. (A) Trabecular bone volume, (B) Trabecular thickness, (C) Trabecular separation and (D) Trabecular number from 12-week old *Dmp1Cre.EfnB2* mice. Data shown are mean ± SEM, n = 7-9/group, +p<0.05, ++p<0.01, +++p<0.001 vs. female *Dmp1Cre.EfnB2*/*w/w* controls, *p<0.05, **p<0.01 vs. sex-matched *Dmp1Cre.EfnB2*/*w/w* controls.
Figure 5-18 Osteoblast parameters measured by histomorphometry in the trabecular secondary spongiosa of 12-week old Dmp1Cre.EfnB2 mice. (A) Number of osteoblasts/Bone perimeter, (B) Osteoblast surface/Bone surface, (C) Osteoid thickness. Data shown are mean ± SEM, n = 7-9/group.
Figure 5-19 Osteoclasts parameters from trabecular secondary spongiosa in 12-week old *Dmp1Cre.EfnB2*<sup>w/w</sup> (w/w) and *Dmp1Cre.EfnB2*<sup>f/f</sup> (f/f) mice.

(A) Number of osteoclasts/Bone perimeter, (B) Osteoblast surface/Bone surface, (C) Osteoclast length and (D) Osteoclast area. Data shown are mean ± SEM, n = 7-9/group, *p<0.05, **p<0.01 vs. *Dmp1Cre.EfnB2*<sup>w/w</sup> mice, +p<0.05 vs. female *Dmp1Cre.EfnB2*<sup>w/w</sup> controls.
Table 5- 8 Additional static and dynamic histomorphometric parameters from tibiae of 12-week old female and male Dmp1Cre.EfnB2 mice

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Female Dmp1Cre.EfnB2&lt;sup&gt;w/w&lt;/sup&gt; (n=7)</th>
<th>Male Dmp1Cre.EfnB2&lt;sup&gt;+/–&lt;/sup&gt; (n=9)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Osteoid Surface/Bone Surface (%)</td>
<td>7.39 ± 1.45</td>
<td>5.96 ± 1.14</td>
</tr>
<tr>
<td>Single labelled surface/Bone Surface (%)</td>
<td>20.44 ± 2.10</td>
<td>26.24 ± 2.20</td>
</tr>
<tr>
<td>Double labelled surface/Bone Surface (%)</td>
<td>24.30 ± 2.60</td>
<td>26.72 ± 2.63</td>
</tr>
<tr>
<td>Mineralising Surface/Bone Surface (%)</td>
<td>34.51 ± 2.52</td>
<td>42.62 ± 1.99</td>
</tr>
<tr>
<td>Mineral Apposition Rate (μm/day)</td>
<td>2.09 ± 0.09</td>
<td>1.61 ± 0.09</td>
</tr>
<tr>
<td>Bone Formation Rate/Bone Surface (μm/μm&lt;sup&gt;2&lt;/sup&gt;/day)</td>
<td>0.71 ± 0.04</td>
<td>0.68 ± 0.04</td>
</tr>
</tbody>
</table>

Data shown are mean ± SEM, where +p<0.05, +++p<0.001 vs. male Dmp1Cre.EfnB2<sup>w/w</sup> controls and *p<0.05 vs. sex-matched Dmp1Cre.EfnB2<sup>+/–</sup> controls.
5.4 Discussion

In this chapter, I report that 12-week old female Dmp1Cre.EfnB2flo/flo femora are more brittle than controls and have altered bone composition, highlighting a stage-specific role of ephrinB2 during osteoblast differentiation in regulating bone material composition and strength.

EphrinB2 knockdown in Dmp1Cre.EfnB2flo/flo mice was confirmed in FACS-sorted GFP+ cells. Bones from 12-week old female Dmp1Cre.EfnB2flo/flo mice exhibited impaired bone strength compared to controls, including significantly lower structural parameters such as ultimate force and deformation and material parameters such as toughness. Indentation distance increase measured by reference point indentation, a parameter that has been previously negatively correlated with reduced bone toughness (260), was greater. Female Dmp1Cre.EfnB2flo/flo mice showed no significant difference in cortical size, width or moment of inertia, mineral density or mineralisation rate compared to controls suggesting that ephrinB2 in late osteoblasts/osteocytes maintains bone strength by regulating bone composition.

sFTIRM and polarised FTIRI were used to assess changes in the two main components of bone: the hydroxyapatite mineral crystal lattice and the collagenous matrix. sFTIRM revealed a high mineral:matrix ratio at the periosteal edge (regions 1 and 2) in Dmp1Cre.EfnB2flo/flo mice compared to controls. Our dynamic histomorphometry demonstrated that the rate at which osteoid mineralisation is initiated was unchanged (periosteal mineral apposition rate). The high mineral:matrix ratio therefore may suggest that as soon as mineralisation is initiated, more mineral is deposited in Dmp1Cre.EfnB2flo/flo mice compared to controls. This was not explained by significant changes in mRNA expression levels of osteocyte products previously reported to regulate mineralisation (Dmp1(97), Bglap (82), Mepe (98) and Sost (99)). This suggests that the greater mineral deposition is regulated by genes not previously known to regulate mineralisation and will be further investigated by RNA sequencing analysis of marrow-flushed bones from control and Dmp1Cre.EfnB2flo/flo mice.
The greater mineral:matrix ratio in *Dmp1Cre.EfnB2*/*f/f* mice contrasts with *OsxCre.EfnB2*/*f/f* mice (described in Chapter 4), where the initiation of mineralisation was delayed but the mineral:matrix ratio on the periosteal edge was unchanged. *OsxCre.EfnB2*/*f/f* mice with deletion of ephrinB2 early in the osteoblast differentiation pathway showed delayed initiation of mineralisation likely due to osteoblast apoptosis at the stage of matrix production (195). In contrast, ephrinB2 would be deleted later in the osteoblast differentiation pathway in *Dmp1Cre.EfnB2*/*f/f* mice, an outcome upheld by the presence of *EfnB2* mRNA in DMP1-GFP–ve cells isolated from collagenase digested *Dmp1Cre.EfnB2*/*f/f* bones. This means that osteoblasts would survive past the checkpoint at which anti-apoptotic action of ephrinB2 is required, confirmed by a lack of any detectable increase in osteoblast apoptosis. I suggest that the normal initiation of mineralisation occurs because *Dmp1Cre.EfnB2*/*f/f* osteoblasts are capable of completing differentiation into late stage osteoblasts and osteocytes, but that their lack of ephrinB2 beyond this point is responsible for increased level of mineralisation.

*Dmp1Cre.EfnB2*/*f/f* femora also showed greater carbonate incorporation within the mineral (carbonate:mineral ratio) with increasing bone age compared to control bones, particularly in the mature regions (region 2 and 3). Hydroxyapatite mineral is composed of calcium, phosphate and hydroxyl (OH) ions which can be replaced by fluoride, chloride or carbonate. The carbonate:mineral ratio represents the amount of carbonate substitution for phosphate or hydroxyl in the hydroxyapatite mineral crystal lattice (Ca₁₀(PO₄)₆(OH)₂) (263). While little is known about the relationship between carbonate content and bone strength, a higher carbonate:mineral ratio in iliac crest cortical bone has been associated with fracture susceptibility in osteoporotic patients compared to control subjects (264). This increase in carbonate:mineral rate has also been observed in postmenopausal osteoporotic bones compared to non-osteoporotic bone (265) indicating that higher carbonate levels may cause bone fragility. Currently no other study has investigated carbonate:mineral ratio with increasing bone tissue age on a bone forming surface. The carbonate:mineral ratio has been examined in human osteonal bone where a
reduction in the carbonate:mineral ratio from the centre of the osteon centre through to the periphery of the osteon was reported (256). This is in contrast to our data which may be due to our region of analysis being on a surface which primarily undergoes bone formation while the osteon undergoes remodelling. Carbonate content has also been measured in young and older rat bone and was reported to be greater in the older animals (266, 267). This is consistent with our observations where a higher carbonate:mineral ratio is also observed in older bone.

Altered collagen matrix properties were indicated by a lower amide I:II ratio in tibiae from *Dmp1Cre.EfnB2*/*f* mice compared to controls. Amide I:II ratio has only been reported previously in articular cartilage as a marker for collagen fibre orientation (223, 268) but not yet in bone tissue. The amide I peak indicates the organic matrix protein content by reflecting the vibration of C=O bonds that run perpendicular to the collagen triple helical axis, while the amide II peak reflects vibration of the C-N bond that run parallel along the collagen triple helical axis (269, 270). The amide I:II ratio declined significantly from the periosteal edge through to the maturing bone indicating that the collagen fibres are either stretching (increasing distance between the C and N molecules) in a parallel direction to the fibre axis or compressing (decreasing distance between the C and O molecules) in width as the bone matrix matures. Whether the collagen fibres stretch or compress in response to hydroxyapatite crystal deposition and growth, or their stretching determines crystal size or orientation is not known. The decline in the amide I:II ratio is due to an increase in the amide II peak, however there are concerns about analysing individual peaks due to variations in sample or section thickness providing false information on the “concentration” of that material. Reduced amide I:II ratio was not observed in *OsxCre.EfnB2*w/w* control bones which may be due to their delayed initiation or progression of mineralisation (195) or the effects of the *OsxCre* transgene which has previously been reported to cause a delay in bone accrual in wildtype mice (212). It may also be due to the lower sample numbers, which were approximately half that of the study of the *Dmp1Cre.EfnB2*/*f* mice.
Dmp1Cre.EfnB2\textsuperscript{f/f} femora also demonstrated a reduction in amide I:II ratio with bone tissue age, and their amide I:II ratio was significantly lower compared to controls, especially in the newly formed bone. This suggests that, in Dmp1Cre.EfnB2\textsuperscript{f/f} the collagen conformation changes to allow a greater level of mineral (indicated by the mineral:matrix ratio) to be incorporated, or results from the accrual of a high mineral content. It is not possible at this stage to discern which is the case.

Collagen crosslinking, represented by the 1660:1690 cm\textsuperscript{-1} ratio, was significantly greater in the intermediate region of Dmp1Cre.EfnB2\textsuperscript{f/f} bones compared to controls. The 1660:1690 cm\textsuperscript{-1} ratio represents post-translational modification of collagen and can also be described as nonreducible:reducible crosslinks (271). The intermolecular cross-linking within collagen fibres provides their tensile strength and viscoelasticity (271). Greater collagen crosslinks have been observed in osteoporotic patients (271) which may be due to the matrix in osteoporotic patients being produced at a faster rate or undergoing post-translational modification for a longer period compared to the bone matrix of normal bone (271). The greater 1660:1690 ratio observed in the intermediate region of bones from Dmp1Cre.EfnB2\textsuperscript{f/f} mice is associated with greater mineral:matrix and carbonate:mineral ratio indicating that cross links mature in the same region in which mineral accumulates. This suggests that the greater mineral accumulation and carbonate substitution in Dmp1Cre.EfnB2\textsuperscript{f/f} bone may result from more mineral packed within the collagen fibrils at the stage of osteoid maturation causing stretching and/or compression of the fibres.

Further investigation into the orientation of collagen fibres from Dmp1Cre.EfnB2\textsuperscript{f/f} tibiae was performed using FTIR imaging coupled with 0\textdegree and 90\textdegree polarising filters. Collagen is composed of fibres which are aligned in different directions throughout the cortical bone. For example, woven bone consists of randomly aligned fibres and lamellar bone consists of an organised alignment of fibres. Each collagen fibre is made up of multiple triple helices of collagen where the triple helical structure of collagen is composed of amide I and amide II bonds (272). The 0\textdegree polarising filter was used to enhance bonds aligned parallel to the tissue section, while the 90\textdegree polarising filter was used to enhance
bonds aligned perpendicular to the tissue section (268). Use of these two polarising filters allowed us to observe the molecular orientation of amide I and amide II bonds within collagen fibres relative to the plane of the tissue section, as well as their distribution throughout the cortical bone. Tibiae from 12-week old Dmp1Cre.EfnB2<sup>w/w</sup> controls showed enrichment of amide I bonds relative to amide II bonds using the 0° polarising filter, which enhanced bonds aligned parallel to the tissue section. This suggested that in a longitudinal section of bone, the majority of collagen fibres are directed upwards (or out towards the observer/microscope), i.e. collagen fibres are oriented concentrically in a transverse direction around the tibiae. In comparison, Dmp1Cre.EfnB<sup>ff</sup> bones demonstrated enhanced amide II bond intensity compared to the intensity of amide II bonds in bones of control mice, indicating that collagen fibres are oriented flat along the tissue section (or parallel to the microscope slide) and therefore less transverse fibres than in controls. This confirms the lower amide I:II ratio in Dmp1Cre.EfnB<sup>ff</sup> bones detected by our bone-age-matched sFTIRM method.

The 90° polarising filter also enriched both amide I and II bonds on the periosteal and the endosteal sides of the tibial cortex in Dmp1Cre.EfnB2<sup>w/w</sup> control bones. This suggested that collagen fibres are more highly organised and oriented perpendicular and parallel to the plane of the tissue section around the periosteal edge and endosteal side of the tibiae of control bones. The centre of the cortex may show much of this highly organised pattern because these are sites of intracortical canals which house blood vessels within mouse bone (273). Bones from Dmp1Cre.EfnB2<sup>ff</sup> mice demonstrated a heterogeneous distribution of amide I and II bonds throughout the tibial cortex, thus the greater mineral deposition and carbonate incorporation may also relate to a heterogeneous distribution of collagen bonds throughout cortical bone.

Collagen fibre organisation has previously been shown to correlate with mechanical properties of bone. For example, longitudinal orientation of collagen fibres was reported to be a predictor of tensile strength and elastic modulus in cortical bone (274, 275). Additionally, a decreased proportion of longitudinally oriented collagen has been reported in the Mov13 mouse which harbours a collagen mutation ultimately reducing
their post-yield deflection leading to brittle bones (231). Another mouse model of osteogenesis imperfecta (oim mice) show altered collagen structure caused by the dysfunctional synthesis of three α1(I) chains to form a homotrimer instead of the normal α1(1)2α2(1) structure in tropocollagen (276). Oim mice also display a brittle bone phenotype (277-279). Although we observed a difference in the distribution of amide I and II bonds measured by FTIRI, polarised light microscopy measures the deposition of collagen fibres and the proportion of woven and lamellar bone was not changed in Dmp1Cre.EfnB2f/f mice. Therefore, the greater mineral accumulation, altered collagen fibre stretch/compression and heterogeneous distribution of amide I and II bonds may all explain the brittleness of bones from Dmp1Cre.EfnB2f/f mice.

Osteocyte lacunar size remained unchanged in femora from Dmp1Cre.EfnB2f/f compared to controls; however the number of osteocyte lacunae per total bone area was significantly greater. Although osteocyte lacunar number may not accurately represent the number of living osteocytes since nuclei cannot be detected by backscattered electron microscopy, this suggests that osteoblasts are incorporated into the bone matrix as osteocytes at a faster rate in Dmp1Cre.EfnB2f/f mice. Since Dmp1Cre targets late osteoblasts at the stage where they become embedded within the newly formed bone (103, 104), deletion of ephrinB2 appears to promote the embedment of late osteoblasts into the bone matrix. In turn, this greater density of osteocytes may contribute to mineral accumulation and carbonate incorporation in Dmp1Cre.EfnB2f/f bones by greater local production of factors such as Dmp1 and Mepe that control the process of mineralisation, even though they were not significantly altered at the whole bone level.

Osteocytes within the cortical bone of Dmp1Cre.EfnB2f/f mice demonstrated abnormal morphology compared to controls. Light microscope images of cortical bone from Dmp1Cre.EfnB2f/f tibiae showed degenerating osteocytes with condensed cytoplasm and either an empty part or with an amorphous matrix, compared to the osteocytes embedded in control bones. Preliminary data by transmission electron microscopy showed osteocytes from Dmp1Cre.EfnB2f/f control mice have a regular structure containing a nucleus but osteocytes from Dmp1Cre.EfnB2f/f mice contained large vacuoles, with
sometimes very small vesicles (Mike Ikegame, personal communication). Vacuolisation of the cytoplasm and pyknotic nuclei have previously been described as a sign of degeneration in osteocytes (280, 281). In contrast to OsxCre.EfnB2\textsuperscript{ff} mice, apoptotic bodies were not observed in osteocytes of Dmp1Cre.EfnB2\textsuperscript{ff} mice and the number of TUNEL positive osteocytes in tibiae from 12-week old Dmp1Cre.EfnB2\textsuperscript{ff} mice was not different compared to controls. This indicates that the abnormal morphology observed by transmission electron microscopy is not related to apoptosis as no apoptotic bodies were found within osteocytes and suggests that the anti-apoptotic role of ephrinB2 may be restricted to earlier stages of osteoblast differentiation. The osteocytes in Dmp1Cre.EfnB2\textsuperscript{ff} mice may be undergoing degeneration that release mineral into the matrix causing the greater mineral content of these mice. The greater number of osteocyte lacunae may also reflect increased osteocyte incorporation to compensate for the abnormal/defective osteocytes of Dmp1Cre.EfnB2\textsuperscript{ff} mice. Overall, these data suggest that the brittle bone phenotype in 12-week old Dmp1Cre.EfnB2\textsuperscript{ff} mice may be due to defective osteocyte function associated with greater mineral deposition and carbonate incorporation as well as altered collagen organisation within their cortical bone.

No significant differences were observed in cortical bone structure or strength in 12-week old male Dmp1Cre.EfnB2\textsuperscript{ff} femora compared to controls. The female-specific phenotype was also observed in OsxCre.EfnB2\textsuperscript{ff} mice (described in Chapter 4) providing further evidence that ephrinB2 may play a sex-specific role in regulating cortical bone structure and strength. It is also possible that age-related differences in growth between male and female mice may play a role in the sex-specificity of the phenotype since 6-week old male Dmp1Cre.EfnB2\textsuperscript{ff} femora showed similar (though milder) mechanical deficits as 12-week old female Dmp1Cre.EfnB2\textsuperscript{ff} mice, including lower force at yield and failure force. Additionally, as female Dmp1Cre.EfnB2\textsuperscript{ff} mice aged to 26 weeks, the mechanical defect resolved, providing further evidence that it may be specific to the smaller cortical bone size of 6-week old males and 12-week old females.
12-week old female \textit{Dmp1Cre.EfnB2}^{f/f} mice also displayed greater tibial trabecular bone volume and a reduction in trabecular separation. This may be due to impaired osteoclast function suggested by enlarged osteoclast size \textit{in vivo}. Impaired osteoclast attachment has previously been demonstrated in mice deficient in the \textit{c-src} gene because they were unable to form proper sealing zones resulting in larger osteoclasts with impaired function leading to osteopetrosis (282). At the light microscope level osteoclasts appeared to not attach to the bone surface properly in \textit{Dmp1Cre.EfnB2}^{f/f} mice (data not shown). Osteoclast attachment or motility may have been modified by the greater mineral deposition and carbonate incorporation into the mineral in bones from \textit{Dmp1Cre.EfnB2}^{f/f} mice however the relationship between carbonate concentration and osteoclast resorption remains unclear. Decreased resorptive capacity of primary rabbit osteoclasts has been observed in a dose dependent manner by presence of carbonate inside a mineral lattice (283). In contrast, the majority of studies have indicated increased osteoclast activity with higher levels of carbonate. For example, enhanced osteoclast resorption was observed on hydroxyapatite material containing high carbonate substitutions (284), a finding that was confirmed \textit{in vivo} by increased osteoclastogenesis near implanted hydroxyapatite with more carbonate substitutions (284). Earlier studies also demonstrated that the formation and activity of osteoclasts was increased on artificially carbonated apatite structures (285, 286). Since the majority of studies suggest increased resorption of higher carbonate containing materials, then bone resorption might be expected to be enhanced in \textit{Dmp1Cre.EfnB2}^{f/f} mice. One possible explanation for the discrepancy in findings may be that the level of carbonate reported \textit{in vitro} is different to what is found \textit{in vivo}. For example, only one of the above studies reported carbonate concentration in their materials, and this consisted of amounts up to 2.35 wt. % (285). Bone contains approximately 7 wt. % carbonate (287) so the changes in carbonate we observed in \textit{Dmp1Cre.EfnB2}^{f/f} are unlikely to be the comparable to those shown to promote resorption as reported \textit{in vitro}.

Osteoclasts at the growth plate of neonate \textit{OsxCre.EfnB2}^{f/f} mice had abnormal morphology with a lack of sealing zones and poor attachment to growth plate cartilage leading to impaired cartilage resorption (288), providing prior evidence that ephrinB2
deletion in the osteoblast lineage can influence osteoclast activity. Cultured chondrocytes from OsxCre.EfnB2\textsubscript{f/f} neonates also showed impaired support of osteoclastogenesis and low mRNA levels of cartilage-degrading enzymes, including ADAMTS4. This suggested that OsxCre-expressing cells, including hypertrophic chondrocytes, are dependent on ephrinB2 for their production of cartilage-degrading enzymes to promote both osteoclast and osteoblast attachment to the cartilage surface (288).

In conclusion, this chapter demonstrates firstly that ephrinB2 within late osteoblasts/osteocytes is required for the formation of strong bone matrix by restraining mineral accrual, incorporation of carbonate into the mineral and by regulating the alignment and distribution of collagen throughout the cortical bone. Osteocytic ephrinB2 may also regulate trabecular bone mass through osteoclast attachment or activity but this requires further investigation.
CHAPTER 6: Effects of intermittent parathyroid hormone (PTH) treatment on bone composition

6.1 Introduction

Previous work using a pharmacological inhibitor of the ephrinB2/EphB4 interaction (sEphB4) showed that this signal is not only required as a checkpoint for osteoblast differentiation in vitro and in vivo (157, 194) but also for the anabolic action of PTH (194). Impairment in the anabolic action of PTH was observed in vivo in the presence of sEphB4 not only due to impaired late stage osteoblast differentiation but also to increased osteoclast formation in vivo (194). The increase in osteoclast formation was reproduced when osteoblasts were co-cultured with osteoclast precursors and treated with sEphB4 suggesting that early stages of osteoblast differentiation may be more supportive of osteoclast formation (194). Inhibiting the ephrinB2/EphB4 interaction in osteoblasts in vitro increased their support of osteoclastogenesis by rapidly promoting RANKL transcription in cells of the osteoblast lineage and by stimulating mRNA levels of other pro-osteoclastic factors, such as interleukin-6 (Il6) and oncostatin M receptor (Osmr) (194).

Since PTH stimulates ephrinB2 expression by osteoblasts (157), mice with osteoblast specific deletion of ephrinB2 (OsxCre.EfnB2\textsuperscript{f/f}) were generated (described in Chapter 4) and treated with anabolic PTH to determine the role of osteoblastic ephrinB2 in the anabolic action of PTH (195). The PTH-induced increase in osteoblast number, osteoid thickness and mineral apposition rate in both trabecular and periosteal bone in male mice was significantly impaired, but not fully blocked, in OsxCre.EfnB2\textsuperscript{f/f} mice (195). Consistent with a limited response to PTH, these mice also showed a deficiency in the usual PTH-induced changes in gene expression. Early osteoblast markers (Runx2, Alpl and Colla1) were not increased, and Sost expression was not reduced. However, the RANKL gene (Tnfsf11) response to PTH was retained (195). This indicated that ephrinB2 signalling within the osteoblast is required for PTH to stimulate osteoblast differentiation
and reduce osteocytic sclerostin expression, but is not required for PTH to increase RANKL expression. This may, at least in part, relate to retention of early osteoblast-lineage cells that have higher levels of RANKL expression, and have reached the stage of PTH receptor expression. Thus, ephrinB2 reverse signalling within the osteoblast lineage is important both for osteoblast survival (as described in Chapter 4) and for the anabolic action of PTH during bone remodelling and periosteal growth.

To determine whether ephrinB2 in osteocytes is also required for PTH action, I examined the effect of anabolic PTH treatment in male mice lacking ephrinB2 in late osteoblasts/osteocytes. Micro-CT analysis of femora and histomorphometric analysis of the tibiae demonstrated that ephrinB2-deficiency in late osteoblasts/osteocytes did not significantly affect the anabolic action of PTH treatment in male mice (Section 6.3). For this reason, and since I had developed a technique for sFTIRM analysis of bone composition (see Chapter 4 and 5) I used samples from this study to investigate the effect of anabolic PTH treatment on bone composition. This is reported in the published manuscript (Section 6.5).

6.2 Methods

Vehicle- and PTH-treated 12-week old male Dmp1Cre.EfnB2\textsuperscript{w/w} and Dmp1Cre.EfnB2\textsuperscript{f/f} mice were analysed by micro-CT as described in Section 2.2 and trabecular histomorphometry in the secondary spongiosa as described in section 2.3.5.1. Male mice were used because their lower level of bone remodelling makes the effects of PTH on osteoblast activity more readily detectable.
6.3 Results

6.3.1 The effect of anabolic PTH treatment in 12-week old male Dmp1Cre.EfnB2\(^{w/w}\) and Dmp1Cre.EfnB2\(^{f/f}\) mice

Anabolic PTH treatment caused a significant increase in cortical thickness in the mid diaphysis of bones from 12-week old male Dmp1Cre.EfnB2\(^{w/w}\) and Dmp1Cre.EfnB2\(^{f/f}\) mice compared to vehicle-treated genotype-matched controls (Figure 6-1A) and two-way ANOVA revealed there was not a significant difference in the magnitude of these effects. Periosteal mineral apposition rate was also significantly increased by PTH treatment in Dmp1Cre.EfnB2\(^{w/w}\) control mice compared to vehicle-treated controls. PTH treatment did not significantly increase periosteal mineral apposition rate in Dmp1Cre.EfnB2\(^{f/f}\) mice, but by two-way ANOVA. This difference in effect between the two genotypes was not significant (p= 0.51) (Figure 6-1B).

![Figure 6-1](image)

**Figure 6-1** Cortical parameters from PTH-treated male 12-week old Dmp1Cre.EfnB2\(^{w/w}\) (w/w) and Dmp1Cre.EfnB2\(^{f/f}\) (f/f) mice.

(A) Cortical thickness assessed by micro-CT and (B) Periosteal mineral apposition rate assessed by dynamic histomorphometry at the mid diaphysis. Data shown are mean + SEM, n = 6-10/group, **p<0.01, ***p<0.001 for effect of PTH treatment vs. vehicle-treated genotype-matched control.
In trabecular bone, PTH-treatment significantly increased osteoid volume, mineral apposition rate and osteoblast number compared to vehicle-treated genotype-matched controls (Figure 6-2A-C). The number of osteoclasts/bone perimeter was significantly increased by PTH treatment in Dmp1Cre.EfnB2\(^{w/w}\) controls but remained unchanged in Dmp1Cre.EfnB2\(^{f/f}\) mice. This difference in effect was not significant by two-way ANOVA (p=0.82) (Figure 6-2D).

Figure 6-2 Trabecular histomorphometric parameters from PTH-treated male 12-week old Dmp1Cre.EfnB2\(^{w/w}\) (w/w) and Dmp1Cre.EfnB2\(^{f/f}\) (f/f) mice.

(A) Osteoid Volume/Bone Volume, (B) Mineral Apposition Rate, (C) Number of Osteoblasts/Bone Perimeter and (D) Number of Osteoclasts/Bone Perimeter. Data shown are mean + SEM, n = 6-10/group, *p<0.05, **p<0.01, ***p<0.001 for effect of PTH treatment vs. vehicle-treated genotype-matched control.
6.4 Discussion

Deletion of ephrinB2 in late osteoblasts/osteocytes did not significantly alter the anabolic effect of PTH. Anabolic PTH treatment caused a significant increase in cortical and trabecular bone mass and formation rates in both control and 12-week old male \textit{Dmp1Cre.EfnB2}\textsuperscript{+/−} mice. Osteoid volume and osteoblast and osteoclast numbers were also not significantly altered in PTH-treated in \textit{Dmp1Cre.EfnB2}\textsuperscript{+/−} mice indicating that ephrinB2 in late osteoblasts/osteocytes is not required for the anabolic action of PTH.

This is in contrast to the impaired anabolic action of PTH observed in \textit{OsxCre.EfnB2}\textsuperscript{+/−} mice (195). Trabecular parameters such as osteoblast number, osteoid volume and mineral apposition rate were significantly greater in control mice but these responses to PTH were significantly impaired in mice lacking ephrinB2 within the entire osteoblast lineage (195). On the periosteum, the PTH-induced increase in mineral apposition rate was also completely blocked in \textit{OsxCre.EfnB2}\textsuperscript{+/−} mice (195). This indicated that osteoblastic ephrinB2 is required for intermittent PTH to stimulate bone formation in both trabecular and periosteal.

