School of Biomedical Sciences

Analysis of Candidate Genes within the 3p14-p22 Region of the Human Genome for Association with Bone Mineral Density Phenotypes

Benjamin H Mullin

This thesis is presented for the Degree of
Doctor of Philosophy
of
Curtin University of Technology

February 2011

To the best of my knowledge and belief this thesis contains no material previously published by any other person except where due acknowledgment has been made. This thesis contains no material which has been accepted for the award of any other degree or diploma in any university.

Preface

The experimental work contained within this thesis was performed in the Department of Endocrinology & Diabetes at Sir Charles Gairdner Hospital under the supervision of Doctor Cyril Mamotte, Associate Professor Scott Wilson, and Professor Richard Prince. All experimental work in this thesis was performed by myself unless otherwise stated.

Benjamin H. Mullin, B.Sc.

Publications arising from this thesis

Mullin, B. H., Prince, R. L., Dick, I. M., Hart, D. J., Spector, T. D., Dudbridge, F. & Wilson, S. G. 2008. Identification of a role for the *ARHGEF3* gene in postmenopausal osteoporosis. *American Journal of Human Genetics,* 82**,** 1262-9.

Mullin, B. H., Prince, R. L., Mamotte, C., Spector, T. D., Hart, D. J., Dudbridge, F. & Wilson, S. G. 2009. Further genetic evidence suggesting a role for the RhoGTPase-RhoGEF pathway in osteoporosis. *Bone*, 45**,** 387-91.

Research grants received during completion of this thesis

Wilson, S. G., Prince, R. L., Mamotte C., Mullin B. H. 2008. Influence of the *ARHGEF3* gene on bone phenotypes. *Arthritis Australia Project Grant* ($14,500).

Presentations

Mullin, B. H., Prince, R. L., Dick, I. M., Hart, D. J., Spector, T. D., Dudbridge, F., Mamotte, C. & Wilson, S. G. Polymorphisms within *ARHGEF3* are associated with bone mineral density in women.

- *Australian and New Zealand Bone and Mineral Society Annual Scientific Meeting. Rydges Lakeland Resort, Queenstown, NZ. 9th – 12th September 2007 (Poster Presentation).*
- *Mark Liveris Health Sciences Research Student Seminar. Curtin University of Technology, Western Australia. 2nd November 2007.*

Mullin, B. H., Prince, R. L., Mamotte, C., Dick, I. M., Hart, D. J., Spector, T. D., Dudbridge, F. & Wilson, S. G. Identification of a role for the *ARHGEF3* gene in postmenopausal osteoporosis.

- *Western Australian Bone and Cartilage Program Journal Club. Dept. of Endocrinology & Diabetes, Sir Charles Gairdner Hospital, Western Australia. 23rd May 2008.*
- *Australian Society for Medical Research - WA Scientific Symposium. Curtin University of Technology, Western Australia. 4th June 2008.*
- *Endocrinology Forum. Dept. of Endocrinology & Diabetes, Sir Charles Gairdner Hospital, Western Australia. 19th June 2008.*
- *Sir Charles Gairdner Hospital Young Investigator Award. Western Australian Institute for Medical Research, Sir Charles Gairdner Hospital, Western Australia. 25th June 2008.*

Mullin, B. H., Prince, R. L., Mamotte, C., Dick, I. M., Hart, D. J., Spector, T. D., Dudbridge, F. & Wilson, S. G. Association between polymorphism in the *RHOA* gene and bone mineral density in Caucasian women.

- *Australian and New Zealand Bone and Mineral Society Annual Scientific Meeting. Hilton on the Park, Melbourne, Victoria. 28th – 30th August 2008 (Poster Presentation).*
- *Mark Liveris Health Sciences Research Student Seminar. Curtin University of Technology, Western Australia. 6th November 2008.*

Mullin, B. H., Mamotte, C., Prince, R. L., Dick, I. M., Hart, D. J., Spector, T. D., Dudbridge, F. & Wilson, S. G. Association between variation in two related genes, *RHOA* and *ARHGEF3,* and bone mineral density in Caucasian women.

- *Western Australia Biomedical Research Institute Postgraduate Symposium*. *Curtin University of Technology, Western Australia*. *30th September 2008*.

Mullin, B. H., Prince, R. L., Mamotte, C., Spector, T. D., Hart, D. J., Dudbridge, F. & Wilson, S. G. Further genetic evidence suggesting a role for the RhoGTPase-RhoGEF pathway in osteoporosis.

- *Australian Society for Medical Research - WA Scientific Symposium*. *Curtin University of Technology, Western Australia*. *3rd June 2009*.
- *Endocrinology Forum*. *Dept. of Endocrinology & Diabetes, Sir Charles Gairdner Hospital, Western Australia*. *2nd July 2009*.
- *Sir Charles Gairdner Hospital Young Investigator Award*. *Western Australian Institute for Medical Research, Sir Charles Gairdner Hospital, Western Australia*. *19th October 2009*.

Mullin, B. H., Prince, R. L., Mamotte, C. & Wilson, S. G. Role of the *ARHGEF3* and *RHOA* genes in postmenopausal osteoporosis.

- *Australian Society for Medical Research - WA Scientific Symposium*. *Curtin University of Technology, Western Australia*. *9th June 2010*.
- *Endocrinology Forum*. *Dept. of Endocrinology & Diabetes, Sir Charles Gairdner Hospital, Western Australia*. *15th July 2010*.

Mullin, B. H., Mamotte, C., Prince, R. L. & Wilson, S. G. Identification of a role for the *ARHGEF3* and *RHOA* genes in postmenopausal osteoporosis.

- *Mark Liveris Health Sciences Research Student Seminar*. *Curtin University of Technology, Western Australia*. *8th November 2010*.

Acknowledgements

Firstly, I would like to express my gratitude to my three supervisors, Doctor Cyril Mamotte, Associate Professor Scott Wilson, and Professor Richard Prince. Thank you for your guidance and support; without it my project would not have been possible. Your expertise has proven to be invaluable and your help in everything from planning experiments to applying for funding has been immense and will not be forgotten.

Secondly, I'd like to thank Professor Jiake Xu and Doctor Taksum Cheng from the Centre for Orthopaedic Research at UWA for performing the bone resorption assays, providing me with research materials and for instruction on how to differentiate and culture osteoclast-like cells. I must also thank Doctor Peter Dallas, Professor Rebecca Mason and Doctor Giuseppe Verdile for providing me with access to cell lines.

Many thanks to Doctor Suzanne Brown for providing me with expert advice related to statistical analyses, particularly with regards to analysing the data from my microarray work.

I would like to acknowledge the financial support provided to me by Curtin University of Technology in the form of a Curtin University Postgraduate Scholarship. I would also like to acknowledge Arthritis Australia for providing me with a Project Grant, which funded a significant amount of the laboratory consumables and reagents that were used during my project.

I would like to say a big thank you to my family, particularly my Mother who passed away during my project. You always provided me with immense support and encouragement over the years and you always believed in me. Thanks Dad for nurturing my interest in science from a young age.

Finally, I would like to say a very special thank you to my partner Shelby. You have kept me smiling when times were tough and motivated when I have been feeling flat. I am eternally grateful for your unwavering support.

Summary

Previous studies have identified the 3p14-p22 chromosomal region as a quantitative trait locus for bone mineral density (BMD). The overall aim of this thesis is to identify the gene or genes from this region that are responsible for the linkage observed. The studies described within are centred on the analysis of polymorphisms within candidate genes from 3p14-p22 for association with BMD phenotypes in Caucasian women. Some functional work has also been performed to provide information on the role of these genes in bone cells. It is hoped that by identifying the genes that regulate BMD, a better understanding of osteoporosis may be gained, more effective treatments can be developed and individuals at increased risk of developing the disease can be identified.

Osteoporosis is a systemic bone disease characterised by low bone density and micro-architectural deterioration which results in fragile bones that are at increased risk of low-trauma fracture. Peak bone mass is attained in early adult life, but declines in postmenopausal women, in particular, due to a reduction in oestrogen production with effects on bone as well as intestinal and renal calcium handling. However, in addition to the effects of oestrogen, calcium and other environmental factors on bone structure and fracture, there is a strong genetic effect on peak bone mass, bone loss and fracture rates in postmenopausal women. Twin and family studies have suggested that 50 – 90 % of the variance in peak bone mass is heritable. Multiple genetic linkage studies have identified the 3p14-p22 region of the human genome as a quantitative trait locus for BMD. Therefore, it was hypothesised that one or more genes in this genomic region are significantly associated with BMD in Caucasian women.

Based on a review of the literature, the *ARHGEF3* gene was identified as a positional candidate in this chromosomal region. Common genetic variation within this gene was analysed by genotyping tagging single nucleotide polymorphisms (SNPs) in two populations of Caucasian women using the Illumina GoldenGate assay and matrix-assisted laser desorption/ionisation time-of-flight (MALDI-ToF) mass spectrometry techniques, the latter of which was performed in-house. Multiple associations were identified between polymorphism within the *ARHGEF3* gene and BMD, with the strongest associations observed between the SNP rs7646054 and various BMD

phenotypes in both populations (*P < 0.001 – 0.038*). This SNP was also found to be significantly associated with fragility fracture rate (*P = 0.026*). The SNP rs7646054 was found to be located within the 5' untranslated region of a recently described transcript variant of *ARHGEF3* designated NM_001128616. *In silico* bioinformatics analysis suggested that polymorphism at rs7646054 affects the folding and stability of NM_001128616.

Based on the associations identified between the *ARHGEF3* gene and BMD phenotypes and a review of the literature, the *RHOA* gene was identified as a second positional candidate in the 3p14-p22 chromosomal region. Common variation within this gene was analysed by genotyping tagging SNPs in the same two populations of Caucasian women that were used in the *ARHGEF3* study using the same genotyping techniques. Multiple associations were identified between polymorphism within the *RHOA* gene and BMD, with the strongest associations observed between the SNP rs17595772 and various BMD phenotypes in both populations (*P = 0.001 – 0.036*). The SNP rs17595772 was found to tag a very large linkage disequilibrium block that spans across several other genes in the region.

Mutation within another gene from the 3p14-p22 chromosomal region, *FLNB* (see below), has been implicated in a variety of human genetic disorders characterised by skeletal malformation. Disruption of the gene in mice also results in severe skeletal abnormalities. A recent publication has identified common sequence variation within this gene as significantly associated with various BMD phenotypes and has also identified five SNPs from the 5' region of the gene as associated with *FLNB* mRNA expression. These five SNPs were genotyped in a population of Caucasian women using the TaqMan technique. Significant associations were observed between the three SNPs rs11720285, rs11130605 and rs9809315 and various BMD phenotypes (*P = 0.004 – 0.043*).

Subsequent to the aforementioned studies, functional studies were performed to examine the role of the *ARHGEF3* and *RHOA* genes in bone cells. Expression of the *ARHGEF3* and *RHOA* genes was identified in both osteoblast-like and osteoclast-like cells, however expression of the *ARHGEF3* transcript variant NM_001128616 was identified in the osteoclast-like cells only. The effect of *ARHGEF3* and *RHOA*

gene knockdown in human osteoblast-like and osteoclast-like cells was then investigated to give clues as to the role of these genes in bone cells. Knockdown of *RHOA* in the Saos-2, hFOB 1.19 and MG-63 osteoblast-like cell lines was consistently found to significantly reduce the expression of alpha 2 actin, smooth muscle (ACTA2) mRNA ($P < 0.001$), indicating a possible role for RHOA signalling in regulation of *ACTA2* gene expression. Knockdown of *ARHGEF3* in the Saos-2 osteoblast-like cell line was found to significantly reduce the expression of osteoprotegerin mRNA ($P < 0.001 – 0.02$), an effect that was replicated in the hFOB 1.19 osteoblast-like cell line ($P = 0.003$). Knockdown of *RHOA* in the Saos-2 osteoblast-like cell line was found to significantly increase the expression of parathyroid hormone 1 receptor mRNA ($P = 0.002$). The protein products of these mRNA transcripts are both involved with the mechanism by which parathyroid hormone stimulates the osteoblast to promote osteoclastogenesis, suggesting a role for the *ARHGEF3* and *RHOA* genes in this process. Knockdown of the *ARHGEF3* and *RHOA* genes in the osteoclast-like cells proved more difficult. However, there was some evidence to suggest that knockdown of *RHOA* significantly reduces the expression of Rho GDP dissociation inhibitor alpha ($P < 0.001 – 0.004$) and ACTA2 mRNA ($P < 0.001 – 0.003$). The effect of *ARHGEF3* and *RHOA* gene knockdown on the bone resorbing capabilities of osteoclast-like cells was then examined. Gene knockdown was performed on osteoclast-like cells cultured on bovine bone slices, with the mean resorption pit volume subsequently calculated for each treatment group. No significant differences were observed between either of the *ARHGEF3* or *RHOA* knockdown groups and the negative control group ($P = 0.47$ and 0.33 respectively).

These studies have identified common variation within the *ARHGEF3* and *RHOA* genes as significantly associated with BMD in Caucasian women. Variation within these genes has not been previously implicated in BMD regulation or osteoporosis. These studies have also provided supporting evidence for association between variation in the *FLNB* gene and BMD in Caucasian women. All three of these genes are involved with cytoskeletal reorganisation and actin polymerisation, mechanisms that have been shown to have a role in osteoblast differentiation and osteoclast function. This suggests a role for genetic regulation of the cell cytoskeleton as a key pathway in osteoporosis susceptibility. In addition to this, the functional studies

performed have highlighted some potential roles for the *ARHGEF3* and *RHOA* genes in bone cells and have provided some leads for future study. Knockdown of these genes in osteoclast-like cells was not found to influence their bone resorptive capabilities, however this could be due to insufficient gene knockdown.

Table of contents

	Page
Preface	**i**
Publications arising from this thesis	**i**
Research grants received during completion of this thesis	**i**
Presentations	**ii**
Acknowledgements	**iv**
Summary	**v**
Table of contents	**ix**
Nomenclature used in this thesis	**xvi**
Abbreviations	**xvii**
Chapter 1 – General introduction and literature review	**1**
1.1 General introduction	1
1.2 Bone structure	1
1.3 Bone remodelling	5
1.4 Osteoporosis and fracture risk	7
1.4.1 Osteoporosis	7
1.4.2 Osteoporotic fracture	9
1.5 The osteoblast	10
1.5.1 Background and function of the osteoblast	10
1.5.1.*i* Intramembranous ossification	11
1.5.1.*ii* Endochondral ossification	12
1.5.2 Influence of parathyroid hormone (PTH) on the osteoblast	12
1.5.3 Transcription factors and signalling molecules involved in osteoblast differentiation and function	14
1.5.3.*i* Runt-related transcription factor 2 (RUNX2)	14
1.5.3.*ii* Osterix (SP7)	18
1.5.3.*iii* Activating transcription factor 4 (ATF4)	19
1.5.3.*iv* Wingless int (Wnt) signalling	20

1.5.3.*v* Indian hedgehog homolog (IHH) 21
1.5.4 Osteoblast-produced bone gamma-carboxyglutamate (gla) protein (osteocalcin or BGLAP) 23
1.6 The osteoclast 23
1.6.1 Background and function of the osteoclast 24
1.6.2 Cytokines and signalling molecules involved in osteoclast differentiation and function 26
1.6.2.*i* Receptor activator of nuclear factor-κB ligand (RANKL) 26
1.6.2.*ii* Osteoprotegerin (OPG) 29
1.6.2.*iii* Macrophage colony stimulating factor (M-CSF or CSF1) 30
1.6.2.*iv* Tumour necrosis factor alpha (TNF) 30
1.6.2.*v* TNF receptor-associated factor 6 (TRAF6) 31
1.6.3 Role of integrins and cytoskeletal dynamics in the osteoclast 31
1.6.3.*i* Integrins 32
1.6.3.*ii* Cytoskeletal dynamics 32
1.7 The osteocyte 34
1.7.1 Background and function of the osteocyte 34
1.7.2 Proteins thought to be involved in osteocyte function 35
1.7.2.*i* Matrix metallopeptidases 14 and 2 (MMP14 and MMP2) 36
1.7.2.*ii* Dentin matrix acidic phosphoprotein 1 (DMP1) 38
1.7.2.*iii* Matrix extracellular phosphoglycoprotein (MEPE) 38
1.7.2.*iv* Klotho (KL) 39
1.7.3 Regulatory effects of the osteocyte on osteoblasts and osteoclasts 39
1.8 Major dietary and environmental influences on bone 40
1.8.1 Calcium 41
1.8.2 Vitamin D 42
1.8.3 BMI and lean body mass 43
1.8.4 Resistance exercise 44

1.8.5 Negative influences on bone 45
1.9 Heritability of BMD 46
1.10 Additional heritable factors that contribute to fracture risk 47
1.11 Efforts at identifying the genes involved in BMD regulation and fracture risk 48
1.11.1 Candidate gene studies 48
1.11.2 Genome-wide linkage (GWL) studies 51
1.11.3 Genome-wide association (GWA) studies 56
1.12 Aims and unifying hypothesis of this thesis 63
1.12.1 Aims 63
1.12.2 Unifying hypothesis of this thesis 64

Chapter 2 – Materials and methods 65
2.1 Materials 65
2.1.1 Whole-genome amplification 65
2.1.2 PicoGreen DNA quantitation 65
2.1.3 SNP genotyping by single nucleotide extension using matrix-assisted laser desorption/ionisation time-of-flight (MALDI-ToF) mass spectrometry 65
2.1.4 Agarose gel electrophoresis 66
2.1.5 TaqMan SNP genotyping 66
2.1.6 Isolation of peripheral blood mononuclear cells 66
2.1.7 RNA extraction 66
2.1.8 Reverse transcription 67
2.1.9 Real-time PCR 67
2.1.10 Cell culture 67
2.1.11 Microarray 68
2.1.12 Statistical and other software packages 68
2.1.13 Other 68
2.1.14 Equipment 69
2.2 General methods 70
2.2.1 Whole-genome amplification 70
2.2.2 PicoGreen DNA quantitation 71
2.2.3 SNP genotyping by single nucleotide extension using

matrix-assisted laser desorption/ionisation time-of-flight (MALDI-ToF) mass spectrometry 73
2.2.4 Agarose gel electrophoresis 80
2.2.5 TaqMan SNP genotyping 80
2.2.6 RNA extraction 81
2.2.7 Reverse transcription 83
2.2.8 Real-time PCR 84

Chapter 3 – Analysis of variation within the *ARHGEF3* gene for association with bone mineral density phenotypes 88
3.1 Introduction 88
3.1.1 Hypothesis 91
3.2 Materials and methods 91
3.2.1 Subjects 91
3.2.1.*i* Discovery cohort 91
3.2.1.*ii* Replication cohort 92
3.2.2 Genotyping 93
3.2.2.*i* Discovery cohort 93
3.2.2.*ii* Replication cohort 93
3.2.3 SNP selection 94
3.2.4 Statistical analysis 94
3.3 Results 95
3.3.1 Discovery cohort 95
3.3.2 Replication study 101
3.4 Discussion 105

Chapter 4 – Analysis of variation within the *RHOA* gene for association with bone mineral density phenotypes 112
4.1 Introduction 112
4.1.1 Hypothesis 115
4.2 Materials and methods 115
4.2.1 Subjects 115
4.2.1.*i* Discovery cohort 115
4.2.1.*ii* Replication cohort 115

4.2.2 Genotyping 115
4.2.2.*i* Discovery cohort 115
4.2.2.*ii* Replication cohort 115
4.2.3 SNP selection 116
4.2.4 Statistical analysis 116
4.3 Results 117
4.3.1 Discovery cohort 117
4.3.2 Replication cohort 121
4.4 Discussion 121

Chapter 5 – Analysis of variation within the *FLNB* gene for association with bone mineral density phenotypes 128
5.1 Introduction 128
5.1.1 Hypothesis 132
5.2 Materials and methods 132
5.2.1 Subjects 132
5.2.2 Genotyping 132
5.2.3 SNP selection 132
5.2.4 Statistical analysis 132
5.3 Results 133
5.4 Discussion 136

Chapter 6 – *ARHGEF3* transcript profiling and effects of *ARHGEF3* and *RHOA* gene knockdown on osteoblast-like and osteoclast-like cells *in vitro* 143
6.1 Introduction 143
6.1.1 Hypotheses 146
6.2 Materials and methods 146
6.2.1 Cell culture 146
6.2.2 Isolation of peripheral blood mononuclear cells and osteoclastogenesis 147
6.2.3 siRNA knockdown 147
6.2.3.*i* Osteoblast-like cells 149
6.2.3.*ii* Peripheral blood mononuclear cells/

osteoclast-like cells 149
6.2.4 Microarray 150
6.2.5 Gene selection 150
6.2.6 Real-time PCR 151
6.2.7 Bone resorption assays 152
6.2.8 TRAP staining 153
6.2.9 Statistical analysis 153
6.2.9.*i* Microarray 153
6.2.9.*ii* Real-time PCR 158
6.2.9.*iii* Bone resorption assays 158
6.3 Results 158
6.3.1 NM_001128616 transcript profiling in bone cells 158
6.3.2 Microarray and real-time PCR in osteoblast-like cells 159
6.3.2.*i* Microarray results 159
6.3.2.*ii* Confirmation of microarray results by real-time PCR 159
6.3.3 Microarray and real-time PCR in osteoclast-like cells 165
6.3.3.*i* Microarray results 165
6.3.3.*ii* Confirmation of microarray results by real-time PCR 167
6.3.4 Bone resorption assays 172
6.4 Discussion 172

Chapter 7 – General discussion and conclusion 178
7.1 Role of the *ARHGEF3*, *RHOA* and *FLNB* genes in BMD regulation 178
7.2 Relevance of the *ARHGEF3*, *RHOA* and *FLNB* genes to the linkage observed between the 3p14-p22 chromosomal region and BMD 179
7.2.1 Other instances of multiple genes contributing to a single linkage peak 181
7.3 Expression of the *ACTA2* gene may be regulated by RHOA signalling in bone cells 182
7.4 A possible role for *ARHGEF3* and *RHOA* in PTH-induced signalling in the osteoblast with subsequent effects on osteoclastogenesis 183
7.5 Limitations of these studies 184

7.5.1 Gene knockdown levels in osteoclast-like cells 184
7.5.2 Bone resorption assays 185
7.6 Future studies 186
7.6.1 Polymorphism at rs7646054 and expression of NM_001128616 186
7.6.2 Polymorphism at rs17595772 and rs17080528 and expression of the *RHOA* gene 187
7.6.3 Investigation of how polymorphism at rs11720285, rs11130605 and rs9809315 regulates expression of the *FLNB* gene 187
7.6.4 Further investigation into the role of the *ARHGEF3* and *RHOA* genes in the osteoblast 188
7.6.5 Investigation of the effects of *ARHGEF3*, *RHOA* and *FLNB* gene knockdown on the osteoclast cytoskeleton 189
7.7 Conclusion 189
7.8 Significance of findings 190

References 192
Appendices 243

Nomenclature used in this thesis

The genetic nomenclature used in this thesis complies with the Guidelines for Human Gene Nomenclature (Wain et al., 2002). When a human gene is referred to, the symbol of the gene is presented in capitalised italicised letters. When a mouse gene is referred to, the symbol of the gene is presented in italicised letters with only the first letter of the symbol capitalised. When a human protein is referred to, the symbol of the protein is presented in capitalised non-italicised letters. When a mouse protein is referred to, the symbol of the protein is presented in non-italicised letters with only the first letter of the symbol capitalised. For example:

Human *ARHGEF3* gene, human ARHGEF3 protein.
Mouse *Arhgef3* gene, mouse Arhgef3 protein.

SNPs are identified by their reference SNP (rs) ID.
Where possible, the Mendelian Inheritance in Man (MIM) reference number for a disease is included.

Abbreviations

$1,25(OH)_2D_3$	1,25-dihydroxyvitamin D3
ACTA2	Alpha 2 actin, smooth muscle
ANCOVA	Analysis of co-variance
ANOVA	Analysis of variance
ARHGEF3	Rho guanine nucleotide exchange factor 3
ATF4	Activating transcription factor 4
BGLAP	Bone gamma-carboxyglutamate (gla) protein
BMD	Bone mineral density
BMI	Body mass index
cDNA	Complimentary deoxyribonucleic acid
CLCN7	Chloride channel 7
cM	Centimorgan
CNV	Copy-number variation
COL1A1	Collagen type I alpha 1
CSF1R	Colony stimulating factor 1 receptor
CT value	Cycle threshold value
CTSK	Cathepsin K
ddNTP	Dideoxyribonucleoside triphosphate
DIAPH1	Diaphanous homolog 1
DMP1	Dentin matrix acidic phosphoprotein 1
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleoside triphosphate
dsDNA	Double-stranded DNA
DXA	Dual energy X-ray absorptiometry
EDTA	Ethylenediaminetetra-acetic acid
ELISA	Enzyme-linked immunosorbent assay
EMSA	Electrophoretic mobility shift assay
ESR1	Oestrogen receptor 1
FLNB	Filamin B
g	Gram
GDI	GDP dissociation inhibitor
GDP	Guanosine diphosphate

GEF	Guanine nucleotide exchange factor
GPCR	G-protein coupled receptor
G-proteins	Guanine nucleotide binding proteins
GTP	Guanosine triphosphate
GWA	Genome-wide association
GWL	Genome-wide linkage
h	Hour
IHH	Indian hedgehog homolog
IL6	Interleukin 6
IL7	Interleukin 7
IU	International units
Kb	Kilobase
kDa	Kilodalton
KL	Klotho
kV	Kilovolts
L	Litre
LD	Linkage disequilibrium
LOD	Logarithm of the odds
LRP5	Low density lipoprotein receptor-related protein 5
LRP6	Low density lipoprotein receptor-related protein 6
M	Molar
MALDI-ToF	Matrix-assisted laser desorption/ionisation time-of-flight
Mb	Megabase
M-CSF	Macrophage colony stimulating factor
MEPE	Matrix extracellular phosphoglycoprotein
mg	Milligram
min	Minute
mL	Millilitre
mM	Millimolar
MMP14	Matrix metallopeptidase 14
MMP2	Matrix metallopeptidase 2
mRNA	Messenger ribonucleic acid
MSC	Mesenchymal stem cell

NCBI	National Center for Biotechnology Information
NFκB	Nuclear factor-κB
ng	Nanogram
nL	Nanolitre
nM	Nanomolar
nsec	Nanosecond
OPG	Osteoprotegerin
OSE2	Osteoblast-specific cis-acting element 2
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
PHA	Phytohaemagglutinin
PTH	Parathyroid hormone
PTH1R	Parathyroid hormone 1 receptor
QTL	Quantitative trait locus
RANK	Receptor activator of nuclear factor-κB
RANKL	Receptor activator of nuclear factor-κB ligand
RHOA	Ras homolog gene family member A
RNA	Ribonucleic acid
ROCK	Rho-associated coiled-coil containing kinase
RUNX2	Runt-related transcription factor 2
s	Second
SD	Standard deviation
shRNA	Short hairpin RNA
siRNA	Small interfering RNA
SNP	Single nucleotide polymorphism
SOST	Sclerostin
SP7	Osterix
TE buffer	Tris-EDTA
TFBS	Transcription factor binding site
TGFB1	Transforming growth factor beta 1
TNF	Tumour necrosis factor alpha
TNFRSF1A	Type 1 receptor p55
TNFRSF1B	Type 2 receptor p75

TRAF6	TNF receptor-associated factor 6
TRAP	Tartrate resistant acid phosphatase
tSNP	Tagging SNP
UTR	Untranslated region
µg	Microgram
µL	Microlitre
µM	Micromolar
V	Volts
VDR	Vitamin D receptor
WHO	World Heatlh Organisation
Wnt	Wingless int

Chapter 1 – General introduction and literature review

1.1 General introduction

Osteoporosis is a common and debilitating bone disease that is characterised by a low bone mineral density (BMD), which leads to an increased risk of fracture (Kanis et al., 1994). The disease is particularly prevalent in postmenopausal women due to a reduction in oestrogen production, with subsequent effects on bone as well as intestinal and renal calcium handling (Prince and Dick, 1997). In addition to the effects of oestrogen, calcium and other environmental factors on bone structure, there is a strong genetic effect on peak bone mass (attained in early adult life), bone loss and fracture rates (Flicker et al., 1995, Michaelsson et al., 2005). Twin and family studies suggest that 50 – 90 % of the variation in peak bone mass is heritable (Evans et al., 1988, Pocock et al., 1987, Seeman et al., 1996). The genome-wide linkage scanning approach has identified at least 11 replicated quantitative trait loci (QTL) for BMD (Ioannidis et al., 2007, Lee et al., 2006, Xiao et al., 2006), suggesting that the genetic effect for common variation in BMD is under polygenic control. The 3p14-p22 region of the human genome has been identified as a QTL for BMD in multiple studies (Lee et al., 2006, Wilson et al., 2003, Wynne et al., 2003, Xiao et al., 2006). It is hoped that by identifying the genes that have a role in BMD regulation, a better understanding of the mechanisms and biological signalling pathways underlying osteoporosis will be attained. This could lead to better treatments for the disease and the development of a panel of genetic marker tests that could identify individuals at increased risk of developing osteoporosis. Once these individuals have been identified, early intervention strategies can be applied to reduce their risk of developing the disease.

This chapter focuses on reviewing the literature relevant to bone. The basic elements of bone structure and remodelling are initially discussed before an overview of osteoporosis and a detailed description of the major cell types involved with maintaining the bone structure is given. Environmental and genetic factors thought to influence bone are then covered with particular attention given to studies aimed at identifying genes that influence bone density. Finally, the aims and unifying hypothesis of the thesis are presented.

1.2 Bone structure

The skeleton is comprised of both bone and cartilage and serves three main functions: to provide mechanical support, protect the internal organs and to act as a storage vessel in mineral homeostasis (particularly relevant for calcium and phosphate) (Ng et al., 1997). Bone is made up of two main constituents: cells and the extracellular matrix. There are three main cell types that are involved in maintaining the bone matrix: osteoblasts, which are responsible for the production of new bone; osteoclasts, which are responsible for the resorption of bone; and osteocytes, which have a role in maintaining the bone structure in response to mechanical forces and damage (Clarke, 2008). The extracellular matrix, which is the most abundant constituent, is comprised of collagen fibres and non-collagenous proteins and is hardened by mineralisation with calcium phosphate in the form hydroxyapatite (Lian et al., 1999).

The bones of the skeleton can be divided into two main groups: long bones, such as the humerus and femur, and flat bones, such as the scapula and ilium (pelvis). Long bones can be divided into 3 main segments: the two wider sections at each end of the bone (the epiphyses), a cylindrical section in the middle (the midshaft or diaphysis) and an intermediate section between them (metaphysis) (Figure 1.1). The epiphysis and the metaphysis are separated in growing bones by a layer of cartilage known as the epiphyseal cartilage or growth plate. This layer contains proliferative cells that are responsible for the longitudinal growth of the long bones (Baron, 1999). Long bones and flat bones are primarily derived by two different processes: endochondral and intramembranous ossification, respectively (Baron, 1999). There are two main types of mature bone: cortical (or compact) bone and cancellous (or trabecular/spongy) bone. These two types of bone are made up of the same constituent cells and matrix elements but display structural and functional differences, particularly relevant with regards to porosity and micro-architecture.

Cortical bone has a dense ordered structure, is found in the shaft of long bones (Figure 1.1) and the surfaces of flat bones and accounts for approximately 85 % of the total bone in the human body (Mundy, 1999). Cortical bone is laid down concentrically around central canals (known as Haversian canals), forming structural units that are known as Haversian systems. Haversian systems contain nerves, connective tissue, blood vessels and lymphatics, with each Haversian canal

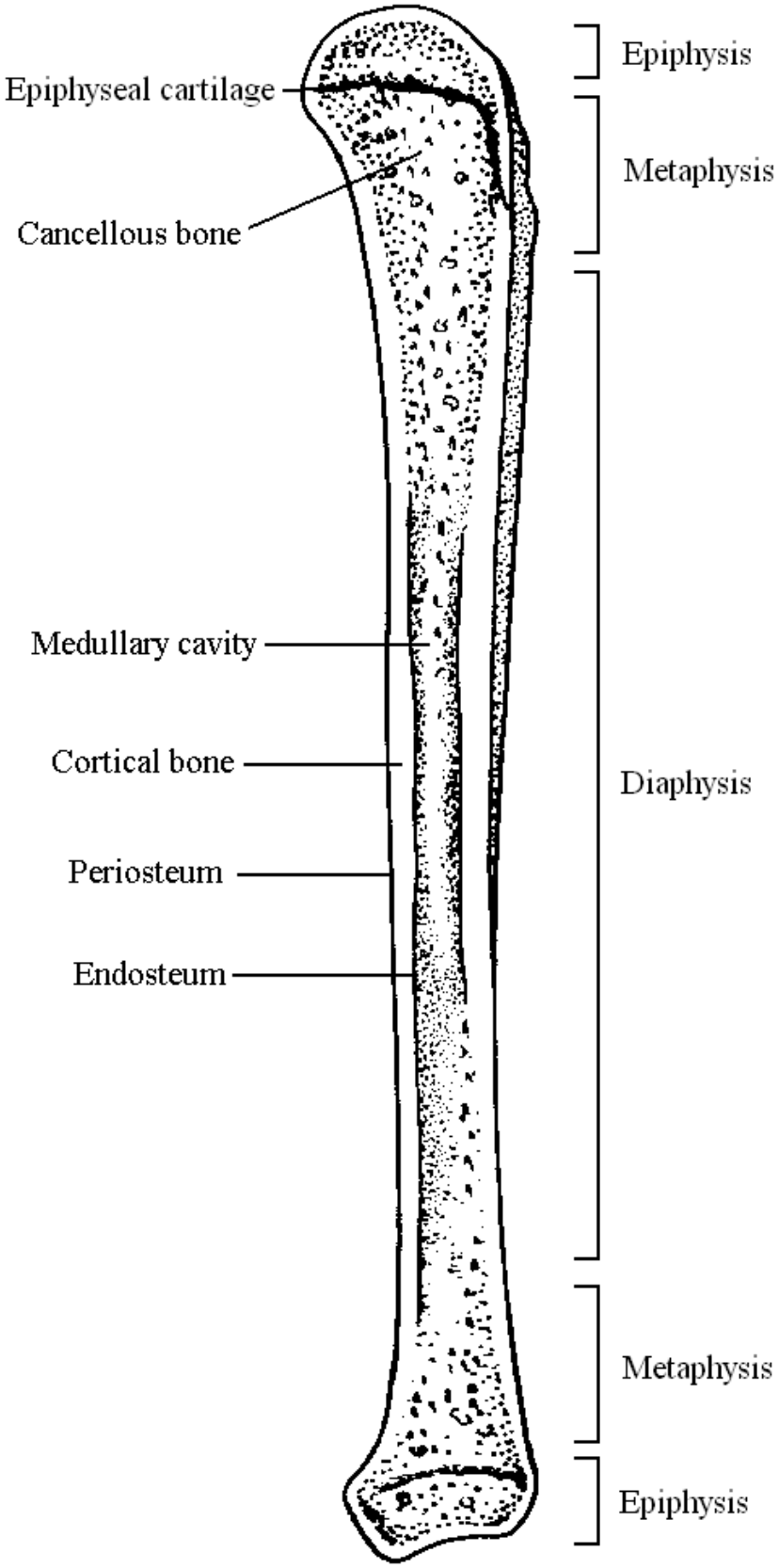

Figure 1.1. A section through a growing long bone with the location of the main anatomical features indicated. Figure adapted from Arthur's Medical Clipart (Arthur, 2008).

surrounded by a concentric layer of rings or lamellae of bone matrix. Each lamella contains tiny spaces called lacunae that contain osteocytes. 80 – 90 % of cortical bone volume is calcified (Baron, 1999); it has great tensile strength and a high resistance to bending and torsion. Cortical bone also acts as a major reservoir for various chemical elements such as calcium, phosphate and magnesium (Broadus, 1999).

Cancellous bone is less dense than cortical bone, is lighter, more elastic and has an irregular structure. It is less common than cortical bone, making up approximately 15 % of the total bone in the human body (Mundy, 1999). Cancellous bone is typically found proximal to joints, in the expanded ends of long bones (Figure 1.1) and in the interior of flat bones. The structure of cancellous bone is comprised of interconnecting beams, rods and bars called trabeculae, which act as a supporting structure for the bone, with intervening marrow. The trabeculae are aligned along lines of stress, which adds considerably to the strength of the bone by providing additional mechanical support (Mosekilde et al., 2000). 15 – 25 % of cancellous bone volume is calcified and the bone is very resistant to compressive stress (Baron, 1999). Cancellous bone is highly vascular, usually contains red bone marrow and is the site of haematopoiesis (Arai et al., 2005).

Each bone generally has an outer layer of cortical bone overlying cancellous bone and the medullary cavity. Cortical bone has an outer membrane called the periosteum (Figure 1.1) which is divided into two layers: an outer fibrous layer and an inner layer which has osteogenic, or bone forming, potential. New bone is formed within this inner layer allowing the bone to enlarge, part of a process known as periosteal apposition (Rauch, 2005). An additional layer present on the inner surface of the cortex of cortical bone is called the endosteum (Figure 1.1).

In bone tissue, a network of collagen fibres is present in the extracellular matrix and is an important element of the bone structure, with the pattern of these fibres having an influence on the mechanical properties of the bone. At the microscopic level, two types of bone can be identified: lamellar bone and woven bone. Lamellar bone has a highly organised structure and contains collagen fibres that are arranged in a parallel fashion into concentric sheets, whereas woven bone contains collagen fibres that are

arranged randomly (Baron, 1999). Lamellar bone has a lower proportion of osteocytes than woven bone and has greater mechanical strength. Woven bone is formed quickly and is primarily found in the growing skeleton, at the site of healing injuries and in various pathological conditions. At the site of a fracture, woven bone is formed initially and is slowly replaced by lamellar bone in a process known as "bony substitution".

1.3 Bone remodelling

Bone is a dynamic organ that undergoes significant remodelling and is constantly being broken down and reformed through the coordinated actions of osteoblasts, osteoclasts and osteocytes. This process is tightly regulated and is essential for repairing damage to the bone tissue as well as for maintaining bone strength and controlling plasma calcium homeostasis (Ng et al., 1997). Bone remodelling activity is regulated by a variety of systemic hormones, local hormones/cytokines and mechanical stimuli. The remodelling activity varies in different types of bone, with cancellous bone having a higher turnover rate than cortical bone (Hadjidakis and Androulakis, 2006). Approximately 20 % of the cancellous bone surface is undergoing remodelling at any one time (Hill, 1998). Bone remodelling takes place in geographically and chronologically separated regions throughout the skeleton, suggesting that activation of the sequence of cellular events that leads to the process is primarily controlled by local mechanisms in the bone microenvironment (Mundy, 1999).

The bone remodelling cycle consists of three consecutive stages: the resorptive phase, when bone is actively resorbed by osteoclasts; the reversal phase, when macrophage-like cells and subsequently osteoblasts replace the osteoclasts within the resorption lacuna; and the formative phase, when the osteoblasts produce new bone (see Figure 1.2 for full cycle) (Hadjidakis and Androulakis, 2006). The remodelling cycle starts with the retraction of the cells lining the endosteal surface, leading to exposure of the endosteal membrane which is subsequently digested by matrix metalloproteinases and collagenases (Meikle et al., 1992). Osteoclast precursors (from the myeloid lineage) are then activated, which fuse to become large multinucleated osteoclasts that resorb the exposed mineralised bone creating a resorption pit. Once the resorption pit has reached its maximum depth, the

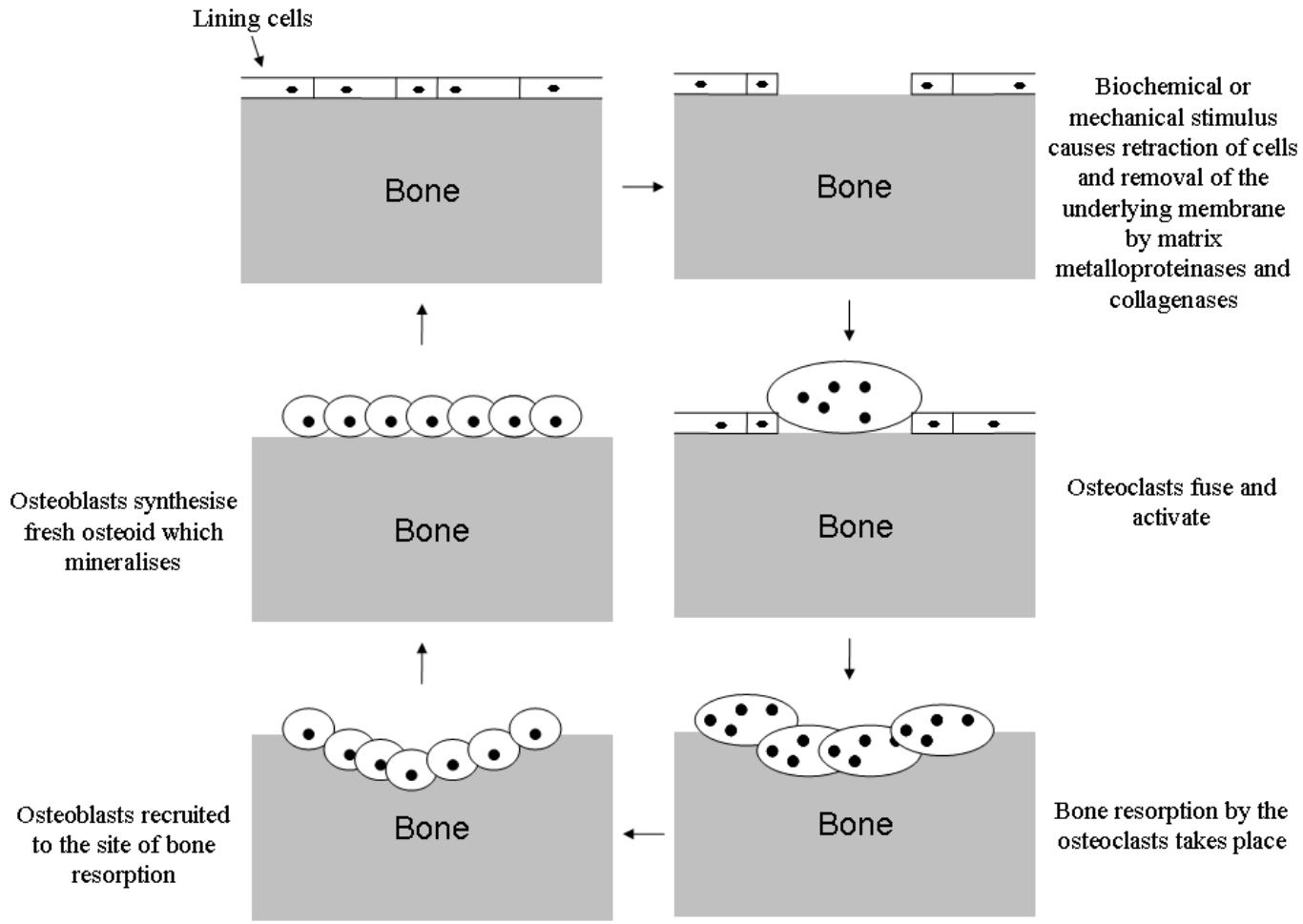

Figure 1.2. The bone remodelling cycle.

osteoclasts are replaced by macrophage-like cells that prepare the bone surface for the arrival of osteoblasts and stimulate osteoblast differentiation and migration (Hadjidakis and Androulakis, 2006). Osteoblasts are then recruited to the bottom of the resorption pit and lay down fresh osteoid which is subsequently mineralised. This is continued until the resorption pit is completely filled. The complete remodelling cycle typically takes from 3 to 6 months (Baron, 1999). Defects in the turnover, structure and density of bone can lead to diseases such as osteoporosis, characterised by reduced bone density with an increased risk of fracture (Sipos et al., 2009), or osteopetrosis, characterised by increased bone density which often results in scoliosis and brittle bones (Stark and Savarirayan, 2009).

1.4 Osteoporosis and fracture risk

1.4.1 Osteoporosis

Osteoporosis is a systemic bone disease characterised by low bone mass and disturbed micro-architecture of bone tissue (Figure 1.3), resulting in increased fragility with a corresponding increase in fracture risk (Kanis et al., 1994). There are four clinical forms of osteoporosis: postmenopausal, idiopathic, steroid-induced and senile (Wingate, 1984). The studies contained within this thesis are primarily concerned with postmenopausal osteoporosis, hence this form will be discussed here. Peak bone mass is usually attained during the third decade of life, after which the bone mass of an individual steadily declines with advancing age (Eastell, 1999). This decline is particularly pronounced in postmenopausal women due to a reduction in oestrogen production with subsequent effects on bone (Prince and Dick, 1997). The World Health Organisation (WHO) has proposed that women with bone density values greater than 2.5 standard deviations (SD) below the young adult mean value are considered osteoporotic (Kanis et al., 1994). According to this definition, based on the use of x-ray densitometry as a non-invasive measurement of bone mass and size, as many as 30 % of postmenopausal women are afflicted with osteoporosis (Melton, 1995). Since postmenopausal osteoporosis is a disease that predominantly affects individuals beyond the reproductive years, it has not been strongly selected against through the process of natural selection.

Bone loss in postmenopausal women occurs primarily because of an increase in the rate of bone remodelling and an imbalance between the activity of osteoblasts and

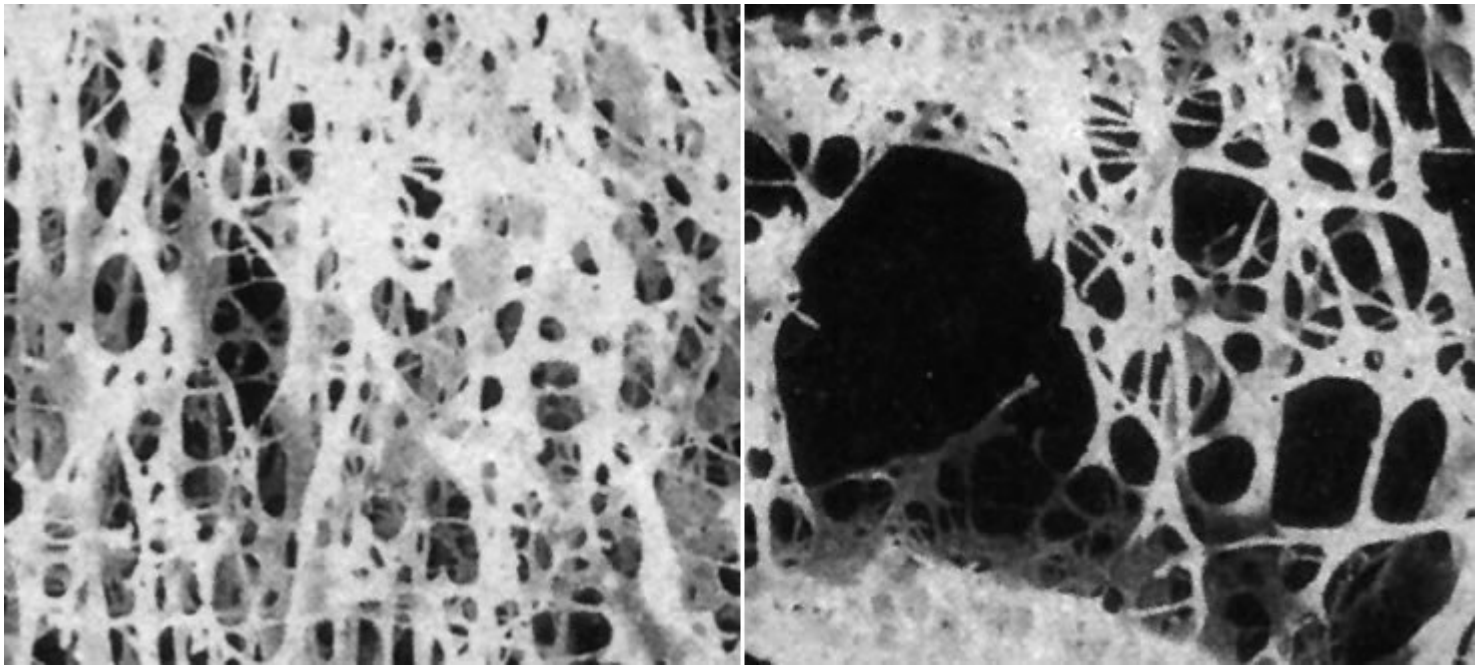

Figure 1.3. An image comparing the micro-architectural differences between normal bone (left) and osteoporotic bone (right). Note the thinning and loss of trabeculae in the osteoporotic bone. Figure adapted from the International Osteoporosis Foundation (IOF, 2010).

osteoclasts (Eastell, 1999). If the process of bone remodelling were perfect, all resorbed bone would be replaced and there would be no overall loss or gain of bone mass. However, miniscule reductions in the amount of bone replaced during each bone remodelling cycle create a remodelling imbalance and accumulate to produce significant reductions in bone mass over time. This bone loss is magnified in postmenopausal women by the increased rate of bone remodelling (Eastell, 1999).

Bone loss in postmenopausal women occurs in two phases: the first is a period of rapid bone loss that lasts for approximately 5 years after menopause and the second is a period of slower bone loss that also affects men (Riggs et al., 1998). The main reason for the first rapid phase of bone loss is oestrogen deficiency, since the circulating quantity of oestradiol decreases by 90 % after menopause (Eastell, 1999). Oestrogen is an inhibitor of bone remodelling (Kanis, 1996), with studies in ovariectomised primates revealing an increase in metabolic activity on bone surfaces (Thompson et al., 1992) and increased circulating levels of bone turnover markers (Hotchkiss et al., 2001). Oestrogen has been shown to directly inhibit osteoclastic bone resorption by inducing osteoclast apoptosis (Kameda et al., 1997). There is also evidence to suggest that oestrogen deficiency stimulates the production of interleukin 6 (IL6) (Manolagas and Jilka, 1992) and interleukin 7 (IL7) (Sato et al., 2007) in bone, both of which have been shown to promote osteoclastogenesis (Lowik et al., 1989, Weitzmann et al., 2000). Oestrogen supplementation in postmenopausal women via hormone therapy has been shown to reduce the loss of bone mass as well as the incidence of vertebral and hip fractures (Cauley et al., 2003, Genant et al., 1997, Lindsay et al., 1976).

1.4.2 Osteoporotic fracture

Osteoporotic fractures predominantly occur at the hip, wrist, forearm and in the vertebral column. It has been estimated that 9 million fractures occurred worldwide in the year 2000, of which 1.6 million were at the hip, 1.7 million at the forearm and 1.4 million were clinical vertebral fractures (Johnell and Kanis, 2006). The prevalence of vertebral fracture is thought to be largely underestimated since the majority of these fractures are clinically silent (Cummings and Melton, 2002). It has been estimated that irrespective of the site of bone density measurement, for every decrease in bone density of one SD, the risk of new vertebral fracture increases by a

factor of 2.0 – 2.4 (Wasnich, 1993). As the population ages and the proportion of people aged 80 years and over increases, the incidence of osteoporotic fracture is also expected to increase, particularly in the developed world (Johnell and Kanis, 2006, Reginster and Burlet, 2006, Riggs and Melton, 1995).

Hip fractures are regarded as being the most severe of the osteoporotic fractures (Johnell and Kanis, 2005), with almost all hip fracture patients requiring hospitalisation (Pasco et al., 2005). Sernbo and Johnell found that only 50 % of hip fracture patients managed to regain their pre-fracture status regarding ability to walk and the need for aids at home to increase mobility (Sernbo and Johnell, 1993). Pasco et al. published similar findings, observing that almost 50 % of women who had suffered a hip fracture had not regained their pre-fracture mobility after 1 year (Pasco et al., 2005). Hip fracture patients also have a high mortality rate, with the age-adjusted relative risk of dying following a hip fracture estimated at 6.7 (Cauley et al., 2000). Vertebral fractures are slightly less common, with estimates suggesting that 1 % of women at the age of 65 years and 2.9 % between the ages of 75 and 79 suffer from a new vertebral fracture each year (2002). However, the reduction in quality of life is almost as severe as for hip fractures (Johnell and Kanis, 2005), with suggestions that approximately 34 % of women who have suffered a vertebral fracture have impaired mobility 1 year later (Pasco et al., 2005). Mortality is also high in patients who have suffered a vertebral fracture, with the age-adjusted relative risk of dying following a clinical vertebral fracture estimated at 8.6 (Cauley et al., 2000).

The economic burden associated with osteoporotic fracture is substantial, with much of the burden attributed to hip fractures (Reginster and Burlet, 2006, Riggs and Melton, 1995). More than 2 million osteoporotic fractures are suspected to have occurred in the US in 2005, and the costs associated with these are predicted to be in the region of US $16.9 billion (Burge et al., 2007). Of this, hip fractures were found to account for 14 % of all fractures and 72 % of the total cost (Burge et al., 2007).

1.5 The osteoblast

1.5.1 Background and function of the osteoblast

Osteoblasts differentiate from mesenchymal stem cells (also known as marrow stromal cells or MSCs), a multipotent stem cell that can differentiate into a variety of cell types including chondroblasts/chondrocytes (cartilage producing cells) and adipocytes (fat storage cells). Osteoblasts are capable of producing a variety of growth factors under a range of stimuli, some of which include: transforming growth factor beta (Canalis et al., 1993b), the bone morphogenic proteins (Chen et al., 2004) and the insulin-like growth factors (Canalis et al., 1993a). They are characterised by a well-developed rough endoplasmic reticulum and the presence of a large circular Golgi complex. The plasma membrane of the cell is typically rich in alkaline phosphatase, the concentration of which, in serum, is often used as a marker of osteoblastic activity or bone formation (Rodan, 1992). The osteoblast is responsible for the formation of new bone, a process that involves, initially, the secretion of osteoid (the organic portion of the bone matrix), which is primarily composed of fibres and ground substance. The primary fibre-type present in the osteoid is type 1 collagen and the ground substance is composed mostly of osteocalcin and chondroitin sulphate (Netter, 1987). The secreting side of the cell is characterised by cytoplasmic processes that extend deep into the osteoid matrix. Approximately 10 days after secretion of the osteoid (a period of time known as the osteoid maturation period), osteoblasts mineralise the matrix through the ordered deposition of hydroxyapatite onto the collagen and matrix lattice (Aubin, 1998).

The functions of osteoblasts and osteoclasts are closely linked. Osteoblasts have been shown to mediate the effects of PTH on osteoclasts (McSheehy and Chambers, 1986) and secrete factors that control osteoclastogenesis, a process that has been well established *in vivo* (Teitelbaum, 2000) and *in vitro* (Kobayashi et al., 2009). This will be discussed later.

1.5.1.i Intramembranous ossification

There are two types of processes involved in the formation of new bones during foetal development: intramembranous ossification and endochondral ossification. During intramembranous ossification, MSCs located within a vascularised region of the embryonic connective tissue differentiate into osteoblasts. These osteoblasts then synthesise woven bone, without the use of a cartilaginous template, which contains many large osteocytes that have differentiated from osteoblasts that have become

buried in the matrix (Franz-Odendaal et al., 2006). MSCs at the periphery of the woven bone continue to differentiate into osteoblasts, while blood vessels that are incorporated between the woven bone trabeculae form the hematopoietic bone marrow. The woven bone is then remodelled and gradually replaced with mature lamellar bone (Baron, 1999). Bones that are formed through the process of intramembranous ossification are known as membrane bones (Franz-Odendaal et al., 2006).

1.5.1.ii Endochondral ossification

Endochondral ossification involves the production of a cartilage template of the bone prior to synthesis of the bone matrix (Olsen, 1999). MSCs first condense at the sites of future bone formation, where they differentiate into chondroblasts and begin secreting cartilaginous matrix. Some of these cells then become embedded in the cartilage matrix that they have produced, where they continue to proliferate and become large (hypertrophic) chondrocytes (Teixeira et al., 2008). MSCs at the periphery of the newly produced cartilage continue to differentiate and proliferate resulting in appositional growth of the cartilage template. A bone collar is produced around the shaft of the developing bone through intramembranous ossification. Vascular invasion of the cartilage subsequently occurs, triggering apoptosis of the chondrocytes. Osteoblasts then migrate from the bone collar into the cartilage and produce woven bone that is subsequently remodelled and gradually replaced with lamellar bone (Baron, 1999).

1.5.2 Influence of parathyroid hormone (PTH) on the osteoblast

PTH is a systemic hormone that is secreted by the parathyroid glands in response to reduced circulating levels of ionised calcium (Ca^{+2}) and is the principal regulator of calcium homeostasis along with calcitonin and 1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$) (Locker, 1996), the biologically active form of vitamin D. In its intact form, PTH is an 84 amino acid peptide, although it also exists in circulation as smaller fragments (D'Amour, 2006). PTH has been shown to regulate blood calcium levels through effects on bone (Parfitt, 1976), the kidney (Friedman, 2000) and indirectly on the intestine through increased synthesis of vitamin D in the kidney (Brenza et al., 1998, Poole and Reeve, 2005). PTH can exert anabolic or catabolic effects on bone depending on the length of exposure, with short exposures to high

levels of the hormone stimulating anabolic responses and sustained exposure to high levels, such as resulting from prolonged hypocalcaemia, resulting in a catabolic response (Poole and Reeve, 2005). PTH binds to the parathyroid hormone receptors 1 and 2, known as PTH1R and PTH2R respectively, with PTH1R the primary receptor expressed in bone (Mannstadt et al., 1999). The effects of PTH on bone appear to be primarily mediated through the osteoblast, although osteocytes and bone lining cells may also have a role in the process (Divieti et al., 2001, Dobnig and Turner, 1995).

The anabolic effects of PTH on bone appear to be a combined result of increased osteoblast precursor proliferation, increased osteoblast differentiation and decreased osteoblast apoptosis. Jilka et al. found that administration of daily PTH injections in mice with either normal bone mass or osteopenia (reduced bone density) stimulated an increase in bone formation through prevention of osteoblast apoptosis (Jilka et al., 1999). This effect was subsequently confirmed *in vitro* using rodent and human osteoblasts (Jilka et al., 1999). A later study by Wang et al. found that continuous exposure to PTH blocked the differentiation of murine calvarial osteoblast cultures whereas transient exposure to PTH ultimately resulted in enhanced osteoblast differentiation (Wang et al., 2005b). Pettway et al. subsequently published evidence suggesting that PTH also promotes osteoblast precursor proliferation (Pettway et al., 2008). In this study, mesenchymal stem cells were implanted into immunocompromised mice before subjecting the mice to 3 weeks of PTH or vehicle treatment initiated at different time-points post-implantation (Pettway et al., 2008). An anabolic response in PTH-treated implants was observed, as assessed by histomorphometry and MicroCT analysis (Pettway et al., 2008).

In addition its anabolic effects on bone, the catabolic effects of PTH on bone are also mediated through the osteoblast and result in increased osteoclastic bone resorption through increased osteoclastogenesis (McSheehy and Chambers, 1986), leading to mobilisation of calcium from the bone (Poole and Reeve, 2005). PTH stimulates production of receptor activator of nuclear factor-κB ligand (RANKL) and inhibits the production of osteoprotegerin (OPG) by osteoblasts (Huang et al., 2004, Lee and Lorenzo, 1999). This increase in RANKL and decrease in OPG production results in

increased osteoclastogenesis. The role of these two molecules in regulating osteoclastogenesis is discussed later.

1.5.3 Transcription factors and signalling molecules involved in osteoblast differentiation and function

Transcription factors are proteins that bind to specific DNA sequences and can stimulate or inhibit the transcription or expression of certain genes, often playing a major role in cellular differentiation (Mitchell and Tjian, 1989). There are a wide variety of interacting transcription factors and related signalling molecules involved in the differentiation, bone production and osteoclast-regulatory functions of the osteoblast. Of these, the RUNX2, SP7 and ATF4 transcription factors are considered to be the major players (Marie, 2008) and are discussed here. The established (canonical) wingless int (Wnt) signalling pathway has also been shown to play an important role in osteoblast differentiation and function (Piters et al., 2008) and is discussed here along with the Indian hedgehog homolog (IHH), an integral component of the Indian hedgehog signalling pathway that has been shown to promote osteoblast differentiation (Day and Yang, 2008). Figure 1.4 presents an overview of the potential interactions and relationships between the regulatory elements and signalling pathways discussed in this section.

1.5.3.i Runt-related transcription factor 2 (RUNX2)

Osteoblastic differentiation is controlled by a variety of transcription factors that are expressed in a specific sequence (Figure 1.5) (Marie, 2008). One of these transcription factors is RUNX2, which appears to have a central role in osteoblast differentiation and function (Komori, 2008). Ducy et al. identified the murine Runx2 protein as a possible osteoblast-specific transcription factor and regulator of osteoblast differentiation (Ducy et al., 1997). They observed that its expression in primary mouse osteoblasts was down-regulated by $1,25(OH)_2D_3$ (Ducy et al., 1997), which supports clinical evidence suggesting that excess $1,25(OH)_2D_3$ may prevent osteoblast terminal differentiation and cause aplastic bone disease (Goodman et al., 1994), a disorder characterised by low bone formation (Andress et al., 1987). Expression of the murine *Runx2* gene is evident during embryonic development and precedes osteoblast differentiation; expression of the gene at this point is restricted to MSCs that progress to become either chondrocytes or osteoblasts (Ducy et al., 1997).