EphrinB2 expressed during different stages of osteoblast differentiation has different effects on bone formation, strength and quality. \textit{OsxCre.EfnB2}\textsuperscript{+/−} and \textit{Dmp1Cre.EfnB2}\textsuperscript{+/−} mice demonstrated completely different strength phenotypes where ephrinB2 in the osteoblast lineage was shown to be required for the maintenance of cortical bone elasticity in female mice. In contrast, ephrinB2 in late osteoblasts/osteocytes was required for the formation of strong bone matrix by restraining mineral accrual, incorporation of carbonate into the mineral and by regulating the alignment and distribution of collagen throughout the cortical bone. In addition, ephrinB2’s requirement for the anabolic action of PTH during osteoblast differentiation was also very different. Only \textit{OsxCre.EfnB2}\textsuperscript{+/−} mice demonstrated an impaired anabolic response to PTH indicating that it is ephrinB2 in osteoblasts that is most important for the anabolic action of PTH rather than ephrinB2 expressed in the osteocyte.
Since ephrinB2 in late osteoblasts/osteocytes did not alter the anabolic action of PTH, I used the developed sFTIRM method to analyse the bone composition of vehicle and PTH-treated *Dmp1Cre.EfnB2<sup>w/w</sup>* controls which is reported in the following published manuscript (Section 6.5).
6.5 Paper
Anabolic action of parathyroid hormone (PTH) does not compromise bone matrix mineral composition or maturation

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A B S T R A C T

Intermittent administration of parathyroid hormone (PTH) is used to stimulate bone formation in patients with osteoporosis. A reduction in the degree of matrix mineralisation has been reported during treatment, which may reflect either production of undermineralised matrix or a greater proportion of new matrix within the bone samples assessed. To explore these alternatives, high resolution synchrotron-based Fourier Transform Infrared Microspectroscopy (sFTIRM) coupled with calcein labelling was used in a region of non-remodelling cortical bone to determine bone composition during anabolic PTH treatment compared with region-matched samples from controls.

8 week old male C57BL/6 mice were treated with vehicle or 50 μg/kg PTH, 5 times/week for 4 weeks (n = 7–9/group). Histomorphometry confirmed greater trabecular and periosteal bone formation and 3-point bending tests confirmed greater femoral strength in PTH-treated mice. Dual calcein labels were used to match bone regions by time-since-mineralisation (bone age) and composition was measured by sFTIRM in six 15 μm² regions at increasing depth perpendicular to the most immature bone on the medial periosteal edge; this allowed in situ measurement of progressive changes in bone matrix during its maturation.

The sFTIRM method was validated in vehicle-treated bones where the expected progressive increases in mineral:matrix ratio and collagen crosslink type ratio were detected with increasing bone maturity. We also observed a gradual increase in carbonate content that strongly correlated with an increase in longitudinal stretch of the collagen triple helix (amide I:amide II ratio). PTH treatment did not alter the progressive changes in any of these parameters from the periosteal edge through to the more mature bone.

These data provide new information about how the bone matrix matures in situ and confirm that bone deposited during PTH treatment undergoes normal collagen maturation and normal mineral accrual.

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1. Introduction

Parathyroid hormone (PTH) is the only current clinically available pharmacological agent that increases bone mass in patients with osteoporosis [1]. This is achieved by direct actions on the osteoblast lineage: promoting precursor differentiation [2], preventing mature osteoblast apoptosis [3], activating lining cells [2], and inhibiting sclerostin production by osteocytes [4,5]. Bone formation involves two sequential events: deposition of Type I collagen-rich organic matrix (osteoid), and mineralisation by hydroxyapatite crystal formation within that matrix [6]. While PTH treatment increases osteoid production, lower mineral levels in bone samples from PTH-treated rats, monkeys and humans have raised concerns that the mineralisation process may be compromised in the presence of PTH [7–9].

Early studies in ovariectomised rats reported a reduction in ash weight in PTH-treated samples compared to controls which seemed paradoxical [7]. Later work using backscattered electron microscopy indicated a lower average degree of bone tissue mineralisation in samples from rats treated with PTH [8]. Cortical bone from PTH-treated monkeys showed a lower degree of mineralisation, crystallinity and collagen crosslinking than untreated controls on both periosteal and endosteal surfaces [9]. Biopsies from patients with osteoporosis treated with PTH showed significantly less matrix mineral, mineral crystallinity and collagen crosslinking in both cortical and trabecular bone compared to biopsies from placebo-treated patients [10]. Low mineralisation density was also observed in trabecular bone from PTH-treated patients [11], and mice showed reduced crystallinity in the cortical and trabecular matrix deposited during PTH treatment [12].
The mineralisation process has two phases. Within ~5–10 days, osteoid undergoes primary mineralisation with initiation being controlled by mature osteoblasts [13]. Over subsequent weeks, months and years, secondary mineralisation occurs with mineral continuing to accumulate at a slower rate [14]. Low mineral content in PTH treated bone may relate to a slower progression of primary or secondary mineralisation. Alternatively, a higher rate of remodelling may not allow sufficient time for secondary mineralisation. More recently, it has been reported that regions with lower mineralisation density in biopsies from PTH-treated patients exist primarily at sites of new bone tissue formation [11]. This provides support for the concept that the lower mineral levels in PTH-treated bone result from a greater proportion of newly formed (less mineralized) bone in each sample, rather than a change in matrix composition per se, since analysis regions used in the above studies were not corrected for a greater proportion of new bone.

We tested whether the progression of bone matrix mineralisation is altered by PTH and accounted for differences in the proportion of newly formed bone by assessing regions matched for bone age. To do this, we combined in vivo calcein-labelling (to identify bone sites commencing mineralisation at specific times) with synchrotron-based Fourier-transform infrared microspectroscopy (sFTIRM). The sFTIRM method allows in situ measurement of mineral composition and collagen structure without protein denaturation or bone maceration [15,16]. The highly focused infrared beam provided by the synchrotron light source [17] allowed us to collect high signal-to-noise IR spectra from smaller regions than those measured by laboratory based instruments. By coupling this to a fluorescence microscope, we are able to measure at sites identified by calcein labels. The calcine labels mark bone that has recently undergone primary mineralisation, thereby allowing us to compare bone at the same level of mineral maturity (bone age) between PTH-treated mice and controls. We have used this approach in murine cortical bone that lacks Haversian remodelling [18], at the tibial diaphyseal periosteum that lacks osteoclasts [19]. This allows measurement of continually maturing bone matrix without changes caused by recent osteoclast activity or remodelling, making it possible to measure un-interrupted primary and secondary mineralisation.

2. Materials and methods

2.1. Animals

8-week old male mice (26.8 ± 0.7 g) on a C57BL/6 background were randomized to two treatment groups; 9 mice were administered vehicle (2% heat inactivated (HI) mouse serum + saline), and 10 mice administered PTH [1–34] (50 μg/kg) (Bachem, Bubendorf, Switzerland) by intraperitoneal injection 5 days a week for 4 weeks, as previously described [20,21]. Mice were kept in an SPF facility, weighed daily and injection volumes adjusted according to weight changes over the 4-week treatment period; all injections were carried out in the morning. Animals were housed in a 12-h light and dark cycle with standard food and water provided ad libitum. Calcein (20 mg/kg) was administered by intraperitoneal (IP) injection 7 and 2 days before tissue collection. After the treatment protocol, mice were fasted for 12 h and one hour after the last injection mice were anesthetized with ketamine/xylazine and killed by cervical dislocation. At tissue collection, all samples were randomized and labelled with non-identifiable numbers so that all subsequent observations were blinded to the treatment group; treatment groups were revealed after all laboratory-based analyses were complete. All animal procedures were conducted with approval from the St. Vincent’s Health Melbourne Animal Ethics Committee and complied with the Australian Code for the Care and Use of Animals for Scientific Purposes. No adverse events were observed.

2.2. Micro-computed tomography (micro-CT)

Ex vivo micro-CT was performed on femora using the SkyScan 1076 system (Bruker-microCT, Kontich, Belgium); one PTH-treated femur was broken during collection and was excluded. Images were acquired using the following settings: 9 μm voxel size, 0.5 mm aluminium filter, 50 kV voltage, and 100 μA current, 2600 ms exposure time, rotation 0.5 degrees, frame averaging = 1. Images were reconstructed and analysed using SkyScan software programs NRecon (version 1.6.9.8), Dataviewer (version 1.4.4) and CT Analyzer (version 1.11.8.0). Cortical analyses were performed in a region that was 15% of total bone length commencing 30% above the distal femoral end toward the femoral mid-shaft. Cortical bone was analysed using global thresholding (81–255) in CT Analyzer.

2.3. 3-point bending

After microCT analysis, anteroposterior (A-P) and mediolateral (M-L) femoral widths were measured with digital calipers at the mid-shaft. Structural and material properties were identified by three-point bending tests, as previously described [22]. Data from one vehicle-treated femur was excluded due to an abnormal loading curve resulting from rotation of the bone during testing. Load was applied to the femoral mid-shaft anterior surface between two supports located 6.0 mm apart. Load-displacement curves were recorded at 1.0 mm/s crosshead speed using an Instron 5565 A dual column material testing system, and Bluehill 2 software (Instron, Norwood, MA, USA). Ultimate force (N), ultimate deformation (mm) and energy absorbed to failure (mJ) were measured from the load–displacement curves. Combining the geometric calculations and the biomechanical test results, material properties were calculated for each bone as described by Schrifer and colleagues [23] to obtain ultimate stress (MPa), elastic modulus (MPa), and toughness (MPa).

2.4. Histomorphometry

Tibiae were fixed by immersion in cold 4% paraformaldehyde overnight and embedded in methyl methacrylate resin as described previously [24]. Periosteal parameters (cortical thickness (Ct.Th), periosteal mineral apposition rate (Ps.MAR), periosteal mineralising surface/bone surface (Ps.MS/BS), and periosteal bone formation rate/bone surface (Ps.BFR/BS)) were measured on sagittal tibial sections commencing 1500 μm distal to the growth plate hypertrophic zone, as previously described [25]. Trabecular parameters (mineral apposition rate (MAR), mineralising surface/bone surface (MS/BS), bone formation rate/bone surface (BFR/BS), osteoid surface/bone surface (OS/BS), osteoid thickness (O.Th), osteoblast surface/bone surface (ObS/BS) and osteoclast surface/bone surface (Ocs/BS)) were measured on the same tibial sections in the proximal secondary spongiosa, commencing 370 μm below the growth plate, in a 1110 μm² region, as previously described [25] (Osteomeasure; Osteometrics, Atlanta, GA, USA).

2.5. Synchrotron-based Fourier Transform Infrared Microspectroscopy (sFTIRM)

sFTIRM data were collected using a Bruker Hyperion 2000 IR microscope coupled to a V80 FTIR spectrometer located at the IR Microspectroscopy beamline at the Australian Synchrotron. The Hyperion microscope was equipped with an epifluorescence accessory suitable for calcine visualisation (excitation/emission ~490 nm / ~515 nm). Thin 3 μm sagittal sections were taken from the same methyl methacrylate (MMA)–embedded tibial blocks used for histomorphometry. Sections were placed on 22 mm diameter × 0.5 mm polished barium fluoride (BaF2) windows (Crystan Limited, UK) for sFTIRM analysis. IR absorbance spectra were recorded in transmission mode and collected in the mid-IR region from 750 to 3850 cm⁻¹ using a mercury cadmium
telluride detector, at 8 cm\(^{-1}\) spectral resolution and 128 co-added scans/pixel spectral resolution. All data acquisition was undertaken with OPUS version 6.5 and data analysis completed with OPUS version 7.2 (Bruker Optik, Germany).

For each tibial section, the microscope video camera was used to image the cortical diaphysis (1500 \(\mu\)m from the base of the growth plate hypertrophic zone), the same location used for histomorphometric periosteal apposition measurements (Fig. 1B) one section was analysed per mouse. We have previously observed that this is a site of bone modelling, with continuous periosteal apposition, from at least 6 weeks of age [19]. Spectra were collected from 3 regions matched for bone age by superimposing regions for measurement on the calcine labels incorporated during mineralisation at 2 days (region 1), and 7 days before tissue collection, respectively, on each sample. An immediate zone equally spaced between the two calcine labels was also selected for analysis (region 2). Additional measurement regions then progressed, evenly spaced, into the bone matrix (regions 4–6) (Fig. 1C). Based on the known periosteal appositional rates, we termed these zones primary mineralisation (regions 1–3) and secondary mineralisation (regions 4–6). sFTIRM mapping was performed with the synchrotron source, using a 15 \(\times\) 15 \(\mu\)m aperture. Data were collected in transmission mode, with a background spectrum collected through clear BaF2. For each sample an additional MMA reference spectrum was collected in transmission mode, with a background spectrum collected through clear BaF2. For each sample an additional MMA reference spectrum was collected from within the embedding material.

After acquisition, raw spectra for each region and sample were baseline corrected using a 3-point baseline at 3 wavenumbers: 1800, 800, and the closest minimum to 1200 cm\(^{-1}\) using OPUS 7.2. Residual MMA absorbance peaks were then subtracted using the relevant MMA reference spectrum for each sample by iterative manual subtraction. A residual 1730 cm\(^{-1}\) MMA band remained that was excluded from analysis. Spectroscopic parameters calculated were integrated peak areas of the following peaks: Phosphate (mineral) (1180–916 cm\(^{-1}\)), amide I (matrix) (1588–1712 cm\(^{-1}\)), amide II (1600–1500 cm\(^{-1}\)) and \(\nu_2\) carbonate (890–852 cm\(^{-1}\)). Ratios of these integrated peak areas were calculated as follows: mineral:matrix ratio [26] (1180–916 cm\(^{-1}\):588–1712 cm\(^{-1}\)), carbonate:mineral ratio [27] (1180–916 cm\(^{-1}\):890–852 cm\(^{-1}\)) and amide I:II ratio [28] (1588–1712 cm\(^{-1}\):1600–1500 cm\(^{-1}\)). Sub-bands from the amide I, amide II and phosphate peaks were analysed by spectral curve fitting using Grams/AI (Version 9.2, Thermo Scientific, USA). Briefly, individual spectra were base corrected according to wavenumber limits of the sub-bands of interest (amide I and II peaks for 1660 cm\(^{-1}\) and 1690 cm\(^{-1}\) sub-bands and phosphate peaks for 1030 cm\(^{-1}\), 1020 cm\(^{-1}\), 1127 cm\(^{-1}\) and 1096 cm\(^{-1}\) sub-bands). The second-derivative of each peak was used to estimate subpeak positions that were then curve-fitted using 500 maximum iterations with an auto limit fix function. Subpeak integrated areas were reported as collagen crosslinking (1660:1690 cm\(^{-1}\)) [29], crystallinity (1030/1020 cm\(^{-1}\)) [30] and acid phosphate content (1127/1096 cm\(^{-1}\)) [31] ratios.

2.6. Statistics

All graphs show mean ± SEM; sample number (n) is reported in the figure legends. Statistical significance was determined by unpaired t-tests for histomorphometric and microCT analysis, and two-way ANOVA with Fisher’s LSD test for sFTIRM-derived data (GraphPad Prism 7 (version 7.0a)), p < 0.05 was considered statistically significant. To relate indices of collagen crosslinking and stretch to mineral accrual, data from vehicle-treated animals, including all regions of analysis was tested with a straight line-of-best fit least-squares model using GraphPad Prism 7 (version 7.0a).

3. Results

3.1. Confirmation of model: PTH treatment increased osteoblast activity, bone size and bone strength

Histomorphometric trabecular bone analysis in the secondary spongia confirmed that 8 week old male mice treated with PTH at 50 \(\mu\)g/kg/day for 4 weeks showed significantly greater trabecular mineral apposition rate (MAR) and bone formation rate (BFR/BS) (Fig. 2A, C) than vehicle treated age- and sex-matched controls as previously reported [13,20,21,25]. PTH treatment was also associated with a greater osteoblast surface (OBS/BS), thicker osteoid (O.Th) and greater osteoid surface (OS/BS) within the trabecular bone than in vehicle-treated controls (Fig. 2D–F). Osteoclast surface (OC/S/BS) was also significantly greater in PTH treated mice (Fig. 2G).

Cortical thickness (Ct.Th), area (Ct.Ar) and anteroposterior (A-P) widths were also greater in PTH-treated mice compared to vehicle-treated controls (Fig. 3A–D). The greater cortical dimensions in PTH-treated mice were reflected in a greater moment of inertia (Fig. 3E) and significantly stronger bones, indicated by a greater ultimate force than vehicle-treated controls by 3-point bending tests (Fig. 3F–G). However, material properties normalized for bone size such as ultimate stress (Fig. 3H), ultimate strain and toughness were not different in PTH treated samples compared to vehicle-treated controls (Table 1).

Periosteal mineral apposition rate (Ps.MAR, mineralizing surface/ bone surface (Ps.MS/BS) and bone formation rate/bone surface (Ps.BFR/BS) were increased in PTH treated mice compared to vehicle-treated controls (Table 1). The greater cortical dimensions in PTH-treated mice were reflected in a greater moment of inertia (Fig. 3E) and significantly stronger bones, indicated by a greater ultimate force than vehicle-treated controls by 3-point bending tests (Fig. 3F–G). However, material properties normalized for bone size such as ultimate stress (Fig. 3H), ultimate strain and toughness were not different in PTH treated samples compared to vehicle-treated controls (Table 1).
Bone from PTH-treated mice demonstrated a significant increase in the mineral:matrix ratio (Fig. 4B) that approximately doubled from regions 1 to 6 (Fig. 4A). When matched for bone age, similar changes were observed in maturing bone from the periosteal edge (Fig. 5A, B). This was observed in bones from both vehicle- and PTH-treated mice and there was no significant effect of treatment.

A similar observation was made in the carbonate:mineral ratio, which reached a plateau within the more mature bone from region 4 onwards in samples from both vehicle- and PTH-treated mice (Fig. 4C). The increase in carbonate:mineral ratio between the most immature and most mature bone did not differ significantly between PTH- and vehicle-treated mice.

Crystallinity, which represents the relative amount of crystal size and perfection [32,33], was highly variable during bone maturation in both treatment groups. Vehicle-treated mice showed a gradual increase (from regions 2–4) then a decrease (from regions 4–5) in the mature regions of bone (Fig. 4D). In contrast to the decreased crystallinity between regions 4 and 5 in vehicle-treated mice, the mature regions of bone (region 5) in PTH-treated mice demonstrated increased crystallinity compared to the immature regions (regions 1 and 3). This crossover in the curves between regions 4 and 5 was significant, as indicated by a significant interaction between region and treatment. A lower level of crystallinity was detected in region 4 compared to vehicle in bones from PTH-treated mice (Fig. 4D). Acid phosphate content was not significantly modified by bone age or by PTH treatment (Fig. 4E).

The amide I:II ratio reduced significantly with increasing depth from the periosteal edge (Fig. 5A, B). This was observed in bones from both vehicle- and PTH-treated mice; there was no significant effect of treatment. From region 4 onwards, collagen crosslinking was significantly greater than at the periosteal edge (Fig. 5C); again, this was observed in bones from both vehicle- and PTH-treated mice and there was no significant effect of treatment.

### 3.3. Relationships between mineral accrual and collagen fibre orientation

The correlation matrix of data obtained from vehicle-treated samples confirmed a significant relationship between the magnitudes of amide I:II, mineral:matrix, carbonate:mineral ratios and collagen crosslinking with the regions of bone analysed (Table 2). In addition to significant relationships that exist between parameters derived from the same peaks (e.g. amide I:II and mineral:matrix both contain data from the amide I peak), we observed a novel significant negative correlation between the amide I:II and carbonate:mineral ratios, and between the amide I:II ratio and acid phosphate content (Fig. 6). The relationship between amide I:II and carbonate:mineral ratios was significant, not only when the full spectrum of regions was included in the analysis, but also when analysis was restricted to regions 2, 4 and 6 (region 2: p = 0.034, R = –0.746; region 4: p = 0.004, R = –0.875; region 6: p = 0.045; R = –0.717).

### Figure 2

Trabecular bone formation was stimulated by PTH treatment. A: Trabecular Mineral Apposition rate (MAR), B: Mineralising Surface/Bone Surface (MS:BS), C: Bone Formation Rate/Bone Surface (BFR/BS), D: Osteoid Surface/Bone Surface (OS:BS), E: Osteoid Thickness (O.Th), F: Osteoblast Surface/Bone Surface (ObS/BS) and G: Osteoclast Surface/Bone Surface (OcS/BS) measured in the secondary spongiosa of tibiae from 8-week old male mice treated with vehicle or PTH (50 μg/kg/day) for 4 weeks. N = 7–9/group. Mean ± SEM, **p < 0.01, ***p < 0.001 vs. vehicle-treated controls.
4. Discussion

Anabolic PTH treatment does not alter the progression of bone matrix maturation. Neither the rate of mineral accumulation, nor mineral composition, collagen crosslinking or collagen fibre alignment was modified in bones from PTH treated mice compared to bone age matched vehicle-treated samples. Three point-bending tests indicated that the greater bone strength induced by PTH in this model could not be detected after normalizing for the altered bone geometry, and was therefore fully explained by the increase in cortical dimensions.

We used timed calcein labels and high resolution synchrotron-based FTIRM to identify newly mineralised bone on the periosteal surface, and to measure bone composition while controlling for the greater amount of bone deposited during PTH treatment. In addition, assessment of this process in murine cortical bone, which does not undergo Haversian remodelling, has allowed us to assess the continual process of matrix maturation, including both primary and secondary mineralisation processes. When accounting for bone age, we observed that PTH did not change the normal progression as bone matrix matures from the periosteal edge through to the more mature cortical bone, and that earlier reports of lower bone mineral content, crystallinity and collagen crosslinking in PTH-treated samples can be explained by their greater proportion of immature bone.

The use of high resolution synchrotron light allows quantification of bone composition in small regions (15 μm²). This has allowed us to study the normal progression of bone matrix maturation in situ, and define the changes in both bone mineral and collagen fibre orientation that occur as bone matures. With respect to bone mineral, we observed a gradual increase in mineral:matrix ratio and in the proportion of carbonate to mineral. The increase in mineral:matrix ratio, indicating mineral incorporation into the bone matrix, has previously been described in rabbit, rat, and baboon cortical bone[14,34,35], and within human osteonal bone[32,36]. The lower slope of this parameter in the more mature regions of bone is consistent with initial rapid primary mineralisation followed by slower accumulation of mineral. The rise in carbonate:mineral ratio with increasing distance from the periosteum in both treatment groups suggests that carbonate content is higher in bone samples from older animals, noted in chicken[37], rat and bovine cortical bone[38,39].

Table 1

<table>
<thead>
<tr>
<th>Property</th>
<th>Vehicle</th>
<th>PTH</th>
<th>p-Values</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ultimate strain (%)</td>
<td>3.05 ± 0.23</td>
<td>2.58 ± 0.23</td>
<td>0.1768</td>
</tr>
<tr>
<td>Toughness (mJ/mm³)</td>
<td>0.34 ± 0.03</td>
<td>0.35 ± 0.05</td>
<td>0.8405</td>
</tr>
<tr>
<td>Energy absorbed to failure (mJ)</td>
<td>1.04 ± 0.11</td>
<td>1.27 ± 0.20</td>
<td>0.3465</td>
</tr>
</tbody>
</table>
Earlier studies of in vitro crystallisation also showed increased carbonate substitution with increasing maturity of apatite crystals [37]. Our data provides the first in situ evidence that carbonate substitution occurs during the normal process of bone matrix maturation in healthy young bone. In contrast, in human osteonal bone, a lowering in the carbonate:mineral ratio has been observed as mineral:matrix ratio increases from the centre of an osteon to the periphery [36]. The reason for this difference is not yet clear. It may relate to differences in the type of bone: the present study measured bone in an area of modelling, without prior resorption, while the human osteon data would reflect changes in bone that has undergone remodelling, and has therefore been resorbed and replaced by less mature matrix.

As bone matrix became mineralised, we also observed changes in the collagen matrix: a decrease in amide I:II ratio in samples from both PTH and vehicle treated mice. This novel observation indicates that collagen fibre orientation changes as bone matrix becomes more heavily mineralised. While the amide I peak reflects the vibration of C=O bonds running perpendicular to the collagen helical axis, the amide II peak reflects vibration of the C—N bond along the collagen triple helix axis [40]. Sensitivity of peak intensities to functional group...
orientation requires the use of polarised infrared radiation, or placement of an infrared polariser between the sample and detector [40]. The observation of collagen orientation reported here arises from the inherent polarisation of the synchrotron IR beam, whereby the electric vector at the microscope stage is aligned approximately perpendicular to the orientation of the femur sections, (left to right in Fig. 1). The amide I:II ratio therefore reflects the proportional change in perpendicular (amide I) to parallel (amide II) stretch of the collagen triple helix (see Fig. 5B) [40]. This suggests that collagen fibres stretch along their longitudinal orientation during the process of mineral accrual, even after mineralisation is initiated. The robust correlation of carbonate:mineral with amide I:II is strongly supportive of this concept (note that although the correlation of mineral:matrix and amide I:II is even stronger, this is largely because the amide I peak is represented in both parameters). Acid phosphate content was also significantly correlated with amide I:II ratio, showing a significant increase with increasing collagen stretch. Whether the collagen fibres stretch in response to hydroxyapatite crystal growth, or their stretching determines crystal size is not known.

The present study demonstrates that with increasing matrix maturity, in addition to longitudinal collagen stretching, there is an increase in collagen crosslinking. This is consistent with earlier work in remodelling human trabecular bone where a gradual increase was observed in samples from normal individuals across a similar distance to our measurements here in non-remodelling murine cortical bone [41]. Collagen’s intermolecular crosslinking pattern contributes to bone’s tensile strength and elasticity [29], but studying collagen crosslinking during bone maturation has been challenging because of the need to extract protein for mass spectroscopic analysis. By quantifying two specific crosslinks in situ, pyridinoline (Pyr) and dehydro-dihydroxylysinoonorleucine (deH-DHLNL) [29], Pyr:deH-DHLNL (1660:1690 cm⁻¹) reflects collagen crosslinking within the bone matrix [29]. Our observations are similar to that of healthy remodelling human bone [41]. In contrast to our results and earlier work in human trabecular bone, biopsies from high turnover osteoporosis patients showed a high 1660:1690 ratio at the mineralised bone edge that declined rapidly, and then increased again [41]. This indicates that regardless of whether it is remodelling or modelling bone, or in the presence or absence of PTH, collagen crosslinking increases with matrix maturity in healthy bone.

The normal composition that we have detected in PTH-treated samples contrasts with previous assessments of bone samples from PTH-treated monkeys [9] and humans [10]. These earlier studies, although of high spatial resolution (6–7 μm) averaged FTIR-derived indices over a 400 μm² area. Such a large area would include a high proportion of less mature, and therefore, less mineralised bone. Indeed, the authors of those papers suggested that this may have been the explanation for the low proportion of mineralized bone in PTH-treated samples. Our analysis, in much smaller regions of 15 μm², coupled with calcein labelling has allowed us to measure immature and mature bone separately, and to correct for the higher proportion of new bone that exists with PTH treatment. With such correction, there is no difference in the matrix composition in bone from PTH- treated and untreated mice.

To account for the significant increase in tibial cortical thickness induced by PTH treatment, our first three regions of analysis began on the calcein labels which labelled 2 and 7-day old bone on the periosteal surface. Regions 4–6 aligned perpendicularly to the 3rd region. Based on the known mineral appositional rate, bone in regions 5 and 6 is likely to have been formed prior to the intervention of PTH or vehicle treatment, which is a limitation to this study. To restrict the measurements to only the bone formed during the treatment protocol, in future studies, calcein (or a differently coloured label) should be administered prior to the commencement of PTH and vehicle treatment.

In human biopsies, trabecular bone histomorphometry showed new bone formation induced by PTH on both modelling and remodelling surfaces, with a predominant effect on remodelling surfaces [42].

![Fig. 6](image-url)

**Fig. 6.** Relationship between collagen stretch with mineral accumulation and carbonate substitution. Scatterplots of individual data points from vehicle-treated mice showing the relationship between (A) Carbonate:Mineral ratio and Amide I:II ratio and (B) Acid phosphate content and Amide I:II ratio. Shades of grey indicate the region of analysis from which data is derived, with the lightest colour indicating the most immature bone. Black line indicates the line of best fit with a linear regression model, and dashed lines indicate 95% confidence intervals. R² and p values are shown within each graph.