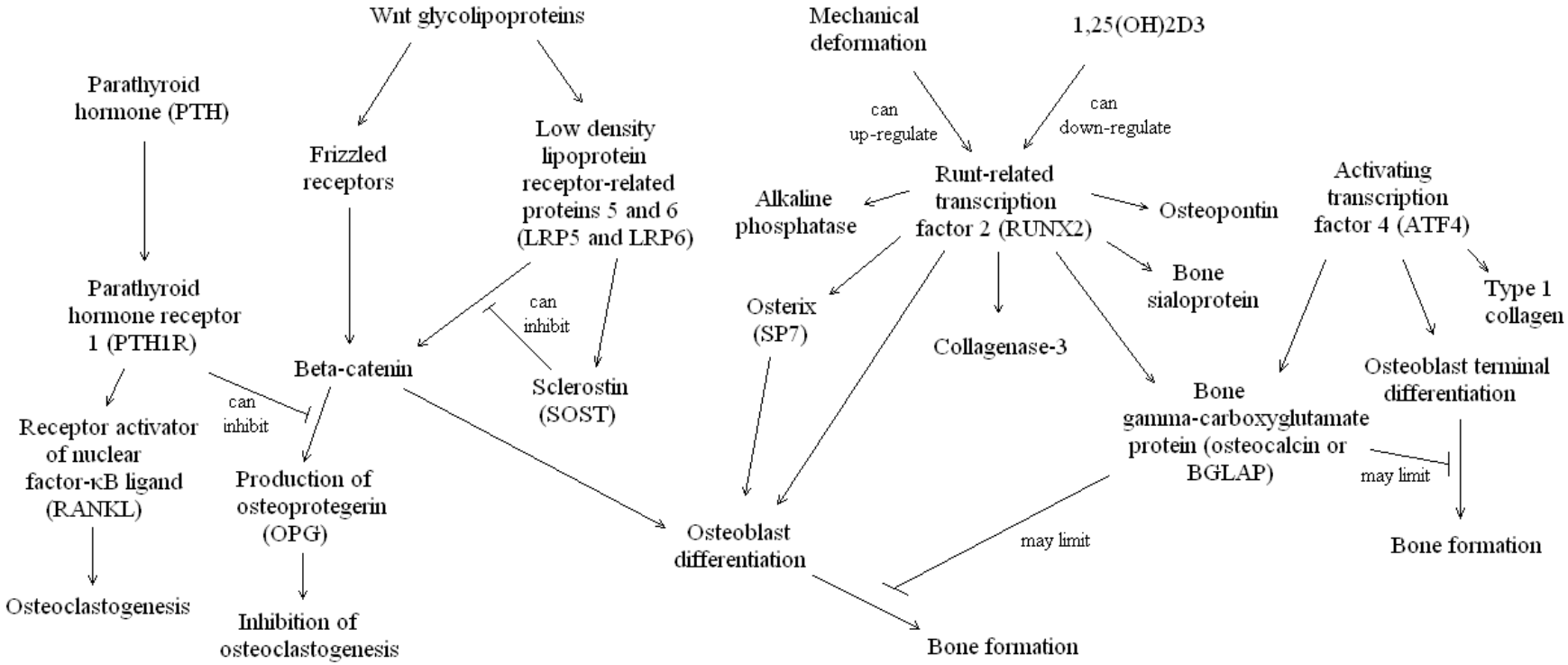

Figure 1.4. Schematic diagram presenting an overview of potential interactions between signalling pathways in the osteoblast. This diagram helps to illustrate the major role that the RUNX2 transcription factor has in osteoblasts. The influence of PTH in stimulating osteoclastogenesis is also of particular note.

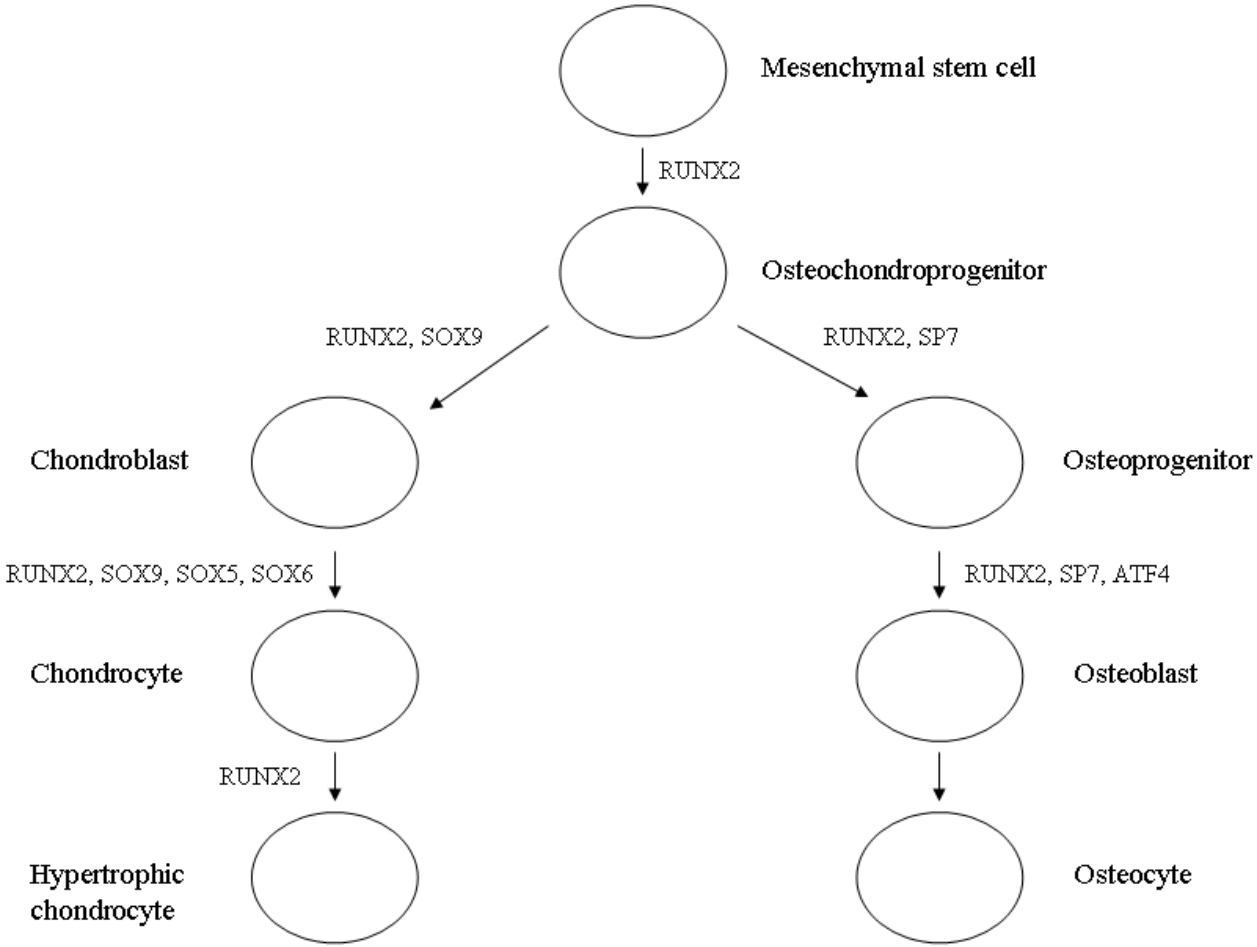

Figure 1.5. Osteoblast and chondrocyte differentiation pathways with associated transcription factors. Note that the *RUNX2* gene is expressed in both differentiation pathways, whereas expression of *SP7* and *ATF4* are more specifically related to osteoblast differentiation.

It was initially suggested that later in development expression is restricted to osteoblasts (Ducy et al., 1997), however various studies have confirmed that RUNX2 also has a role in the differentiation of chondrocytes (Enomoto et al., 2000, Komori, 2002, Kim et al., 1999) (Figure 1.5) and odontoblasts (dentin synthesising tooth homologs of osteoblasts) (D'Souza et al., 1999). *Runx2*-deficient mice are born with a deficiency of osteoblasts and osteoclasts and possess a skeleton composed completely of cartilage (Komori et al., 1997, Otto et al., 1997). Functional studies have found that the murine Runx2 protein binds to the osteoblast-specific cis-acting element 2 (OSE2) (Ducy and Karsenty, 1995). OSE2 is found in the promoter region of many genes essential to osteoblast function, including those encoding: type 1 collagen, bone gamma-carboxyglutamate (gla) protein (osteocalcin or BGLAP), osteopontin, bone sialoprotein, alkaline phosphatase, and collagenase-3, as well as the *Runx2* gene itself (Ducy et al., 1997, Harada et al., 1999, Jimenez et al., 1999).

In addition to its proposed role in osteoblast differentiation, RUNX2 also appears to have a role in bone formation by differentiated osteoblasts. Ducy et al. generated transgenic mice that over-expressed the DNA binding domain of Runx2 in differentiated osteoblasts postnatally (Ducy et al., 1999). This DNA binding domain has a higher affinity for DNA than wild-type Runx2, has no transcriptional activity and acts in a dominant-negative manner (Ducy et al., 1999). Mice over-expressing this domain were born with a normal skeleton but developed osteopenia thereafter, caused by a decrease in bone formation despite a normal osteoblast count (Ducy et al., 1999). Expression analysis revealed that many osteoblast-related transcripts, including several encoding bone extracellular matrix proteins, were virtually absent and that the osteoblasts expressing the modified Runx2 protein were less active (Ducy et al., 1999). In addition to this, Ziros et al. found that low level mechanical deformation, or stretching, of human osteoblasts directly up-regulated the expression of RUNX2 and its binding to OSE2 (Ziros et al., 2002). This suggests that mechanical stimulation may promote osteoblastogenesis and/or osteoblast function through effects on RUNX2.

Mutation in the human *RUNX2* gene has been implicated in multiple cases of Cleidocranial Dysplasia (Bergwitz et al., 2001, Lee et al., 1997, Machuca-Tzili et al., 2002, Mundlos et al., 1997, Zhou et al., 1999). This is an autosomal dominant

condition characterised by a variety of skeletal phenotypes including underdeveloped or absent collarbones, delayed closing of spaces between the bones of the skull, shortened fingers and forearms, scoliosis of the spine, dental abnormalities and reduced BMD (Chitayat et al., 1992, Dore et al., 1987, Jensen, 1990, Morava et al., 2002, Vaughan et al., 2002).

1.5.3.ii Osterix (SP7)

SP7 is another transcription factor that is thought to have a role in osteoblast differentiation. Nakashima et al. detected expression of the *Sp7* gene specifically in the developing bones of mouse embryos and subsequently generated *Sp7*-null mice (Nakashima et al., 2002). The mice died within 1 hour of birth and a complete absence of endochondral ossification in the skeletal elements was observed, that is, calcification/mineralisation of the cartilaginous skeleton, resulting in a lack of bone formation (Nakashima et al., 2002). Intramembranous ossification was also affected, with cells in the skeletal elements unable to differentiate into osteoblasts (Nakashima et al., 2002). Kaback et al. analysed the expression of Sp7 during endochondral ossification in mice, finding that it is expressed in osteochondroprogenitor cells and mature osteoblasts but not in hypertrophic chondrocytes (Figure 1.5) (Kaback et al., 2008). Over-expression of Sp7 in mouse embryonic limb derived clonal cells was found to promote osteoblast differentiation, whereas inhibition of Sp7 expression in these cells using small interfering RNA (siRNA) was found to promote chondrocyte differentiation (Kaback et al., 2008). Baek et al. subsequently examined the effect of Sp7 in the bones of adult mice by inactivation of the *Sp7* gene in osteoblasts after bone collar formation at embryonic day 14.5 (Baek et al., 2009). Newborn mice showed no significant abnormalities, however by adulthood the mice exhibited an osteopenic phenotype including delayed osteoblast maturation (Baek et al., 2009). Overall, these results point to a role for SP7 in osteoblast differentiation and bone formation in both developing and adult bone.

In terms of hierarchical relationships, there is evidence in the literature to suggest that SP7 acts downstream of RUNX2 in the pathway of osteoblast differentiation and that RUNX2 is required for expression of SP7. Nishio et al. performed an analysis of the murine *Sp7* gene promoter and identified a putative Runx2 binding site (Nishio et al., 2006). They subsequently found that over-expression of Runx2 caused up-

regulation of this region of the *Sp7* promoter 2-fold (Nishio et al., 2006). This supports previous evidence published by Nakashima et al., who found that osteoblasts obtained from *Sp7*-null mice continued to express Runx2 at levels comparable to the wild-type but that *Runx2*-null mice failed to express Sp7 (Nakashima et al., 2002).

1.5.3.iii Activating transcription factor 4 (ATF4)

Yang et al. identified the transcription factor ATF4 as a critical regulator of osteoblast terminal differentiation (Figure 1.5) and osteoblast-specific gene expression (Yang et al., 2004). They demonstrated that ATF4 regulates the synthesis of type 1 collagen and that deficiency of Atf4 in mice causes delayed bone formation during embryonic development and reduced bone mass throughout adult life (Yang et al., 2004). They also presented evidence suggesting that the protein may have a role in the pathophysiology of Coffin-Lowry syndrome (MIM 303600) (Yang et al., 2004), a rare genetic disease characterised by intellectual disability and skeletal abnormalities (Coffin et al., 1966, Lowry et al., 1971). In a subsequent study, Yang and Karsentry demonstrated that ATF4 is regulated post-translationally and has the ability to induce the expression of the osteoblast-specific gene *Bglap* (which encodes osteocalcin) in mouse non-osteoblastic cells (Yang and Karsenty, 2004). Zhang et al. found that deficiency of Atf4 in murine osteoblasts and mouse primary MSCs resulted in severe proliferative defects including delay of cell cycle progression and an increased rate of apoptosis (Zhang et al., 2008).

There is evidence in the literature to suggest that ATF4 has a role in mediating the effects of PTH on osteoblasts. Yu et al. found that treatment of murine osteoblasts and mouse primary MSCs with PTH increased *Atf4* gene expression and that Atf4 protein was required for PTH-stimulated *Bglap* gene expression in these cells, suggesting that ATF4 is a downstream target of PTH action in osteoblasts (Yu et al., 2008). A more recent study by Yu et al. produced supporting evidence for this when they found that the anabolic effects of PTH on bone are severely impaired in *Atf4*-null mice (Yu et al., 2009). They also found that PTH-induced stimulation of *Sp7* gene expression in murine osteoblasts was completely abolished by Atf4 protein deficiency (Yu et al., 2009).

1.5.3.iv Wingless int (Wnt) signalling

Many studies have indicated a role for Wnt signalling and in particular the canonical Wnt signalling pathway in the processes of bone formation, osteoblast differentiation and skeletal development. The canonical Wnt signalling pathway is activated when secreted Wnt glycolipoproteins bind to transmembrane receptors of the Frizzled family and their co-receptors, low density lipoprotein receptor-related proteins 5 and 6 (LRP5 and LRP6), ultimately resulting in regulation of the transcriptional co-activator beta-catenin (MacDonald et al., 2009). Gong et al. identified mutation within the *LRP5* gene as responsible for osteoporosis-pseudoglioma syndrome (MIM 259770) (Gong et al., 2001), a genetic disorder characterised by severe juvenile-onset osteoporosis and blindness (Gong et al., 1996). They also found that the LRP5 protein is able to mediate Wnt signalling via the canonical pathway *in vitro* and that dominant-negative mutants of LRP5 can interfere with this signalling pathway and cause a reduction in bone thickness in murine calvarial explant cultures (Gong et al., 2001). The authors also demonstrated that the *Lrp5* gene is expressed by murine osteoblasts and that expression of the gene was found to increase upon osteoblastic differentiation of a pluripotent mesenchymal cell line (Gong et al., 2001). Ellies et al. subsequently found that the product of the *sclerostin* (*SOST*) gene physically interacts with LRP5 and LRP6 to inhibit the canonical Wnt signalling pathway (Ellies et al., 2006). Mutation in the *SOST* gene has been identified as responsible for the rare bone disease sclerosteosis (MIM 269500) (Balemans et al., 2001, Brunkow et al., 2001), which is an autosomal-recessive disorder characterised by hyperactive osteoblasts and progressive bone overgrowth (Hamersma et al., 2003).

Additional evidence for a role of the canonical Wnt signalling pathway in the osteoblast was published by Day et al., who demonstrated that expression of beta-catenin promotes the differentiation of osteoblasts in preference to chondrocytes from mesenchymal progenitors (Day et al., 2005). This finding led the authors to suggest that the canonical Wnt signalling pathway may act as a molecular switch in mesenchymal cells, determining whether they differentiate towards an osteoblast or chondrocyte fate (Day et al., 2005). In addition to this, Glass et al. found that beta-catenin promotes the ability of differentiated osteoblasts to inhibit osteoclast differentiation and therefore bone resorption through production of OPG (Glass et al., 2005).

1.5.3.v Indian hedgehog homolog (IHH)

The IHH is an integral component of the Indian hedgehog signalling pathway that has been shown to control various aspects of skeletal development (Day and Yang, 2008). A study published by St-Jacques et al. examined mice deficient in *Ihh* (St-Jacques et al., 1999), a gene that had previously been implicated in chondrocyte differentiation (Vortkamp et al., 1996). The authors found that in addition to reduced chondrocyte proliferation and maturation, the *Ihh*-null mice were born with severely impaired skeletal development as a result of an absence of osteoblast development in the endochondral bones (St-Jacques et al., 1999). Long et al. produced supporting evidence for this when they found that mouse embryos with defective Ihh signalling presented with impaired osteoblast differentiation which led to formation of a defective bone collar during endochondral ossification (Long et al., 2004). To examine the role of IHH postnatally, Maeda et al. generated tamoxifen-inducible conditional *Ihh*-knockout mice (Maeda et al., 2007). They observed a continued loss of trabecular bone over time in the knockout mice with reduced Wnt signalling in the osteoblastic cells (Maeda et al., 2007). In addition to this, dwarfism was observed in the knockout mice (Figure 1.6) that appeared to be caused by premature fusion of the growth plates of various endochondral bones (Maeda et al., 2007). These results would suggest that in addition to its role in endochondral ossification, IHH also has a role in osteoblast function and maintenance of the skeleton postnatally.

Additional evidence for a role of IHH in skeletal development has come from studies implicating mutation within the human *IHH* gene in two genetic diseases with skeletal phenotypes. Gao et al. identified 3 heterozygous missense mutations within the *IHH* gene in 3 unrelated large Chinese families with a history of type A1 brachydactyly (MIM 112500) (Gao et al., 2001), a condition characterised by the shortening or absence of the middle fingers (Bell, 1951). Mutations within this gene were subsequently identified in many other instances of the disease (Kirkpatrick et al., 2003, Liu et al., 2006, McCready et al., 2002). Hellemans et al. identified 2 missense mutations within the *IHH* gene in two consanguineous families with multiple instances of the genetic disease acrocapitofemoral dysplasia (MIM 607778) (Hellemans et al., 2003). This disease is characterised by cone shaped epiphyses in the hands and hips and shortened limbs with brachydactyly (Mortier et al., 2003).

Figure 1.6. Wild-type and *Ihh*-null mice. 8-week old littermates: the mouse on the right has had induced knockout of the *Ihh* gene at postnatal day 0 while the mouse on the left is wild-type. Note the smaller size of the knockout mouse thought to be caused by premature fusion of the growth plates of various endochondral bones. Figure adapted from Maeda et al. (Maeda et al., 2007).

1.5.4 Osteoblast-produced bone gamma-carboxyglutamate (gla) protein (osteocalcin or BGLAP)

BGLAP is a small highly-conserved non-collagenous protein (Carr et al., 1981) that is exclusively produced by osteoblasts and binds strongly to hydroxyapatite (Boivin et al., 1990). It is associated with the mineralised matrix of bone, is the most abundant non-collagenous protein in the bone extracellular matrix (Boivin et al., 1990, Weinreb et al., 1990) and is secreted into the general circulation where it can be used as a clinical indicator of bone formation (Pagani et al., 2005). To investigate the role of BGLAP in bone, Ducy et al. generated *Bglap*-null mice and observed increased bone mass relative to wild type mice as a result of an increase in bone formation without impairment of bone resorption (Ducy et al., 1996). They also found that bone mineralisation was unaffected in *Bglap*-null mice, prompting the authors to suggest that BGLAP works to limit bone formation without impairing bone resorption or mineralisation (Ducy et al., 1996). Boskey et al. also generated *Bglap*-null mice and observed differences in bone mineral ratios relative to wild type mice (Boskey et al., 1998), indicating a role for BGLAP in stimulating bone mineral maturation. However, despite these early findings the exact role of BGLAP in bone remains to be determined.

Interestingly, in addition to its apparent role in bone, osteoblast-produced BGLAP appears to have a systemic role in glucose metabolism. Ducy et al. noted that mice null for *Bglap* appeared mildly hypoglycaemic and had increased visceral fat (Ducy et al., 1996). Lee et al. observed similar effects in that mice null for *Bglap* displayed decreased pancreatic β-cell proliferation, glucose intolerance and insulin resistance (Lee et al., 2007). They also demonstrated that BGLAP stimulates insulin expression in pancreatic β-cells and adiponectin in adipocytes (Lee et al., 2007). Supporting evidence for this was published by Ferron et al., who found that the Bglap protein is involved in the regulation of glucose metabolism and fat mass in mice (Ferron et al., 2008). Several recent clinical studies have reported links between BGLAP levels and plasma glucose as well as various measures of adiposity (Im et al., 2008, Kindblom et al., 2009, Pittas et al., 2009). It therefore appears that in addition its role in bone turnover, the osteoblast may have a role in energy homeostasis.

1.6 The osteoclast

1.6.1 Background and function of the osteoclast

The mature osteoclast is a large cell that can contain from four to twenty nuclei (Baron, 1999). It is responsible for the resorption of bone and is usually found in contact with a calcified bone surface or within a resorption lacuna. The cytoplasm of the osteoclast typically contains many vacuoles, Golgi complexes and transport vesicles that contain lysosomal enzymes which are essential to the bone resorptive capabilities of the cell. Walker was the first to demonstrate that the osteoclast is of hematopoietic origin (Walker, 1975a, Walker, 1975b, Walker, 1975c). Udagawa et al. published evidence to suggest that the osteoclast is derived from the monocyte/macrophage family and that the principal precursor is located in the bone marrow (Udagawa et al., 1990). Subsequent studies have determined that the osteoclast is derived from mononuclear precursors in the myeloid lineage of hematopoietic cells (Boyce and Xing, 2008).

When an osteoclast comes into contact with bone matrix, the plasma membrane forms deep foldings in the area facing the bone (Baron, 1999). A ruffled border in the centre of this is surrounded by a ring of contractile proteins that is known as the sealing zone. The sealing zone is responsible for the attachment of the cell to the bone surface (Marchisio et al., 1984) and for the creation of an isolated compartment between the cell and the bone surface. Integrin receptors bind to specific sequences within the matrix proteins which results in attachment of the cell to the matrix. The lysosomal enzymes synthesised in the osteoclast, including cathepsin K (CTSK) and tartrate resistant acid phosphatase (TRAP), are secreted through the ruffled border into the isolated extracellular compartment (Figure 1.7) (Baron, 1999). The osteoclast then acidifies this compartment by pumping protons, present at a high concentration in the cytosol as a result of the action of carbonic anhydrase, across the ruffled border membrane via an electrogenic H^+ ATPase (proton pump) (Figure 1.7) (Blair et al., 1989). Mutations in the genes encoding the protein subunits of this pump have been identified as a common cause of autosomal recessive osteopetrosis (Frattini et al., 2000). The cell avoids alkalinisation of the cytosol by exchanging bicarbonate (HCO_3^-) for chloride (Cl^-) at the basolateral membrane (Figure 1.7) (Baron, 1989). These Cl^- ions are then transported to the isolated extracellular compartment through a chloride channel known as chloride channel 7 (CLCN7) (Figure 1.7) where they are charge coupled to the H^+ ATPase, producing HCl and

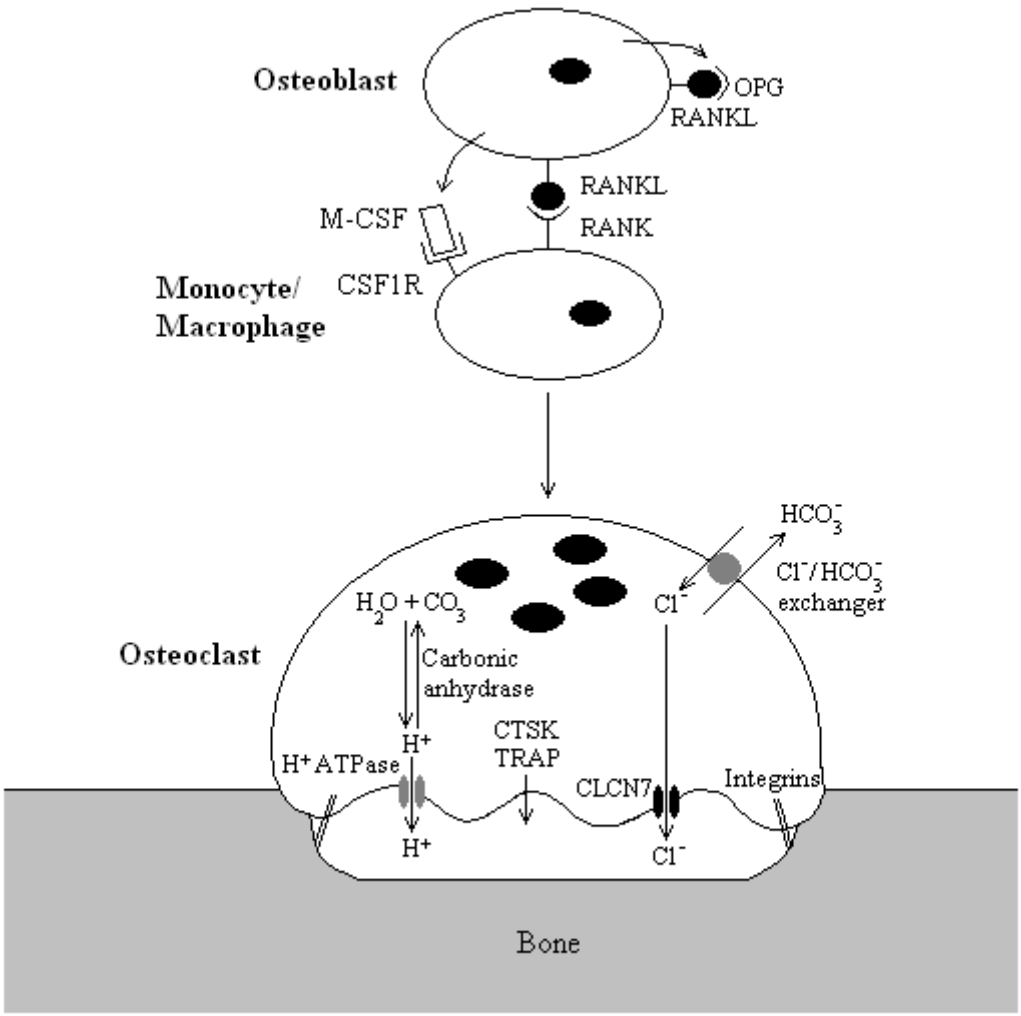

Figure 1.7. The processes of osteoclastogenesis and subsequent bone resorption induced by stimulation of monocytes/macrophages with the osteoblast-produced cytokines M-CSF and RANKL.

lowering the pH of the compartment to around 4.5 (Schlesinger et al., 1997). Mutations to the *CLCN7* gene have been known to cause decreased bone resorption leading to osteopetrosis (Blair et al., 1986). The bone matrix is exposed when the low pH in the isolated extracellular compartment first degrades the hydroxyapatite-collagen links, then subsequently dissolves the hydroxyapatite crystals. The lysosomal enzymes, which are now at optimal pH, proceed in degrading the proteinaceous bone matrix (Blair et al., 1986). The elements released during bone resorption are either internalised, transported through the osteoclast for release at the basolateral membrane, or released directly from the resorption pit during periods of relapse of the sealing zone (Baron, 1999).

1.6.2 Cytokines and signalling molecules involved in osteoclast differentiation and function

There are many different cytokines and interacting signalling molecules involved in osteoclast differentiation and function. As mentioned previously, PTH has a major role in promoting osteoclastogenesis through regulation of RANKL and OPG production by the osteoblast (Huang et al., 2004, Lee and Lorenzo, 1999). The role of these and other cytokines in regulating osteoclastogenesis will be discussed here, including the pro-osteoclastogenic cytokines macrophage colony stimulating factor (M-CSF or CSF1) (Asagiri and Takayanagi, 2007), tumour necrosis factor alpha (TNF) (Teitelbaum, 2007) and the intracellular signal transducer TNF receptor-associated factor 6 (TRAF6) (Blair et al., 2005). Figure 1.8 presents an overview of the potential interactions and relationships between the regulatory elements and signalling pathways discussed in this section.

1.6.2.i Receptor activator of nuclear factor-κB ligand (RANKL)

RANKL is encoded for by the *TNFSF11* gene (Anderson et al., 1997), is a member of the tumour necrosis factor superfamily and is considered to be the primary osteoclastogenic cytokine. It had been suggested as early as 1981 that osteoblastic cells may have a role in osteoclast recruitment and activity (Rodan and Martin, 1981). A study by Udagawa et al. found that osteoclastogenesis was dependent upon physical contact between osteoclast precursor cells and certain mesenchymal cells such as osteoblasts or their precursors (Udagawa et al., 1990). This finding led to the discovery of RANKL (Lacey et al., 1998, Yasuda et al., 1998). RANKL resides on

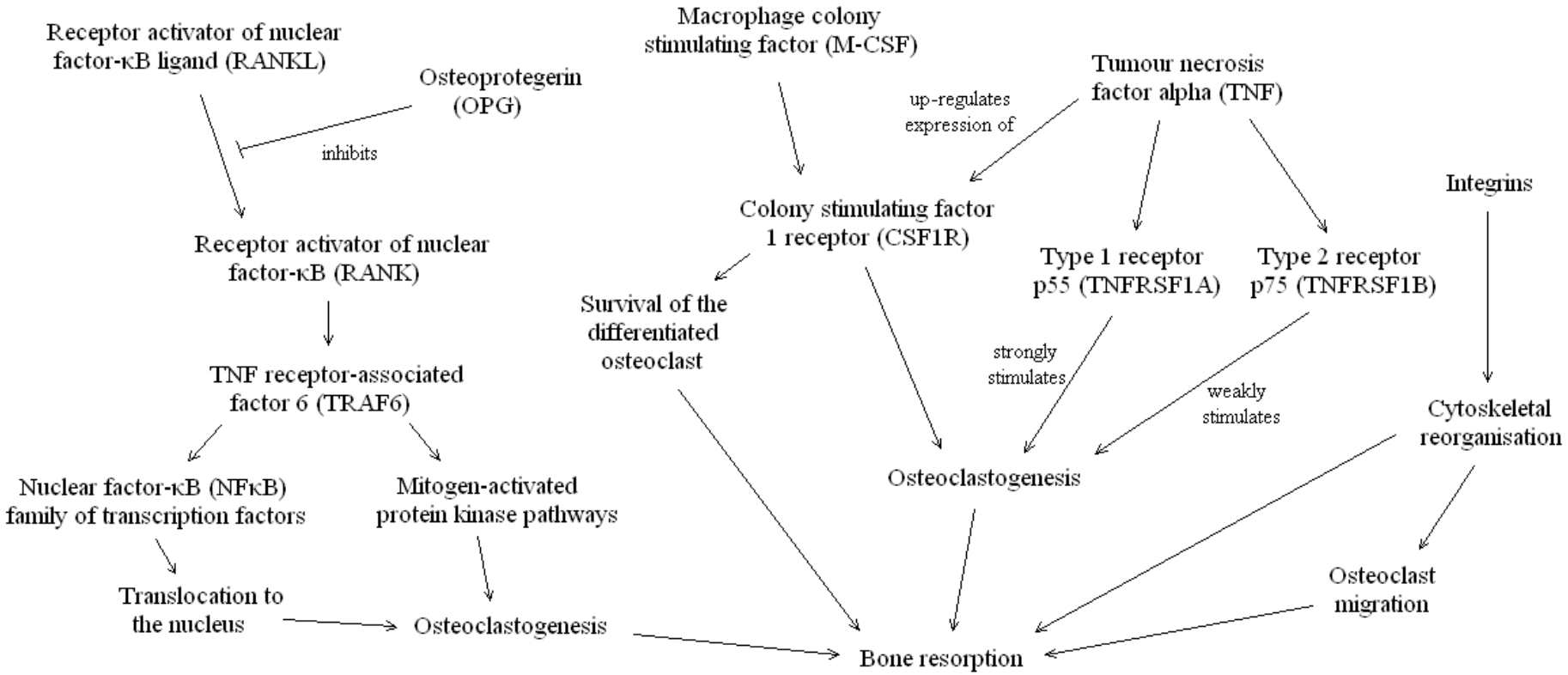

Figure 1.8. Schematic diagram presenting an overview of potential interactions between signalling pathways in the osteoclast. The cytokines RANKL and M-CSF both have prominent roles in stimulating osteoclastogenesis. Also of note are the different influences of the TNF cytokine on osteoclastogenesis depending on the receptor that has been activated.

the surface of cells of the osteoblast lineage and is also produced in both membrane-bound and soluble forms by T lymphocytes during states of skeletal inflammation such as rheumatoid arthritis or osteomyelitis (infection of the bone) (Kong et al., 1999). Kong et al. generated *Tnfsf11*-null mice and observed severe osteopetrosis with shortening of the long bones resulting from a bone-remodelling defect (Kong et al., 1999). The mice also suffered from failure of tooth eruption, a typical finding in osteopetrosis since bone resorption is required to open a pathway through the bone of the jaw through which the teeth can erupt. Hematopoietic precursor cells obtained from the spleens of the *Tnfsf11*-null mice were found to be capable of undergoing osteoclastogenesis when cultured with M-CSF and RANKL *in vitro* (Kong et al., 1999). Subsequent studies have identified soluble RANKL as capable of inducing severe skeletal catabolism in rats (Yuan et al., 2008) and loss of function mutations in the human *TNFRSF11* gene have been implicated in osteoclast-poor autosomal recessive osteopetrosis (Sobacchi et al., 2007).

RANKL stimulates osteoclastogenesis in hematopoietic cells by binding to receptor activator of nuclear factor-κB (RANK) (Nakagawa et al., 1998), which is encoded for by the *TNFRSF11A* gene (Anderson et al., 1997). Dougall et al. generated *Tnfrsf11a*-null mice and observed pronounced osteopetrosis with shortened limbs and failure of tooth eruption which appeared to result from a complete absence of osteoclasts (Dougall et al., 1999). Hematopoietic cells from the *Tnfrsf11a*-null mice cultured with M-CSF and RANKL failed to differentiate into osteoclasts (Dougall et al., 1999). Li et al. also generated *Tnfrsf11a*-null mice, again observing osteopetrosis along with a complete absence of osteoclasts (Li et al., 2000). Transfection of hematopoietic cells from these mice with a functional *Tnfrsf11a* gene was found to rescue the osteoclastogenic capabilities of the cells (Li et al., 2000).

The intracellular signalling mechanism by which RANKL stimulates osteoclastogenesis is thought to occur as follows: activation of RANK by RANKL results in the recruitment of an adaptor protein known as TRAF6 (discussed later). TRAF6 acts as a second messenger to activate various mitogen-activated protein kinase pathways and the nuclear factor-κB (NFκB) family of transcription factors (Ross, 2000). Once activated, NFκB transcription factors translocate to the nucleus where they bind to DNA target sites (Lee and Kim, 2003). Mice lacking NFκB

signalling present with osteopetrosis as a result of impaired osteoclast differentiation (Iotsova et al., 1997). See Figure 1.8 for an overview of this pathway.

1.6.2.ii Osteoprotegerin (OPG)

OPG, which is encoded for by the *TNFRSF11B* gene (Morinaga et al., 1998), is a soluble member of the TNF receptor family that acts as a decoy receptor for RANKL that competes with RANK (Hofbauer and Schoppet, 2004). Simonet et al. identified OPG as a secreted glycoprotein, over-expression of which causes osteopetrosis in mice (Simonet et al., 1997). The authors also observed that OPG inhibits osteoclastogenesis of hematopoietic cells *in vitro* (Simonet et al., 1997). Bucay et al. subsequently generated *Tnfrsf11b*-null mice and observed decreased bone density characterised by porosity of the trabecular and cortical bones with a high incidence of fracture (Bucay et al., 1998). Mizuno et al. also generated *Tnfrsf11b*-null mice, which presented with severe osteoporosis and reduced bone strength as a result of increased osteoclastogenesis (Mizuno et al., 1998). In addition to its role in inhibiting osteoclastogenesis, various studies have identified a role for OPG in suppressing osteoclast survival (Akatsu et al., 1998), activity (Hakeda et al., 1998) and attachment to bone surfaces (O'Brien et al., 2000). OPG has been shown to exist in monomeric and homodimeric forms, with evidence suggesting that the homodimeric arrangement has a greater affinity for RANKL (Schneeweis et al., 2005).

Mutation within the *TNFRSF11B* gene has been identified as responsible for juvenile Paget's disease (also known as idiopathic or hereditary hyperphosphatasia) (MIM 239000), a disorder characterised by increased bone turnover, loss of bone mineralisation, broadened bone shafts, osteopenia, increased susceptibility to fracture and increased levels of alkaline phosphatase in the blood, the latter of which possibly reflects increased osteoblast activity (Caffey, 1972, Golob et al., 1996). Whyte et al. discovered that homozygous deletion of *TNFRSF11B* was responsible for juvenile Paget's disease in two unrelated patients of Navajo Indian ancestry (Whyte et al., 2002). Cundy et al. subsequently identified a homozygous inactivating deletion within the *TNFRSF11B* gene as responsible for juvenile Paget's disease in a consanguineous family of Iraqi origin (Cundy et al., 2002). A study by Chong et al. analysed the *TNFRSF11B* gene in 8 patients suffering from juvenile Paget's disease, identifying mutations in 5 of the subjects (Chong et al., 2003).

1.6.2.iii Macrophage colony stimulating factor (M-CSF or CSF1)

M-CSF, which is encoded for by the *CSF1* gene (Morris et al., 1991) and is produced by osteoblasts and their precursors (Takahashi et al., 1991), has been shown to be essential for macrophage survival and proliferation as well as regulation of osteoclastogenesis (Yoshida et al., 1990). M-CSF exists in both membrane-bound and secreted forms (Cosman et al., 1988) and promotes the proliferation of osteoclast precursors and survival of the differentiated osteoclast through effects on the colony stimulating factor 1 receptor (also known as c-FMS or CSF1R) (Faccio et al., 2003b), expression of which has been found to be up-regulated by TNF (Yao et al., 2006).

Yoshida et al. identified a nonsense (protein-truncating) mutation within the murine *Csf1* gene as responsible for a form of recessive osteopetrosis in mice (Yoshida et al., 1990). These mice present with a restricted bone remodelling ability and are deficient in mature osteoclasts and macrophages (Marks and Lane, 1976). Administration of recombinant human M-CSF to the mice was found to correct their impaired bone resorption (Felix et al., 1990). Dobbins et al. identified a similar mutation in the *Csf1* gene as responsible for a form of osteopetrosis in rats (Dobbins et al., 2002). Subsequent studies have also implicated M-CSF in pathological bone loss during inflammatory osteolysis, a disorder characterised by excessive bone resorption by osteoclasts and can result in joint collapse, with blockade of M-CSF signalling found to arrest the condition (Kitaura et al., 2005).

1.6.2.iv Tumour necrosis factor alpha (TNF)

The inflammatory cytokine TNF, which exists in both membrane-bound and soluble forms, appears to have a synergistic relationship with RANKL and has been shown to promote osteoclastogenesis in both postmenopausal osteoporosis (Weitzmann and Pacifici, 2005) and inflammatory osteolysis (Teitelbaum, 2006). Lam et al. published evidence suggesting that TNF could not induce osteoclastogenesis of murine osteoclast precursors in the absence of RANKL, although the cytokine was found to augment RANKL-stimulated osteoclastogenesis in a dose-dependent manner *in vitro* (Lam et al., 2000). Kim et al. subsequently found that TNF can stimulate osteoclastogenesis of murine hematopoietic precursor cells of myeloid lineage in the

absence of RANKL, although this could only occur in cells treated with M-CSF and transforming growth factor beta 1 (TGFB1) (Kim et al., 2005). Other groups have presented evidence suggesting that TNF can activate fully differentiated osteoclasts in a mechanism that is independent of RANK signalling (Fuller et al., 2006, Fuller et al., 2002).

The TNF cytokine binds to two different membrane receptors, the type 1 receptor p55 (TNFRSF1A) and the type 2 receptor p75 (TNFRSF1B), with the osteoclastogenic effects of the cytokine differing depending on the receptor that has been activated. Abu-Amer et al. found that bone marrow from mice expressing only the Tnfrsf1a receptor produced substantially more osteoclasts than the wild type, whereas marrow from mice only expressing the Tnfrsf1b receptor produced substantially fewer than the wild type (Abu-Amer et al., 2000). They also found that the soluble form of the cytokine preferentially bound to the Tnfrsf1a receptor, whereas the membrane-bound form preferentially bound to Tnfrsf1b (Abu-Amer et al., 2000).

1.6.2.v TNF receptor-associated factor 6 (TRAF6)

TRAF6 is an intracellular signalling molecule that is activated upon binding of RANKL to RANK (Asagiri and Takayanagi, 2007) and appears to be involved in both osteoclastogenesis (Naito et al., 1999, Ye et al., 2002) and osteoclast function (Lomaga et al., 1999). Naito et al. generated *Traf6*-deficient mice and observed an osteopetrotic phenotype that appeared to be a result of impaired osteoclast differentiation (Naito et al., 1999). Without *Traf6*, the murine osteoclast precursor cells were unable to differentiate into osteoclasts after stimulation with RANKL (Naito et al., 1999). Ye et al. published supporting evidence for this after demonstrating that cell-permeable peptides containing the TRAF6-binding motif inhibit TRAF6 signalling and prevent osteoclastogenesis *in vitro* (Ye et al., 2002). Lomaga et al. published evidence suggesting that TRAF6 may also be involved with osteoclast function when they generated mice deficient in *Traf6* and observed an osteopetrotic phenotype with defects in bone remodelling that appeared to be due to impaired osteoclastic bone resorptive capabilities (Lomaga et al., 1999).

1.6.3 Role of integrins and cytoskeletal dynamics in the osteoclast

Communication between the integrin receptors (also known as integrins) and the cytoskeleton is an important mechanism in osteoclasts as it is essential for the successful attachment of the cell to the bone surface and subsequent resorption of the bone. Integrins located on the surface of an osteoclast can recognise specific bone matrix proteins and transmit signals to the interior of the cell that bring about cytoskeletal responses (Zou and Teitelbaum, 2010). These processes are relevant to themes that appear in subsequent chapters in this thesis, therefore they are discussed here.

1.6.3.i Integrins

Integrins, which aid in the attachment of the osteoclast to bone matrix as well as the transmission of matrix-derived signals to the interior of the cell (outside-in signalling) (Miyauchi et al., 1991), are made up of alpha and beta heterodimers. The heterodimer usually consists of a long extracellular domain and a relatively short intracellular domain. Outside-in signalling by integrins can mediate numerous intracellular events, one of the most prominent being reorganisation of the actin cytoskeleton. Various integrins have been implicated in osteoclast function. McHugh et al. demonstrated that the αvβ3 integrin complex, made up of the alpha V and beta 3 integrins, is essential for normal osteoclast function in mice (McHugh et al., 2000). They found that osteoclasts lacking in the complex were characterised by abnormal formation of the ruffled membrane and actin rings *in vivo* in addition to failure of the cells to spread *in vitro* (McHugh et al., 2000). This supported previous findings that the expression of the αvβ3 integrin replaces that of αvβ5 during osteoclastogenesis (Inoue et al., 1998) and that the integrin is essential to the resorptive process (Crippes et al., 1996, Horton et al., 1991, Ross et al., 1993). Zhao et al. published evidence suggesting that unoccupied αvβ3 integrins transmit a positive death signal resulting in osteoclast apoptosis (Zhao et al., 2005). Other integrins that have been implicated in osteoclast function include the alpha L and alpha 3 integrins, both of which have been found to be significantly up-regulated during osteoclastogenesis of peripheral blood mononuclear cells (Day et al., 2004).

1.6.3.ii Cytoskeletal dynamics

To achieve the various tasks required of an osteoclast during bone resorption, substantial and rapid cytoskeletal reorganisation is necessary. Actin is one of the

most abundant proteins found in eukaryotic cells, is the major component of the cytoskeleton and has a role in a variety of cellular events including: determination of cell shape, cell division, motility and DNA transcription (Oda and Maeda, 2010). Actin in monomeric form is known as globular or G-actin, which can be assembled into a polymeric form known as filamentous or F-actin (Pollard, 2007). When an osteoclast comes into contact with bone, it forms a large circular ring of actin which becomes the sealing zone, enabling the secure attachment of the cell to the bone surface (Luxenburg et al., 2007, Vaananen et al., 2000). This actin ring surrounds the ruffled membrane and is responsible for the isolation of the acidified extracellular compartment in which bone resorption occurs (Faccio et al., 2003a).

Cytoskeletal reorganisation in the osteoclast can be triggered through outside-in integrin signalling, occurring when the extracellular region of the integrin complex comes into contact with Arg-Gly-Asp (RGD)-containing proteins in the bone matrix such as bone sialoprotein 1 and 2, vitronectin and fibronectin (Helfrich et al., 1992, Horton et al., 1995). The intracellular regions of integrin complexes often reside within podosomes, which are located within the actin ring (Faccio et al., 2003a). Podosomes were first described by Marchisio et al. and are dynamic dot-like structures composed of an F-actin core surrounded by the integrin and a ring of scaffolding proteins which can include vinculin, paxillin and talin (Marchisio et al., 1984). Osteoclasts possess unique podosomal arrangements and can reorganise their podosomes into superstructures. Binding of the extracellular portion of the integrin to a ligand causes the intracellular portion of the integrin complex to dissociate from the podosome and move to the lamellipodia, which is a cytoskeletal actin projection located on the mobile edge of the cell and is responsible for mediating osteoclast motility (Small et al., 2002). The integrin then becomes localised in the ruffled membrane during bone resorption.

It has been suggested that Rho family GTPases, which are regulators of intracellular actin dynamics, may also mediate integrin activation. Faccio et al. published evidence suggesting that the vav 3 guanine nucleotide exchange factor (VAV3), which activates the RhoGTPases RHOA, RHOG and RAC1, is essential for stimulated osteoclast activation (Faccio et al., 2005). They found that mice deficient in *Vav3* presented with increased bone mass and were protected from bone loss

mediated by systemic bone resorption stimuli such as treatment with PTH or RANKL (Faccio et al., 2005). Osteoclasts deficient in *Vav3* demonstrated impaired actin cytoskeletal organisation, polarisation, spreading and resorptive capabilities resulting from impaired signalling downstream of the M-CSF receptor and αvβ3 integrin (Faccio et al., 2005).

1.7 The osteocyte

1.7.1 Background and function of the osteocyte

Osteocytes are terminally differentiated osteoblasts that reside within microscopic spaces in the bone matrix called lacunae. They form when an osteoblast becomes trapped in the mineralised matrix that it has produced; various estimates suggest that 10 – 20 % of osteoblasts differentiate into osteocytes (Aubin and Turksen, 1996) and that osteocytes make up over 90 % of bone cells in the adult (Knothe Tate et al., 2004). Osteocytes have much longer lifespans than active osteoblasts, are non-proliferative and have a significantly lower bone forming activity than osteoblasts (Manolagas, 2000). A large number of changes occur to the cell during the osteoblast-osteocyte differentiation process including reduction in susceptibility to apoptosis, permanent cell cycle arrest and production of dendritic processes (Noble, 2008).

Osteocytes play an important role in the determination and maintenance of bone structure (Bonewald, 2007, Heino et al., 2002, Knothe Tate et al., 2004). Despite being isolated from each other by the bone matrix, osteocytes are able to communicate with each other as well as bone-lining cells at the periosteal and endosteal surfaces of the bone through an elaborate network of linked dentritic processes that reside within canaliculi. It has been suggested that this network of processes can be reorganised (Bonewald, 2007) and has the potential to stimulate bone resorption (Tatsumi et al., 2007, Zhao et al., 2002). Altered osteocyte numbers have been noted in many disease states, with an increase in osteocyte density observed in osteoporosis (Mullender et al., 1996), osteogenesis imperfecta (Sarathchandra et al., 1996) and $1,25(OH)_2D_3$ deficiency (Malluche et al., 1980).

Osteocytes are thought to play a role in bone remodelling in response to mechanical stimuli. Evidence has been published suggesting that osteocytes respond to

mechanical stimuli by increasing production of various molecules including: nitric oxide (Pitsillides et al., 1995), prostaglandin E2 (Ajubi et al., 1996, Cherian et al., 2005), insulin-like growth factor 1 (Lean et al., 1995, Reijnders et al., 2007) and collagen type I (Sun et al., 1995). Some of these molecules have been reported to have a role in regulating osteoclastogenesis (MacIntyre et al., 1991, Mochizuki et al., 1992, Take et al., 2005). While being the predominant cell type found in bone, it has been observed that the number of empty osteocyte lacunae increases with age (Frost, 1960) and that fewer osteocyte lacunae are associated with a reduced ability to repair accumulated microdamage (Burr, 1993).

Interestingly, the availability of oxygen may have a role in osteocyte differentiation. It is likely that oxygen supply to the osteocyte *in vivo* is relatively low, particularly considering that the cells do not directly receive blood flow and instead rely on the extracellular fluid contained within the lacuna-canalicular system as a sole provider of oxygen and nutrients (Knothe Tate, 2003). It has been demonstrated that growing murine MC3T3-E1 osteoblast-like cells in hypoxic conditions (5 % O_2) stimulates osteocytogenesis (Hirao et al., 2007). This is supported by the fact that expression of the protein hypoxia up-regulated 1 (HYOU1) is higher in osteocyte-like cells than in osteoblast-like cells (Guo et al., 2005). This protein has been detected in several different cell types subjected to oxygen deprivation (Kuwabara et al., 1996) and may play a role in the cytoprotective mechanisms necessary for survival in hypoxic conditions, having been shown to suppress apoptosis (Ozawa et al., 1999).

1.7.2 Proteins thought to be involved in osteocyte function

The osteocyte produces a variety of enzymes that are essential to the role of the cell in determining and maintaining bone structure (Klein-Nulend et al., 2003). The matrix metallopeptidases 14 and 2 (MMP14 and MMP2) and the dentin matrix acidic phosphoprotein 1 (DMP1) are produced by the osteocyte and appear to have a role in modifying the extracellular matrix (Holmbeck et al., 2005, Inoue et al., 2006, Narayanan et al., 2003). These are discussed here, along with the matrix extracellular phosphoglycoprotein (MEPE) and klotho (KL), which are both thought to play an important role in the osteocyte (Igarashi et al., 2002, Suzuki et al., 2005) although their exact function in this cell type remains to be determined. Figure 1.9 presents an

overview of the potential interactions and relationships between the regulatory elements and signalling pathways discussed in this section.

1.7.2.i Matrix metallopeptidases 14 and 2 (MMP14 and MMP2)

Many genes have been suggested to have a role in osteocyte function, including several encoding matrix metallopeptidases. Holmbeck et al. published evidence suggesting that deficiency of MMP14 leads to disruption of collagen cleavage in osteocytes, resulting in a loss of formation of osteocyte processes with no effects on osteocyte number or viability (Holmbeck et al., 2005). The MMP14 protein has been shown to activate MMP2 (Sato et al., 1994) and can act as a collagenase. It appears that this collagen-cleaving activity is essential for osteocyte function and that a degree of bone matrix destruction is necessary for maintenance of the canalicular network. This theory is supported by the observation that in the absence of live osteocytes the canalicular network gradually fills with mineralised matrix, a process known as micropetrosis (Frost, 1960). In addition to this, it has been demonstrated that osteocyte viability is reduced and canaliculae number decreased in animals containing a targeted mutation which results in the production of collagenase-resistant type 1 collagen (Inoue et al., 2006, Zhao et al., 2000).

Inoue et al. produced evidence to suggest that MMP2 also plays a crucial role in the formation and maintenance of the osteocytic canalicular network, when they found that mice deficient in *Mmp2* displayed opposing bone phenotypes with reduced BMD in the long bones and increased calvarial BMD (Inoue et al., 2006). These effects appeared to be caused by disruptions to the canalicular network with associated osteocyte death (Inoue et al., 2006). The authors suggested that the opposing bone phenotypes were a result of differences in the extent of canalicular network impairment in each bone type: the long bones presenting with reduced canaliculi and the calvariae presenting with a completely disrupted canalicular network (Inoue et al., 2006). In addition to this, Martignetti et al. identified mutations within the *MMP2* gene as responsible for an autosomal recessive disorder called nodulosis, arthropathy and osteolysis (NAO) syndrome (also known as Torg-Winchester syndrome) (MIM 259600) (Martignetti et al., 2001), which is characterised by joint erosion, osteolysis, facial abnormalities and generalised osteoporosis (Al-Mayouf et al., 2000, Al-Otaibi et al., 2002).

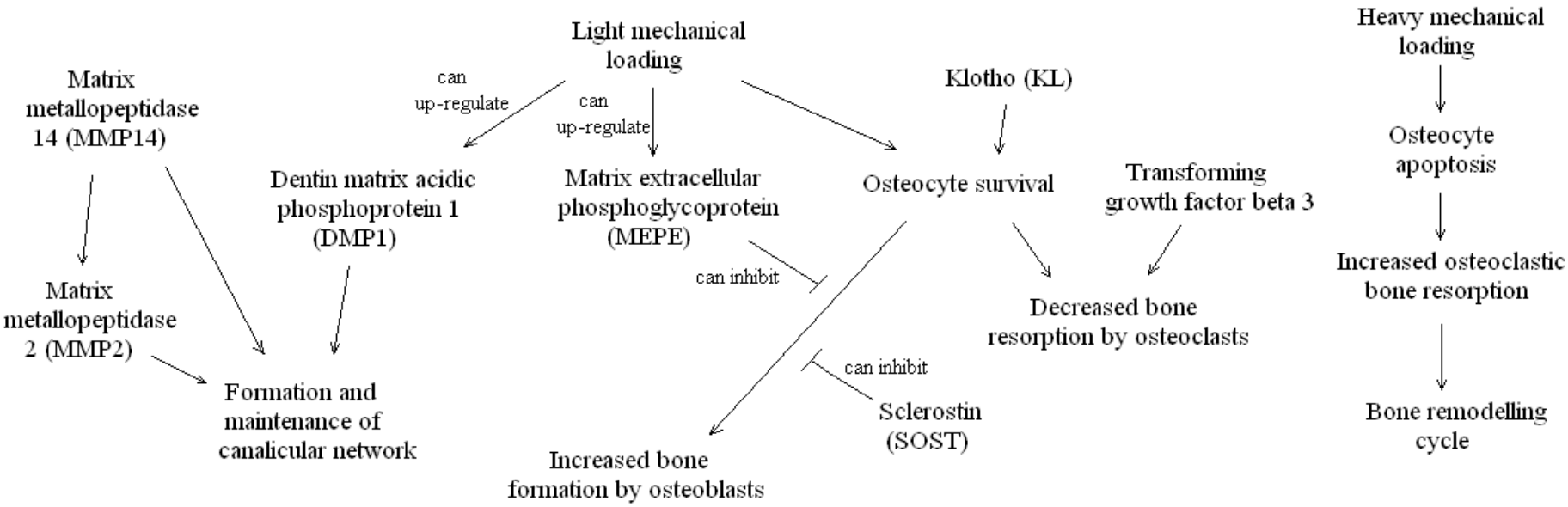

Figure 1.9. Schematic diagram presenting an overview of potential interactions between signalling pathways in the osteocyte. Light mechanical loading tends to stimulate the osteocyte into promoting bone formation by osteoblasts and inhibiting bone resorption by osteoclasts. Heavy mechanical loading can lead to osteocyte apoptosis, which stimulates osteoclastic bone resorption and thus the bone remodelling cycle.

1.7.2.ii Dentin matrix acidic phosphoprotein 1 (DMP1)

Another protein thought to have a role in osteocyte function is DMP1. Toyosawa et al. identified the DMP1 protein as highly expressed in chicken and rat osteocytes and preosteocytes (Toyosawa et al., 2001). Gluhak-Heinrich et al. subsequently found that expression of *Dmp1* is up-regulated in mouse osteocytes obtained from bone that has been subjected to mechanical loading (Gluhak-Heinrich et al., 2003), suggesting that osteocytes modify their matrix microenvironment in response to mechanical stimuli. The Dmp1 protein has also been shown to be produced in undifferentiated mouse osteoblasts, where it functions as a nuclear protein involved in the expression of osteoblastic genes (Narayanan et al., 2003). However, as the cell matures and reaches the later stages of osteoblast differentiation, the murine Dmp1 protein is phosphorylated after production and exported to the extracellular matrix where it regulates the nucleation of hydroxyapetite (Narayanan et al., 2003). Lorenz-Depiereux et al. and Feng et al. identified loss-of-function mutations within the *DMP1* gene in five families demonstrating autosomal recessive hypophosphatemic rickets (MIM 241520) (Feng et al., 2006, Lorenz-Depiereux et al., 2006). Feng et al. also found that *Dmp1*-null mice presented with defective osteocyte maturation, which resulted in pathological changes in bone mineralisation and the development of osteomalacia (softening of the bones) (Feng et al., 2006). The authors concluded that lack of functional DMP1 leads to altered skeletal mineralisation and disturbed phosphate homeostasis as a result of defective osteocyte function (Feng et al., 2006).

1.7.2.iii Matrix extracellular phosphoglycoprotein (MEPE)

There is evidence to suggest that the glycoprotein MEPE may also have a role in the osteocyte, although its exact function is yet to be determined. Igarashi et al. found that the *Mepe* gene is first expressed in mice at day 17 of embryogenesis in a small number of mature osteoblasts (Igarashi et al., 2002). Expression of the gene was subsequently seen in osteocytes during ossification of the skeleton and production of the protein seemed to correlate with increased cell differentiation to osteocytes (Igarashi et al., 2002). The authors concluded that Mepe represents an important marker of the osteocyte phenotype in mice and probably has a role in regulating osteocyte function (Igarashi et al., 2002). Gowen et al. studied mice deficient in *Mepe* and observed a phenotype characterised by increased bone mass, suggesting that the protein plays an inhibitory role in bone formation (Gowen et al., 2003).

Particularly high expression of the *MEPE* gene was subsequently found in osteocytes residing within mineralised bone (Nampei et al., 2004). Gluhak-Heinrich et al. found that the expression of *Mepe* in mice was stimulated by mechanical loading in a delayed manner (Gluhak-Heinrich et al., 2007).

1.7.2.iv Klotho (KL)

The *KL* gene encodes a type-I membrane protein related to beta-glucosidases and has been of much interest to the scientific and medical community over the last few years due to its suspected role in a variety of age-related diseases in mice (Kuro-o et al., 1997, Suga et al., 2000, Utsugi et al., 2000). Kuro-o et al. generated transgenic mice with an insertional mutation of the *Kl* gene (Kuro-o et al., 1997). Mice homozygous for this mutated gene presented with various age-related diseases such as arteriosclerosis, skin atrophy, ectopic calcification, emphysema and osteoporosis, as well as growth retardation, shortened lifespan and infertility (Kuro-o et al., 1997). Impairment of osteoblast and osteoclast differentiation resulting in low-turnover osteopenia was subsequently identified in *Kl*-deficient mice (Kawaguchi et al., 1999). Suzuki et al. conducted a study of the histological and ultrastructural features of bone matrix obtained from *Kl*-deficient mice, observing an abundance of empty osteocytic lacunae as well as evidence of increased osteoblastic and osteocytic apoptosis (Suzuki et al., 2005). This suggests a possible role for *Kl* in the osteocyte as well as raising the possibility that the osteoporosis seen in *Kl*-deficient mice is partly due to effects in osteocytes.

1.7.3 Regulatory effects of the osteocyte on osteoblasts and osteoclasts

There is evidence to suggest that the osteocyte has a role in regulating osteoblast function. Multiple studies have demonstrated that osteocytes produce sclerostin (SOST) (van Bezooijen et al., 2005, Poole et al., 2005, Winkler et al., 2003), which is a Wnt signalling pathway antagonist and negative regulator of bone formation through inhibiting the production of bone by osteoblasts (Moester et al., 2010). As mentioned previously, various mutations within the *SOST* gene have been implicated in Sclerosteosis, an autosomal-recessive bone disease that is characterised by hyperactive osteoblasts (Balemans et al., 2001, Brunkow et al., 2001). This would suggest that osteocytes are capable of negatively regulating osteoblast function through the production of SOST.

In addition to its apparent influence on osteoblast function, there is a significant amount of evidence in the literature to suggest that live osteocytes negatively regulate osteoclast function whereas osteocyte apoptosis stimulates osteoclastic bone resorption. Verborgt et al. found that osteocyte apoptosis induced by bone fatigue triggers osteoclastic bone resorption (Verborgt et al., 2000). Heino et al. subsequently demonstrated that osteocyte-like cells have an active inhibitory role on osteoclastic bone resorption (Heino et al., 2002). This effect was particularly strong after treatment of the osteocytes with oestrogen and appeared to be mediated through production of transforming growth factor beta 3 (Heino et al., 2002). Noble et al. subsequently studied the effects on osteocyte viability of short periods of mechanical loading applied to the ulnae of rats, observing a decrease in osteocyte apoptosis with light loading and an eightfold increase in osteocyte apoptosis with heavy loading (Noble et al., 2003). The authors speculated that the lighter loading stimulated adaptive bone remodelling characterised by decreased osteocyte apoptosis, decreased bone resorption and increased bone formation (Noble et al., 2003). However, the heavier loading caused micro-damage to the bone resulting in increased osteocyte apoptosis, which in turn stimulated osteoclastic bone resorption and thus the remodelling cycle in an effort to repair the damage (Noble et al., 2003). Tatsumi et al. published supporting evidence for this after observing large-scale bone resorption in a transgenic mouse model subjected to targeted osteocyte death (Tatsumi et al., 2007). Ikeda subsequently developed a transgenic mouse model in which osteocytes can be specifically ablated *in vivo* (Ikeda, 2008). This was found to trigger osteoclast invasion of cortical bone with subsequent osteoclastic bone resorption (Ikeda, 2008).

1.8 Major dietary and environmental influences on bone

Maintenance of healthy bone requires a good supply of a number of nutrients from the diet. In addition to this, a number of environmental factors have an influence on bone health. Vitamin D (which can be obtained from both the diet and exposure to sunlight) and calcium and are perhaps the most well known dietary/environmentally acquired factors involved in bone maintenance, and deficiency of either can result in detrimental effects on BMD. Other factors such as body mass index (BMI), regular resistance/weight bearing exercise and the avoidance of various negative influences

on bone such as high alcohol intake and tobacco use also play a role in the regulation of BMD.

1.8.1 Calcium

Dietary calcium intake has a major influence on bone metabolism. Calcium is a major component of bone mass and without sufficient calcium intake it is not possible to build and maintain an adequate skeleton. In addition to its structural role, bone also serves as a calcium store for the body. If the calcium requirements of the body are not fulfilled through dietary absorption, it will be resorbed from the bone through the actions of PTH (Juppner et al., 1999) to the detriment of the structure and strength of the bone (Heaney, 2002). Therefore, sufficient calcium must be obtained from the diet to maintain bone strength. However, it is but one of a number of factors which can influence BMD and studies into dietary calcium supplementation have had mixed results. A meta-analysis of 19 studies into calcium supplementation in children found that it increased total body BMD, however the effects were small and were not observed at the spine or hip sites (Winzenberg et al., 2006). Studies into the effect of calcium supplementation on fracture rates have also had mixed results. A meta-analysis published by Reid et al. found that although total fracture numbers are diminished in women supplemented with calcium, the probability of sustaining a hip fracture is in fact increased (Reid et al., 2008). The authors hypothesised that this is due to reduced periosteal expansion in the women using calcium supplementation (Reid et al., 2008).

It is plausible that the inconsistent results observed in these studies are merely due to the fact that calcium supplementation is only of benefit to individuals that have low calcium levels. Lambert et al. published the results of a study into calcium supplementation over an 18-month period in 96 adolescent girls from the UK that were judged to have low habitual calcium intake (Lambert et al., 2008). They observed a significant increase in BMD at all sites studied in girls supplemented with calcium. However, they also noted that the positive effects disappeared within two years of withdrawal of the supplement (Lambert et al., 2008). A recent study into calcium supplementation in pregnant Chinese women with habitual low calcium intake also found a dose-dependent relationship between calcium intake and BMD (Liu et al., 2010). BMD values at the whole body and spine were found to be

significantly higher in the calcium supplemented group relative to the control group, although these results should be treated with caution due to the small sample sizes used in the study (Liu et al., 2010).

1.8.2 Vitamin D

Vitamin D can be obtained from the diet through consumption of such foods as oily fish and eggs, however the main source for the body is obtained through its production in the skin stimulated by exposure to sunlight (Holick, 1999). Vitamin D_3 produced by the skin does not have significant biological activity and is first converted to 25-hydroxyvitamin D_3 in the liver. The 25-hydroxyvitamin D_3 is then transported to the kidney where it is converted to $1,25(OH)_2D_3$, the biologically active form of vitamin D. This compound has a major role in maintaining serum calcium levels by increasing the efficiency of intestinal calcium absorption (Holick, 1999). Elderly individuals tend to suffer from diminished calcium absorption from the intestine which, when coupled with decreased serum $1,25(OH)_2D_3$ resulting from an age-related reduction in the ability of the skin to produce vitamin D_3 when exposed to sunlight (Holick et al., 1989), results in high PTH levels leading to mobilisation of calcium from the bone in an effort to maintain plasma calcium levels (Duque and Troen, 2008). In addition to the effects on plasma calcium regulation, Duque et al. found that treatment with $1,25(OH)_2D_3$ decreased osteoclast number while stimulating osteoblastogenesis and osteoblast activity resulting in enhanced formation of new bone in a mouse model of senile osteoporosis (Duque et al., 2005). It has also been reported that $1,25(OH)_2D_3$ reduces bone marrow adipogenesis and stimulates osteogenic gene expression in the same mouse model (Duque et al., 2004b) as well as reducing apoptosis in human osteoblasts (Duque et al., 2004a).

A meta-analysis published by Bischoff-Ferrari et al. suggested that oral vitamin D supplementation between 700-800 IU per day reduces the risk of hip and non-vertebral fractures in ambulatory or institutionalised elderly persons (Bischoff-Ferrari et al., 2005). However, the effect was not seen with a lower dose of 400 IU per day (Bischoff-Ferrari et al., 2005). Interestingly, it has also been observed that supplementation with 700-1000 IU per day vitamin D reduces the risk of falling among elderly individuals by 19 %, although doses lower than this may not have any effect (Bischoff-Ferrari et al., 2009). This reduced risk of falling could partially

explain the reduction in fracture rate seen by Bischoff-Ferrari et al. in individuals supplemented with vitamin D (Bischoff-Ferrari et al., 2005).

Studies have suggested that supplementation with both calcium and vitamin D has a positive effect on bone health. Zhu et al. published the results of a 5-year randomised controlled trial into the effects of calcium alone and calcium with vitamin D supplementation in elderly ambulatory Australian women (Zhu et al., 2008). They found that hip BMD was preserved in both the calcium alone and calcium with vitamin D supplementation groups at year 1, however at the year 3 and year 5 time points hip BMD was preserved only in the calcium with vitamin D group (Zhu et al., 2008). A recent meta-analysis of 29 randomised trials found that supplementation with either calcium alone or calcium and vitamin D in people aged 50 years or older can reduce fracture risk by 24 % and significantly reduce the rate of bone loss at the spine and hip, as long as the compliance rate is high (Tang et al., 2007).

1.8.3 BMI and lean body mass

There is a significant amount of evidence in the literature to suggest that BMI and lean body mass are involved in the regulation of BMD as well as being major factors in fracture risk. Heavier individuals subject their weight-bearing bones to greater loads than lighter individuals, which can result in the development of greater bone density through the action of osteocytes (Lozupone et al., 1996). In addition to this, increased fat mass can result in a greater production of oestrogens since adipose tissue serves as the site of conversion of androstenedione to the oestrogenic hormone, oestrone (Schindler et al., 1972). Since adipose tissue is the major source of oestrogen production in postmenopausal women (Cleland et al., 1985), it could explain why obese women do not lose bone as quickly as non-obese women after the menopause (Dawson-Hughes et al., 1987, Ribot et al., 1987, Ribot et al., 1994).

Felson et al. published the results of a study to examine the effects of body weight and BMI on BMD in elderly males and females (Felson et al., 1993). They found that BMI accounted for a substantial proportion of the variance in BMD at all sites in women, but only at weight-bearing sites such as the femur and spine in men (Felson et al., 1993). An Australian study published in 1996 found that BMD was associated with lean mass independently of fat mass and height in 112 female twin pairs with a

mean age of 45 (Seeman et al., 1996). The authors observed a 5 – 10 % increment of BMD to be associated with a 10 – 20 % increment in lean mass (Seeman et al., 1996). Joakimsen et al. published data from a study into height, weight and BMI in relation to non-vertebral fracture risk in middle-aged Norwegians (Joakimsen et al., 1998). They observed an increased risk of low-energy fracture in persons with a lower BMI (Joakimsen et al., 1998). These findings were supported by data from De Laet et al., who conducted a meta-analysis incorporating almost 60,000 subjects examining BMI as a predictor of BMD and fracture risk (De Laet et al., 2005). They found that a low BMI is associated with a substantial increase in fracture risk, particularly with hip fracture, in both men and women that was largely dependent upon BMD (De Laet et al., 2005). However, BMI was still found to be a significant risk factor for hip fracture even after adjustment of fracture risk for BMD (De Laet et al., 2005). This could be due to the thickness of padding over the greater trochanter (Nilsson, 1970), nutritional deficiencies resulting in muscle weakness (Bischoff et al., 2003) or a greater risk of falling (Willig et al., 2003). A recent study by Morin et al. incorporating over 8,000 women aged 40 – 59 again found that low BMI predicted an increased fracture risk that was largely dependent on BMD (Morin et al., 2009).

1.8.4 Resistance exercise

Regular resistance training has been shown to increase BMD. Hind et al. published a review of clinical trials examining the effects of exercise and bone mineral accrual in children and adolescents (Hind and Burrows, 2007). They found that exercise did appear to enhance bone mineral accrual in children, particularly those in early puberty, however it was unclear as to the optimal exercise program (Hind and Burrows, 2007). Winters-Stone et al. examined the response of bone to exercise at specific skeletal sites in premenopausal women (Winters-Stone and Snow, 2006). They observed a site-specific positive response of hip and spine BMD to lower and upper body exercise respectively (Winters-Stone and Snow, 2006). Martyn-St James et al. published the results of a meta-analysis into high-intensity resistance training and postmenopausal bone loss, finding a general trend towards an increase in BMD following resistance training which reached statistical significance at the lumbar spine site (Martyn-St James and Carroll, 2006). These findings were supported by a recent study in Brazilian postmenopausal women which found that strength training

over 24 weeks preserved BMD at the lumbar spine and femoral neck sites when compared to untrained controls (Bocalini et al., 2009). Maddalozzo et al. found that resistance training was more effective than hormone replacement therapy in attenuating bone loss at the spine in early postmenopausal women (Maddalozzo et al., 2007).

1.8.5 Negative influences on bone

Several negative lifestyle influences on bone health have been identified. An Australian twin study published in 1995 found that lifetime tobacco use was independently associated with a reduced BMD at the lumbar spine, total hip and forearm sites in postmenopausal women (Flicker et al., 1995). A meta-analysis of the effects of cigarette smoking on BMD was published by Ward et al., who found that smokers had a significantly reduced BMD at all sites examined when compared to non-smokers (Ward and Klesges, 2001). These effects were particularly pronounced at the hip where smokers had on average one-third of an SD less bone mass than non-smokers (Ward and Klesges, 2001). A recent study in premenopausal Japanese women also found a negative effect of smoking on BMD at the lumbar spine (Tamaki et al., 2009). Similar results have been found in studies investigating smoking and fracture risk. A meta-analysis published by Kanis et al. looking at over 59,000 men and women found that current smokers have a significantly higher risk of fracture than non-smokers (Kanis et al., 2005b). The highest increase in fracture risk was observed at the hip and persisted even after taking BMD into account (Kanis et al., 2005b). Taes et al. found that smoking from a young age in men has a negative effect on bone geometry, density and fracture rate (Taes et al., 2009). The authors speculated that this could be caused by interactions with the action of sex steroids (Taes et al., 2009).

High alcohol intake has also been reported to have a negative effect on bone health (Kanis et al., 2005, Lalor et al., 1986, Santori et al., 2008, Saville, 1965). Kanis et al. examined BMD and fracture risk relative to alcohol intake in a cohort comprised of almost 17,000 men and women (Kanis et al., 2005). They observed significant associations between alcohol intake and the risk of osteoporotic and hip fracture, although this was not caused by effects on BMD (Kanis et al., 2005). A recent study in male alcoholics found that the skeletal damage caused by alcohol abuse seems to

occur through negative effects on bone formation (Santori et al., 2008). It has also been found that various factors thought to negatively influence bone health, such as impaired vitamin D metabolism and malnutrition, are common among alcoholics (Pitts and Van Thiel, 1986, Stransky and Rysava, 2009).

1.9 Heritability of BMD

In addition to dietary and environmental influences on bone structure and fracture, there is a strong genetic effect on peak bone mass, bone loss and fracture rates (Flicker et al., 1995, Krall and Dawson-Hughes, 1993, Michaelsson et al., 2005, Pocock et al., 1987). Studies using both monozygotic and dizygotic twin pairs have proven very useful for examining the heritability of bone mass. Smith et al. analysed bone mass and width in 71 juvenile and 80 adult twin pairs, observing greater phenotype discordance between dizygotic twins when compared to monozygotic twins which increased with age (Smith et al., 1973). Supporting evidence for this was published by Pocock et al., who also observed greater correlation between BMD in monozygotic twins than in dizygotic twins (Pocock et al., 1987). They found that the genetic effect seemed to be strongest at the spine and slightly weaker at the proximal femur and forearm (Pocock et al., 1987). Evans et al. conducted a case-control study using 24 patients that had suffered an osteoporotic fracture and 35 asymptomatic relatives (Evans et al., 1988). They concluded that bone mass is lower in healthy young and middle-aged relatives of osteoporotic patients than in individuals with no family history of osteoporosis (Evans et al., 1988).

Krall et al. analysed familial resemblance in BMD at 5 skeletal sites among 160 adult members of 40 families (Krall and Dawson-Hughes, 1993). After adjustment of the BMD data for age, height, weight and significant lifestyle or environmental factors, they concluded that 46 – 62 % of the variance in BMD is heritable (Krall and Dawson-Hughes, 1993). Flicker et al. ran a cross-sectional twin study aimed at determining the influence of lifestyle and genetic factors on BMD in elderly women (Flicker et al., 1995). In 69 female twin pairs aged 60 – 89 years examined it was found that 20 – 33 % of the variation in BMD was accounted for by age, lifestyle and body composition factors, with 75 % of the residual variation in BMD at the non-forearm sites accounted for by genetic factors (Flicker et al., 1995). Seeman et al. published the results of a study in 56 monozygotic and 56 dizygotic female twins

with a mean age of 45 and concluded that 60 – 80 % of the individual variance in femoral neck BMD can be attributed to genetic factors (Seeman et al., 1996). A later study published by Hunter et al. examined BMD and heel ultrasound variables in 360 pairs of female monozygotic twins and 885 pairs of female dizygotic twins (Hunter et al., 2001). The authors found that their heritability estimates for BMD at most body sites was lower in the postmenopausal women, although they produced evidence to suggest that the same genes are involved either side of the menopause (Hunter et al., 2001).

Michaëlsson et al. ran a large study in over 33,000 Swedish twins to examine the genetic contribution to fracture risk in the elderly (Michaelsson et al., 2005). They found that the genetic influence on fracture risk differed considerably depending on the type of fracture and age of the patient (Michaelsson et al., 2005). The proportion of age-adjusted fracture variance explained by genetic variation for the entire cohort was 0.16 for any fracture, 0.27 for osteoporotic fracture and 0.48 for hip fracture (Michaelsson et al., 2005). These figures increased slightly when looking specifically at the female subset of the cohort (Michaelsson et al., 2005). The proportion of age-adjusted hip fracture variance explained by genetic variation was 0.68 for those individuals younger than 69 years of age, 0.47 for those between 69 – 79 years of age and 0.03 for those older than 79 years of age (Michaelsson et al., 2005). The authors concluded that the genetic influence on fracture is greater in females than in males and decreases with advancing age (Michaelsson et al., 2005).

A recent study by Zhai et al. looked at the heritability of bone loss in 712 postmenopausal Caucasian women (Zhai et al., 2009). Monozygotic and dizygotic twin pairs were measured at baseline and followed-up for an average of 8 years (Zhai et al., 2009). After adjustment of the data for age at baseline and weight change at follow-up, the heritability estimates for bone loss at the femoral neck, lumbar spine and forearm were 0.47, 0.44 and 0.56 respectively (Zhai et al., 2009).

1.10 Additional heritable factors that contribute to fracture risk

It is important to keep in mind that BMD is not the only heritable phenotype that contributes to fracture risk (Albagha and Ralston, 2003). Studies have demonstrated that some of the heritable component of fracture risk is independent of BMD (Deng

et al., 2002, Kanis et al., 2007), which is supported by the identification of polymorphic variants that contribute to variation in BMD but have no impact on fracture risk (Andrew et al., 2005). Hip bone geometry phenotypes such as hip axis length, which are critical components of bone strength and have been shown to be under strong genetic control (Demissie et al., 2007, Shen et al., 2005, Xiong et al., 2006a), have been demonstrated as important risk factors for hip fracture (Brownbill and Ilich, 2003). Differences in femoral neck geometry have in fact been suggested as partially responsible for the differences in the rate of hip fracture between Caucasians and other ethnic groups (Cummings et al., 1994). Biochemical markers of bone turnover, including the bone formation marker undercarboxylated osteocalcin and the bone resorption markers urinary C-telopeptide cross-links of collagen type I and free urinary deoxypyridinoline, have also been shown to predict fracture risk independently of BMD (Garnero et al., 1996b, Szulc et al., 1993). In addition, a recent study has highlighted impaired balance as a heritable phenotype that contributes to fracture risk (Wagner et al., 2009).

1.11 Efforts at identifying the genes involved in BMD regulation and fracture risk

Identifying the specific genes that influence BMD and fracture risk has proven a daunting task. Bone mass is a polygenic trait in that it appears to be influenced by many different genes, each contributing a small amount to the heritable portion of the phenotype. To complicate things further, there is evidence to suggest that the genes that regulate BMD and influence fracture risk do so in an age-specific (Kammerer et al., 2003, Karasik et al., 2003) and gender-specific (Peacock et al., 2005, Ralston et al., 2005) manner. It has also been suggested that different genetic variants are responsible for osteoporosis susceptibility in different ethnic groups (Dvornyk et al., 2003, Lei et al., 2006). These are all factors that must be taken into consideration when examining the genetics of osteoporosis.

1.11.1 Candidate gene studies

Early efforts to identify the genes that influence bone mass centred around candidate genes, which are selected for study based solely on the function of the gene product and its role in bone. Candidate gene studies have the advantage of higher statistical power and easier sample recruitment when compared to linkage and genome-wide

association studies (Xu et al., 2010). The gene encoding the vitamin D receptor (*VDR*), which mediates the action of $1,25(OH)_2D_3$ on cells, was the first candidate gene to be studied in relation to osteoporosis (Ralston, 2003). Four early publications reported significant associations between polymorphism in the *VDR* gene and BMD (Fleet et al., 1995, Krall et al., 1995, Morrison et al., 1994, Riggs et al., 1995), although subsequent publications produced conflicting results (Alahari et al., 1997, Garnero et al., 1996a, Gross et al., 1996, Houston et al., 1996, Lim et al., 1995, Tokita et al., 1996, Tsai et al., 1996, Viitanen et al., 1996). More recent studies examining the relationship between variation in the *VDR* gene and BMD have been conducted using large cohorts. Macdonald et al. genotyped five common polymorphisms in the *VDR* gene in a population of 3,100 women from the UK but found no significant associations with bone mass, bone loss or fracture rates (Macdonald et al., 2006). Uitterlinden et al. subsequently analysed the same five *VDR* gene polymorphisms in a meta-analysis incorporating 26,242 participants from European background, of which 18,405 were women (Uitterlinden et al., 2006). No significant associations were observed between any of the five polymorphisms and BMD, although one of the polymorphisms was found to be marginally associated with risk of vertebral fracture (Uitterlinden et al., 2006).

Another early candidate gene to be studied in relation to osteoporosis was the gene encoding the oestrogen receptor 1 (*ESR1*) (Ralston and Uitterlinden, 2010). An article published in late 1995 reported significant associations between a dinucleotide repeat polymorphism lying upstream of this gene and BMD as well as various markers of bone turnover (BGLAP, urinary pyridinoline and urinary deoxypyridinoline) in postmenopausal Japanese women (Sano et al., 1995). However, an obvious limitation of this study was the small size of the cohort – only 144 women were used in the study and only 15 of these carried the allele of interest (Sano et al., 1995). A study published the following year by Kobayashi et al. produced supporting evidence for a role of the *ESR1* gene in BMD regulation in postmenopausal Japanese women (Kobayashi et al., 1996), although subsequent studies in varying ethnic groups produced mixed results (Bagger et al., 2000, Deng et al., 1998, Han et al., 1999, Salmen et al., 2000). Ioannidis et al. conducted a meta-analysis that examined the role of three common *ESR1* polymorphisms in a population of 18,917 individuals from European background, of which 14,622 were

women (Ioannidis et al., 2004). They found that none of the polymorphisms examined were significantly associated with BMD parameters, although one of the polymorphisms was significantly associated with vertebral and overall fracture risk (Ioannidis et al., 2004).

The gene encoding collagen type I alpha 1 (*COL1A1*) has been extensively studied as a candidate gene for BMD regulation. Grant et al. published data suggesting that a polymorphism located in the regulatory region of the *COL1A1* gene is associated with bone mass and vertebral fracture risk in two populations of British women with a combined total of 299 individuals (Grant et al., 1996). This polymorphism was reported to lie within a recognition site for the transcription factor Sp1, suggesting that variation at this site could affect transcription of the gene (Grant et al., 1996). A small study in Swedish postmenopausal women published in 1998 cast doubt over these findings (Liden et al., 1998) and subsequent studies again produced conflicting results (Heegaard et al., 2000, Nakajima et al., 1999, Weichetova et al., 2000). Ralston et al. conducted a meta-analysis that examined the role of the Sp1 transcription factor site polymorphism in BMD regulation in a population of 20,786 individuals from European background, of which 14,675 were women (Ralston et al., 2006). They found that the polymorphism was significantly associated with both femoral neck and lumbar spine BMD, in addition to a nominally significant association with incident vertebral fracture in females (Ralston et al., 2006).

A large number of additional genes have been reported as significantly associated with BMD in candidate gene studies. Some of the more commonly studied include those encoding: low density lipoprotein receptor-related protein 5 (*LRP5*) (Mizuguchi et al., 2004, van Meurs et al., 2008), transforming growth factor beta 1 (*TGFB1*) (Yamada et al., 1998), bone morphogenic protein 2 (*BMP2*) (Xiong et al., 2006b), sclerostin (*SOST*) (Uitterlinden et al., 2004), runt-related transcription factor 2 (*RUNX2*) (Doecke et al., 2006, Vaughan et al., 2002), RANK (*TNFSF11A*) (Koh et al., 2007), RANKL (*TNFSF11*) (Hsu et al., 2006), osteoprotegerin (*TNFRSF11B*) (Arko et al., 2002, Zhao et al., 2005b) and interleukin 6 (*IL6*) (Moffett et al., 2004, Murray et al., 1997, Tsukamoto et al., 1999).

A recent study by Richards et al. analysed more than 36,000 common single nucleotide polymorphisms (SNPs) selected from the International HapMap Project (Phase 2 dataset) (Frazer et al., 2007) from the CEU population (Utah residents with Northern and Western European ancestry) in 150 genes previously proposed as candidates for osteoporosis predisposition (Richards et al., 2009). These SNPs were analysed in relation to BMD in over 19,000 European individuals and fracture risk in almost 6,000 people from the Netherlands (Richards et al., 2009). They found that SNPs from only 9 of the 150 gene loci were significantly associated with BMD at the femoral neck and lumbar spine (Richards et al., 2009). These 9 genes were: *ESR1*, *LRP4* (encoding low density lipoprotein receptor-related protein 4), *ITGA1* (encoding alpha 1 integrin), *LRP5*, *SOST*, *SPP1* (encoding secreted phosphoprotein 1), *TNFRSF11A*, *TNFRSF11B* and *TNFSF11* (Richards et al., 2009).

1.11.2 Genome-wide linkage (GWL) studies

GWL studies use genetic markers (often microsatellites) to identify chromosomal segments that are shared between family members carrying a trait of interest (Dawn Teare and Barrett, 2005). This information can be used to identify regions of the genome potentially containing one or more genes influencing a particular trait. Candidate genes can then be selected from the region for analysis. As opposed to candidate gene studies, GWL studies are robust with regards to population admixture/stratification (Xu et al., 2010) and can be used to study both monogenic gene disorders (parametric linkage) and complex gene disorders (non-parametric linkage). Evidence for linkage of a particular trait to a region of the genome is usually expressed as a logarithm of the odds (LOD) score. Despite the recent popularity of genome-wide association (GWA) studies (discussed below), information obtained from GWL studies remains important, as GWL studies are capable of capturing information from rare polymorphisms/haplotypes and copy number variations unlike many GWA studies that only genotype common, low-effect variants (Karasik et al., 2010). A large number of disease-associated genes have been identified through information provided by GWL studies. Some of these are thought to have a role in various polygenic diseases, including: Crohn's disease (Beckly et al., 2008, Ogura et al., 2001), schizophrenia (Macintyre et al., 2010, Pimm et al., 2005), chronic obstructive pulmonary disease (Hersh et al., 2009), attention deficit hyperactivity disorder (Wigg et al., 2008) and asthma (Van Eerdewegh et al., 2002).

A number of GWL studies aimed at identifying quantitative trait loci (QTL) for BMD phenotypes have been performed. One of the first was published by Johnson et al. who identified the chromosomal locus 11q12-13 as linked to a very high spinal bone density phenotype in a single extended pedigree comprised of 22 individuals (Johnson et al., 1997). They observed a LOD score of 5.74 with a marker in 11q12-13, a genomic region that had also been linked with osteoporosis-pseudoglioma syndrome (MIM 259770) (Gong et al., 1996). Subsequent genotyping of additional markers and a systematic search for mutations that segregated with the phenotype revealed that an amino acid change within the *LRP5* gene, which is located within the 11q12-13 chromosomal region, was responsible for the high spinal bone density phenotype in this family (Little et al., 2002).

Devoto et al. published the results of a GWL study aimed at identifying regions of the genome likely to contain genes predisposing to low BMD (Devoto et al., 1998). 330 DNA markers spread across the autosomal genome were analysed in relation to spine and hip BMD in 149 members of 7 large pedigrees with recurrence of low BMD (Devoto et al., 1998). They found evidence of linkage to the following chromosomal regions: 1p36 for hip BMD (LOD score 3.51), 2p23-24 for spine BMD (LOD score 2.07) and 4qter for both hip and spine BMD (LOD scores > 2.5) (Devoto et al., 1998). Devoto et al. published a follow-up study confirming and extending the finding of linkage with 1p36 in an expanded sample of 42 families, observing linkage of the region with femoral neck BMD (LOD score 3.53) (Devoto et al., 2001).

Subsequent GWL studies have identified many different QTL for BMD phenotypes, some of which include: 1q21, 2p21, 3p14-p22, 5q33, 6p21, 10q21, 20p12, 20q13 and 21q22 (Williams and Spector, 2007). Of these, the 3p14-p22 chromosomal region has proven to be one of the most replicated (see Table 1.1 for a summary). Duncan et al. published the first evidence suggesting linkage between 3p14-p22 and BMD (Duncan et al., 1999). They analysed 64 genetic markers around 23 candidate genes for BMD regulation in a cohort of 614 individuals from 115 families (Duncan et al., 1999). These markers were analysed in relation to lumbar spine and femoral neck BMD (Duncan et al., 1999). They observed the strongest evidence of linkage with

Table 1.1. Summary of linkage findings between the 3p14-p22 chromosomal region and BMD.

Study	Population	Phenotype	Markers	LOD score
Duncan et al. (1999)	258 males and 356 females from 115 families	Lumbar spine BMD	D3S3559	1.3 – 1.6
		Femoral neck BMD	D3S3559, D3S1289	2.7 – 3.5
Wilson et al. (2003)	2,775 females from 1,348 families	Lumbar spine BMD	D3S1298 to D3S1285	2.1 – 2.7
Wynne et al. (2003)	26 males and 402 females from 175 families	Lumbar spine BMD	D3S3559, D3S1289	1.1 – 1.6
Xiao et al. (2006)	1816 males and 2682 females from 451 families	Total hip BMD	D3S2384 / D3S2409	2.3

two markers in the 3p14-p22 genomic region, which were linked with both lumbar spine (LOD scores 1.3 – 1.6) and femoral neck BMD (LOD scores 2.7 – 3.5) (Duncan et al., 1999).

Wilson et al. compared the results between two independent GWL screens: one in unselected female dizygotic twin pairs aged 18 – 80 years (1,094 pedigrees) and one in extremely discordant or concordant (EDAC) sibling pairs aged 25 – 83 years (254 pedigrees) (Wilson et al., 2003). Lumbar spine and total hip BMD were analysed in both cohorts and whole-body BMD was analysed in the unselected twin pairs (Wilson et al., 2003). Maximum evidence of linkage for both the unselected twins and EDAC siblings was observed for lumbar spine BMD at 3p14-p22 (LOD scores of 2.7 and 2.1 respectively) (Figure 1.10) (Wilson et al., 2003). Evidence of linkage in the unselected twin cohort was also observed with whole-body BMD at 1p36 (LOD score 2.4) (Wilson et al., 2003).

Two later studies and a meta-analysis also found linkage between the 3p14-p22 region of the human genome and BMD phenotypes. Wynne et al. analysed 24 genetic markers across 7 chromosomal loci in 175 Irish families containing probands with low BMD (Wynne et al., 2003). They found that 2 markers within the 3p14-p22 genomic region were linked with lumbar spine BMD (LOD scores > 1.0) (Wynne et al., 2003). These are the same two markers from this genomic region identified by Duncan et al. as linked with BMD (Duncan et al., 1999). Xiao et al. genotyped 4,126 European individuals from 451 families for a large-scale GWL scan (Xiao et al., 2006). They observed linkage between the 3p14-p22 chromosomal region and total hip BMD (LOD score 2.29) (Xiao et al., 2006). A meta-analysis of 11 GWL scans performed by Lee et al. provided convincing support for linkage between this region and BMD (Lee et al., 2006). The study incorporated 12,685 individuals from 3,097 families and divided the autosomal genome into 120 bins that were ranked according to maximum evidence for linkage within each bin (Lee et al., 2006). The 3p14-p22 chromosomal region was part of one of 7 bins that were found to lie above the 95 % confidence level (Lee et al., 2006).

Mouse genetic studies can provide data relevant to human disease gene studies since there are regions of the mouse genome that are homologous with regions of the

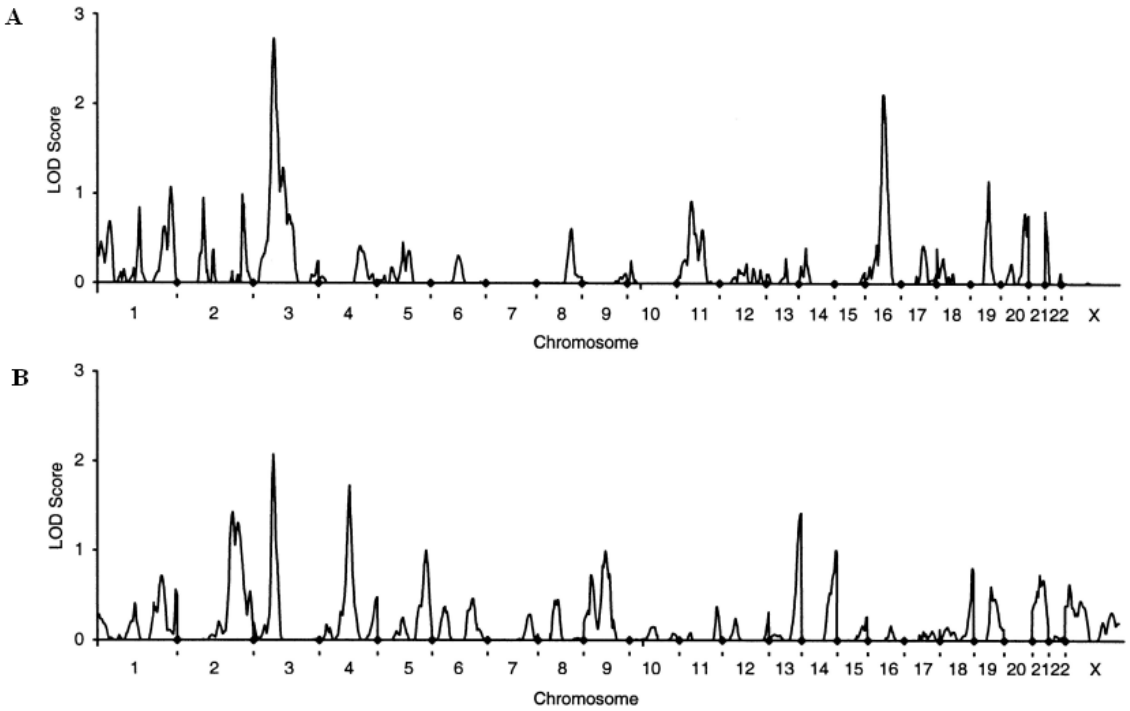

Figure 1.10. Genome-wide linkage scans for quantitative trait loci that regulate lumbar spine BMD in: A) 1,094 twin pairs, and B) 254 EDAC sibling pairs. Note the strong linkage peaks on chromosome 3 in each population. Figure adapted from Wilson et al. (Wilson et al., 2003).

human genome and these can provide supporting evidence for the importance of a region. Mouse models help to eliminate many of the complicating environmental influences on a trait so that a more refined QTL can be identified. The region 3p14-p22 in the human genome shares homology with mouse chromosome 14 (approximately 2 – 10 cM). Klein et al. performed a GWL study for whole-body BMD in two genetically distinct strains of laboratory mice that were raised under strict environmental control (Klein et al., 1998). They observed a strong genetic influence on the trait, indicating an estimated heritability of 0.35 – 0.6 and observed linkage of the phenotype with 10 chromosomal sites (Klein et al., 1998). One of these sites was on mouse chromosome 14 (approximately 2 – 10 cM) (Klein et al., 1998), a region that is syntenic with the human chromosomal region 3p14-p22.

Collectively, the literature reviewed here provides compelling evidence for linkage between the 3p14-p22 chromosomal region and BMD. This linkage has been observed in multiple studies in both humans and in syntenic chromosomal regions of the mouse genome. Previous studies have attempted to identify the genes within the 3p14-p22 region that are responsible for the linkage observed. The *PTH1R* gene, which is located within this chromosomal region and encodes the parathyroid hormone 1 receptor, represents an attractive candidate. However, previous studies on the gene have reported mixed results (Lei et al., 2005, Scillitani et al., 2006, Tenne et al., 2008, Vilarino-Guell et al., 2007, Zhang et al., 2006), suggesting that the main gene or genes responsible for the linkage between the 3p14-p22 chromosomal region and BMD remain to be identified.

1.11.3 Genome-wide association (GWA) studies

GWA studies utilise a non-hypothesis-driven approach whereby a large number of study subjects are genotyped for dense genetic markers (usually in the form of SNPs) covering the genome in an unbiased fashion. These markers are often selected for their tagging properties, allowing for capture of maximal genetic variation with a minimal marker set which results in cost benefits (Magi et al., 2007). GWA studies are well-suited to identifying novel genes with modest influences on complex diseases/traits (Hirschhorn and Daly, 2005). However, there are many issues that must be carefully considered when interpreting the results from GWA studies, including: use of appropriate sample sizes, correction for multiple testing (which is

of course very stringent in GWA studies due to the large number of genetic markers analysed), use of appropriate marker density, potential population stratification and adequate replication of findings (Xu et al., 2010). GWA studies have identified genes thought to have a role in a variety of diseases/traits, including: height (Estrada et al., 2009, Tonjes et al., 2009), hair/skin colour (Han et al., 2008), postmenopausal breast cancer (Hunter et al., 2007), prostate cancer (Eeles et al., 2008, Thomas et al., 2008), psoriasis (Zhang et al., 2009) and Parkinson's disease (Satake et al., 2009, Simon-Sanchez et al., 2009).

The results from the first GWA study aimed at identifying genes associated with bone mass phenotypes was published in 2007 (Kiel et al., 2007). The authors genotyped more than 70,000 autosomal SNPs in 1,141 individuals (495 men and 646 women with a total mean age of 62.5 years) for association with ten primary quantitative traits including various measures of BMD (femoral neck, trochanter and lumber spine BMD), calcaneal ultrasound, and geometric indices of the hip (Kiel et al., 2007). They found that out of the top 40 SNPs with the greatest number of significantly associated BMD traits, one half to two-thirds were in or near genes that had not been previously implicated in osteoporosis (Kiel et al., 2007). Using the additive generalised estimating equation (GEE) and the family-based association test (FBAT) models, the authors identified 12 and 2 associations respectively with the ten primary phenotypes using a cut-off of $P < 1.0 \times 10^{-6}$ (Kiel et al., 2007). The polymorphisms responsible for these associations were located in the genes encoding: ELKS/RAB6-interacting/CAST family member 2 (*ERC2*), zinc finger protein 366 (*ZNF366*), neuregulin 1 (*NRG1*) and nuclear receptor subfamily 5, group A, member 2 (*NR5A2*) (Kiel et al., 2007).

Following the initial study published by Kiel et al., there was a flurry of publications reporting the results from GWA studies investigating the genetics of bone mass regulation. Strykarsdottir et al. genotyped over 300,000 SNPs and tested these for association with hip and lumbar spine BMD in a discovery population comprised of 5,861 Icelandic men and women, subsequently genotyping a subset of 74 SNPs in 3 replication cohorts comprised of 4,165, 2,269 and 1,491 individuals from Iceland, Denmark and Australia respectively (Styrkarsdottir et al., 2008). The authors identified and replicated significant associations between variation in 5 genomic

regions and BMD (Styrkarsdottir et al., 2008). The variants within these 5 regions lay within or around the genes: *TNFSF11*, *TNFRSF11B*, *ESR1*, *ZBTB40* (encoding zinc finger and BTB domain containing 40) and the major histocompatibility complex region (6p21) (Styrkarsdottir et al., 2008). Collectively, these variants were found to account for approximately 3 % of the total variation in hip and spine BMD (calculated from the replication cohorts) (Styrkarsdottir et al., 2008). The loci at the genes *TNFRSF11B, ZBTB40* and the major histocompatibility complex region were also significantly associated with osteoporotic fracture (Styrkarsdottir et al., 2008). By enlarging the discovery and replication cohorts to 6,865 and 8,510 (total) individuals respectively, four additional loci significant at the genome-wide level were identified near the genes *SOST*, *MARK3* (encoding MAP/microtubule affinity-regulating kinase 3), *SP7* (encoding osterix) and *TNFRSF11A* (Styrkarsdottir et al., 2009).

Richards et al. analysed over 300,000 SNPs for association with lumbar spine and femoral neck BMD in a population of 2,094 women from the UK (mean age of 49.7 years), with replication of significant findings attempted in 6,463 individuals from 3 other cohorts in Western Europe (Richards et al., 2008). Associations with BMD significant at a genome-wide level ($P < 5 \times 10^{-8}$) were identified for two SNPs located near to the *TNFRSF11B* and *LRP5* genes (Richards et al., 2008). Polymorphism at the SNP near *TNFRSF11B* was also found to be associated with an increased risk of osteoporosis and expression levels of the *TNFRSF11B* gene in lymphoblast cell lines (Richards et al., 2008). Polymorphism at the SNP in *LRP5* causes a non-synonymous coding change (Ala > Val) and this SNP was also found to be significantly associated with osteoporotic fracture at the lumbar spine and femoral neck sites (Richards et al., 2008). Collectively, the variance in BMD accounted for by polymorphism at these two SNP sites was 1 % at the lumbar spine and 0.6 % at the femoral neck (Richards et al., 2008). The presence of both risk alleles at these two SNPs was found to increase the risk of osteoporotic fracture independently of BMD (Richards et al., 2008).

Xiong et al. genotyped over 370,000 SNPs to test for association with BMD at the spine and hip in a discovery cohort of 1,000 unrelated Caucasian men and women (Xiong et al., 2009). This GWA study was somewhat unique as the authors decided

to attempt replication of the most significant findings in four populations of varying ethnic background, including: a cohort comprised of 593 Caucasian families (n = 1,972), a Chinese hip fracture population (n = 700), a Chinese BMD population (n = 2,955) and a population from Tobago of African ancestry (n = 908) (Xiong et al., 2009). In the discovery cohort the authors identified significant associations between BMD and polymorphism in two genes, *ADAMTS18* (encoding ADAM metallopeptidase with thrombospondin type 1 motif, 18) and *TGFBR3* (encoding transforming growth factor, beta receptor III) (Xiong et al., 2009). These associations were subsequently replicated and polymorphism in *ADAMTS18* was found to be associated with hip fracture in the Chinese sample (Xiong et al., 2009). The results from this study indicate a role for the *ADAMTS18* and *TGFBR3* genes in BMD regulation in multiple ethnic groups (Xiong et al., 2009).

Yang et al. performed a slightly different GWA study that involved genome-wide copy-number variation (CNV) analysis in 700 elderly Chinese individuals, 350 of whom had suffered from osteoporotic hip fracture (mean age of 69.4 years) and 350 of whom were controls (mean age of 69.5 years) (Yang et al., 2008). A genomic map was completed containing 727 CNV regions in Chinese individuals and association with osteoporotic fracture was identified for a deletion variant of the *UGT2B17* gene (encoding UDP glucuronosyltransferase 2 family, polypeptide B17) on 4q13.2, the product of which is an enzyme that catabolises steroid hormones (Yang et al., 2008). This association was subsequently replicated in an independent Chinese cohort comprised of 399 individuals that had suffered from hip osteoporotic fracture and 400 negative controls (Yang et al., 2008). Associations were also identified with hip BMD and femoral-neck bone geometry in additional Chinese (n = 689) and Caucasian populations (n = 1000), with the *UGT2B17* CNV accounting for 1.4 % and 0.7 % of the variation in BMD in each of these populations respectively (Yang et al., 2008). Deletion of the *UGT2B17* gene was also found to be associated with higher serum testosterone and oestrogen in a population of 236 young Chinese males (Yang et al., 2008).

Meta-analysis of GWA data can greatly enlarge study populations and improves the power of the study to detect associations. Rivadeneira et al. conducted a large-scale collaborative meta-analysis of five GWA studies incorporating 19,195 subjects of

Northern European background (age range of 18 – 96 years) for loci associated with femoral neck and lumbar spine BMD (Rivadeneira et al., 2009). They identified 20 loci that achieved genome-wide significance ($P < 5 \times 10^{-8}$), of which 13 were located in genomic regions not previously associated with BMD (Table 1.2) (Rivadeneira et al., 2009). The remaining 7 were located in genomic regions that have previously been identified as significantly associated with BMD and included the regions containing the genes *LRP5, TNFSF11, TNFRSF11A* and *TNFRSF11B* (Rivadeneira et al., 2009). 15 of the 20 loci were significantly associated with lumbar spine BMD and 10 were significantly associated with femoral neck BMD (Table 1.2) (Rivadeneira et al., 2009). A highly significant linear decrease in the mean lumbar spine and femoral neck BMD was seen with increasing number of low BMD risk alleles (Rivadeneira et al., 2009). A risk allele analysis was then conducted using the top associated SNPs from the 15 lumbar spine and 10 femoral neck BMD loci (Rivadeneira et al., 2009). They found that when combined, the 15 lumbar spine SNPs accounted for around 2.9 % of the variance in lumbar spine BMD and the 10 femoral neck SNPs accounted for around 1.9 % of the variance in femoral neck BMD (Rivadeneira et al., 2009).

One of the 20 loci identified by Rivadeneira et al. as significantly associated with BMD at genome-wide significance is located within the 3p22 chromosomal region (Table 1.2) (Rivadeneira et al., 2009). The polymorphism within this region that showed maximal association with BMD is situated approximately 103 Kb upstream of the nearest gene, *CTNNB1*, which encodes beta-catenin (Rivadeneira et al., 2009). As discussed earlier, beta-catenin is an integral component of the Wnt signalling pathway (Wagner et al., 2010) and thus presents as an excellent candidate for regulation of BMD. The *CTNNB1* gene is located within the support intervals defined for the 3p14-p22 chromosomal region by Wilson et al. as linked with BMD by GWL (Wilson et al., 2003). It is therefore possible that this gene is responsible for the linkage observed between this chromosomal region and BMD. However, the *CTNNB1* gene is located approximately 10 Mb from the centre of this linkage region, which would suggest that one or more additional genes located within this region also contribute to the linkage observed between 3p14-p22 and BMD.

Table 1.2. Twenty loci identified by Rivadeneira et al. as significantly associated with femoral neck and/or lumbar spine BMD in 19,195 subjects of Northern European background.

Locus	Site	Candidate genes in region	Gene product
1p31.3	FN and LS	*WLS*	Wntless homolog
1p36	FN and LS	*ZBTB40*	Zinc finger and BTB domain containing 40
2p21	LS	*SPTBN1*	Spectrin, beta, non-erythrocytic 1
3p22	FN	*CTNNB1*	Catenin (cadherin-associated protein), beta 1, 88 kD
4q21.1	LS	*MEPE*	Matrix extracellular phosphoglycoprotein
5q14	FN	*MEF2C*	Myocyte enhancer factor 2C
6q25	FN and LS	*ESR1*	Oestrogen receptor 1
7p14	LS	*STARD3NL*	STARD3 N-terminal like
7q21.3	FN and LS	*FLJ42280*	Hypothetical LOC401388
8q24	FN and LS	*TNFRSF11B*	Osteoprotegerin
11p11.2	FN	*ARHGAP1*	Rho GTPase activating protein 1
		LRP4	Low density lipoprotein receptor-related protein 4
11p14.1	LS	*DCDC5*	Doublecortin domain containing 5
		DCDC1	Doublecortin domain containing 1
11p15	FN	*SOX6*	SRY (sex determining region Y)-box 6
11q13.4	LS	*LRP5*	Low density lipoprotein receptor-related protein 5
12q13	LS	*SP7*	Osterix
13q14	LS	*TNFSF11*	Tumour necrosis factor (ligand) superfamily, member 11 (RANKL)
16q24	LS	*FOXC2*	Forkhead box C2 (MFH-1, mesenchyme forkhead 1)
		FOXL1	Forkhead box L1
17q12	LS	*CRHR1*	Corticotropin releasing hormone receptor 1
17q21	FN	*HDAC5*	Histone deacetylase 5
		C17orf53	Chromosome 17 open reading frame 53
18q21	LS	*TNFRSF11A*	Tumour necrosis factor receptor superfamily, member 11a, NFKB activator (RANK)

The results from these GWA studies have helped to highlight the importance of the Wnt signalling and RANK-RANKL-OPG pathways in the genetic regulation of bone mass as well as the complex architecture that underlies variation in BMD. They have also proved useful for identifying genes that may have a role in BMD regulation that do not stand out as strong candidate genes, such as *ZBTB40*. However, the genetic variants identified as significantly associated with BMD in these studies account for only a small amount of the variance of each phenotype. This would suggest that there are still a large number of BMD-influencing genes in the human genome that are yet to be identified, a notion supported by the fact that genes strongly associated with BMD are yet to be identified for many of the chromosomal regions that have been linked with BMD in GWL studies.

1.12 Aims and unifying hypothesis of this thesis

1.12.1 Aims

The broad aims of this thesis are to identify the gene or genes located within the 3p14-p22 chromosomal region that are responsible for the linkage observed between the region and BMD, and further, to explore the role of these genes in bone metabolism. The thesis incorporates a series of genetic and functional studies, a number of which are follow-up studies to findings generated during the course of the work.

The specific aims are:

1) Determine whether common polymorphism within a candidate gene from the 3p14-p22 chromosomal region, *ARHGEF3*, is associated with bone density in Caucasian women.

2) Determine whether common polymorphism within a second candidate gene from the 3p14-p22 chromosomal region, *RHOA*, is associated with bone density in Caucasian women.

3) Analyse variation at five polymorphic sites located in the *FLNB* gene (also situated in the 3p14-p22 chromosomal region), which have been identified as significantly associated with *FLNB* mRNA expression, for associated with bone density phenotypes in Caucasian women.

4) Analyse expression of the NM_001128616 transcript variant of *ARHGEF3* in human osteoblast-like and osteoclast-like cells.

5) Identify genes, from a list of candidates, whose expression is influenced by *ARHGEF3* and *RHOA* gene knockdown in human osteoblast-like and osteoclast-like cells.

7) Determine whether knockdown of the *ARHGEF3* and *RHOA* genes influences the bone-resorptive capabilities of osteoclast-like cells.

1.12.2 Unifying hypothesis of this thesis

One or more genes in the human genomic region 3p14-p22 are significantly associated with bone mineral density in Caucasian women.

Specific hypotheses are contained within each chapter.

Chapter 2 – Materials and methods

2.1 Materials

2.1.1 Whole-genome amplification

Item	Supplier
REPLI-g Midi Kit	QIAGEN, USA
- Buffer D1	
- Buffer N1	
- 4X REPLI-g Buffer	
- REPLI-g DNA Polymerase	
96-Well PCR Microplate	Axygen, USA
8-Cap Strips	Axygen, USA

2.1.2 PicoGreen DNA quantitation

Item	Supplier
Quant-iT PicoGreen dsDNA Assay Kit	Invitrogen, USA
- Quant-iT PicoGreen dsDNA Reagent	
- Lambda DNA Standard (100 µg/mL)	
96-Well OptiPlate	PerkinElmer, USA

2.1.3 SNP genotyping by single nucleotide extension using matrix-assisted laser desorption/ionisation time-of-flight (MALDI-ToF) mass spectrometry

Item	Supplier
Multiplex PCR Kit	QIAGEN, USA
- 2X Multiplex PCR Master Mix	
Shrimp Alkaline Phosphatase (1 unit/µL)	USB Corporation, USA
Shrimp Alkaline Phosphatase Dilution Buffer	USB Corporation, USA
Dideoxynucleotide Triphosphate (ddNTP) Set 5 mM	Amersham Biosciences, USA
Oligonucleotide primers 100 µM	Sigma-Aldrich, USA
Thermo Sequenase (32 units/µL)	Amersham Biosciences, USA
Thermo Sequenase Dilution Buffer	Amersham Biosciences, USA
384-Well Hard-Shell PCR Plate	MJ Research, USA

Microseal ‘A’ Film	Bio-Rad, USA
AG 501-X8(D) 20-50 Mesh Ionic Exchange Resin	Bio-Rad, USA
3-Hydroxypicolinic Acid (≥ 99 %)	Fluka, Switzerland
Acetonitrile	EM Science, USA
Ammonium Citrate	Sigma-Aldrich, USA

2.1.4 Agarose gel electrophoresis

Item	**Supplier**
DNA Grade Agarose	Progen Biosciences, Australia
DirectLoad Wide Range DNA Marker	Sigma-Aldrich, USA
Ethidium Bromide	Sigma-Aldrich, USA
1Kb DNA Ladder	Promega, USA

2.1.5 TaqMan SNP genotyping

Item	**Supplier**
10X PCR Buffer II	Applied Biosystems, USA
25mM $MgCl_2$	Applied Biosystems, USA
dNTP set 100mM	Promega, USA
AmpliTaq Gold DNA Polymerase (5 units/µL)	Applied Biosystems, USA
384-Well Hard-Shell PCR Plate (Black)	Bio-Rad, USA
Microseal ‘A’ Film	Bio-Rad, USA
TaqMan SNP Genotyping Assay	Applied Biosystems, USA

2.1.6 Isolation of peripheral blood mononuclear cells

Item	**Supplier**
Vacutainer K2E 10 mL	Becton, Dickinson and Company, USA
Ficoll-Paque	Pharmacia, Sweden

2.1.7 RNA extraction

Item	**Supplier**
RNeasy Mini Kit	QIAGEN, USA

- Buffer RLT
- Buffer RW1
- Buffer RPE
- RNeasy Spin Column

2.1.8 Reverse transcription

Item	**Supplier**
QuantiTect Reverse Transcription Kit	QIAGEN, USA
- gDNA Wipeout Buffer	
- 5X QuantiScript Reverse Transcription Buffer	
- Reverse Transcription Primers	
- QuantiScript Reverse Transcriptase	
96-Well PCR Microplate	Axygen, USA
8-Cap Strips	Axygen, USA

2.1.9 Real-time PCR

Item	**Supplier**
QuantiFast SYBR Green PCR Kit	QIAGEN, USA
- 2X QuantiFast SYBR Green Master Mix	
96-Well iCycler iQ PCR Plates	Bio-Rad, USA
Microseal 'B' Film	Bio-Rad, USA
QuantiTect Primer Assay	QIAGEN, USA
Oligonucleotide primers 100 µM	Sigma-Aldrich, USA

2.1.10 Cell culture

Item	**Supplier**
Dulbecco's Modified Eagle's Medium (DMEM)	Sigma-Aldrich, USA
α-Modified Eagle's Medium (α-MEM)	Invitrogen, USA
Opti-Modified Eagle's Medium (Opti-MEM)	Invitrogen, USA

Foetal Bovine Serum (FBS)	Sigma-Aldrich, USA
Penicillin/Streptomycin 100X (10,000 units penicillin and 10 mg streptomycin/mL)	Sigma-Aldrich, USA
Trypsin-EDTA Solution 1X (0.25 % trypsin, 0.1% EDTA)	Sigma-Aldrich, USA
Small interfering RNA (siRNA)	QIAGEN, USA
HiPerFect Transfection Reagent	QIAGEN, USA
Recombinant Human RANKL	Invitrogen, USA
Recombinant Human M-CSF	Invitrogen, USA
25 cm^2 Tissue Culture Flasks	DKSH, Switzerland
75 cm^2 Tissue Culture Flasks	DKSH, Switzerland
24-Well Tissue Culture Plates	Greiner Bio-One, Germany
96-Well Tissue Culture Plates	Greiner Bio-One, Germany
Bovine Bone	Obtained from butcher
Cell Dissociation Solution Non-Enzymatic 1X	Sigma-Aldrich, USA
Sodium Bicarbonate ($NaHCO_3$)	Ajax Finechem, Australia
HEPES	Sigma-Aldrich, USA

2.1.11 Microarray

Item	**Supplier**
TotalPrep RNA Amplification Kit	Applied Biosystems, USA
HumanHT-12 v3 Kit	Illumina Inc., USA

2.1.12 Statistical and other software packages

Item	**Supplier**
PASW Statistics 17	SPSS Inc., USA
Statistica v8.0	StatSoft Inc., USA
BeadStudio v3.4.0	Illumina Inc., USA
MetroPro v8.2.0	Zygo Corp., USA
REST2008 v2.0.7	Corbett Research Pty. Ltd., Australia

2.1.13 Other

Item	Supplier
Ethanol	Thermo Fisher Scientific, USA
Gloves, Non-Sterile, Latex	Ansell, USA
Trizma Base ($NH_2C(CH_2OH)_3$)	Sigma-Aldrich, USA
Ethylenediaminetetra-Acetic Acid (EDTA)	Sigma-Aldrich, USA
Sodium Hydroxide (NaOH)	APS Finechem, Australia
Glacial Acetic Acid	Biolab, New Zealand
Bromophenol Blue	Sigma-Aldrich, USA
Xylene Cyanol	Sigma-Aldrich, USA
Glycerol	Ajax Finechem, Australia
Sodium Chloride (NaCl)	Sigma-Aldrich, USA
Potassium Chloride (KCl)	Merck Pty Ltd., Australia
Sodium Phosphate Dibasic (Na_2HPO_4)	Sigma-Aldrich, USA
Potassium Phosphate (KH_2PO_4)	Sigma-Aldrich, USA
Paraformaldehyde	Merck Pty Ltd., Australia
Sodium Acetate ($C_2H_3NaO_2$)	BDH Chemicals, Australia
Sodium Tartrate Dihydrate ($C_4H_8Na_2O_8$)	Ajax Finechem, Australia
2-Ethoxyethanol ($C_4H_{10}O_2$)	Sigma-Aldrich, USA
Fast Red Violet LB Salt	Sigma-Aldrich, USA
0.8 µm Filter	Millipore, USA
Naphthol AS-MX Phosphate Disodium Salt	Sigma-Aldrich, USA

2.1.14 Equipment

Item	Supplier
PTC-220 DNA Engine Dyad Peltier Thermal Cycler	MJ Research, USA
iQ5 Multicolor Real-Time PCR Detection System	Bio-Rad, USA
2100 Bioanalyzer	Agilent Technologies, USA
ND-1000 Spectrophotometer	NanoDrop Technologies, USA
DNA Electrophoresis Cell Tank	Bio-Rad, USA

CO_2 Incubator – Hera Cell 150	Forma Scientific, USA
MicroCL 21R Centrifuge	Thermo Electron Corporation, USA
Orbital 420 Centrifuge	Clements Medical Equipment, Australia
Vortex, ST19	Sentra, USA
Victor^2 Multilabel Plate Reader	Wallac, USA
PowerPack 3000	Bio-Rad, USA
Voyager-DE PRO 6066 Mass Spectrometer	Applied Biosystems, USA
384-Well MALDI-ToF Array	Applied Biosystems, USA
Ultraviolet Transilluminator (UVT) 100	International Biotechnologies Inc., USA
Pipettes – P1000, P200, P20, P2	Gilson Inc., USA
Phase Contrast Microscope, IMT-2	Olympus, Japan
pH and Temperature Meter, 900-P	TPS Pty Ltd., Australia
10 mL Transfer Pipette	Falcon, USA
3.5 mL Transfer Pipette	Sarstedt, Germany
Polaroid Camera, Model QSP	International Biotechnologies Inc., USA
Orbital 900 Centrifuge	Clements Medical Equipment, Australia
Plate Rotator, IKA-VIBRAX-VXR	Wiarton, Canada
Light Microscope, CH-2	Olympus, Japan
LaborLux II Light Microscope	Leitz, Germany
DXC-390P 3CCD Colour Video Camera	Sony, Japan
XL30 Scanning Electron Microscope	Philips, Netherlands
NewView 6300 3D Optical Profilometer	Zygo Corp., USA

2.2 General methods

2.2.1 Whole-genome amplification

Whole-genome amplification was performed as a way of increasing the quantity of template DNA available for SNP genotyping from a genomic DNA sample. This was carried out using the REPLI-g Kit (QIAGEN), which uses exonuclease-resistant primers to non-specifically amplify all DNA sequence within a sample in a highly uniform manner (Hosono et al., 2003). Each genomic DNA sample was first diluted to a concentration of 20 ng/µL in 1X TE buffer (Tris-EDTA buffer, Appendix I). 2.5 µL of each template DNA sample was then added to a 96-well PCR plate (Axygen). One well on each plate was used for a negative control and received 2.5 µL of 1X TE

buffer instead of template DNA and one well received a duplicate sample. Therefore, each 96-well PCR plate contained 94 different genomic DNA samples (1 of which was present in duplicate) and 1 negative control. 2.5 µL of Buffer D1 (denaturation buffer) was then added to each well on the plate and the samples were mixed thoroughly by vortexing before being left to stand at room temperature for 3 minutes. 5 µL of Buffer N1 (neutralisation buffer) was subsequently added to each sample. A master mix was then prepared by combining the following components while keeping the mixture chilled on ice:

Component	**Volume per reaction**
4X REPLI-g Buffer (QIAGEN) (contains primers)	12.5 µL
Distilled-deionised water	27 µL
REPLI-g DNA Polymerase (QIAGEN)	0.5 µL
Total volume	**40 µL**

Each sample was chilled using ice before 40 µL of this master mix was added to each. The microplate was then capped, mixed by vortexing and centrifuged before being incubated in a thermal cycler using the following temperature programme:

Temperature	**Duration**	**Cycles**
30 °C	8 h	1
65 °C	3 min	1

Lid temperature tracking at 1 °C above block temperature

The mean quantity (± standard deviation) of whole-genome amplified DNA produced in each 50 µL reaction was 31 (± 10) µg, as assessed by PicoGreen quantitation (described below). An example of whole-genome amplified DNA electrophoresed on an agarose gel is shown in Figure 2.1.

2.2.2 PicoGreen DNA quantitation

Whole-genome amplified DNA samples were quantitated using the Quant-iT PicoGreen dsDNA Assay Kit (Invitrogen). PicoGreen is an ultrasensitive nucleic acid stain that fluoresces when bound to double-stranded DNA (dsDNA), making it

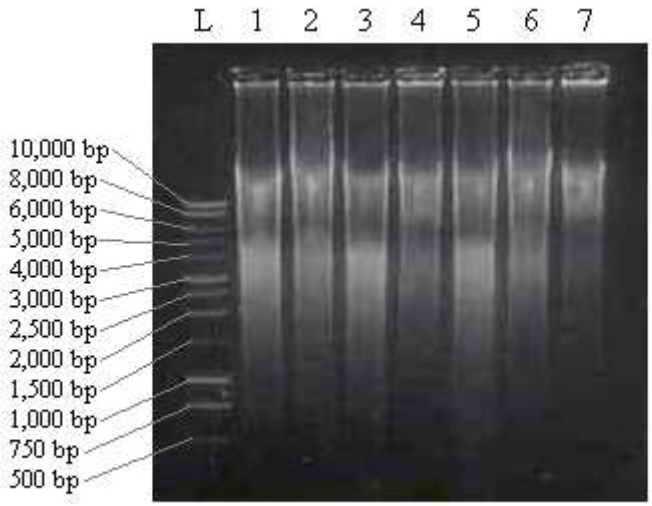

Figure 2.1. Seven whole-genome amplified DNA samples electrophoresed alongside a 1 Kb DNA ladder in a 1 % agarose gel. This image illustrates the large size of the amplified DNA fragments, the majority of which are over 1 Kb in length.

ideal for specific quantitation of dsDNA in the presence of other single-stranded nucleic acids (Ahn et al., 1996). Compared to other DNA quantitation techniques, the PicoGreen method is more sensitive than the Hoechst (bisbenzimide) dye (Singer et al., 1997) and is more specific for dsDNA than spectrophotometric absorbance at 260 nm (Ahn et al., 1996).

All quantitations were performed in a darkened room to prevent photodegredation of the PicoGreen reagent. 1 µL of each whole-genome amplified DNA sample was first diluted in 9 µL of 1X TE buffer. 2 µL of this dilution was then added to a single well in a 96-well OptiPlate (PerkinElmer) containing 1 µL of Quant-iT PicoGreen dsDNA Reagent diluted in 194 µL 1X TE buffer. The contents of the well were then mixed by pipetting and the fluorescence measured using a Victor^2 Multilabel Plate Reader (Wallac). Each 96-well plate analysed contained two blanks (1X TE buffer instead of whole-genome amplified DNA) and duplicate sets of 7 dsDNA standards at the following concentrations: 75 ng/µL, 50 ng/µL, 25 ng/µL, 12.5 ng/µL, 6.25 ng/µL, 3.125 ng/µL, 1.5262 ng/µL. The fluorescence from each standard was then plotted against concentration to create a standard curve from which the concentration of each sample could be read in a linear model. All samples were quantitated in duplicate with the mean of the two values obtained used for downstream work.

2.2.3 SNP genotyping by single nucleotide extension using matrix-assisted laser desorption/ionisation time-of-flight (MALDI-ToF) mass spectrometry

The MALDI-ToF mass spectrometry method of SNP genotyping involves two PCR steps followed by analysis of the reaction products on a MALDI-ToF mass spectrometer (Ross et al., 1998). The first of the two PCR steps uses a forward and reverse oligonucleotide primer pair to amplify the genomic region containing the SNP site. The second PCR step is a single-base primer extension reaction. This is achieved using a sequence specific oligonucleotide sequencing primer that binds to the amplified template DNA sequence in such a way that the 3' end of the primer is bound to the base immediately adjacent to the SNP site. This primer is then extended by a single nucleotide, the identity of which is determined by the base present at the SNP site on the template sequence (Figure 2.2). Dideoxynucleoside triphosphates (ddNTPs) are used in this reaction instead of deoxynucleoside triphosphates (dNTPs) because they do not contain an OH group on their 3' carbon atom. As a result,

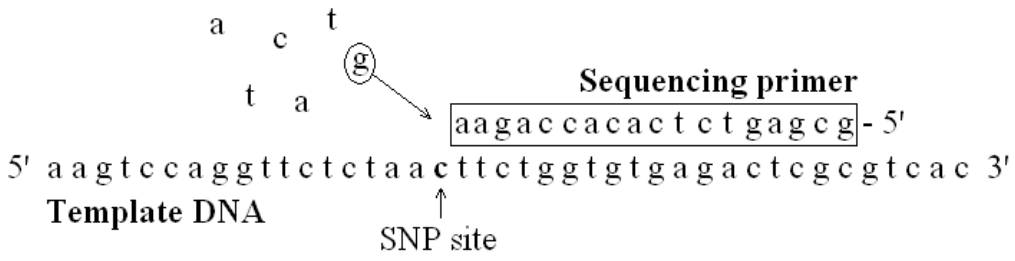

Figure 2.2. Illustration of how the single nucleotide extension step works in the MALDI-ToF mass spectrometry SNP genotyping technique. Note: due to the use of dideoxynucleoside triphosphates the reaction will be terminated after the addition of the *G* nucleotide by the Thermo Sequenase enzyme.

ddNTPs cannot bind to additional incoming nucleotides and hence the sequencing primer is extended by a single base only. The products from the second PCR step are de-salted before being analysed on a MALDI-ToF mass spectrometer. By determining the mass of the sequencing primer plus single base extension molecule, the identity of the ddNTP that was attached to the sequencing primer and hence the identity of the SNP allele can be determined.