### Table 2

<table>
<thead>
<tr>
<th>Region</th>
<th>Mineral: matrix</th>
<th>Carbonate: mineral</th>
<th>Collagen crosslinking</th>
<th>Crystallinity</th>
<th>Acid phosphate content</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amide I:II</td>
<td>r² = 0.875</td>
<td>p = 4.4 × 10⁻¹⁶</td>
<td>r² = 0.632</td>
<td>r² = 0.051</td>
<td>r² = 0.483</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>r² = 2.465 × 10⁻⁷</td>
<td>p = 0.732</td>
<td>p = 0.001</td>
</tr>
<tr>
<td></td>
<td>r² = 0.587</td>
<td>p = 1.164 × 10⁻⁵</td>
<td>r² = 0.001</td>
<td>r² = 0.064</td>
<td>r² = 0.424</td>
</tr>
<tr>
<td></td>
<td>r² = 0.471</td>
<td>r² = 0.027</td>
<td></td>
<td>r² = 0.604</td>
<td>r² = 0.306</td>
</tr>
<tr>
<td>Carbonate: mineral</td>
<td>r² = 0.251</td>
<td>r² = 0.085</td>
<td></td>
<td>p = 0.854</td>
<td>p = 0.043</td>
</tr>
<tr>
<td>Collagen crosslinking</td>
<td>r² = 0.081</td>
<td>r² = 0.181</td>
<td>r² = 0.181</td>
<td>p = 0.241</td>
<td>r² = 0.014</td>
</tr>
<tr>
<td>Crystallinity</td>
<td>r² = 0.582</td>
<td>r² = 0.125</td>
<td></td>
<td>r² = 0.582</td>
<td>r² = 0.334</td>
</tr>
<tr>
<td>Acid phosphate content</td>
<td>r² = 0.027</td>
<td>r² = 0.064</td>
<td></td>
<td>r² = 0.081</td>
<td>r² = 0.424</td>
</tr>
</tbody>
</table>
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accounting perhaps for N 70% of new bone [43]. Modelling, the process
that we have assessed here, also contributes to PTH action, both in
humans [43] and in rats and mice [2,44,45]. Whether bone deposited
on modelling surfaces differs in composition from that deposited on remodelling surfaces is not yet known. Since calcein labels are short-lived
in mouse trabecular bone, which has very low mineralisation levels,
replicating the present study in remodelling murine trabecular bone
would be technically challenging. Therefore, it would be useful to attempt a similar study in larger species to measure bone maturation in
the presence and absence of PTH in osteonal cortical bone, and in
more highly mineralised trabecular matrix. While we observed no
change in bone matrix deposited in the presence of PTH in young
healthy male animals, the question of whether the quality of bone laid
down in the presence of PTH is of the same quality as compromised
bone matrix deposited in aged estrogen deﬁcient animals, or aged patients remains unanswered. This too would be a useful follow-up study.
In summary, this study quantiﬁed changes in bone composition during bone matrix maturation in normal growth and during PTH treatment in modelling murine bone. We conclude that mineralisation
levels, collagen crosslinking and collagen ﬁbre longitudinal stretch all
increase during matrix maturation on a modelling surface, and their
progression is not altered by PTH treatment. These data conﬁrm that
lower mineralisation levels previously observed in PTH-treated bone reﬂect a higher proportion of newly formed bone, and that bone deposited
on the murine periosteal surface during PTH treatment is not inadequately mineralised but instead undergoes a normal collagen maturation process and normal mineral accrual as vehicle-treated bones.
Author contributions
Study design: NAS. Study conduct: CV, TAP, ARB, KRB, MJT, and NAS.
Data collection: CV, ARB, and NAS. Data analysis: CV, TAP, KRB, and NAS.
Data interpretation: CV, MRF, KRB, LPP, MJT, TJM, and NAS. Drafting
manuscript: CV, NAS. Revising manuscript content: CV, KRB, MRF,
KRB, MJT, TJM, and NAS. All authors approved the ﬁnal version of the
manuscript. NAS takes responsibility for the integrity of the data
analysis.
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undertaken on the infrared microspectroscopy (IRM) beamline at the
Australian Synchrotron, Victoria, Australia.
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CHAPTER 7: General Discussion

This project aimed to understand mechanisms by which parathyroid hormone (PTH) increases bone mass, strength and quality by investigating two of its downstream targets: ephrinB2 and gp130. Work conducted prior to my PhD demonstrated that PTH upregulated gp130 and ephrinB2 mRNA and protein levels in osteoblasts (156, 157). In addition, both of these downstream targets are required for normal levels of bone formation in physiological conditions (194, 252, 288) and for the anabolic response to PTH (181, 195). Their roles in osteoblasts and osteocytes were examined using three different mouse models and showed that although both are downstream targets of PTH; they have different effects on bone formation, strength and bone composition. The three mouse models also revealed that these downstream targets contribute differently to the anabolic action of PTH on bone.

Work conducted prior to my PhD showed that gp130 deletion throughout the osteoblast lineage (OsxCre.gp130f/f) resulted in low trabecular bone formation rate and mass. Similarly, deletion of gp130 in late osteoblasts/osteocytes (Dmp1Cre.gp130f/f) resulted in the same phenotype, indicating that the osteocyte is the key cell through which gp130 controls trabecular bone formation (252). Dmp1Cre.gp130f/f mice also demonstrated an impaired anabolic response to PTH and low levels of PTH receptor in gp130-deficient osteoblasts (181). My PhD studies specifically examined the cortical bone phenotype in Dmp1Cre.gp130f/f mice and found that 26-week old male Dmp1Cre.gp130f/f mice had wider femora compared to controls (Chapter 3). This opposing phenotype of low trabecular bone formation and greater cortical dimensions caused by gp130 deletion in osteocytes is unique, and contrasts with other osteocyte-specific knockouts of factors that stimulate bone formation, such as β-catenin and insulin-like growth factor 1 which result in low bone mass in both cortical and trabecular bone due to reduced bone formation at both sites (289, 290). Femora from Dmp1Cre.gp130f/f mice also showed decreased intrinsic bone strength, such as elastic modulus and ultimate stress. The decline in material quality was attributed to altered collagen production (collagen type α1 and type α2 mRNA levels) and organisation (a higher proportion of woven bone) in
Despite this, the mechanical strength was maintained likely due to an increase in periosteal dimensions, as a compensatory mechanism for poor bone material quality. This work indicated an essential role of gp130 in osteocytes to promote the deposition of lamellar bone within the cortical matrix that preserves the material strength of cortical bone. As gp130 expression is increased by PTH and gp130 signalling in late osteoblasts/osteocytes is required for the anabolic action of PTH (156, 181), these data indicate that the role of osteocytic gp130 in PTH action is to stimulate trabecular bone formation and control the production and organisation of collagen during bone formation.

The second downstream target of PTH investigated during my PhD studies was ephrinB2. Previous work in our lab demonstrated that 12-week old female mice with genetic deletion of ephrinB2 within the osteoblast lineage (OsxCre.EfnB2\textsuperscript{f/f}) resulted in delayed initiation of mineralisation and increased osteoblast apoptosis (288). During my PhD, I found that OsxCre.EfnB2\textsuperscript{f/f} mice had slender femora and thinner cortices, resulting in greater femoral flexibility (Chapter 4). This indicated a role for ephrinB2 in the osteoblast lineage to maintain adequate modelling of cortical bone for normal bone strength. In contrast, deletion of ephrinB2 later in osteoblast differentiation (Dmp1Cre.EfnB2\textsuperscript{f/f}) resulted in femora that were more brittle, requiring less work to fracture than their controls (Chapter 5). Synchrotron-based Fourier Transform Infrared Microscopy (sFTIRM) analysis of bones from Dmp1Cre.EfnB2\textsuperscript{f/f} mice demonstrated a more rapid progression of mineralisation (mineral:matrix ratio), greater incorporation of carbonate into the mineral (carbonate:mineral ratio) and parallel stretching/compression of collagen fibres (amide I:II ratio). This confirmed that ephrinB2 in late osteoblasts/osteocytes is required to restrain incorporation of carbonate into the mineral and to regulate collagen fibre orientation. In contrast, bones from OsxCre.EfnB2\textsuperscript{f/f} mice did not display any alterations in mineral and matrix composition suggesting that deletion of ephrinB2 within the osteoblast lineage does not affect the quality of the bone that is laid down and mineralised. Despite their opposite flexibility phenotypes, both mouse models required less force to cause permanent damage to their bones indicating that their bones are both more fragile than controls.
The significant differences in strength phenotypes between the OsxCre.EfnB2^f/f and Dmp1Cre.EfnB2^f/f mouse models indicated that ephrinB2 has stage-specific effects during osteoblast differentiation in regulating bone size, bone formation and material composition. OsxCre.EfnB2^f/f osteoblasts underwent apoptosis rather than differentiating into fully mature cells that promote osteoid mineralisation. The subsequent delay in the initiation of mineralisation resulted in compliant bones, indicating an anti-apoptotic role of osteoblastic ephrinB2 necessary for ongoing osteoblast differentiation, initiation of mineralisation and restriction of bone elasticity at the stage where collagen matrix production and mineralisation is most important. In contrast, bones from Dmp1Cre.EfnB2^f/f mice showed no delay in initiation of mineralisation, but deposition of excessive mineral as soon as the mineralisation process started. This indicated the ephrinB2 in late osteoblasts/osteocytes promotes bone strength by limiting bone matrix mineralisation and carbonate substitution into the mineral. The reason we did not observe composition changes in bones from OsxCre.EfnB2^f/f mice may be due to that fact that the osteoblast apoptosis and delayed initiation of mineralisation observed in mice with earlier deletion of ephrinB2 would be a dominant phenotype that would mask the effects of the later deletion of ephrinB2 in Dmp1Cre.EfnB2^f/f mice.

The altered mineral composition in Dmp1Cre.EfnB2^f/f mice detected by sFTIRM was associated not only with high mineral levels and carbonate, but also with greater collagen crosslinks, an indicator of increased collagen maturity (271). This suggested that the greater maturation of the collagen fibres might mediate the increased packing of mineral within the collagen fibrils, providing the first indication that matrix-embedded osteocytes may not only control bone matrix composition, but may also control the structure of the organic matrix that surrounds them. This was also suggested by the sFTIRM data and polarised FTIR imaging, which showed a relative enhancement of amide II bonds in bones from Dmp1Cre.EfnB2^f/f mice, and lower amide I:II ratio compared to controls under 0° polarisation. This technique has only ever been applied to determine spatial distribution of collagen orientation in tendon (268) and cartilage (223). A heterogeneous distribution of amide I and II throughout the tibial cortex of Dmp1Cre.EfnB2^f/f mice under the 90° polarising filter highlighted altered distribution of collagen fibres compared
to controls. This still requires quantitative analysis, but the qualitative observations suggest altered collagen distribution may accelerate mineral deposition and carbonate incorporation throughout cortical bone.

To find a mechanism for the brittle bone phenotype in 12-week old female \textit{Dmp1Cre.EfnB2}^{f/f} mice we next investigated their osteocyte properties. Although this aspect of the work is still at an early stage, backscattered electron microscopy revealed that the number of osteocyte lacunae per total bone area in \textit{Dmp1Cre.EfnB2}^{f/f} mice was significantly greater than controls, which suggests that a greater number of osteocytes are becoming embedded within the newly formed bone. This may contribute to the greater mineral accumulation, but does not reveal whether there are viable osteocytes within the lacunae. TUNEL staining indicated that the potentially defective osteocytes in \textit{Dmp1Cre.EfnB2}^{f/f} mice are not undergoing apoptosis which is in contrast to \textit{OsxCre.EfnB2}^{f/f} mice where increased apoptosis was detected both by TEM and by a greater number of TUNEL+ osteoblasts than in controls. Preliminary transmission electron microscopy images suggest abnormal osteocyte morphology within their lacunae. Together these data suggest that the brittle bone phenotype in 12-week old \textit{Dmp1Cre.EfnB2}^{f/f} mice may be due to defective osteocytes that are unable to inhibit mineral deposition and carbonate incorporation. To further investigate the mechanism behind the greater mineral and carbonate content, we will perform RNA sequencing to compare gene expression from control and \textit{Dmp1Cre.EfnB2}^{f/f} mice.

EphrinB2 deletion in late osteoblasts/osteocytes also resulted in greater trabecular bone volume in 12-week old female mice which was accompanied by enlarged osteoclasts, suggesting impaired osteoclast function (Chapter 5). We hypothesized that the greater carbonate content in \textit{Dmp1Cre.EfnB2}^{f/f} mice may modify osteoclast resorptive activity however it is not clear whether previously reported \textit{in vitro} studies would reflect carbonate levels \textit{in vivo} in \textit{Dmp1Cre.EfnB2}^{f/f} mice. Impaired osteoclast attachment has previously been reported at growth plate, but not in bone of \textit{OsxCre.EfnB2}^{f/f} neonates therefore further investigation into osteoclast morphology, attachment and activity is
required to determine whether impaired osteoclast function is causing the greater trabecular bone volume in $Dmp1\text{Cre}.EfnB2^{\text{ff}}$ mice.

Since deletion of ephrinB2 late in osteoblast differentiation ($Dmp1\text{Cre}.EfnB2^{\text{ff}}$ mice) resulted in a completely different phenotype to the earlier deletion, we then determined whether ephrinB2 in late osteoblasts/osteocytes is also required for the anabolic action of PTH in male mice. Just as ephrinB2 expression during different stages of osteoblast differentiation showed differing effects on bone formation, strength and quality, their requirement for the anabolic action of PTH during osteoblast differentiation was also very different. PTH increased ephrinB2 expression in both osteoblasts and osteocytes (157) but only deletion of ephrinB2 in the entire osteoblast lineage impaired the anabolic effect of PTH (195). In contrast, deletion of ephrinB2 in late osteoblasts/osteocytes did not impair the anabolic action of PTH (Chapter 6) indicating that ephrinB2 signalling in osteocytes may contribute to the regulation of mineral accrual and bone composition without contributing to the anabolic action of PTH. This suggests it is osteoblastic ephrinB2 which is most important for the anabolic action of PTH by preventing osteoblast apoptosis.

Since there was no significant modification in the effect of PTH in the $Dmp1\text{Cre}.EfnB2^{\text{ff}}$ mice, I used sFTIRM to analyse bone composition from vehicle-treated $Dmp1\text{Cre}.EfnB2^{\text{ww}}$ mice to examine the effects of anabolic PTH treatment on bone composition. A reduction in the degree of mineralisation has been reported during treatment which may reflect production of under-mineralised matrix or a greater proportion of new matrix within a large area of measurement (131, 148, 149). My analysis corrected for tissue age by using fluorescent labels on non-remodelling periosteal bone to detect changes in bone matrix composition and collagen conformation during the process of bone mineralisation. We identified that intermittent parathyroid hormone (PTH) treatment does not alter mineral accrual during bone formation and that newly formed bone deposited during PTH treatment undergoes normal collagen maturation and normal mineral accrual similar to that of vehicle-treated controls. The low bone
mineralization levels with PTH observed in larger regions may relate simply to a greater proportion of new bone.

Deletion of two different osteocytic downstream targets of PTH (gp130 and ephrinB2) also demonstrated different roles for these proteins in the anabolic action of PTH. Impaired anabolic action of PTH in Dmp1Cre.gp130<sup>−/−</sup> mice occurred by inhibition of PTH receptor expression indicating that osteocytic gp130 maintains PTH receptor expression within the osteoblast lineage (181). As Dmp1Cre.gp130<sup>−/−</sup> mice had wider bones with more woven bone collagen fibres, the contribution of gp130 signalling in late osteoblasts/osteocytes to the anabolic action of PTH may be to increase bone mass and size, control collagen deposition and improve bone strength. In contrast, ephrinB2 deletion in late osteoblasts/osteocytes resulted in greater mineral and carbonate content but its deletion did not alter the anabolic action of PTH. This indicates that in addition to ephrinB2 signalling in osteoblasts contributing to the anabolic action of PTH, osteocytes also contribute to the anabolic action of PTH through gp130 signalling.

Both OsxCre.EfnB2<sup>−/−</sup> and Dmp1Cre.EfnB2<sup>−/−</sup> mouse models demonstrated a bone phenotype in females only suggesting either that EphrinB2 is regulated by sex steroids, or that the role of ephrinB2 is more important during higher states of remodelling or restricted bone growth (both of these occur in females compared to males). This latter suggestion was supported by the observation of similar mechanical deficits in 6-week old male Dmp1Cre.EfnB2<sup>−/−</sup> and 12-week old female Dmp1Cre.EfnB2<sup>−/−</sup> mice. In contrast to the female-specific phenotype of ephrinB2-deficient mice, the strength phenotype of Dmp1Cre.gp130<sup>−/−</sup> mice was more apparent in males than in females. This may suggest that ephrinB2 signalling is more important in females and gp130 signalling is more important in males for regulating bone formation and strength. This may also indicate that PTH has sex-specific effects on bone however the female-specific phenotype in OsxCre.EfnB2<sup>−/−</sup> mice and impaired the anabolic response to PTH in male OsxCre.EfnB2<sup>−/−</sup> mice suggests that the anabolic effect of PTH is not sex-specific.

Sex-specific differences in controls were observed in all 3 mouse models, consistent with the greater periosteal apposition that occurs in males than in females and greater
endosteal resorption in females than in males (44, 45). Differences in Cre controls were noted: 12 week old male OsxCre controls were capable of deforming more before fracturing while 12 week old male Dmp1Cre controls required more force to fracture compared to female controls. This could be due to the effects of the Cre transgene on the skeleton; while we have not found significant changes in bone structure in our OsxCre control mice (195), the OsxCre transgene has been reported to cause delayed weight gain and a transient delay in cortical bone growth at 6 weeks of age compared to wild-type controls (212).

In conclusion, my study has shown that downstream targets of PTH (ephrinB2 and gp130) expressed either in osteoblasts or osteocytes have very different effects on bone formation, strength and quality. I have demonstrated that although ephrinB2 and gp130 are two downstream targets of PTH, only osteoblastic expression of ephrinB2 (OsxCre.EfnB2f/f) and osteocytic expression of gp130 (Dmp1Cre.gp130f/f) are required for the anabolic action of PTH. Both of these mouse models showed impaired bone formation highlighting that the anabolic effect of PTH primarily regulates bone size, formation and woven and lamellar bone deposition. Osteocytic ephrinB2 deletion did not impair the anabolic action of PTH but revealed new functions of osteocytes in restricting carbonate deposition and collagen fibre stretch/compression, and possibly regulating osteoclast activity by changing matrix composition. Additionally, Dmp1Cre.gp130f/f mice demonstrated that osteocytes regulate woven and lamellar deposition of collagen and periosteal growth. Overall, investigating gp130 and ephrinB2 as downstream targets of PTH highlighted the different roles that osteoblasts and osteocytes play in contributing to bone formation, mineralisation and strength both during normal bone formation and in the anabolic action of PTH, which will contribute to our knowledge for targeting specific cell types that alter either bone mass or quality. My project also involved the development of a novel sFTIRM method which demonstrated that anabolic PTH treatment resulted in bone with normal composition. This technique provides a new avenue for investigating the causes of bone fragility which could lead to improved diagnostic tests and the development of different personalised treatments.
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Appendix 1
Regulation of cortical and trabecular bone mass by communication between osteoblasts, osteocytes and osteoclasts

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ABSTRACT

The size and strength of bone is determined by two fundamental processes. One process, bone remodelling, renews the skeleton throughout life. In this process existing bone is resorbed by osteoclasts and replaced, in the same location, by osteoblasts. The other process is bone modelling, where bone formation and resorption occur at different sites so that the shape of bone is changed. Recent data suggests that both remodelling and modelling are controlled by signals between the cells that carry out these two processes. Osteoclasts both resorb bone, and provide inhibitory and stimulatory signals, including cardiotrophin-1 and sphingosine-1-kinase, to the osteoblast lineage thereby regulating their differentiation and activity on both trabecular and cortical surfaces. In addition, the osteoblast lineage, including osteoblast progenitors, matrix-producing osteoblasts, bone lining cells, and matrix-embedded osteocytes, produce both inhibitory and stimulatory factors that stimulate osteoclast differentiation. We will discuss the roles of osteoblast- and osteocyte-derived RANKL, and paracrine, autocrine and endocrine factors, such as ephrinB2, the IL-6/gp130 family of cytokines, parathyroid hormone, and its related peptide, PTHrP. These factors not only stimulate RANKL production, but also stimulate osteoblast differentiation and activity. This review will focus on recent data, generated from pharmacological and genetic studies of mouse models and what these data reveal about these pathways at different stages of osteoblast differentiation and their impact on both bone remodelling and modelling in trabecular and cortical bone.

Introduction

The skeleton is constantly remodelled by repeated cycles of cellular activity occurring asynchronously throughout the skeleton in which tiny packets of bone are resorbed and then replaced. This process always occurs in the same sequence: bone resorption by osteoclasts followed by bone matrix production by osteoblasts. This is the fundamental process by which the skeleton changes in response to hormonal and mechanically-induced stresses.

In addition to this process, bone also adapts by the process of modelling; here bone formation and resorption do not occur in sequence at the same site. Modelling occurs during growth, and in response to mechanical loading; it can also be induced by pharmacological agents that promote bone formation without a requirement for prior resorption [1]. Modelling is also responsible for cortical expansion, where osteoblasts on the periosteal surface continue to form bone at the diaphysis of the long bones (Fig. 1). The mechanisms that determine why some bone surfaces remodel while others model are not known, but understanding the relationships between the cells involved in modelling and remodelling holds great potential for developing therapeutics that can restore bone strength in osteoporosis.

Originally, the basic multicellular unit (BMU)1 responsible for remodelling was thought to consist of two classes of specialized cells on the bone surface, osteoclasts and osteoblasts, which contribute to remodelling by bone resorption and formation, respectively. Although osteoclasts are derived from the hemopoietic lineage, and osteoblasts from the mesenchymal lineage, these cell types act in close apposition and regulate the function of the other lineage by appropriate production of both inhibitory and stimulatory factors [2,3]. Over the past 50 years, this concept has been refined, and a

1 Abbreviations used: BMU, basic multicellular unit; RANKL, Receptor Activator of NFKB Ligand; OPG, osteoprotegerin; PTH, parathyroid hormone; BMD, bone mineral density; CTQC1, collagen triple helix repeat containing 1; CT-1, Cardiotrophin-1; VDR, vitamin D receptor; PTHrP, PTH receptor; LIF, leukemia inhibitory factor; OSM, oncostatin M; CNTF, ciliary neurotrophic factor.
number of regulatory factors have been identified, some of which we discuss below [4–7]. The best understood example of this intercellular regulation is the production of both the osteoclast stimulus Receptor Activator of NFκB Ligand (RANKL) and its decoy receptor inhibitor osteoprotegerin (OPG) by cells of the osteoblast lineage [8]. It is therefore, the same cell lineage that both forms bone matrix and regulates osteoclast differentiation in response to paracrine and endocrine stimuli, including parathyroid hormone (PTH), 1,25-dihydroxyvitamin-D$_3$ and cytokines [9–11]. The osteoblast lineage includes committed osteoblast precursors, matrix-producing osteoblasts, lining cells and matrix-embedded osteocytes; the major contributing cells to these two activities are unlikely to be at the same stage of differentiation within the lineage, and this concept is discussed below. Similarly, osteoclasts produce a range of “coupling factors”. This is achieved both by releasing factors from the bone matrix itself during the process of resorption, and by production of soluble, and possibly membrane bound, regulators of bone formation (for recent reviews see [4,7,12]).

Although the initial concept of remodelling focussed on the cells on the bone surface, we now understand that there are many other cellular contributors that regulate bone formation and resorption within the BMU. These include osteocytes, terminally differentiated osteoblast lineage cells that reside in an interconnected network that extends throughout the bone matrix, and multiple cell types in the marrow space (e.g., haemopoietic precursors, macrophages, T-cells, natural killer cells and adipocytes) [13]. Furthermore, different stages of osteoblast differentiation are now understood to play distinct roles in regulating the activities of osteoclasts [13], and each other [14]. This is particularly relevant for the initiation of the bone remodelling cycle, where osteocytes and osteoprogenitors produce the RANKL required for osteoclastogenesis [7].

The identification of a bone remodelling canopy that lifts from the bone surface when osteoclastic resorption initiates the remodelling cycle to enclose the BMU in an isolated environment is a concept that has been explored at length in human specimens by the Delaissé laboratory [15,16]. This would provide a controlled locale in which osteoblast lineage cells, osteoclasts, and potentially other contributing marrow cells, may exchange factors and influence precursors provided by the associated vasculature. However, experimental interrogation of its contribution to the actions of specific coupling factors using genetically altered mouse models is limited because this anatomical structure has not been observed in the mouse, the model that has been used most extensively for defining the intercellular signalling pathways that modify bone remodelling.

Much work using genetically altered mice has focussed on the overall influence of these pathways on the internal trabecular network (Fig. 1), including the quantity of trabecular bone and the level of trabecular remodelling. However, major questions remain about the effects of the intercellular signalling pathways that regulate the cortical bone (Fig. 1), and cortical bone matrix quality and strength. Since it is now understood that intra-cortical remodelling and cortical bone loss are contributors to skeletal fragility [1], more attention is beginning to be paid to differences in effect of signalling pathways in cortical vs. trabecular bone. This review will focus on some notable intercellular pathways that control bone mass and bone strength in cortical and trabecular bone, as examples of
a wider range of factors at play: osteoclast-derived coupling factors (cardiotrophin-1 and sphingosine-1-phosphate), osteoblast line-
age-derived RANKL, IL-6 family cytokines, and ephrinB2.

**Interpreting data on bone mass and remodelling in mouse models**

Studies of bone strength, mass and remodelling in humans rely mainly on surrogate markers such as bone mineral density (BMD) and serum biochemical markers since biopsies are rarely obtained. The use of mouse models, particularly genetically altered mice, allows direct measurement of changes in bone mass, remodelling and strength when specific factors are removed from the body. Now that cell-specific deletion is possible, the contributions of individual cell types to bone mass can also be determined. It is now commonly accepted that genetically altered mice should be backcrossed onto an inbred strain before analysis, and that littermate controls should be used for all skeletal analysis, since different inbred strains have significantly different bone trabecular and cortical bone mass, shape, strength, and level of remodelling [17–19], but equal consideration needs to be given to the sex and age of the animals analysed.

Male and female mammals, including humans, differ significantly in their rates of bone remodelling, their cortical dimensions (determined by modelling) and their bone strength. Briefly, after sexual maturity, male mice have higher trabecular bone mass [20,21], a lower level of bone remodelling [20,22], greater perios-
teal diameter and ultimate bone strength [23,24] than females. For this reason, phenotypes of low bone remodelling are more readily detectable in female mice, since the level of bone remodelling is already low in the male [25,26], and phenotypes demonstrat-
ing high levels of bone remodelling are more readily detectable in male mice [27].

In mice, as in humans [28], periosteal expansion (modelling) continues throughout life at the mid-diaphysis [20] while trabecu-
lar bone volume continues to reduce [20], and bone remodelling levels reduce with age on trabecular surfaces in rodents [21]. Tra-
becular bone volume peaks at 3 months of age, and at this age, periosteal apposition is still occurring in the tibiae and femora, making this an ideal time for analysis of genetically altered mice [20].

Although both humans and mice demonstrate increased perios-
teal apposition with age, cortical structure is vastly different in these two species. Mice lack the characteristic Haversian systems that give cortical bone its structure in larger mammals. These sys-
tems comprise multiple concentric rings surrounding central blood vessels, but the smaller thickness of cortical bone in the mouse means that such organisation is not required. Although mice do not possess Haversian systems, they are a useful model of perios-
teal growth, and of growth-related remodelling of the cortical bone from the rapidly deposited woven bone, that contains disorganised collagen fibres, to the more mechanically competent lamellar bone, in which collagen fibres are oriented to maximise bone strength.

**Osteoclast-derived coupling factors in bone: roles in modelling and remodelling**

The osteoclast lineage, including mature, resorbing osteoclasts and their precursors, provide coupling factors that match bone for-
mation to the level of bone resorption [4,7]. These include some released from the bone matrix during bone resorption, such as IGF-1 and TGFβ [29,30], and an increasing number of factors secreted by both inactive and active osteoclasts, including cardio-
trophin-1 [31], sphingosine-1-phosphate [32], BMP6 and Wnt10b [32], collagen triple helix repeat containing 1 (CTHRCT1) [33], and Sema4D [34]. Since none of these factors are expressed exclusively by osteoclasts, in vivo studies to determine the role of osteoclastic release of them will require the generation of cell specific knock-
outs of each factor.

Cardiotrophin-1 (CT-1) is a member of the IL-6 family of cyt-
okines. While it is expressed in a number of different organs, within the skeleton its expression is restricted to the osteoclast [31]. When osteoblasts are exposed to it, either in vitro or in vivo, their differentiation and bone forming activity are increased [31]. Fur-
thermore, expression of the osteocyte-derived bone formation inhibitor, sclerostin is reduced by CT-1 [25]. When mice with glo-
al deletion of CT-1 were studied, although osteoclast numbers were increased, osteoclast activity was reduced. This low level of resorption resulted in a high bone mass phenotype, termed osteo-
petrosis. Furthermore, in neonate and adult male mice, bone for-
mation on trabecular surfaces was lower in the absence of CT-1 pointing to a lack of coupling factor activity in bone remodelling [31].

In studies of other osteopetrotic mutants, Karsdal et al. [35] noted that trabecular bone formation was frequently maintained in mice with osteosteat-rich osteopetrosis (such as c-src deficiency) [36,37], in which osteoclasts were present but showed impaired function. In contrast, osteoclast-poor osteopetrosis, in which bone resorption was low because of a lower number of osteoclasts, displayed impaired bone formation on trabecular surfaces [38–40]. This suggested that, although both matrix-derived and osteo-
clast-derived factors play a part, it is the osteoclast itself, not neces-
sarily its resorptive action, which is the key source of coupling factors for trabecular osteoblasts.