The two PCR steps were performed in a 384-well PCR plate (MJ Research). Reactions were multiplexed so that multiple SNP regions were amplified in a single reaction. The Multiplex PCR Kit (QIAGEN) was used to perform the first PCR step with sequence specific oligonucleotide primers (refer to section 2.1.16 for primer sequences). A mixture of the following components was made up at room temperature:

Component	**Volume per reaction**
2X Multiplex PCR Master Mix (QIAGEN)	8.25 µL
2 µM forward and reverse primer solution	1.65 µL
Distilled-deionised water	5.6 µL
Total volume	**15.5 µL**

15.5 µL of this master mix was combined with 2.5 µL genomic DNA (20 ng/µL) in each well of the 384-well plate. The plate was then covered with Microseal 'A' Film (Bio-Rad) before being vortexed and centrifuged. The samples were then incubated in a thermal cycler using the following temperature programme:

Temperature	**Duration**	**Cycles**
95 °C	15 min	1
98 °C	15 s	
60 °C	1.5 min	45
72 °C	1.5 min	
72 °C	10 min	1

Lid temperature constant at 85 °C

Randomly selected samples from each 384-well PCR plate were subsequently electrophoresed on an agarose gel to confirm specific amplification of correct target sequences (example displayed in Figure 2.3). After the first PCR step each reaction mix was dephosphorylated using shrimp alkaline phosphatase (USB Corp.) to hydrolyse residual oligonucleotide primers and nucleotides. This involves preparation of a mixture at room temperature containing the following components:

Component	**Volume per reaction**
Shrimp Alkaline Phosphatase Dilution Buffer (USB Corp.)	2 µL
Shrimp Alkaline Phosphatase (1 unit/µL) (USB Corp.)	1 µL
Total volume	**3 µL**

3 µL of this mixture was added to each sample in the 384-well PCR plate. The plate was then covered with Microseal 'A' Film, vortexed and centrifuged. The samples were incubated in a thermal cycler using the following temperature programme:

Temperature	**Duration**	**Cycles**
37 °C	1 h	1
80 °C	15 min	1
Lid temperature constant at 100°C		

Subsequent to dephosphorylation, the samples were subjected to a second PCR step involving a single-base primer extension reaction (refer to section 2.1.16 for primer sequences). A master mix for this step was prepared at room temperature containing:

Component	**Volume per reaction**
ddNTP mix (1.25 mM of each ddNTP)	0.4 µL
Sequencing primer (100 µM)	0.1 µL - 0.18 µL
Thermo Sequenase Dilution Buffer (Amersham Biosciences, USA)	0.28 µL
Thermo Sequenase (Amersham	0.01 µL

Biosciences, USA)	
Distilled-deionised water	Variable
Total volume	**4.5 µL**

4.5 µL of this master mix was added to each sample before the 384-well PCR plate was covered using Microseal 'A' Film, vortexed and centrifuged. The samples were then incubated in a thermal cycler using the following temperature programme:

Temperature	**Duration**	**Cycles**
94 °C	2 min	1
98 °C	7 s	
37 °C (ramp to 37 °C at 1.2 °C/s)	30 s	50
72 °C (ramp to 72 °C at 1.2 °C/s)	10 s	
16 °C	5 min	1
Lid temperature constant at 100°C		

Following the second PCR step, each sample was de-salted to allow for analysis using the MALDI-ToF mass spectrometer. This was carried out by adding 10 mg of AG 501-X8(D) 20-50 Mesh Ionic Exchange Resin (Bio-Rad) to each sample and incubating for 15 min with rotation. A batch of fresh MALDI-ToF matrix was then prepared by combining 6 mg 3-hydroxypicolinic acid (Fluka), 12.5 µL ammonium citrate (50 mg/mL) and 100 µL acetonitrile. 1 µL of this matrix was added to each well of a 384-well MALDI-ToF sample analysis array and left to dry before 1 µL of sample was added to the dried matrix. The sample array was then analysed on a Voyager-DE PRO 6066 mass spectrometer. Each matrix element of the sample array was sequentially irradiated with ultraviolet laser pulses leading to ionisation of the analyte molecules. The ions were then accelerated through a detection region at a velocity that was inversely proportional to their mass-to-charge ratio. Spectra were obtained using an accelerating voltage of 25 kV and extraction delay time of 300 nsec with 20 laser pulses averaged. Spectra were visualised using Data Explorer Software v4.0 and were processed using a macro developed to handle accumulated spectra and apply noise removal and baseline correction functions (example displayed in Figure 2.4).

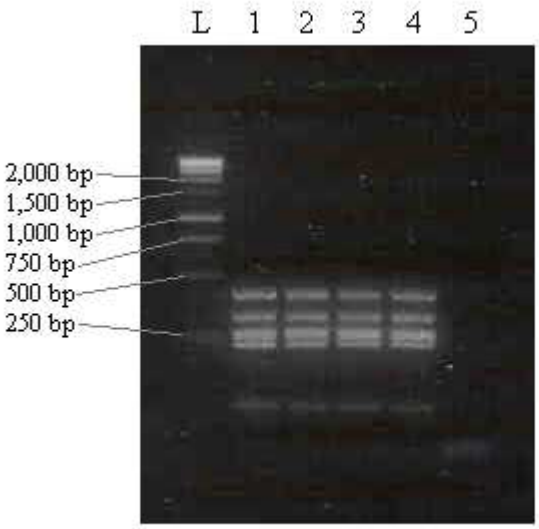

Figure 2.3. Multiplexed PCR products. Reaction products from four DNA samples (lanes 1 – 4) and one negative control (lane 5) multiplexed with five primer pairs electrophoresed alongside a 1 Kb DNA ladder in a 3 % agarose gel. The five product sizes are (in bp): 101, 241, 285, 339 and 435. Note the faint band of unused primer at the bottom of the negative control lane (~ 20 bp).

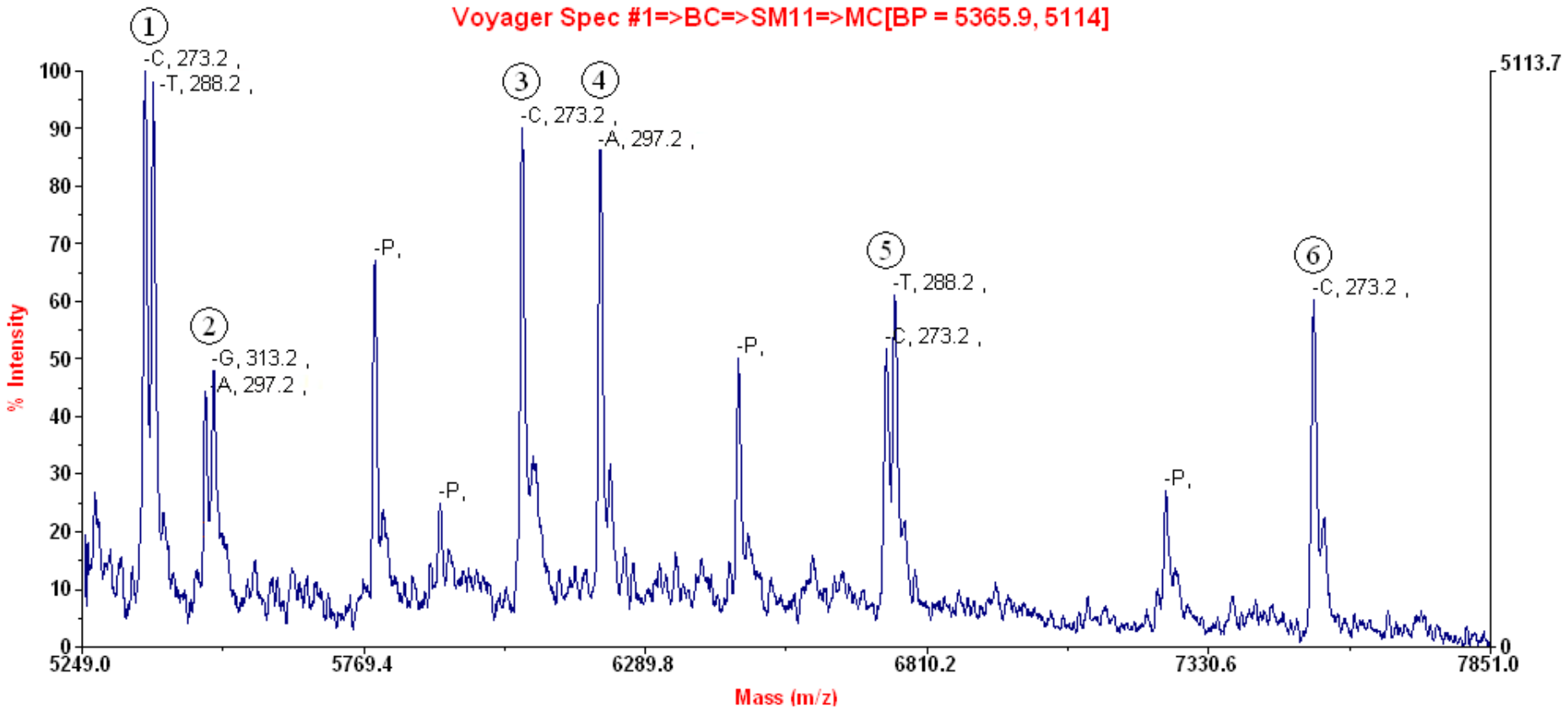

Figure 2.4. An example of a processed MALDI-ToF spectrum showing the genotypes for six SNPs in a single multiplexed reaction. SNP 1 = *TC*, SNP 2 = *AG*, SNP 3 = *CC*, SNP 4 = *AA*, SNP 5 = *TC*, SNP 6 = *CC*.

2.2.4 Agarose gel electrophoresis

1 – 3 % agarose gels containing 500 ng/mL ethidium bromide were prepared in 1X TAE buffer. 2 µL of loading buffer was mixed with 6 µL of sample and loaded onto the gel before being electrophoresed at 100 – 120 V for 20 – 50 min, depending on DNA fragment size. The gel was then viewed using an ultraviolet transilluminator (UVT) and photographed using a Polaroid camera fitted to the UVT housing.

2.2.5 TaqMan SNP genotyping

TaqMan SNP genotyping reactions were performed in a 384-well PCR plate (Bio-Rad) using a TaqMan SNP genotyping assay (Applied Biosystems) specific for each SNP. Each TaqMan SNP genotyping assay contained four oligonucleotides: two as forward and reverse primers to amplify the region containing the SNP site and two allele specific oligonucleotide TaqMan probes that hybridise to their respective targets at the SNP site and which differ in sequence by a single base. Each of these probes is labelled with either a FAM or VIC reporter dye at their 5' end and a quencher at their 3' end. Only one of the two probes will bind to a template DNA fragment depending on the identity of the base at the SNP site. Once the forward and reverse primers have bound to a DNA fragment, extension will occur through incorporation of nucleotides which is catalysed by the polymerase enzyme. Upon reaching the probe, the 5' label is cleaved by the polymerase which separates it from the quencher and causes it to fluoresce. This label-specific fluorescence can then be used to determine which probe has bound to the template DNA and hence the identity of the allele at the SNP site.

To perform SNP genotyping using the TaqMan method, a master mix was first prepared at room temperature containing the following components:

Component	**Volume per reaction**
10X PCR Buffer II (Applied Biosystems)	0.5 µL
25 mM $MgCl_2$ (Applied Biosystems)	0.7 µL
dNTP mix (25 mM of each dNTP)	0.04 µL
TaqMan SNP Genotyping Assay (including four oligonucleotides) (Applied Biosystems)	0.125 µL

AmpliTaq Gold DNA Polymerase (5 units/µL) (Applied Biosystems)	0.06 µL
Distilled-deionised water	3.075 µL
Total volume	**4.5 µL**

4.5µL of this master mix was added to each well of the 384-well PCR plate prior to the addition of 0.5 µL of genomic DNA (20 ng/µL). The 384-well PCR plate was then covered using Microseal 'A' Film, vortexed and centrifuged before being incubated in a thermal cycler using the following temperature programme:

Temperature	**Duration**	**Cycles**
50 °C	2 min	1
95 °C	10 min	1
92 °C	15 s	35
60 °C	1 min	
Lid temperature constant at 100°C		

Following PCR, fluorescence from the 384-well PCR plate was detected using a Victor^2 Multilabel Plate Reader (Wallac). A scatter plot was then created using the data on fluorescence emitted from each of the two probes (Figure 2.5) and genotypes were assigned to each sample.

2.2.6 RNA extraction

Total RNA was extracted from cells in culture using the RNeasy Mini Kit (QIAGEN). The first step involved lysis of the cultured cells by adding 350 µL of Buffer RLT (lysis buffer) directly to the cell culture vessel, with the lysate mixed and homogenised by pipetting up and down at least 5 times. 350 µL of 70 % ethanol was added to the lysate to precipitate the RNA with the pipetting step repeated to mix. The sample was then transferred to an RNeasy Spin Column (QIAGEN) placed in a 2 mL collection tube. The column was centrifuged at ≥ 10,000 rpm for 15 s and the flow-through discarded. 700 µL Buffer RW1 (wash buffer 1) was then added to the spin column and the centrifugation step was repeated with the flow-through discarded (care was taken at this point not to let the spin column contact the flow-through). 500 µL Buffer RPE (wash buffer 2) was subsequently added to the spin

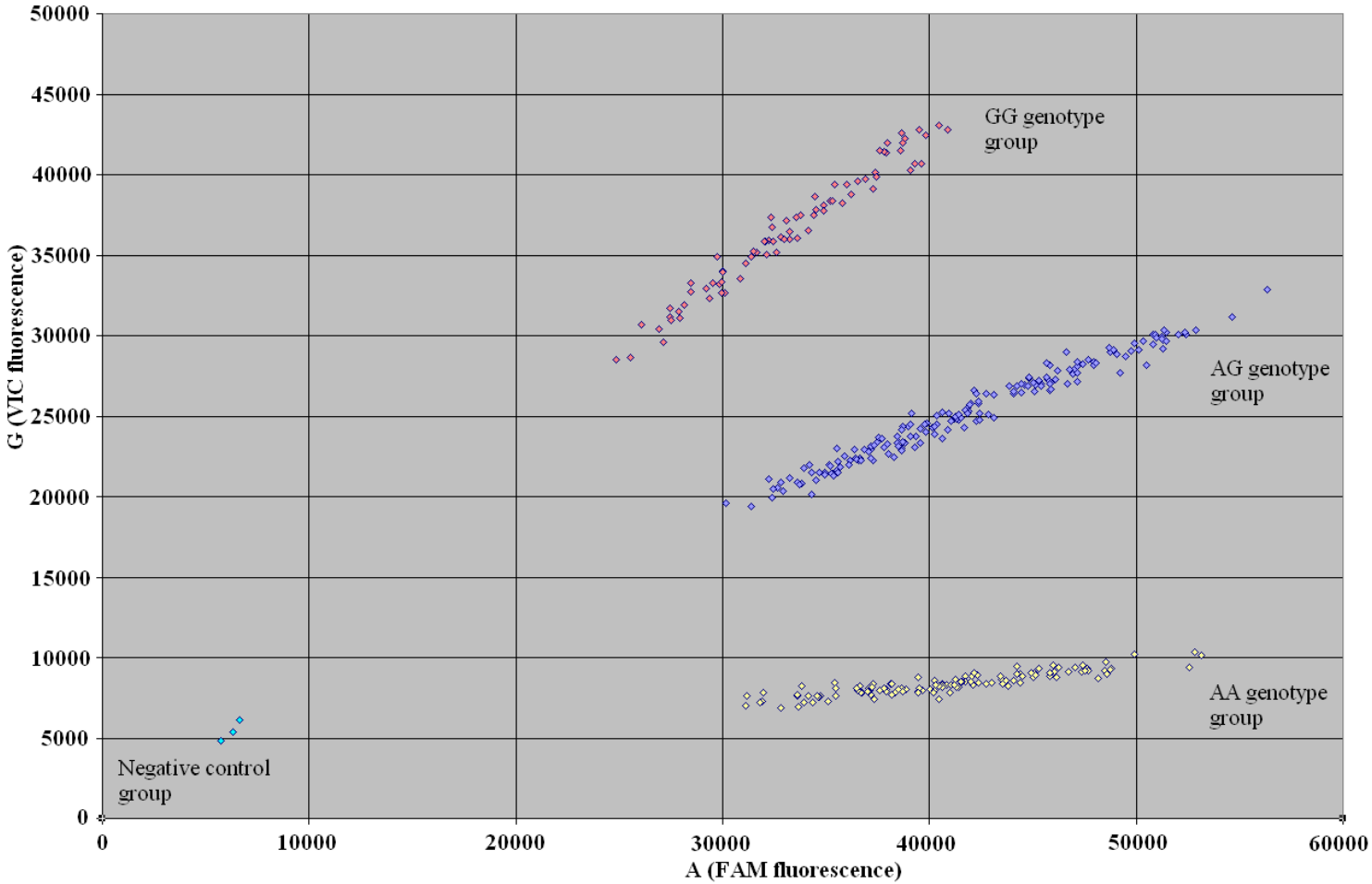

Figure 2.5. An example of a TaqMan scatter plot with the *GG*, *AG* and *AA* genotype groups indicated. For each sample, the fluorescence emitted from the VIC reporter (*G* allele) is plotted against the fluorescence emitted from the FAM reporter (*A* allele).

column and the centrifugation step repeated with the flow-through discarded. Another 500 µL aliquot of Buffer RPE was added to the spin column with centrifugation proceeding at ≥ 10,000 rpm for 2 min. The collection tube was discarded with the flow-through after this step and a new collection tube was attached to the spin column. The spin column was then centrifuged at ≥ 11,000 rpm for 1 min before being placed in a clean 1.5 mL collection tube. 30 – 50 µL of RNase-free water was subsequently added directly to the spin column membrane and centrifugation at ≥ 10,000 rpm for 1 min followed to elute total RNA. RNA was quantitated using an ND-1000 spectrophotometer (NanoDrop Technologies) and was kept chilled using ice while being used or at - 80 °C for long term storage.

2.2.7 Reverse transcription

Reverse transcription of RNA was performed using the QuantiTect Reverse Transcription Kit (QIAGEN). This kit removes contaminating genomic DNA from the RNA sample before using random hexamer primers to produce cDNA from all regions of the template RNA in an unbiased manner. RNA samples and reagents were thawed while chilled on ice before ≤ 1 µg of RNA was added to a single well in a 96-well PCR plate (Axygen) made up to a total volume of 12 µL with RNase-free water. 2 µL of gDNA Wipeout Buffer was then added to each sample while chilled on ice before the samples were capped, mixed by vortexing and centrifuged. The samples were then incubated at 42 °C for 2 min then chilled on ice. A reverse-transcription master mix was then made up while chilled on ice containing:

Component	**Volume per reaction**
5X QuantiScript Reverse Transcription Buffer (QIAGEN)	4 µL
Reverse Transcription Primers (QIAGEN)	1 µL
QuantiScript Reverse Transcriptase (QIAGEN)	1 µL
Total volume	**6 µL**

6 µL of this master mix was added to each sample while chilled on ice before the 96-well PCR plate was capped, mixed by vortexing and centrifuged. The samples were incubated in a thermal cycler using the following temperature programme:

Temperature	Duration	Cycles
42 °C	15 min	1
95 °C	3 min	1

Lid temperature tracking at 1 °C above block temperature

2.2.8 Real-time PCR

Real-time PCR was carried out using SYBR green, which is an asymmetrical cyanine dye that fluoresces when bound to DNA (Cosa et al., 2001). The dye preferentially binds to double-stranded DNA and can be used in non-specific DNA quantitation. Real-time PCR incorporating SYBR green technology can be used to quantitate the amount of template RNA, DNA or cDNA in a sample by amplifying a region of interest within the template sequence using a standard PCR primer pair and thermal cycler protocol (Arya et al., 2005). During the extension step of each PCR cycle a fluorescence reading is taken, with the cycle number at which the SYBR green fluorescence rises above a specific threshold (C_T value) used to calculate the concentration of the target sequence in the original sample (Figure 2.6a). Relative quantitation can be performed by comparing a test sample against a control, or absolute quantitation can be performed by comparing a test sample against a standard of known concentration.

Real-time PCR was carried out using the QuantiFast SYBR Green Kit (QIAGEN). The following master mix was made up at room temperature:

Component	Volume per reaction
2X QuantiFast SYBR Green Master Mix (QIAGEN)	7.5 µL
10 µM Forward and Reverse Primer Mix	1.5 µL
Distilled-deionised water	4.5 µL
Total volume	**13.5 µL**

13.5 µL of this master mix was combined with 1.5 µL of template cDNA in a single well of a 96-well iCycler iQ PCR plate (Bio-Rad). Reactions were then covered using Microseal 'B' Film (Bio-Rad), mixed by vortexing and centrifuged before

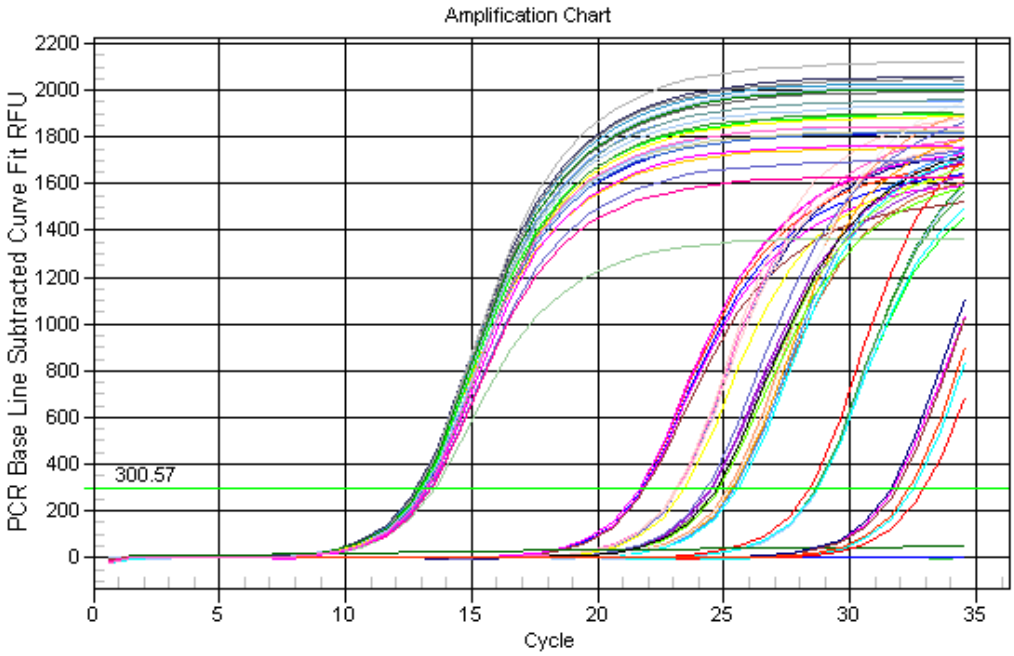

Figure 2.6a. An example of a real-time PCR amplification plot with the baseline threshold value set at 300.57. The C_T values generated by this amplification chart range from ~ 12.5 to ~ 33.

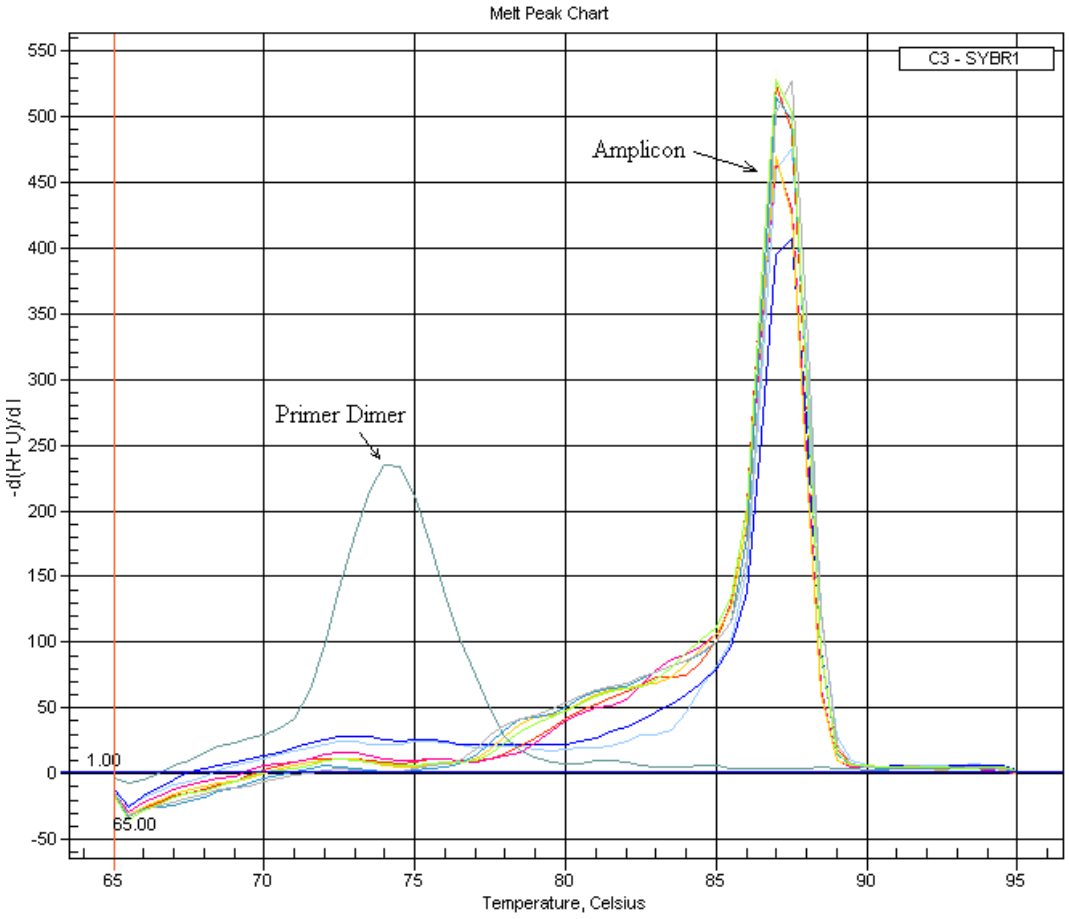

Figure 2.6b. An example of a melting-curve analysis for a set of samples subjected to real-time PCR. The target sequence has been amplified in all samples except one, where amplification of a primer-dimer has occurred (indicated). Note that the melting temperature of the amplified primer-dimer is significantly lower than that of the amplified PCR product (~ 74 °C compared to ~ 87 °C).

being incubated in an iQ5 Multicolor Real-Time PCR Detection System (Bio-Rad) using the following temperature programme:

Temperature	**Duration**	**Cycles**
95 °C	5 min	1
95 °C	10 s	35
60 °C (fluorescence data collection point)	30 s	
Lid temperature constant at 100°C		

Upon completion of the PCR protocol, a melting-curve analysis was performed by the real-time PCR thermal cycler (Figure 2.6b). This can indicate whether a single sequence was amplified in each sample during the thermal cycling. To achieve this, the temperature was slowly increased from 65 °C to 95 °C with a fluorescence reading taken at every 0.5 °C interval. This allows the user to identify the melting temperature of the amplified sequence within each sample, since there is a large decrease in SYBR green fluorescence when the amplified DNA within a sample becomes single-stranded. The amplification of primer-dimers can also be easily detected using this technique, as they usually have a low melting temperature due to their short length. As an additional quality control step, PCR product from randomly selected samples was electrophoresed on an agarose gel to confirm specific amplification of the correct target sequence.

Chapter 3 – Analysis of variation within the *ARHGEF3* gene for association with bone mineral density phenotypes

3.1 Introduction

The *ARHGEF3* gene encodes the Rho guanine nucleotide exchange factor (GEF) 3. Thiesen et al. identified the *ARHGEF3* transcript as composed of 3,561 nucleotides including a large 3' untranslated region (UTR) of 1,853 nucleotides (Thiesen et al., 2000). The transcript encodes a protein of 526 amino acids, expression of which was detected in wide variety of tissues including the heart, brain, kidney, lung, pancreas, spleen and skeletal muscle (Thiesen et al., 2000). The *ARHGEF3* gene was mapped to the 3p14-p22 chromosomal region (Thiesen et al., 2000) and represents a strong candidate gene for BMD regulation.

The function of *ARHGEF3* was first discussed by Arthur et al., who stated that it was a Rho family GEF containing two domains: a Dbl homology (DH) domain which is responsible for catalytic activity and a pleckstrin homology (PH) domain (Arthur et al., 2002) which is thought to provide a site for regulation by phospholipids and localise the GEFs to the plasma membrane where they can regulate their downstream targets (Schmidt and Hall, 2002). The product of the *ARHGEF3* gene specifically activates two members of the RhoGTPase family: RHOA (Arthur et al., 2002), known to play a role in bone (Chellaiah et al., 2000, Meyers et al., 2005), and RHOB (Arthur et al., 2002), with a potential role in osteoarthritis (Gebhard et al., 2004, Mahr et al., 2006). When expressed in fibroblasts *ARHGEF3* has been shown to cause the assembly of more robust stress fibres and focal adhesions than in fibroblasts not expressing the protein through specific activation of the RHOA and RHOB GTPases (Arthur et al., 2002).

Members of the RhoGEF family have been found to have a role in bone cells. A study published by Brazier et al. used gene arrays and quantitative real-time PCR to identify genes encoding members of the RhoGEF and RhoGTPase families that are up-regulated during RANKL-mediated osteoclastogenesis in mouse cells (Brazier et al., 2006). They identified the RhoGTPase gene *Rhou* and the RhoGEF genes *Net1* (also known as *Arhgef8*) and *Dock5* as significantly up-regulated by RANKL treatment (Brazier et al., 2006). Silencing of the *Rhou* and *Net1* genes was subsequently found to inhibit osteoclastogenesis and silencing of *Dock5* was lethal in

osteoclast precursors (Brazier et al., 2006). The product of the murine *Net1* gene has been identified as a Rhoa-specific exchange factor (Alberts and Treisman, 1998).

There is evidence in the literature to suggest that members of the RhoGEF family are involved in human genetic disease with skeletal phenotypes. The *FGD1* gene is located in the p11 region of the X chromosome (Glover et al., 1993, Pasteris et al., 1994) and encodes a RhoGEF protein that specifically activates the Rho family GTPase CDC42 (Zheng et al., 1996). Mutation in this gene has been linked with the genetic disease faciogenital dysplasia (also known Aarskog-Scott syndrome) (Figure 3.1) (MIM 305400) (Kaname et al., 2006, Orrico et al., 2005, Orrico et al., 2004, Orrico et al., 2000, Orrico et al., 2007, Pasteris et al., 1994, Schwartz et al., 2000, Shalev et al., 2006, Bedoyan et al., 2009, Bottani et al., 2007, Orrico et al., 2010). This disorder is rare, having first being described by Aarskog with additional characterisation by Scott (Aarskog, 1970, Scott, 1971). The disorder is characterised by a variety of skeletal abnormalities including short stature, hypertelorism, brachydactyly and retarded bone age (Orrico et al., 2007, Schwartz et al., 2000). Pasteris et al. was the first to identify a point mutation within the *FGD1* gene as responsible for the disorder in a small family with two affected male siblings (Pasteris et al., 1994). This mutation was identified as a single base insertion within exon 7 of the gene, resulting in a frameshift and the formation of a premature stop codon causing the production of a truncated FGD1 protein (Pasteris et al., 1994). Orrico et al. analysed 13 independent patients suffering from faciogenital dysplasia and identified a missense mutation (altering the amino acid sequence of the protein) located within exon 10 of the *FGD1* gene in one patient (Orrico et al., 2000). This mutation was found to reside within one of the two PH domains of the *FGD1* gene and cause the substitution of a highly conserved residue (Orrico et al., 2000). Since these early studies, many incidences of mutation to the *FGD1* gene in patients suffering from faciogenital dysplasia have been reported (Kaname et al., 2006, Orrico et al., 2005, Orrico et al., 2004, Orrico et al., 2007, Shalev et al., 2006, Bedoyan et al., 2009, Bottani et al., 2007, Orrico et al., 2010). These mutations seem to be randomly distributed across all domains of the FGD1 protein (Orrico et al., 2010), some of which have been found to affect the intracellular localisation of the protein and/or its GEF activity (Bedoyan et al., 2009, Orrico et al., 2004, Orrico et al., 2000, Schwartz et al., 2000).

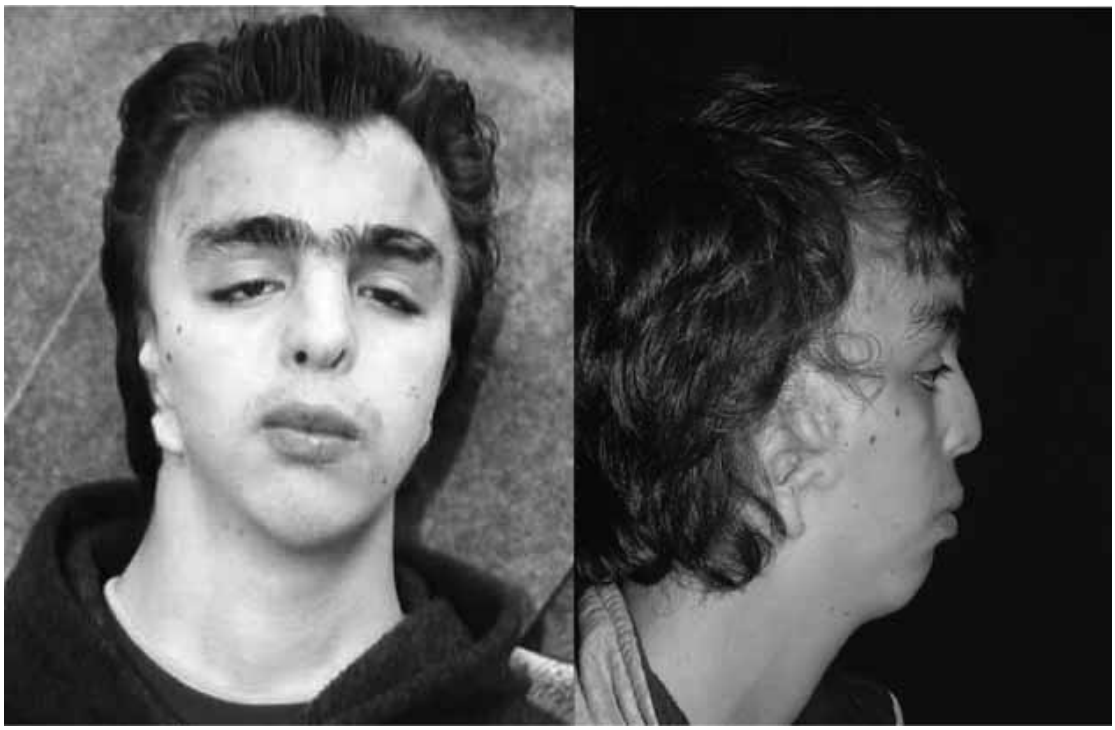

Figure 3.1. A case of faciogenital dysplasia caused by mutation to the *FGD1* gene. Note the clinical craniofacial features: palpebral ptosis (drooping of the upper eyelid), downslanting palpebral fissures (separation between the upper and lower eyelids), long philtrum and low-set ears. Figure adapted from Orrico et al. (Orrico et al., 2007).

The pattern of *Fgd1* expression has been examined in mice and has been found to correlate with faciogenital dysplasia skeletal manifestations (Gorski et al., 2000). Initial expression of *Fgd1* was found during the onset of embryonic ossification in regions of active bone formation and up-regulation of the gene was found to cause an increase in expression of osteopontin (Gorski et al., 2000), a protein synthesised by osteoblasts at the onset of matrix mineralisation (Bianco et al., 1991). This would suggest that *FGD1* plays a role in ossification and bone formation and not in earlier stages of skeletogenesis. Evidence has also been presented to suggest that FGD1 is preferentially associated with the *trans*-Golgi network (TGN) and that reduced expression of FGD1 causes a reduction in post-Golgi transport of various cargoes including bone-specific proteins in osteoblasts (Egorov et al., 2009). These effects were found to be dependent upon CDC42 activation by FGD1 and appear to be caused by impairment of TGN membrane extension along microtubules (Egorov et al., 2009).

Due to the apparent role of members of the RhoGEF family in bone and the location of the *ARHGEF3* gene in the 3p14-p22 chromosomal region, the aim of the work described in this chapter was to analyse common variation within the *ARHGEF3* gene for association with BMD parameters in a discovery cohort of Caucasian women. Replication of significant findings was subsequently performed in an independent replication cohort.

3.1.1 Hypothesis

Polymorphism within the *ARHGEF3* gene is significantly associated with bone density in Caucasian women.

3.2 Materials and methods

3.2.1 Subjects

3.2.1.i Discovery cohort

A total of 769 women from 335 families were recruited in Australia and the UK. This family-based population included siblings recruited in 1998 for a study of the genetics of osteoporosis (Wilson et al., 2003). BMD Z-score values for the proband at the spine, total hip and femoral neck sites were Z = - 1.54 (1.03), - 1.00 (0.95) and - 1.03 (1.05) respectively (median (interquartile range)). Sibships within the cohort

included two-hundred and sixty-four families with 2 siblings, forty-nine with 3, seventeen with 4, four with 5 and one with 7 siblings. The median difference in Z score between sibs with extreme BMD measurements were: spine Z = 2.2, total hip Z = 1.5 and femoral neck Z = 1.6. Exclusion criteria were applied where possible and included the presence of bone cancer, hyperparathyroidism, unstable thyroid disease, long term steroid use (> 5 mg/day for more than 6 months and presently on therapy), chronic immobility, rheumatoid arthritis, anorexia nervosa, osteomalacia, amenorrhea for > 6 months, premature cessation of regular menstruation or surgical oophorectomy +/- HRT (age < 40 yrs), and epilepsy with use of anticonvulsant medication for > 1 year. All subjects from the study provided written informed consent, and the ethics committees of participating institutions including the Human Research Ethics Committee at Curtin University of Technology approved the experimental protocols.

At a clinic visit data including age, height, weight, medical, gynaecological, and lifestyle data were recorded and a blood sample collected. Dual energy X-ray absorptiometry (DXA) BMD was assessed (Hologic Inc., Bedford, MA, USA) at the lumbar spine L1-L4 and the total hip that includes an area from the femoral neck to just below the lesser trochanter. Within this area the femoral neck sub-region is widely used in clinical practice for prediction of fracture propensity and was the phenotype chosen for use in this study. Due to the range of ages in this cohort, BMD data were adjusted for age prior to analysis by conversion to BMD Z-scores. The correlations between the BMD Z scores obtained from different sites were r = 0.82 (total hip and femoral neck), r = 0.63 (total hip and spine) and r = 0.52 (femoral neck and spine).

3.2.1.ii Replication cohort

This group of subjects was recruited in 1988 to participate in a longitudinal epidemiological study of rheumatic diseases (The Chingford Study). Women between the ages of 45 and 64 were recruited from a single large general practice in Chingford, North-East London using a population-based method. All women within this age range that were on a register of > 11,000 patients were invited to participate in the study. No exclusion criteria were applied. This cohort has similar demographics and anthropometry to the general UK population regarding height,

weight, smoking status and hysterectomy rates (Arden et al., 1996, Hart and Spector, 1993). Demographic and lifestyle factors data were obtained by questionnaires completed in 1988. DNA samples were obtained from 780 individuals. Bone density measurements were undertaken using a Hologic QDR-2000 densitometer (Hologic Inc., Bedford, MA, USA) in 1998 approximately 10 years after the subjects were initially recruited, with hip and spine DXA BMD data obtained from 775 and 779 individuals respectively. The correlations between the BMD measurements obtained from different sites were r = 0.89 (total hip and femoral neck), r = 0.7 (total hip and spine) and r = 0.67 (femoral neck and spine). Subjects were categorised as fracture-free or having had a previous fracture as described previously (Keen et al., 1999), with fractures sustained at any skeletal site up to 2003 included in the analysis excluding those caused by high-impact trauma. All subjects from the study provided written informed consent, and the ethics committees of participating institutions including the Human Research Ethics Committee at Curtin University of Technology approved the experimental protocols.

3.2.2 Genotyping

3.2.2.i Discovery cohort

Genomic DNA was extracted and purified from EDTA whole blood obtained from each subject (Johns and Paulus-Thomas, 1989). Whole-genome amplification was performed on each DNA sample using the REPLI-g Midi Kit (QIAGEN) (see *Chapter 2* for a full description of the technique). Genotyping was then performed on the amplified DNA using the Illumina GoldenGate assay on an Illumina BeadStation 500 GX, utilising bead array hybridisation (Illumina, 2004). This part of the analysis was performed by staff at Illumina Inc. (San Diego, USA). The genotype call rate using this technique was 99.8 % with an estimated error rate of < 0.1 %, as assessed by comparing genotype calls between 8 samples genotyped in duplicate.

3.2.2.ii Replication cohort

Genotyping in the replication cohort was performed in-house using the MALDI-ToF mass spectrometry technique (Wise et al., 2003) (see *Chapter 2* for a full description of the technique). Genotyping reactions were performed using sequence specific oligonucleotide primers designed using Primer3 software (Rozen and Skaletsky, 2000) in a multiplex PCR (see Appendix II for primer sequences). The volume of

each genotyping primer used was optimised prior to analysis of the test samples. Using this technique the genotype call rate was 96.6 % and the estimated error rate was < 0.1 %, as assessed by comparing genotype calls between 8 samples genotyped in duplicate.

3.2.3 SNP selection

17 SNPs were selected in the region of the *ARHGEF3* gene for genotyping in the discovery cohort. Tagging SNPs (tSNPs) were initially selected across the region using the Perlegen Genome Browser (European American population) (Hinds et al., 2005), which at the time was considered to be one of the most comprehensive resources available for viewing patterns of linkage disequilibrium (LD) across the human genome. The Perlegen Genome Browser identifies tSNPs as being in linkage LD of $r^2 \geq 0.8$ with all other SNPs in the LD bin. An effort was made to tag all 13 LD bins identified within the gene and the 50 Kb region immediately 5' of the gene that contained 2 or more SNPs. Due to assay design issues, 9 of the 13 LD bins were able to be tagged. The remaining SNPs genotyped were selected from the Perlegen Genome Browser (European American population) (Hinds et al., 2005) as independent SNPs not belonging to any LD bin, or were selected from the dbSNP database.

One SNP from the 17 genotyped in the discovery cohort was initially selected for genotyping in the replication cohort. In subsequent studies, made possible by the release of more detailed LD data for the European American population in the Perlegen Genome Browser (Hinds et al., 2005), three additional tSNPs around the 5' end of *ARHGEF3* were selected for genotyping in the replication cohort using a tagging strength of $r^2 \geq 0.8$. Two extra SNPs from the 17 genotyped in the discovery cohort were also analysed at this time.

3.2.4 Statistical analysis

Analysis of the data from the discovery cohort was performed using the FBAT (Family Based Association Tests) software to test for association within sib-pairs (Laird et al., 2000). The empirical variance estimator was used to allow for prior linkage to the region. Correction for multiple testing was performed by randomly permuting phenotypes within sibships and repeating all FBAT tests on the permuted

datasets. The minimum P-values were recorded for 10,000 random reassignments of the data (using an automated script written in Perl), with an adjusted P-value ≤ 0.05 considered significant. This approach was used to correct for tests of multiple SNPs within each phenotype, and also for tests of multiple SNPs across multiple phenotypes. For the latter, correlation between phenotypes was preserved by permuting the whole phenotype vector for each subject. For individual BMD scores, adjusting for multiple SNPs, this corresponds to an unadjusted P-value of about 0.0037. For adjustment for testing BMD scores at three sites, the corresponding unadjusted P-value was 0.002. To examine the effect of reducing the number of correlated traits, principal component analysis was carried out. The effect of menopausal status on BMD in the discovery cohort was analysed using multiple linear regression implemented in Statistica v8.0.

QPDTPHASE v2.404, which is part of the UNPHASED software suite (Dudbridge, 2003), is a program for association analysis of multilocus haplotypes from unphased genotype data and was used to estimate the genetic effect size in the discovery cohort. This program was also used to perform a haplotype analysis. Throughout, two-tailed P-values are reported, with $P \leq 0.05$ considered significant. LD between the different SNPs was evaluated using the JLIN software (Carter et al., 2006) and Graphical Overview of Linkage Disequilibrium (GOLD) (Abecasis and Cookson, 2000).

Statistical analysis of the data from the replication cohort was performed using Statistica v8.0. One-way analysis of variance (ANOVA) was used to test for differences between genotype groups. BMD data were adjusted for age and weight using analysis of co-variance (ANCOVA). QTPHASE v2.404, which is also part of the UNPHASED software suite (Dudbridge, 2003), was used to perform an allelic association test for the replication cohort. Genotype effects on the prevalent fracture rate were examined using a Chi-square test.

3.3 Results

3.3.1 Discovery cohort

All SNPs genotyped were in Hardy-Weinberg equilibrium (χ^2 test, $P < 0.05$). The demographic and morphometric characteristics of the populations are detailed in

Table 3.1. The discovery cohort recorded a lower mean BMD than the replication cohort at each site studied despite a younger mean age, which was expected due to the high proportion of osteoporotic individuals in this population. The discovery cohort also recorded a lower mean weight than the replication cohort. For the total hip and femoral neck sites, menopausal status was not a significant predictor of BMD. At the spine, menopausal status was found to account for < 1% of the variance in BMD.

The chromosomal position and allele distribution of the 17 SNPs genotyped is detailed in Table 3.2 with the location of each SNP relative to the various splice variants of *ARHGEF3* displayed in Figure 3.2. Using FBAT, significant associations were seen between the SNPs rs4681928, rs1344142, rs1110866, rs7646054 and rs3772219 and various measures of BMD Z score ($P < 0.001 – 0.041$). The strongest associations were observed with rs7646054, which was associated with BMD Z score at the total hip ($P = 0.006$), femoral neck ($P < 0.001$) and spine ($P = 0.006$). Among the other SNPs, rs4681928 was significantly associated with BMD Z score of the femoral neck ($P = 0.01$) and spine ($P = 0.03$), rs1344142 with spine ($P = 0.04$), rs1110866 with femoral neck ($P = 0.02$) and rs3772219 with spine ($P = 0.03$). The significant association between rs7646054 and femoral neck BMD Z score persisted after adjustment of the data for testing multiple SNPs ($P = 0.007$), as did the association corrected further for testing multiple anatomical sites ($P = 0.024$).

Principal component analysis was also applied to the BMD trait group. The first two principal components explained 94.5 % of the trait variance. However, using the eigenvalue > 1 criterion, only the first component, which explained 77.3 % of the variance in BMD, should be retained. This component showed maximal association with rs7646054 ($P = 0.002$).

The more common *G* allele at rs7646054 is associated with a lower BMD Z score at each site studied (Table 3.3), indicating that this allele has a negative effect on BMD. Note that the mean BMD Z scores reported by QPDTPHASE are loosely interpreted as the expected trait value for an individual carrying that particular allele, and are not interpretable as additive effects on the mean.

Table 3.1. Demographics and bone density parameters of the discovery and replication populations.

Variable	Discovery	Replication
Age (years)	54.2 ± 12.7 (769)	62.5 ± 5.9 (780)
Weight (Kg)	62.7 ± 11.27 (699)	69.1 ± 12.6 (778)
Prevalent fractures (%)	-	34 (780)
Total Hip DXA BMD (mg/cm^2)	801 ± 136 (760)	869 ± 128 (775)
Total Hip BMD Z Score	- 0.420 ± 0.992 (760)	0.489 ± 0.994 (775)
Femoral Neck DXA BMD (mg/cm^2)	700 ± 133 (749)	747 ± 119 (775)
Femoral Neck BMD Z Score	- 0.355 ± 1.050 (749)	0.276 ± 1.019 (775)
Spine L1-L4 DXA BMD (mg/cm^2)	855 ± 158 (767)	955 ± 155 (779)
Spine BMD Z Score	- 0.669 ± 1.252 (767)	0.745 ± 1.384 (779)

Results are given as *mean ± SD (number of measurements)*.

Table 3.2. Position and allele distribution of all *ARHGEF3* SNPs genotyped.

SNP	Chromosome Position*	Function/ Location*	Genotype Distribution in the Discovery Cohort (%)
rs17288993	56940107	5' region	AA (79.7), AG (18.8), GG (1.4)
rs4681928	56901206	5' region	AA (63.6) (65.7)†, AG (33.5) (30)†, GG (2.9) (4.3)†
rs1039381	56849769	5' region	AA (61.8), AG (35), GG (3.1)
rs9311623	56846106	5' region	TT (56.8), TC (38), CC (5.2)
rs9842692	56844958	5' region	CC (73.1), TC (24.7), TT (2.2)
rs1344142	56832473	5' region	GG (26.4) (30.1)†, AG (52.6) (48.1)†, AA (21) (21.8)†
rs17288922	56826427	5' region	GG (68.4), AG (29.4), AA (2.2)
rs1566487	56822871	5' region	TT (36.8)†, TC (49.6)†, CC (13.6)†
rs4681898	56811780	5' region	CC (44.1), TC (43.6), TT (12.2)
rs13434307	56809329	Intron 1	AA (75.9), AG (22.8), GG (1.3)
rs6803697	56806588	Intron 1	GG (49)†, AG (42)†, AA (9)†
rs4681888	56805127	Intron 1	TT (70.1), TC (26.4), CC (3.5)
rs12632941	56804703	Intron 1	GG (70.8)†, AG (26.8)†, AA (2.4)†
rs1110866	56801364	Intron 1	AA (41.2), AC (47.2), CC (11.6)
rs7646054	56784668	Intron 1	GG (29.1) (33.2)†, AG (52.1) (47.4)†, AA (18.8) (19.4)†
rs2171855	56764783	Intron 2	TT (73.8), TC (23.3), CC (2.9)
rs3821412	56762491	Intron 4	AA (24.3), AG (53.1), GG (22.6)
rs3772219	56746291	Exon 8 Change of aa 335 (Leu to Val)	TT (45.6), TG (42.5), GG (11.9)
rs11919130	56732292	3' region	CC (73), TC (25.4), TT (1.7)
rs2317248	56714619	3' region	TT (33.9), AT (48.6), AA (17.6)

* From GenBank reference sequence NM_019555, Genome Build 36.2.

† Allele distribution in the replication cohort.

aa – amino acid.

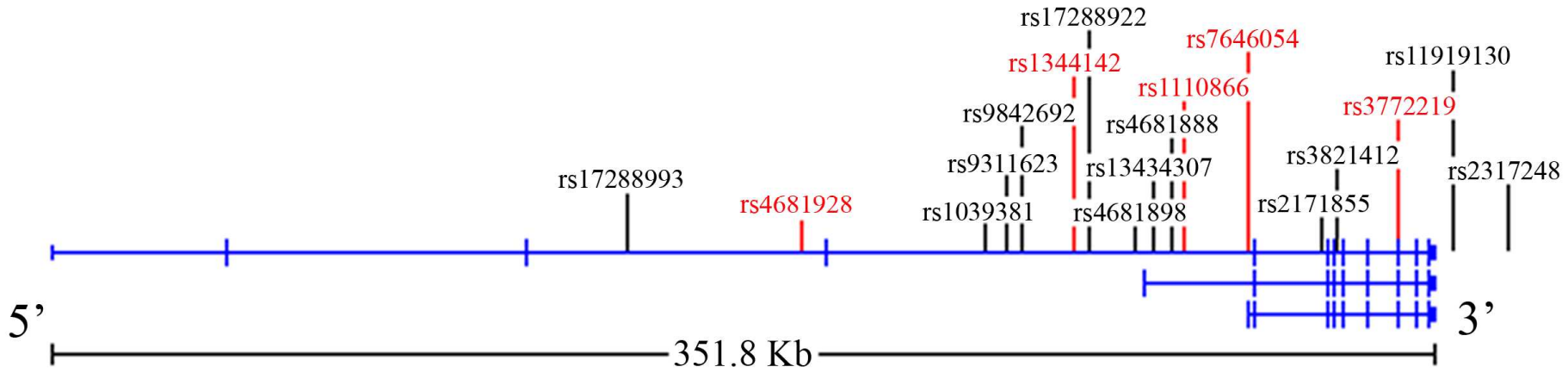

Figure 3.2. 17 SNPs genotyped in the *ARHGEF3* gene. Diagram showing the localisation of the 17 SNPs in *ARHGEF3* genotyped in the discovery cohort relative to the 3 RefSeq splice variants of the gene according to the University of California - Santa Cruz (UCSC) Genome Browser (February 2009 assembly). The SNPs highlighted in red are those that are significantly associated with BMD parameters in the discovery cohort prior to adjustment for multiple testing.

Table 3.3. Association between rs7646054 and BMD Z scores in the discovery cohort.

BMD Z Score Phenotype	Mean BMD Z Score		*P*
	G allele	*A allele*	
Total hip	- 0.437 ± 1.235 (837)	- 0.399 ± 1.222 (683)	**0.013**
Femoral neck	- 0.383 ± 1.325 (825)	- 0.322 ± 1.264 (673)	**0.001**
Spine	- 0.686 ± 1.619 (842)	- 0.650 ± 1.538 (692)	**0.008**

Results are given as *mean ± SD (number of alleles contributing to the mean)*, derived from QPDTPHASE v2.404.

Pair-wise linkage disequilibrium D' and r^2 values for the five SNPs are shown in Figure 3.3. A haplotype analysis was undertaken on the femoral neck and spine BMD Z score data using UNPHASED to determine whether any haplotypes were more strongly associated with either phenotype than individual SNPs. Each haplotype analysis incorporated only the 3 SNPs that were most significantly associated with the phenotype in the FBAT analysis. For femoral neck BMD Z score the SNPs included in the haplotype analysis were rs4681928, rs1110866 and rs7646054 whereas the SNPs rs4681928, rs7646054 and rs3772219 were included in the spine BMD Z score analysis. Significant associations were observed with both phenotypes (Table 3.4), including a stronger overall association with spine BMD Z score than in the individual SNP analysis suggesting independent effects of the SNPs on BMD. In the femoral neck BMD haplotype analysis the significance of the overall association did not surpass that of the individual SNP analysis. However, a very strong association was observed between femoral neck BMD Z score and the most common *AAA* haplotype (588 alleles) in a post hoc analysis ($P < 0.001$).

3.3.2 Replication study

Because rs7646054 was the strongest predictor of spine and hip bone density, it was taken forward to the replication study to determine whether the effect would be detectable in a population-based cohort of postmenopausal women. Significant associations were observed between rs7646054 and spine and total hip BMD including the femoral neck area, all of which persisted after adjustment of the BMD data for the covariates age and weight (Table 3.5). Consistent with the results for the discovery cohort, subjects homozygous for the *G* allele compared to individuals homozygous for the *A* allele had lower BMD at the total hip, femoral neck and spine sites (- 3.7 %, - 3.3 %, and - 3.5 % respectively). Compared to heterozygous individuals with the *AG* genotype, *GG* individuals again had lower BMD at the three sites (- 1.8 %, - 2.4 % and - 3.7 % respectively). No significant associations between genotype and the covariates age or weight were found.

An allelic association test of the replication cohort using BMD Z score as the phenotype was then carried out using QTPHASE to confirm replication with the same type of association test and the same phenotype as that used for the discovery cohort. Significant associations were observed between rs7646054 and total hip

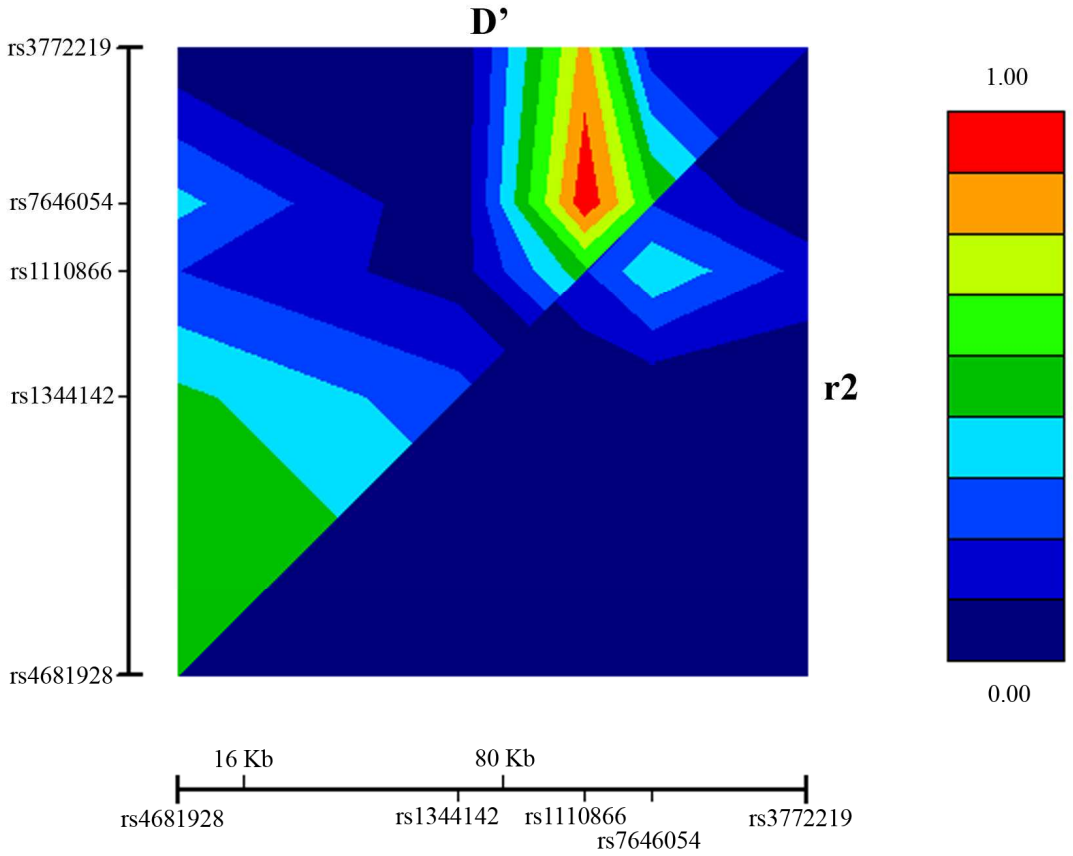

Figure 3.3. Pairwise linkage disequilibrium plot for the five SNPs in the *ARHGEF3* gene associated with BMD in the discovery population. Different colours represent the strength of LD according to the scale shown on the right.

Table 3.4. BMD Z-scores for *ARHGEF3* 3-SNP haplotypes in the discovery cohort.

BMD Z Score Phenotype	Haplotype (rs4681928, rs1110866, rs7646054)						
	AAA allele	*ACG allele*	*AAG allele*	*GCG allele*	*GAA allele*	*GAG allele*	*P*
Femoral neck	- 0.341 ± 1.188 (588)[c]	- 0.376 ± 1.162 (374)	- 0.408 ± 1.129 (239)	- 0.358 ± 0.974 (144)[a]	- 0.205 ± 1.072 (81)	- 0.359 ± 0.781 (65)	**0.003**
	Haplotype (rs4681928, rs7646054, rs3772219)						
	AGT allele	*AAT allele*	*AAG allele*	*AGG allele*	*GGT allele*	*GGG allele*	*P*
Spine	- 0.677 ± 1.378 (450)[a]	- 0.573 ± 1.299 (375)[a]	- 0.720 ± 1.187 (228)[a]	- 0.665 ± 1.171 (177)	- 0.725 ± 1.161 (151)[b]	- 0.722 ± 0.864 (61)	**0.002**

Results are given as *mean ± SD (number of alleles)*, derived from QPDTPHASE v2.404.

[a]*P < 0.05*, [b]*P < 0.01* and [c]*P < 0.001* for individual haplotypes compared to all others.

Table 3.5. Osteodensitometry parameters and fracture rate in relation to the genotype distribution of rs7646054 in the replication cohort.

Phenotype	*AA* Genotype	*AG* genotype	*GG* genotype	*P*
Total Hip DXA BMD (mg/cm^2)	889 ± 128[a] (150)	872 ± 128[a] (354)	857 ± 122[b] (252)	**0.013**
Femoral Neck DXA BMD (mg/cm^2)	759 ± 115[a] (150)	753 ± 120[a] (354)	735 ± 117[b] (252)	**0.038**
Spine DXA BMD (mg/cm^2)	967 ± 172[a] (150)	969 ± 148[a] (356)	934 ± 152[b] (253)	**0.007**
Prevalent Fracture (%)	36.4 (151)	29.1 (358)	39.1 (253)	**0.026**

Results are given as *mean ± SD (number of measurements)*.

The BMD data are adjusted for age and weight.

[a] significantly different from [b] in post hoc analysis ($P < 0.05$).

BMD Z score (*P = 0.007*), femoral neck BMD Z score (*P = 0.02*) and spine BMD Z score (*P = 0.02*).

In the replication cohort 265 subjects had suffered a fracture prior to 2004, giving a prevalent fracture rate of 34 %. rs7646054 was found to be significantly associated with fracture rate, *GG* individuals having an increased fracture rate (Table 3.5).

As mentioned previously, more comprehensive genome-wide LD data became available for the European American population in the Perlegen Genome Browser (Hinds et al., 2005). Subsequently, a more detailed pattern of LD across the *ARHGEF3* gene in the European American population became apparent. In an effort to tag additional variation around the 5' region of the gene, three additional tSNPs around the 5' end of *ARHGEF3* were selected for genotyping in the replication cohort. These SNPs are rs1566487, rs6803697 and rs12632941. In addition to this, two SNPs from the 17 genotyped in the discovery cohort that were significantly associated with BMD phenotypes were also analysed for replication: rs4681928 and rs1344142. No significant associations were observed between any of the additional 5 SNPs genotyped and BMD phenotypes. The chromosomal position and allele distribution of these SNPs in this cohort is detailed in Table 3.2.

3.4 Discussion

The data presented in this chapter provide evidence that variation within the *ARHGEF3* gene affects BMD in women aged 40 to 70 in the peri and early postmenopausal phase of life, at the time that fracture risk is rising. Strong evidence was found for an influence of individual SNPs and haplotypes on spine and femoral neck BMD Z score. The SNP rs7646054, located within intron 1 of the *ARHGEF3* gene, was the best predictor of bone density and the results were replicated in a second cohort including a statistically significant association with fracture rate. In general, fracture risk rises by about two times for a reduction in hip BMD of one standard deviation (15 % of the mean) (Marshall et al., 1996). We saw decreases in BMD of about 3 % that equate to an increase in fracture risk for the "disease" genotype of about 20 %, in keeping with the observed fracture rate in the replication cohort.

Using the genetic power calculator developed by Purcell and colleagues (Purcell et al., 2003), with QTL variance set as 0.05, QTL and marker frequency 0.5 and 0.55 respectively, D' = 0.9 and sib correlation of 0.4, the power of the discovery cohort is 0.91 at a type-1 error rate of 0.05. If the QTL variance is only 0.03, then the power is 0.72. Similarly for the replication cohort, if the QTL variance is set as 0.05 then the power for this study is 0.99, whilst for a QTL variance of 0.03 the power is estimated as 0.98.

Since rs7646054 is located within an intron, it is unlikely that polymorphism at this site would result in structural changes to the ARHGEF3 protein. According to updated LD data provided by the International HapMap Project (Phase 3 dataset) (Altshuler et al., 2010), rs7646054 is in strong LD ($r^2 > 0.8$) with the SNPs rs983739 and rs4681859 in the CEU population (Utah residents with Northern and Western European ancestry). These two SNPs are both located 5' of rs7646054, with the LD between the three SNPs spanning a region of approximately 13.7 Kb located within the first intron of the predominantly expressed *ARHGEF3* transcript (RefSeq accession number NM_019555) (Figure 3.4). The region is adjacent to exon 2 of this splice variant, although no polymorphisms within this exon have been identified to date. The region is, however, positioned immediately 5' of a recently described transcript variant of *ARHGEF3* (RefSeq accession number NM_001128616) (Totoki et al., 2007). Indeed, the rs7646054 SNP is located within the 5' UTR of this transcript. Since mRNA UTRs have been identified as an integral component of post-transcriptional regulation of gene expression (Mignone et al., 2002), the 5' UTR sequence from the NM_001128616 transcript was analysed using the Vienna RNA Websuite (Gruber et al., 2008) using both the *A* and *G* alleles at the rs7646054 SNP site (Figures 3.5a and 3.5b respectively). The minimum free energy, which is the difference in free energy between the unfolded and folded mRNA states (Ringner and Krogh, 2005), was 3.5 kcal/mol lower for the 5' UTR containing the *A* allele than for the 5' UTR containing the *G* allele. This would suggest that the mRNA transcript containing the *A* allele has a more stable secondary structure, a feature that is associated with lower translation rates and higher transcript turnover in yeast (Muhlrad et al., 1995, Ringner and Krogh, 2005). Indeed, this effect has been observed with differences in minimum free energy as small as 1 kcal/mol (Ringner and Krogh, 2005). Collectively, these data would suggest that polymorphism at

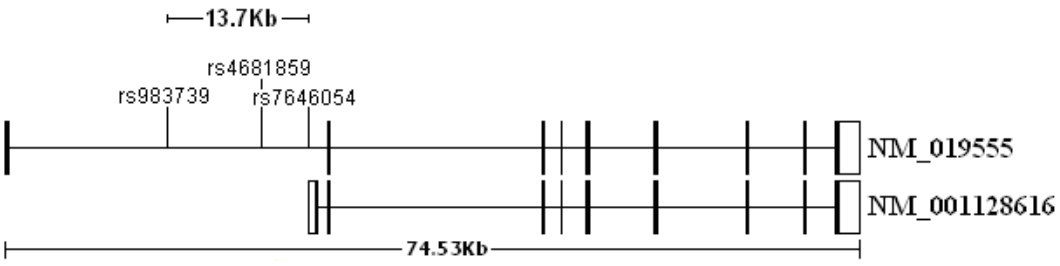

Figure 3.4. The location of 3 SNPs that are in strong LD, relative to two splice variants of *ARHGEF3*. The solid boxes represent exons and the empty boxes represent untranslated regions (UTRs).

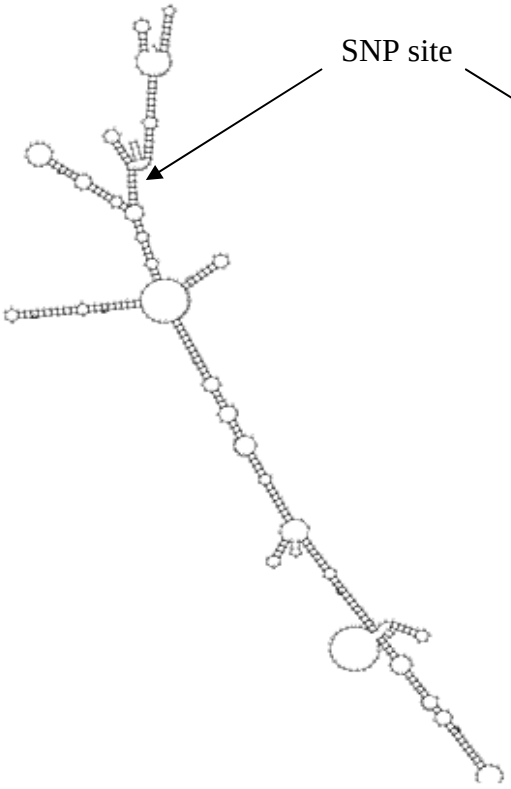

Figure 3.5a. Schematic of the 5' UTR of NM_001128616 containing the less common *A* allele at rs7646054. Minimum free energy: -165.1 kcal/mol.

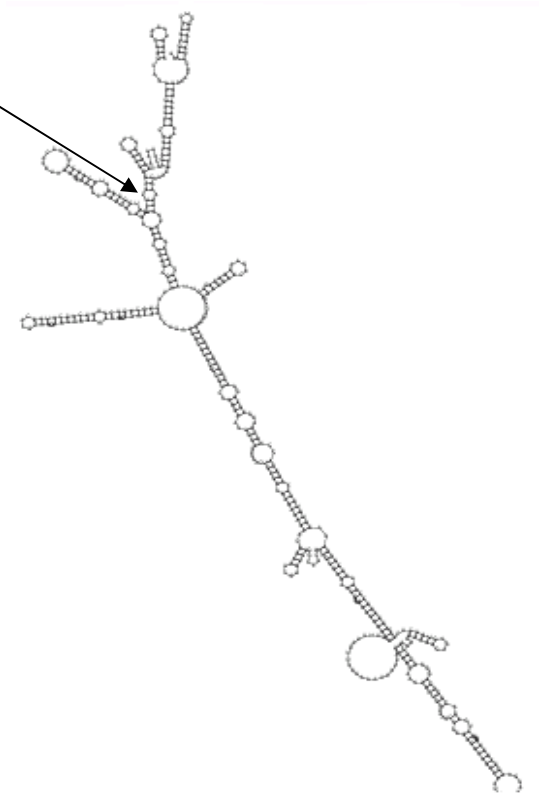

Figure 3.5b. Schematic of the 5' UTR of NM_001128616 containing the more common *G* allele at rs7646054. Minimum free energy: -161.6 kcal/mol.

rs7646054 could influence the stability and hence the expression of the NM_001128616 mRNA transcript of the *ARHGEF3* gene. Future studies could investigate whether polymorphism at this SNP site is associated with NM_001128616 mRNA levels (discussed in *Chapter 7*).

A recent study published by Li et al. analysed the rs7646054 SNP from *ARHGEF3* for association with BMD parameters in a case-control population of 1,080 Chinese females, 533 of whom were postmenopausal (Li et al., 2010). The authors did not observe any associations between the rs7646054 SNP and BMD (Li et al., 2010). Comparisons between this study and the results presented in this chapter must be done with caution due to differences in study design as well as differences in the ethnicity of the study subjects. There are well documented differences in typical bone mass and bone turnover phenotypes between different ethnic groups (Araujo et al., 2007, Davis et al., 1994, Finkelstein et al., 2002, Nelson et al., 2000, Tobias et al., 1994) and it has been suggested that these groups may also have different genetic susceptibility loci for osteoporosis (Dvornyk et al., 2003, Lei et al., 2006). There is also a substantial difference in the frequency of the *A* and *G* alleles at the rs7646054 SNP site in Caucasian and Chinese populations. According to the International HapMap Project (Phase 3 dataset) (Altshuler et al., 2010) the frequency of the *A* and *G* alleles in the CEU population is 0.46 and 0.54 respectively, which is similar to those seen in our study populations (0.45 and 0.55 respectively in our discovery cohort; 0.43 and 0.57 respectively in our replication cohort). Li et al. did not publish the allele frequencies that they observed for rs7646054 in their study population (Li et al., 2010), however according to the International HapMap Project (Phase 3 dataset) (Altshuler et al., 2010) the frequency of the *A* and *G* alleles for rs7646054 in the CHB population (Han Chinese in Beijing, China) is 0.19 and 0.81 respectively.

The primary role of the RhoGEFs such as ARHGEF3 is to activate the RhoGTPases, key regulators of actin dynamics that cycle between an inactive GDP-bound and an active GTP-bound state (Figure 3.6). RhoGEFs achieve this by catalysing the exchange of GDP for GTP through stabilisation of the nucleotide-free state (Rossman et al., 2005). GTP then spontaneously binds and renders the protein active. The RHOA GTPase, which is activated by the product of the *ARHGEF3* gene (Arthur et al., 2002), has been implicated in osteoblast differentiation and osteoclast

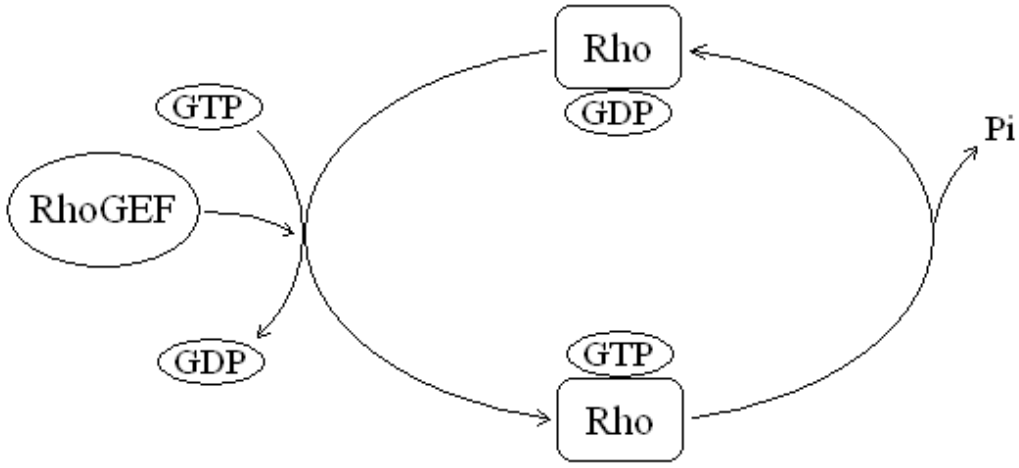

Figure 3.6. Mechanism of action of the RhoGEFs. The RhoGEFs work to activate the RhoGTPases, which cycle between an inactive GDP-bound and an active GTP-bound state. The RhoGEFs achieve this by catalysing the exchange of GDP for GTP.

function (discussed in *Chapter 4*). The RHOB GTPase, which is also activated by the product of the *ARHGEF3* gene (Arthur et al., 2002), seems to have a role in cartilage biology, having been linked to osteoarthritis in which a chondrocyte disorder plays a major role (Gebhard et al., 2004, Mahr et al., 2006).

In conclusion, it has been shown that genetic variation within the *ARHGEF3* gene is associated with variation in BMD in Caucasian women, possibly through effects on mRNA folding and stability. Therefore, the hypothesis has been supported by the data. There is evidence in the literature to suggest that the two RhoGTPases that are specifically activated by the product of the *ARHGEF3* gene, RHOA and RHOB, have a role in bone and cartilage cell biology.

Chapter 4 – Analysis of variation within the *RHOA* gene for association with bone mineral density phenotypes

4.1 Introduction

The Rho genes were first isolated from a cDNA library created from RNA obtained from abdominal ganglia of molluscs (Aplysia) (Madaule and Axel, 1985). Evolutionarily conserved counterparts encoding proteins of around 21 kDa in size were subsequently identified in flies, yeast cells, rats and humans (Madaule and Axel, 1985). Indeed, these homologs were so conserved that amino-acid sequence analysis revealed 96.8 % homology between the human and Aplysia proteins (Madaule and Axel, 1985). The mammalian Rho family of GTPases belongs to the Ras superfamily of small GTP-binding proteins. It contains 20 members from eight subfamilies (Boureux et al., 2007) that cycle between an inactive GDP-bound and an active GTP-bound state. The three most commonly studied members of the Rho family are RHOA, CDC42 and RAC1, and these three proteins have been shown to interact with each other (Nobes and Hall, 1995). The Rho family of proteins can be distinguished from other small GTPases by the presence of the Rho insert domain which is located between the fifth β strand and the fourth α helix in the small GTPase domain (Valencia et al., 1991). There are three types of regulatory protein that have been shown to influence the activation state of the RhoGTPases: the GEFs, of which ARHGEF3 is a member, which catalyse the exchange of GDP for GTP thus activating the RhoGTPases (Hart et al., 1996); the GDIs (GDP dissociation inhibitors), which inhibit the release of GDP, thereby keeping the RhoGTPases in an inactive state (Fukumoto et al., 1990); and the GAPs (GTPase activating proteins), which increase the hydrolysis rate of GTP by the Rho proteins and thus the rate at which they become inactivated (Diekmann et al., 1991). The human *RHOA* gene, which encodes the Ras homolog gene family member A, is situated within the 3p14-p22 chromosomal region (Cannizzaro et al., 1990, Kiss et al., 1997). The human RHOA protein is 193 amino acids in length (Avraham and Weinberg, 1989, Yeramian et al., 1987) and shares 88 % homology with the RHOB protein (Avraham and Weinberg, 1989). It is located primarily in the cytosol with smaller amounts bound to the plasma membrane (Adamson et al., 1992).

Paterson et al. injected a constitutively active GTP-bound form of RHOA into Swiss 3T3 cells, observing contraction of the cell bodies and formation of finger-like

processes at the cell periphery within minutes (Paterson et al., 1990). Subsequent actin staining with phalloidin revealed strong actin stress fibres extending from these finger-like processes across the cell, leading the authors to suggest that the RHOA protein has a role in cytoskeletal organisation (Paterson et al., 1990). A later study also using Swiss 3T3 cells found that RHOA could be activated by the addition of extracellular ligands (for example, lysophosphatidic acid) and that activation of this protein lead to the assembly of contractile actin-myosin stress fibres and focal adhesion complexes (Ridley and Hall, 1992). There is also evidence to suggest that RHOA activity is essential to maintain focal adhesions in attached cells (Hotchin and Hall, 1995). These combined data suggest that the RhoGTPase family members are key regulatory molecules that link cell surface receptors with the actin cytoskeleton (Hall, 1998).

There is evidence from gene knockout work in mice that the RhoGTPases have a role in skeletal development. Wang et al. generated mice with a targeted disruption of the *Arhgap1* gene (Wang et al., 2005a), the product of which has been shown to deactivate the RhoGTPase CDC42 by catalysing GTP hydrolysis of the protein (Moon and Zheng, 2003). The authors found that embryonic and neonatal mice homozygous for the disruption were approximately 25 – 40 % smaller than those that were wild-type and suffered severe growth retardation (Wang et al., 2005a). A subsequent study by the same group found that *Arhgap1*-knockout mice presented with shortened lifespan, multiple premature aging-like phenotypes, osteoporosis and severe lordokyphosis (Figure 4.1) (Wang et al., 2007). This led the authors to speculate that CDC42 has a role in DNA damage repair ability and aging related physiology (Wang et al., 2007).

Due to the location of the *RHOA* gene in the 3p14-p22 chromosomal region, its role in bone cells (discussed below), the apparent role of the RhoGTPase CDC42 in bone and the associations observed between polymorphism in the *ARHGEF3* gene and BMD phenotypes, the aim of the work described in this chapter was to analyse polymorphism within the *RHOA* gene for association with BMD parameters in a discovery cohort of Caucasian women. Replication of significant findings was subsequently performed in an independent replication cohort. These are the same cohorts as those used in the *ARHGEF3* study.

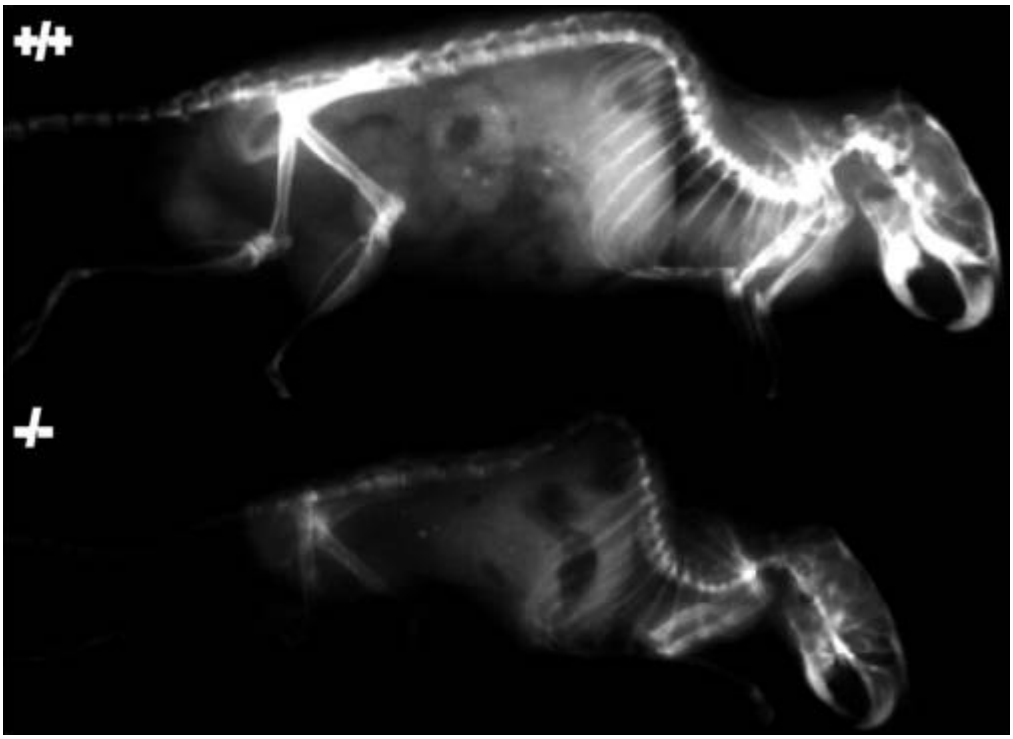

Figure 4.1. X-rays of 15-month old male wild type and *Arhgap1* knockout mice. Note the severe lordokyphosis and reduced bone density in the knockout mouse. Figure adapted from Wang et al. (Wang et al., 2007).

4.1.1 Hypothesis

Polymorphism within the *RHOA* gene is significantly associated with bone density in Caucasian women.

4.2 Materials and methods

4.2.1 Subjects

4.2.1.i Discovery cohort

For a full description of the discovery cohort refer to *Chapter 3*, section 3.2.1.*i*. Briefly, a total of 769 women from 335 families were recruited in Australia and the UK. This family-based population included siblings recruited in 1998 for a study of the genetics of osteoporosis (Wilson et al., 2003). All subjects from the study provided written informed consent, and the ethics committees of participating institutions including the Human Research Ethics Committee at Curtin University of Technology approved the experimental protocols.

4.2.1.ii Replication cohort

For a full description of the replication cohort refer to *Chapter 3*, section 3.2.1.*ii*. Briefly, women between the ages of 45 and 64 were recruited using a population-based method. DNA samples were obtained from 780 individuals, with hip and spine DXA BMD data obtained from 775 and 779 individuals respectively. All subjects from the study provided written informed consent, and the ethics committees of participating institutions including the Human Research Ethics Committee at Curtin University of Technology approved the experimental protocols.

4.2.2 Genotyping

4.2.2.i Discovery cohort

Genotyping in the discovery cohort was performed as described in *Chapter 3*, sections 3.2.2.*i*. Briefly, genotyping was performed using the Illumina GoldenGate assay (Illumina, 2004) on whole-genome amplified DNA. The genotype call rate using this technique was 99.6 % with an estimated error rate of < 0.1 %.

4.2.2.ii Replication cohort

Genotyping in the discovery cohort was performed as described in *Chapter 3*, sections 3.2.2.*i*. Briefly, genotyping was performed in-house using the MALDI-ToF

mass spectrometry technique (Wise et al., 2003) (see Appendix II for primer sequences). Using this technique the genotype call rate was 97.1 % and the estimated error rate was < 0.1 %.

4.2.3 SNP selection

Nine SNPs were selected in the region of the *RHOA* gene for genotyping in the discovery cohort. Tagging SNPs (tSNPs) were initially selected across the region using the Perlegen Genome Browser (European American population) (Hinds et al., 2005) as described in *Chapter 3*. We were able to tag all 3 LD bins identified within the immediate vicinity of the *RHOA* gene (Perlegen LD bins 1050962, 1046850 and 1046281). The remaining SNPs genotyped were selected from the dbSNP database (Build 132), which does not currently contain any record of exonic polymorphisms within the human *RHOA* gene with a minor allele frequency > 1 % (discussed later).

Two SNPs from the nine genotyped in the discovery cohort were selected for genotyping in the replication cohort.

4.2.4 Statistical analysis

For a full description of the statistical analysis performed in the discovery cohort refer to *Chapter 3*, section 3.2.4. Briefly, the data from the discovery cohort were analysed using the FBAT (Family Based Association Tests) software to test for association within sib-pairs (Laird et al., 2000). Correction for multiple testing was performed to correct for tests of multiple SNPs within each phenotype, and also for tests of multiple SNPs across multiple phenotypes.

To estimate the genetic effect size, the genotype-specific adjustment to the trait values that leads to an FBAT statistic of zero for the residuals was calculated (Vansteelandt et al., 2008). This gives an estimate of the additive effect on the mean that is consistent with the testing method used. Haplotype analysis used QPDTPHASE v2.404, which is part of the UNPHASED software suite (Dudbridge, 2003). Throughout, two-tailed *P*-values are reported, with $P \leq 0.05$ considered significant. LD between the different SNPs was evaluated using the JLIN software (Carter et al., 2006).

Statistical analysis of the data from the replication cohort was performed in Statistica v8.0 using ANOVA for differences between genotype groups. BMD Z-score data were adjusted for weight by ANCOVA. Genotype effects on fracture rate were examined using a Chi-square test.

4.3 Results

4.3.1 Discovery cohort

All SNPs genotyped were in Hardy-Weinberg equilibrium (χ^2 test, $P < 0.05$). Of the 9 SNPs genotyped in the discovery cohort, one was found to be monomorphic (rs6446270), that is, only one allele was identified in the entire cohort. The remaining 8 fell into 3 distinct LD blocks as defined by a tagging strength of $r^2 \geq 0.8$ (Figure 4.2). The chromosomal position and allele distribution of all 9 SNPs genotyped is detailed in Table 4.1. LD block 1 contained the SNPs rs6446272 and rs17080528 (Perlegen LD bin 1050962); block 2 contained rs4855877, rs17595772 and rs17650792 (Perlegen LD bin 1046850); block 3 contained rs940045, rs10155014 and rs4955426 (Perlegen LD bin 1046281). Using FBAT, significant associations were observed between various measures of BMD Z-score and the SNPs in block 1 (rs6446272, rs17080528) and block 2 (rs4855877, rs17595772, rs17650792) (*P = 0.001 – 0.036*). No significant associations were seen between the SNPs in block 3 (rs940045, rs10155014, rs4955426) and BMD phenotypes (*P = 0.46 – 0.8*). The SNP rs17080528 was selected to represent block 1 and rs17595772 was selected to represent block 2, since these two SNPs demonstrated the strongest associations with BMD parameters. The SNP rs17080528 was significantly associated with BMD Z-scores for the total hip (*P = 0.026*) and femoral neck (*P = 0.003*) whereas rs17595772 was significantly associated with BMD Z-scores for the total hip (*P = 0.014*), femoral neck (*P = 0.001*) and spine sites (*P = 0.036*). Significant associations for rs17595772 with total hip and femoral neck sites were maintained after correction for testing multiple SNPs (*P = 0.038* and *0.002* respectively), and the association with femoral neck was maintained after further correction for testing multiple anatomical sites (*P = 0.007*). Estimates of the additive genetic effect suggest that the less common *T* allele at rs17080528 and the more common *G* allele at rs17595772 are associated with a reduced BMD Z-score (Table 4.2a and 4.2b).

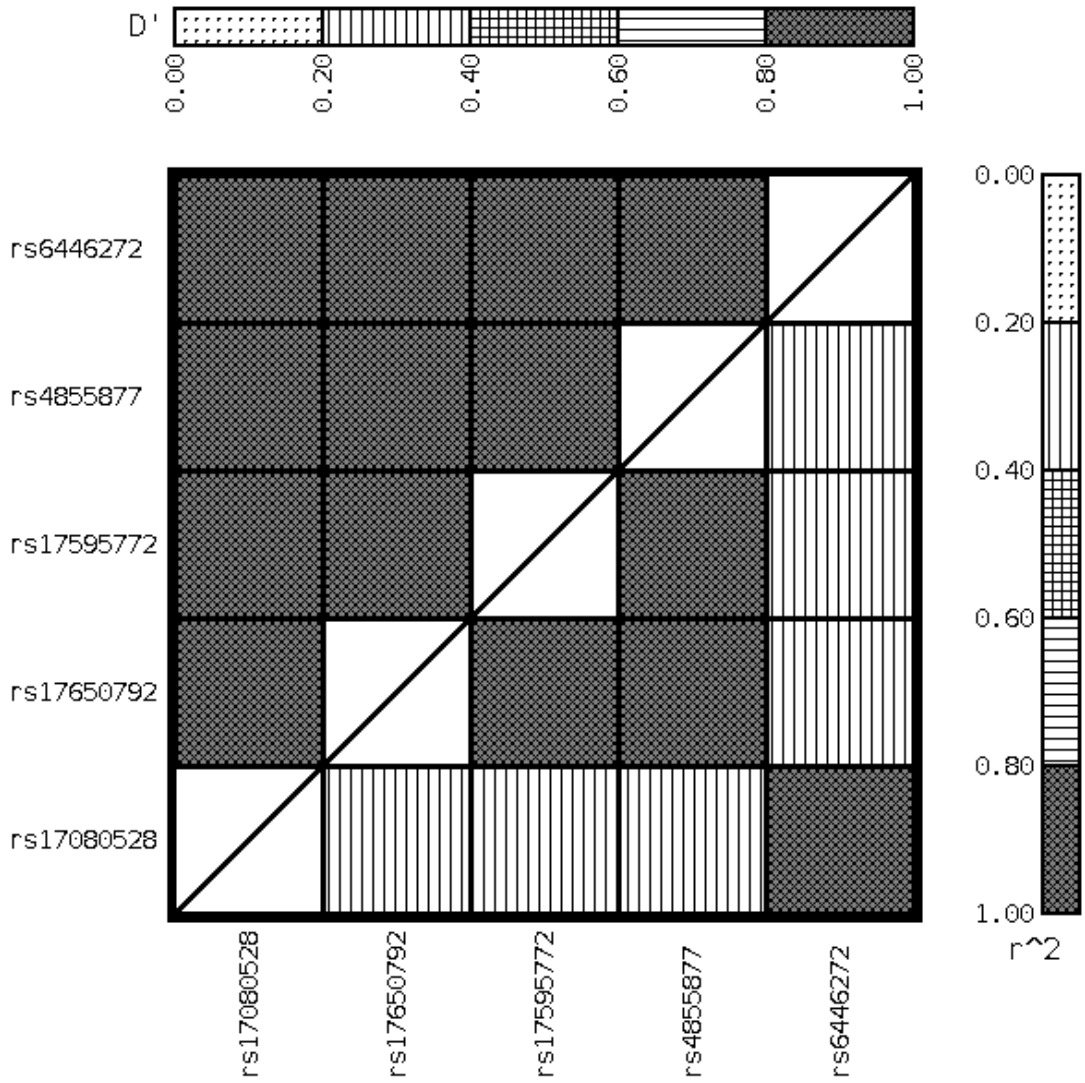

Figure 4.2. LD analysis of the 5 SNPs significantly associated with BMD parameters in the discovery cohort.

Table 4.1. Position and allele distribution of the *RHOA* SNPs investigated.

SNP	Chromosome Position*	Function/ Location*	Genotype Distribution in the Discovery Cohort (%)
rs6446272	49438291	5’ region	GG (45.8), AG (45), AA (9.3)
rs940045	49424642	5’ region	TT (54.6), TC (39.1), CC (6.3)
rs4855877	49423531	Intron 1	AA (32.4), AG (51), GG (16.5)
rs6446270	49420781	Intron 1	CC (100)
rs10155014	49407325	Intron 1	TT (54.3), TC (39.3), CC (6.4)
rs17595772	49384708	Intron 2	GG (32.1) (29.9)†, AG (50.9) (50.0)†, AA (17.0) (20.1)†
rs17650792	49365254	3’ region	AA (32.4), AG (50.4), GG (17.2)
rs17080528	49364846	3’ region	CC (45.8) (50.9)†, TC (45.2) (41.3)†, TT (9.0) (7.8)†
rs4955426	49118442	3’ region	TT (60.7), TG (33.7), GG (5.6)

* From GenBank reference sequence NM_001664, Genome Build 36.3.

† Allele distribution in the replication cohort.

Table 4.2a. Additive effect of the *T* allele at rs17080528 on BMD Z-score phenotypes in the discovery cohort.

BMD Z-score Phenotype	Additive effect of *T* allele
Total hip	- 0.12
Femoral neck	- 0.15
Spine	- 0.13

Results are given as the effect size that gives a residual FBAT statistic of zero.

Table 4.2b. Additive effect of the *G* allele at rs17595772 on BMD Z-score phenotypes in the discovery cohort.

BMD Z-score Phenotype	Additive effect of *G* allele
Total hip	- 0.20
Femoral neck	- 0.25
Spine	- 0.20

Results are given as the effect size that gives a residual FBAT statistic of zero.

A 2-SNP haplotype analysis was undertaken on rs17080528 and rs17595772 to determine whether haplotypes of the LD blocks tagged by each SNP would prove to be more significantly associated with BMD Z-scores than either block individually. The *TA* haplotype had an estimated frequency of zero, suggesting a lack of historical recombination. The association of the *CA* haplotype was therefore equivalent to that of the *A* allele of rs17595772, and the association of *TG* equivalent to that of the *T* allele of rs17080528. The *CG* haplotype had no significant association, so it was concluded that no additional information was obtained from the haplotype analysis.

4.3.2 Replication cohort

The SNPs rs17080528 and rs17595772 were genotyped in the replication cohort. The allele distribution of these two SNPs in this cohort is detailed in Table 4.1. No significant associations between rs17080528 and BMD Z-scores were observed ($P = 0.21 – 0.42$). However, rs17595772 demonstrated significant associations with BMD Z-scores for the total hip, femoral neck and spine sites after adjustment of the BMD Z-score data for the covariate weight (Table 4.3). Consistent with the results for the discovery cohort, subjects homozygous for the *G* allele at rs17595772 compared to individuals homozygous for the *A* allele had lower BMD at the total hip, femoral neck and spine sites (- 3.4 %, - 3.8 %, and - 5.9 % respectively). Compared to heterozygous individuals with the *AG* genotype, *GG* individuals again had lower BMD at the three sites (- 1.1 %, - 1.8 % and - 2.7 % respectively). No significant association between rs17595772 genotype and the covariate weight was found.

In the replication cohort 265 subjects had suffered a fracture prior to 2004, giving a prevalent fracture rate of 34 %. Neither rs17080528 nor rs17595772 were significantly associated with fracture rate.

4.4 Discussion

The results presented here suggest that genetic variation in the *RHOA* gene, in addition to that in the *ARHGEF3* gene, may influence BMD in women. These genes are both situated in the 3p14-p22 region of the genome, an area that has been linked with BMD in multiple studies (Duncan et al., 1999, Wilson et al., 2003, Wynne et al., 2003, Klein et al., 1998, Xiao et al., 2006, Lee et al., 2006). This certainly raises the possibility that more than one gene from this region is responsible for the linkage

Table 4.3. BMD Z-scores in relation to the allele distribution of rs17595772 in the replication cohort.

Phenotype	*AA* Genotype	*AG* genotype	*GG* genotype	*P*
Total Hip BMD Z score	0.7 ± 1.1^{a} (149)	0.5 ± 1.0^{b} (371)	0.4 ± 0.9^{b} (225)	**0.034**
Femoral Neck BMD Z score	0.4 ± 1.1^{a} (149)	0.3 ± 1.0 (371)	0.2 ± 0.9^{b} (225)	**0.036**
Spine BMD Z score	1.1 ± 1.5^{a} (150)	0.8 ± 1.5^{b} (374)	0.6 ± 1.2^{b} (225)	**0.002**

Results are given as *mean ± SD (number of measurements)*.

The BMD Z-score data are adjusted for weight.

[a] significantly different from [b] in post hoc analysis ($P < 0.05$).

observed, a phenomenon that has been reported previously with genetic susceptibility to tuberculosis (Jamieson et al., 2004) and essential-hypertension (chronically elevated blood-pressure with no identifiable cause) (Chang et al., 2007). This is discussed further in *Chapter 7*.

According to updated LD data provided by the International HapMap Project (Phase 3 dataset) (Altshuler et al., 2010), for the CEU population the two SNPs rs17080528 and rs17595772 tag two large LD blocks that span a region approximately 219 Kb on chromosome 3 (Figure 4.3). Seven genes have been identified in this region (including *RHOA*) and it is possible that variation within any one of these is responsible for the associations observed. After interrogation of the literature to investigate the function of each gene product (Table 4.4), it was found that the *TCTA*, *GPX1* and *USP4* genes all have potential roles in bone metabolism. It cannot be ruled out that polymorphism within these two LD blocks influences BMD through effects on these genes. However, *RHOA* is considered to be the most likely candidate among these for a role in BMD regulation especially when considering the associations identified in these same populations between the *ARHGEF3* gene and BMD phenotypes (refer to *Chapter 3*).

The RHOA GTPase has been implicated in osteoblast differentiation. McBeath et al. found that cell shape regulates the commitment of human mesenchymal stem cells (hMSCs) to the adipocyte or osteoblast lineages by modulating endogenous RHOA activity (McBeath et al., 2004). They also demonstrated that RHOA expression committed hMSCs to an osteoblastic fate whereas expression of dominant-negative RHOA caused adipogenesis (McBeath et al., 2004). These effects overrode the presence of differentiation factors in the media and are thought to occur through regulation of cytoskeletal tension (McBeath et al., 2004). Meyers et al. found that over-expression of RHOA restored actin cytoskeletal arrangement, enhanced the expression of osteoblastic genes and suppressed the expression of adipocytic genes in hMSCs cultured in modelled microgravity (MMG) (Meyers et al., 2005). Interestingly, it was found that the quantity of activated RHOA protein dropped by 88 ± 2% in hMSCs cultured in MMG whereas the total expression of RHOA did not change significantly (Meyers et al., 2005), indicating that the RhoGEFs responsible for activating RHOA could have had a role in the effect. A recent publication by

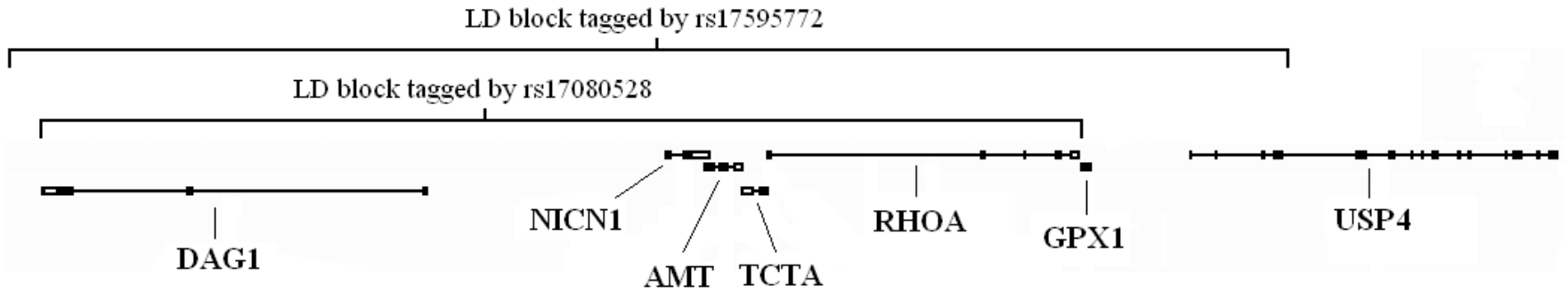

Figure 4.3. LD blocks tagged by rs17080528 and rs17595772 according to the International HapMap Project (Phase 3 dataset) (Altshuler et al., 2010).

Table 4.4. Role of gene products, including any potential role in bone metabolism, for each of the 6 genes (in addition to *RHOA*) covered by the linkage disequilibrium blocks tagged by rs17080528 and rs17595772.

Gene	Role of gene product
DAG1	Encodes dystroglycan 1, an integral component of the dystrophin-glycoprotein complex. This complex provides a link between the subsarcolemmal cytoskeleton and the basement membrane (Barresi and Campbell, 2006). Reduced glycosylation of dystroglycan has been implicated in some cases of muscular dystrophy (Mercuri et al., 2009).
NICN1	Encodes nicolin 1, which is a nuclear protein (Backofen et al., 2002). Not a great deal is known about this protein, however the murine *Nicn1* gene has been highlighted as a possible candidate gene for alcohol preference (Mulligan et al., 2006).
AMT	Encodes aminomethyltransferase, which is part of a complex involved with glycine cleavage (Nanao et al., 1994). Mutations in this gene have been implicated in nonketotic hyperglycinemia, a condition characterised by severe neurological symptoms resulting from disturbed glycine metabolism (Toone et al., 2001).
TCTA	Encodes T-cell leukemia translocation altered gene protein, which was originally implicated in T-cell acute lymphoblastic leukemia (Aplan et al., 1995). A recent study has suggested that this protein may play an important role in the cellular fusion that takes place during osteoclastogenesis (Kotake et al., 2009).
GPX1	Encodes glutathione peroxidase 1, a selenium-containing antioxidant enzyme that appears to have a role in metabolising hydrogen peroxide (Lei et al., 2007) and has been implicated in various types of cancer (Hu et al., 2005). Overexpression of Gpx1 in a mouse macrophage cell line has been found to impair osteoclastogenesis (Lean et al., 2005) and a recent study has identified associations between polymorphism in *GPX1* and BMD in humans (Mlakar et al., 2010).
USP4	Encodes ubiquitin specific peptidase 4, which is a deubiquitinating enzyme that has been implicated in lung cancer (Frederick et al., 1998, Gray et al., 1995). USP4 has recently been identified as a member of the Wnt signalling pathway, with suppression of USP4 having been found to activate beta-catenin dependent transcription (Zhao et al., 2009).

Wang and Stern has also suggested a possible role for RHOA in the production of RANKL and OPG by osteoblasts in response to PTH stimulation (discussed in *Chapter 7*) (Wang and Stern, 2010).

In addition to its role in osteoblast differentiation, the RHOA GTPase has also been implicated in osteoclast function. Osteoclasts are highly motile cells that rely on rapid changes to their cytoskeleton to achieve the movement and attachment that is required for bone resorption (Kanehisa and Heersche, 1988, Lakkakorpi et al., 1993, Lakkakorpi and Vaananen, 1991, Lakkakorpi and Vaananen, 1996). Chellaiah et al. identified RHOA as playing a major role in this process (Chellaiah et al., 2000). By transducing active and inactive RHOA into avian osteoclasts they demonstrated that the protein is essential for podosome assembly, stress fibre formation, osteoclast motility and bone resorption (Chellaiah et al., 2000). In addition, farnesyl pyrophosphate synthase, the specific target of nitrogen-containing bisphosphonates widely used in the treatment of osteoporosis, is essential for the prenylation and therefore activity of RhoGTPases including RHOA (Reszka and Rodan, 2004). Bisphosphonates cause loss of osteoclast activity and induction of apoptosis, possibly through the inactivation of RhoGTPases (Denoyelle et al., 2003). Zhang et al. found that the actin ring structure that is typical of a resorbing osteoclast is disrupted by ADP-ribosyltransferase C3 (C3 exoenzyme) (Zhang et al., 1995). This exoenzyme is produced by *Clostridium botulinum* and is specifically involved in ADP-ribosylation of Rho proteins, thereby inactivating them (Sekine et al., 1989, Narumiya and Morii, 1993, Nemoto et al., 1991).

As mentioned previously, the RHOA protein appears to be very highly conserved across multiple species. Indeed, the dbSNP database (Build 132) does not presently contain any record of exonic polymorphisms within the human *RHOA* gene with a minor allele frequency > 1 %. There is also no evidence of polymorphism within the gene that could influence splicing. This suggests that the function of the RHOA protein is critical for survival, a theory supported by the fact that RHOA is involved with a wide range of biological processes in multiple tissue types (Etienne-Manneville and Hall, 2002). As such, all of the SNPs genotyped in this study are either intronic or located 5’ or 3’ of the *RHOA* gene. When considering this, it seems likely that if the SNPs genotyped in this study do influence BMD, they do so through

mild regulatory effects on the *RHOA* gene as opposed to structural changes to the RHOA protein, the latter of which could result in profound phenotypic changes. Future studies could investigate whether polymorphism at rs17595772 or rs17080528 is associated with *RHOA* mRNA levels (discussed in *Chapter 7*).

In conclusion, the evidence presented here suggests that common genetic variation within the *RHOA* gene region is associated with BMD in Caucasian women. Therefore, the hypothesis has been supported by the data. There is a substantial amount of evidence to suggest that RHOA has a role in the biology of osteoblasts and osteoclasts. These data further support a role for the RhoGTPase-RhoGEF pathway in osteoporosis.

Chapter 5 – Analysis of variation within the *FLNB* gene for association with bone mineral density phenotypes

5.1 Introduction

Mammals carry three filamin genes, encoding: filamin A (*FLNA*), filamin B (*FLNB*) and filamin C (*FLNC*). The *FLNC* gene appears to be predominantly expressed in muscle, whereas the *FLNA* and *FLNB* genes are widely expressed (Brocker et al., 1999, Chakarova et al., 2000). A comparison between the *FLNA, FLNB* and *FLNC* genes revealed a highly conserved intron-exon structure, with significant differences observed in the 32nd exon of each gene encoding the hinge 1 region (Chakarova et al., 2000). Filamins exist *in vivo* as dimers, with around 70 % homology existing between the FLNA, FLNB and FLNC protein structures (van der Flier and Sonnenberg, 2001). The FLNB monomer is a 278 kDa protein (Xu et al., 1998) consisting of an N-terminal actin-binding domain composed of two calponin homology domains (CH1 and CH2) (Gorlin et al., 1990), followed by a series of 24 β-sheet repeats that bind to various cytoplasmic and transmembrane proteins (Gorlin et al., 1990, Xu et al., 1998). The C-terminal β-sheet repeat (repeat number 24) is known as the dimerisation domain and allows for the formation of FLNB homodimers (Weihing, 1988). The *FLNB* gene is located in the p14-p22 region of human chromosome 3 (Brocker et al., 1999) and presents as another strong candidate for BMD regulation. The gene is made up of 45 exons and 44 introns, spanning around 80 Kb of genomic DNA (Chakarova et al., 2000).

Mutations to the *FLNB* gene have been implicated in a variety of genetic disorders characterised by skeletal malformation. Steiner et al. performed a linkage study in three consanguineous families and one non-consanguineous family with high incidences of spondylocarpotarsal synostosis (SCT) syndrome (MIM 272460) (Steiner et al., 2004), a rare autosomal recessive disorder characterised by fusions of the vertebrae as well as the carpal and tarsal bones (Langer et al., 1994). They observed linkage of the disorder with the 3p14 region of the human genome (LOD 6.49) (Steiner et al., 2004). Krakow et al. subsequently examined the *FLNB* gene for mutations in these four families, identifying a unique nonsense mutation (creating a premature stop codon) in each (Krakow et al., 2004). The authors concluded that SCT syndrome results from the absence or truncation of the *FLNB* gene product (Krakow et al., 2004). This theory was supported by two later studies that identified

nonsense *FLNB* mutations in two individuals suffering from SCT syndrome (Brunetti-Pierri et al., 2008, Mitter et al., 2008).

Larsen syndrome (LS) is a genetic disease characterised by craniofacial abnormalities in addition to multiple dislocations of the large joints such as the knees, elbows and hip joints (Larsen et al., 1950). The condition can be inherited as an autosomal dominant (MIM 150250) or recessive (MIM 245600) form, with the recessive form arguably the more severe (Bonaventure et al., 1992, Chen et al., 1982). Vujic et al. published the results of a linkage study in a large Swedish pedigree suffering from autosomal dominant LS (Vujic et al., 1995). This study highlighted the 3p14-p21 region of the human genome as potentially harbouring the gene responsible for the disorder (LOD 13.4) (Vujic et al., 1995). Krakow et al. analysed the *FLNB* gene for mutations in four individuals with sporadically occurring LS and one family with a dominantly inherited form of the condition (Krakow et al., 2004). They identified a unique *de novo* missense mutation within the *FLNB* gene in each of the five cases (Krakow et al., 2004). Bicknell et al. subsequently identified *FLNB* mutations in 20 unrelated individuals suffering from LS, these mutations seemed to cluster in the actin-binding domain of the FLNB protein and in the β-sheet repeats 13-17 (Bicknell et al., 2007).

Atelosteogenesis is a heritable lethal chondrodysplasia characterised by insufficient ossification of various bones including the humerus, femur, thoracic spine and hand bones (Maroteaux et al., 1982). The disease shares many phenotypic features with LS (Nishimura et al., 1997, Schultz et al., 1999). Krakow et al. examined the *FLNB* gene in five unrelated individuals suffering from atelosteogenesis: three suffering from atelosteogenesis type I (AOI) (MIM 108720) and two suffering from atelosteogenesis type III (AOIII) (MIM 108721) (Krakow et al., 2004). They observed missense mutations in the *FLNB* gene in all five individuals, with one individual suffering from AOI and another suffering from AOIII carrying the same mutation (Krakow et al., 2004). Farrington-Rock et al. subsequently identified 14 novel *FLNB* missense mutations in 15 unrelated patients with AOI and AOIII (Farrington-Rock et al., 2006). Most of these mutations were found to reside within exons 2 and 3 which encode part of the actin-binding region of the protein, with the remainder found in exons 28 and 29 which encode the β-sheet repeats 14 and 15

(Farrington-Rock et al., 2006). Some of these mutations are thought to act in a gain-of-function mechanism, resulting in increased actin-binding affinity (Sawyer et al., 2009).

Boomerang dysplasia (MIM 112310) is a perinatal lethal osteochondrodysplasia characterised by insufficient or non-existent ossification of the limb bones and vertebrae (Kozlowski et al., 1985). Bicknell et al. identified missense *FLNB* mutations in two individuals suffering from the disorder (Figure 5.1) (Bicknell et al., 2005). These mutations were found to reside within regions of the gene that are evolutionarily well conserved and encode parts of the actin-binding domain of the protein (Bicknell et al., 2005).

Wilson et al. published the results of the first study into common variation within the *FLNB* gene and BMD (Wilson et al., 2009). The four SNPs rs7637505, rs9822918, rs2177153 and rs2001972, were identified as significantly associated with age-adjusted femoral neck BMD (BMD Z-score) in a discovery population of 767 women from 334 families of European background (Wilson et al., 2009). Each of these four SNPs is located in either an intron or 5' of the *FLNB* gene. Of these four SNPs, rs7637505 was significantly associated with femoral neck and lumbar spine BMD Z-score in a replication population of 1,085 female twins from the UK, while rs9822918 and rs2177153 were significantly associated with femoral neck BMD Z-score in an additional replication population comprised of 1,315 unrelated postmenopausal Australian women (Wilson et al., 2009). Subsequent gene expression analysis in 96 human osteoblast cell cultures derived from 96 unrelated donors revealed that rs9822918, rs2177153 and rs2001972 did not reveal any association with *FLNB* mRNA expression levels (rs7637505 was not represented on the analysis chip) (Wilson et al., 2009). However, five SNPs located either 5' or in intron 1 of the *FLNB* gene, all in moderate LD (D' > 0.5) with either rs7637505 or rs2177153, were identified as significantly associated with *FLNB* mRNA expression: rs11130605, rs839230, rs11720285, rs1718481 and rs9809315 (Wilson et al., 2009). The aim of the work described in this chapter was to analyse these 5 SNPs for association with BMD phenotypes in a population of Caucasian women.

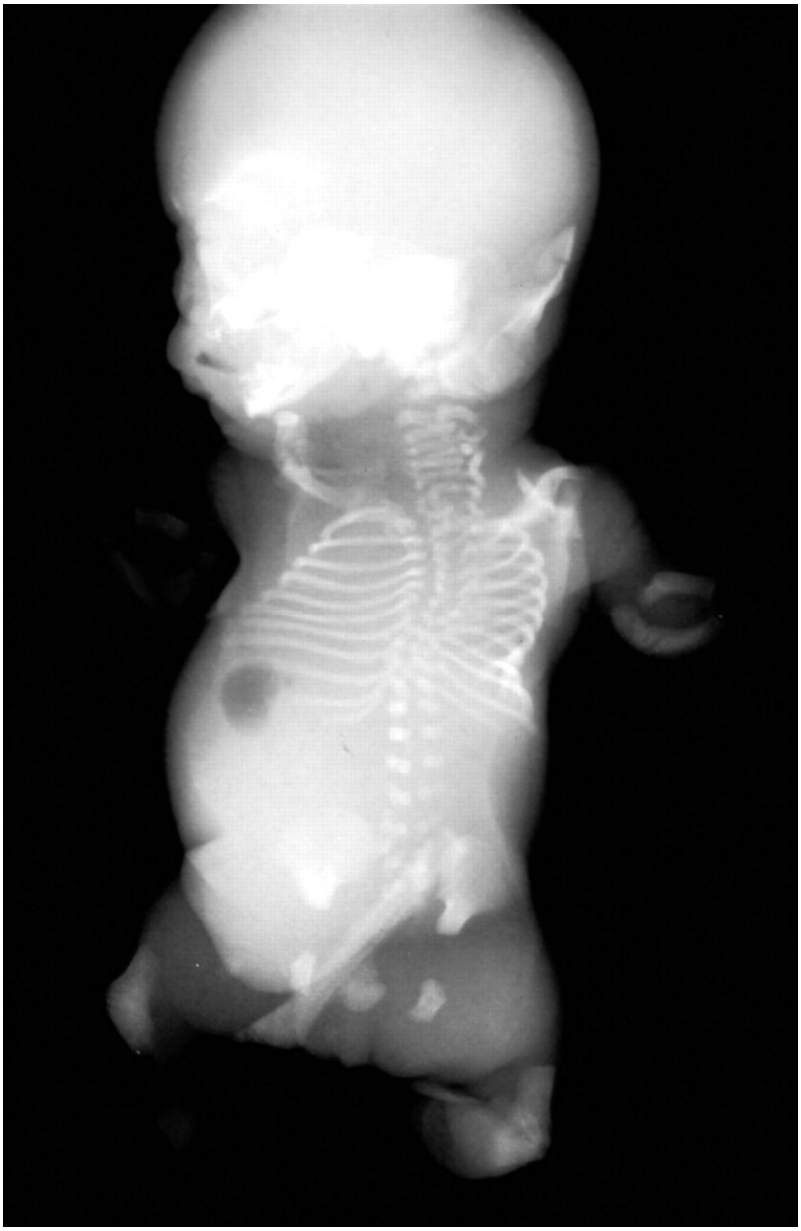

Figure 5.1. A male foetus delivered at 22 weeks suffering from boomerang dysplasia. This radiograph reveals unossified humeri, absent femora and a complete lack of ossification in the hands and feet. Figure adapted from Bicknell et al. (Bicknell et al., 2005).

5.1.1 Hypothesis

Variation at one or more of the polymorphic sites rs11130605, rs839230, rs11720285, rs1718481 and rs9809315 in the *FLNB* gene is significantly associated with bone density in Caucasian women.

5.2 Materials and methods

5.2.1 Subjects

For a full description of the cohort used in this study refer to *Chapter 3*, section 3.2.1.*i*. Briefly, a total of 769 women from 335 families were recruited in Australia and the UK. This family-based population included siblings recruited in 1998 for a study of the genetics of osteoporosis (Wilson et al., 2003) and is the same cohort as used in the "Discovery Study" by Wilson et al. (Wilson et al., 2009) with the inclusion of one additional sibling pair. All subjects from the study provided written informed consent, and the ethics committees of participating institutions including the Human Research Ethics Committee at Curtin University of Technology approved the experimental protocols.

5.2.2 Genotyping

Genomic DNA was extracted and purified from EDTA whole blood obtained from each subject (Johns and Paulus-Thomas, 1989). Genotyping was performed on genomic DNA using the TaqMan assay, which utilises fluorogenic 5' nuclease chemistry, in 384-well PCR plate format (see *Chapter 2* for a full description of the technique). Using this technique the genotype call rate was 99.3 % and the estimated error rate was < 0.1 %, as assessed by comparing genotype calls between 8 samples genotyped in duplicate.

5.2.3 SNP selection

5 SNPs from the 5' region of the *FLNB* gene were selected for genotyping. These were selected based on previously published data suggesting that all five SNPs are significantly associated with expression of *FLNB* mRNA in 96 human osteoblast cell cultures (Wilson et al., 2009).

5.2.4 Statistical analysis

For a full description of the statistical analysis performed in this cohort refer to *Chapter 3*, section 3.2.4 (discovery cohort). Briefly, the data from the cohort were analysed using the FBAT (Family Based Association Tests) software to test for association within sib-pairs (Laird et al., 2000). Correction for multiple testing was performed to correct for tests of multiple SNPs within each phenotype, and also for tests of multiple SNPs across multiple phenotypes.

UNPHASED v3.1.3 was used to estimate the genetic effect size in this cohort by generating an additive value as an estimate to how each allele influences the trait value relative to the most common allele (Dudbridge, 2003). This program was also used to perform a haplotype analysis. Throughout, two-tailed P-values are reported, with $P \leq 0.05$ considered significant. LD between the different SNPs was evaluated using the JLIN software (Carter et al., 2006).

5.3 Results

All 5 SNPs genotyped were in Hardy-Weinberg equilibrium (χ^2 test, $P < 0.05$). LD analysis revealed that none of the 5 SNPs genotyped were in LD of $r^2 > 0.8$ with each other (Figure 5.2). An additional LD analysis revealed that one of the 5 SNPs genotyped in this study, rs839230, is in strong LD ($r^2 > 0.8$) with rs704529 which was genotyped by Wilson et al. (Wilson et al., 2009). However, none of the other 4 SNPs genotyped in this study were in LD of $r^2 > 0.8$ with any of the 13 SNPs genotyped by Wilson et al. (Figure 5.2) (Wilson et al., 2009). The chromosomal position and allele distribution of the 5 SNPs genotyped is detailed in Table 5.1.

Using FBAT, significant associations were seen for femoral neck BMD Z-score with the SNPs rs11720285, rs11130605 and rs9809315 ($P = 0.005$, 0.043 and 0.004 respectively). These three SNPs were also found to be significantly associated with total hip BMD Z-score ($P = 0.014$, 0.026 and 0.022 respectively). No significant associations were observed between any of the 5 SNPs examined and spine BMD Z-score. After correction of the data for testing 5 SNPs across 3 anatomical sites, the significant association between rs9809315 and femoral neck BMD Z-score was maintained ($P = 0.028$). Estimates of the additive genetic effect suggest that the less common *C* allele at rs11720285, the *T* allele at rs11130605 and the *T* allele at

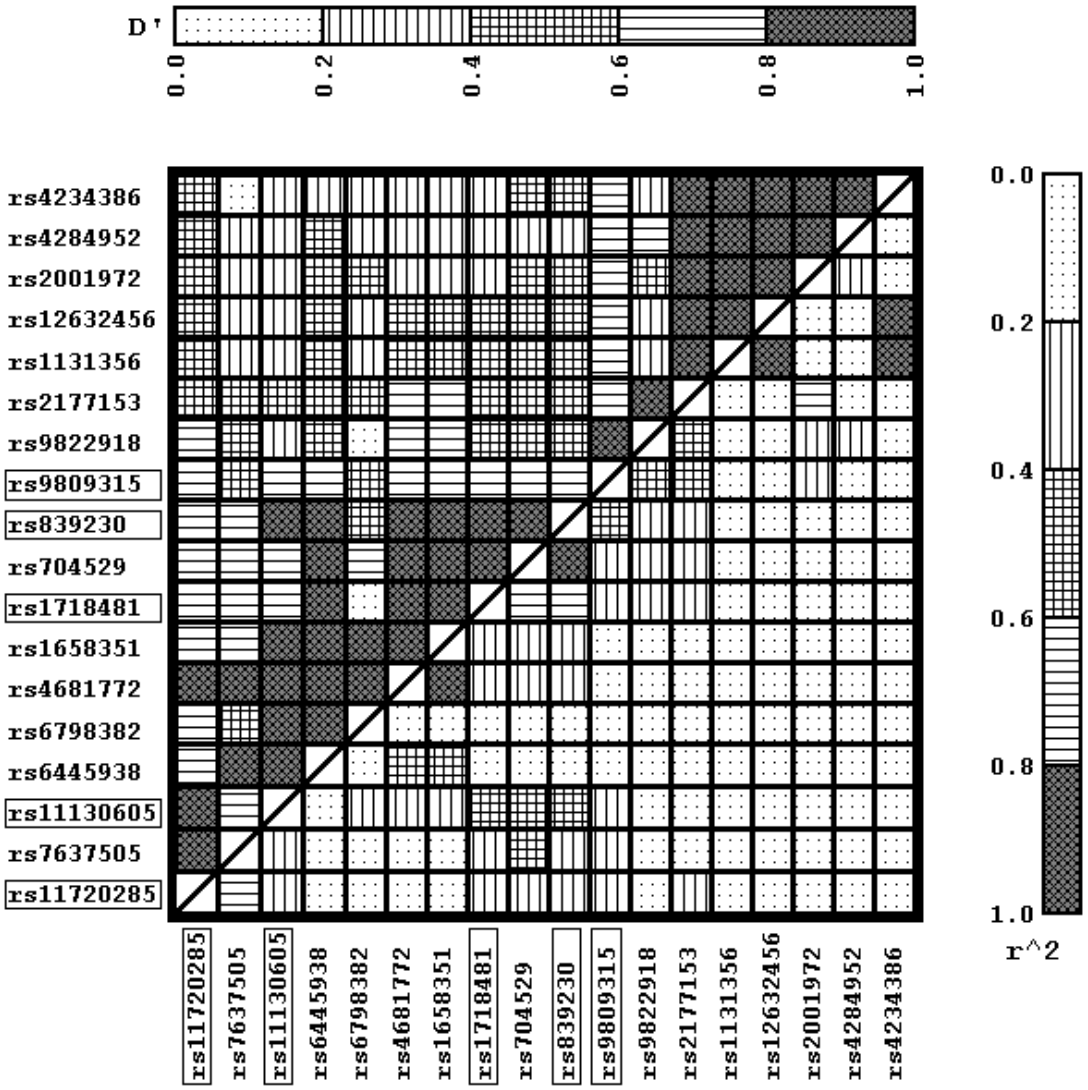

Figure 5.2. LD analysis of the 13 SNPs in *FLNB* genotyped by Wilson et al. (Wilson et al., 2009), plus the additional 5 SNPs genotyped in this study (highlighted).

Table 5.1. Position and allele distribution of the 5 SNPs genotyped around the 5' region of the *FLNB* gene.

SNP	Chromosome Position*	Function/ Location*	Location relative to transcription start site*	Genotype Distribution (%)
rs11720285	57936410	5' region	32,757 bp upstream	AA (54.9), AC (39.8), CC (5.2)
rs11130605	57964209	5' region	4,958 bp upstream	CC (36.5), TC (48.6), TT (14.9)
rs1718481	58000943	Intron 1	31,776 bp downstream	GG (32.2), AG (50.3), AA (17.5)
rs839230	58011832	Intron 1	42,665 bp downstream	GG (40.1), AG (47.4), AA (12.5)
rs9809315	58025305	Intron 1	56,138 bp downstream	CC (49.5), TC (40.7), TT (9.8)

* Relative to GenBank reference sequence NM_001457, Genome Build 36.3.

rs9809315 are associated with an increased BMD Z-score at both the total hip and femoral neck sites (Table 5.2).

A 3-SNP haplotype analysis was carried out using rs11720285, rs11130605 and rs9809315 to determine whether haplotypes of the LD blocks tagged by each SNP would prove to be more significantly associated with femoral neck or total hip BMD Z-score than in the individual SNP analysis. 6 haplotypes with a frequency greater than 4 % in the population were identified. Significant associations were observed with femoral neck BMD Z-score only (Table 5.3), although the level of significance did not surpass that observed in the individual SNP analysis for this phenotype. The *CTT* haplotype was found to have a strong positive influence on femoral neck BMD Z-score.

5.4 Discussion

The results presented here suggest that genetic variation within the 5' region of the *FLNB* gene has a role in determining bone density in Caucasian women. Wilson et al. identified 5 SNPs from the 5' region of the *FLNB* gene that were strongly associated with expression levels of *FLNB* mRNA, 3 of which have been shown here to be significantly associated with age-adjusted total hip and femoral neck BMD in Caucasian women (Wilson et al., 2009). The less common allele at each of these 3 SNP sites was associated with an increased BMD Z-score in this study. *FLNB* is the third gene from the 3p14-p22 region of the human genome that has been implicated in the regulation of bone density in this population of women. This supports the theory raised in *Chapter 4* that more than one gene from this chromosomal region is responsible for the linkage observed between 3p14-p22 and BMD.

A recent study published by Li et al. analysed seven SNPs from the *FLNB* gene (Li et al., 2010), including the SNPs rs9822918 and rs2177153 genotyped by Wilson et al. (Wilson et al., 2009), for association with BMD parameters in a case-control population of 1,080 Chinese females, 533 of whom were postmenopausal (Li et al., 2010). The authors were not able to replicate the findings for rs2177153 (probably due to the fact that this SNP had a minor allele frequency of only 0.02 in their population), however they did observe significant associations between the rs9822918 SNP and BMD at the total hip (Li et al., 2010). Although providing more

Table 5.2. Additive value of the less common allele at rs11720285, rs11130605 and rs9809315 relevant to BMD Z-score.

SNP	BMD Z Score Phenotype	Additive value of less common allele
rs11720285	Femoral neck	+ 0.306 (366)
	Total hip	+ 0.274 (373)
rs11130605	Femoral neck	+ 0.241 (575)
	Total hip	+ 0.268 (592)
rs9809315	Femoral neck	+ 0.304 (435)
	Total hip	+ 0.254 (448)

Results are given as *additive effect on the trait of the less common allele relative to the more common allele (number of alleles included in analysis)*, derived from UNPHASED v3.1.3.

Table 5.3. Haplotype analysis and additive value of each haplotype relevant to femoral neck BMD Z-score.