Local release of soluble factors from osteoclasts provides a simple mechanism to control bone formation in the context of bone remodelling, where osteoblasts and their precursors are in close proximity, but the question of whether osteoclast-derived coupling factors also influence cortical bone remains puzzling because this is a surface on which osteoblasts and osteoclasts are not in direct apposition. This particular question has not been studied at length in mouse models. However, when RANKL was provided by lym-
phocytes in a rescue of the osteoclast-deficient osteopetrotic RANKL null mouse, cortical remodelling, and periosteal growth was restored even though osteoclasts were only restored to the endocortical surfaces [41]. This indicates that coupling factors from endocortical osteoclasts are able to signal to the osteoblast lineage on the periosteal surface (Fig. 1). The coupling factors that are most important for this set of signals are not fully described. Further-
more, in most models in which putative coupling factors have been deleted, there is a lack of data describing cortical dimensions, the woven or lamellar nature of the cortical bone, or the level of bone formation on the periosteal surface. In only two coupling factor-
null mice have cortical dimensions been reported: CT-1 null mice show no defect in periosteal remodelling [31], but mice that lack coupling factor BMP6 show impaired periosteal growth [42], sug-
gesting that the latter may be a coupling factor that stimulates periosteal growth. However, the BMP6 null mice also have reduced longitudinal growth due to loss of BMP6 at the growth plate, and this would also limit periosteal growth.

Recent experiments using cathepsin K inhibitors to reduce osteoclast activity in preclinical models have shown that bone for-
mation on both trabecular and cortical surfaces is retained when osteoclast activity is reduced (for review see [43]). This suggests that coupling factors released by the cathepsin K-deficient osteo-
clast may continue to be available to periosteal bone-forming oste-
oblasts. Mice were generated that lack cathepsin K specifically in osteoclasts to investigate whether this increase in bone formation related to a change in osteoclast activity or signalling. These mice (both males and females) exhibited significantly greater trabecular bone formation and increased femoral cross-sectional area,
implying a higher level of periosteal bone formation [44]. Osteoclasts isolated from these mice demonstrated increased support for osteoblast differentiation; this effect was inhibited by a spingosine-1-phosphate antagonist. This suggests that even when non-functional, osteoclasts may release coupling factors, including spingosine-1-phosphate, and thereby stimulate both periosteal and trabecular bone formation. Other coupling factors may also be involved, since it has been reported that osteoclast-mediated release of matrix-derived IGF-1 and BMP-2 also occurs when osteoclasts are treated with cathepsin K inhibitors in vitro [45]. These findings suggest potential mechanisms by which osteoclast activity can be inhibited by cathepsin K inhibitors for osteoporosis treatment while bone formation is simultaneously increased.

How are periosteal osteoclasts exposed to coupling factors produced by distant osteoclasts on the endocortical surface? This question remains unanswered, but an appealing model is that stable coupling factors required for periosteal bone formation may be transported through the osteocyte lacuna-canalicular network. Alternatively, the primary cellular target of coupling factors may be the osteocyte; an example of this is the way that CT-1 inhibits osteocytic sclerostin production [31]. This could then increase bone formation on the adjacent bone surface. The question of how sclerostin is directed to the appropriate bone surface on which modelling is required also remains unanswered.

Osteoclast-derived coupling factors may also contribute to bone formation when it is stimulated by parathyroid hormone (PTH). PTH is classically regarded as a hormone that stimulates bone resorption. This action is mediated by its ability to stimulate production of RANKL by osteoblast lineage cells [9], including osteocytes [46]. In contrast to this catabolic action, daily injection of PTH can be used therapeutically to increase bone formation and bone mass in postmenopausal women [47]. This action depends also on direct PTH action on the osteoblast lineage where it also promotes differentiation of committed osteoblast precursors [48], inhibits osteoblast apoptosis [49,50] and reduces osteocytic production of the bone formation inhibitor sclerostin [51,52]. Since PTH stimulates both bone formation and resorption, and the activities of both osteoclasts and osteoblasts are linked through the normal process of bone remodelling [53,54], it has been proposed that anabolic action of PTH depends, at least in part, on its ability to stimulate osteoclast activity [4]. This was first suggested many years ago, when rapid morphological changes in osteoclasts were observed in rats injected with PTH [55].

A role for osteoclasts in the anabolic action of PTH was further indicated by the reduction in the anabolic effect with concomitant bisphosphonate treatment [56], a lack of anabolic response in young mice with osteoclast-poor osteopetrosis [57], but retention of anabolic response in young mice with osteoclast-rich osteopetrosis [58]. This suggested that the osteoclast contribution to PTH anabolic action may not require bone resorption, just the presence of osteoclasts and their coupling factors. In addition, this suggests that osteoclasts of patients treated with cathepsin K inhibitors might still provide sufficient signal for PTH anabolic action to be retained. In addition to the effects of long-term osteoclast inhibition described above, we found that transient inhibition of osteoclasts by co-administration of low dose sCT blocked PTH anabolic response in normal young female rats [59]. This seemed to accord with the hypothesis that active osteoclasts are required for the anabolic effect of PTH. However, in addition to inhibiting coupling-mediated actions, sCT treatment also increased osteocytic expression of the bone formation inhibitor sclerostin [59]. This suggested an alternative mechanism by which sCT may inhibit the PTH anabolic effect and by which endogenous CT may stimulate bone formation. Surprisingly, when the same experiment was carried out in 6 month old sham-operated and ovariectomized rats, with a lower level of bone remodelling, only the stimulation of serum P1NP levels was reduced by sCT co-administration [60]. The transient inhibition of osteoclast activity therefore only partially inhibited the anabolic effect of PTH in older rats, suggesting that the role of osteoclast-derived coupling factors in the anabolic action of PTH may be most important in states of high bone remodelling, as exists in the young animal compared to the adult. It is also possible that while transient osteoclast inhibition is sufficient to block PTH anabolic action in young animals, any osteoclastic contribution to PTH-induced bone formation in older animals may be prevented only by a constant blockade of osteoclast activity, as occurs in humans treated with therapeutic osteoclast inhibitors, such as bisphosphonates or anti-RANKL.

Clinical studies in postmenopausal women that have combined osteoclast inhibitors with anabolic PTH therapy provide conflicting results, and are complicated by the limitation that bone biopsies are rarely available in such studies so data is limited to BMD and biochemical markers. Early work showed that the combination of a bisphosphonate and PTH limited the effect of PTH on BMD [61,62]. A recent study indicates that the combination of anti-RANKL treatment with PTH has a cumulative effect on BMD, but the PTH-induced increase in bone formation itself was blocked, as indicated by significant reductions in the serum markers P1NP and osteocalcin [63].

Thus, the osteoclast provides coupling factors to both trabecular and periosteal surfaces to stimulate bone formation in both physiological modelling and remodelling. They also contribute to the anabolic action of PTH, and possibly its paracrine analog PTHrP [14], most significantly in states of high remodelling, as in the young animal.

**EphrinB2:EphB4: osteoblast: osteoclast communication or an inter-osteoblast-lineage signal?**

EphrinB2 is a membrane-bound receptor tyrosine kinase that, in bone, is expressed at all stages of osteoblast differentiation and in osteoclasts [64,65]. To induce signalling above baseline levels of phosphorylation, EphrinB2 must interact with a membrane bound receptor, such as EphB4, which is expressed by the osteoblast lineage, but not by osteoclasts [64,65]. Two distinct features of membrane-bound Ephs and ephrins are: (1) their requirement for direct cell-to-cell interaction, and (2) their ability to generate bidirectional signals where forward signalling through the Eph receptor and reverse signalling through the ephrin ligand occur at the same time [66].

In vitro data has indicated that when osteoclasts and osteoblasts come into contact, subsequent signalling through EphrinB2 in the osteoclast lineage restricts their differentiation, while signalling in the osteoblast through EphB4 stimulates bone formation [64]. This suggested that ephrinB2 may act as a membrane-bound osteoclastic coupling factor that stimulates bone formation in osteoblasts via EphB4 intracellular signalling. However, osteoclast lineage-specific deletion of EphrinB2 presented no detectable bone phenotype [64]. This suggests that any inhibitory role of ephrinB2 signalling in the osteoblast lineage is redundant with other pathways, or may indicate that contact between osteoclasts and osteoblasts, as exists in cell culture conditions is a rare event in physiological conditions. For example, in bone remodelling osteoclasts and osteoblasts act on the same surface but at different times [67], while in bone remodelling, they act on different surfaces.

The osteoblast lineage, including osteoblast precursors and osteocytes, expresses both ephrinB2 and EphB4 [65]. In addition, there is extensive contact among these cells that is required for bone formation [68–70]. Although ligand-independent phosphorylation is possible, the high degree of contact within the osteoblast lineage is likely to explain the endogenous phosphorylation of both
tyrosine kinases in these cells in vitro [65]. A key role for osteoblastic ephrinB2 signalling in bone formation was suggested by its strong and specific upregulation by parathyroid hormone (PTH) and its related paracrine protein PTHrP [65]. The importance of the ephrinB2:EphB4 interaction was shown by inhibition of late stage osteoblast differentiation and mineralisation in vitro [65,71,72], a finding that was reproduced in vivo, both in the presence and absence of PTH [71]. In vivo, this inhibition of osteoblast differentiation resulted in an accumulation of early-stage osteoblasts, indicated by high mRNA levels of Runx2, Osx, Alpl, Col1a1 and PTHHR [71]. These data suggested that ephrinB2:EphB4 interactions between bone-surface osteoblasts, and possibly osteocytes, are required for the progression of osteoblasts to full maturity, and for full expression of late osteoblast/osteocyte markers.

Because of the nature of bidirectional signalling, s EphB4 blocks both EphB4 (reverse) and ephrinB2 (forward) signalling, making it impossible to discern from these pharmacological experiments whether forward or reverse signalling is most important for osteoblast differentiation. Early data using a mouse that overexpresses EphB4 in the osteoblast lineage demonstrated a significant increase in osteoblast activity in female mice, suggesting that forward signalling may be most important [64]. However, in our analysis of these mice, backcrossed to C57BL/6, there was no difference in their bone mass or bone formation activity compared to littermate wild type males or females (F.M. Takyar, H.J. Brennan, N.A. Sims, unpublished observations), suggesting that promoting EphB4 forward signalling does not stimulate bone formation in remodelling in all strains of mice.

Therefore, ephrinB2:EphB4 signalling within the osteoblast lineage provides a check-point through which osteoblasts must pass to reach late stages of osteoblast differentiation. The key question of whether it is forward or reverse signalling that is most important during bone remodelling, and relative roles in trabecular vs. cortical bone remain unresolved.

What are the respective roles of preosteoblast- and osteocyte-derived RANKL?

Inhibition of the ephrinB2:EphB4 interaction in vivo and in vitro also promoted RANKL mRNA levels in cultured osteoblast lineage cells, and enhanced their support of osteoclast formation [65,71,72]. Consistent with this finding, in the context of fracture healing, EphB4 overexpression in the osteoblast lineage was associated with a lower number of osteoclasts at the fracture site [73]. The greater number of early-stage osteoblasts and increased support of osteoclast differentiation in vitro and in vivo without an increase in their bone forming activity suggests that inhibition of the ephrinB2:EphB4 interaction in the osteoblast lineage has separated the osteoblast’s bone forming effort from its support of osteoclastogenesis. Further, this suggests that late stage osteoblasts are less supportive of osteoclast function. This is consistent with early in vitro studies that showed greater RANKL expression in less differentiated osteoblasts cultured in vitro [74], however, more recent studies have suggested that late-stage osteoblast lineage cells, particularly osteocytes, are also a physiologically significant source of RANKL.

The most highly studied mechanism by which the osteoblast lineage controls osteoclast formation is their production of RANKL. RANKL expression in the osteoblast lineage is directly stimulated by a wide range of factors, including PTH [9], 1,25-dihydroxyvitamin-D$_3$ [75] and IL-6 family cytokines [76]. It has long been thought that the major contributor of RANKL to bone remodelling is the osteoblast lineage, most likely osteoblast precursors [2], although RANKL has been known for many years to be expressed by newly embedded osteocytes as well [77].

Recent in vivo studies suggest that fully differentiated and matrix-embedded osteocytes are also a significant source of RANKL. Cell-specific knockouts demonstrated osteopetrosis in mice that lack RANKL throughout the osteoblast lineage, and a significant, but more mild, osteoprototic phenotype, including increased trabecular bone mass, in mice with RANKL deletion targeted to late osteoblasts and osteocytes only [46,78]. Although cortical bone was not a focus of that work, the reduction in cortical thickness associated with hindlimb unloading, suggesting that osteocytic RANKL may be of importance in endocortical resorption of disuse-associated bone loss [77]. Cortical thickness may also be greater in osteocyte-specific RANKL null mice, but statistical analysis of this was not reported, nor the change in endocortical resorption.

When RANKL deletion was induced in the osteoblast lineage in 26 week old mice, no detectable change in trabecular bone mass was detected, suggesting that the contribution of early osteoblast-derived RANKL to trabecular osteoclastogenesis may be most important during in trabecular bone during growth [46]. Notably, this difference in trabecular bone phenotypes between induced osteoblast- and osteocyte-specific deletion of RANKL after development was not reproduced in a recent manuscript that achieved a similar level of RANKL knockdown, albeit in younger mice [79]. In the latter work, data was reported only for male mice, and the earlier work did not state which sex was analysed. Additionally, in direct contrast to the findings of Nakashima et al. [78] RANKL mRNA levels were higher in osteoblast-rich cell preparations of the later paper compared to osteocyte-rich preparations, a finding consistent with early studies of differentiating osteoblasts in vitro [74].

Some insights into the paracrine and endocrine pathways that depend on osteocyte-specific RANKL may be gained from mice with osteocyte-specific deletion of pathways known to stimulate RANKL. In three examples, osteocyte-specific deletion of the vitamin D receptor (VDR), the PTH receptor (PTHHR1) or the IL-6 family co-receptor subunit gp130 revealed no osteopetrosis, nor any alteration in RANKL expression in bone [80–82]. Furthermore, and in the case of the VDR null mice, a robust RANKL response to 1α,25-dihydroxyvitamin-D$_3$ administration was observed. This suggests that the main source of RANKL produced in response to these particular stimulatory factors in bone remodelling of the adult trabecular bone is not the osteocyte.

The mechanism by which osteoblast-lineage cells make RANKL available to osteoclast precursors is not yet known, particularly since early in vitro studies indicated that cell–cell contact between cultured osteoblast-lineage cells and osteoclast precursors was required for RANKL to support osteoclast formation [83]. Osteoblast precursors are not tethered to the bone surface, so their contact with osteoclast progenitors can occur directly in the marrow space. For matrix-embedded osteocytes, it has been suggested that soluble RANKL, or membrane-bound RANKL on exosomes may provide a mechanism [84]. While this may be quite tightly controlled during bone remodelling under the canopy of the BMU, it remains to be established how RANKL may be provided to sites distant from osteoblast lineage cells, where osteoclasts contribute to bone modelling, such as the metaphyseal periosteum.

Are signals to osteoclasts maintained when osteoblasts or osteocytes are reduced?

If signals from the osteoblast are key to maintaining osteoclast numbers, what happens when osteoblasts are depleted? A number of genetic and pharmacological models provide clues. While some osteoblast-lineage specific deletions that lead to low osteoblast numbers, such as PTHHR [83], also cause low levels of osteoclasts,
there are others, such as deletion of β-catenin, where osteoblast numbers are reduced and osteoclast formation is increased [86]. There are still other instances where osteoblast numbers are low, but osteoclast numbers are retained at normal levels, as when gp130 or osterix were deleted in osteoblasts [82,87] (see below). These differences may relate to a shift in the numbers of osteoblasts at different stages of differentiation, and therefore RANKL expression, as observed with sEphB4 treatment (see above). Alternatively, they may indicate a loss of paracrine stimulation of RANKL by each of these factors.

In more extreme examples, such as when osteoblasts are completely ablated in adult mice by stem cell mobilisation therapies [88,89] the surprising finding was that there was no reduction in osteoclast numbers. Similar observations were made when genetically altered mice were generated with a thymidine kinase-mediated deletion of osteocalcin-expressing osteoblasts [90]. The latter model suggests that the cells in the osteoblast lineage required to support osteoclast formation are not the matrix-producing osteoblasts. Rather, it is osteoblast precursors and/or non-dividing cells, such as osteocytes that support trabecular bone remodelling.

In contrast to the osteoblast ablation studies, diphtheria toxin-induced deletion of osteocytes [91] led to a high level of resorption. This suggests either that osteocytes inhibit osteoclast formation (suggesting that their expression of OPG is more significant than their expression of RANKL), or supports the concept that osteocyte apoptosis, induced in this case, by diphtheria toxin, stimulates osteoclast formation, as occurs with microdamage [92].

Role of osteocytic IL-6 family cytokines in trabecular and cortical bone

Another example of low bone formation in the presence of normal osteoclastogenesis is mice in which gp130, the IL-6 cytokine family transducing receptor subunit, was specifically deleted in the osteoblast lineage [82]. The IL-6 family is a large family of cytokines, including IL-6, interleukin 11 (IL-11), leukemia inhibitory factor (LIF), cardiotrophin-1 (CT-1) and oncostatin M (OSM). Each of these cytokines acts by forming a complex that includes a common transmembrane receptor subunit, glycoprotein 130 (gp130) [93].

Most members of the IL-6 family of cytokines have been shown to robustly stimulate osteoclast formation in vitro [94,95], an action mediated by stimulation of RANKL expression by cultured osteoblasts [11,96]. These findings led to the understanding that these cytokines are largely pro-osteoclastic. However, many of these same cytokines also stimulate bone formation [25,31,93,97–100].

The anabolic action of IL-6 cytokines appears to rely on two key mechanisms, firstly stimulating osteoblast commitment at the expense of adipogenesis [25,31] while also inhibiting expression of the osteocyte-specific Wnt signalling antagonist sclerostin [25]. However, it seems that the osteocytic pathway of action is most important for bone formation during bone remodelling, since mice lacking gp130 in the full osteoblast lineage and mice lacking gp130 only in osteocytes demonstrate essentially the same phenotype [82]. In both models, trabecular bone mass was low due to a low level of trabecular bone formation, while periosteal diameter was increased, indicating region-specific roles for gp130 cytokines in bone formation.

In neither male nor female mice was there a significant change in osteocalcification in either the osteocyte or osteoblast lineage knockouts for gp130 [82]. This suggests that the stimulatory actions of IL-6 family cytokines on RANKL expression and osteoclast generation by the osteoblast lineage, while impressive in vitro, may be of little relevance to physiological bone remodelling. Their key role is likely to be one that mediates pathological bone resorption, consistent with previous reports that IL-6 null mice do not show increased osteoclastogenesis in response to ovarietomy [101] or inflammatory arthritis [102]. The low osteoclast numbers observed in mice with global deletion of individual IL-6 family members, OSMR, IL-11R [25,26], are therefore likely to relate to signals from non-osteoblast lineage cells. Surprisingly though, when osteocalcification was stimulated from OSMR or IL-11R null bone marrow macrophages in vitro, there was no impairment [25,26]. The key source of pro-osteoclastogenic factors that respond to IL-6 family cytokines in normal bone remodelling remains unknown.

The increased periosteal growth in the osteocyte-specific gp130 null model [82] indicates that IL-6 family cytokines play an inhibitory role in modelling periosteal bone. One cytokine that may be capable of this is ciliary neurotrophic factor (CNTF), the only IL-6 family cytokine reported to inhibit bone formation [103]. Notably, CNTF is expressed at high levels in skeletal muscle, suggesting that the increased periosteal growth in the absence of osteocytic gp130 may relate to reduced paracrine signals from the adjacent muscle, a control mechanism specific to the periosteal surface [104].

The findings of a low level of trabecular bone formation and increased cortical dimensions elicited by gp130 deletion in osteocytes contrasts with osteocyte-specific knockouts of other factors that stimulate bone formation (β-catenin, Pkd1 and IGF-1); these all resulted in low bone mass of both cortical and trabecular bone due to reduced bone formation at both sites [105–107]. It is also possible that the increased periosteal growth of the osteocyte-specific gp130 null mouse occurs to compensate for poor bone quality; cortical bone demonstrated reduced yield strength, there was a greater proportion of woven bone, and production of collagen type I subunits and osteocalcin was lower than controls [82]. Thus signalling of IL-6 family cytokines in osteocytes controls bone formation in opposite ways on trabecular and periosteal surfaces; in addition this family of cytokines regulates the collagen distribution in cortical bone through the osteocyte, possibly by limiting remodelling of cortical bone.

Concluding comments

In conclusion, the field has advanced to the point that we are now beginning to have a better appreciation of the key intercellular signals that control bone formation and resorption both in the context of bone remodelling and modelling. Since the 1950s, our view of the BMU has extended beyond the immediate interactions of cells on the surface of bone. Osteoclasts are still understood to produce coupling factors that stimulate remodelling on trabecular surfaces; it now seems that they can also signal, through the cortical bone, to stimulate bone modelling on the periosteum, although their method of access still needs to be defined. In addition, the contributions of RANKL derived from cells at specific stages of osteoblast differentiation (including osteocytes) to bone remodelling and modelling, and their regulation at each stage by endocrine and paracrine factors, remains controversial. Finally, we note that IL-6 family cytokines can have both a stimulatory effect on bone formation in remodelling, but inhibitory effects on remodelling at the periosteum, a difference likely to be mediated by cells present in the marrow or on the periosteum that release factors that are yet to be identified.

References

Appendix 2
EphrinB2 Signalling in Osteoblast Differentiation, Bone Formation and Endochondral Ossification

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Abstract The main functions of the osteoblast lineage (pre-osteoblasts, osteoblasts and osteocytes) are the production of bone matrix (osteoid), its mineralisation and the support of osteoclast formation. EphrinB2 and its contact-dependent receptor, EphB4, are stably expressed through all these stages of the lineage, and their expression of ephrinB2 is stimulated by parathyroid hormone (PTH), an agent that stimulates bone formation. Recent work has shown that the ephrinB2/EphB4 interaction is required for late stages of osteoblast differentiation and PTH action. Furthermore, specific deletion of ephrinB2 within the osteoblast lineage delays the process of bone mineralisation resulting in impaired bone strength and its response to anabolic PTH in both trabecular and periosteal bone. EphrinB2 and EphB4 are also expressed in growth plate chondrocytes, cells that also support osteoclast formation in development and fracture healing. This review will discuss the roles of forward and reverse signalling through EphB4 and ephrinB2, respectively, in bone growth and remodelling.

Keywords EphrinB2 · Osteoblast · Osteoclast · Coupling · Apoptosis · Bone formation

Introduction

Skeletal structure depends on two key processes. In bone modelling, bone formation and resorption occur on different surfaces, resulting in changes in the size and shape of the skeleton, including, for example, bone formation on the periosteal (outer) surface of the cortical bone [1]. Secondly, in bone remodelling, bone formation follows prior resorption on the same surface. Bone remodelling occurs asynchronously at many sites throughout the skeleton and throughout life to replace old or damaged bone with new bone matrix and thereby maintain the structural integrity of the skeleton. The sequence of events by which bone formation follows bone resorption is termed “coupling”, and for bone mass to be maintained, balance between these two activities is necessary. The process of coupling, and its optimal balance, involves communication between the bone resorbing cells (osteoclasts) and those that form bone (osteoblasts) [2] and within the osteoblast lineage itself [3]. Recent studies suggest a cell-contact-dependent receptor tyrosine kinase ephrinB2, and its interaction with the receptor EphB4, mediates communication within the osteoblast lineage both during bone remodelling and periosteal growth (modelling).

Three Functions of the Osteoblast Lineage

The osteoblast lineage includes osteoblast precursors, bone matrix-producing osteoblasts and matrix-embedded osteocytes (Fig. 1). This lineage carries out three major functions to determine skeletal structure: bone matrix (osteoid) production, regulation of osteoid mineralisation and support of osteoclast formation. While they are known to contribute to other biological activities, such as maintenance of the haematopoietic stem cell niche [4], phosphate homeostasis...
The osteoblast lineage derives from multipotent mesenchymal progenitors, which can also differentiate into chondrocytes or adipocytes [7]. The expression of early osteoblast markers, such as runt-related transcription factor 2 (Runx2) and osterix (Osx), allows mesenchymal stem cells to become committed osteo-chondro-progenitors with restricted differentiation capacity [8, 9]. Once osteoblasts mature to the stage of actively forming new bone matrix (osteoid), they express high levels of alkaline phosphatase (Alpl), type 1 collagen (Col1a1) and parathyroid hormone 1 receptor (Pthr1), among other markers [10]. Matrix-producing osteoblasts exist as groups of cells on the bone surface, and they require cell-cell contact to produce matrix [11–13]. During later stages of osteoblast differentiation, matrix-producing osteoblasts express osteocalcin (Bglap) [14] and osteopontin (Spp1) [15], which inhibit the process of mineralisation.

After depositing osteoid matrix, osteoblasts may undergo one of three fates: (1) become bone lining cells, (2) undergo apoptosis or (3) become embedded in the matrix and differentiate into osteocytes. Differentiated osteocytes form an extensive intercellular network throughout the bone matrix and regulate both bone formation and resorption. Cell-cell contact is also a feature of this network: multiple dendritic projections from each cell form a total of ~3.7 trillion connections throughout the adult skeleton [16]. In addition to controlling osteoblast activity on the bone surface by the release of local factors such as sclerostin (Sost) [17], and oncostatin M (Osm) [18], osteocytes also regulate bone matrix mineralisation by expressing factors such as dentin matrix protein 1 (Dmp1) [19] and matrix extracellular phosphoglycoprotein (Mepe) [20].

Bone structure is influenced not only by matrix production and its mineralisation but also by the process of osteoclastic bone resorption. During bone remodelling, osteoclast generation depends on production of macrophage colony-stimulating factor (M-CSF) and receptor activator of nuclear factor kappa-B ligand (RANKL) by cells of the osteoblast lineage [21], although whether the key source of RANKL is the osteocyte or the preosteoblast remains controversial [22–24]. During bone development and growth, hypertrophic chondrocytes are also a source of RANKL [23]. They support the formation of osteoclasts that resorb the calcified cartilage matrix, leaving a template on which trabecular bone formation occurs.

**EphrinB2 in Osteoblasts and Osteocytes**

Parathyroid hormone (PTH) acts via its receptor (PTHR1) in osteoblasts both to stimulate bone formation [25, 26] and to stimulate their production of RANKL, thereby also increasing support of osteoclast formation [27]. Intermittent administration of PTH is the only currently available therapeutic agent that can increase bone mass [25]. It achieves this by promoting differentiation of committed osteoblast precursors [28, 29], inhibiting osteoblast and osteocyte apoptosis [30] and inhibiting production of the osteocytic bone formation inhibitor, sclerostin [31]. This anabolic effect of PTH reproduces the action of PTH-related protein (PTHrP), a locally produced paracrine stimulus...
of bone formation [32]. PTHrP not only acts through the same receptor but also has autocrine and nuclear activity [33].

In a microarray analysis of the effects of PTH and PTHrP on cultured osteoblasts, ephrinB2 was rapidly upregulated [34]. This effect was reproduced in PTH-treated rats in vivo [34]. EphrinB2 expression by osteoblasts and osteocytes was confirmed in rat bone [34] and human bone [35] by immunohistochemistry and was particularly prominent in osteoblasts on the surface of mature, lamellar bone, undergoing the process of remodelling.

EphrinB2 is a member of the largest family of receptor tyrosine kinases [36]. This family has the unique ability to generate simultaneous bidirectional signals: forward signalling through Eph receptors and reverse signalling through the ephrin ligand [37]. For signalling to occur, direct cell-cell contact must be made since both receptor and ligand are membrane-bound [36], although soluble forms of some family members exist [38–40]. The Eph/ephrin family can be divided into two groups, A and B. EphinA ligands are membrane-anchored proteins, while ephrinB ligands are transmembrane proteins. These ephrin ligands bind to EphA and EphB receptors, with some promiscuity. Although many members of this family are expressed in osteoblasts in vitro and in vivo [34, 41, 42], only ephrinB2 was stimulated by PTH and PTHrP [34].

Given the extensive connections within the osteoblast lineage, the contact-dependent communication system of ephrins and the specific stimulation of one family member by PTH/PTHrP suggested a possible role in the process of bone formation.

The EphrinB2/EphB4 Interaction is a Checkpoint for Osteoblast Differentiation

Osteoblasts and osteocytes in both human and mouse also express EphB4 [35, 43]. The importance of the particular interaction of ephrinB2 with one of its receptors, EphB4, was identified in studies using inhibitors of this interaction: a peptide antagonist, TNYLFSPNGPIARAW (TNYL-RAW) [43], or the recombinant extracellular domain of EphB4 (sEphB4) [43–44]. Because they block the interaction, both directions of signalling are blocked [45, 46]: this was confirmed in osteoblasts, where both reagents inhibited both ephrinB2 and EphB4 phosphorylation [43]. Addition of either reagent to cultured osteoblasts or a murine stromal cell line inhibited osteoblast differentiation, as indicated by reduced mRNA levels of late-stage osteoblast markers, such as Dmp1, Mepe and Sost [34, 43] without any change in early osteoblast markers such as Runx2, Colla1 and Alpl [43]. Inhibition of the interaction of ephrinB2 with its other receptors EphB2 and EphA4 did not show the same effect [43, 47]. In addition, TNYL-RAW addition to human mesenchymal stem cells inhibited their ability to form mineral in vitro, a finding consistent with impaired osteoblast differentiation [35]. This suggested that the specific interaction between ephrinB2 and EphB4 within the osteoblast lineage is a checkpoint through which the differentiating osteoblast must pass for continued differentiation in vitro (Fig. 1). This interaction did not require the presence of any other cell type indicating that signalling within the osteoblast lineage is necessary for late stages of osteoblast differentiation.