BMD Z score phenotype	Haplotype (rs11720285, rs11130605, rs9809315)						
	ACC allele	*CTT allele*	*ATC allele*	*ACT allele*	*ATT allele*	*CTC allele*	*P*
Femoral neck	0 (769)	+ 0.72 (260)	+ 0.184 (148)	+ 0.044 (83)	- 0.442 (82)	- 0.164 (76)	**0.006**

Results are given as *additive effect on the trait of each haplotype relative to the most common haplotype (number of alleles included in the analysis)*, derived from UNPHASED v3.1.3.

support of a role for the *FLNB* gene in osteoporosis, comparisons with this study and that performed by Wilson et al. must be done with caution due to differences in study design as well as differences in the ethnicity of the study subjects (Wilson et al., 2009), as discussed in *Chapter 3*.

Filamins are promiscuous proteins, with over 70 binding partners identified to date (Popowicz et al., 2006, Sarkisian et al., 2008). They are thought to have a role in stabilising the actin cytoskeleton, providing a link between the actin network and the cellular membranes, and mediating interactions between actin and transmembrane receptors (Stossel et al., 2001). Filamins act to maintain the structural integrity of cells by crosslinking the actin cytoskeleton into 3D gels (Hartwig and Shevlin, 1986, Stossel, 1989, Stossel et al., 2001). The ability of filamin proteins to bind actin at their N-terminus and form tail-to-tail homodimers at their C-terminus allows them to create the orthogonal actin networks and bundles that result in gelation (Gorlin et al., 1990). It has also been proposed that filamins are important during foetal development, regulating the communication between extracellular signals and the cytoskeleton to guide migration of cells into the correct anatomical sites (Stossel et al., 2001).

There is a large amount of evidence to suggest that FLNB has a role in bone in humans, particularly when considering the various genetic diseases with skeletal phenotypes thought to be caused by mutation to the *FLNB* gene (Bicknell et al., 2007, Bicknell et al., 2005, Bonaventure et al., 1992, Brunetti-Pierri et al., 2008, Krakow et al., 2004, Mitter et al., 2008). In addition to this, there is evidence to suggest that the murine *Flnb* gene has a role in bone. *Flnb* expression has been detected in vertebral bodies obtained from mouse embryos, and it has been suggested that the gene plays a role in vertebral segmentation, joint formation and endochondral ossification (Krakow et al., 2004). Zhou et al. generated mice with a targeted disruption of the *Flnb* gene and observed impaired development of the microvasculature and skeletal systems in *Flnb*-deficient embryos, few of which reached term (Zhou et al., 2007). Those that were born were very small and had severe skeletal malformations including scoliotic and kyphotic spines, fusion of vertebral bodies, lack of intervertebral discs and reduced hyaline matrix in the extremities, thorax and vertebrae (Zhou et al., 2007). Interestingly, the authors also

noted that the expression of the *Rhoa* gene increased > 3-fold in *Flnb*-deficient fibroblasts.

Additional support for the role of FLNB in bone has been generated through studies on the highly homologous FLNA protein. Leung et al. recently published evidence suggesting a major role for the *FLNA* gene in osteoclast differentiation (Leung et al., 2010). Osteoclastogenesis in *Flna*-null mice was severely impaired as a result of decreased migratory ability of monocyte precursors (Leung et al., 2010). Activation of the Rho proteins Rac1, Cdc42 and Rhoa were partially or completely abolished in these monocytes, with addition of constitutively active Rac1 and Cdc42 found to rescue their migratory capabilities (Leung et al., 2010). These results suggest that FLNA has a major role in osteoclastogenesis through regulation of the cytoskeleton via RhoGTPases that are required for the migratory capabilities of osteoclast precursors. Interestingly, the *Flna*-null mice generated by Leung et al. presented with osteoporosis, suggesting that additional bone-related mechanisms are at work (Leung et al., 2010).

There are two non-synonymous coding changes within the *FLNB* gene that have a minor allele frequency > 1 % in Caucasians, both of which were genotyped in the study by Wilson et al. and neither of which demonstrated significant associations with BMD parameters (Wilson et al., 2009). It is highly likely that the associations observed here between polymorphism at rs11720285, rs11130605 and rs9809315 and BMD in women are a result of regulatory effects to the gene as opposed to structural changes to the FLNB protein. Firstly, these three SNPs are all located in either the 5' region or intron 1 of the *FLNB* gene. Secondly, all three of these SNPs have been reported as significantly associated with *FLNB* mRNA expression (Wilson et al., 2009). Interrogation of this genomic region using the Eponine program (Down and Hubbard, 2002), the SwitchGear Transcription Start Site (TSS) prediction algorithm (Trinklein et al., 2003) and the Cold Spring Harbor Laboratory Transcriptional Regulatory Element Database (TRED) (Zhao et al., 2005a) revealed the presence of 4, 1 and 2 predicted regulatory elements respectively. However, all 7 of these predicted regulatory elements are located within a 650 bp region surrounding the 5' UTR and first exon of the *FLNB* gene (Figure 5.3).

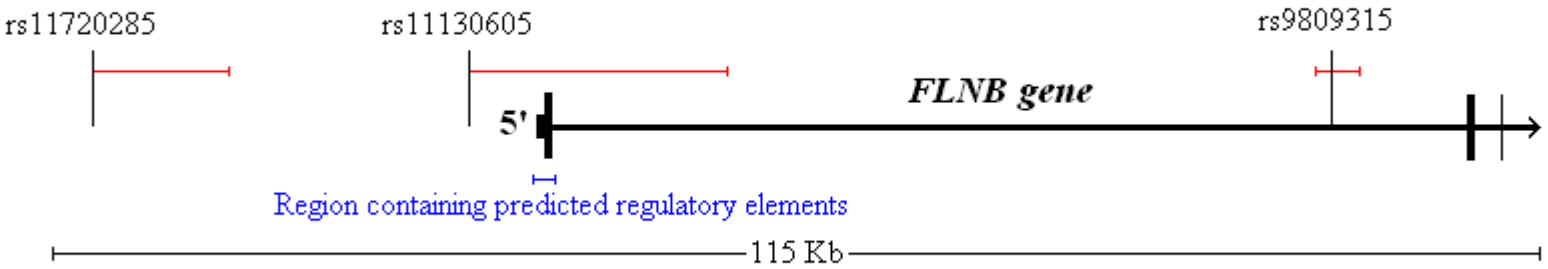

Figure 5.3. Diagram of the 5' end of the *FLNB* gene with significant SNPs and predicted regulatory elements. The approximate locations of the three SNPs significantly associated with BMD phenotypes, the LD blocks that they are a part of (red), and the 650 bp region containing the predicted regulatory regions (blue) are indicated.

An analysis of the LD surrounding the SNPs rs11720285, rs11130605 and rs9809315 was carried out using data for the CEU population from the International HapMap Project (Phase 3 dataset) (Altshuler et al., 2010). The SNP rs11720285 was found to be in strong LD ($r^2 \geq 0.8$) with the SNPs rs4681770, rs7637505 and rs6445937; rs11130605 was in strong LD with rs7631741 and rs7634753; and rs9809315 was in strong LD with rs13096731 and rs13071918. The location of these LD blocks is displayed in Figure 5.3. The LD block tagged by rs11720285 stretches to within 24 Kb of the 650 bp region containing the predicted regulatory elements and the LD block tagged by rs11130605 spans across this 650 bp region. The LD block tagged by rs9809315 on the other hand is quite small and is located well away from this area, near the 3' end of intron 1. These data would suggest that polymorphism within the LD block tagged by rs11130605 is the most likely candidate of the three as being directly responsible for the regulatory effects seen on the gene. This is further supported by the fact that rs11130605 was also identified as the SNP most strongly associated with *FLNB* mRNA levels in the study by Wilson et al. (Wilson et al., 2009). If this were the case, the associations seen between rs11720285 and rs9809315 and BMD phenotypes would probably be a result of the moderate LD between these two SNPs and rs11130605. However, it is also possible that polymorphism tagged by the rs11720285 and rs9809315 SNPs influences *FLNB* mRNA expression and hence BMD independently of the rs11130605 LD block through regulatory elements that were not identified in the above analysis. This theory is supported by the results from the haplotype analysis, which identified a strong positive influence on femoral neck BMD Z-score of the *CTT* haplotype which could indicate independent contributions from each of the three alleles.

In conclusion, polymorphism at the SNPs rs11720285, rs11130605 and rs9809315, all of which are located in either the 5' region or intron 1 of the *FLNB* gene, is significantly associated with BMD in Caucasian women. Therefore, the hypothesis has been supported by the data. The associations observed are almost certainly due to regulatory effects on the expression of the gene. Future studies could investigate exactly how these polymorphisms regulate expression of the *FLNB* gene (discussed in *Chapter 7*).

Chapter 6 – *ARHGEF3* transcript profiling and effects of *ARHGEF3* and *RHOA* gene knockdown on osteoblast-like and osteoclast-like cells *in vitro*

6.1 Introduction

Rho proteins are thought to be involved in multiple biological processes in numerous tissue types, some of which include: gene transcription, vesicle trafficking, microtubule dynamics, establishment of cell polarity, integrin signalling, cell cycle progression and actin cytoskeletal reorganisation (Etienne-Manneville and Hall, 2002). Activation of the RHOA protein can occur in response to extracellular stimuli from a number of cytokines, growth factors, hormones, integrins and adhesion molecules. Most of these activate RHOA through G-protein coupled receptors (GPCRs), a large family of transmembrane receptors that transfer extracellular signals into the interior of a cell. The GPCRs use various guanine nucleotide binding proteins (G-proteins) for signal transduction, some of which activate RHOA though regulation of the RhoGEFs (Siehler, 2009, Meyer et al., 2008). RHOA can also be activated by the ephrin A receptors through the neuronal GEF (NGEF) (Shamah et al., 2001), the insulin-like growth factor 1 receptor through forming a complex with ARHGEF12 (Becknell et al., 2003), and by the transmembrane receptor kinectin 1 (Santama et al., 2004). Once activated, RHOA has been shown to interact with a wide variety of downstream effectors, potentially initiating a vast array of signalling cascades.

Much evidence has been presented as to the mechanisms by which activated RHOA influences the actin cytoskeleton. Watanabe et al. identified diaphanous homolog 1 (DIAPH1) as a downstream effector of RHOA (Watanabe et al., 1997). DIAPH1 is a member of the formin group of proteins that are involved in producing long, straight actin filaments by catalysing actin nucleation and polymerisation (Goode and Eck, 2007). DIAPH1 was also found to bind profilin (Watanabe et al., 1997), a protein that promotes the polymerisation of actin (Cao et al., 1992, Pantaloni and Carlier, 1993). RHOA, DIAPH1 and profilin were found to co-localise in the membrane ruffles of rapidly spreading cells, suggesting that DIAPH1 acts as a molecular link between RHOA and profilin in a cytoskeletal mechanism that appears to be involved with cell motility (Watanabe et al., 1997). A later study by Peck et al. identified and characterised a ubiquitously expressed RHOA-binding protein designated Rhophilin-2 (Peck et al., 2002). Through studies in the HeLa cervical cancer cell line, the

authors produced evidence to suggest that Rhophilin-2 acts as a scaffold protein that increases filamentous actin turnover or limits stress fibre formation in the absence of high levels of RHOA activity (Peck et al., 2002).

One of the main downstream effectors of RHOA is the Rho-associated coiled-coil containing kinase (ROCK), which controls a number of different signalling pathways involved with cytoskeletal reorganisation. ROCK is a serine/threonine kinase that phosphorylates various substrates (Riento and Ridley, 2003). One of these substrates is the myosin-binding subunit of myosin phosphatase, which is deactivated upon phosphorylation by ROCK (Kimura et al., 1996, Uehata et al., 1997). When this is coupled with the ROCK-induced phosphorylation of myosin light chain (Amano et al., 1996), actin cross-linking by myosin is stimulated which enhances actomyosin contractility (Narumiya et al., 2009). ROCK has also been found to stabilise existing actin filaments by phosphorylating LIM-kinase, which subsequently phosphorylates and deactivates cofilin (Maekawa et al., 1999), a small protein that stimulates actin filament disassembly (Lappalainen and Drubin, 1997). A study by Hirakawa et al. in human umbilical vein endothelial cells has also highlighted a possible role for RHOA – ROCK signalling in actin cytoskeletal reorganisation mediated through tyrosine phosphorylation of focal adhesion kinase and paxillin (Hirakawa et al., 2004). Figure 6.1 presents an overview of the RhoGEF/RHOA signalling pathways discussed in this section.

As mentioned in previous chapters, the *RHOA* gene has been shown to have a role in both osteoblasts (McBeath et al., 2004, Meyers et al., 2005) and osteoclasts (Chellaiah et al., 2000). Given the results in this thesis showing association between polymorphism in the *ARHGEF3* and *RHOA* genes and BMD in postmenopausal women, the role of the products of these genes in osteoblasts and osteoclasts was investigated. The first aim of the work presented in this chapter was to analyse expression of the NM_001128616 transcript variant of the *ARHGEF3* gene, which carries the SNP rs7646054 (see *Chapter 3*), in osteoblast-like and osteoclast-like cells using quantitative real-time PCR. The second aim was to identify genes, from a list of candidates, whose expression was influenced by knockdown of the *ARHGEF3* and *RHOA* genes in osteoblast-like and osteoclast-like cells using microarray. Quantitative real-time PCR was then used to validate some of the more significant

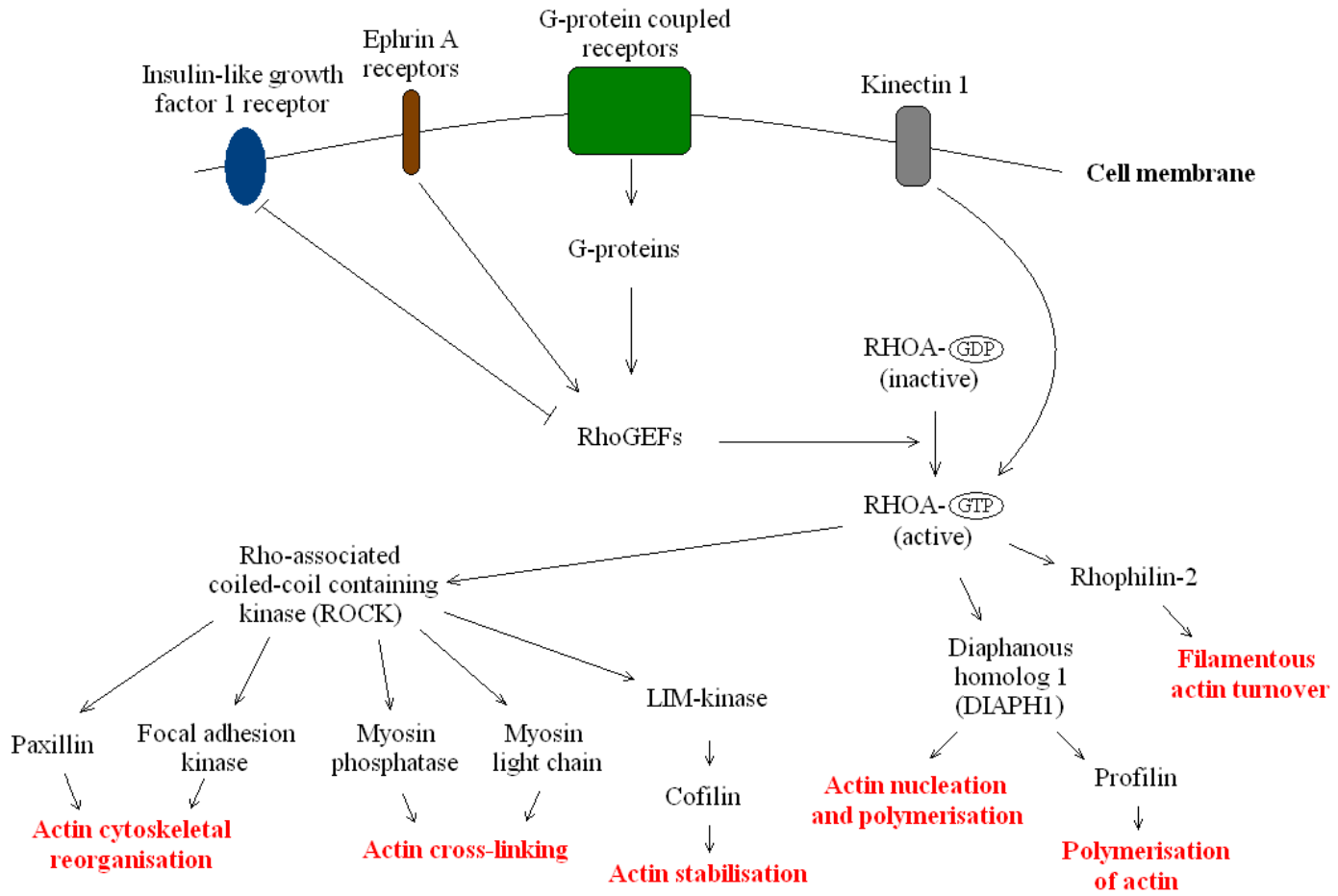

Figure 6.1. Schematic diagram of proposed mechanisms by which the RhoGEF/RHOA signalling pathways control cytoskeletal actin dynamics.

microarray findings, since this technique is considered to be an appropriate method of confirming microarray-generated data (Provenzano and Mocellin, 2007). The third aim of this chapter was to determine whether knockdown of the *ARHGEF3* and *RHOA* genes influences the resorptive capabilities of osteoclast-like cells.

6.1.1 Hypotheses

The NM_001128616 transcript variant of the *ARHGEF3* gene is expressed in bone cells.

Knockdown of the *ARHGEF3* and *RHOA* genes in osteoblast-like and osteoclast-like cells results in significant changes to the expression of genes involved with bone cell function or the RHOA signalling pathway.

Knockdown of the *ARHGEF3* and *RHOA* genes in osteoclast-like cells results in significant changes to their bone-resorptive capabilities.

6.2 Materials and methods

6.2.1 Cell culture

The human osteoblast-like cell lines Saos-2 (derived from osteosarcoma tissue), hFOB 1.19 (immortalised foetal osteoblasts) and MG-63 (derived from osteosarcoma tissue) were used for the gene knockdown work. These cell lines were cultured in DMEM (Sigma-Aldrich) pH 7.4 supplemented with 4.77 g/L HEPES, 3.7 g/L $NaHCO_3$, 10 % FBS and 1 % penicillin/streptomycin (100 units penicillin and 100 µg streptomycin per mL of media). The human BE(2)-M17 cell line (derived from neuroblastoma tissue) was cultured in Opti-MEM (Invitrogen) pH 7.4 supplemented with 2.4 g/L $NaHCO_3$, 5 % foetal bovine serum (FBS) and 1 % penicillin/streptomycin. Peripheral blood mononuclear cells/osteoclast-like cells (described below) were cultured in α-MEM (Invitrogen) pH 7.4 supplemented with 2.2 g/L $NaHCO_3$, 10 % FBS and 1 % penicillin/streptomycin. All cell culture media was sterilised prior to addition of FBS by filtration through a 0.8 µm filter. All cells were cultured at 37 ºC with 5 % CO_2 and the medium was changed every 2 – 3 days. Total RNA was harvested from each culture using the RNeasy Mini Kit (QIAGEN) and reverse transcription of the RNA was performed using the QuantiTect Reverse Transcription Kit (QIAGEN) (see *Chapter 2* for a full description of these

techniques). Quantitation of total RNA was performed using an ND-1000 spectrophotometer (NanoDrop Technologies).

6.2.2 Isolation of peripheral blood mononuclear cells and osteoclastogenesis

Osteoclast-like cells were differentiated from peripheral blood mononuclear cells isolated from 4 donors: a 47 year old Caucasian male (donor 1), a 28 year old Caucasian male (donor 2), a 56 year old Caucasian male (donor 3) and a 62 year old Caucasian male (donor 4). Each batch of cells was isolated from 30 mL whole blood collected in 10 mL K_2EDTA Vacutainer tubes (Becton, Dickinson and Company). Anti-coagulated whole blood samples were centrifuged at 2,200 rpm for 10 min at room temperature before buffy coats were collected and diluted to a total volume of 4 mL with 1X PBS (phosphate buffered saline, Appendix I). The cell suspension was then gently layered over 3 mL of Ficoll-Paque (Pharmacia) before being centrifuged again at 1,600 rpm for 40 min at room temperature. The top layer of plasma was then removed before the lymphocyte layer was collected and transferred to a clean tube. These cells were gently re-suspended in 6 mL 1X PBS and centrifuged at 800 rpm for 10 min at room temperature. The supernatant was then removed and this wash step was repeated. The washed cells were re-suspended in 5 mL medium supplemented with 10 ng/mL macrophage colony stimulating factor (M-CSF) and seeded directly into either a 24-well tissue culture plate or 25 cm^2 tissue culture flask. After two days, the medium was replaced with medium supplemented with 10 ng/mL M-CSF and 100 ng/mL RANKL. The cells were then grown using this medium formulation for 17 days while osteoclastogenesis occurred (Figure 6.2).

6.2.3 siRNA knockdown

Transfection of cells with siRNA sequences was used to knockdown expression of each of the *ARHGEF3* and *RHOA* genes. Two different siRNA sequences were used in tandem to knockdown expression of each gene. There is evidence to suggest that the RHOA protein has a half-life of up to 31 hours (Backlund, 1997), therefore a minimum gene knockdown period of 48 hours was used to ensure that the reduction in expression of the *RHOA* gene was sufficiently prolonged so as to have an effect at the protein level. Negative controls treated with AllStars Negative Control siRNA (QIAGEN) were included in each experiment. This negative control siRNA is a tested and validated non-silencing siRNA that has no homology to any known

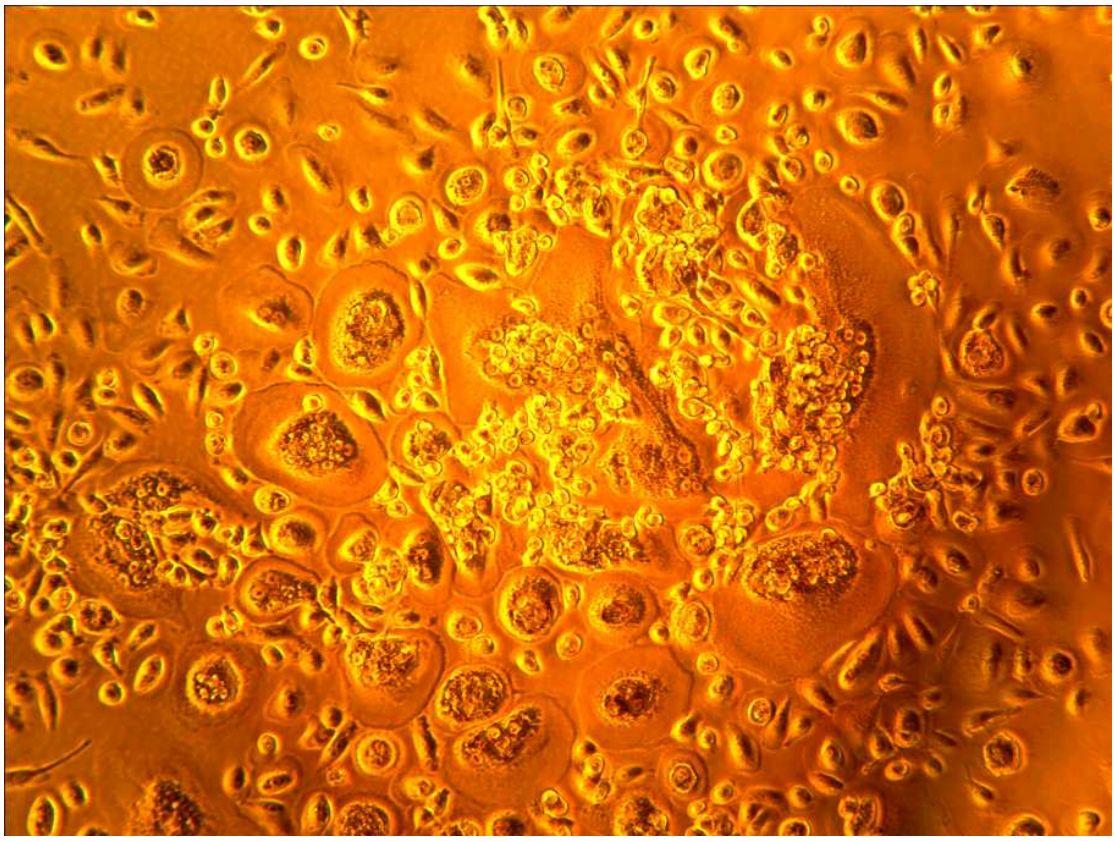

Figure 6.2. Peripheral blood mononuclear cells undergoing osteoclastogenesis as viewed under a phase contrast microscope (100X magnification). These cells were cultured for 16 days. Note the presence of multiple nuclei in some of the cells.

mammalian genes and has minimal off target effects as confirmed with Affymetrix GeneChip arrays (QIAGEN, 2010). All knockdown experiments were performed in triplicate.

6.2.3.i Osteoblast-like cells

siRNA knockdown experiments were performed in 24-well tissue culture plates with each well containing 500 µL of the aforementioned DMEM-based culture medium. For the Saos-2, hFOB 1.19 and MG-63 osteoblast-like cell lines, each well was seeded with 5 x 10^4 cells. Cells were grown for 24 hours before fresh medium was added to each culture and transfections were performed using a final siRNA concentration of 30 nM for transfection. The mix used had the following composition and was prepared at room temperature:

Component	**Volume (per well)**
10 µM siRNA	1.82 µL of each of the 2 siRNA sequences
FBS-free DMEM	96.36 µL
HiPerFect Transfection Reagent (QIAGEN)	6 µL
Total volume	**106 µL**

The above transfection mix was left at room temperature for 10 min to allow formation of transfection complexes before 106 µL was added dropwise to each well. Cells in each well were incubated with the transfection mix for 48 hours at 37 ºC prior to washing with 1X PBS (at room temperature) and subsequent extraction of total RNA.

6.2.3.ii Peripheral blood mononuclear cells/osteoclast-like cells

500 µL of freshly isolated peripheral blood mononuclear cells were aliquotted into each of 9 wells in a 24-well tissue culture plate. After osteoclastogenesis had occurred, as observed microscopically and confirmed biochemically (discussed later), siRNA knockdown experiments were performed using a final siRNA concentration of 100 nM for transfection. The mixture used had the following composition and was prepared at room temperature:

Component	**Volume (per well)**
10 µM siRNA	6.07 µL of each of the 2 siRNA sequences
FBS-free α-MEM	87.86 µL
HiPerFect Transfection Reagent (QIAGEN)	6 µL
Total volume	**106 µL**

The above transfection mix was left at room temperature for 10 min before 106 µL was added dropwise to each well. Cells in each well were incubated with the transfection mix for 48 hours at 37 ºC prior to washing with 1X PBS (at room temperature) and subsequent extraction of total RNA.

6.2.4 Microarray

A microarray analysis was performed on the RNA samples extracted from the Saos-2 cells and osteoclast-like cells from donor 1 that had been treated with *ARHGEF3*, *RHOA* and negative control siRNA. This was performed to identify genes that are differentially expressed as a direct result of knockdown of *ARHGEF3* and *RHOA*. Microarray analysis was performed using the HumanHT-12 v3 Expression BeadChip Kit (Illumina Inc.) and the TotalPrep RNA Amplification Kit (Applied Biosystems). The HumanHT-12 BeadChip profiles the expression of more than 25,000 annotated genes derived from NCBI RefSeq (Build 36.2) (Illumina, 2008). An outline of the approach used is given below:

Nine RNA samples extracted from Saos-2 cell cultures and 9 RNA samples extracted from osteoclast-like cell cultures (donor 1) were analysed by microarray. Each set of 9 samples was comprised of: 3 cultures treated with siRNA specific for *ARHGEF3*; 3 cultures treated with siRNA specific for *RHOA*; and 3 cultures treated with negative control siRNA. The quality and quantity of all RNA samples was checked prior to microarray analysis using a 2100 Bioanalyzer (Agilent Technologies). 10 µL of each RNA sample was subsequently processed for amplification and analysis by microarray. These analyses were performed by staff located in the Molecular Biology department at PathWest Laboratory Medicine WA.

6.2.5 Gene selection

While data were generated for most of the 25,000 genes included on the microarray, only 264 candidate genes were selected for statistical analysis in order to limit the potential for false positives. These candidate genes were selected on the basis of belonging to any of the following categories: genes thought to have a role in osteoblast function (45), genes thought to have a role in osteoclast function (62) or genes thought to be a part of the RHOA signalling pathway (157). Appendix III has a full list of the candidate genes analysed.

6.2.6 Real-time PCR

Quantitative real-time PCR was used to check for expression of the NM_001128616 transcript variant of the *ARHGEF3* gene, determine gene knockdown levels achieved and to validate microarray results for selected targets (see *Chapter 2* for a full description of the technique). This was performed on cDNA template using SYBR Green technology. cDNA samples were diluted in 1X TE buffer (Tris-EDTA buffer, Appendix I) before analysis. QuantiTect Primer Assays (QIAGEN) were used to amplify most gene transcript sequences. Bioinformatics analysis revealed that the QuantiTect Primer Assay for the *ACTA2* gene only amplifies one of the two transcript variants for this gene. Therefore, custom primer pairs were designed for this gene and the NM_001128616 transcript from the *ARHGEF3* gene using the web-based Primer3 software package (Rozen and Skaletsky, 2000) (see Appendix II for primer sequences). The human 18S ribosomal RNA gene (*RRN18S*), which has been validated as a suitable internal reference (housekeeping gene) for quantitative real-time PCR in a variety of human tissues (Aerts et al., 2004, Banda et al., 2008, Catalan et al., 2007), was selected as an internal reference for this work. The purpose of the internal reference is to allow for normalisation of the data for variations in the quantity of cDNA added to each reaction. The reaction efficiency of each primer pair was calculated by amplifying a 10-fold dilution series of target sequence across 5 orders of magnitude. This was performed to confirm that the reaction efficiency of each gene of interest is no more than 10 % from that of the internal reference as recommended by Schmittgen and Livak (Schmittgen and Livak, 2008). The log template dilution (x-axis) was plotted against the cycle threshold (C_T) value obtained for each dilution (y-axis) with the slope of the line put into the equation $m = -(1 / \log E)$, where m is the slope of the line and E is the reaction efficiency. A reaction efficiency of 2.0 equates to a perfect doubling of amplicon product during each PCR

cycle. All reactions were performed in triplicate with the mean C_T value used in the statistical analysis. The reaction efficiencies derived from these experiments are shown in Table 6.1. Melting-curve analysis was performed on all real-time PCR products to confirm amplification of a single sequence (see *Chapter 2* for a full description of this technique). A random selection of PCR products were also subjected to agarose gel electrophoresis for additional confirmation of the specificity of amplification.

6.2.7 Bone resorption assays

Essentially, this involved incubation of osteoclast-like cells on bone slices and monitoring of the subsequent resorption pit formation. Osteoclast-like cells from donor 1 were grown on bovine bone slices to determine whether knockdown of the *ARHGEF3* or *RHOA* genes affects their resorption capabilities. Peripheral blood mononuclear cells were isolated from 30 mL whole blood and seeded into a 25 cm^2 tissue culture flask. Osteoclastogenesis was induced for 10 – 14 days (as described previously), by which time the cells had differentiated into small multinucleated osteoclasts. At this point, the cells were detached from the tissue culture flask using Cell Dissociation Solution (Sigma-Aldrich), since if left to differentiate for longer they become very difficult to detach. Processing of the cells for resorption studies was then performed according to the standard protocols established by Prof Jiake Xu and colleagues (Centre for Orthopaedic Research, The University of Western Australia). The detached osteoclasts were pelleted by centrifugation at 1,500 rpm for 5 min and resuspended in fresh medium supplemented with 10 ng/mL M-CSF and 100 ng/mL RANKL. 100 µL of the cell suspension was then seeded into each of 18 wells containing a slice of bovine bone in a 96-well tissue culture plate. The cells were left for 2 hours to attach to the bone slices before the medium was removed and replaced with 50 µL of fresh medium. Transfection was then performed using a final siRNA concentration of 100 nM. The mix used had the following composition and was prepared at room temperature:

Component	**Volume (per well)**
10 µM siRNA	1 µL of each of the 2 siRNA sequences

FBS-free α-MEM	47 µL
HiPerFect Transfection Reagent (QIAGEN)	1 µL
Total volume	**50 µL**

The above transfection mix was left at room temperature for 10 min before 50 µL was added dropwise to each well. Cells in each well were incubated with the transfection mix for 96 hours at 37 ºC. Each bone slice was then removed and either stained for the osteoclast marker, tartrate resistant acid phosphatase (TRAP), or gently scrubbed to remove the osteoclast-like cells and analysed for resorption pit formation. The resorption pit analyses were performed on 2 bone chips for each treatment. Light microscopy was used to confirm the presence of resorption pits on each bone chip (Figure 6.3) and a NewView 6300 3D Optical Profilometer (Zygo Corp.) was used in conjunction with the MetroPro v8.2.0 (Zygo Corp.) software package to quantitate the volume of each resorption pit (Figure 6.4). The volume of each resorption pit was measured 3 times with the mean of these 3 readings used in the statistical analysis.

6.2.8 TRAP staining

Osteoclast-like cells were TRAP stained (using a chromogenic TRAP enzyme substrate) to confirm production of the TRAP enzyme as an indicator of the osteoclast phenotype. This involved washing the cells with 1X PBS, fixation with 4 % paraformaldehyde for 15 min, washing 3 times with 1X PBS before incubation with filtered TRAP stain solution (Appendix I) at 37 °C for 25 min. The stained cells were then washed 3 times with 1X PBS prior to visualisation using light microscopy (Figure 6.5).

6.2.9 Statistical analysis

6.2.9.i Microarray

Differential expression analysis of the microarray data using the Illumina custom error model was performed using the BeadStudio v3.4.0 (Illumina Inc.) software package. Samples treated with the negative control siRNA were specified as the reference group. The raw microarray gene expression data were normalised using the quantile normalisation algorithm (Bolstad et al., 2003), which adjusts the sample signals to minimise the influence of variation arising from non-biological factors (eg.

Table 6.1. Quantitative real-time PCR primer pair reaction efficiencies.

Template transcript	Reaction efficiency
ARHGEF3	1.92
RHOA	1.84
TNFRSF11B	1.93
ALPL	1.87
PTH1R	1.95
ACTA2	1.85
CCL5	1.94
OSCAR	1.94
ARHGDIA	1.86
RRN18S	1.88

A reaction efficiency of 2.0 is equivalent to a perfect doubling of target sequence during each PCR cycle.

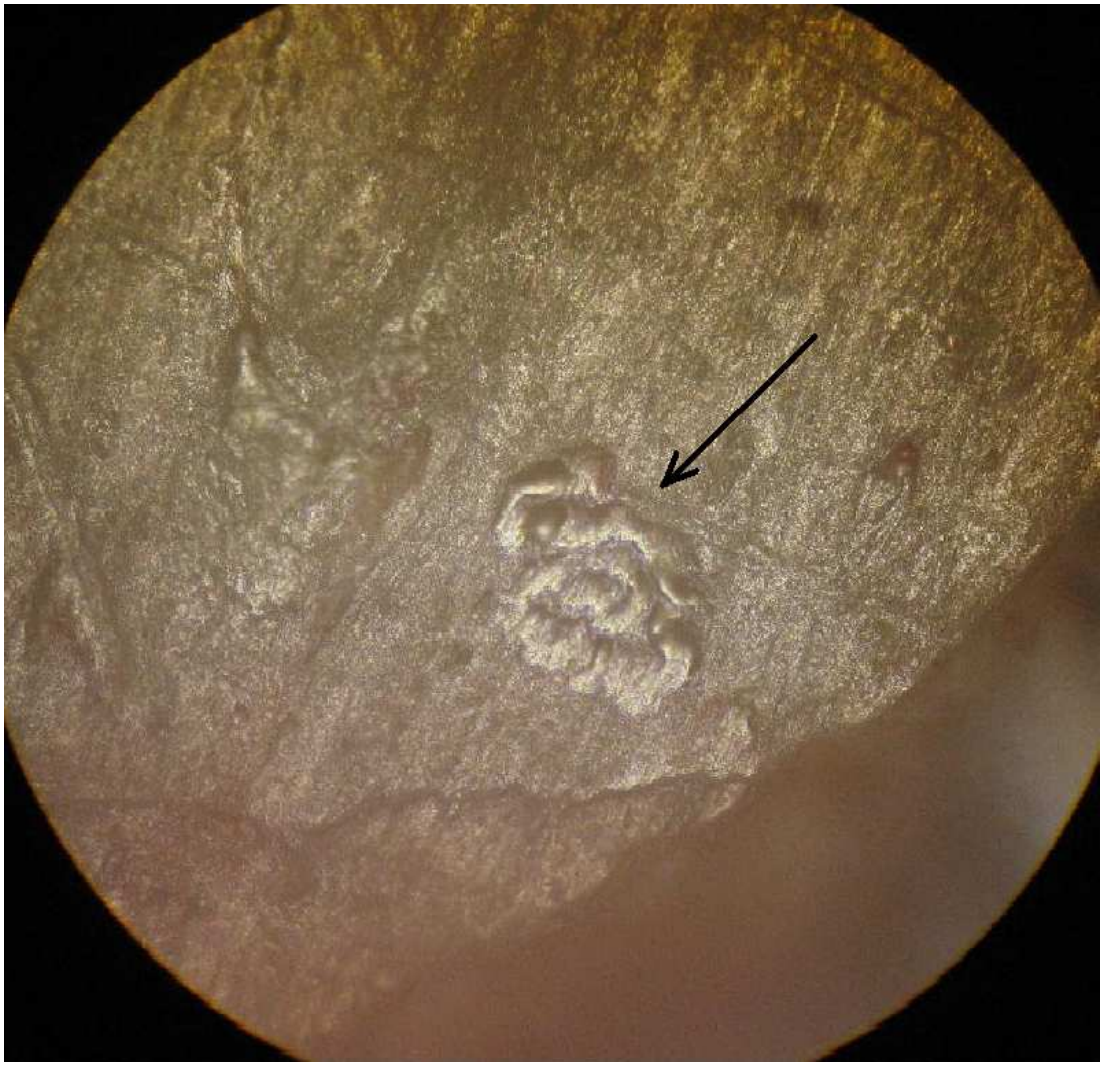

Figure 6.3. Image of an osteoclast resorption pit as viewed under a light microscope using 200X magnification. The location of the resorption pit is indicated by the arrow.

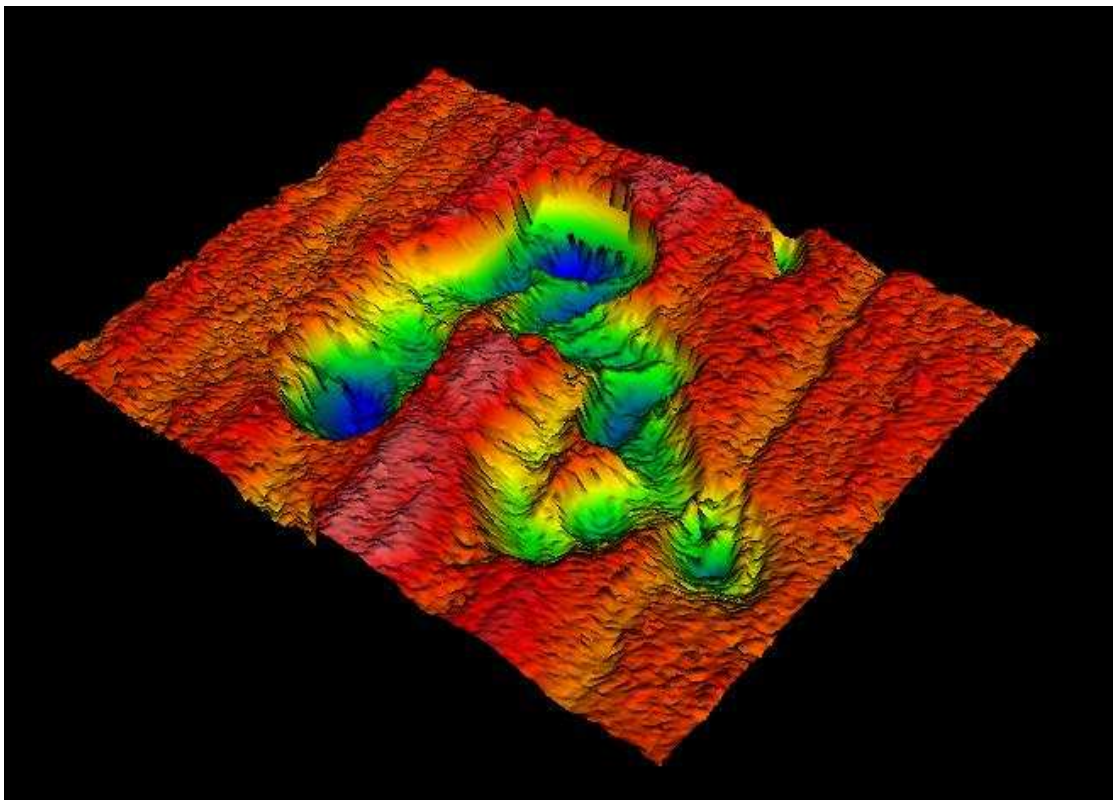

Figure 6.4. Image of an osteoclast resorption pit as viewed using a NewView 6300 3D Optical Profilometer. The depth of the pit is delineated by colour, blue being the deepest, and orange/yellow being the shallowest.

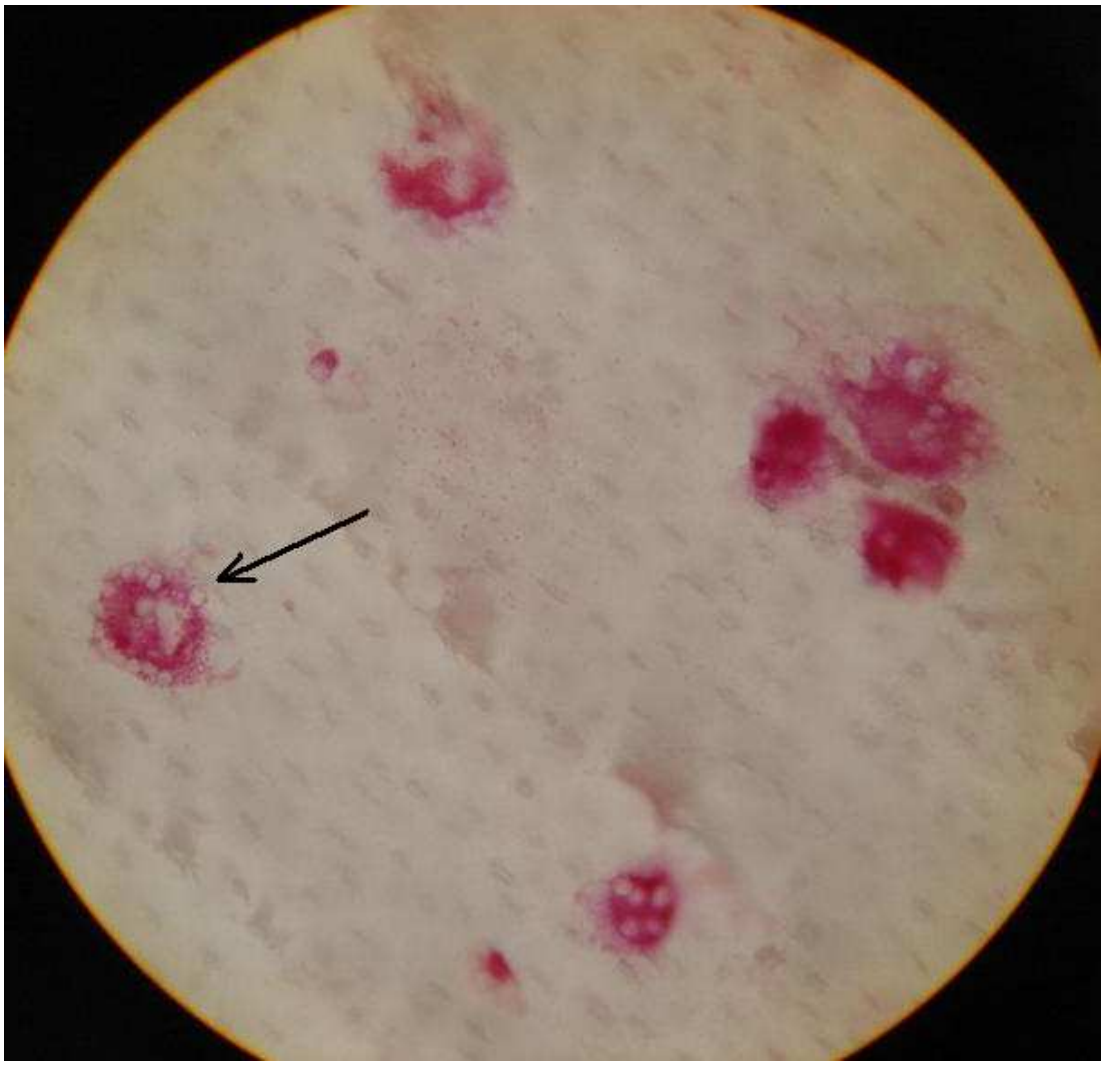

Figure 6.5. Image of TRAP stained osteoclast-like cells attached to a bovine bone slice as viewed under a light microscope (250X magnification). The location of a TRAP stained osteoclast-like cell is indicated by the arrow.

pipetting variation) (Illumina, 2007). Background subtraction was performed on the data to minimise the variation in background noise between arrays and to remove signal resulting from non-specific hybridisation (Dunning et al., 2008). Once background subtraction has been performed on the data, the expected signal for unexpressed targets is zero. The data were corrected for multiple testing using the Benjamini-Hochberg False Discovery Rate algorithm (Benjamini and Hochberg, 1995).

6.2.9.ii Real-time PCR

Gene expression ratios were calculated using the comparative C_T method as described by Schmittgen and Livak (Schmittgen and Livak, 2008). Briefly, the ΔC_T (C_T of the test gene - C_T of the internal reference) was calculated for each gene of interest in each sample in the test and control groups. This figure was then entered into the equation $2^{-\Delta CT}$ with the mean ± standard deviation calculated for each of the test and control groups. The mean of the test group was then divided by the mean of the control group to generate the expression ratio. An unpaired *t*-test was performed comparing the $2^{-\Delta CT}$ data from the test group with the control group to determine whether differences in expression observed were statistically significant.

6.2.9.iii Bone resorption assays

The volume of each resorption pit recorded for each of the knockdown groups was compared with the negative control group using an unpaired *t*-test. Throughout, two-tailed P-values are reported, with $P \leq 0.05$ considered significant.

6.3 Results

6.3.1 NM_001128616 transcript profiling in bone cells

As mentioned in *Chapter 3*, the *ARHGEF3* SNP rs7646054 that was found to be significantly associated with BMD is located within the 5' UTR of a recently described transcript variant of the gene designated NM_001128616 (Totoki et al., 2007). To determine whether this transcript variant is expressed in osteoblast-like cells, cDNA from the Saos-2 cell line was subjected to real-time PCR using 2 primer pairs: one designed to amplify all *ARHGEF3* transcript variants and one designed to amplify a sequence that is specific for the 5' UTR of the NM_001128616 transcript. While expression of the *ARHGEF3* gene was confirmed in this cell type, no

NM_001128616-specific amplification was detected. Since the NM_001128616 transcript variant was originally discovered in brain tissue (Totoki et al., 2007), cDNA from the neuroblastoma cell line BE(2)-M17 was checked for presence of the transcript as this cell line more closely resembles the cell type found in brain tissue. Again, expression of the *ARHGEF3* gene was confirmed in this cell type but no NM_001128616-specific amplification was detected. Finally, cDNA from each of the 4 batches of osteoclast-like cells (obtained from 4 different donors) and the hFOB 1.19 and MG-63 osteoblast-like cell lines were also checked for presence of the transcript. All 6 of these cell types were found to express the *ARHGEF3* gene. Neither of the hFOB 1.19 or MG-63 osteoblast-like cell lines were found to express NM_001128616, however all 4 batches of osteoclast-like cells were found to express the transcript. Therefore, the osteoclast-like cells were the only cell type found to express the NM_001128616 transcript variant of the *ARHGEF3* gene.

6.3.2 Microarray and real-time PCR in osteoblast-like cells

6.3.2.i Microarray results

Knockdown of the *ARHGEF3* and *RHOA* genes was confirmed by quantitative real-time PCR prior to microarray analysis. For the *ARHGEF3* and *RHOA* genes, a mean knockdown of 81 % and 79 % was achieved respectively in the Saos-2 cells (Table 6.2a and 6.2b). Out of the 202 candidate genes examined in the osteoblast-like cells, *ARHGEF3* and *RHOA* knockdown resulted in significant changes in expression of 8 and 2 genes respectively after adjustment for multiple testing (Table 6.3 and Table 6.4). For the *ARHGEF3* knockdown cultures, the genes that were found to be influenced by knockdown include: *TNFRSF11B*, *SP7*, *ALPL*, *ANGPTL2*, *GNA11*, *MYO9B*, *GNAI2* and *PFN1*. For the *RHOA* knockdown cultures, the genes that were found to be influenced by knockdown include: *PTH1R* and *ACTA2*.

6.3.2.ii Confirmation of microarray results by real-time PCR

Two of the differentially regulated genes from each of the *ARHGEF3* and *RHOA* knockdown groups were selected for confirmation and replication analysis. These genes were selected based on a number of factors including their potential relevance in bone metabolism (Table 6.5), their level of expression in the cell type and the size and statistical significance of the regulatory effect. The 2 genes selected from the *ARHGEF3* knockdown experiments were *TNFRSF11B* and *ALPL*; those selected

Table 6.2a. Quantitative real-time PCR validation of *ARHGEF3* gene knockdown in each cell type.

Cell type	$2^{-\Delta C_T}$ test group	$2^{-\Delta C_T}$ control group	Expression ratio	*P*
Saos-2 (first batch)	$7.8 \times 10^{-6} \pm 7.8 \times 10^{-7}$	$4.1 \times 10^{-5} \pm 5.2 \times 10^{-6}$	0.19	< 0.001
Saos-2 (second batch)	$6.6 \times 10^{-6} \pm 7.9 \times 10^{-7}$	$2.6 \times 10^{-5} \pm 2.1 \times 10^{-6}$	0.25	< 0.001
hFOB 1.19	$5.9 \times 10^{-6} \pm 7.8 \times 10^{-7}$	$2.4 \times 10^{-5} \pm 2.1 \times 10^{-6}$	0.25	< 0.001
MG-63	$3.1 \times 10^{-6} \pm 2.6 \times 10^{-7}$	$1.9 \times 10^{-5} \pm 1.4 \times 10^{-6}$	0.16	< 0.001
Osteoclast-like (donor 1)	$8.9 \times 10^{-5} \pm 8.5 \times 10^{-6}$	$2.4 \times 10^{-4} \pm 5.8 \times 10^{-6}$	0.37	< 0.001
Osteoclast-like (donor 2)	$5.5 \times 10^{-4} \pm 9.9 \times 10^{-5}$	$9.2 \times 10^{-4} \pm 2.8 \times 10^{-5}$	0.59	0.003
Osteoclast-like (donor 3)	$3.2 \times 10^{-4} \pm 9.1 \times 10^{-5}$	$6.8 \times 10^{-4} \pm 1.1 \times 10^{-4}$	0.48	0.01
Osteoclast-like (donor 4)	$6.7 \times 10^{-4} \pm 5.7 \times 10^{-5}$	$8.8 \times 10^{-4} \pm 2.1 \times 10^{-4}$	0.75	0.15

$2^{-\Delta C_T}$ values are given as *mean* ± *SD*.

Expression ratios are given as expression of the gene in the knockdown cultures relative to the negative control cultures.

Table 6.2b. Quantitative real-time PCR validation of *RHOA* gene knockdown in each cell type.

Cell type	$2^{-\Delta C_T}$ test group	$2^{-\Delta C_T}$ control group	Expression ratio	*P*
Saos-2 (first batch)	$1.4 \times 10^{-3} \pm 1.1 \times 10^{-4}$	$6.5 \times 10^{-3} \pm 4.4 \times 10^{-4}$	0.21	< 0.001
Saos-2 (second batch)	$1.5 \times 10^{-3} \pm 2.3 \times 10^{-4}$	$4.7 \times 10^{-3} \pm 2.5 \times 10^{-4}$	0.32	< 0.001
hFOB 1.19	$1.1 \times 10^{-3} \pm 1.8 \times 10^{-4}$	$4.9 \times 10^{-3} \pm 4.1 \times 10^{-4}$	0.23	< 0.001
MG-63	$6.4 \times 10^{-4} \pm 2.6 \times 10^{-4}$	$3.8 \times 10^{-3} \pm 2.9 \times 10^{-4}$	0.17	< 0.001
Osteoclast-like (donor 1)	$8.4 \times 10^{-4} \pm 6.7 \times 10^{-5}$	$5.4 \times 10^{-3} \pm 2.0 \times 10^{-4}$	0.16	< 0.001
Osteoclast-like (donor 2)	$4.2 \times 10^{-3} \pm 3.6 \times 10^{-4}$	$6.6 \times 10^{-3} \pm 2.8 \times 10^{-4}$	0.64	< 0.001
Osteoclast-like (donor 3)	$2.4 \times 10^{-3} \pm 2.7 \times 10^{-4}$	$4.4 \times 10^{-3} \pm 3.2 \times 10^{-4}$	0.55	0.001
Osteoclast-like (donor 4)	$3.5 \times 10^{-3} \pm 3.6 \times 10^{-4}$	$5.2 \times 10^{-3} \pm 3.4 \times 10^{-4}$	0.68	0.004

$2^{-\Delta C_T}$ values are given as *mean* ± *SD*.

Expression ratios are given as expression of the gene in the knockdown cultures relative to the negative control cultures.

Table 6.3. List of genes significantly influenced by *ARHGEF3* knockdown in Saos-2 osteoblast-like cells as determined by microarray analysis.

Gene	Gene product	Mean signal – knockdown (fluorescence units)	Mean signal – control (fluorescence units)	Expression ratio	*P*
TNFRSF11B	Osteoprotegerin	235	308	0.76	< 0.001
SP7	Osterix	434	381	1.14	0.007
ALPL	Alkaline phosphatase	19262	17173	1.12	0.03
ANGPTL2	Angiopoietin-like 2	81	50	1.64	< 0.001
GNA11	Guanine nucleotide binding protein alpha 11	352	469	0.75	0.002
MYO9B	Myosin IXB	165	212	0.78	0.005
GNAI2	Guanine nucleotide binding protein alpha inhibiting activity polypeptide 2	1106	1330	0.83	0.006
PFN1	Profilin 1	7458	8220	0.91	0.02

Expression ratios are given as expression of the gene in the knockdown cultures relative to the negative control cultures.

P values have been adjusted for multiple testing.

Table 6.4. List of genes significantly influenced by *RHOA* knockdown in Saos-2 osteoblast-like cells as determined by microarray analysis.

Gene	Gene product	Mean signal – knockdown (fluorescence units)	Mean signal – control (fluorescence units)	Expression ratio	*P*
PTH1R	Parathyroid hormone 1 receptor	1166	492	2.37	0.002
ACTA2	Alpha 2 actin, smooth muscle	3059	7812	0.39	< 0.001

Expression ratios are given as expression of the gene in the knockdown cultures relative to the negative control cultures.

P values have been adjusted for multiple testing.

Table 6.5. Genes selected for confirmation and replication analysis in the osteoblast-like cells.

Gene	Potential relevance of gene product in bone metabolism
TNFRSF11B	OPG is an osteoblast-produced decoy receptor for RANKL that primarily works to inhibit osteoclastogenesis (Hofbauer and Schoppet, 2004). OPG has also been shown to have a role in suppressing osteoclast survival (Akatsu et al., 1998), activity (Hakeda et al., 1998) and attachment to bone surfaces (O'Brien et al., 2000). Polymorphism within the *TNFRSF11B* gene has been identified as significantly associated with BMD in multiple studies (Richards et al., 2009, Richards et al., 2008, Rivadeneira et al., 2009, Styrkarsdottir et al., 2008). Refer to *Chapter 1*, section 1.4.2.*ii* for more information.
ALPL	Alkaline phosphatase is a membrane-bound enzyme that is expressed at high levels in osteoblasts and is thought to play a role in tissue mineralisation (Rodan, 1992). Increased circulating levels of the enzyme are often seen in bone disorders such as Paget's disease (Golob et al., 1996) and osteomalacia (softening of the bones due to defective bone mineralisation) (Rendina et al., 2009).
PTH1R	A receptor for PTH and PTH-related protein that is expressed on the surface of osteoblasts and is responsible for mediating some of the effects of PTH on bone (discussed in *Chapter 1*). Mutations in the *PTH1R* gene have been implicated in Ollier disease (Rendina et al., 2009), which is characterised by the presence of multiple enchondromas (cartilaginous tumours) in the bone marrow (Silve and Juppner, 2006).
ACTA2	A cytoskeletal protein that appears to be a part of the RHOA signalling pathway (Mack et al., 2001, Zhao et al., 2007), expression of which has been found to be reduced during osteochondrogenesis of smooth muscle cells (Speer et al., 2010, Zebboudj et al., 2003).

from the *RHOA* knockdown experiments were *PTH1R* and *ACTA2*. Quantitative real-time PCR data confirmed the microarray findings in these samples except for the increased expression of the *ALPL* gene in the *ARHGEF3* knockdown Saos-2 cells, which could not be confirmed as statistically significant (Table 6.6).

A second set of *ARHGEF3* and *RHOA* knockdown experiments was performed in Saos-2 cells in an effort to validate the quantitative real-time PCR results. Mean knockdown levels of 75 % and 68 % were achieved for the *ARHGEF3* and *RHOA* genes respectively as determined by quantitative real-time PCR (Table 6.2a and 6.2b). Out of the 4 genes selected for confirmation and replication analysis (2 from the *ARHGEF3* and 2 from the *RHOA* knockdown groups), the significant findings for the *TNFRSF11B*, *PTH1R* and *ACTA2* genes were replicated (Table 6.6). The *ALPL* gene, which was found to be significantly up-regulated in response to *ARHGEF3* knockdown in the microarray analysis, was found to be significantly down-regulated by *ARHGEF3* knockdown in the second batch of Saos-2 cells (Table 6.6).

Two additional osteoblast-like cell lines, hFOB 1.19 and MG-63, were also used for further replication analyses. In the hFOB 1.19 cell line, a mean knockdown of 75 % and 77 % was achieved for the *ARHGEF3* and *RHOA* genes respectively (Table 6.2a and 6.2b). In the MG-63 cell line, a mean knockdown of 84 % and 83 % was achieved for the *ARHGEF3* and *RHOA* genes respectively (Table 6.2a and 6.2b). Out of the 4 genes selected for confirmation and replication analysis, the down-regulation of the *TNFRSF11B* gene in response to *ARHGEF3* knockdown was replicated in the hFOB 1.19 cells and the down-regulation of the *ACTA2* gene in response to *RHOA* knockdown was replicated in both the hFOB 1.19 and MG-63 cells (Table 6.6). The up-regulation of the *ALPL* gene in response to *ARHGEF3* knockdown in the batch of Saos-2 cells analysed by microarray was replicated in the MG-63 cells (Table 6.6). Expression of the *PTH1R* gene was not detected in the hFOB 1.19 or MG-63 cell lines, therefore possible regulatory effects could not be studied. Overall, the down-regulation of the *ACTA2* gene in response to *RHOA* knockdown was the most consistent result observed.

6.3.3 Microarray and real-time PCR in osteoclast-like cells

6.3.3.i Microarray results

Table 6.6. Quantitative real-time PCR validation of gene regulation in osteoblast-like cells in response to *ARHGEF3* and *RHOA* gene knockdown.

Cell type	Knockdown	Gene amplified	$2^{-\Delta C_T}$ test group	$2^{-\Delta C_T}$ control group	Expression ratio	*P*
Saos-2 (first batch)	*ARHGEF3*	*TNFRSF11B*	$5.9 \times 10^{-4} \pm 5.0 \times 10^{-5}$	$7.7 \times 10^{-4} \pm 6.1 \times 10^{-5}$	0.76	0.02
		ALPL	$2.9 \times 10^{-2} \pm 1.0 \times 10^{-3}$	$2.7 \times 10^{-2} \pm 2.7 \times 10^{-3}$	1.09	0.21
	RHOA	*PTHR1*	$3.9 \times 10^{-4} \pm 6.0 \times 10^{-5}$	$2.0 \times 10^{-4} \pm 1.7 \times 10^{-5}$	1.98	0.006
		ACTA2	$5.3 \times 10^{-4} \pm 5.4 \times 10^{-5}$	$1.7 \times 10^{-3} \pm 8.7 \times 10^{-5}$	0.32	< 0.001
Saos-2 (second batch)	*ARHGEF3*	*TNFRSF11B*	$4.4 \times 10^{-4} \pm 3.2 \times 10^{-5}$	$5.9 \times 10^{-4} \pm 4.0 \times 10^{-5}$	0.73	0.006
		ALPL	$1.6 \times 10^{-2} \pm 1.1 \times 10^{-3}$	$2.2 \times 10^{-2} \pm 1.4 \times 10^{-3}$	0.75	0.007
	RHOA	*PTHR1*	$2.3 \times 10^{-4} \pm 2.6 \times 10^{-5}$	$1.4 \times 10^{-4} \pm 3.6 \times 10^{-5}$	1.60	0.03
		ACTA2	$7.8 \times 10^{-4} \pm 4.6 \times 10^{-5}$	$2.2 \times 10^{-3} \pm 1.8 \times 10^{-5}$	0.35	< 0.001
hFOB 1.19	*ARHGEF3*	*TNFRSF11B*	$2.6 \times 10^{-4} \pm 1.0 \times 10^{-5}$	$3.7 \times 10^{-4} \pm 2.6 \times 10^{-5}$	0.72	0.003
		ALPL	$5.7 \times 10^{-5} \pm 1.8 \times 10^{-6}$	$6.2 \times 10^{-5} \pm 6.9 \times 10^{-6}$	0.92	0.29
	RHOA	*PTHR1*	-	-	-	-
		ACTA2	$2.1 \times 10^{-4} \pm 1.2 \times 10^{-5}$	$4.2 \times 10^{-4} \pm 4.0 \times 10^{-5}$	0.50	< 0.001
MG-63	*ARHGEF3*	*TNFRSF11B*	$8.2 \times 10^{-3} \pm 1.2 \times 10^{-3}$	$8.0 \times 10^{-3} \pm 7.4 \times 10^{-4}$	1.02	0.83
		ALPL	$4.3 \times 10^{-5} \pm 2.7 \times 10^{-6}$	$3.7 \times 10^{-5} \pm 2.3 \times 10^{-6}$	1.16	0.04
	RHOA	*PTHR1*	-	-	-	-
		ACTA2	$4.2 \times 10^{-5} \pm 1.3 \times 10^{-5}$	$1.3 \times 10^{-4} \pm 8.5 \times 10^{-6}$	0.34	< 0.001

$2^{-\Delta C_T}$ values are given as $mean \pm SD$.

Expression ratios are given as expression of the gene in the knockdown cultures relative to the negative control cultures.

The second batch of Saos-2 cells was used to validate the results obtained from the first before proceeding with the additional cell lines.

In the osteoclast-like cells, knockdown of the *ARHGEF3* and *RHOA* genes was confirmed by quantitative real-time PCR prior to microarray analysis. For the *ARHGEF3* and *RHOA* genes, a mean knockdown of 63 % and 84 % was achieved respectively in the osteoclast-like cells from donor 1 (Table 6.2a and 6.2b). Expression of the genes encoding the osteoclastic biochemical markers TRAP (*ACP5*), cathepsin K (*CTSK*) and calcitonin receptor (*CALCR*) was confirmed from the microarray output. The *ACP5* and *CTSK* genes were found to be expressed at particularly high levels in this cell type (mean microarray signal > 14,000 fluorescence units). Out of the 219 candidate genes examined in the osteoclast-like cells, *ARHGEF3* and *RHOA* knockdown resulted in significant changes in expression of 12 and 9 genes respectively after adjustment for multiple testing (Table 6.7 and Table 6.8). For the *ARHGEF3* knockdown cultures, the genes that were found to be influenced by knockdown include: *CCL5*, *HLA-C*, *SNCA*, *TNF*, *OSCAR*, *CD44*, *BIRC3*, *ITGB7*, *ITGAE*, *ITGAL*, *ITGA3* and *ITGAM*. For the *RHOA* knockdown cultures, the genes that were found to be influenced by knockdown include: *TNF*, *THBS2*, *CCL5*, *ITGB7*, *ARHGDIA*, *IGF1*, *ACTA2*, *MYL9* and *ITGAE*. Of these, the influence of *RHOA* knockdown on expression of the *ACTA2* gene was also observed in the osteoblast-like cells.

6.3.3.ii Confirmation of microarray results by real-time PCR

Two of the differentially regulated genes from each of the *ARHGEF3* and *RHOA* knockdown experiments were selected for confirmation and replication analysis. These genes were selected based on a number of factors including their potential relevance in bone metabolism (Table 6.9), their level of expression in the cell type and the size and statistical significance of the regulatory effect. The 2 genes selected from the *ARHGEF3* knockdown experiments were *CCL5* and *OSCAR*; those selected from the *RHOA* knockdown experiments were *ARHGDIA* and *ACTA2*. Quantitative real-time PCR was able to confirm the microarray findings in these samples (Table 6.10).

Replication of the regulatory effects observed in response to *ARHGEF3* and *RHOA* knockdown in the osteoclast-like cells from donor 1 was attempted in osteoclast-like cells from 3 additional donors. In the cells obtained from donor 2, a mean knockdown of 41 % and 36 % was achieved for the *ARHGEF3* and *RHOA* genes

Table 6.7. List of genes significantly influenced by *ARHGEF3* knockdown in osteoclast-like cells as determined by microarray analysis.

Gene	Gene product	Mean signal – knockdown (fluorescence units)	Mean signal – control (fluorescence units)	Expression ratio	*P*
CCL5	Chemokine ligand 5	4402	588	7.48	< 0.001
HLA-C	Major histocompatibility complex, class I, C	235	87	2.71	< 0.001
SNCA	Synuclein, alpha	129	260	0.49	< 0.001
TNF	Tumour necrosis factor alpha	420	156	2.69	< 0.001
OSCAR	Osteoclast associated immunoglobulin-like receptor	1950	1244	1.57	0.01
CD44	CD44 molecule	2279	3360	0.68	0.03
BIRC3	Baculoviral IAP repeat-containing 3	496	321	1.55	0.04
ITGB7	Integrin, beta 7	588	106	5.54	< 0.001
ITGAE	Integrin, alpha E	256	483	0.53	< 0.001
ITGAL	Integrin, alpha L	145	79	1.85	0.003
ITGA3	Integrin, alpha 3	107	221	0.48	0.003
ITGAM	Integrin, alpha M	1033	1699	0.61	0.004

Expression ratios are given as expression of the gene in the knockdown cultures relative to the negative control cultures.

P values have been adjusted for multiple testing.

Osteoclast-like cells derived from donor 1.

Table 6.8. List of genes significantly influenced by *RHOA* knockdown in osteoclast-like cells as determined by microarray analysis.

Gene	Gene product	Mean signal – knockdown (fluorescence units)	Mean signal – control (fluorescence units)	Expression ratio	*P*
TNF	Tumour necrosis factor alpha	345	156	2.21	< 0.001
THBS2	Thrombospondin 2	89	43	2.05	< 0.001
CCL5	Chemokine ligand 5	891	588	1.52	0.006
ITGB7	Integrin, beta 7	246	106	2.32	< 0.001
ARHGDIA	Rho GDP dissociation inhibitor alpha	579	998	0.58	< 0.001
IGF1	Insulin-like growth factor 1	121	42	2.86	< 0.001
ACTA2	Alpha 2 actin, smooth muscle	545	965	0.56	0.001
MYL9	Myosin, light chain 9, regulatory	22	49	0.46	0.04
ITGAE	Integrin, alpha E	342	483	0.71	0.04

Expression ratios are given as expression of the gene in the knockdown cultures relative to the negative control cultures.

P values have been adjusted for multiple testing.

Osteoclast-like cells derived from donor 1.

Table 6.9. Genes selected for confirmation and replication analysis in the osteoclast-like cells.

Gene	Potential relevance of gene product in bone metabolism
CCL5	The product of the *CCL5* gene is a chemoattractant cytokine (Schall et al., 1990) that has been implicated in rheumatoid arthritis, osteoarthritis (Lisignoli et al., 2002, Volin et al., 1998) and osteomyelitis (infection of the bone) (Wright and Friedland, 2002). Expression of *CCL5* has been shown to be induced during osteoclast differentiation (Day et al., 2004) and there is evidence to suggest that CCL5 produced by osteoclasts induces osteoblast chemotaxis (Yano et al., 2005).
OSCAR	An osteoclast-associated receptor that is expressed in preosteoclasts and mature osteoclasts and may play a critical role in osteoclast differentiation (Kim et al., 2002). OSCAR expression has been found to be up-regulated in circulating monocytes obtained from rheumatoid arthritis patients and expression of the receptor seems to be related to the inflammatory activity of the disease (Herman et al., 2008).
ARHGDIA	The product of the *ARHGDIA* gene is a Rho GDP dissociation inhibitor that inhibits RHOA activation (DerMardirossian and Bokoch, 2005), effectively working in an opposite manner to ARHGEF3. A recent study has suggested that expression of *ARHGDIA* is significantly up-regulated in rat osteoblasts treated with PTH *in vivo* and *in vitro* (Sun et al., 2009).
ACTA2	See Table 6.5.

Table 6.10. Quantitative real-time PCR validation of gene regulation in osteoclast-like cells in response to *ARHGEF3* and *RHOA* gene knockdown.

Cell source	Knockdown	Gene amplified	$2^{-\Delta C_T}$ test group	$2^{-\Delta C_T}$ control group	Expression ratio	*P*
Donor 1	*ARHGEF3*	*CCL5*	$4.1 \times 10^{-3} \pm 1.3 \times 10^{-3}$	$5.5 \times 10^{-4} \pm 1.2 \times 10^{-4}$	7.49	0.008
		OSCAR	$1.1 \times 10^{-3} \pm 3.9 \times 10^{-5}$	$8.9 \times 10^{-4} \pm 8.4 \times 10^{-5}$	1.27	0.01
	RHOA	*ARHGDIA*	$1.0 \times 10^{-3} \pm 1.2 \times 10^{-4}$	$1.8 \times 10^{-3} \pm 1.4 \times 10^{-4}$	0.57	0.002
		ACTA2	$1.1 \times 10^{-4} \pm 9.0 \times 10^{-6}$	$1.9 \times 10^{-4} \pm 1.8 \times 10^{-5}$	0.62	0.003
Donor 2	*ARHGEF3*	*CCL5*	$1.6 \times 10^{-3} \pm 2.6 \times 10^{-4}$	$2.3 \times 10^{-3} \pm 1.1 \times 10^{-4}$	0.67	0.009
		OSCAR	$1.8 \times 10^{-4} \pm 2.6 \times 10^{-5}$	$2.2 \times 10^{-4} \pm 1.7 \times 10^{-5}$	0.84	0.13
	RHOA	*ARHGDIA*	$1.2 \times 10^{-3} \pm 9.4 \times 10^{-5}$	$1.7 \times 10^{-3} \pm 8.2 \times 10^{-5}$	0.74	0.004
		ACTA2	$7.7 \times 10^{-4} \pm 1.1 \times 10^{-4}$	$7.8 \times 10^{-4} \pm 4.7 \times 10^{-5}$	0.98	0.86
Donor 3	*ARHGEF3*	*CCL5*	$1.9 \times 10^{-3} \pm 3.8 \times 10^{-4}$	$5.3 \times 10^{-3} \pm 8.9 \times 10^{-4}$	0.36	0.004
		OSCAR	$4.0 \times 10^{-4} \pm 6.2 \times 10^{-5}$	$5.0 \times 10^{-4} \pm 1.2 \times 10^{-4}$	0.81	0.30
	RHOA	*ARHGDIA*	$8.6 \times 10^{-4} \pm 1.4 \times 10^{-4}$	$1.1 \times 10^{-3} \pm 1.2 \times 10^{-4}$	0.81	0.14
		ACTA2	$5.8 \times 10^{-4} \pm 1.1 \times 10^{-5}$	$7.1 \times 10^{-4} \pm 1.3 \times 10^{-5}$	0.82	< 0.001
Donor 4	*ARHGEF3*	*CCL5*	$2.2 \times 10^{-3} \pm 7.4 \times 10^{-4}$	$3.0 \times 10^{-3} \pm 5.9 \times 10^{-4}$	0.72	0.19
		OSCAR	$2.9 \times 10^{-4} \pm 3.7 \times 10^{-5}$	$3.1 \times 10^{-4} \pm 3.1 \times 10^{-5}$	0.96	0.66
	RHOA	*ARHGDIA*	$8.8 \times 10^{-4} \pm 8.3 \times 10^{-5}$	$1.0 \times 10^{-3} \pm 1.0 \times 10^{-4}$	0.85	0.11
		ACTA2	$8.7 \times 10^{-4} \pm 6.1 \times 10^{-5}$	$1.0 \times 10^{-3} \pm 7.5 \times 10^{-5}$	0.88	0.09

$2^{-\Delta C_T}$ values are given as $mean \pm SD$.

Expression ratios are given as expression of the gene in the knockdown cultures relative to the negative control cultures.

respectively (Table 6.2a and 6.2b). In the cells obtained from donor 3, a mean knockdown of 52 % and 45 % was achieved for the *ARHGEF3* and *RHOA* genes respectively (Table 6.2a and 6.2b). In the cells obtained from donor 4, a mean knockdown of 25 % and 32 % was achieved for the *ARHGEF3* and *RHOA* genes respectively (Table 6.2a and 6.2b). Consistent replication of the findings from the microarray analysis proved difficult in this cell type. However, the down-regulation of the *ARHGDIA* and *ACTA2* genes observed in response to *RHOA* knockdown was replicated in the cells obtained from donor 2 and donor 3 respectively (Table 6.10). The *CCL5* gene, which was found to be significantly up-regulated in response to *ARHGEF3* knockdown in the cells obtained from donor 1, was found to be significantly down-regulated in the cells obtained from donors 2 and 3 (Table 6.10).

6.3.4 Bone resorption assays

The mean resorption pit volume observed ± standard deviation was 54,441 μm^3 ± 36,693 μm^3, 34,593 μm^3 ± 22,194 μm^3 and 45,282 μm^3 ± 33,416 μm^3 for the *ARHGEF3* knockdown, *RHOA* knockdown and negative control cultures respectively. No significant differences were observed between either of the treatment groups and the negative control group (*P = 0.47* and *0.33* for the *ARHGEF3* and *RHOA* knockdown groups respectively; Figure 6.6).

6.4 Discussion

The main objectives of the work described in this chapter were to determine whether the NM_001128616 transcript variant of *ARHGEF3* is expressed in bone cells, examine the influence of *ARHGEF3* and *RHOA* knockdown on expression of genes relevant to bone metabolism and the RHOA signalling pathway in osteoblast-like and osteoclast-like cells, and to determine whether knockdown of *ARHGEF3* and *RHOA* influences the bone resorptive capabilities of osteoclast-like cells.

Concerning the first objective, expression of the *ARHGEF3* gene was detected in all cell types examined, but was substantially higher in osteoclast-like cells than in osteoblast-like cells (~19 fold higher in the osteoclast-like cells based on the microarray output). However, expression of the NM_001128616 transcript variant of the *ARHGEF3* gene was detected only in the osteoclast-like cells. It is possible that the NM_001128616 transcript variant is expressed in the osteoblast-like cells but that

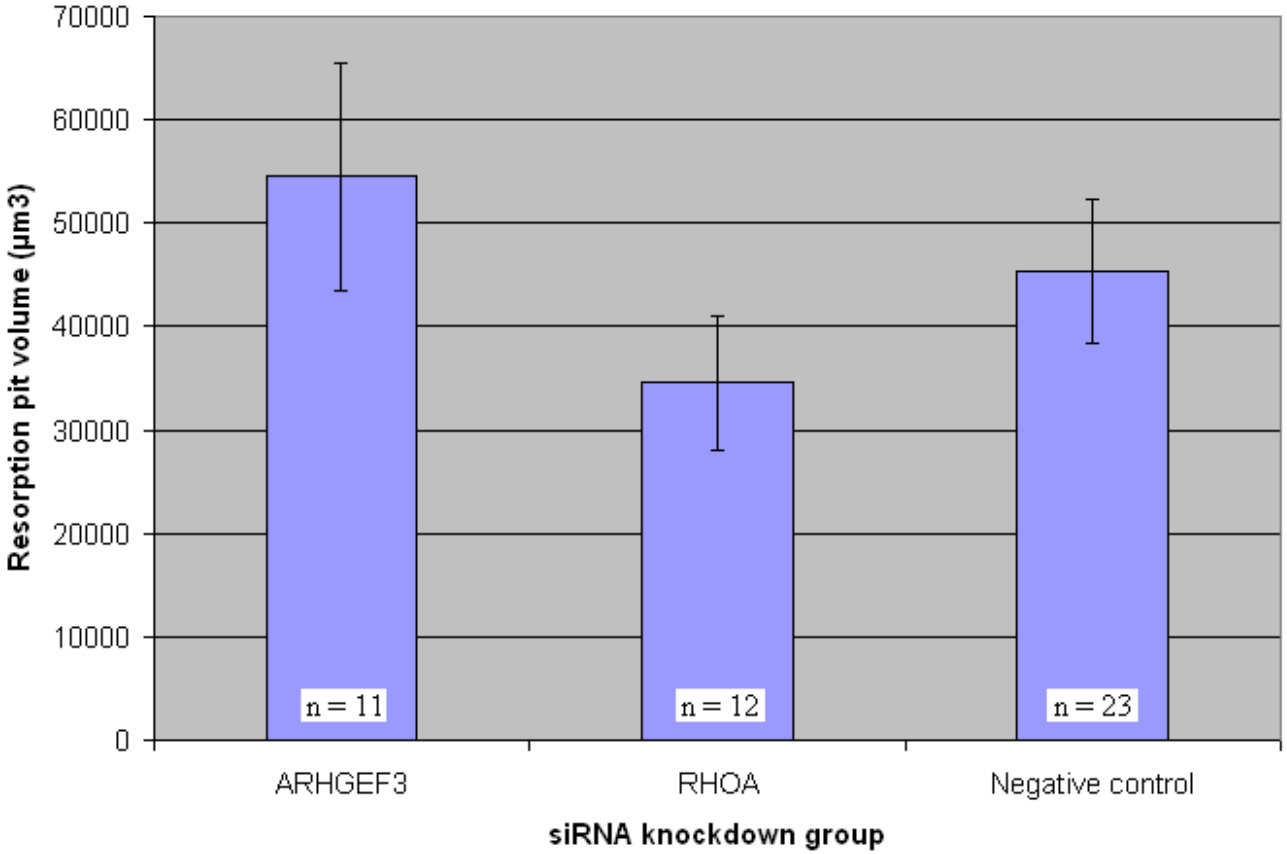

Figure 6.6. Bone resorption pit formation of osteoclasts treated with *ARHGEF3, RHOA* and negative control siRNA. Neither the *ARHGEF3* nor *RHOA* siRNA groups are significantly different from the negative control siRNA group. Values are given as *mean* ± *SEM*. n = number of pits analysed.

its expression was below detectable levels, but it is also possible that osteoblasts do not express this particular transcript. In any case, if polymorphism at rs76546054 influences BMD through effects on the NM_001128616 transcript variant, it seems more likely that this effect is mediated through the osteoclast than the osteoblast, although an influence through the osteoblast cannot be totally excluded. Further studies could focus on allele-specific expression analysis of the NM_001128616 transcript in cells obtained from individuals that are heterozygous for polymorphism at rs7646054 (discussed in *Chapter 7*).

Due to the associations identified in this thesis between polymorphism in the *ARHGEF3* and *RHOA* genes and bone density in women, the effects of knockdown of these two genes on expression of selected candidate genes in bone cells was investigated. Greater gene knockdown levels were achieved in the osteoblast-like cells than in the osteoclast-like cells (78.7 % versus 45.2 % mean knockdown for the *ARHGEF3* gene; and 76.7 % versus 49.3 % mean knockdown for the *RHOA* gene, as determined by quantitative real-time PCR). Concerning the studies performed in the osteoblast-like cells, expression of the *ACTA2* gene was found to be significantly down-regulated by *RHOA* knockdown in all three osteoblast-like cell lines examined (Saos-2, hFOB 1.19 and MG-63), with an average expression ratio of 0.38 seen in knockdown cell cultures relative to control cell cultures by quantitative real-time PCR. The *ACTA2* gene encodes an alpha actin cytoskeletal protein, and was found to be expressed at high levels in the Saos-2 cell line as determined by microarray and quantitative real-time PCR analysis. This supports expressed sequence tag (EST) profile data for the gene from the NCBI database which suggest that *ACTA2* mRNA is expressed in human bone and bone marrow. There have been very few studies on the role of the *ACTA2* gene product in bone metabolism, with most studies having been carried out in smooth muscle cells. It is a major component of the smooth muscle cell contractile apparatus, accounting for around 40 % of the total protein and around 70 % of the total actin in smooth muscle cells (Fatigati and Murphy, 1984, Owens, 1995). There is evidence in the literature to suggest that the *ACTA2* gene is regulated by RHOA signalling, this is discussed further in *Chapter 7*.

Knockdown of the *ARHGEF3* gene in both batches of Saos-2 cells resulted in consistent and significant down-regulation of the levels of *TNFRSF11B* (OPG)

mRNA. This effect was replicated in the hFOB 1.19 cells, but not in the MG-63 cell line. It is not clear why this effect was not seen in the MG-63 cells, it may be an effect particular to the cell line. In addition to these findings, knockdown of the *RHOA* gene in both batches of Saos-2 cells resulted in significant up-regulation of *PTH1R* (parathyroid hormone 1 receptor) mRNA levels, although expression of this gene was not detected in the hFOB 1.19 or MG-63 cell lines. These genes both have a major role in the mechanism by which osteoblasts stimulate osteoclastogenesis upon exposure to PTH, suggesting that the *ARHGEF3* and *RHOA* genes may be involved in this process. Supporting evidence for this theory comes from a recent publication by Wang and Stern, who found that the osteoclastogenic activity of UMR-106 rat osteoblast-like cells was inhibited when the cells were transfected with constitutively active Rhoa (Wang and Stern, 2010). This is discussed further in *Chapter 7*.

Caution is advised in drawing any conclusions from the gene expression data in the osteoclast-like cells, since consistently high gene knockdown (> 60 %) was not achieved in the cells obtained from donors 2, 3 and 4. Nevertheless, some interesting results were obtained. Expression of the *ARHGDIA* gene was found to be significantly reduced in response to *RHOA* gene knockdown in the osteoclast-like cells obtained from donors 1 and 2. The product of the *ARHGDIA* gene is a Rho GDP dissociation inhibitor (GDI) that is a negative regulator of several of the RhoGTPases (DerMardirossian and Bokoch, 2005). RhoGDIs keep the Rho proteins in their inactive GDP-bound state by inhibiting the exchange of GDP for GTP (Fukumoto et al., 1990) and sequestering them in the cytosol (Olofsson, 1999). The down-regulation of *ARHGDIA* expression seen in the *RHOA* knockdown osteoclast-like cells in this study could be a compensatory mechanism for the reduced expression of the *RHOA* gene. Expression of the *ACTA2* gene was found to be significantly down-regulated in response to *RHOA* knockdown in the osteoclast-like cells obtained from donors 1 and 3. This adds further support to the earlier suggestion that expression of this gene is regulated by the RHOA signalling pathway. The inconsistent nature of the results described here for the *ARHGDIA* and *ACTA2* genes is probably due to the variable gene knockdown that was achieved in this cell type. However, it does appear that there is a trend towards down-regulation of these genes in response to *RHOA* knockdown.

Finally, some preliminary studies were conducted on the influence of *ARHGEF3* and *RHOA* gene knockdown on osteoclast activity, as measured by resorption pit formation on bone slices. The osteoclast-like cells were generated *in vitro* from peripheral blood mononuclear cells and demonstrated the multi-nucleated morphology that is characteristic of the osteoclast cell type with production of TRAP. That the cells could also form resorption pits was further verification that the differentiation of osteoclast-like cells had been successful. However, there were no significant differences observed between the bone resorptive capabilities of the osteoclast-like cells treated with *ARHGEF3* or *RHOA* siRNA and those treated with negative control siRNA. There are several possible explanations for this, one of which is the large amount of variation observed within each treatment group during the resorption pit volume analysis. Another possible explanation is the disappointing level of gene knockdown achieved in this cell type, as alluded to previously. As such, one cannot come to any firm conclusion from this negative finding. Alternative techniques that could be employed in future studies to achieve greater gene knockdown in osteoclast-like cells are discussed in *Chapter 7*.

In conclusion, the total expression of all *ARHGEF3* transcript variants appears to be much higher in the osteoclast-like cells examined than in the osteoblast-like cell lines. Expression of the NM_001128616 transcript of *ARHGEF3* was detected in the osteoclast-like cells, therefore the hypothesis that this transcript variant is expressed in bone cells has been supported by the data. The fact that this transcript only appeared to be expressed by the osteoclast-like cells indicates that if variation at the rs7646054 SNP site is directly responsible for the associations observed with BMD via this transcript, the effect is likely to be mediated through the osteoclast. Knockdown of the *ARHGEF3* and *RHOA* genes in the three osteoblast-like cell lines revealed important regulatory changes, including significant down-regulation of the *ACTA2* gene in response to *RHOA* knockdown in all three cell lines, significant down-regulation of the *TNFRSF11B* gene in response to *ARHGEF3* knockdown in the Saos-2 and hFOB 1.19 cell lines, and significant up-regulation of the *PTH1R* gene in response to *RHOA* knockdown in the Saos-2 cell line. These results suggest that the *RHOA* gene product may regulate the expression of the *ACTA2* gene, and that the *ARHGEF3* and *RHOA* genes may have a role in PTH-mediated signalling in

the osteoblast, which could have downstream effects on osteoclastogenesis. In the osteoclast-like cells, greater than 60 % gene knockdown was achieved only in the cells obtained from one of the donors (donor 1). Some interesting regulatory effects were observed in these cells, including down-regulation of both the *ARHGDIA* and *ACTA2* genes in response to *RHOA* knockdown. The hypothesis that knockdown of the *ARHGEF3* and *RHOA* genes in osteoblast-like and osteoclast-like cells influences the expression of genes involved with bone cell function has been supported to some degree (for example, the influence of *RHOA* knockdown on *ACTA2* expression in the osteoblast-like cells), although clearly additional validation is required for a number of the genes examined, especially in the osteoclast-like cells. No significant effects on the bone-resorptive capabilities of the osteoclast-like cells were observed as a result of *ARHGEF3* or *RHOA* knockdown, therefore the hypothesis that knockdown of these genes would influence their bone resorptive capabilities has not been supported by the data. However, this could be due to insufficient gene knockdown. Future studies could attempt replication of some of the other significant gene regulatory findings from the microarray analysis.

Chapter 7 – General discussion and conclusion

7.1 Role of the *ARHGEF3, RHOA* and *FLNB* genes in BMD regulation

The studies presented in this thesis provide evidence to suggest that common polymorphism within the *ARHGEF3*, *RHOA* and *FLNB* genes may have a role in BMD regulation. These three genes are all located within the 3p14-p22 chromosomal region, which has been identified by multiple genome-wide linkage (GWL) studies as a potential quantitative trait locus for BMD (Duncan et al., 1999, Lee et al., 2006, Wilson et al., 2003, Wynne et al., 2003, Xiao et al., 2006). Interestingly, all three of these genes appear to have a role in cytoskeletal dynamics and actin polymerisation (Stossel et al., 2001, Arthur et al., 2002, Etienne-Manneville and Hall, 2002). There is evidence in the literature to suggest that RHOA and the filamin proteins do in fact interact and are involved in the same intracellular signalling pathways (Bellanger et al., 2000, Bourguignon et al., 2006, Leung et al., 2010), which suggests that the associations observed between these genes and BMD are mediated through common mechanisms or pathways.

Variants in multiple genes within biochemical pathways such as the Wnt signalling and RANK-RANKL-OPG pathways have been shown to play a role in susceptibility to osteoporosis (Richards et al., 2008, Rivadeneira et al., 2009, Styrkarsdottir et al., 2008). The evidence presented here suggests that the ARHGEF3-RHOA-FLNB pathway also has multiple genetic variants that may influence an individual's risk of developing the disease. This would not be the first time that multiple genes involved with a particular function or pathway have been identified in the same chromosomal region. Prokaryotes possess characteristic gene clusters that are known as operons, where multiple genes that function together in the same biochemical pathway are located close together in the genome and their expression is co-regulated (Osbourn and Field, 2009). Functional gene clusters have also been identified in eukaryotes, including humans, and this phenomenon has been suggested to have evolutionary benefits (Hurst et al., 2004). For example, the major histocompatibility complex, which is a cluster of genes that are essential to the immune system, has been localised to the 6p21 region of the human genome (1999). Another example would be the close proximity of the gene encoding glutamine phosphoribosyl pyrophosphate amidotransferase, which is involved in the initial step in *de novo* purine synthesis, and that encoding phosphoribosylamidoimidazole-

succinocarboxamide synthase, which is involved in the same pathway (Brayton et al., 1994). It is possible that the 3p14-p22 region of the human genome contains a non-random clustering of genes involved with cytoskeletal dynamics and actin polymerisation, with subsequent effects on bone density regulation. However, it is debatable whether the *ARHGEF3*, *RHOA* and *FLNB* genes are in close enough proximity to constitute a gene cluster. More evidence is required to give this theory credibility.

As discussed in previous chapters, cytoskeletal dynamics have a well documented role in both the differentiation of osteoblasts (McBeath et al., 2004, Meyers et al., 2005) and the bone resorption function of osteoclasts (Chellaiah et al., 2000). It is therefore plausible that common variation within the *ARHGEF3*, *RHOA* and *FLNB* genes could result in small changes to the cytoskeleton with subsequent effects on the lineage commitment of mesenchymal stem cells and the bone resorptive capabilities of osteoclasts. These subtle effects could result in miniscule changes to the bone resorptive cycle that accumulate over the course of an individual's lifetime and result in a detrimental effect on BMD.

7.2 Relevance of the *ARHGEF3, RHOA* and *FLNB* genes to the linkage observed between the 3p14-p22 chromosomal region and BMD

That the *ARHGEF3*, *RHOA* and *FLNB* genes are collectively responsible for the linkage observed between the 3p14-p22 chromosomal region and BMD could explain why a single gene from this region with a large influence on BMD has not previously been identified, an outcome that perhaps would have been anticipated based on the strength of the linkage observed (Wilson et al., 2003, Xiao et al., 2006). Indeed, this phenomenon could hold true for other regions of the genome linked with BMD in which no single gene with a large influence on BMD has been identified, a plausible theory considering the large number of genes thought to have a minor influence on the BMD phenotype. Analysis of the LD between the three SNPs located in the *ARHGEF3*, *RHOA* and *FLNB* genes that demonstrated the most significant associations with BMD parameters (rs7646054, rs17595772 and rs9809315 respectively) using JLIN (Carter et al., 2006) suggests that the LD between these three polymorphisms is very low (D' and $r^2 < 0.2$) (Figure 7.1). This would strongly suggest that the associations reported in this thesis between variation

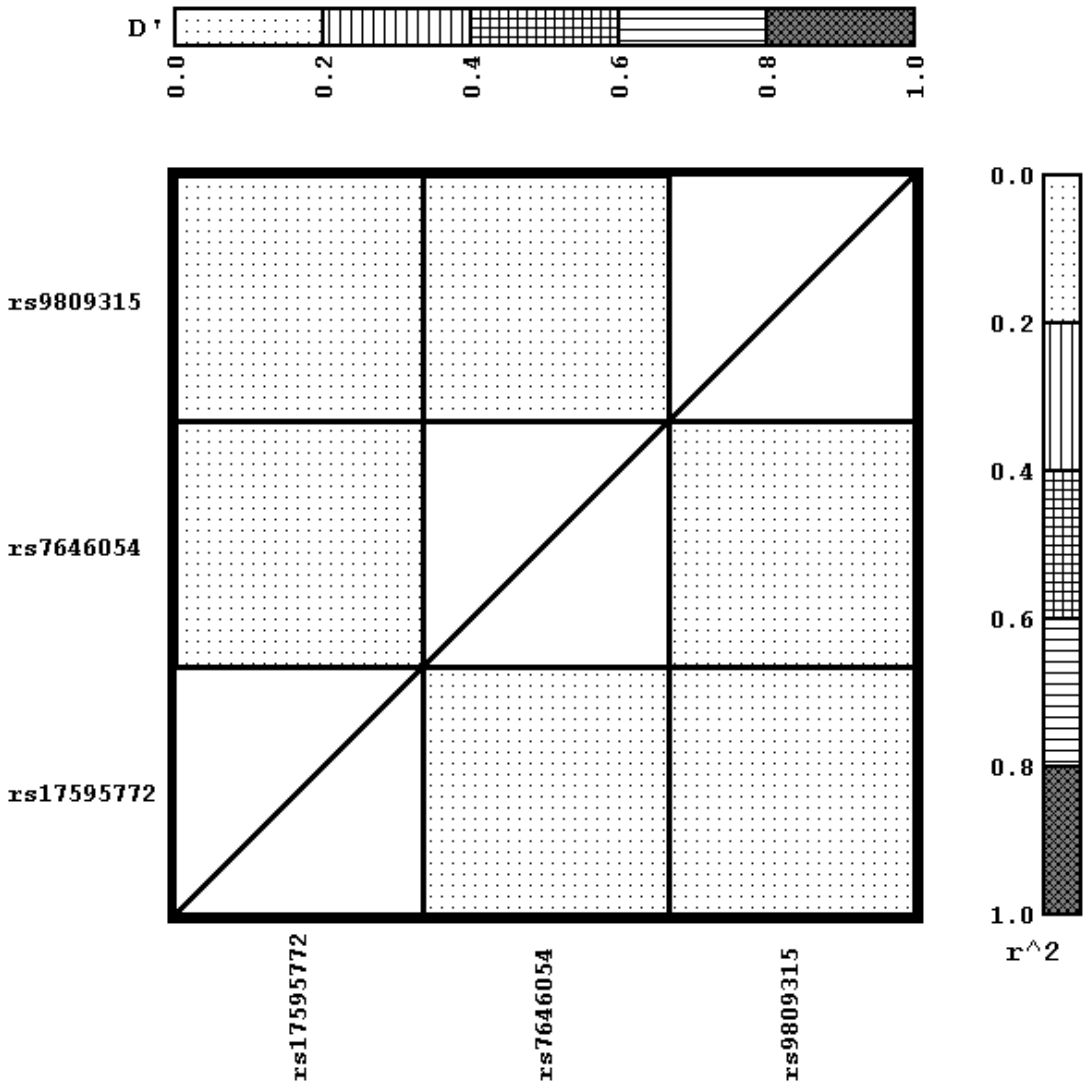

Figure 7.1. LD analysis of rs17595772 (*RHOA*), rs7646054 (*ARHGEF3*) and rs9809315 (*FLNB*) in the discovery cohort.

in each of these genes and BMD phenotypes are independent. In addition to this, Rivadeneira et al. identified significant associations between variation in the *CTNNB1* gene (Rivadeneira et al., 2009), which encodes an integral component of the Wnt signalling pathway (beta-catenin) (Wagner et al., 2010), and BMD in a large-scale meta-analysis of five genome-wide association studies (discussed in *Chapter 1*). This gene is located within the support intervals defined for the 3p14-p22 chromosomal region by Wilson et al. as linked with BMD, which is flanked by the markers D3S1298 and D3S1285, so therefore may also contribute to the linkage observed (Wilson et al., 2003).

7.2.1 Other instances of multiple genes contributing to a single linkage peak

If the *ARHGEF3*, *RHOA* and *FLNB* genes are collectively responsible for the linkage seen between the 3p14-p22 chromosomal region and BMD, it would not be the first instance of multiple genes contributing to a single linkage peak. Jamieson et al. genotyped 16 microsatellites located across the 17q genomic region in 92 multi-case tuberculosis families (627 individuals) from Brazil (Jamieson et al., 2004). The authors decided to examine this region for linkage with tuberculosis susceptibility after a region on mouse chromosome 11, which is syntenic with 17q in humans, was found to carry susceptibility genes to cutaneous leishmaniasis (a type of skin infection caused by a single-celled parasite) (Mock et al., 1993, Roberts et al., 1993). A single linkage peak for tuberculosis susceptibility located in the chromosomal region 17q12 was observed (Jamieson et al., 2004). The authors then genotyped 49 SNPs with a minor allele frequency ≥ 0.1 located in various candidate genes throughout the region, identifying significant associations between polymorphism in 4 genes and susceptibility to tuberculosis (Jamieson et al., 2004). Subsequent stepwise conditional logistic regression analysis of the data suggested that the 4 genes contribute separate main effects, prompting the authors to speculate that the 4 genes may collectively account for the linkage seen at 17q12 (Jamieson et al., 2004).

A more recent publication by Chang et al. describes a GWL study aimed at identifying regions of the human genome linked with essential-hypertension (chronically elevated blood-pressure with no identifiable cause) (Chang et al., 2007). Five sets of microsatellite markers with an average intermarker distance of 9.2 cM were genotyped in 1,010 European American (EA) and 816 African American (AA)

subjects from 592 families (Chang et al., 2007). The authors observed strong linkage of diastolic blood pressure with chromosome 1q in the EA subset, a genomic region that had been previously linked with blood pressure-related phenotypes (Hunt et al., 2002, James et al., 2003, Perola et al., 2000). Sixteen additional microsatellite markers were then genotyped within this chromosomal region, with the original linkage peak being resolved into two distinct peaks, located at 175.6 cM and 218.5 cM (Chang et al., 2007). The authors selected 9 candidate genes from this region for further analysis, with 58 SNPs located in or around these 9 genes genotyped in both the EA and AA subsets (Chang et al., 2007). Variation within two of these candidate genes was found to be significantly associated with both systolic and diastolic blood pressure in both subsets, while variation in a third gene was found to be associated with systolic blood pressure in the AA subset (Chang et al., 2007). Variation within each of these three genes was then analysed in two replication populations, with significant associations observed between each gene and blood pressure phenotypes in at least one of the replication groups (Chang et al., 2007). For each SNP that was significantly associated with blood pressure in more than one cohort, the same allele was associated with higher blood pressure in each cohort (Chang et al., 2007). Analysis of the pairwise linkage disequilibrium between the SNPs located in the three genes that demonstrated associations with blood pressure phenotypes suggested that each gene is independently associated with blood pressure (all pairwise r^2 values < 0.02) (Chang et al., 2007). The authors suggested that since the susceptibility alleles for most complex diseases have modest affects, the linkage signals uncovered by most GWL studies are likely to arise from multiple genes located in the same region that have a combined effect (Chang et al., 2007). They also speculated that the clustering of multiple genes that influence a specific phenotype may be evolutionarily advantageous (Chang et al., 2007).

7.3 Expression of the *ACTA2* gene may be regulated by RHOA signalling in bone cells

The most consistent regulatory effect seen in the gene knockdown work described in *Chapter 6* was the down-regulation of *ACTA2* mRNA expression seen in all three osteoblast-like cell lines (Saos-2, hFOB 1.19 and MG-63) in response to *RHOA* knockdown. The effect was also seen in the osteoclast-like cells obtained from donors 1 and 3. There is evidence in the literature to suggest that expression of the

ACTA2 gene is influenced by RHOA activity. Mack et al. found that expression of constitutively active Rhoa in rat smooth muscle cell cultures significantly increased the activity of the *Acta2* promoter, whereas inhibition of Rhoa decreased the activity of the promoter (Mack et al., 2001). They also found that stimulation of actin polymerisation in these smooth muscle cells by treatment with jasplakinolide, a cyclic peptide isolated from marine sponge that has been shown to bind to and stabilise filamentous actin *in vivo* (Bubb et al., 1994), increased the activity of the *Acta2* promoter by 13-fold (Mack et al., 2001). Zhao et al. published evidence suggesting that static tensile forces applied to rat fibroblasts stimulates the promoter activity of the *Acta2* gene through the Rho signalling pathway (Zhao et al., 2007). Collectively, these data suggest that expression of the *ACTA2* gene may be regulated through the RHOA signalling pathway, and the results presented in this thesis support this.

7.4 A possible role for *ARHGEF3* and *RHOA* in PTH-induced signalling in the osteoblast with subsequent effects on osteoclastogenesis

The cell culture work described in *Chapter 6* found that knockdown of the *ARHGEF3* gene in two of the three osteoblast-like cell lines studied resulted in the down-regulation of *TNFRSF11B* mRNA (encoding OPG), and that knockdown of the *RHOA* gene stimulated up-regulation of *PTH1R* mRNA (encoding parathyroid hormone 1 receptor) in the Saos-2 cell line. These genes are both central to the mechanism by which PTH stimulates osteoclast differentiation through the osteoblast. As discussed in *Chapter 1*, treatment of osteoblasts with PTH, which binds to the receptor encoded by the *PTH1R* gene, has been shown to stimulate osteoclastogenesis through increasing the production of RANKL (by promoting expression of the *TNFSF11* gene) and decreasing the production of OPG (by inhibiting expression of the *TNFRSF11B* gene) (Lee and Lorenzo, 1999, Huang et al., 2004). There is a large amount of evidence in the literature to suggest that RHOA is a signalling intermediate for PTH in osteoblasts. Radeff et al. found that treatment of UMR-106 rat osteoblast-like cells with *Clostridium difficile* toxin B (Radeff et al., 2004), which specifically inhibits the Rho proteins (including RHOA) through glucosylation of the nucleotide binding site (Wilkins and Lyerly, 1996), reduced PTH-induced expression of the *Il6* gene, the product of which has been shown to promote osteoclastogenesis (Lowik et al., 1989). The authors concluded that the Rho

proteins are an important component of PTH signalling in osteoblasts and may have a role in the activation of the intracellular messenger protein kinase C alpha (Radeff et al., 2004). A recent study published by Wang and Stern stably transfected UMR-106 rat osteoblast-like cells with either constitutively active Rhoa (caRhoa) or dominant negative Rhoa (dnRhoa) and co-cultured them with RAW 264.7 mouse monocyte/macrophage-like cells to examine the effects on hormone-stimulated osteoclastogenesis (Wang and Stern, 2010). They found that when the cells transfected with dnRhoa were treated with PTH and/or calcitriol, the production of *TNFSF11* mRNA (encoding RANKL) increased and production of *TNFRSF11B* mRNA (encoding OPG) decreased, stimulating osteoclastogenesis of the RAW 264.7 cells (Wang and Stern, 2010). However, when the cells transfected with caRhoa were treated with PTH and/or calcitriol, the levels of *TNFSF11* and *TNFRSF11B* mRNA did not change significantly and osteoclastogenesis of the RAW 264.7 cells failed to occur (Wang and Stern, 2010). When the UMR-106 cells transfected with caRhoa were treated with the ROCK inhibitor Y27632, the ability of the cells to stimulate osteoclastogenesis of the RAW 264.7 cells was restored (Wang and Stern, 2010). These results led the authors to suggest that RHOA signalling can inhibit hormone-stimulated osteoclastogenesis through effects on RANKL and OPG expression in osteoblasts (Wang and Stern, 2010). The results presented in this thesis add to the body of evidence that exists in the literature suggesting a role for RHOA in PTH-induced signalling in the osteoblast.

7.5 Limitations of these studies

7.5.1 Gene knockdown levels in osteoclast-like cells

A significant limitation to the *ARHGEF3* and *RHOA* gene knockdown work performed was the poor levels of gene knockdown achieved in the osteoclast-like cells. The failure to obtain maximal gene knockdown confounds the interpretation of results obtained from experiments performed in these cells, particularly where the results are negative. Several strategies could be employed to increase the level of gene knockdown achieved. siRNA knockdown could be carried out at an earlier stage of osteoclast differentiation when the cells are smaller and have a higher ratio of surface area to cell volume. Additional methods of delivering siRNA to the cells could also be explored, such as electroporation techniques (Stroh et al., 2010) or the use of a viral vector (Chen et al., 2005). Viral vectors can also be used in the nuclear

delivery of gene expression cassettes that express short hairpin RNA (shRNA), which act as endogenous interfering RNA and have the potential to achieve stable gene knockdown (Sliva and Schnierle, 2010). An additional siRNA delivery method is the magnetofection technique, which involves the targeting of siRNA associated with magnetic nanoparticles to the cell surface through application of a gradient magnetic field (Mykhaylyk et al., 2008). Magnetofection has been found to yield high transfection efficiencies with low cytotoxic effects (Ensenauer et al., 2010). Alternatively, experiments could be conducted on osteoclasts obtained from mice in which the gene of interest has been knocked out through either introduction of a targeted mutation or transfection with a dominant-negative construct. These latter techniques have the potential to yield cells that are completely deficient in functional gene product, as opposed to gene knockdown techniques in which some of the functional gene product remains. However, the major limitation here is that the cells are not from human origin.

7.5.2 Bone resorption assays

There were two major limitations to the bone resorption assays performed in this thesis. Firstly, the level of gene knockdown achieved was poor, as already alluded to. Secondly, there was a large amount of variation seen in the resorption pit volumes observed within each treatment group. The latter resulted in large standard deviations within each treatment group and made it difficult to interpret the data. The problem could be due to inaccuracies in the three-dimensional quantification of the osteoclast resorption pits. This process is made difficult by the unevenness of the bone slice surface (caused by the cutting of the bone) and the erroneous contribution to the resorption pit volumes made by pre-existing exposed osteocyte lacunae and blood vessel spaces. To avoid this problem, an alternative method of assessing the bone resorptive capabilities of osteoclasts could be employed. Foged et al. developed an enzyme-linked immunosorbent assay (ELISA) for quantitating the bone resorption of osteoclasts cultured *in vitro* by quantifying the amount of type 1 collagen released into the culture medium during the resorptive process (Foged et al., 1996). This technique has the advantage of not relying on volumetric resorption pit measurements (Foged et al., 1996).

7.6 Future studies

7.6.1 Polymorphism at rs7646054 and expression of NM_001128616

Since rs7646054 is located within the 5' UTR of the NM_001128616 transcript variant of *ARHGEF3*, the allele present at rs7646054 is carried on the NM_001128616 mRNA. A real-time PCR approach could therefore be used to quantitate the amount of NM_001128616 mRNA transcript carrying each allele to examine the influence of polymorphism at rs7646054 on expression of this transcript. This technique was considered for use in this project when the associations between variation at rs7646054 and BMD phenotypes were first identified and an *in silico* bioinformatics analysis suggested that polymorphism at this SNP site alters the folding and stability of the 5' UTR of this transcript (discussed in *Chapter 3*). However, at that stage of the project the NM_001128616 transcript variant had only been recently described and real-time PCR could not detect this transcript in either the Saos-2 osteoblast-like cell line or the BE(2)-M17 neuroblastoma cell line, hence its existence could not be confirmed. It was not until much later in the project that this transcript was detected using real-time PCR in the osteoclast-like cells (discussed in *Chapter 6*).

To perform this technique, blood samples would first need to be collected from a number of individuals that are heterozygous at the rs7646054 SNP site. Since they are heterozygous at this polymorphic site, these individuals will express one copy of the NM_001128616 transcript variant carrying each allele for rs7646054. The peripheral blood mononuclear cells (PBMCs) can be isolated from the whole blood using the Ficoll-Paque centrifugal separation technique described in *Chapter 6*. The PBMCs can then be cultured for 4 days with phytohaemagglutinin (PHA), which is a lectin that has been shown to stimulate proliferation as well as mRNA and protein expression in lymphocytes (Jagus-Smith and Kay, 1976). This PHA treatment is important as it will up-regulate the expression of the NM_001128616 mRNA transcript variant, allowing greater quantities to be available for subsequent analysis. Alternatively, the PBMCs could be stimulated to differentiate into osteoclast-like cells as described in *Chapter 6*, since expression of the transcript has been confirmed in this cell type. After culture, total RNA can be extracted from the cells and reverse transcribed. The resulting cDNA could then be subjected to quantitative real-time PCR utilising two allele-specific TaqMan probes for the rs7646054 SNP site. If the

expression of one allele is higher than the other, it will be reflected in the quantity of template carrying each allele that is detected by the real-time PCR assay. These results can be compared against results obtained using the same real-time PCR assay on genomic DNA samples obtained from individuals that are heterozygous at rs7646054, as these samples would be expected to have a 50/50 ratio of each allele. A standard curve can also be created using serial dilutions of two DNA samples, one homozygous for the *A* allele at rs7646054 and one homozygous for the *G* allele, mixed together in different proportions. This can be used to accurately calculate the expression ratio of each allele. Differences in the quantity of transcript carrying one allele compared to the other could indicate differences in the transcription rate of the two alleles or, as previously suggested, differences in the mRNA stability of the two transcripts.

7.6.2 Polymorphism at rs17595772 and rs17080528 and expression of the *RHOA* gene

A similar principal to that described above could be used to investigate whether polymorphism at rs17595772 or rs17080528, which are both located in the *RHOA* gene and were found to be significantly associated with BMD parameters in this thesis (see *Chapter 4*), influences expression of *RHOA* mRNA. Blood samples would first be collected from a number of individuals that are homozygous for the major and minor alleles at these polymorphic sites. PBMCs can then be isolated from these samples and cultured with PHA before total RNA is extracted and reverse transcribed. Gene expression ratios representing the ratio of expression of the *RHOA* gene relative to an internal reference (such as the 18S ribosomal RNA gene) can then be calculated for each sample using quantitative real-time PCR in conjunction with the comparative C_T statistical method described by Schmittgen and Livak (discussed in *Chapter 6*) (Schmittgen and Livak, 2008). An unpaired *t*-test can then be used to determine whether there is a significant difference between the gene expression ratios from each homozygote group.

7.6.3 Investigation of how polymorphism at rs11720285, rs11130605 and rs9809315 regulates expression of the *FLNB* gene

The three *FLNB* SNPs rs11720285, rs11130605 and rs9809315 have been identified as significantly associated with *FLNB* mRNA expression (Wilson et al., 2009) and

BMD parameters in women (see *Chapter 5*). The exact mechanism by which these SNPs regulate expression of the *FLNB* gene remains to be determined. It is possible that polymorphism tagged by one or more of these SNPs influences the binding of transcription factors to regulatory sequences predicted to lie within a 650 bp region surrounding the 5' UTR and first exon of the *FLNB* gene (discussed in *Chapter 5*). It would be interesting to sequence this 650 bp region plus the 2 Kb of sequence immediately 5' of the *FLNB* transcription start site for polymorphisms in strong linkage disequilibrium with the rs11720285, rs11130605 and rs9809315 SNPs. If such polymorphisms were discovered, any potential changes to transcription factor binding sites (TFBS) could be analysed *in silico* using the bioinformatics software packages F-SNP (Lee and Shatkay, 2008) and PROMO (Messeguer et al., 2002). Any potential changes to TFBS identified by this analysis could then be verified *in vitro* using an electrophoretic mobility shift assay (EMSA). This is performed by generating oligonucleotide probes containing the predicted TFBS sequence with either the wild-type or polymorphic allele present. These probes are then incubated with nuclear extract from a human cell line known to express the *FLNB* gene. The reaction mixtures are electrophoresed on either an agarose or polyacrylamide gel and the banding patterns examined to determine whether transcription factor binding has occurred. An oligonucleotide probe that has bound to a transcription factor will migrate through the gel more slowly than unbound probe.

7.6.4 Further investigation into the role of the *ARHGEF3* and *RHOA* genes in the osteoblast

The findings published by Wang and Stern (Wang and Stern, 2010), coupled with the evidence presented in this thesis, suggests that the *ARHGEF3* and *RHOA* genes may have a role in regulating the osteoclastogenic capabilities of the osteoblast. Therefore, the influence of knockdown of the *ARHGEF3* and *RHOA* genes on expression of RANKL and OPG in human osteoblast-like cells in response to treatment with PTH could be examined. Out of the three osteoblast-like cell lines used in this thesis, the Saos-2 was the only one that was found to express the PTH1R receptor. Hence, this cell line would be a good candidate for use in this work. It would also be interesting to examine the effects of *FLNB* gene knockdown in the Saos-2 cells under these conditions, as this could provide clues as to whether the *FLNB* gene is also involved in this mechanism.

7.6.5 Investigation of the effects of *ARHGEF3, RHOA* and *FLNB* gene knockdown on the osteoclast cytoskeleton

It would be interesting to attempt one of the alternative knockdown techniques described above (section 7.5.1) in actively resorbing osteoclast-like cells differentiated from human PBMCs. The effects of the gene knockdown on the intracellular actin structures of the cells could then be analysed by staining with fluorescent phalloidin (Wulf et al., 1979) followed by visualisation using confocal microscopy (Small et al., 1999). Phalloidin, a member of the phallotoxin family of poisonous bicyclic heptapeptides derived from the poisonous mushroom *Amanita phalloides* (Wieland and Faulstich, 1978), binds tightly to filamentous actin (Estes et al., 1981) and is commonly used to visualise actin filaments in both living and fixed cells (Small et al., 1999). This technique could reveal whether the actin cytoskeleton of resorbing human osteoclast-like cells is significantly disrupted by knockdown of the *ARHGEF3*, *RHOA* and *FLNB* genes.

7.7 Conclusion

This thesis has identified common variation within the *ARHGEF3* and *RHOA* genes as significantly associated with BMD in Caucasian women. Polymorphism within these genes has not been previously implicated in BMD regulation or osteoporosis. In addition to these results, this thesis has produced supporting evidence for a previous publication that reported significant associations between polymorphism in the *FLNB* gene and BMD in Caucasian women (Wilson et al., 2009). Out of five SNPs previously identified as associated with *FLNB* mRNA levels, three were found to be significantly associated with BMD in Caucasian women in this thesis. The *ARHGEF3, RHOA* and *FLNB* genes are all thought to have a role in cytoskeletal reorganisation (Arthur et al., 2002, Hall, 1998, Stossel et al., 2001), a mechanism that has been implicated in osteoblast differentiation (McBeath et al., 2004, Meyers et al., 2005) and osteoclast function (Chellaiah et al., 2000). These three genes are all located within the 3p14-p22 region of the human genome, a region that has been linked with BMD in multiple studies (Duncan et al., 1999, Wilson et al., 2003, Wynne et al., 2003, Xiao et al., 2006, Lee et al., 2006). Therefore, the unifying hypothesis that one or more genes in the human genomic 3p14-p22 region are

significantly associated with bone mineral density in Caucasian women has been supported by the data.

One of the polymorphisms found to influence BMD in this thesis, rs7646054, is located within the 5' UTR of a transcript variant of the *ARHGEF3* gene termed NM_001128616. In the functional studies performed in this thesis, expression of this transcript variant was detected in osteoclast-like cells but not osteoblast-like cells. This suggests that if polymorphism at rs76546054 influences BMD through effects on the NM_001128616 transcript variant, it seems likely that this effect is mediated through the osteoclast. Expression of mRNA encoding alpha 2 actin, smooth muscle (ACTA2) was found to be consistently down-regulated by *RHOA* gene knockdown in osteoblast-like cells, supporting evidence in the literature suggesting that expression of the *ACTA2* gene is regulated by the RHOA signalling pathway. Expression of OPG mRNA was found to be significantly down-regulated by knockdown of the *ARHGEF3* gene in two out of three osteoblast-like cell lines examined, whereas parathyroid hormone 1 receptor mRNA was found to be significantly up-regulated by *RHOA* gene knockdown in Saos-2 osteoblast-like cells. These results could suggest a role for the *ARHGEF3* and *RHOA* genes in PTH-mediated signalling in the osteoblast. Successful knockdown of the *ARHGEF3* and *RHOA* genes was found to be difficult in osteoclast-like cells, although there was some evidence to suggest that knockdown of *RHOA* reduces the expression of Rho GDP dissociation inhibitor alpha and ACTA2. Knockdown of the *ARHGEF3* and *RHOA* genes was not found to significantly influence the bone-resorptive capabilities of osteoclast-like cells. However, the lack of effect could be due to insufficient gene knockdown in this cell type.

7.8 Significance of findings

The findings presented in this thesis are significant because while *ARHGEF3* and *RHOA* were considered to be candidate genes for bone density regulation, this is the first demonstration that variation within these genes is significantly associated with BMD in Caucasian women and therefore may have a role in osteoporosis. Cytoskeletal dynamics has a well established role in osteoblast differentiation and osteoclast function, however it had not been previously considered a major factor in the development of osteoporosis. This thesis also provides supporting evidence of a

role for another gene involved with the cytoskeleton, *FLNB*, and BMD regulation. These results not only contribute to the medical and scientific community's understanding of osteoporosis and the genetic architecture behind the heritable component of the disease, but they also provide the basis for further studies in the area. They could also assist in the future development of a panel of genetic marker tests to identify individuals at increased risk of developing osteoporosis. This panel could take the form of a diagnostic array which includes other genetic loci that have been identified as contributing to variance in BMD. Once individuals have been identified as susceptible to osteoporosis due to their genetic makeup, early intervention strategies can be applied to reduce their risk of developing the disease. The cell culture work performed in this thesis is the first to examine the effects of *ARHGEF3* and *RHOA* gene knockdown in bone cells using siRNA. The findings from this work highlight the NM_001128616 transcript variant of *ARHGEF3*, the influence of RHOA signalling on expression of the *ACTA2* gene, and the PTH-induced signalling pathway in osteoblasts as potential areas for further research.

References

1999. Complete sequence and gene map of a human major histocompatibility complex. The MHC sequencing consortium. *Nature,* 401**,** 921-3.

2002. Incidence of vertebral fracture in europe: results from the European Prospective Osteoporosis Study (EPOS). *J Bone Miner Res,* 17**,** 716-24.

AARSKOG, D. 1970. A familial syndrome of short stature associated with facial dysplasia and genital anomalies. *J Pediatr,* 77**,** 856-61.

ABECASIS, G. R. & COOKSON, W. O. 2000. GOLD--graphical overview of linkage disequilibrium. *Bioinformatics,* 16**,** 182-3.

ABU-AMER, Y., ERDMANN, J., ALEXOPOULOU, L., KOLLIAS, G., ROSS, F. P. & TEITELBAUM, S. L. 2000. Tumor necrosis factor receptors types 1 and 2 differentially regulate osteoclastogenesis. *J Biol Chem,* 275**,** 27307-10.

ADAMSON, P., PATERSON, H. F. & HALL, A. 1992. Intracellular localization of the P21rho proteins. *J Cell Biol,* 119**,** 617-27.

AERTS, J. L., GONZALES, M. I. & TOPALIAN, S. L. 2004. Selection of appropriate control genes to assess expression of tumor antigens using real-time RT-PCR. *Biotechniques,* 36**,** 84-6, 88, 90-1.

AHN, S. J., COSTA, J. & EMANUEL, J. R. 1996. PicoGreen quantitation of DNA: effective evaluation of samples pre- or post-PCR. *Nucleic Acids Res,* 24**,** 2623-5.

AJUBI, N. E., KLEIN-NULEND, J., NIJWEIDE, P. J., VRIJHEID-LAMMERS, T., ALBLAS, M. J. & BURGER, E. H. 1996. Pulsating fluid flow increases prostaglandin production by cultured chicken osteocytes--a cytoskeleton-dependent process. *Biochem Biophys Res Commun,* 225**,** 62-8.

AKATSU, T., MURAKAMI, T., NISHIKAWA, M., ONO, K., SHINOMIYA, N., TSUDA, E., MOCHIZUKI, S., YAMAGUCHI, K., KINOSAKI, M., HIGASHIO, K., YAMAMOTO, M., MOTOYOSHI, K. & NAGATA, N. 1998. Osteoclastogenesis inhibitory factor suppresses osteoclast survival by interfering in the interaction of stromal cells with osteoclast. *Biochem Biophys Res Commun,* 250**,** 229-34.

AL-MAYOUF, S. M., MAJEED, M., HUGOSSON, C. & BAHABRI, S. 2000. New form of idiopathic osteolysis: nodulosis, arthropathy and osteolysis (NAO) syndrome. *Am J Med Genet,* 93**,** 5-10.

AL-OTAIBI, L., AL-MAYOUF, S. M., MAJEED, M., AL-EID, W., BAHABRI, S. & HUGOSSON, C. O. 2002. Radiological findings in NAO syndrome. *Pediatr Radiol,* 32**,** 523-8.

ALAHARI, K. D., LOBAUGH, B. & ECONS, M. J. 1997. Vitamin D receptor alleles do not correlate with bone mineral density in premenopausal Caucasian women from the southeastern United States. *Metabolism,* 46**,** 224-6.

ALBAGHA, O. M. & RALSTON, S. H. 2003. Genetic determinants of susceptibility to osteoporosis. *Endocrinol Metab Clin North Am,* 32**,** 65-81, vi.

ALBERTS, A. S. & TREISMAN, R. 1998. Activation of RhoA and SAPK/JNK signalling pathways by the RhoA-specific exchange factor mNET1. *EMBO J,* 17**,** 4075-85.

ALTSHULER, D. M., GIBBS, R. A., PELTONEN, L., DERMITZAKIS, E., SCHAFFNER, S. F., YU, F., BONNEN, P. E., DE BAKKER, P. I., DELOUKAS, P., GABRIEL, S. B., GWILLIAM, R., HUNT, S., INOUYE, M., JIA, X., PALOTIE, A., PARKIN, M., WHITTAKER, P., CHANG, K., HAWES, A., LEWIS, L. R., REN, Y., WHEELER, D., MUZNY, D. M.,

BARNES, C., DARVISHI, K., HURLES, M., KORN, J. M., KRISTIANSSON, K., LEE, C., MCCARROL, S. A., NEMESH, J., KEINAN, A., MONTGOMERY, S. B., POLLACK, S., PRICE, A. L., SORANZO, N., GONZAGA-JAUREGUI, C., ANTTILA, V., BRODEUR, W., DALY, M. J., LESLIE, S., MCVEAN, G., MOUTSIANAS, L., NGUYEN, H., ZHANG, Q., GHORI, M. J., MCGINNIS, R., MCLAREN, W., TAKEUCHI, F., GROSSMAN, S. R., SHLYAKHTER, I., HOSTETTER, E. B., SABETI, P. C., ADEBAMOWO, C. A., FOSTER, M. W., GORDON, D. R., LICINIO, J., MANCA, M. C., MARSHALL, P. A., MATSUDA, I., NGARE, D., WANG, V. O., REDDY, D., ROTIMI, C. N., ROYAL, C. D., SHARP, R. R., ZENG, C., BROOKS, L. D. & MCEWEN, J. E. 2010. Integrating common and rare genetic variation in diverse human populations. *Nature,* 467**,** 52-8.

AMANO, M., ITO, M., KIMURA, K., FUKATA, Y., CHIHARA, K., NAKANO, T., MATSUURA, Y. & KAIBUCHI, K. 1996. Phosphorylation and activation of myosin by Rho-associated kinase (Rho-kinase). *J Biol Chem,* 271**,** 20246-9.

ANDERSON, D. M., MARASKOVSKY, E., BILLINGSLEY, W. L., DOUGALL, W. C., TOMETSKO, M. E., ROUX, E. R., TEEPE, M. C., DUBOSE, R. F., COSMAN, D. & GALIBERT, L. 1997. A homologue of the TNF receptor and its ligand enhance T-cell growth and dendritic-cell function. *Nature,* 390**,** 175-9.

ANDRESS, D. L., MALONEY, N. A., COBURN, J. W., ENDRES, D. B. & SHERRARD, D. J. 1987. Osteomalacia and aplastic bone disease in aluminum-related osteodystrophy. *J Clin Endocrinol Metab,* 65**,** 11-6.

ANDREW, T., ANTIONIADES, L., SCURRAH, K. J., MACGREGOR, A. J. & SPECTOR, T. D. 2005. Risk of wrist fracture in women is heritable and is influenced by genes that are largely independent of those influencing BMD. *J Bone Miner Res,* 20**,** 67-74.

APLAN, P. D., JOHNSON, B. E., RUSSELL, E., CHERVINSKY, D. S. & KIRSCH, I. R. 1995. Cloning and characterization of TCTA, a gene located at the site of a t(1;3) translocation. *Cancer Res,* 55**,** 1917-21.

ARAI, F., HIRAO, A. & SUDA, T. 2005. Regulation of hematopoiesis and its interaction with stem cell niches. *Int J Hematol,* 82**,** 371-6.

ARAUJO, A. B., TRAVISON, T. G., HARRIS, S. S., HOLICK, M. F., TURNER, A. K. & MCKINLAY, J. B. 2007. Race/ethnic differences in bone mineral density in men. *Osteoporos Int,* 18**,** 943-53.

ARDEN, N. K., GRIFFITHS, G. O., HART, D. J., DOYLE, D. V. & SPECTOR, T. D. 1996. The association between osteoarthritis and osteoporotic fracture: the Chingford Study. *Br J Rheumatol,* 35**,** 1299-304.

ARKO, B., PREZELJ, J., KOMEL, R., KOCIJANCIC, A., HUDLER, P. & MARC, J. 2002. Sequence variations in the osteoprotegerin gene promoter in patients with postmenopausal osteoporosis. *J Clin Endocrinol Metab,* 87**,** 4080-4.