When such inhibition of the ephrinB2/EphB4 interaction was tested in vivo, using sEphB4, late stages of osteoblast differentiation were again impaired [43]. The evidence of this was twofold. Firstly, there was the observation of a change in osteoblast function: sEphB4 treatment in mice increased the number of osteoblasts and the level of osteoid production, but the rate of bone mineralisation was not increased. Secondly, mRNA extracted from bones of mice treated with sEphB4 showed high levels of early osteoblast markers with no modification in late stage osteoblast markers [43]. This suggested an accumulation of matrix-producing osteoblasts with reduced ability to fully differentiate. These data showed the ephrinB2/EphB4 interaction is not required for osteoblasts to produce osteoid matrix and confirmed its requirement for osteoblasts to pass a checkpoint into the later stages of differentiation that promote osteoid mineralisation.

Inhibition of the ephrinB2/EphB4 interaction in vivo also impaired the anabolic action of PTH, but not only by impairing late stages of osteoblast differentiation [43]. EphrinB2/EphB4 inhibition in the presence of PTH (but not in its absence) also increased osteoclast formation in vivo. This was reproduced in co-culture studies of osteoblasts with osteoclast precursors, suggesting the early stages of osteoblast differentiation are more supportive of osteoclast formation. In addition, in vitro studies showed that sEphB4 or TNYL-RAW treatment of osteoblasts rapidly increased their support of osteoclastogenesis by promoting RANKL transcription in cells of the osteoblast lineage and rapidly stimulated mRNA levels of other pro-osteoclastic factors, such as interleukin-6 (IL6) and oncostatin M receptor (Osmr) [43]. Therefore, the interaction of ephrinB2 and EphB4 might inhibit the production of a range of osteoclast inhibitors in response to PTH. Surprisingly, this contrasted with other works using a similar co-culture system showing that TNYL-RAW treatment suppressed osteoclastogenesis in a RANKL-independent manner when insulin-like growth factor 1 (IGF-I) was used as a stimulus of osteoclast formation in vitro [48].

The Role of EphrinB2 in Osteoclast Formation

EphrinB2 is expressed not only by osteoblast lineage cells but also in osteoclasts and their precursors [41]. However, ephrinB2 signalling does not play a cell-lineage autonomous role in osteoclasts, since these cells do not express any Eph receptors that bind ephrinB2 [41]. This was confirmed by a lack of effect of ephrinB2/EphB4 inhibition on osteoclast formation in vitro in the absence of osteoblasts [43]. However,
when exogenous EphB4-Fc was used to stimulate ephrinB2 signalling in osteoclast precursors, their ability to form osteoclasts in vitro was impaired [41]. This suggested that interaction of osteoclast precursors with EphB4-expressing cells, such as osteoblasts, could suppress osteoclast formation by activating ephrinB2 signalling in osteoclast precursors [41]. The increased osteoclast formation we observed in response to sEphB4 in vivo and in co-cultures could be explained by this alternative model, except for the finding that sEphB4 treatment retained its ability to stimulate osteoclast formation in co-cultures of ephrinB2-expressing osteoblasts with ephrinB2-deficient osteoclasts [47••]. This confirmed that suppression of ephrinB2 reverse signalling within the osteoclast lineage did not cause the increased osteoclast formation induced by sEphB4. Rather, the osteoclast formation was induced by increased production of RANKL by osteoclasts in which ephrinB2/EphB4 signalling was suppressed. Therefore, ephrinB2/EphB4 signalling in osteoclasts either limits the expression of osteoclastic RANKL or maintains osteoclasts at an immature stage in which they produce more RANKL.

Defective osteoclastogenesis in mice with RANKL deficiency at specific stages of osteoblast and osteocyte differentiation have led to the suggestion that osteocytes are an important source of RANKL expression [23, 24]. Although early studies reported conflicting evidence of whether RANKL mRNA levels are higher in osteocytes than osteoblasts [24, 49, 50], recent work using more specific purification indicates that RANKL mRNA levels are higher in osteoblasts than in osteocytes [22]. Thus, the interaction between ephrinB2 and EphB4 was required for the continued differentiation of osteoblasts; without it, osteoblasts are held at an immature stage when their support of osteoclastogenesis is high and their ability to produce factors that promote bone mineralisation is low.

EphrinB2 Signalling Within the Osteoblast Lineage Promotes Osteoblast Differentiation by Limiting Apoptosis

Pharmacological inhibition of the ephrinB2/EphB4 interaction impaired late stages of osteoblast differentiation both in vivo and in cultured osteoblasts [43•]. However, two questions remained unanswered: (1) which signalling direction (through ephrinB2 or EphB4) is most important and (2) whether all effects observed resulted from inhibiting an osteoblast-lineage specific interaction? While effects were consistent between the two systems, the in vivo effects may also have resulted from inhibition of ephrinB2 and EphB4 signalling in other cells such as embryonic stem cells [51], endothelial cells [52] T cells [53] and neurons [54], all of which are known to influence bone remodelling. The specific role of ephrinB2 within the osteoblast lineage was further investigated using Osx1-Cre-directed deletion of ephrinB2 [47••].

In the absence of ephrinB2 in adult female Osx1-Cre.ephrinB2f/f mice, osteoblast numbers were greater than in controls, but bone formation rate was not increased [47••]. This phenotype is similar to the effects of sEphB4 treatment in vivo [43•]. Furthermore, while osteoid thickness was greater in Osx1-Cre.ephrinB2f/f mice, a reduction in mineral apposition rate in these mice indicated that, rather than osteoid production being increased, it was the progression of bone mineralisation that was delayed [47••], a similar observation to the clinical condition of osteomalacia [55].

This phenotype was not restricted to a change in trabecular bone formation. These mice also exhibited thinner cortical bone, smaller femoral width and reduced polar moment of inertia compared to controls. This and the osteomalacia-like phenotype resulted in reduced bone stiffness, allowing a greater level of bone deformation before fracture by 3-point bending tests and reduced loading and unloading slopes by reference point indentation; in simplest terms, they had soft, rubbery bones [47••].

mRNA from Osx1-Cre.ephrinB2f/f bones revealed higher expression levels of early osteoblast markers and lower levels of late osteoblast/osteocyte markers [47••], again consistent with the impaired late stage osteoblast differentiation observed with sEphB4 treatment [43•]. One mechanism identified that maybe responsible for the impairment in late-stage osteoblast differentiation was a higher level of osteoblast and osteocyte apoptosis in Osx1-Cre.ephrinB2f/f bones [47••]. This was identified by TUNEL staining and electron microscopy in situ and higher levels of caspase 8-mediated apoptosis in cultured ephrinB2-deficient osteoblasts [47••]. This supported the model of an ephrinB2/EphB4 checkpoint in osteoblast differentiation indicated by the pharmacological inhibition studies. Without expression of anti-apoptotic ephrinB2, osteoblasts undergo apoptosis rather than making the transition to the fully mature stages expressing Dmp1, Mepe and Sost that promote osteoid mineralisation.

Does EphB4 Forward or EphrinB2 Reverse Signalling Promote Osteoblast Differentiation?

Due to the bidirectional signalling induced within the osteoblast lineage by the ephrinB2/EphB4 interaction, identifying the signalling direction that promotes osteoblast differentiation has been challenging. For example, when Osx1-Cre.ephrinB2f/f osteoblasts were analysed by Western blot and immunofluorescence, although the EphB4 phosphorylation response (forward signalling) to exogenous ephrinB2 remained intact, their basal levels of EphB4 phosphorylation were low, presumably due to the lack of ephrinB2 reverse signalling in neighbouring cells [47••]. This implied that the phenotype might relate to impairments of both EphB4 and ephrinB2 signalling.
That EphB4 signalling within the osteoblast lineage might promote osteoblast differentiation was suggested when EphB4 phosphorylation was stimulated in cultured osteoblasts by treatment with clustered ephrinB2-Fc [41]. This treatment increased expression of early osteoblast markers such as Runx2, Osx, Alpl, Coll1a1 and Bglap, suggesting that EphB4 forward signalling within osteoblasts enhanced osteoblast differentiation. In addition, trabecular bone volume was increased in transgenic mice overexpressing EphB4 in the osteoblast lineage [41]. Compact bone and marrow isolated from these mice also exhibit greater numbers of osteoprogenitor cells [56•] and, thus, increased support of haematopoiesis [57•]. Although it was suggested that this phenotype occurred due to increased EphB4 forward signalling, it should be noted that EphB4 overexpression in osteoblasts would also stimulate ephrinB2 signalling.

To determine which direction of signalling is most important for osteoblast differentiation, we compared the effects of ephrinB2 knockdown to knockdown of EphB4. Surprisingly, although the ephrinB2 knockdown reproduced the effects of sEphB4 treatment on osteoblast differentiation, EphB4 knockdown had the opposite effect in cultured osteoblasts: mineralized nodule formation and early markers of osteoblast differentiation were increased [47••]. The contrasting effects of EphB4 knockdown and ephrinB2 deletion in osteoblasts supports the conclusion that reverse signalling through ephrinB2 mediates the ephrinB2/EphB4 checkpoint through which osteoblasts pass to reach late stages of differentiation.

### EphrinB2 as a Mediator of PTH and IGF-I Anabolic Actions

Since PTH stimulates ephrinB2 expression, the response of Osx1-Cre.ephrinB2<sup>ΔΔF</sup> mice to anabolic PTH treatment was also investigated. The PTH-induced increase in osteoblast number, osteoid thickness and mineral apposition rate in both trabecular and periosteal bone was impaired, but not fully blocked, in Osx1-Cre.ephrinB2<sup>ΔΔF</sup> mice [47••]. Consistent with a limited response to PTH, the ephrinB2-deficient mice also showed a deficiency in the usual PTH-induced changes in gene expression. Early osteoblast markers (Runx2, Alpl and Col1a1) were not increased, and Sost expression was not reduced. However, the RANKL gene response to PTH was retained [47••]. This indicated that ephrinB2 signalling within the osteoblast is required for the anabolic response of PTH to stimulate osteoblast differentiation and reduce osteocytic sclerostin expression, but is not required for the RANKL response to PTH. Again, this may relate to retention of early osteoblast-lineage cells that have higher levels of RANKL expression, but have reached the stage of PTH receptor expression.

It has also been suggested that ephrinB2/EphB4 signalling may mediate IGF-I-induced bone formation since global genetic deletion of IGF-I, and osteoblast-targeted insulin-like growth factor receptor (IGF-IR) deletion in mice resulted in low levels of both ephrinB2 and EphB4 [48•]. Cell culture studies reported that siRNA deletion of EphB4 and TNYL-RAW treatment blocked the IGF-I-induced increase in mRNA levels of osteoblast markers, Runx2, Alphi and Bglap [58]. It is possible that the effects of IGF-I on ephrinB2 expression may be required for PTH anabolic action, since the PTH-induced increase in ephrinB2 was not observed in IGF-I-deficient bone marrow stromal cells [48•], and PTH anabolic action is reduced in IGF-I-deficient mice [59]. The interdependency of these pathways has not yet been shown nor has the requirement for ephrinB2/EphB4 signalling in the increased bone formation resulting from IGF-I treatment in vivo.

### What is the Role of EphrinB2 Signalling in the Osteoclast Lineage?

The possible involvement of ephrinB2 signalling within the osteoclast in bone remodelling has been a question that is difficult to answer. Since osteoclasts express only ephrinB2, but not EphB4, the signalling of ephrinB2 in osteoclasts depends on an interaction with a different cell type that expresses ephrinB2. The earliest model, based on results from in vitro co-culture studies, proposed that the main interaction of ephrinB2-expressing osteoclasts that suppressed their differentiation was an interaction with EphB4-expressing osteoblasts [41]. This proposed that this direct interaction provided a mechanism for coupling balance, by which the activity of osteoblasts is matched to the prior activity of osteoclasts on the same surface. However, since there is a time delay of some weeks between the processes of bone resorption and formation on bone remodelling surfaces, termed the “reversal phase” [60], a direct interaction between mature osteoclasts and osteoblasts on the bone surface, is rare. Alternative proposals have been suggested, including the possibility that the interaction occurs between osteoclast precursors and osteoblast precursors or between osteoclast precursors and some other ephrinB2-expressing cells such as endothelial cells [52, 61]. Finally, recent work has identified that the cells that line the bone surface during the reversal phase are osteoblast precursors [62]; if these express ephrinB2 and interact directly with EphB4-expressing osteoclasts, these could provide a mechanism, at the cellular basis, by which this might work. However, co-culture studies using osteoclasts deficient in ephrinB2 showed that the stimulatory effects of sEphB4 on osteoclast differentiation did not result from modifying this cell-specific pathway [47••], questioning the role of osteoclastic ephrinB2 in osteoblast-stimulated osteoclastogenesis.
Since haematopoietic specific knockout of ephrinB2 did not result in a detectable bone phenotype [41, 47], the situations in which ephrinB2 signalling within the osteoclast lineage inhibits osteoclast differentiation remain obscure and may be restricted to an in vitro phenomenon.

A very recent study of mice with an osteoclast-driven deletion of the NF-xB inhibitor CHMP5 has suggested the phenotype of exuberant “Pagetic” periosteal remodelling in these mice results from an interaction of ephrinB2 in osteoclasts with EphB4 in osteoblasts [63]. In this work, high ephrinB2 mRNA levels were noted in cultured osteoclasts from these mice, likely a simple reflection of greater osteoclast numbers (also indicated by high mRNA levels of osteoclast markers). Addition of EphB4-Fc in osteoclast-osteoblast co-cultures mildly reduced Alizarin Red staining. This was interpreted as evidence that ephrinB2/EphB4 signalling between osteoclasts and osteoblasts supported osteoclast differentiation, based on the incorrect assumption that EphB4-Fc acted as an inhibitor. The levels of ephrinB2 and EphB4 phosphorylation in cells from these mice were not assessed nor the effects of EphB4-Fc in the co-culture system. This highlights the complexity of studies of ephrinB2/EphB4 interactions and the need for careful examination of the effects of pharmacological stimuli and inhibitors on both directions of signalling.

**EphrinB2/EphB4 Signalling During Endochondral Ossification**

In addition to regulating osteoblast differentiation in periosteal growth and bone remodelling, ephrinB2/EphB4 signalling has also been suggested as a regulator of endochondral ossification. This process occurs during bone development and longitudinal growth at the metaphyseal growth plate (Fig. 1) and describes the way in which a cartilage model is resorbed by osteoclasts and replaced with bone by osteoblasts.

Chondrocytes and osteoblasts share common mesenchymal stem cell progenitors, and like osteoblasts, chondrocytes express both ephrinB2 and EphB4 during the proliferating and hypertrophic stages at the growth plate [48], as well as during fracture healing [64], in articular cartilage [65] and in the ATDC5 chondrocyte cell line [64]. This suggests a role of ephrinB2 and EphB4 in endochondral ossification, although chondrocytes are not cells that have been previously noted to depend on cell-cell contact for their activity.

In vitro studies using human multipotent mesenchymal stem cells and a small peptide of the interaction of ephrinB2 with EphB2, but not EphB4 significantly inhibited their ability to produce glycosaminoglycans, a major component of the cartilage matrix [35]. More recently, however, work with the committed chondrocytic ATDC5 cell line showed that TNYL-RAW treatment blunted the normal progression of chondrocyte differentiation, usually indicated by increased type II collagen followed by type X collagen mRNA levels [48]. This suggests that ephrinB2/EphB4 signalling, and possibly ephrinB2/EphB2 signalling, stimulates the differentiation of committed chondrocytes, and ephrinB2/EphB2 signalling may promote chondrocytic commitment.

The ephrinB2/EphB4 interaction has also been reported to mediate IGF-I/IGF-IR signalling in the growth plate chondrocytes [48]. IGF-I promotes chondrocyte proliferation during longitudinal bone growth [66]. Immunohistochemistry of wild-type bones showed ephrinB2 and EphB4 protein expression in the prehypertrophic chondrocytes of the growth plate, and levels were reduced in mice with germline IGF-I deletion. RNA extracted from these growth plates also showed reduced ephrinB2, Epha4 and RANKL mRNA levels. This suggested that IGF-I/IGF-IR signalling is either required for chondrocytes to reach the stage of ephrinB2/EphB4 expression or that IGF-I signalling directly stimulates ephrinB2 and EphB4 in growth plate chondrocytes, a regulatory pathway that may also exist in osteoblasts. A direct regulatory pathway was supported by the finding that chondrocytic ATDC5 cells treated with IGF-I significantly increased ephrinB2 and EphB4 mRNA levels [48].

Since chondrocytes also support osteoclast differentiation, it might be expected that ephrinB2/EphB4 inhibition in chondrocytes would promote RANKL, as observed in osteoblasts [43]. However, TNYL-RAW treatment of co-cultures of ATDC-5 cells with osteoclast precursors reduced the formation of osteoclasts when stimulated with IGF-I and did not stimulate their production of RANKL [48]. Although different to the effects of sEphB4 treatment in osteoclasts, the result was consistent with the impaired ability of osteoblasts from Osx1Cre.EphrinB2fl/fl mice to support osteoclast differentiation when stimulated with 1,25-dihydroxyvitamin-D3 or oncostatin M [47]. There are two possibilities to explain these effects. One is that, since expression of RANKL increases as chondrocytes differentiate [67], and TNYL-RAW treatment suppresses chondrocyte differentiation, these late stages of chondrocyte differentiation may not be reached. Alternatively, the authors suggested that the ephrinB2/EphB4 interaction might mediate RANKL-independent osteoclastogenesis in the co-culture system used. In vivo studies are required to resolve this question and determine how this system might influence endochondral ossification both during the process of bone development and in fracture healing.

The role of the ephrinB2/EphB4 interaction in endochondral ossification has also been investigated in the context of fracture healing in transgenic mice overexpressing EphB4 in osteoblasts [56]. Fractured femora from EphB4 transgenic mice displayed a significantly larger extent of cartilaginous tissue matrix in intermediate and distal regions compared to controls. This delay in endochondral remodelling may result from impaired resorption of the cartilage matrix due to reduced osteoclast formation [56], which in turn could result either from
enhanced ephrinB2 signalling in the osteoclast lineage [41] or greater EphB4-mediated suppression of RANKL production by osteoblasts [47••]. The authors also suggested that this may result from increased chondrocyte proliferation induced by EphB4, which they observed in cultured chondrocytes, but since EphB4 overexpression in the in vivo model was directed to the osteoblast, this conclusion seems less likely. Nevertheless, these two studies provide intriguing evidence for a role of ephrinB2/EphB4 signalling in endochondral ossification, both in growth and in fracture healing.

Conclusion

The current body of knowledge suggests that ephrinB2 and EphB4 expressed on the cell membrane of osteoblast lineage cells interact with each other to promote osteoblast differentiation and mineralisation (Fig. 1). It appears that ephrinB2 reverse signalling within the osteoblast lineage is most important for osteoblast survival and the anabolic action of PTH during bone remodelling and peristomal growth. Forward signalling through EphB4 may also play a role, but this is difficult to discern from the currently available data. EphrinB2 reverse signalling within osteoclasts, while inhibitory in vitro does not yet have a clear role in vivo, although a number of possible EphB4-expressing cells that may interact with osteoclastic ephrinB2 are being identified. Finally, a similar EphrinB2/EphB4 interaction in chondrocytes may also play a role in regulating longitudinal bone growth or the process of endochondral ossification in fracture healing.

Compliance with Ethics Guidelines

Conflict of Interest  Christina Vrahnas and Natalie A. Sims declare that they have no conflict of interest.

Human and Animal Rights and Informed Consent  This article does not contain any studies with human or animal subjects performed by any of the authors.

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This work suggests that EphrinB2/EphB4 signalling within osteoblasts can promote early stages of callus formation and bone formation in fracture repair.


Appendix 3
The Primary Function of gp130 Signaling in Osteoblasts Is To Maintain Bone Formation and Strength, Rather Than Promote Osteoclast Formation

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ABSTRACT
Interleukin-6 (IL-6) family cytokines act via gp130 in the osteoblast lineage to stimulate the formation of osteoclasts (bone resorbing cells) and the activity of osteoblasts (bone forming cells), and to inhibit expression of the osteocyte protein, sclerostin. We report here that a profound reduction in trabecular bone mass occurs both when gp130 is deleted in the entire osteoblast lineage (Osx1Cre gp130 f/f) and when this deletion is restricted to osteocytes (DMP1Cre gp130 f/f). This was caused not by an alteration in osteoclastogenesis, but by a low level of bone formation specific to the trabecular compartment. In contrast, cortical diameter increased to maintain ultimate bone strength, despite a reduction in collagen type I production. We conclude that osteocytic gp130 signaling is required for normal trabecular bone mass and proper cortical bone composition. © 2014 American Society for Bone and Mineral Research.

KEY WORDS: OSTEOBLASTS; CYTOKINES; CELL/TISSUE SIGNALING; ENDOCRINE PATHWAYS; MATRIX MINERALIZATION; BONE MATRIX

Introduction

Osteoporosis results from imbalanced bone remodeling in which the level of bone resorption, carried out by osteoclasts, exceeds that of bone formation, carried out by osteoblasts. This leads to reduced bone mass in both the trabecular and cortical bone compartments. Both osteoblast and osteoclast formation are stimulated by cytokines that signal through glycoprotein 130 (gp130), a signal transducer utilized by many different interleukin-6 (IL-6) family cytokines, including IL-6, IL-11, leukemia inhibitory factor (LIF), oncostatin M (OSM), ciliary neurotrophic factor (CNTF), and cardiotrophin-1 (CT-1).(11) Cytokine-specific knockout mouse models have established unique and necessary roles for each of these cytokines in physiological regulation of longitudinal growth, periosteal expansion, and trabecular structure,(2-9) as well as bone loss associated with inflammation(10) and estrogen deficiency.(11)

Although gp130 is ubiquitously expressed,(11) stimulation of osteoclast formation by IL-6, IL-11, LIF, CT-1, and OSM depends on the presence of osteoblasts in vitro(12,13) and is mediated by increased osteoclast receptor activator of NF-κB ligand (RANKL) mRNA expression.(12-15) Furthermore, stimulation of osteoclast formation by other agents such as IL-1, parathyroid hormone (PTH), and 1,25-dihydroxyvitamin D3, are also mediated, at least in part, by gp130.(13) However, the role of osteoblast lineage gp130 signaling in osteoclast formation is not well defined, and has been complicated by conflicting effects on osteoclast formation by genetic deletion of individual cytokines that signal via gp130 and phenotypic differences between the sexes in vivo.(15) For example, systemic deletion of OSM receptor (OSMR)(6) inhibited osteoclast formation in male and female mice. A similar phenotype was reported in male mice with systemic deletion of IL-11 receptor (IL-11R)(10) but females showed only a reduction in osteoblastogenesis. In contrast, CT-1 knockout mice(9) had increased formation of osteoclasts with impaired activity in both males and females. Further complicating the data, systemic LIF,(5) LIF receptor (LIFR), (17) or gp130 deletion(18) resulted in increased numbers of large, active osteoclasts clustered near the growth plate in neonate mice. The contrasting nature of these in vivo osteoclast phenotypes are remarkable, given the strong stimulatory effects of OSM, IL-6, CT-1, and IL-11 on osteoclast formation in vitro.(12,13)

IL-6 family cytokines also act on the osteoblast lineage to stimulate bone formation. IL-6, IL-11, CT-1, and OSM all promote osteoblast differentiation in vitro(6,9,19) and OSM, CT-1, and LIF stimulate bone formation in vivo. (6,9,20) OSM, CT-1, LIF, and IL-11
also inhibit adipocyte differentiation,\(^{5,6,9}\) suggesting an influence of these cytokines on early osteoblast precursor commitment. Furthermore, OSM, CT-1, IL-11, and LIF influence terminally differentiated osteoblasts embedded within the bone matrix (osteocytes), by suppressing their production of sclerostin,\(^{90}\) a potent inhibitor of Wnt signaling and bone formation.\(^{21}\) This indicates that IL-6 family cytokines act on osteoblasts at all stages of differentiation (eg, early osteoblasts, late osteoblasts, or osteocytes), but the relevant stages at which they support bone formation, bone resorption, or adipogenesis are unknown.\(^{22}\)

To determine the stage-specific roles of gp130 in the osteoblast lineage in both bone modeling and remodeling, we have generated two mouse models where gp130 was conditionally deleted either from the entire osteoblast lineage (Osx1Cre) or osteoblast lineage in both bone modeling and remodeling, we obtained from Rodger McEver (Oklahoma Medical Research Foundation) and DMP1Cre mice via subcutaneous injection as described.\(^{9}\) Calvariae were harvested upon sacrifice, 10 days after the last injection, and analyzed by histomorphometry as described.\(^{9}\)

### Histomorphometry and \(\mu CT\)

Histomorphometry was performed on tibial sections as described.\(^{29}\) Ex vivo \(\mu CT\) was performed on femoral, vertebal, and calvarial specimens using the SkyScan 1076 system (Bruker-microCT, Kontich, Belgium). Images were acquired using the following settings: 9-\(\mu\)m voxel resolution, 0.5-mm aluminum filter, 50-kV voltage, and 100-\(\mu\)A current, 2600 ms exposure time, rotation 0.5 degrees, frame averaging = 1. Images were reconstructed and analyzed using SkyScan software programs NRecon (version 1.6.3.3), DataViewer (version 1.4.4), and CT Analyzer (version 1.12.0.0). Femoral trabecular analysis region of interest (ROI) was determined by identifying the distal end of the femur and calculating 15% of the total femur length toward the femora mid-shaft, where we then analyzed an ROI of 12.6% of the total femur length. Analysis of bone structure was completed using adaptive thresholding (mean of minimum and maximum values) in CT Analyzer. Thresholds for analysis were determined manually based on grayscale values (0-255, where 0 = black and 255 = white) for each experimental group as follows: Trabecular bone 12-week-old mice: Osx1Cre.gp130, 38 to 255; DMP1Cre.gp130, 42 to 255. Trabecular bone 26-week-old mice: Osx1Cre.gp130 and DMP1Cre.gp130, 42 to 255. Cortical analyses were performed 35% above the distal end of the femur toward the femora mid-shaft, also with a 12.6% ROI with the threshold values set as follows: Cortical bone, 12-week-old mice: Osx1Cre.gp130, 63 to 255; DMP1Cre.gp130, 57 to 255. Cortical bone, 26-week-old mice: Osx1Cre.gp130 and DMP1Cre.gp130, 100 to 255. Vertebral trabecular bone was evaluated in the L\(_6\) vertebral body of 12-week-old mice, where the ROI was defined as a cylinder measuring 50% of the total height and 66% of the width of the vertebral column at the midpoint. Thresholds were as follows: Osx1Cre.gp130, 42 to 255; DMP1Cre.gp130, 50 to 255. Polarized light microscopy was performed on 100-\(\mu\)-mm-thick transverse sections collected approximately 500 \(\mu\)m from the tip of the distal femur using an Isomet Saw (Buehler, Lake Bluff, IL, USA), and measurements included the entire bone interface.

### Semiquantitative real-time PCR

cDNA synthesis from 0.1 to 1 \(\mu\)g Dnase-treated RNA was performed using AffinityScript (Agilent Technologies, Santa Clara, CA, USA) per the manufacturer’s instructions. Stock cDNA was diluted 1:5 to 1:10 and semiquantitative real-time PCR (qPCR) was performed using an in-house master mix of 10 \(\times\) AmpliTaq Gold with SYBR Green nucleic acid gel stain (Life Technologies, Carlsbad, CA, USA).
Technologies). Primers were used as published (7,30) or designed using Primer Blast (NCBI; http://www.ncbi.nlm.nih.gov/tools/primer-blast) and reported in Table 1. Samples were dispensed onto an optically clear 96-well plate (Thermo Scientific) and run on a Stratagene MX3000P (Agilent Technologies). Cycling conditions were as follows: (95°C for 10 minutes), (95°C for 30 seconds, 58°C for 1 minute, 72°C for 30 seconds) × 40 cycles, followed by dissociation step (95°C for 1 minute, 55°C for 30 seconds, 95°C for 30 seconds). Post-run samples were analyzed using Stratagene software MxPro and reported using linear delta threshold cycle (ΔCT) values normalized to β-2 microglobulin (β2M) or hypoxanthine phosphoribosyltransferase 1 (HPRT1) for primary mouse cells or hydroxymethylbilane synthase (HMBS) for femoral extracts.

Mechanical testing

Femurs from 26-week-old male and female DMP1Cre.gp130 mice (males n = 8 w/w, n = 8 f/f; females n = 5 w/w, n = 7 f/f) were tested at the mid-shaft by three-point bending at room temperature. Load was applied in the anteroposterior (AP) direction midway between two supports that were 6.0 mm apart. Load-displacement curves were recorded at a crosshead speed of 1.0 mm/s using an Instron 5565A dual column materials testing system, using Bluehill 2 software (Instron, Norwood, MA, USA). Prior to testing they were kept moist in gauze swabs soaked in phosphate buffered saline (PBS). Ultimate force (FU; N), yielding force (FY; N), stiffness (S; N/mm), and energy (work) to failure (U; mJ) were calculated from the load-displacement curves as described (31) The yield point was determined from the load deformation curve at the point at which the curve deviated from linearity. Widths of the cortical mid-shaft in the mediolateral (ML) and AP directions were measured using digital calipers, and the average cortical thickness was determined by μCT. Combining the geometric calculations and the biomechanical test results, the material properties of each bone were calculated as described by Schriefer and colleagues (32) to obtain ultimate strength (τ, MPa); elastic modulus (E, MPa), and modulus of toughness (u, MPa). Average load-deformation and stress-strain curves for each sex and genotype were also generated.

Reference point indentation

Local bone material properties at the femur mid-shafts from 12-week-old male DMP1Cre.gp130 mice (males n = 5 w/w, n = 9 f/f; females n = 5 w/w, n = 5 f/f) were determined by reference point indentation (RPI) using a BP2 probe assembly apparatus (Biodent Hfc, Active Life Scientific Inc., Santa Barbara, CA, USA). The BP2 assembly includes a 90-degree cono-spherical test probe with a ≤5-μm radius point and a flat bevel reference probe with ~5-mm cannula length and friction <0.1 N. To assure consistency between measurements a line 6 mm from the femoral condyle was marked with pencil to indicate the initial probe position. To achieve a maximum indentation force of 2 N, a specific load of 300 g (reference force) was manually applied onto the femur. Two Newtons of force were applied for 10 cycles. Samples were kept partially hydrated with 70% ethanol during measurements. Internal friction, defined as the force resisting motion between the test and reference probe, was identified by the size and shape of the force-distance graph and kept at a constant of <0.3 N to ensure that disruptions within the probe assembly would not affect results. Test measurements were taken pre-experiment and post-experiment on polymerized methyl methacrylate (MMA) to ensure that measurements were consistent and that there were no probe faults through the course of the study. Data was discarded if graphs displayed high friction, or disruptions in the curves of the loading and unloading slopes. The distance the probe travels into the bone (total indentation distance (TDI)) is a measure of the bone’s resistance to fractures; indentation distance increase (ID1) is the indentation distance in the last cycle relative to the first cycle and is correlated to bone tissue roughness; average unloading slope indicates the compressibility of the bone and can be used as a measure of stiffness (33).

Statistics

All graphs are represented as the mean/genotype or the mean of all biological replicates. The number of animals (n) is reported for each graph, on the graph or in the figure legends. For in vitro experiments, three biological replicates were performed and averaged. All error bars are standard error of the mean. Statistical significance was considered p < 0.05. All statistics were calculated using unpaired Student’s t test or one-way ANOVA (GraphPad software) or as indicated in the figure legends.

Results

Confirmation of deletion of functional gp130

Knockdown of gp130 was confirmed in FACS-sorted Osx1Cre-GFP-expressing neonate calvarial osteoblasts, which indicated a 70% reduction in gp130 expression in Osx1Cre.gp130f/f cells compared to controls (Fig. 1A). Because the DMP1Cre construct does not contain a reporter element, Gp130 knockdown of approximately 50% was verified in RNA from whole femurs, flushed of marrow, from both male and female 12-week-old mice (Fig. 1B).

To confirm deletion of functional response of gp130, DMP1Cre.gp130f/f female mice and their controls were injected with OSM over the calvaria to stimulate bone formation (6). Although DMP1Cre.gp130w/w mice formed additional bone in response to OSM, DMP1Cre.gp130f/f mice did not (Fig. 1C). Furthermore, the increase in calvarial thickness and greater mineral apposition rate (MAR) in response to OSM in DMP1Cre.gp130w/w mice was completely ablated by osteocytic deletion of gp130 (Fig. 1C).

In the osteoblast lineage, gp130 maintains trabecular bone volume

μCT analysis of trabecular bone structure revealed a significantly lower trabecular bone volume (BV/TV) and trabecular number (Tb.N) (Fig. 2A, C), with no change in trabecular thickness (Tb.Th).

| Table 1. Primer Sequences for Semiquantitative Real-Time PCR |
|-----------------|-----------------|-----------------|
| **Gene name**   | **Direction**   | **Sequence**    |
| Gp130 (exon 15)| Forward         | 5’-AGAAGGCAATGCTGCTGGTGTG-3’|
|                 | Reverse         | 5’-AAAGCAAGAAGACGAGCCCGAC-3’|
| Tnfsf11         | Forward         | 5’-TGTGCGGCGGCTGGTTCTTCA-3’|
|                 | Reverse         | 5’-CCTGGATGGTCTGATGTGC-3’|
| Mepe            | Forward         | 5’-AGGCTGCTGTGGTGGAGCTG-3’|
|                 | Reverse         | 5’-CGTGGTTCTGGTCGCTTCCATG-3’|
| Hmbs            | Forward         | 5’-CCTGACCGCCATTACCCCG-3’|
|                 | Reverse         | 5’-CATCCCAATGACCCATTGG-3’|

All sequences were designed against the mouse genome.
heterozygote mice demonstrated no significant alteration in trabecular structure at 12 weeks of age (data not shown). Six-week-old Osx1Cre;gp130f/f mice did not exhibit a skeletal phenotype (Supporting Fig. 2), suggesting gp130 signaling in the osteoblast lineage is not required for bone growth.

To determine the stage of osteoblast differentiation at which gp130 is most important for maintaining trabecular bone structure, we used an identical approach to assess trabecular bone structure in 12-week-old and 26-week-old DMP1Cre;gp130f/f males (Fig. 3). μCT evaluation of femoral (Fig. 3A) and vertebral (Supporting Fig. 3) trabecular bone structure revealed a low BV/TV (Fig. 3A) and Tb.N, and (D) Tb.Sp in the metaphysis of the distal femur. μCT images were produced in ParaView (E) and represent the average value for trabecular bone structures in A–D (12-week-old mice: n = 8 w/w, n = 7 f/f; 26-week-old mice: n = 8 w/w and n = 8 f/f). For all graphs, single plot points represent mean/group and error bars indicate standard error mean, where **p < 0.01, ***p < 0.001, ****p < 0.0001. See also Supporting Figs. 1 and 2. BV/TV = % bone volume/tissue volume; Tb.Th = trabecular thickness; Tb.N = trabecular number; Tb.Sp = trabecular spacing.

(Fig. 2B), and significantly greater trabecular spacing (Tb.Sp) in the femur (Fig. 2D,E) and vertebrae (Supporting Fig. 1) of male Osx1Cre;gp130f/f mice compared to Osx1Cre;gp130w/w controls at 12 and 26 weeks of age. Histomorphometric analysis of the proximal tibia confirmed this phenotype (Supporting Fig. 2), and detected significantly lower trabecular thickness in Osx1Cre; gp130f/f 12-week-old mice (Supporting Fig. 2B). Osx1Cre;gp130w/w

Fig. 1. Verification of gp130 functional knockdown by Osx1Cre and DMP1Cre. (A) Osterix1Cre GFP-expressing neonatal calvarial cells from Osx1Cre;gp130w/w and Osx1Cre;gp130f/f mice were freshly isolated and FACs sorted for the GFP-positive population. Knockdown of gp130 was assessed by qPCR using primers targeted to the transmembrane domain (exon 15) of gp130 (n = 2 w/w and n = 3 f/f litters of pooled pups collected in independent experiments). (B) Femurs collected from 12-week-old DMP1Cre;gp130w/w and DMP1Cre;gp130f/f male and female mice flushed of bone marrow were assessed for gp130 knockdown by qPCR with exon 15-directed primers (males, n = 8 mice/group, females, n = 5 w/w and n = 8 f/f mice/group). (C) mOSM (0.2 μg in 25 μL) was administered for 5 sequential days over the calvaria of DMP1Cre;gp130w/w and DMP1Cre;gp130f/f 6-week-old females. Calvaria were collected 10 days following the last injection (gp130w/w mice: n = 8 vehicle, n = 7 mOSM; gp130 f/f mice: n = 9 vehicle, n = 8 mOSM). For all graphs, columns represent mean/group and error bars indicate standard error mean, where *p < 0.05, **p < 0.01, ***p < 0.001. GFP = green fluorescent protein; FACs = fluorescence-activated cell sorting; mOSM = mouse oncostatin M; calvarial Th. = calvarial thickness; MS/BS = mineralizing surface/bone surface; MAR = mineral apposition rate; BFR/BS = bone formation rate/bone surface.

Fig. 2. Conditional deletion of gp130 throughout the osteoblast lineage reduces trabecular bone in adult male mice. Trabecular bone structure was assessed by μCT (SkyScan) in 12-week-old and 26-week-old male Osx1Cre;gp130 mice for (A) BV/TV, (B) Tb.Th, (C) Tb.N, and (D) Tb.Sp in the metaphysis of the distal femur. μCT images were produced in ParaView (E) and represent the average value for trabecular bone structures in A–D (12-week-old mice: n = 8 w/w, n = 7 f/f; 26-week-old mice: n = 8 w/w and n = 8 f/f). For all graphs, single plot points represent mean/group and error bars indicate standard error mean, where **p < 0.01, ***p < 0.001, ****p < 0.0001. See also Supporting Figs. 1 and 2. BV/TV = % bone volume/tissue volume; Tb.Th = trabecular thickness; Tb.N = trabecular number; Tb.Sp = trabecular spacing.

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Demonstrated a significant increase in tibial Tb.Th (by 12.7%, p < 0.001) and Tb.Sp (by 34.5%, p < 0.01) by μCT (data not shown).

Neonate Osx1Cre.gp130f/f and DMP1Cre.gp130f/f mice did not show any significant skeletal or morphological defects, indicating that gp130 in the osteoblast lineage is not required for normal bone development.

Conditional deletion of gp130 in osteocytes and osteoblasts inhibits trabecular bone formation, but not osteoclast formation

Dynamic histomorphometry revealed that the low bone mass of DMP1Cre.gp130f/f and Osx1Cre.gp130f/f mice was associated with a low rate of bone formation. Both DMP1Cre.gp130f/f (Fig. 4A) and Osx1Cre.gp130f/f (Fig. 4B) male mice demonstrated 30% lower trabecular bone formation rate (BFR) compared to their respective w/w controls. This was attributed to significantly lower mineralizing surface (dLS/BS), not a low MAR (Fig. 4), suggesting impaired osteoblast differentiation in both strains of mice. However, no significant changes in osteoblast number (N.

Ob/B.Pm), osteoblast surface/bone surface (Ob.S/BS), osteoid surface (Os/BS), or osteoid volume (OV/BV) were observed in male DMP1Cre.gp130f/f or Osx1Cre.gp130f/f mice (Table 2). This may relate to the already low baseline values of these parameters in 12-week-old male mice, making any further reduction difficult to detect.

In 12-week-old female mice, where osteoblast numbers and extent of osteoid are significantly greater than in male mice, we detected significantly lower N.Ob/B.Pm and Ob.S/BS (Table 2) as well as reduced BFR in DMP1Cre.gp130f/f (Fig. 4C) and Osx1Cre.gp130f/f (Fig. 4D) mice compared to controls, confirming reduced
osteoblast differentiation in the absence of osteocyte gp130. To determine whether this was associated with diversion toward the adipocyte lineage, marrow adipocyte numbers were evaluated in Osx1Cre.gp130f/f mice, and were at wild-type levels in both male and female 12-week-old mice (data not shown).

Despite the known influence of IL-6 family cytokines on osteoclastogenesis, osteoclast number (N.Oc/B.Pm), and surface/bone surface (Oc.S/BS) were not significantly different in DMP1Cre.gp130f/f or Osx1Cre.gp130f/f male or female mice compared to controls (Table 2).

Cortical circumference is increased with gp130 deletion in the osteoblast lineage

The cortical bone phenotypes of DMP1Cre.gp130 12-week-old and 26-week-old male mice (Fig. 5A) were strikingly different to those observed in trabecular bone. Although there was no change in bone length (Supporting Fig. 5) or cortical thickness (data not shown), femoral periosteal perimeter (Ps.Pm), mean cross-sectional moment of inertia (CSMI), and marrow area were all significantly greater in DMP1Cre.gp130f/f male mice (Fig. 5A) compared to controls. This reflected increased bone width in both the AP and lateral-medial directions (Fig. 5B). A similar cortical phenotype was observed in female DMP1Cre.gp130f/f mice at 26 weeks of age (data not shown), in 12-week-old male Osx1Cre.gp130f/f mice (Fig. 5C), and in 12-week-old and 26-week-old female Osx1Cre.gp130f/f mice (data not shown). At 26 weeks of age there was no difference in cortical bone mass between Osx1Cre.gp130f/f and w/w controls mice owing to delayed bone accrual in the w/w group, an effect of the Osx1Cre transgene that has been reported.35

In contrast to their low trabecular BFR, DMP1Cre.gp130f/f and Osx1Cre.gp130f/f mice formed periosteal bone at the same rate and along the same extent of surface as their respective w/w controls at 12 weeks (Fig. 5D, E) and at 6 and 26 weeks (data not shown); this was also found in transverse sections of femurs from 12-week-old DMP1Cre.gp130 male mice (data not shown). There was also no detectable difference in endocortical mineralizing surface, MAR, BFR, osteoclast number, or osteoclast surface in 12-week-old mice (data not shown). This indicates that the larger cortical diameter is caused by a level of bone formation slightly greater than control throughout bone growth, but at a level too low to be detectably greater at any single time point (ie, 6, 12, and 26 weeks).

In osteocytes, gp130 maintains cortical material properties

Because CSMI and marrow area were significantly greater in the absence of gp130 in both Osx1Cre.gp130f/f and DMP1Cre.gp130f/f mice, mechanical properties of the femoral mid-shaft were evaluated in 26-week-old DMP1Cre.gp130f/f and DMP1Cre.gp130f/f mice by three-point bending test. Elastic modulus (intrinsic stiffness), ultimate strength, and yield strength (level of stress at which permanent damage is initiated in the bone) were all significantly lower in male DMP1Cre.gp130f/f mice (Fig. 6A–C), and elastic modulus was significantly lower in female DMP1Cre.gp130f/f mice (Fig. 6A). Toughness (amount of energy required to fracture the bone) was slightly lower in the males, but this was not statistically significant (Fig. 6D). Parameters that are not corrected for the altered size and shape of the bone (stiffness, failure force, deformation at failure, and energy absorbed at failure) were not significantly different in DMP1Cre.gp130f/f males or females compared to w/w controls (Table 3), indicating that the structural integrity of the whole bone remained intact due to the increased cortical dimensions. The load-deformation graphs confirm no difference in response to mechanical load in either the male or female mice prior to normalization for bone geometry (Fig. 6E, F). In contrast, the average stress-strain curves demonstrate that after normalization for bone geometry, the DMP1Cre.gp130f/f male bones are more compliant and withstand less stress than their wild-type counterparts (Fig. 6G, H). Postyield stress (the ratio of stress the bone withstands after yield) was also significantly reduced in male DMP1Cre.gp130f/f mice only (Fig. 6I). Because the bones from the DMP1Cre.gp130f/f males had poorer material properties, tissue mineral density was assessed by μCT.

### Table 2. Bone Cell Parameters of DMP1Cre.gp130 and Osx1Cre.gp130 Male and Female Mice at 12 Weeks of Age

<table>
<thead>
<tr>
<th>Parameters</th>
<th>DMP1Cre.gp130</th>
<th>Osx1Cre.gp130</th>
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<tbody>
<tr>
<td></td>
<td>Males</td>
<td>Females</td>
</tr>
<tr>
<td></td>
<td>w/w (n = 9)</td>
<td>f/f (n = 11)</td>
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<tr>
<td>N.Ob/B.Pm (1/mm)</td>
<td>5.74 ± 1.15</td>
<td>4.60 ± 1.21</td>
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<td></td>
<td>16.61 ± 2.73</td>
<td>15.23 ± 2.31†</td>
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<td></td>
<td>14.24 ± 0.97</td>
<td>8.12 ± 1.06***</td>
</tr>
<tr>
<td>Ob.S/BS (%)</td>
<td>8.83 ± 1.83</td>
<td>7.35 ± 1.87</td>
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<tr>
<td></td>
<td>24.10 ± 5.35</td>
<td>13.62 ± 1.55†</td>
</tr>
<tr>
<td></td>
<td>18.6 ± 1.65</td>
<td>10.33 ± 1.71**</td>
</tr>
<tr>
<td>OS/BS (%)</td>
<td>8.37 ± 1.83</td>
<td>7.08 ± 1.97</td>
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<td>0.87 ± 0.23</td>
<td>0.91 ± 0.34</td>
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<td>3.09 ± 0.38</td>
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<tr>
<td>OV.Oc/B.Pm (1/mm)</td>
<td>1.56 ± 0.25</td>
<td>1.83 ± 0.27</td>
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<td>2.05 ± 0.17</td>
<td>2.46 ± 0.46</td>
</tr>
<tr>
<td>Oc.S/BS (%)</td>
<td>5.98 ± 1.04</td>
<td>7.21 ± 1.35</td>
</tr>
<tr>
<td></td>
<td>6.56 ± 0.81</td>
<td>6.46 ± 0.73</td>
</tr>
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</table>

Values were manually counted and quantified in the proximal tibia of histological sections from 12-week-old male and female mice. Values/group are mean ± standard error and n/group.

N.Oc/B.Pm = osteoblast number/bone perimeter; Ob.S/BS = osteoblast surface/bone surface; OS/BS = osteoid surface; OV/BV = osteoid volume/bone volume; N.Oc/B.Pm = osteoclast number; Oc.S/BS = osteoclast surface.

†Significant at p < 0.05.

**Significant at p < 0.01.

***Significant at p < 0.001.
Fig. 5. Cortical diameter is increased with gp130 deletion. The femoral diaphysis of 12-week-old and 26-week-old male mice was analyzed by μCT (SkyScan) for cortical bone structure. Ps.Perim, CSMI, and marrow area are reported for (A) DMP1Cre.gp130 (12-week-old mice: n = 8 w/w, n = 12 f/f; 26-week-old mice: n = 8 w/w and n = 8 f/f) and (C) Osx1Cre.gp130 (12-week-old mice: n = 8 w/w, n = 8 f/f; 26-week-old mice: n = 8 w/w and n = 8 f/f) mice. (B) Anterior-posterior and lateral-medial width was measured for each bone by digital calipers at the femoral mid-shaft of 26-week-old DMP1Cre.gp130w/w (males n = 8, females n = 5) and DMP1Cre.gp130f/f (males n = 8, females n = 8) mice. (D, E) Periosteal bone formation was quantified by dynamic markers of bone formation in the tibia midshaft using Osteomasure. Calcein was injected into mice 10 and 3 days prior to cull and transaxial histological sections from the tibia of (D) DMP1Cre.gp130 (n = 10 w/w, n = 11 f/f) and (E) Osx1Cre.gp130 mice (n = 6 w/w, n = 10 f/f) were assessed for mineralizing surface (dLS/BS + 1/2sLS/BS), dLS/BS, sLS/BS, BFR/BS, and Ps.MAR on the periosteal surface. For all graphs, the columns or single plot points represent mean/group and error bars indicate standard error, where *p < 0.05, **p < 0.01, ***p < 0.001. See also Supporting Figure 5. Ps.Perim = periosteal perimeter; CSMI = cross-sectional moment of inertia; dLS/BS = double-labeled surface/bone surface; sLS/BS = single-labeled surface/bone surface; BFR/BS = bone formation rate/bone surface; Ps.MAR = periosteal mineral apposition rate.
Fig. 6. gp130 Deletion in osteocytes reduces material properties of cortical bone in male mice. Twenty-six-week-old male (*n = 8 w/w, n = 8 f/f) or female (*n = 5 w/w, n = 7 f/f) DMP1Cre.gp130 mice were subjected to three-point bending test for assessment of (A) elastic modulus, (B) ultimate strength, (C) yield strength, and (D) work (toughness). (E, F) Average Load-deformation curves and (G, H) stress-strain curves for male and female mice were also generated from three-point bending test and represent the average/group. (I) Postyield stress (the ration of stress the bone withstands after yield) as assessed by three-point bending test. (J) Proportion of lamellar versus woven bone in 12-week-old male DMP1Cre.gp130 mice (*n = 9 w/w, n = 8 f/f) assessed by polarized light microscopy with representative images. White arrows indicate woven bone, gray arrows indicate lamellar bone; scale bars represent 100 μm. For all graphs, columns represent mean/group and error bars indicate standard error, where *p < 0.05, **p < 0.01. See also Supporting Fig. 6.
on bones from 12-week-old mice (Supporting Fig. 6A) but no difference between w/w and f/f mice was detected. Notably, female mice had a significantly higher baseline tissue mineral density than male mice (p < 0.0001, Supporting Fig. 6A).

To determine whether the poor matrix material properties were due to differences in the proportion of woven versus lamellar bone in DMP1Cre.gp130f/f mice, 100-μm transverse sections of 12-week-old male femurs were analyzed by polarized light microscopy. DMP1Cre.gp130f/f mice showed a significantly higher proportion of disorganized woven bone compared to lamellar bone matrix in the proximal femur (Fig. 6J).

When 12-week-old bones from these mice were analyzed by RPI, there was no difference in total indentation distance, average loading or unloading slopes, or indentation distance increase after loading (Supporting Fig. 6B), suggesting that the poor material properties of DMP1Cre.gp130f/f bones do not relate to a defect at the microindentation level.

Osteocyte density is greater in the trabecular compartment of gp130 osteocyte conditional knockout mice, resulting in greater local production of sclerostin

The trabecular compartment–specific reduction in BFR in the absence of gp130 in osteocytes suggests that osteoblasts in the trabecular bone and periosteum respond differently to gp130 signals controlling bone formation. Because OSM, LIF, IL-11, and CT-1 all inhibit osteocyte production of the bone formation inhibitor sclerostin, the density of total osteocytes and sclerostin-positive osteocytes was assessed in 12-week-old male Osx1Cre.gp130 and DMP1Cre.gp130 cortical and trabecular bone to determine whether region-specific changes in osteocyte number and sclerostin production could explain the region-specific phenotype. Osteocyte density was no different in the trabecular versus cortical bone of the Osx1Cre.gp130f/f mice (Fig. 7A). However, Osx1Cre.gp130f/f mice had significantly more sclerostin-positive osteocytes in both trabecular and cortical bone than their w/w controls (Fig. 7B), suggesting that gp130 deletion early in the osteoblast lineage increases the number of osteocytes that express sclerostin throughout trabecular and cortical bone.

In contrast to the Osx1Cre.gp130f/f mice, there was no significant difference in total osteocyte density in trabecular bone between DMP1Cre.gp130w/w and DMP1Cre.gp130f/f mice. However, within DMP1Cre.gp130f/f mice, there was a substantially greater numerical density of osteocytes in the trabecular bone compared to the cortical bone of the same mice (Fig. 7A). Again, this was in contrast to the Osx1Cre.gp130f/f mice. Although the percent of sclerostin-positive osteocytes was not different between DMP1Cre.gp130w/w and DMP1Cre.gp130f/f mice in the

![Fig. 7. gp130 Deletion in osteoblasts increases the proportion of sclerostin-positive osteocytes. Histological sections from 12-week-old male DMP1Cre.gp130 (n = 6 w/w, n = 7 f/f) and Osx1Cre.gp130 mice (n = 12 w/w, n = 9 f/f) were evaluated for (A) total osteocyte number/mm² of bone in femoral metaphyseal trabecular and cortical bone. Histological sections were stained for sclerostin by immunohistochemistry and (B) the number of sclerostin-positive osteocytes in trabecular (proximal tibia) and cortical bone (tibia midshaft) were counted manually and normalized to bone area. Paired statistical analyses were used for intra-mouse comparisons. Data are mean ± standard error and *p < 0.05, **p < 0.01, ***p < 0.001. N.Oy = number of osteocytes; N.SOST+ Oy = number of sclerostin-positive osteocytes.

**Table 3. Ultimate Properties of DMP1Cre.gp130 26-Week-Old Mice**

<table>
<thead>
<tr>
<th></th>
<th>Male</th>
<th>Female</th>
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<tbody>
<tr>
<td></td>
<td>w/w (n = 8)</td>
<td>f/f (n = 8)</td>
</tr>
<tr>
<td>Stiffness (N/mm)</td>
<td>117.6 ± 6.92</td>
<td>119.2 ± 5.48</td>
</tr>
<tr>
<td>Failure force (N)</td>
<td>19.10 ± 1.51</td>
<td>19.01 ± 0.77</td>
</tr>
<tr>
<td>Deformation at failure (mm)</td>
<td>0.164 ± 0.01</td>
<td>0.164 ± 0.01</td>
</tr>
<tr>
<td>Energy absorbed at failure (J)</td>
<td>1.48 × 10⁻³ ± 2 × 10⁻⁴</td>
<td>1.47 × 10⁻³ ± 3 × 10⁻⁴</td>
</tr>
</tbody>
</table>

Femurs from 26-week-old male and female DMP1Cre.gp130 mice were subjected to three-point bending and evaluated for stiffness and ultimate properties (to failure point).
trabecular or cortical bone (data not shown), the greater total osteocyte density in trabecular bone compared to cortical bone resulted in significantly more sclerostin-positive osteocytes in trabecular bone compared to cortical bone in DMP1Cre.gp130\(^ff\) mice (Fig. 7B), a difference that was not observed in control mice. Thus, the increased number of sclerostin-positive osteocytes is only because of an increase in the total number of osteocytes, not an increase in the proportion of sclerostin-producing osteocytes, and suggests other mechanisms are responsible for the differences in bone volume between DMP1Cre.gp130\(^w/w\) and DMP1Cre.gp130\(^ff\) mice.

Because osteocyte sclerostin production was increased in both the trabecular and cortical bone, but only in Osx1Cre.gp130\(^ff\) mice, these data also indicate that region-specific patterns of sclerostin production are not responsible for the region-specific phenotype observed in both Osx1Cre.gp130\(^ff\) and DMP1Cre.gp130\(^ff\) mice.

Osteocyte deletion of gp130 reduces osterix, collagen type 1-\(\alpha\)1, and osteocalcin gene expression

To assess the effects of gp130 deletion on gene expression in bone, we determined differences in osteoblast and osteocyte marker genes in femurs of DMP1Cre.gp130\(^ff\) male and female mice with verified gp130 knockdown (Fig. 18). mRNA levels of the early osteoblast marker osterix (Fig. 8A) and mature osteoblast marker osteocalcin (Fig. 8B), as well as collagen type 1-\(\alpha\)1 (Fig. 8C), were all significantly lower (\(-50\%\)) in bones from DMP1Cre.gp130\(^ff\) male mice compared to DMP1Cre.gp130\(^w/w\) mice. Notably, collagen type 1-\(\alpha\)2 mRNA levels were not significantly changed in \(ff\) bones (Fig. 8D). Thus the ratio of collagen type 1-\(\alpha\)1:collagen type 1-\(\alpha\)2, which normally exists in a 2:1 ratio at the protein level \(^{[26]}\) was reduced (Fig. 8E). Osx, Ocn, Col1-\(\alpha\)1, and Col1-\(\alpha\)2 were all at wild-type levels in flushed femora from female DMP1Cre.gp130\(^ff\) mice (data not shown). Neither receptor activator of NF-\(\kappa\)B ligand (RANKL; Tnfsf11) nor Sost mRNA levels were significantly different in DMP1Cre.gp130\(^ff\) male or female mice compared to controls (Fig. 8F; female data not shown).

**Discussion**

This work demonstrates that the key cell through which gp130 controls trabecular bone formation and cortical bone strength is the osteocyte. Genetic deletion of gp130 in osteocytes results in very low trabecular BFR and mass, and larger cortical bone diameter that compensates for significantly degraded cortical bone material properties and low collagen production. This regionally divergent phenotype was confirmed in a second model where gp130 was deleted in the entire osteoblast lineage. In neither model of gp130 deficiency was osteoclastogenesis altered. This leads us to conclude that the increased RANKL production in the osteoblast lineage that occurs in response to IL-6 family cytokines does not play a key role in physiological bone growth and remodeling, but it is the regulation of osteoblast differentiation and matrix production through the osteocyte that is the key role of gp130 in the osteoblast lineage (Fig. 9).

The findings of a low level of trabecular bone formation and increased cortical dimensions elicited by gp130 deletion in osteocytes is unique. It contrasts with osteocyte-specific knockouts of other pathways that stimulate bone formation (\(\beta\)-catenin, polycystin 1 [Pkd1], and insulin-like growth factor 1 [IGF-1]).

Fig. 8. Osteocytic deletion of gp130 reduces OSX, COL1-\(\alpha\)1, and OCN gene expression. Femurs collected from 12-week-old DMP1Cre.gp130\(^w/w\) and DMP1Cre.gp130\(^ff\) male mice and flushed of bone marrow were examined for osteoblast and osteocyte markers of gene expression by qPCR (\(n = 8\) mice/group). Statistical significance between w/w and \(ff\) bones was reached in (A–C) for osterix, osteocalcin, and collagen type 1-\(\alpha\)1 mRNA levels. (D) Collagen type 1-\(\alpha\)2 was not significantly altered. (E) Ratio of mRNA levels for collagen type 1-\(\alpha\)1:collagen type 1-\(\alpha\)2 in flushed femurs is significantly lower in \(ff\) mice. (F) No statistical significance between genotypes was detected for Runx2, Alpl, Tnfsf11, Tnfsf11b, Mepe, or Sost gene expression, all normalized to Hmbs. For all graphs, columns represent mean/group and error bars indicate standard error, where \(^*p < 0.05, \,**p < 0.01, \,**p < 0.001\). Runx2 = runt-related transcription factor 2; Alpl = alkaline phosphatase; Tnfsf11 = receptor activator of NF-\(\kappa\)B ligand; Tnfsf11b = osteoporogenin; Mepe = matrix extracellular phosphoglycoprotein; Sost = sclerostin; Hmbs = hydroxymethylbilane synthase; Osx = osterix; Col1-\(\alpha\)1 = collagen type 1-\(\alpha\)1; Ocn = osteocalcin.

which result in low bone mass in both cortical and trabecular bone owing to reduced bone formation at both sites.\(^{[37–39]}\) These data suggest that stimulating gp130 signaling in osteocytes may increase trabecular bone formation and trabecular bone mass, without increasing osteoclast formation, a phenomenon that could be exploited therapeutically.
All IL-6 family members, apart from those that signal through CNTF receptor (CNTFR), stimulate osteoclast differentiation. This influence has been understood for many years to depend on the ability of these cytokines to stimulate RANKL production by osteoblast-lineage cells. This is supported by in vitro coculture studies, where osteoclast formation in response to IL-6 family cytokines depended on the presence of osteoblasts, and osteoclast formation in response to other cytokines and hormones such as IL-1, PTH, and 1,25-dihydroxyvitamin-D$_3$ was partially dependent on gp130 signaling. It was surprising, then, that we observed no change in osteoclast differentiation or RANKL expression when gp130 was deleted in the osteoblast lineage, or in osteocytes alone. In previous work, adult mice with global deletion of either OSMR or IL-11R demonstrated low levels of osteoclast formation, whereas increased numbers of osteoclasts with impaired activity were observed in mice lacking CT-1. OSMR-null osteoblasts were less supportive of osteoclast formation when stimulated with 1,25-dihydroxyvitamin-D$_3$, but supported enhanced osteoclastogenesis in response to PTH. Suggesting stimulus-dependent roles of osteoblastic gp130 signaling in support of osteoclastogenesis. In the cases of IL-11R and CT-1 deletion, altered osteoclast formation levels were intrinsic to the hemopoietic lineage. Importantly, in all of these mouse knockout models, the IL-6 family cytokine was deleted systemically, and therefore it is unclear whether the effects on osteoclastogenesis in these mice was because of the absence of gp130 signaling in the osteoblast lineage. The data presented here indicate that osteoclast formation in physiological bone remodeling does not require gp130 signaling in the committed osteoblast lineage (Fig. 9A), and this is the first instance in which the absence of gp130 signaling was restricted to the osteoblast lineage. This supports the concept that RANKL expression induced by IL-6 family cytokines in the osteoblast lineage may be most important in specific conditions of elevated osteoclast formation such as estrogen deficiency or inflammatory arthritis. In addition, during bone development and growth, control of osteoclast formation by the IL-6 family may be restricted to the growth plate, as observed in the neonate-lethal LIFR and gp130-null mice and adult LIF-deficient mice. In this region, gp130-dependent osteoclastogenesis may be more directly controlled by hypertrophic chondrocytes than by the osteoblast lineage. This is further supported by a lack of bone phenotype in neonate or 6-week-old Osx1Cre.gp130$^{/-}$ mice, indicating that gp130 signaling in the osteoblast lineage is not required for normal bone development; rather, its key role is to maintain bone formation in the physiological process of bone remodeling in the adult skeleton.

The low level of trabecular bone formation, lower osteoblast number, and reduced osteinix, type 1 collagen, and osteocalcin mRNA levels in DMP1Cre.gp130$^{/-}$ bones confirms a critical role for osteocyte gp130 in promoting osteoblast differentiation (Fig. 9B). This confirms a physiological role for gp130 cytokines that stimulate bone formation in vivo and modify osteocyte gp130 signaling, such as OSM, CT-1, and LIF. This is further supported by the complete absence of an anabolic response to supracalvarial injections of OSM in DMP1Cre.gp130$^{/-}$ mice. This is also consistent with the known stimulatory effects of the IL-6 family cytokines on bone formation, and low levels of trabecular bone formation reported in mice with global deletion of LIF, CT-1, OSMR, or IL-11R. Although IL-6 family...
members that stimulate bone formation also inhibit adipogenesis in vitro,\(^5,6,9,16\) no change in marrow adipogenesis was observed in the osteoblast-specific or osteocyte-specific knockouts (data not shown). This indicates that the anti-adipogenic action of IL-6 family cytokines is mediated by gp130 signaling in non-committed osteoblast precursors prior to osteoblast expression (Fig. 9A).

Deletion of gp130 in osteocytes led to decreased intrinsic bone strength (ie, ultimate strength) in DMP1Cre.gp130\(^{f/f}\) mice and increased the diaphyseal dimensions such that ultimate bending load remained unchanged. This means that the fracture resistance of the bones was retained despite a significant reduction in their material properties. This mechanical maintenance was achieved by an increase in periosteal dimensions, hence increased moment of inertia, to compensate for the decline in bone material quality. The modest increase in trabecular thickness, detected only by \(\mu\)CT, may also reflect this compensatory mechanism. Alternatively, this slight increase in trabecular thickness was specific for the DMP1Cre model, which suggests that gp130 signaling in the osteocyte may limit the thickness of new trabeculae formed at the growth plate, and that this limiting effect is negated when gp130 signaling is deleted throughout the osteoblast lineage. Accordingly, the low trabecular BFR reported here and measured in the secondary spongiosa reflects a lower rate of bone remodeling, not trabecular formation at the growth plate. Furthermore, the reduced material properties of the cortical bone in osteocytic gp130 deficiency suggests that gp130 signaling in osteocytes may contribute to collagen deposition during the osteoid production phase, or mineral deposition during matrix maturation (Fig. 9B,C). Because no significant alterations in tissue mineral density or periosteal MAR were detected in 12-week-old DMP1Cre.gp130\(^{f/f}\) mice, the poor material properties of the bone matrix likely reflect a defect in collagen deposition rather than altered mineralization. Indeed, this was supported by our findings that the ratio of collagen 1 type \(\alpha1\) to type \(\alpha2\) mRNA levels was altered in male mice, and there was a higher proportion of woven bone in cortical samples of the male mice. However, we cannot rule out an alteration in the distribution of mineral in the bones of DMP1Cre.gp130\(^{f/f}\) male mice, particularly given the lower osteocalcin mRNA levels.

Although the DMP1Cre.gp130\(^{f/f}\) female bones were less stiff than their wild-type littermates, only male DMP1Cre.gp130\(^{f/f}\) bones had lower ultimate strength, yield strength, and postyield stress. These data indicate an altered long bone phenotype, because it is the collagen matrix that provides the postyield fracture resistance.\(^{42}\) Previous work has demonstrated that slender bones have a higher degree of mineralization and tissue mineral density to compensate for their smaller bone size;\(^{43}\) as we have observed in the smaller diameter and significantly higher tissue mineral density of the female DMP1Cre.gp130\(^{f/f}\) and control bones compared to males. The higher mineral content in the female bones may therefore provide some protection against the effect of gp130 deletion on bone stiffness.

Increased periosteal circumference has not been observed in any global gp130 knockout mouse models. In fact, IL-6, IL-11R, CT-1, and OSM knockout models each show reduced periosteal circumference.\(^{2,3,6,9}\) This suggests that maintenance of cortical bone strength by increased periosteal apposition depends on influences of IL-6 family cytokines on other cell types that stimulate increased activity of periosteal osteoblasts. The increased periosteal expansion in DMP1Cre.gp130\(^{f/f}\) mice is likely to be a response to altered mechanical loading resulting from the poor material integrity. We were unable to detect an increase in periosteal bone formation on transverse and coronal sections at any single time point, which suggests that the periosteal expansion in these bones occurred slowly and over a long period of time, which may be indicative of a cumulative mechanical response over the life of the mouse. This may also indicate that, even though OSM, OSMR, and IL-11 mRNA levels in bone are increased in response to mechanical load,\(^{44}\) periosteal bone formation that is likely induced by mechanical forces may not require gp130 signaling in osteocytes.

More sclerostin-positive osteocytes were observed in both trabecular and cortical bone of Osx1Cre.gp130\(^{f/f}\) mice compared to controls, but not in DMP1Cre.gp130\(^{f/f}\) mice. This suggests that the inhibitory paracrine action of endogenous gp130 cytokines on sclerostin expression, previously described by pharmacological studies in vivo and in vitro,\(^{60}\) is mediated indirectly by an action on early osteoblasts. This increase in sclerostin-positive osteocytes was only observed in Osx1Cre.gp130\(^{f/f}\) mice, suggesting it may contribute to the normal trabecular thickness in these mice, a phenotype that was not observed in the DMP1Cre.gp130\(^{f/f}\) mice. Because both Osx1Cre.gp130\(^{f/f}\) and DMP1Cre.gp130\(^{f/f}\) mice exhibited low trabecular bone formation and large periosteal circumference, regulation of sclerostin production can be excluded as the primary driving force behind the change in bone structure observed in these mice.

The greater osteocyte density and low BFR found specifically within the trabecular bone of the DMP1Cre.gp130\(^{f/f}\) mice is consistent with reports in human bone, where trabecular osteocyte density and BFR were negatively correlated.\(^{45}\) This suggests that IL-6 family cytokines, in addition to reducing sclerostin,\(^{40}\) may also determine the rate at which osteocytes become incorporated within the trabecular network. It is also possible that in the absence of osteocytic gp130 osteoblast apoptosis is impaired, and more osteoblasts survive through to osteocyte differentiation, consistent with previous in vitro reports of an antiapoptotic role of IL-6 family cytokines.\(^{46}\) However, it remains unclear why this was observed only in DMP1Cre.gp130\(^{f/f}\) bones when the Osx1Cre.gp130\(^{f/f}\) mice also exhibited low trabecular bone mass.

Therapeutic use of IL-6 inhibition for inflammation is widespread,\(^{47}\) and new IL-6 family inhibitors are actively being pursued for a wide range of disorders.\(^{48}\) Clinical trials have begun to test the efficacy of a soluble form of gp130 (sgp130-Fc) that selectively blocks IL-6 trans-signaling by sequestering the IL-6:IL-6R complex and preventing it from binding membrane-bound IL-6R.\(^{49,50}\) These pathologic conditions already exhibit increased fracture risk, and our findings of detrimental effects of osteocyte gp130 inhibition on trabecular bone mass and cortical bone material properties indicates that careful skeletal monitoring of patients enrolled in future clinical trials is warranted.

In conclusion, we report here an essential role for osteocyte gp130 signaling to support trabecular bone formation and healthy composition of the cortical bone matrix. These data also suggest that stimulation of gp130 signaling targeted to the osteocyte may provide therapeutic benefit by stimulating trabecular bone formation and preserving material properties in the cortical bone.

**Disclosures**

All authors state that they have no conflicts of interest.
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Appendix 4
EphrinB2 signaling in osteoblasts promotes bone mineralization by preventing apoptosis


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ABSTRACT Cells that form bone (osteoblasts) express both ephrinB2 and EphB4, and previous work has shown that pharmacological inhibition of the ephrinB2/EphB4 interaction impairs osteoblast differentiation in vitro and in vivo. The purpose of this study was to determine the role of ephrinB2 signaling in the osteoblast lineage in the process of bone formation. Cultured osteoblasts from mice with osteoblast-specific ablation of ephrinB2 showed delayed expression of osteoblast differentiation markers, a finding that was reproduced by ephrinB2, but not EphB4, RNA interference. Microcomputed tomography, histomorphometry, and mechanical testing of the mice lacking ephrinB2 in osteoblasts revealed a 2-fold delay in bone mineralization, a significant reduction in bone stiffness, and a 50% reduction in osteoblast differentiation induced by anabolic parathyroid hormone (PTH) treatment, compared to littermate sex- and age-matched controls. These defects were associated with significantly lower mRNA levels of late osteoblast differentiation markers and greater levels of osteoblast and osteocyte apoptosis, indicated by TUNEL staining and transmission electron microscopy of bone samples, and a 2-fold increase in annexin V staining and 7-fold increase in caspase 8 activation in cultured ephrinB2 deficient osteoblasts. We conclude that osteoblast differentiation and bone strength are maintained by antiapoptotic actions of ephrinB2 signaling within the osteoblast lineage.—Tonna, S., Takyar, F. M., Vrahnas, C., Crimeen-Irwin, B., Ho, P. W. M., Poulton, I. J., Brennan, H. J., McGregor, N. E., Allan, E. H., Nguyen, H., Forwood, M. R., Tatarczuch, L., Mackie, E. J., Martin, T. J., Sims, N. A. EphrinB2 signaling in osteoblasts promotes bone mineralization by preventing apoptosis. FASEB J. 28, 4482–4496 (2014). www.fasebj.org

Key Words: osteocyte · osteoclast · bone matrix · bone strength

Originally discovered as transmembrane proteins in an erythropoietin-producing human hepatocellular carcinoma cell line (1), ephrins and the Eph receptor ligands (Ephs) constitute the largest family of receptor tyrosine kinases (RTKs) (2). They are distinct from other RTKs in that receptor-ligand interactions generate simultaneous bidirectional signals: forward signaling through the Eph receptor and reverse signaling through the Eph ligand (3). Further, both receptor and ligand are membrane-bound; as such, their signaling requires direct cell-cell contact (2). Ephrin/Eph family members are recognized as local mediators of cell function through contact-depen...
dent processes as disparate as cell migration and adhesion, tumorigenesis, vasculogenesis, axon guidance, and synaptic plasticity.

Some members of this family, including ephrinB1, ephrinB2, EphB4, EphB2, and EphA4, are expressed in osteoblasts and in osteocytes, terminally differentiated osteoblasts embedded within the bone matrix itself (4–8). Osteoblasts are the cells responsible for bone formation, a process that occurs throughout life. In the adult skeleton, bone formation is a component of bone remodeling, an essential mechanism in which bone matrix is continuously renewed. This process is required for the achievement and maintenance of skeletal strength. Bone remodeling takes place asynchronously in discrete sites known as basic multicellular units (BMUs; refs. 9, 10). In each of these, the bone remodeling sequence begins with the formation of osteoclasts that resorb bone, after which osteoblast progenitor cells differentiate and form new bone matrix. For bone mass to be maintained, this process requires balanced levels of activity of the two cell types, a process termed coupling (11).

In studying actions of parathyroid hormone (PTH), an agent that stimulates bone formation when administered intermittently (12) and PTH-related protein (PTHrP), which is a necessary paracrine stimulus of bone formation (13), we found that treatment with either agent resulted in substantially increased ephrinB2 production in osteoblasts in vitro and bone in vivo, with no regulation of any other ephrin ligand or Eph receptor (7). Furthermore, pharmacological blockade of ephrinB2/EphB4 interaction impaired late stages of osteoblast differentiation both in vivo and in vitro (7, 8, 14). EphrinB2 is also expressed in bone-resorbing osteoclasts, and it has been suggested that direct interaction of ephrinB2 on the osteoclast cell surface and EphB4 on the surface of the bone-forming osteoblast couples bone formation to resorption (5). However, this proposal has been controversial, since the activities of osteoclasts and osteoblasts at the BMU occur at different times, meaning these cells are rarely in contact in vivo. To determine which cell type is the most important source of ephrinB2 in the process of bone remodeling, we have studied the skeletal phenotype of mice with osteoblast-specific deletion of ephrinB2, and their response to anabolic treatment with PTH. The results, and comparisons with in vitro knockdown of EphB4, indicate that ephrinB2 signaling within the osteoblast lineage is required for complete differentiation of the osteoblast, and that early apoptosis due to ephrinB2 deficiency results in reduced bone strength and impaired anabolic response to PTH.

MATERIALS AND METHODS

Animals

Tg(Sp7-cre, TetO-EGFP/cre)1Ame (Osx1-GFP::Cre), Efnb2H11350/+, and CtskH11350/Cre reporter transgenic mice backcrossed to C57Bl/6 for ≥5 generations were obtained from Andrew MacMahon (Harvard University, Cambridge, MA, USA; ref. 15), David J. Anderson (Howard Hughes Medical Institute, California Institute of Technology, Pasadena, CA, USA; ref. 16), and Shigeaki Kato (Fukushima University, Fukushima, Japan; ref. 17), respectively. These mice were interbred to produce osteoblast-lineage specific Osx1.Efnb2+/− and osteoclast-lineage specific Ctsk.Efnb2+/− strains. St. Vincent’s Health Melbourne Animal Ethics Committee approved all animal work.

Specialist reagents

Synthetic DNA encoding the extracellular domain of mouse EphB4 [murine soluble Eph4B (msEphB4)], incorporating a C-terminal His affinity tag and flanking EcoRI and Notl/Eagl restriction sites, was designed from Swiss-Prot sequence P54761, based on the extracellular domain of human sEphB4, as described previously (18). After subcloning into a mammalian pME18s expression vector, msEphB4 was expressed and purified by Drs. Tim Adams and Judith Scoble (Commonwealth Scientific and Industrial Research Organisation, Parkville, VIC, Australia) as described previously (19). Human sEphB4 was obtained from Vascgene Therapeutics (Los Angeles, CA, USA). Recombinant hPTH(1–34) was purchased from Bachem (Budendorf, Switzerland). SNEWIQPREPQH17 (SNEW), the selective inhibitor of ephrinB2:EphB2 receptor interaction (20), and KLYPVWPLSSL (KYL), the selective inhibitor of the ephrinB2:EphA4 interaction (3), were synthesized by Auspep (Parkville, VIC, Australia). Affinity-purified antibodies to mouse EphB4, phospho-tyrosine (4G10), and recombinant mouse ephrinB2-Fc and EphB4-Fc were obtained from R&D Systems (Minneapolis, MN, USA). Phospho-ephrinB antibodies were obtained from Cell Signaling Technologies (Danvers, MA, USA).

Bone histomorphometry, micro–computed tomography (microCT), reference point indentation, and TUNEL staining

Phenotypic analysis was carried out on 12- and 26-wk-old littermates of Osx1.Efnb2+/− and Osx1.Efnb2+//− mice. PTH treatment was carried out in 8-wk-old male Osx1.Efnb2+/− or Efnb2+/− littermates randomly allocated to control or PTH (1–34) treatment. The expression of Osteo1-GFP::Cre was not associated with any significant alteration in bone structure or turnover at 12 wk of age (Supplemental Table S1). hPTH(1–34) at 30 μg/kg or vehicle (20 mM Tris-HCl; 150 mM NaCl, pH 8, and 2% heat-inactivated mouse serum) were administered 5 d/wk for 4 wk by intraperitoneal injection as described previously (8, 21). Mice were weighed daily, and injection amounts were adjusted according to weight. All mice were injected with calcein at 7 and 2 d before death, as described previously (21), and a blood sample was collected by cardiac puncture exsanguination. RNA was prepared from femora flushed of marrow as described previously (8). For histomorphometric analysis, tibiae were fixed in 4% paraformaldehyde, embedded in methyl methacrylate, and sectioned and stained as described previously (8). The secondary spiongosis of the proximal tibia was analyzed by histomorphometry (Osteomeasure; Osteometrics, Atlanta, GA, USA). MicroCT analysis, reference point indentation, and 3-point bending tests were carried out on femora and vertebrae that were fixed in 4% paraformaldehyde/ PBS overnight and stored in 70% ethanol. Femora were analyzed by microCT (Skyscan 1174; Skyscan, Aartselaar, Belgium) as described previously (8, 22). Local bone material properties at the femoral midshaft were examined by reference point indentation using a BP2 probe.
assembly apparatus (Biodent Hf; Active Life Scientific Inc., Santa Barbara, CA, USA) as described previously (22). Mechanical testing by 3-point bending was performed on femora as described previously (22).

The extent of apoptosis was determined in decalcified paraffin-embedded tibial sections from 12-wk-old male Osx1.EfnfB2β/α and EfnfB2β/β littermate mice using a DeadEnd Colorimetric TUNEL kit (Promega, Madison, WI, USA) according to the manufacturer’s instructions.

Transmission electron microscopy (TEM)

Femurs from 9- and 12-wk-old Osx1.EfnfB2β/α, Osx1.EfnfB2β/β, and Osx1.EfnfB2β/β mice (n = 3 each) were used for TEM analysis. After decalcification in 10% EDTA for 14 d, samples were postfixed in 1% osmium tetroxide and 1.5% potassium ferrocyanide for 5 h and embedded in Spurr’s resin. Ultrathin sections were stained with uranyl acetate and Reynolds’ lead citrate and examined with a Philips 300 transmission electron microscope (Philips, Amsterdam, The Netherlands) at 60 kV.

Cell culture

Primary calvarial osteoblasts were prepared from 1- to 3-d-old Osx1.EfnfB2β/α, Osx1.EfnfB2β/β, EfnfB2β/β, and C57Bl/6 (lacking Osx1-GFP::Cre) mice as described previously (8), with the following modifications. After sequential digestions, the supernatants were pooled and plated in complete medium (αMEM and 10% FBS) and left overnight at 37°C with 5% CO₂. The following day, the dishes were plated into 5% CO₂, 5%O₂, and 90% N₂ in a sealed hypoxia incubator chamber (Billups-Rothenberg Inc., Del Mar, CA, USA) at 37°C for 48 h. Cultures were expanded to subconfluence in 175 cm² flasks at 37°C under 5% CO₂ and cryopreserved in 10% dimethyl sulfoxide (DMSO) until use. To study alkaline phosphatase (ALP) activity, mRNA and protein expression, and cAMP activity during differentiation, reanimated cells were differentiated at confluence in αMEM, 15% heat-inactivated FBS, and 50 µg/ml ascorbate (Sigma-Aldrich, St. Louis, MO, USA). For mineralization studies, confluent primary calvarial osteoblasts and Kusa 4b10 cells (23) were grown in osteoblast differentiation medium containing 10 mM β-glycerophosphate, and differentiated for 21 d. ALP activity and Alizarin Red staining were assessed as described previously (23).

The potential of bone marrow to form osteoblasts was determined by treatment with receptor activator of NFκB ligand (RANKL; Oriental Yeast Company, Osaka, Japan) and macrophage-colony-stimulating factor (M-CSF) as described previously (24). Osteoclast formation in the presence of primary calvarial osteoblasts or Kusa 4b10 cells was studied using a previously described coculture system (24).

EphB4-ALP (EphB4-AP) binding

Mouse EphB4-AP fusion protein (GeneHunter, Nashville, TN, USA) was used to transiently transfected HEK 293T cells as described previously (25) and conditioned culture medium (containing secreted EphB4-AP) was filtered and stored at −80°C. To assess EphB4-AP binding, confluent Osx1.EfnfB2β/α and Osx1.EfnfB2β/β calvarial cells, differentiated in osteoblast differentiation medium for 15 d, were thrice washed for 5 min in ice-cold PBS, blocked for 30 min at room temperature in αMEM plus 10% FBS and 0.1% NaN₃, and incubated with EphB4-AP (10 nM in αMEM) overnight at 4°C. After washing twice in PBS for 5 min, samples were prepared for colorimetric detection of EphB4-AP as previously (25), with minor modifications: 0.8 mM Levamisole (Dako, Carpinteria, CA, USA) was added to block endogenous ALP, and samples were left to develop overnight at 37°C. EphB4-AP was quantified using ImageJ software (U.S. National Institutes of Health, Bethesda, MD, USA).

Proliferation and cAMP assays

Proliferation of undifferentiated primary calvarial cells (αMEM and 10%FBS) was determined using a ClickIT-EDU Microplate assay (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s instructions. cAMP activity was measured in primary calvarial osteoblasts differentiated for 3 d in response to treatments as listed, as described previously (26).

Immunofluorescence and Western blotting

For immunofluorescence and Western blotting, Osx1.EfnfB2β/α and Osx1.EfnfB2β/β primary calvarial osteoblasts were grown to confluence and starved of serum for 3 h in αMEM. Total EphB4, tyrosine phosphorylation, and phosphorylated ephrinB were detected as described previously (8) in untreated cells, and in cells treated with EphB4-Fc or ephrinB2-Fc, at concentrations indicated in the figures. Quantification of Western blot band intensities was performed on Metamorph 7.8.6.0 software (Molecular Devices Corp., Sunnyvale, CA, USA) using region segmentation and thresholding tools. Since total EphB4 levels varied, phospho-EphB4 intensities are expressed as a ratio to total EphB4 intensity.

Generation of stable EphB4-knockdown cell lines

A retroviral system (27) was used to knock down EphB4 in the Kusa 4b10 stromal cell line (23). Briefly, a set of 10 small hairpin RNAs (shRNAs) was designed, validated by sequencing, and ligated into a viral LMP plasmid containing a microRNA30 sequence flanked by both puromycin resistance and green fluorescent protein (GFP)-reporter genes. LMP expressing a hairpin targeting Renilla was prepared the same way and used as control. After transfection into Phoenix cells [American Type Culture Collection (ATCC), Manassas, VA, USA], media containing the retroviral-packaged shRNAs were used to transfect undifferentiated Kusa 4b10 cells. Puromycin was used to select cells, and GFP reporter expression was confirmed using an Olympus IX-81 live-cell imager (Olympus, Tokyo, Japan). Level of knockdown was assessed by quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR) and Western blot, and the two most effective shRNAs were used for further study as follows: 1714, sense 5′-GAATGGTGTGTCTAC-3′; and 4241, sense 5′-GGAAGTGTTGTGTTGTCAACA-3′ and antisense 5′-TTGACCACACCCATTCAA-3′; and antisense 5′-TTGACCACACCCATTCAA-3′.

Lenti-Cre mediated recombination of efnB2 in vitro

A lentiviral vector (5 µg) containing both Cre recombinase and ZsGreen under control of the CMV promoter and 3 plasmids encoding HIV-Rev (5 µg), HIV-gag/pol (12 µg) and ectopic envelope (2 µg) (courtesy of Dr. Carl R. Walkley, St Vincent’s Institute, Melbourne, VIC, Australia) were used to cotransfect HEK293T cells by the calcium phosphate precipitation method. Cells were seeded to 10 cm² dishes 24 h prior to transfection and maintained in DMEM containing 10% FBS and 0.2× penicillin streptomycin glutamine (PSG) and incubated for 24 h at 37°C in 5% CO₂. Supernatants harvested 48 h after transfection were filtered through 0.45 µm
Small interfering RNA (siRNA) knockdown of ephrinB2 and EphB4

Phoenix E cells were transfected at 70% confluence with plasmid DNA containing empty vector, EphB2 siRNA-969, or EphB4 siRNA-877, kindly provided by Dr. Koichi Matsuo (Keio University, Tokyo, Japan; ref. 5) with FuGene 6 (Promega) at 3:1 to DNA. Medium was replaced 24 h post-transfection with fresh medium and cultured at 32°C to enhance retroviral titer. Retroviral supernatants were collected at 48 h post-transfection, filtered, and stored at −80°C. Kasa4b10 osteoblasts were infected with retroviral supernatant (30% v/v) in the presence of polybrene (8 μg/ml, Sigma-Aldrich). Infected cells were sorted for GFP by flow cytometry. Sorted GFP+ cells were cultured in osteoblast differentiation medium and mineralization assessed as above.

RNA quantitation

Total RNA from cells and femoral samples was prepared using Trizol (Life Technologies; Invitrogen) according to the manufacturer’s instructions, as described previously (8). RNA samples were DNase treated with Ambion Turbo DNA-free kit (Ambion Life Technologies, Austin, TX, USA), and 1 μg of RNA was converted to cDNA as described previously (8). For <500,000 cells, RNA was precipitated using 100 mg/ml of glycerogen (Ambion Life Technologies). qRT-PCR was performed using a MX3000P (Stratagene; La Jolla, CA, USA) and SVBR Green (Invitrogen). Primer sequences used for real-time PCR have been previously described (7, 8); EphB2 primers directed to the targeted region were: 5′-AGAAC-TGGGAGCAGGCTTG-3′, 5′-TGGCCACAGTGGTAGTGGC-3′, and smooth muscle actin (Acta2) primers were: TTCAT-ACTGGAGGACTAGCAGTTTTAGAGTCC-5′, 5′-TCATGTTGTCGCTGGTGGC-GAGG-3′. Data shown in Figs. 1A–C, 2D, and 5A–C and Supplemental Fig. S2A were calculated by normalizing to housekeeping genes β-2 microglobulin (B2m) or hypoxanthine-guanine phosphoribosyltransferase 1 (Hprt1) using the ΔΔCt method, and fold-change calculations for Figs. 1B, 2C, J, and 6C were performed by the ΔΔCt method (28).

Flow cytometry

Detection of GFP+ and ZsGreen+ osteoblasts and assessment of apoptosis was performed on cell populations previously described above. Apoptosis was simulated by 1 μM staurosorpin for 24 h, and activation of caspase 8 by 50 μg/ml mTNF-α (R&D Systems, Minneapolis, MN, USA) for 48 h. Cells were trypsinized and resuspended in annexin binding buffer (10 mM HEPES, 140 mM NaCl, and 2.5 mM CaCl2, pH 7.4) for staining with annexin V–Pacific Blue conjugate (Life Technologies; Compark Circuit, Melbourne, VIC, Australia), and 7-AAD (BD Biosciences, San Jose, CA, USA) or anti-cleaved caspase 8 (Cell Signaling Technologies). Samples were acquired using either a BD LSRRFortessa or BD FACSaria (BD Biosciences) and analyzed with FlowJo 10 (TreeStar Inc., Ashland, OR, USA).

RESULTS

We have previously reported that inhibition of the high-affinity interaction of ephrinB2 and EphB4 with EphB4 reduces osteocalcin (Bglap) and stimulates RANKL [tumor necrosis factor (ligand) superfamily member 11 (Tnfsf11)] mRNA levels (8). In the present study, specific inhibition of the interactions of ephrinB2 with its lower affinity-receptors EphA4 or EphB2 (29) did not reproduce this effect (Fig. 1A). This indicates that, within the osteoblast lineage, it is the interaction between ephrinB2 and EphB4 that is most important for osteoblast differentiation, leading us to focus on investigating this interaction.

Osteoblast-specific EphB2 knockdown and the level of EphB4 signaling were assessed by multiple methods. Loss of EphB2 mRNA (80% reduction) was verified in GFP+ Osx1.EfnB2+Δ osteoblasts compared to Osx1.EfnB2+Δ osteoblasts (Fig. 1B). Related ephrin and Eph family member mRNA levels were not significantly different from control (Fig. 1B). Since fluorescent activated cell sorting (FACS)-sorted Osx1.Cre GFP+ cells did not proliferate well in vitro, as others have noted (30), calvarial osteoblasts were differentiated from individual Osx1.EfnB2f/f and Osx1.EfnB2f/f littermates for further study. EphB2 mRNA levels were not changed during differentiation in wild-type cells, as previously observed (7); but were significantly less in Osx1.EfnB2fΔ cells compared to Osx1.EfnB2fΔ+Δ cells at all time points (Fig. 1C). A 60% reduction in membrane-bound ephrinB2 protein was confirmed in Osx1.EfnB2fΔ+Δ osteoblasts compared to controls by EphB4-AP Fusion protein binding (ref. 31 and Fig. 1D). Consistent with that, treatment of Osx1.EfnB2fΔ+Δ osteoblasts with EphB4-Fc did not increase ephrinB phosphorylation (Fig. 1E). The extent of basal EphB4 phosphorylation was slightly lower in Osx1.EfnB2fΔ+Δ primary calvarial osteoblast cultures compared to control cells (Fig. 1A), but not completely absent. This low level of EphB4 phosphorylation may result from ligand-independent EphB4 phosphorylation, as reported previously in other cell types (32). But also, since primary osteoblast cultures are impure, they contain more ephrinB-expressing cells. Stimulation with ephrinB2-Fc increased EphB4 phosphorylation, rescuing Osx1.EfnB2fΔ+Δ levels to those observed in stimulated control cells, indicating that EphB4 response to exogenous ephrinB2 was maintained in Osx1.EfnB2fΔ+Δ osteoblasts (Fig. 1F). Since ephrinB2 is also expressed in the osteoclast lineage (5), we confirmed that EphB2 mRNA levels were not altered in osteoclast precursors [bone marrow macrophages (BMMs)] nor in differentiated osteoclasts from Osx1.EfnB2fΔ+Δ mouse compared to Osx1.EfnB2fΔ+Δ (Supplemental Fig. S1A).

Statistics

Statistical differences were determined by Student’s t test or 1- or 2-way ANOVA, followed by Tukey post hoc test, using Prism 6.0e (GraphPad, San Diego, CA, USA); P < 0.05 was considered statistically significant.
EphrinB2 is required for osteoblast differentiation and support of osteoclastogenesis in vitro

We used 4 approaches to establish the effect of ephrinB2 deficiency on osteoblast differentiation. First, differentiating Osx.1.EfnB2Δ/Δ calvarial cells demonstrated impaired function, indicated by lower ALP activity (Fig. 2A) and impaired mineralization (Fig. 2B) with no alteration in proliferation (Supplemental Fig. S1B). Second, while there was no difference in expression of the early mesenchymal marker Acta2 (33) in FACS-purified GFP+ Osx.1.EfnB2Δ/Δ cells (Fig. 2C), mRNA levels of all osteoblast differentiation markers assessed were significantly lower than in controls (Fig. 2C). This included early [Runx-related transcription factor 2 (Runx2) and osterix (Osx)], mature [alkaline phosphatase (Alpl), PTH receptor 1 (Pthr1)] and late-stage [Bglap and sclerostin (Sost)] markers of osteoblast differentiation. Third, when unpurified Osx.1.EfnB2Δ/Δ and Osx.1.EfnB2Δ/Δ calvarial osteoblasts were differentiated, Acta2 levels were unchanged, but increases in osteoblast marker genes were delayed in Osx.1.EfnB2Δ/Δ cells, and did not reach the levels attained by Osx.1.EfnB2Δ/Δ controls (Fig. 2D). Consistent with low Pthr1 mRNA levels in Osx.1.EfnB2Δ/Δ osteoblasts, their cAMP response to PTH was significantly lower than Osx.1.EfnB2Δ/Δ controls (Supplemental Fig. S1C), while responses to other adenylyl cyclase stimuli (forskolin, prostaglandin E2, and isoproterenol) were maintained (Supplemental Fig. S1C). Fourth, siRNA knockdown of ephrinB2 in Kusa 4b10 osteoblasts impaired their mineralization compared to control (Supplemental Fig. S1D). Although osteoblast progenitors can also differentiate to adipocytes (34), mRNA levels of adipocyte markers were similar in Osx.1.EfnB2Δ/Δ calvarial osteoblasts and controls, as was narrow adipocyte number in tibial specimens from these mice (Supplemental Fig. S1E). These findings indicate that ephrinB2 deletion specifically impairs osteoblast differentiation without changing precursor commitment toward the adipocyte lineage.
Osteoblasts support osteoclast formation by expressing RANKL/Tnfsf11. Both GFP+/Osx1.Efnb2<sup>Δ/Δ</sup> cells and unpurified cultured calvarial osteoblasts expressed lower levels of Tnfsf11 mRNA compared to Oxsl.Efnb2<sup>Δ/Δ</sup> controls (Fig. 2C, D). Tumor necrosis factor receptor superfamily member 11B [Tnfsf11b; osteoprotegerin (OPG)] levels were not significantly different (Fig. 2C, D). Consistent with low Tnfsf11 levels, Oxsl.Efnb2<sup>Δ/Δ</sup> cells were less supportive of osteoclast formation when cocultured with BMMs regardless of whether they were stimulated with oncostatin M (OSM; Fig. 2E) or 1,25-dihydroxy-vitamin-D3 (Fig. 2F). Furthermore, osteoblastic deletion of ephrinB2 did not alter the inherent ability of bone marrow-derived macrophages from Oxsl.Efnb2<sup>Δ/Δ</sup> mice to generate osteoclasts, whether this was in cocultures (Fig. 2E, F) or by treatment with RANKL and M-CSF (Fig. 2G). Thus, ephrinB2 in osteoblasts contributes to their support of osteoclast formation by maintaining RANKL expression.

Although response to exogenous EphrinB2 was maintained, EphB4 phosphorylation in Oxsl.Efnb2<sup>Δ/Δ</sup> osteoblasts was reduced, suggesting the reduced osteoblast differentiation could relate to reduced EphB4 forward signaling (5). We therefore investigated whether the effects of ephrinB2 deletion could be reproduced by...
shRNA knockdown of EphB4 in Kusa 4b10 stromal cells. EphB4 mRNA levels and protein were significantly reduced, and maintained at low levels throughout the culture period, but EphB2, EphB2, EfnB1, and EphA4 levels were not altered (Fig. 2I and Supplemental Fig. S1F). EphB4 knockdown did not inhibit any markers of osteoblast differentiation compared to control at d 1 (Fig. 2I). In contrast to the effects of EfnB2 knockdown (Fig. 1C), EphB4 knockdown increased Osx and Alpl mRNA levels at d 1 (Fig. 2I), and increased mineralized nodule formation after 21 d of culture (Fig. 2I). siRNA-mediated knockdown of EphB4 also increased mineralized nodule formation compared to control (Supplemental Fig. S1G), confirming that the reduced differentiation of Osx1EfnB2^−/− osteoblasts is not replicated by EphB4 knockdown.

In contrast to previous report that osteoblastic EphB4 limits osteoclast formation by interacting with ephrinB2 in the osteoclast lineage (5), shRNA knockdown of EphB4 in Kusa 4b10 cells did not modify their ability to support osteoclast formation (Fig. 2K). To confirm this, when ephrinB2-deficient osteoclasts were generated from Ctsk.EfnB2^−/− BMMs (Supplemental Fig. S2A), the number of osteoclasts formed from Ctsk.EfnB2^−/− and Ctsk.EfnB2^+/− BMMs were similar (Supplemental Fig. S2B), and addition of clustered EphB4-Fc to these cultures did not inhibit osteoclast formation. Furthermore, when the soluble extracellular domain of sEphB4 was used to block ephrinB2/EphB4 interaction, it stimulated osteoclast formation from both Ctsk.EfnB2^−/− and Ctsk.EfnB2^+/− BMMs to the same extent (Supplemental Fig. S2C). We conclude that EphB4-induced ephrinB2 signaling in the osteoclast lineage does not restrain osteoclast formation.

Osteoblastic ephrinB2 maintains bone formation, mineralization, and stiffness

Adult 12-wk-old female Osx1.EfnB2^−/− mice demonstrated 30% fewer osteoclasts per unit bone perimeter (N.Oc/B.Pm) than controls (Fig. 3A). In contrast, their number of osteoclasts per unit bone perimeter (N.Ob/B.Pm) was significantly higher than controls (Fig. 3B). Despite this, mineralizing surface per unit bone surface (MS/BS) was not significantly altered (Fig. 3C) and both mineral apposition rate (MAR) and bone formation rate per unit bone surface (BFR/BS) were significantly lower in Osx1.EfnB2^−/− mice compared to controls, and osteoid thickness (O.Th) was higher (Fig. 3B, C). This low MAR and thick osteoid resulted in a prolonged mineralization lag time (MLT), indicating delayed osteoid maturation in the absence of osteoblast ephrinB2, consistent with the delayed osteoblast differentiation observed in vitro.

The lower levels of bone mineralization and osteoclastogenesis resulted in a gradual accrual of trabecular bone mass, observed at 26 wk of age, but not earlier (Fig. 3D). Three point bending studies revealed that Osx1.EfnB2^−/− femora were more flexible: they were significantly less stiff, with greater toughness and ultimate deformation, and lower yield force compared to control (Fig. 3E). This increased compliance was not associated with a significant alteration in cortical tissue mineral density (TMD), but cortical thickness, polar moment of inertia, and medial-lateral femoral width were all lower in Osx1.EfnB2^−/− femora than controls (Fig. 3F). The greater flexibility of the Osx1.EfnB2^−/− femora can thus be attributed to both delayed osteoid maturation and reduced diaphyseal dimensions.

Osteoblastic ephrinB2 is required for normal response to anabolic PTH treatment

Since PTH stimulates ephrinB2 expression in osteoblasts (7), we investigated the response of male Osx1.EfnB2^−/− mice to anabolic PTH treatment. Male mice were used because their lower level of bone remodeling makes the effects of PTH on osteoblast activity more readily detectable and because they did not exhibit a significant change in trabecular bone structure or remodeling indices with ephrinB2 deletion at either 12 or 26 wk (Supplemental Table S2, and data not shown). PTH treatment of control mice resulted in significantly greater N.Ob/B.Pm, osteoid surface per unit bone surface (OS/BS), O.Th, osteoid volume per unit bone volume (OV/BV) and MAR compared to vehicle treatment (Fig. 4A, B), confirming efficacy of intermittent PTH treatment. These responses to PTH were all significantly impaired in Osx1.EfnB2^−/− mice (Fig. 4A, B), although the serum marker P1NP response was not. There was no significant effect of PTH treatment on N.Oc/B.Pm in Osx1.EfnB2^−/− mice or control mice (Fig. 4C). In the short (4 wk) treatment period and the moderate does chosen, PTH did not significantly increase trabecular bone mass (Fig. 4D). On the periosseum, the PTH-induced increases in MAR and BFR/BS were also completely blocked in Osx1.EfnB2^−/− mice (Fig. 4E). These results indicate that osteoblastic ephrinB2 is required for intermittent PTH to stimulate bone formation in both trabecular and periosteal bone.

Microindentation did not detect any significant effect of PTH treatment in either genotype, but both average loading slope (Avg LS) and unloading slope (Avg US) were significantly lower in Osx1.EfnB2^−/− bones compared to control (Fig. 4F), confirming that bone produced by Osx1.EfnB2^−/− osteoblasts is softer and more flexible than control.

Analysis of mRNA levels of osteoblast markers in bones collected from Osx1.EfnB2^−/− male mice revealed higher levels of early markers Runx2 and collagen I (ColIa1) compared to control (Fig. 5A), while late osteoblast and osteocyte markers Bglap and Sost were more than 50% lower (Fig. 5B), consistent with an impairment in late osteoblast differentiation. Although Sost mRNA levels were low, there was no significant alteration in osteocyte density or number of osteocytes expressing sclerostin in the Osx1.EfnB2^−/− bones (data not shown).

Bones from PTH-treated control mice, sampled 1 h after last injection, demonstrated higher levels of Alpl, ColIa1 and Tnsf11 (Fig. 5B, C) and lower Sost and
TNFRSF11B levels than vehicle-treated, consistent with previous observations (35, 36). These effects of PTH on osteoblast marker genes were altered in Osx1.EfnB2Δ/Δ femora: no PTH-induced increases in Runx2, Alpl and Col1a1 mRNAs were observed, and the reduction in Sost levels was reversed (Fig. 5A, B). These changes were not caused by a change in Pthr1 mRNA (Fig. 5C), and the PTH-induced increase in TNFSF11 and reduction in TNFSF11B observed in controls were retained in Osx1.EfnB2Δ/Δ femora. (Fig. 5C). Thus, while ephrinB2 deletion in osteoblasts did not modify the PTH response of genes that regulate osteoclastogenesis, it blocked the
PTH response of early osteoblast marker genes. This is consistent with the impaired osteoblast activity observed in the presence of normal osteoclastogenesis in response to PTH in ephrinB2-deficient mice.

EphrinB2 protects osteoblasts from extrinsic apoptotic stimuli

Having established that ephrinB2 is required for late stage osteoblast differentiation, gene expression, function and response to anabolic PTH, we investigated responsible mechanisms. While Osx1.EfnB2w/w osteoblasts demonstrated typical cuboidal morphology, with cytoplasm enriched with rough endoplasmic reticulum, residing on osteoid (Fig. 6A, Supplemental Fig. S3A), a high proportion of Osx1.EfnB2+/H9004 and Osx1.EfnB2w/H9004 osteoblasts showed abnormal morphology by TEM. This included irregular shape, disorganized organelles, dilated rough endoplasmic reticulum (Fig. 6A and Supplemental Fig. S3A), and more frequent observations of apoptotic osteoblasts and apoptotic bodies being phagocytosed by adjacent osteoblasts (Fig. 6A and Supplemental Fig. S3A). Apoptotic osteocytes were also observed in Osx1.EfnB2+/w and Osx1.EfnB2w/w but not Osx1.EfnB2+/w samples (Fig. 6A and Supplemental Fig. S3B). Increased apoptosis was confirmed by significantly more TUNEL+ osteoblasts in Osx1.EfnB2Δ/Δ tibial sections compared to control (Fig. 6B).

To identify the key proapoptotic pathway at play in the absence of osteoblastic ephrinB2, EfnB2f/f calvarial osteoblasts were infected with ZsGreen+Lenti-Cre particles to delete ephrinB2 (Fig. 6C). More ZsGreen+Lenti-Cre.EfnB2Δ/Δ calvarial osteoblasts underwent apoptotic cell death, as indicated by annexin V staining compared to control cells both at baseline and in response to staurosporine-induced apoptosis; notably, ZsGreen+Lenti-Cre.EfnB2Δ/Δ showed an increased number of cells that were positive for annexin V and highly positive for 7-AAD. This high number of late apoptotic cells suggests a more rapid response to staurosporine by EfnB2-deficient osteoblasts (Fig. 6D). Furthermore, ZsGreen+Lenti-Cre.EfnB2Δ/Δ calvarial osteoblasts exhibited ~10-fold more caspase 8-expressing cells than controls (Fig. 6E), indicating that ephrinB2 deletion increases programmed cell death of osteoblasts by caspase 8-activated apoptosis.

**DISCUSSION**

We show here that ephrinB2 signaling in the osteoblast lineage is essential for complete differentiation of os-
teoblasts in bone remodeling, and for the anabolic action of PTH. A striking antiapoptotic role for ephrinB2 is evident in the osteoblast lineage in vivo and recapitulated in vitro. This role is required for the faithful progression of late-stage osteoblast differentiation, indicating that ephrinB2 in the osteoblast lineage controls mineralization and bone strength in trabecular and cortical bone, both in normal remodeling and in response to anabolic PTH treatment.

The Eph/ephrin family constitutes the largest family of receptor tyrosine kinases, notable because both receptor and ligand are membrane bound, and both forward (through receptor) and reverse (through ligand) signaling take place on their interaction (2). We observed that while ephrinB2 is capable of interacting with other receptors in osteoblasts, it is only inhibition of its interaction with the EphB4 receptor that significantly reduces osteocalcin expression, prompting us to focus on the relative roles of ephrinB2 and EphB4 in osteoblast differentiation.

Bone formation by osteoblasts is highly dependent on cell-cell contact. Teams of osteoblasts first produce osteoid matrix, and in the second phase, the matrix slowly becomes mineralized. The time between these two processes is the MLT, which is prolonged in ephrinB2-deficient mice. These mice also exhibit a 30% lower rate of bone mineralization, and 20% increase in osteoid thickness, two hallmarks of osteomalacia (37). The distended endoplasmic reticulum detected in ephrinB2-deficient osteoblasts by electron microscopy resembles observations in patients with osteogenesis imperfecta (38) and in mouse models with defects in collagen folding and secretion (39–41), leading us to suggest that the delayed mineralization is secondary to a collagen defect. This osteomalacia-like phenotype and reduced bone formation led to bones with slender diaphysis and significantly greater flexibility, indicated by both 3-point bending tests and reference point indentation. Clearly, osteoblasts in the late stages of differentiation, including osteocytes, regulate the maturation and mineralization of osteoid to achieve optimal bone stiffness, and this is impaired in the ephrinB2-deficient mice, resulting in compromised bone strength.

Delayed mineralization and reduced bone stiffness were associated with increased osteoblast apoptosis in Osx1.EfnB2Δ/Δ mice and increased sensitivity to proapoptotic agents in Osx1.EfnB2Δ/Δ osteoblasts. Apoptosis is one of 4 possible activities of differentiated osteoblasts, with the other three being active bone formation, encapsulation in the bone matrix and differentiation to osteocytes, or conversion to lining cells that reside on the bone surface. A high level of osteoblast apoptosis has been observed in mice treated with glucocorticoids, oxidative stress, and unloading (42–44), as well as genetic PTHrP deficiency (13); each of the earlier reports observed lower osteoblast numbers on trabecular bone. In contrast, while we observed reduced osteoblast differentiation in vitro, osteoblast numbers were significantly greater in vivo in female Osx1.EfnB2Δ/Δ mice, and normal in males. By electron microscopy these cells appear as clearly differentiated osteoblasts, although many of them showed abnormal morphology with unusually distended rough endoplasmic reticu-
Figure 6. Osteoblastic ephrinB2 protects osteoblasts from apoptosis. A) Representative transmission electron micrographs at low power (top panels) and high power (bottom panels) showing osteoblast (Ob) morphology on trabecular bone (B) and newly deposited osteoid (Os) in 9-wk-old Osx1.EfnB2<sup>+/−</sup> (left panels) and Osx1.EfnB2<sup>−/−</sup> mice (middle and right panels). Middle panels: an apoptotic body (AB) showing intense homogeneous chromatin condensation undergoing phagocytosis by an adjacent osteoblast. Right panels: an Osx1.EfnB2<sup>−/−</sup> apoptotic osteocyte (AOy) recently embedded in osteoid. LC, bone lining cell. Images are representative of 3 mice/group. B) Quantification of percentage of TUNEL<sup>+</sup> osteoblasts on trabecular surfaces of femoral secondary spongiosa in 12-wk-old male Osx1.EfnB2<sup>−/−</sup> mice (EfnB2<sup>−/−</sup>) compared to Cre<sup>−</sup> littermate controls (EfnB2<sup>+/+</sup>), n = 9–10 mice/group. *P < 0.05 vs. control. C) mRNA levels of EfnB2, Runx2, Alpl, and Bglap in FACS-purified ZsGreen.Lenti-Cre<sup>−</sup>EfnB2<sup>−/−</sup> (Δ/Δ) osteoblasts compared to ZsGreen.Lenti-Cre<sup>−</sup>C57BL/6 osteoblasts (w/w). No EfnB2 deletion was observed in ZsGreen-cells from the same culture. *P < 0.05, **P < 0.01 vs. w/w ZsGreen<sup>+</sup>. D) Representative FACS plots of annexin V (AnV<sup>−</sup>)/7AAD<sup>−</sup> staining (left and right panels), and fold change in percentage of cells (middle panel) that are AnV<sup>−</sup>/7AAD<sup>−</sup>, 7AAD<sup>+</sup> or AnV<sup>−</sup> w/w and Δ/Δ ZsGreen<sup>−</sup> osteoblasts both untreated (center panel, left bars) and after 1 μM staurosporine (SS) treatment (center panel, right bars). *P < 0.05, **P < 0.01 vs. untreated w/w control without SS; "<sup>−</sup>" P < 0.01 vs. SS-treated w/w cells. E) Top panel: representative FACS histograms of caspase 8<sup>+</sup> cells in w/w ZsGreen<sup>−</sup> osteoblasts (blue) and Δ/Δ ZsGreen<sup>−</sup> osteoblasts (red); TNF-α used as a positive control (green). Bottom panel: quantification of activated caspase 8<sup>+</sup> cells in w/w ZsGreen<sup>−</sup> osteoblasts, and w/w and Δ/Δ ZsGreen<sup>−</sup> osteoblasts; TNF-α used as a positive control. *P < 0.01; ***P < 0.0001 vs. untreated Lenti-Cre-matched control. Data are means ± se of 3 independent experiments.

Lum. Osteoblasts were observed undergoing apoptosis on the bone surface, and being phagocytosed by neighboring healthy osteoblasts, as previously reported (45). It is important to note that at the light microscopy level, such apoptotic cells would not be distinguishable from an active osteoblast, and would be counted with active osteoblasts by histomorphometry; this may account for the higher osteoblast number in female Osx1.EfnB2<sup>−/−</sup> mice. Gene expression data from the bones of the male Osx1.EfnB2<sup>−/−</sup> mice also provide some clues. While Runx2 expression in vivo is high, mRNA levels of the late osteoblast and osteocyte markers Bglap and Sost, both regulators of mineralization (46, 47), were significantly lower in Osx1.EfnB2<sup>−/−</sup> mice. This implies that apoptosis of ephrinB2-deficient osteoblasts may be restricted, in vivo, to those that have reached the stage at which these late osteoblast and osteocyte marker genes are expressed, with the increased number of osteoblasts observed in vivo also reflecting a compensatory mechanism. This osteoblast phenotype is consistent with our earlier observations with a systemic inhibitor of ephrinB2/EphB4. In that case decreased osteoblast activity was also observed both in vivo and in vitro, despite an increase in early osteoblast marker genes and osteoblast numbers in vivo (8). Our data here confirms that the effect relates specifically to impaired signaling in the osteoblast lineage.
The finding that ephrinB2 signaling is antiapoptotic in osteoblasts both in the basal state and in response to a nonselective apoptotic stimulus (staurosporine) suggests that ephrinB2 may protect osteoblasts from apoptosis-inducing agents such as cytokines, growth factors, toxins, or nitric oxide, a finding that may be applicable to other cell systems in which ephrinB2 signaling contributes. Indeed, an antiapoptotic role of ephrinB2 is consistent with increased TUNEL staining in breast cancer (18) and Kaposi sarcoma (48) xenograft models treated with the sEphB4 inhibitor of ephrinB2/EphB4 interactions. Increased apoptosis has also been observed in ephrinB2Δ/Δ mammary epithelial cells during lactation (49); our study shows that this antiapoptotic role of ephrinB2 reverse signaling is also at play in the osteoblast. In keeping with these observations, A375 melanoma tumor cells expressing high levels of sEphB4 (and therefore with high levels of ephrinB2 reverse signaling) demonstrated less apoptosis (50). Knockdown of ephrinB2 by siRNA in Kaposi sarcoma increased caspase 8-mediated apoptosis (51), similar to our observations in osteoblasts. However, whether stimulation of ephrinB2 reverse signaling can prevent osteoblast apoptosis in vivo is not clear; certainly this was not the case in colon cancer cells (52).

Although caspase 8 is an initiator caspase involved in the extrinsic cell death pathway, it can also be modified by intrinsic apoptosis regulators that include Bcl2, BclXL, Mcl1, A1 (antiapoptotic) and Bak, Bax, Bid, and Bim (proapoptotic) (53). Mice with global Bcl2 deletion demonstrate a skeletal phenotype with some similarities to our observations in osteoblasts. However, whether stimulation of ephrinB2 reverse signaling can prevent osteoblast apoptosis is not clear; certainly this was not the case in colon cancer cells (52).

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creased and support of osteoclast formation in coculture systems was impaired in Osx1.EfnB2Δ/Δ osteoblasts, regardless of the source of osteoclast progenitors. Receptor blockade in osteoblasts increased RANKL production in cocultures, increased osteoclast formation in response to stimuli (8), and enhanced osteoclast formation in PTH-treated mice sufficiently to increase resorption and abrogate the anabolic effect of PTH. Those findings are consistent with the proposal of Zhao et al. (5) that osteoblast-derived EphB4 might inhibit osteoclast formation, although we suggest that rather than occurring via direct interaction with osteoclast-expressed ephrinB2, this results from impaired osteoblastic RANKL expression. The physiological importance of this pathway remains uncertain; Zhao et al. (5) reported no osteoclast phenotype in mice with myeloid-specific knockout of ephrinB2, nor did we find any difference in numbers of osteoclasts generated from Csk.EfnB2Δ/Δ precursors. The new data presented here suggest that that inhibition of RANKL production by EphB4 forward signaling may remain intact in Osx1.EfnB2Δ/Δ osteoblasts, since EphB4 signaling in response to ephrinB2 from other cellular sources in the bone microenvironment is retained.

Other members of the Eph/ephrin family are also involved in the process of bone formation, but it appears that their roles are distinct. EphrinB1 deletion in osteoblasts also leads to impaired osteoblast differentiation, including a significant reduction in trabecular and calvarial bone mass (55). The latter observation suggests that ephrinB1 may stimulate those early stages of osteoblast differentiation involved in matrix production, while ephrinB2 stimulates the progression of osteoblasts through to the later stages of differentiation that regulate the process of mineralization.

In summary, our results support a novel physiological role of osteoblastic ephrinB2 signaling in bone. We suggest that ephrinB2 within the osteoblast lineage promotes the late stages of osteoblast differentiation and their ability to appropriately mineralize bone by limiting their apoptosis (Fig. 7); this role is required for normal bone strength, and for the full anabolic action of PTH. Notably, the availability of ephrinB2 for these purposes is determined by its paracrine regulation, a feature not yet shown in other tissues that are sites of ephrin action. Given that there is much current interest in the development of drugs influencing the ephrin/Eph pathway, specific inhibition of EphB4, rather than ephrinB2, in antiangiogenic, neuroregenerative, and anticancer therapies could have fewer detrimental side effects on the skeleton than dual-direction antagonists (18). The ability to specifically stimulate ephrinB2 signaling may be attractive for conditions where addi-

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**Figure 7.** Model of osteoblastic ephrinB2 reverse signaling in bone remodeling. EphrinB2 and EphB4 are expressed throughout osteoblast differentiation, including progenitor cells, preosteoblasts, and matrix-producing osteoblasts and osteocytes, and in each of these cells, their interaction generates phosphorylation (P) and subsequent signaling of both ephrinB2 (reverse), and EphB4 (forward). EphrinB2 signals in the osteoblast lineage (yellow arrows) are critical for a number of key actions: they maintain RANKL expression, thereby supporting the formation of bone resorbing osteoclasts via its interaction with RANK in osteoclast progenitors (action 1); they support continued differentiation of the osteoblast lineage (action 2) by inhibiting osteoclast apoptosis (action 3), and consequently maintaining the expression of osteoblast and osteocyte proteins (action 4) that promote mineralization (green lines) of the newly formed osteoid matrix (blue).
tional bone formation is needed, as in osteoporosis, osteogenesis imperfecta, and fracture healing.

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