ARTHUR. 2008. *Arthur's Medical Clipart* [Online]. Available: http://www.arthursclipart.org/medical/humanbody/page_01.htm [Accessed 27th January 2011].

ARTHUR, W. T., ELLERBROEK, S. M., DER, C. J., BURRIDGE, K. & WENNERBERG, K. 2002. XPLN, a guanine nucleotide exchange factor for RhoA and RhoB, but not RhoC. *J Biol Chem,* 277**,** 42964-72.

ARYA, M., SHERGILL, I. S., WILLIAMSON, M., GOMMERSALL, L., ARYA, N. & PATEL, H. R. 2005. Basic principles of real-time quantitative PCR. *Expert Rev Mol Diagn,* 5**,** 209-19.

ASAGIRI, M. & TAKAYANAGI, H. 2007. The molecular understanding of osteoclast differentiation. *Bone,* 40**,** 251-64.

AUBIN, J. E. 1998. Advances in the osteoblast lineage. *Biochem Cell Biol,* 76**,** 899-910.

AUBIN, J. E. & TURKSEN, K. 1996. Monoclonal antibodies as tools for studying the osteoblast lineage. *Microsc Res Tech,* 33**,** 128-40.

AVRAHAM, H. & WEINBERG, R. A. 1989. Characterization and expression of the human rhoH12 gene product. *Mol Cell Biol,* 9**,** 2058-66.

BACKLUND, P. S., JR. 1997. Post-translational processing of RhoA. Carboxyl methylation of the carboxyl-terminal prenylcysteine increases the half-life of Rhoa. *J Biol Chem,* 272**,** 33175-80.

BACKOFEN, B., JACOB, R., SERTH, K., GOSSLER, A., NAIM, H. Y. & LEEB, T. 2002. Cloning and characterization of the mammalian-specific nicolin 1 gene (NICN1) encoding a nuclear 24 kDa protein. *Eur J Biochem,* 269**,** 5240-5.

BAEK, W. Y., LEE, M. A., JUNG, J. W., KIM, S. Y., AKIYAMA, H., DE CROMBRUGGHE, B. & KIM, J. E. 2009. Positive regulation of adult bone formation by osteoblast-specific transcription factor osterix. *J Bone Miner Res,* 24**,** 1055-65.

BAGGER, Y. Z., JORGENSEN, H. L., HEEGAARD, A. M., BAYER, L., HANSEN, L. & HASSAGER, C. 2000. No major effect of estrogen receptor gene polymorphisms on bone mineral density or bone loss in postmenopausal Danish women. *Bone,* 26**,** 111-6.

BALEMANS, W., EBELING, M., PATEL, N., VAN HUL, E., OLSON, P., DIOSZEGI, M., LACZA, C., WUYTS, W., VAN DEN ENDE, J., WILLEMS, P., PAES-ALVES, A. F., HILL, S., BUENO, M., RAMOS, F. J., TACCONI, P., DIKKERS, F. G., STRATAKIS, C., LINDPAINTNER, K., VICKERY, B., FOERNZLER, D. & VAN HUL, W. 2001. Increased bone density in sclerosteosis is due to the deficiency of a novel secreted protein (SOST). *Hum Mol Genet,* 10**,** 537-43.

BANDA, M., BOMMINENI, A., THOMAS, R. A., LUCKINBILL, L. S. & TUCKER, J. D. 2008. Evaluation and validation of housekeeping genes in response to ionizing radiation and chemical exposure for normalizing RNA expression in real-time PCR. *Mutat Res,* 649**,** 126-34.

BARON, R. 1989. Polarity and membrane transport in osteoclasts. *Connect Tissue Res,* 20**,** 109-20.

BARON, R. 1999. Anatomy and Ultrastructure of Bone. *In:* FAVUS, M. J. (ed.) *Primer on the Metabolic Bone Diseases and Disorders of Mineral Metabolism.* 4th ed. Philidelphia: Lippincott Williams & Wilkins.

BARRESI, R. & CAMPBELL, K. P. 2006. Dystroglycan: from biosynthesis to pathogenesis of human disease. *J Cell Sci,* 119**,** 199-207.

BECKLY, J. B., HANCOCK, L., GEREMIA, A., CUMMINGS, J. R., MORRIS, A., COONEY, R., PATHAN, S., GUO, C. & JEWELL, D. P. 2008. Two-stage candidate gene study of chromosome 3p demonstrates an association between nonsynonymous variants in the MST1R gene and Crohn's disease. *Inflamm Bowel Dis,* 14**,** 500-7.

BECKNELL, B., SHEN, T., MAGHRABY, E., TAYA, S., KAIBUCHI, K., CALIGIURI, M. A. & MARCUCCI, G. 2003. Characterization of leukemia-associated Rho guanine nucleotide exchange factor (LARG) expression during murine development. *Cell Tissue Res,* 314**,** 361-6.

BEDOYAN, J. K., FRIEZ, M. J., DUPONT, B. & AHMAD, A. 2009. First case of deletion of the faciogenital dysplasia 1 (FGD1) gene in a patient with Aarskog-Scott syndrome. *Eur J Med Genet,* 52**,** 262-4.

BELL, J. 1951. On brachydactyly and symphalangism. *In:* PENROSE, L. S. (ed.) *Treasury of Human Inheritance.* London: Cambridge University Press.

BELLANGER, J. M., ASTIER, C., SARDET, C., OHTA, Y., STOSSEL, T. P. & DEBANT, A. 2000. The Rac1- and RhoG-specific GEF domain of Trio targets filamin to remodel cytoskeletal actin. *Nat Cell Biol,* 2**,** 888-92.

BENJAMINI, Y. & HOCHBERG, Y. 1995. Controlling the False Discovery Rate: a Practical and Powerful Approach to Multiple Testing. *Journal of the Royal Statistical Society: Series B,* 57**,** 289-300.

BERGWITZ, C., PROCHNAU, A., MAYR, B., KRAMER, F. J., RITTIERODT, M., BERTEN, H. L., HAUSAMEN, J. E. & BRABANT, G. 2001. Identification of novel CBFA1/RUNX2 mutations causing cleidocranial dysplasia. *J Inherit Metab Dis,* 24**,** 648-56.

BIANCO, P., FISHER, L. W., YOUNG, M. F., TERMINE, J. D. & ROBEY, P. G. 1991. Expression of bone sialoprotein (BSP) in developing human tissues. *Calcif Tissue Int,* 49**,** 421-6.

BICKNELL, L. S., FARRINGTON-ROCK, C., SHAFEGHATI, Y., RUMP, P., ALANAY, Y., ALEMBIK, Y., AL-MADANI, N., FIRTH, H., KARIMI-NEJAD, M. H., KIM, C. A., LEASK, K., MAISENBACHER, M., MORAN, E., PAPPAS, J. G., PRONTERA, P., DE RAVEL, T., FRYNS, J. P., SWEENEY, E., FRYER, A., UNGER, S., WILSON, L. C., LACHMAN, R. S., RIMOIN, D. L., COHN, D. H., KRAKOW, D. & ROBERTSON, S. P. 2007. A molecular and clinical study of Larsen syndrome caused by mutations in FLNB. *J Med Genet,* 44**,** 89-98.

BICKNELL, L. S., MORGAN, T., BONAFE, L., WESSELS, M. W., BIALER, M. G., WILLEMS, P. J., COHN, D. H., KRAKOW, D. & ROBERTSON, S. P. 2005. Mutations in FLNB cause boomerang dysplasia. *J Med Genet,* 42**,** e43.

BISCHOFF-FERRARI, H. A., DAWSON-HUGHES, B., STAEHELIN, H. B., ORAV, J. E., STUCK, A. E., THEILER, R., WONG, J. B., EGLI, A., KIEL, D. P. & HENSCHKOWSKI, J. 2009. Fall prevention with supplemental and active forms of vitamin D: a meta-analysis of randomised controlled trials. *Bmj,* 339**,** b3692.

BISCHOFF-FERRARI, H. A., WILLETT, W. C., WONG, J. B., GIOVANNUCCI, E., DIETRICH, T. & DAWSON-HUGHES, B. 2005. Fracture prevention with vitamin D supplementation: a meta-analysis of randomized controlled trials. *JAMA,* 293**,** 2257-64.

BISCHOFF, H. A., STAHELIN, H. B., DICK, W., AKOS, R., KNECHT, M., SALIS, C., NEBIKER, M., THEILER, R., PFEIFER, M., BEGEROW, B., LEW, R. A. & CONZELMANN, M. 2003. Effects of vitamin D and calcium supplementation on falls: a randomized controlled trial. *J Bone Miner Res,* 18**,** 343-51.

BLAIR, H. C., KAHN, A. J., CROUCH, E. C., JEFFREY, J. J. & TEITELBAUM, S. L. 1986. Isolated osteoclasts resorb the organic and inorganic components of bone. *J Cell Biol,* 102**,** 1164-72.

BLAIR, H. C., ROBINSON, L. J. & ZAIDI, M. 2005. Osteoclast signalling pathways. *Biochem Biophys Res Commun,* 328**,** 728-38.

BLAIR, H. C., TEITELBAUM, S. L., GHISELLI, R. & GLUCK, S. 1989. Osteoclastic bone resorption by a polarized vacuolar proton pump. *Science,* 245**,** 855-7.

BOCALINI, D. S., SERRA, A. J., DOS SANTOS, L., MURAD, N. & LEVY, R. F. 2009. Strength training preserves the bone mineral density of postmenopausal women without hormone replacement therapy. *J Aging Health,* 21**,** 519-27.

BOIVIN, G., MOREL, G., LIAN, J. B., ANTHOINE-TERRIER, C., DUBOIS, P. M. & MEUNIER, P. J. 1990. Localization of endogenous osteocalcin in neonatal rat bone and its absence in articular cartilage: effect of warfarin treatment. *Virchows Arch A Pathol Anat Histopathol,* 417**,** 505-12.

BOLSTAD, B. M., IRIZARRY, R. A., ASTRAND, M. & SPEED, T. P. 2003. A comparison of normalization methods for high density oligonucleotide array data based on variance and bias. *Bioinformatics,* 19**,** 185-93.

BONAVENTURE, J., LASSELIN, C., MELLIER, J., COHEN-SOLAL, L. & MAROTEAUX, P. 1992. Linkage studies of four fibrillar collagen genes in three pedigrees with Larsen-like syndrome. *J Med Genet,* 29**,** 465-70.

BONEWALD, L. F. 2007. Osteocytes as dynamic multifunctional cells. *Ann N Y Acad Sci,* 1116**,** 281-90.

BOSKEY, A. L., GADALETA, S., GUNDBERG, C., DOTY, S. B., DUCY, P. & KARSENTY, G. 1998. Fourier transform infrared microspectroscopic analysis of bones of osteocalcin-deficient mice provides insight into the function of osteocalcin. *Bone,* 23**,** 187-96.

BOTTANI, A., ORRICO, A., GALLI, L., KARAM, O., HAENGGELI, C. A., FEREY, S. & CONRAD, B. 2007. Unilateral focal polymicrogyria in a patient with classical Aarskog-Scott syndrome due to a novel missense mutation in an evolutionary conserved RhoGEF domain of the faciogenital dysplasia gene FGD1. *Am J Med Genet A,* 143A**,** 2334-8.

BOUREUX, A., VIGNAL, E., FAURE, S. & FORT, P. 2007. Evolution of the Rho family of ras-like GTPases in eukaryotes. *Mol Biol Evol,* 24**,** 203-16.

BOURGUIGNON, L. Y., GILAD, E., BRIGHTMAN, A., DIEDRICH, F. & SINGLETON, P. 2006. Hyaluronan-CD44 interaction with leukemia-associated RhoGEF and epidermal growth factor receptor promotes Rho/Ras co-activation, phospholipase C epsilon-Ca2+ signaling, and cytoskeleton modification in head and neck squamous cell carcinoma cells. *J Biol Chem,* 281**,** 14026-40.

BOYCE, B. F. & XING, L. 2008. Functions of RANKL/RANK/OPG in bone modeling and remodeling. *Arch Biochem Biophys,* 473**,** 139-46.

BRAYTON, K. A., CHEN, Z., ZHOU, G., NAGY, P. L., GAVALAS, A., TRENT, J. M., DEAVEN, L. L., DIXON, J. E. & ZALKIN, H. 1994. Two genes for de novo purine nucleotide synthesis on human chromosome 4 are closely linked and divergently transcribed. *J Biol Chem,* 269**,** 5313-21.

BRAZIER, H., STEPHENS, S., ORY, S., FORT, P., MORRISON, N. & BLANGY, A. 2006. Expression profile of RhoGTPases and RhoGEFs during RANKL-stimulated osteoclastogenesis: identification of essential genes in osteoclasts. *J Bone Miner Res,* 21**,** 1387-98.

BRENZA, H. L., KIMMEL-JEHAN, C., JEHAN, F., SHINKI, T., WAKINO, S., ANAZAWA, H., SUDA, T. & DELUCA, H. F. 1998. Parathyroid hormone

activation of the 25-hydroxyvitamin D3-1alpha-hydroxylase gene promoter. *Proc Natl Acad Sci U S A,* 95**,** 1387-91.

BROADUS, A. E. 1999. Mineral Balance and Homeostasis. *In:* FAVUS, M. J. (ed.) *Primer on the Metabolic Bone Diseases and Disorders of Mineral Metabolism.* 4th ed. Philadelphia: Lippincott Williams & Wilkins.

BROCKER, F., BARDENHEUER, W., VIETEN, L., JULICHER, K., WERNER, N., MARQUITAN, G., MICHAEL, D., OPALKA, B. & SCHUTTE, J. 1999. Assignment of human filamin gene FLNB to human chromosome band 3p14.3 and identification of YACs containing the complete FLNB transcribed region. *Cytogenet Cell Genet,* 85**,** 267-8.

BROWNBILL, R. A. & ILICH, J. Z. 2003. Hip geometry and its role in fracture: what do we know so far? *Curr Osteoporos Rep,* 1**,** 25-31.

BRUNETTI-PIERRI, N., ESPOSITO, V., DE BRASI, D., MATTIACCI, D. M., KRAKOW, D., LEE, B. & SALERNO, M. 2008. Spondylocarpotarsal synostosis: long-term follow-up of a case due to FLNB mutations. *Am J Med Genet A,* 146A**,** 1230-3.

BRUNKOW, M. E., GARDNER, J. C., VAN NESS, J., PAEPER, B. W., KOVACEVICH, B. R., PROLL, S., SKONIER, J. E., ZHAO, L., SABO, P. J., FU, Y., ALISCH, R. S., GILLETT, L., COLBERT, T., TACCONI, P., GALAS, D., HAMERSMA, H., BEIGHTON, P. & MULLIGAN, J. 2001. Bone dysplasia sclerosteosis results from loss of the SOST gene product, a novel cystine knot-containing protein. *Am J Hum Genet,* 68**,** 577-89.

BUBB, M. R., SENDEROWICZ, A. M., SAUSVILLE, E. A., DUNCAN, K. L. & KORN, E. D. 1994. Jasplakinolide, a cytotoxic natural product, induces actin polymerization and competitively inhibits the binding of phalloidin to F-actin. *J Biol Chem,* 269**,** 14869-71.

BUCAY, N., SAROSI, I., DUNSTAN, C. R., MORONY, S., TARPLEY, J., CAPPARELLI, C., SCULLY, S., TAN, H. L., XU, W., LACEY, D. L., BOYLE, W. J. & SIMONET, W. S. 1998. osteoprotegerin-deficient mice develop early onset osteoporosis and arterial calcification. *Genes Dev,* 12**,** 1260-8.

BURGE, R., DAWSON-HUGHES, B., SOLOMON, D. H., WONG, J. B., KING, A. & TOSTESON, A. 2007. Incidence and economic burden of osteoporosis-related fractures in the United States, 2005-2025. *J Bone Miner Res,* 22**,** 465-75.

BURR, D. B. 1993. Remodeling and the repair of fatigue damage. *Calcif Tissue Int,* 53 Suppl 1**,** S75-80; discussion S80-1.

CAFFEY, J. 1972. Familial hyperphosphatasemia with ateliosis and hypermetabolism of growing membranous bone; review of the clinical, radiographic and chemical features. *Bull Hosp Joint Dis,* 33**,** 81-110.

CANALIS, E., PASH, J., GABBITAS, B., RYDZIEL, S. & VARGHESE, S. 1993a. Growth factors regulate the synthesis of insulin-like growth factor-I in bone cell cultures. *Endocrinology,* 133**,** 33-8.

CANALIS, E., PASH, J. & VARGHESE, S. 1993b. Skeletal growth factors. *Crit Rev Eukaryot Gene Expr,* 3**,** 155-66.

CANNIZZARO, L. A., MADAULE, P., HECHT, F., AXEL, R., CROCE, C. M. & HUEBNER, K. 1990. Chromosome localization of human ARH genes, a ras-related gene family. *Genomics,* 6**,** 197-203.

CAO, L. G., BABCOCK, G. G., RUBENSTEIN, P. A. & WANG, Y. L. 1992. Effects of profilin and profilactin on actin structure and function in living cells. *J Cell Biol,* 117**,** 1023-9.

CARR, S. A., HAUSCHKA, P. V. & BIEMANN, K. 1981. Gas chromatographic mass spectrometric sequence determination of osteocalcin, a gamma-carboxyglutamic acid-containing protein from chicken bone. *J Biol Chem,* 256**,** 9944-50.

CARTER, K. W., MCCASKIE, P. A. & PALMER, L. J. 2006. JLIN: a java based linkage disequilibrium plotter. *BMC Bioinformatics,* 7**,** 60.

CATALAN, V., GOMEZ-AMBROSI, J., ROTELLAR, F., SILVA, C., RODRIGUEZ, A., SALVADOR, J., GIL, M. J., CIENFUEGOS, J. A. & FRUHBECK, G. 2007. Validation of endogenous control genes in human adipose tissue: relevance to obesity and obesity-associated type 2 diabetes mellitus. *Horm Metab Res,* 39**,** 495-500.

CAULEY, J. A., ROBBINS, J., CHEN, Z., CUMMINGS, S. R., JACKSON, R. D., LACROIX, A. Z., LEBOFF, M., LEWIS, C. E., MCGOWAN, J., NEUNER, J., PETTINGER, M., STEFANICK, M. L., WACTAWSKI-WENDE, J. & WATTS, N. B. 2003. Effects of estrogen plus progestin on risk of fracture and bone mineral density: the Women's Health Initiative randomized trial. *JAMA,* 290**,** 1729-38.

CAULEY, J. A., THOMPSON, D. E., ENSRUD, K. C., SCOTT, J. C. & BLACK, D. 2000. Risk of mortality following clinical fractures. *Osteoporos Int,* 11**,** 556-61.

CHAKAROVA, C., WEHNERT, M. S., UHL, K., SAKTHIVEL, S., VOSBERG, H. P., VAN DER VEN, P. F. & FURST, D. O. 2000. Genomic structure and fine mapping of the two human filamin gene paralogues FLNB and FLNC and comparative analysis of the filamin gene family. *Hum Genet,* 107**,** 597-611.

CHANG, Y. P., LIU, X., KIM, J. D., IKEDA, M. A., LAYTON, M. R., WEDER, A. B., COOPER, R. S., KARDIA, S. L., RAO, D. C., HUNT, S. C., LUKE, A., BOERWINKLE, E. & CHAKRAVARTI, A. 2007. Multiple genes for essential-hypertension susceptibility on chromosome 1q. *Am J Hum Genet,* 80**,** 253-64.

CHELLAIAH, M. A., SOGA, N., SWANSON, S., MCALLISTER, S., ALVAREZ, U., WANG, D., DOWDY, S. F. & HRUSKA, K. A. 2000. Rho-A is critical for osteoclast podosome organization, motility, and bone resorption. *J Biol Chem,* 275**,** 11993-2002.

CHEN, D., ZHAO, M. & MUNDY, G. R. 2004. Bone morphogenetic proteins. *Growth Factors,* 22**,** 233-41.

CHEN, H., CHANG, C. H., PERRIN, E. & PERRIN, J. 1982. A lethal, Larsen-like multiple joint dislocation syndrome. *Am J Med Genet,* 13**,** 149-61.

CHEN, M., DU, Q., ZHANG, H. Y., WAHLESTEDT, C. & LIANG, Z. 2005. Vector-based siRNA delivery strategies for high-throughput screening of novel target genes. *J RNAi Gene Silencing,* 1**,** 5-11.

CHERIAN, P. P., SILLER-JACKSON, A. J., GU, S., WANG, X., BONEWALD, L. F., SPRAGUE, E. & JIANG, J. X. 2005. Mechanical strain opens connexin 43 hemichannels in osteocytes: a novel mechanism for the release of prostaglandin. *Mol Biol Cell,* 16**,** 3100-6.

CHITAYAT, D., HODGKINSON, K. A. & AZOUZ, E. M. 1992. Intrafamilial variability in cleidocranial dysplasia: a three generation family. *Am J Med Genet,* 42**,** 298-303.

CHONG, B., HEGDE, M., FAWKNER, M., SIMONET, S., CASSINELLI, H., COKER, M., KANIS, J., SEIDEL, J., TAU, C., TUYSUZ, B., YUKSEL, B. & LOVE, D. 2003. Idiopathic hyperphosphatasia and TNFRSF11B mutations: relationships between phenotype and genotype. *J Bone Miner Res,* 18**,** 2095-104.

CLARKE, B. 2008. Normal bone anatomy and physiology. *Clin J Am Soc Nephrol,* 3 Suppl 3**,** S131-9.

CLELAND, W. H., MENDELSON, C. R. & SIMPSON, E. R. 1985. Effects of aging and obesity on aromatase activity of human adipose cells. *J Clin Endocrinol Metab,* 60**,** 174-7.

COFFIN, R., PHILLIPS, J. L., STAPLES, W. I. & SPECTOR, S. 1966. Treatment of lead encephalopathy in children. *J Pediatr,* 69**,** 198-206.

COSA, G., FOCSANEANU, K. S., MCLEAN, J. R., MCNAMEE, J. P. & SCAIANO, J. C. 2001. Photophysical properties of fluorescent DNA-dyes bound to single- and double-stranded DNA in aqueous buffered solution. *Photochem Photobiol,* 73**,** 585-99.

COSMAN, D., WIGNALL, J., ANDERSON, D., TUSHINSKI, J., GALLIS, B., URDAL, D. & CERRETTI, D. P. 1988. Human macrophage colony stimulating factor (M-CSF): alternate RNA splicing generates three different proteins that are expressed on the cell surface and secreted. *Behring Inst Mitt***,** 15-26.

CRIPPES, B. A., ENGLEMAN, V. W., SETTLE, S. L., DELARCO, J., ORNBERG, R. L., HELFRICH, M. H., HORTON, M. A. & NICKOLS, G. A. 1996. Antibody to beta3 integrin inhibits osteoclast-mediated bone resorption in the thyroparathyroidectomized rat. *Endocrinology,* 137**,** 918-24.

CUMMINGS, S. R., CAULEY, J. A., PALERMO, L., ROSS, P. D., WASNICH, R. D., BLACK, D. & FAULKNER, K. G. 1994. Racial differences in hip axis lengths might explain racial differences in rates of hip fracture. Study of Osteoporotic Fractures Research Group. *Osteoporos Int,* 4**,** 226-9.

CUMMINGS, S. R. & MELTON, L. J. 2002. Epidemiology and outcomes of osteoporotic fractures. *Lancet,* 359**,** 1761-7.

CUNDY, T., HEGDE, M., NAOT, D., CHONG, B., KING, A., WALLACE, R., MULLEY, J., LOVE, D. R., SEIDEL, J., FAWKNER, M., BANOVIC, T., CALLON, K. E., GREY, A. B., REID, I. R., MIDDLETON-HARDIE, C. A. & CORNISH, J. 2002. A mutation in the gene TNFRSF11B encoding osteoprotegerin causes an idiopathic hyperphosphatasia phenotype. *Hum Mol Genet,* 11**,** 2119-27.

D'AMOUR, P. 2006. Circulating PTH molecular forms: what we know and what we don't. *Kidney Int Suppl***,** S29-33.

D'SOUZA, R. N., ABERG, T., GAIKWAD, J., CAVENDER, A., OWEN, M., KARSENTY, G. & THESLEFF, I. 1999. Cbfa1 is required for epithelial-mesenchymal interactions regulating tooth development in mice. *Development,* 126**,** 2911-20.

DAVIS, J. W., NOVOTNY, R., ROSS, P. D. & WASNICH, R. D. 1994. The peak bone mass of Hawaiian, Filipino, Japanese, and white women living in Hawaii. *Calcif Tissue Int,* 55**,** 249-52.

DAWN TEARE, M. & BARRETT, J. H. 2005. Genetic linkage studies. *Lancet,* 366**,** 1036-44.

DAWSON-HUGHES, B., SHIPP, C., SADOWSKI, L. & DALLAL, G. 1987. Bone density of the radius, spine, and hip in relation to percent of ideal body weight in postmenopausal women. *Calcif Tissue Int,* 40**,** 310-4.

DAY, C. J., KIM, M. S., STEPHENS, S. R., SIMCOCK, W. E., AITKEN, C. J., NICHOLSON, G. C. & MORRISON, N. A. 2004. Gene array identification of osteoclast genes: differential inhibition of osteoclastogenesis by cyclosporin A and granulocyte macrophage colony stimulating factor. *J Cell Biochem,* 91**,** 303-15.

DAY, T. F., GUO, X., GARRETT-BEAL, L. & YANG, Y. 2005. Wnt/beta-catenin signaling in mesenchymal progenitors controls osteoblast and chondrocyte differentiation during vertebrate skeletogenesis. *Dev Cell,* 8**,** 739-50.

DAY, T. F. & YANG, Y. 2008. Wnt and hedgehog signaling pathways in bone development. *J Bone Joint Surg Am,* 90 Suppl 1**,** 19-24.

DE LAET, C., KANIS, J. A., ODEN, A., JOHANSON, H., JOHNELL, O., DELMAS, P., EISMAN, J. A., KROGER, H., FUJIWARA, S., GARNERO, P., MCCLOSKEY, E. V., MELLSTROM, D., MELTON, L. J., 3RD, MEUNIER, P. J., POLS, H. A., REEVE, J., SILMAN, A. & TENENHOUSE, A. 2005. Body mass index as a predictor of fracture risk: a meta-analysis. *Osteoporos Int,* 16**,** 1330-8.

DEMISSIE, S., DUPUIS, J., CUPPLES, L. A., BECK, T. J., KIEL, D. P. & KARASIK, D. 2007. Proximal hip geometry is linked to several chromosomal regions: genome-wide linkage results from the Framingham Osteoporosis Study. *Bone,* 40**,** 743-50.

DENG, H. W., LI, J., LI, J. L., JOHNSON, M., GONG, G., DAVIS, K. M. & RECKER, R. R. 1998. Change of bone mass in postmenopausal Caucasian women with and without hormone replacement therapy is associated with vitamin D receptor and estrogen receptor genotypes. *Hum Genet,* 103**,** 576-85.

DENG, H. W., MAHANEY, M. C., WILLIAMS, J. T., LI, J., CONWAY, T., DAVIES, K. M., LI, J. L., DENG, H. & RECKER, R. R. 2002. Relevance of the genes for bone mass variation to susceptibility to osteoporotic fractures and its implications to gene search for complex human diseases. *Genet Epidemiol,* 22**,** 12-25.

DENOYELLE, C., HONG, L., VANNIER, J. P., SORIA, J. & SORIA, C. 2003. New insights into the actions of bisphosphonate zoledronic acid in breast cancer cells by dual RhoA-dependent and -independent effects. *Br J Cancer,* 88**,** 1631-40.

DERMARDIROSSIAN, C. & BOKOCH, G. M. 2005. GDIs: central regulatory molecules in Rho GTPase activation. *Trends Cell Biol,* 15**,** 356-63.

DEVOTO, M., SHIMOYA, K., CAMINIS, J., OTT, J., TENENHOUSE, A., WHYTE, M. P., SEREDA, L., HALL, S., CONSIDINE, E., WILLIAMS, C. J., TROMP, G., KUIVANIEMI, H., ALA-KOKKO, L., PROCKOP, D. J. & SPOTILA, L. D. 1998. First-stage autosomal genome screen in extended pedigrees suggests genes predisposing to low bone mineral density on chromosomes 1p, 2p and 4q. *Eur J Hum Genet,* 6**,** 151-7.

DEVOTO, M., SPECCHIA, C., LI, H. H., CAMINIS, J., TENENHOUSE, A., RODRIGUEZ, H. & SPOTILA, L. D. 2001. Variance component linkage analysis indicates a QTL for femoral neck bone mineral density on chromosome 1p36. *Hum Mol Genet,* 10**,** 2447-52.

DIEKMANN, D., BRILL, S., GARRETT, M. D., TOTTY, N., HSUAN, J., MONFRIES, C., HALL, C., LIM, L. & HALL, A. 1991. Bcr encodes a GTPase-activating protein for p21rac. *Nature,* 351**,** 400-2.

DIVIETI, P., INOMATA, N., CHAPIN, K., SINGH, R., JUPPNER, H. & BRINGHURST, F. R. 2001. Receptors for the carboxyl-terminal region of pth(1-84) are highly expressed in osteocytic cells. *Endocrinology,* 142**,** 916-25.

DOBBINS, D. E., SOOD, R., HASHIRAMOTO, A., HANSEN, C. T., WILDER, R. L. & REMMERS, E. F. 2002. Mutation of macrophage colony stimulating factor (Csf1) causes osteopetrosis in the tl rat. *Biochem Biophys Res Commun,* 294**,** 1114-20.

DOBNIG, H. & TURNER, R. T. 1995. Evidence that intermittent treatment with parathyroid hormone increases bone formation in adult rats by activation of bone lining cells. *Endocrinology,* 136**,** 3632-8.

DOECKE, J. D., DAY, C. J., STEPHENS, A. S., CARTER, S. L., VAN DAAL, A., KOTOWICZ, M. A., NICHOLSON, G. C. & MORRISON, N. A. 2006. Association of functionally different RUNX2 P2 promoter alleles with BMD. *J Bone Miner Res,* 21**,** 265-73.

DORE, D. D., MACEWEN, G. D. & BOULOS, M. I. 1987. Cleidocranial dysostosis and syringomyelia. Review of the literature and case report. *Clin Orthop Relat Res***,** 229-34.

DOUGALL, W. C., GLACCUM, M., CHARRIER, K., ROHRBACH, K., BRASEL, K., DE SMEDT, T., DARO, E., SMITH, J., TOMETSKO, M. E., MALISZEWSKI, C. R., ARMSTRONG, A., SHEN, V., BAIN, S., COSMAN, D., ANDERSON, D., MORRISSEY, P. J., PESCHON, J. J. & SCHUH, J. 1999. RANK is essential for osteoclast and lymph node development. *Genes Dev,* 13**,** 2412-24.

DOWN, T. A. & HUBBARD, T. J. 2002. Computational detection and location of transcription start sites in mammalian genomic DNA. *Genome Res,* 12**,** 458-61.

DUCY, P., DESBOIS, C., BOYCE, B., PINERO, G., STORY, B., DUNSTAN, C., SMITH, E., BONADIO, J., GOLDSTEIN, S., GUNDBERG, C., BRADLEY, A. & KARSENTY, G. 1996. Increased bone formation in osteocalcin-deficient mice. *Nature,* 382**,** 448-52.

DUCY, P. & KARSENTY, G. 1995. Two distinct osteoblast-specific cis-acting elements control expression of a mouse osteocalcin gene. *Mol Cell Biol,* 15**,** 1858-69.

DUCY, P., STARBUCK, M., PRIEMEL, M., SHEN, J., PINERO, G., GEOFFROY, V., AMLING, M. & KARSENTY, G. 1999. A Cbfa1-dependent genetic pathway controls bone formation beyond embryonic development. *Genes Dev,* 13**,** 1025-36.

DUCY, P., ZHANG, R., GEOFFROY, V., RIDALL, A. L. & KARSENTY, G. 1997. Osf2/Cbfa1: a transcriptional activator of osteoblast differentiation. *Cell,* 89**,** 747-54.

DUDBRIDGE, F. 2003. Pedigree disequilibrium tests for multilocus haplotypes. *Genet Epidemiol,* 25**,** 115-21.

DUNCAN, E. L., BROWN, M. A., SINSHEIMER, J., BELL, J., CARR, A. J., WORDSWORTH, B. P. & WASS, J. A. 1999. Suggestive linkage of the parathyroid receptor type 1 to osteoporosis. *J Bone Miner Res,* 14**,** 1993-9.

DUNNING, M. J., BARBOSA-MORAIS, N. L., LYNCH, A. G., TAVARE, S. & RITCHIE, M. E. 2008. Statistical issues in the analysis of Illumina data. *BMC Bioinformatics,* 9**,** 85.

DUQUE, G., EL ABDAIMI, K., HENDERSON, J. E., LOMRI, A. & KREMER, R. 2004a. Vitamin D inhibits Fas ligand-induced apoptosis in human osteoblasts by regulating components of both the mitochondrial and Fas-related pathways. *Bone,* 35**,** 57-64.

DUQUE, G., MACORITTO, M., DION, N., STE-MARIE, L. G. & KREMER, R. 2005. 1,25(OH)2D3 acts as a bone-forming agent in the hormone-independent senescence-accelerated mouse (SAM-P/6). *Am J Physiol Endocrinol Metab,* 288**,** E723-30.

DUQUE, G., MACORITTO, M. & KREMER, R. 2004b. Vitamin D treatment of senescence accelerated mice (SAM-P/6) induces several regulators of stromal cell plasticity. *Biogerontology,* 5**,** 421-9.

DUQUE, G. & TROEN, B. R. 2008. Understanding the mechanisms of senile osteoporosis: new facts for a major geriatric syndrome. *J Am Geriatr Soc,* 56**,** 935-41.

DVORNYK, V., LIU, X. H., SHEN, H., LEI, S. F., ZHAO, L. J., HUANG, Q. R., QIN, Y. J., JIANG, D. K., LONG, J. R., ZHANG, Y. Y., GONG, G., RECKER, R. R. & DENG, H. W. 2003. Differentiation of Caucasians and Chinese at bone mass candidate genes: implication for ethnic difference of bone mass. *Ann Hum Genet,* 67**,** 216-27.

EASTELL, R. 1999. Pathogenesis of Postmenopausal Osteoporosis. *In:* FAVUS, M. J. (ed.) *Primer on the Metabolic Bone Diseases and Disorders of Mineral Metabolism.* 4th ed. Philidelphia: Lippincott Williams & Wilkins.

EELES, R. A., KOTE-JARAI, Z., GILES, G. G., OLAMA, A. A., GUY, M., JUGURNAUTH, S. K., MULHOLLAND, S., LEONGAMORNLERT, D. A., EDWARDS, S. M., MORRISON, J., FIELD, H. I., SOUTHEY, M. C., SEVERI, G., DONOVAN, J. L., HAMDY, F. C., DEARNALEY, D. P., MUIR, K. R., SMITH, C., BAGNATO, M., ARDERN-JONES, A. T., HALL, A. L., O'BRIEN, L. T., GEHR-SWAIN, B. N., WILKINSON, R. A., COX, A., LEWIS, S., BROWN, P. M., JHAVAR, S. G., TYMRAKIEWICZ, M., LOPHATANANON, A., BRYANT, S. L., HORWICH, A., HUDDART, R. A., KHOO, V. S., PARKER, C. C., WOODHOUSE, C. J., THOMPSON, A., CHRISTMAS, T., OGDEN, C., FISHER, C., JAMIESON, C., COOPER, C. S., ENGLISH, D. R., HOPPER, J. L., NEAL, D. E. & EASTON, D. F. 2008. Multiple newly identified loci associated with prostate cancer susceptibility. *Nat Genet,* 40**,** 316-21.

EGOROV, M. V., CAPESTRANO, M., VORONTSOVA, O. A., DI PENTIMA, A., EGOROVA, A. V., MARIGGIO, S., AYALA, M. I., TETE, S., GORSKI, J. L., LUINI, A., BUCCIONE, R. & POLISHCHUK, R. S. 2009. Faciogenital dysplasia protein (FGD1) regulates export of cargo proteins from the golgi complex via Cdc42 activation. *Mol Biol Cell,* 20**,** 2413-27.

ELLIES, D. L., VIVIANO, B., MCCARTHY, J., REY, J. P., ITASAKI, N., SAUNDERS, S. & KRUMLAUF, R. 2006. Bone density ligand, Sclerostin, directly interacts with LRP5 but not LRP5G171V to modulate Wnt activity. *J Bone Miner Res,* 21**,** 1738-49.

ENOMOTO, H., ENOMOTO-IWAMOTO, M., IWAMOTO, M., NOMURA, S., HIMENO, M., KITAMURA, Y., KISHIMOTO, T. & KOMORI, T. 2000.

Cbfa1 is a positive regulatory factor in chondrocyte maturation. *J Biol Chem*, 275**,** 8695-702.

ENSENAUER, R., HARTL, D., VOCKLEY, J., ROSCHER, A. & FUCHS, U. 2010. Efficient and gentle siRNA delivery by magnetofection. *Biotech Histochem*.

ESTES, J. E., SELDEN, L. A. & GERSHMAN, L. C. 1981. Mechanism of action of phalloidin on the polymerization of muscle actin. *Biochemistry*, 20**,** 708-12.

ESTRADA, K., KRAWCZAK, M., SCHREIBER, S., VAN DUIJN, K., STOLK, L., VAN MEURS, J. B., LIU, F., PENNINX, B. W., SMIT, J. H., VOGELZANGS, N., HOTTENGA, J. J., WILLEMSEN, G., DE GEUS, E. J., LORENTZON, M., VON ELLER-EBERSTEIN, H., LIPS, P., SCHOOR, N., POP, V., DE KEIJZER, J., HOFMAN, A., AULCHENKO, Y. S., OOSTRA, B. A., OHLSSON, C., BOOMSMA, D. I., UITTERLINDEN, A. G., VAN DUIJN, C. M., RIVADENEIRA, F. & KAYSER, M. 2009. A genome-wide association study of northwestern Europeans involves the C-type natriuretic peptide signaling pathway in the etiology of human height variation. *Hum Mol Genet*, 18**,** 3516-24.

ETIENNE-MANNEVILLE, S. & HALL, A. 2002. Rho GTPases in cell biology. *Nature*, 420**,** 629-35.

EVANS, R. A., MAREL, G. M., LANCASTER, E. K., KOS, S., EVANS, M. & WONG, S. Y. 1988. Bone mass is low in relatives of osteoporotic patients. *Ann Intern Med*, 109**,** 870-3.

FACCIO, R., NOVACK, D. V., ZALLONE, A., ROSS, F. P. & TEITELBAUM, S. L. 2003a. Dynamic changes in the osteoclast cytoskeleton in response to growth factors and cell attachment are controlled by beta3 integrin. *J Cell Biol*, 162**,** 499-509.

FACCIO, R., TAKESHITA, S., ZALLONE, A., ROSS, F. P. & TEITELBAUM, S. L. 2003b. c-Fms and the alphavbeta3 integrin collaborate during osteoclast differentiation. *J Clin Invest*, 111**,** 749-58.

FACCIO, R., TEITELBAUM, S. L., FUJIKAWA, K., CHAPPEL, J., ZALLONE, A., TYBULEWICZ, V. L., ROSS, F. P. & SWAT, W. 2005. Vav3 regulates osteoclast function and bone mass. *Nat Med*, 11**,** 284-90.

FARRINGTON-ROCK, C., FIRESTEIN, M. H., BICKNELL, L. S., SUPERTI-FURGA, A., BACINO, C. A., CORMIER-DAIRE, V., LE MERRER, M., BAUMANN, C., ROUME, J., RUMP, P., VERHEIJ, J. B., SWEENEY, E., RIMOIN, D. L., LACHMAN, R. S., ROBERTSON, S. P., COHN, D. H. & KRAKOW, D. 2006. Mutations in two regions of FLNB result in atelosteogenesis I and III. *Hum Mutat*, 27**,** 705-10.

FATIGATI, V. & MURPHY, R. A. 1984. Actin and tropomyosin variants in smooth muscles. Dependence on tissue type. *J Biol Chem*, 259**,** 14383-8.

FELIX, R., CECCHINI, M. G. & FLEISCH, H. 1990. Macrophage colony stimulating factor restores in vivo bone resorption in the op/op osteopetrotic mouse. *Endocrinology*, 127**,** 2592-4.

FELSON, D. T., ZHANG, Y., HANNAN, M. T. & ANDERSON, J. J. 1993. Effects of weight and body mass index on bone mineral density in men and women: the Framingham study. *J Bone Miner Res*, 8**,** 567-73.

FENG, J. Q., WARD, L. M., LIU, S., LU, Y., XIE, Y., YUAN, B., YU, X., RAUCH, F., DAVIS, S. I., ZHANG, S., RIOS, H., DREZNER, M. K., QUARLES, L. D., BONEWALD, L. F. & WHITE, K. E. 2006. Loss of DMP1 causes rickets and osteomalacia and identifies a role for osteocytes in mineral metabolism. *Nat Genet*, 38**,** 1310-5.

FERRON, M., HINOI, E., KARSENTY, G. & DUCY, P. 2008. Osteocalcin differentially regulates beta cell and adipocyte gene expression and affects the development of metabolic diseases in wild-type mice. *Proc Natl Acad Sci U S A,* 105**,** 5266-70.

FINKELSTEIN, J. S., LEE, M. L., SOWERS, M., ETTINGER, B., NEER, R. M., KELSEY, J. L., CAULEY, J. A., HUANG, M. H. & GREENDALE, G. A. 2002. Ethnic variation in bone density in premenopausal and early perimenopausal women: effects of anthropometric and lifestyle factors. *J Clin Endocrinol Metab,* 87**,** 3057-67.

FLEET, J. C., HARRIS, S. S., WOOD, R. J. & DAWSON-HUGHES, B. 1995. The BsmI vitamin D receptor restriction fragment length polymorphism (BB) predicts low bone density in premenopausal black and white women. *J Bone Miner Res,* 10**,** 985-90.

FLICKER, L., HOPPER, J. L., RODGERS, L., KAYMAKCI, B., GREEN, R. M. & WARK, J. D. 1995. Bone density determinants in elderly women: a twin study. *J Bone Miner Res,* 10**,** 1607-13.

FOGED, N. T., DELAISSE, J. M., HOU, P., LOU, H., SATO, T., WINDING, B. & BONDE, M. 1996. Quantification of the collagenolytic activity of isolated osteoclasts by enzyme-linked immunosorbent assay. *J Bone Miner Res,* 11**,** 226-37.

FRANZ-ODENDAAL, T. A., HALL, B. K. & WITTEN, P. E. 2006. Buried alive: how osteoblasts become osteocytes. *Dev Dyn,* 235**,** 176-90.

FRATTINI, A., ORCHARD, P. J., SOBACCHI, C., GILIANI, S., ABINUN, M., MATTSSON, J. P., KEELING, D. J., ANDERSSON, A. K., WALLBRANDT, P., ZECCA, L., NOTARANGELO, L. D., VEZZONI, P. & VILLA, A. 2000. Defects in TCIRG1 subunit of the vacuolar proton pump are responsible for a subset of human autosomal recessive osteopetrosis. *Nat Genet,* 25**,** 343-6.

FRAZER, K. A., BALLINGER, D. G., COX, D. R., HINDS, D. A., STUVE, L. L., GIBBS, R. A., BELMONT, J. W., BOUDREAU, A., HARDENBOL, P., LEAL, S. M., PASTERNAK, S., WHEELER, D. A., WILLIS, T. D., YU, F., YANG, H., ZENG, C., GAO, Y., HU, H., HU, W., LI, C., LIN, W., LIU, S., PAN, H., TANG, X., WANG, J., WANG, W., YU, J., ZHANG, B., ZHANG, Q., ZHAO, H., ZHOU, J., GABRIEL, S. B., BARRY, R., BLUMENSTIEL, B., CAMARGO, A., DEFELICE, M., FAGGART, M., GOYETTE, M., GUPTA, S., MOORE, J., NGUYEN, H., ONOFRIO, R. C., PARKIN, M., ROY, J., STAHL, E., WINCHESTER, E., ZIAUGRA, L., ALTSHULER, D., SHEN, Y., YAO, Z., HUANG, W., CHU, X., HE, Y., JIN, L., LIU, Y., SUN, W., WANG, H., WANG, Y., XIONG, X., XU, L., WAYE, M. M., TSUI, S. K., XUE, H., WONG, J. T., GALVER, L. M., FAN, J. B., GUNDERSON, K., MURRAY, S. S., OLIPHANT, A. R., CHEE, M. S., MONTPETIT, A., CHAGNON, F., FERRETTI, V., LEBOEUF, M., OLIVIER, J. F., PHILLIPS, M. S., ROUMY, S., SALLEE, C., VERNER, A., HUDSON, T. J., KWOK, P. Y., CAI, D., KOBOLDT, D. C., MILLER, R. D., PAWLIKOWSKA, L., TAILLON-MILLER, P., XIAO, M., TSUI, L. C., MAK, W., SONG, Y. Q., TAM, P. K., NAKAMURA, Y., KAWAGUCHI, T., KITAMOTO, T., MORIZONO, T., NAGASHIMA, A., OHNISHI, Y., SEKINE, A., TANAKA, T., TSUNODA, T., et al. 2007. A second generation human haplotype map of over 3.1 million SNPs. *Nature,* 449**,** 851-61.

FREDERICK, A., ROLFE, M. & CHIU, M. I. 1998. The human UNP locus at 3p21.31 encodes two tissue-selective, cytoplasmic isoforms with deubiquitinating activity that have reduced expression in small cell lung carcinoma cell lines. *Oncogene,* 16**,** 153-65.

FRIEDMAN, P. A. 2000. Mechanisms of renal calcium transport. *Exp Nephrol,* 8**,** 343-50.

FROST, H. M. 1960. Micropetrosis. *J Bone Joint Surg Am,* 42-A**,** 144-50.

FUKUMOTO, Y., KAIBUCHI, K., HORI, Y., FUJIOKA, H., ARAKI, S., UEDA, T., KIKUCHI, A. & TAKAI, Y. 1990. Molecular cloning and characterization of a novel type of regulatory protein (GDI) for the rho proteins, ras p21-like small GTP-binding proteins. *Oncogene,* 5**,** 1321-8.

FULLER, K., KIRSTEIN, B. & CHAMBERS, T. J. 2006. Murine osteoclast formation and function: differential regulation by humoral agents. *Endocrinology,* 147**,** 1979-85.

FULLER, K., MURPHY, C., KIRSTEIN, B., FOX, S. W. & CHAMBERS, T. J. 2002. TNFalpha potently activates osteoclasts, through a direct action independent of and strongly synergistic with RANKL. *Endocrinology,* 143**,** 1108-18.

GAO, B., GUO, J., SHE, C., SHU, A., YANG, M., TAN, Z., YANG, X., GUO, S., FENG, G. & HE, L. 2001. Mutations in IHH, encoding Indian hedgehog, cause brachydactyly type A-1. *Nat Genet,* 28**,** 386-8.

GARNERO, P., BOREL, O., SORNAY-RENDU, E., ARLOT, M. E. & DELMAS, P. D. 1996a. Vitamin D receptor gene polymorphisms are not related to bone turnover, rate of bone loss, and bone mass in postmenopausal women: the OFELY Study. *J Bone Miner Res,* 11**,** 827-34.

GARNERO, P., HAUSHERR, E., CHAPUY, M. C., MARCELLI, C., GRANDJEAN, H., MULLER, C., CORMIER, C., BREART, G., MEUNIER, P. J. & DELMAS, P. D. 1996b. Markers of bone resorption predict hip fracture in elderly women: the EPIDOS Prospective Study. *J Bone Miner Res,* 11**,** 1531-8.

GEBHARD, P. M., SODER, S., BAU, B. & AIGNER, T. 2004. Down-regulation of the GTPase RhoB might be involved in the pre-apoptotic phenotype of osteoarthritic chondrocytes. *Front Biosci,* 9**,** 827-33.

GENANT, H. K., LUCAS, J., WEISS, S., AKIN, M., EMKEY, R., MCNANEY-FLINT, H., DOWNS, R., MORTOLA, J., WATTS, N., YANG, H. M., BANAV, N., BRENNAN, J. J. & NOLAN, J. C. 1997. Low-dose esterified estrogen therapy: effects on bone, plasma estradiol concentrations, endometrium, and lipid levels. Estratab/Osteoporosis Study Group. *Arch Intern Med,* 157**,** 2609-15.

GLASS, D. A., 2ND, BIALEK, P., AHN, J. D., STARBUCK, M., PATEL, M. S., CLEVERS, H., TAKETO, M. M., LONG, F., MCMAHON, A. P., LANG, R. A. & KARSENTY, G. 2005. Canonical Wnt signaling in differentiated osteoblasts controls osteoclast differentiation. *Dev Cell,* 8**,** 751-64.

GLOVER, T. W., VERGA, V., RAFAEL, J., BARCROFT, C., GORSKI, J. L., BAWLE, E. V. & HIGGINS, J. V. 1993. Translocation breakpoint in Aarskog syndrome maps to Xp11.21 between ALAS2 and DXS323. *Hum Mol Genet,* 2**,** 1717-8.

GLUHAK-HEINRICH, J., PAVLIN, D., YANG, W., MACDOUGALL, M. & HARRIS, S. E. 2007. MEPE expression in osteocytes during orthodontic tooth movement. *Arch Oral Biol,* 52**,** 684-90.

GLUHAK-HEINRICH, J., YE, L., BONEWALD, L. F., FENG, J. Q., MACDOUGALL, M., HARRIS, S. E. & PAVLIN, D. 2003. Mechanical loading stimulates dentin matrix protein 1 (DMP1) expression in osteocytes in vivo. *J Bone Miner Res,* 18**,** 807-17.

GOLOB, D. S., MCALISTER, W. H., MILLS, B. G., FEDDE, K. N., REINUS, W. R., TEITELBAUM, S. L., BEEKI, S. & WHYTE, M. P. 1996. Juvenile Paget disease: life-long features of a mildly affected young woman. *J Bone Miner Res,* 11**,** 132-42.

GONG, Y., SLEE, R. B., FUKAI, N., RAWADI, G., ROMAN-ROMAN, S., REGINATO, A. M., WANG, H., CUNDY, T., GLORIEUX, F. H., LEV, D., ZACHARIN, M., OEXLE, K., MARCELINO, J., SUWAIRI, W., HEEGER, S., SABATAKOS, G., APTE, S., ADKINS, W. N., ALLGROVE, J., ARSLAN-KIRCHNER, M., BATCH, J. A., BEIGHTON, P., BLACK, G. C., BOLES, R. G., BOON, L. M., BORRONE, C., BRUNNER, H. G., CARLE, G. F., DALLAPICCOLA, B., DE PAEPE, A., FLOEGE, B., HALFHIDE, M. L., HALL, B., HENNEKAM, R. C., HIROSE, T., JANS, A., JUPPNER, H., KIM, C. A., KEPPLER-NOREUIL, K., KOHLSCHUETTER, A., LACOMBE, D., LAMBERT, M., LEMYRE, E., LETTEBOER, T., PELTONEN, L., RAMESAR, R. S., ROMANENGO, M., SOMER, H., STEICHEN-GERSDORF, E., STEINMANN, B., SULLIVAN, B., SUPERTI-FURGA, A., SWOBODA, W., VAN DEN BOOGAARD, M. J., VAN HUL, W., VIKKULA, M., VOTRUBA, M., ZABEL, B., GARCIA, T., BARON, R., OLSEN, B. R. & WARMAN, M. L. 2001. LDL receptor-related protein 5 (LRP5) affects bone accrual and eye development. *Cell,* 107**,** 513-23.

GONG, Y., VIKKULA, M., BOON, L., LIU, J., BEIGHTON, P., RAMESAR, R., PELTONEN, L., SOMER, H., HIROSE, T., DALLAPICCOLA, B., DE PAEPE, A., SWOBODA, W., ZABEL, B., SUPERTI-FURGA, A., STEINMANN, B., BRUNNER, H. G., JANS, A., BOLES, R. G., ADKINS, W., VAN DEN BOOGAARD, M. J., OLSEN, B. R. & WARMAN, M. L. 1996. Osteoporosis-pseudoglioma syndrome, a disorder affecting skeletal strength and vision, is assigned to chromosome region 11q12-13. *Am J Hum Genet,* 59**,** 146-51.

GOODE, B. L. & ECK, M. J. 2007. Mechanism and function of formins in the control of actin assembly. *Annu Rev Biochem,* 76**,** 593-627.

GOODMAN, W. G., RAMIREZ, J. A., BELIN, T. R., CHON, Y., GALES, B., SEGRE, G. V. & SALUSKY, I. B. 1994. Development of adynamic bone in patients with secondary hyperparathyroidism after intermittent calcitriol therapy. *Kidney Int,* 46**,** 1160-6.

GORLIN, J. B., YAMIN, R., EGAN, S., STEWART, M., STOSSEL, T. P., KWIATKOWSKI, D. J. & HARTWIG, J. H. 1990. Human endothelial actin-binding protein (ABP-280, nonmuscle filamin): a molecular leaf spring. *J Cell Biol,* 111**,** 1089-105.

GORSKI, J. L., ESTRADA, L., HU, C. & LIU, Z. 2000. Skeletal-specific expression of Fgd1 during bone formation and skeletal defects in faciogenital dysplasia (FGDY; Aarskog syndrome). *Dev Dyn,* 218**,** 573-86.

GOWEN, L. C., PETERSEN, D. N., MANSOLF, A. L., QI, H., STOCK, J. L., TKALCEVIC, G. T., SIMMONS, H. A., CRAWFORD, D. T., CHIDSEY-FRINK, K. L., KE, H. Z., MCNEISH, J. D. & BROWN, T. A. 2003. Targeted

disruption of the osteoblast/osteocyte factor 45 gene (OF45) results in increased bone formation and bone mass. *J Biol Chem,* 278**,** 1998-2007.

GRANT, S. F., REID, D. M., BLAKE, G., HERD, R., FOGELMAN, I. & RALSTON, S. H. 1996. Reduced bone density and osteoporosis associated with a polymorphic Sp1 binding site in the collagen type I alpha 1 gene. *Nat Genet,* 14**,** 203-5.

GRAY, D. A., INAZAWA, J., GUPTA, K., WONG, A., UEDA, R. & TAKAHASHI, T. 1995. Elevated expression of Unph, a proto-oncogene at 3p21.3, in human lung tumors. *Oncogene,* 10**,** 2179-83.

GROSS, C., ECCLESHALL, T. R., MALLOY, P. J., VILLA, M. L., MARCUS, R. & FELDMAN, D. 1996. The presence of a polymorphism at the translation initiation site of the vitamin D receptor gene is associated with low bone mineral density in postmenopausal Mexican-American women. *J Bone Miner Res,* 11**,** 1850-5.

GRUBER, A. R., LORENZ, R., BERNHART, S. H., NEUBOCK, R. & HOFACKER, I. L. 2008. The Vienna RNA Websuite. *Nucleic Acids Res.*

GUO, D., GUTHRIE, J., ZHAO, J., BARRAGAN, L., HARRIS, S. & BONEWALD, L. 2005. Proteomic comparison of osteoblasts and osteocytes reveals unique protein expression patterns. *J Bone Miner Res,* 20(Suppl1)**,** S150.

HADJIDAKIS, D. J. & ANDROULAKIS, II 2006. Bone remodeling. *Ann N Y Acad Sci,* 1092**,** 385-96.

HAKEDA, Y., KOBAYASHI, Y., YAMAGUCHI, K., YASUDA, H., TSUDA, E., HIGASHIO, K., MIYATA, T. & KUMEGAWA, M. 1998. Osteoclastogenesis inhibitory factor (OCIF) directly inhibits bone-resorbing activity of isolated mature osteoclasts. *Biochem Biophys Res Commun,* 251**,** 796-801.

HALL, A. 1998. Rho GTPases and the actin cytoskeleton. *Science,* 279**,** 509-14.

HAMERSMA, H., GARDNER, J. & BEIGHTON, P. 2003. The natural history of sclerosteosis. *Clin Genet,* 63**,** 192-7.

HAN, J., KRAFT, P., NAN, H., GUO, Q., CHEN, C., QURESHI, A., HANKINSON, S. E., HU, F. B., DUFFY, D. L., ZHAO, Z. Z., MARTIN, N. G., MONTGOMERY, G. W., HAYWARD, N. K., THOMAS, G., HOOVER, R. N., CHANOCK, S. & HUNTER, D. J. 2008. A genome-wide association study identifies novel alleles associated with hair color and skin pigmentation. *PLoS Genet,* 4**,** e1000074.

HAN, K., CHOI, J., MOON, I., YOON, H., HAN, I., MIN, H., KIM, Y. & CHOI, Y. 1999. Non-association of estrogen receptor genotypes with bone mineral density and bone turnover in Korean pre-, peri-, and postmenopausal women. *Osteoporos Int,* 9**,** 290-5.

HARADA, H., TAGASHIRA, S., FUJIWARA, M., OGAWA, S., KATSUMATA, T., YAMAGUCHI, A., KOMORI, T. & NAKATSUKA, M. 1999. Cbfa1 isoforms exert functional differences in osteoblast differentiation. *J Biol Chem,* 274**,** 6972-8.

HART, D. J. & SPECTOR, T. D. 1993. The relationship of obesity, fat distribution and osteoarthritis in women in the general population: the Chingford Study. *J Rheumatol,* 20**,** 331-5.

HART, M. J., SHARMA, S., ELMASRY, N., QIU, R. G., MCCABE, P., POLAKIS, P. & BOLLAG, G. 1996. Identification of a novel guanine nucleotide exchange factor for the Rho GTPase. *J Biol Chem,* 271**,** 25452-8.

HARTWIG, J. H. & SHEVLIN, P. 1986. The architecture of actin filaments and the ultrastructural location of actin-binding protein in the periphery of lung macrophages. *J Cell Biol,* 103**,** 1007-20.

HEANEY, R. P. 2002. The importance of calcium intake for lifelong skeletal health. *Calcif Tissue Int,* 70**,** 70-3.

HEEGAARD, A., JORGENSEN, H. L., VESTERGAARD, A. W., HASSAGER, C. & RALSTON, S. H. 2000. Lack of influence of collagen type Ialpha1 Sp1 binding site polymorphism on the rate of bone loss in a cohort of postmenopausal danish women followed for 18 years. *Calcif Tissue Int,* 66**,** 409-13.

HEINO, T. J., HENTUNEN, T. A. & VAANANEN, H. K. 2002. Osteocytes inhibit osteoclastic bone resorption through transforming growth factor-beta: enhancement by estrogen. *J Cell Biochem,* 85**,** 185-97.

HELFRICH, M. H., NESBITT, S. A., DOREY, E. L. & HORTON, M. A. 1992. Rat osteoclasts adhere to a wide range of RGD (Arg-Gly-Asp) peptide-containing proteins, including the bone sialoproteins and fibronectin, via a beta 3 integrin. *J Bone Miner Res,* 7**,** 335-43.

HELLEMANS, J., COUCKE, P. J., GIEDION, A., DE PAEPE, A., KRAMER, P., BEEMER, F. & MORTIER, G. R. 2003. Homozygous mutations in IHH cause acrocapitofemoral dysplasia, an autosomal recessive disorder with cone-shaped epiphyses in hands and hips. *Am J Hum Genet,* 72**,** 1040-6.

HERMAN, S., MULLER, R. B., KRONKE, G., ZWERINA, J., REDLICH, K., HUEBER, A. J., GELSE, H., NEUMANN, E., MULLER-LADNER, U. & SCHETT, G. 2008. Induction of osteoclast-associated receptor, a key osteoclast costimulation molecule, in rheumatoid arthritis. *Arthritis Rheum,* 58**,** 3041-50.

HERSH, C. P., HANSEL, N. N., BARNES, K. C., LOMAS, D. A., PILLAI, S. G., COXSON, H. O., MATHIAS, R. A., RAFAELS, N. M., WISE, R. A., CONNETT, J. E., KLANDERMAN, B. J., JACOBSON, F. L., GILL, R., LITONJUA, A. A., SPARROW, D., REILLY, J. J. & SILVERMAN, E. K. 2009. Transforming growth factor-beta receptor-3 is associated with pulmonary emphysema. *Am J Respir Cell Mol Biol,* 41**,** 324-31.

HILL, P. A. 1998. Bone Remodelling. *British Journal of Orthodontics,* 25**,** 101-107.

HIND, K. & BURROWS, M. 2007. Weight-bearing exercise and bone mineral accrual in children and adolescents: a review of controlled trials. *Bone,* 40**,** 14-27.

HINDS, D. A., STUVE, L. L., NILSEN, G. B., HALPERIN, E., ESKIN, E., BALLINGER, D. G., FRAZER, K. A. & COX, D. R. 2005. Whole-genome patterns of common DNA variation in three human populations. *Science,* 307**,** 1072-9.

HIRAKAWA, M., OIKE, M., KARASHIMA, Y. & ITO, Y. 2004. Sequential activation of RhoA and FAK/paxillin leads to ATP release and actin reorganization in human endothelium. *J Physiol,* 558**,** 479-88.

HIRAO, M., HASHIMOTO, J., YAMASAKI, N., ANDO, W., TSUBOI, H., MYOUI, A. & YOSHIKAWA, H. 2007. Oxygen tension is an important mediator of the transformation of osteoblasts to osteocytes. *J Bone Miner Metab,* 25**,** 266-76.

HIRSCHHORN, J. N. & DALY, M. J. 2005. Genome-wide association studies for common diseases and complex traits. *Nat Rev Genet,* 6**,** 95-108.

HOFBAUER, L. C. & SCHOPPET, M. 2004. Clinical implications of the osteoprotegerin/RANKL/RANK system for bone and vascular diseases. *JAMA,* 292**,** 490-5.

HOLICK, M. F. 1999. Vitamin D: Photobiology, Metabolism, Mechanism of Action, and Clinical Applications. *In:* FAVUS, M. J. (ed.) *Primer on the Metabolic Bone Diseases and Disorders of Mineral Metabolism - Fourth Edition.* Philadelphia: Lippincott Williams & Wilkins.

HOLICK, M. F., MATSUOKA, L. Y. & WORTSMAN, J. 1989. Age, vitamin D, and solar ultraviolet. *Lancet,* 2**,** 1104-5.

HOLMBECK, K., BIANCO, P., PIDOUX, I., INOUE, S., BILLINGHURST, R. C., WU, W., CHRYSOVERGIS, K., YAMADA, S., BIRKEDAL-HANSEN, H. & POOLE, A. R. 2005. The metalloproteinase MT1-MMP is required for normal development and maintenance of osteocyte processes in bone. *J Cell Sci,* 118**,** 147-56.

HORTON, M. A., NESBIT, M. A. & HELFRICH, M. H. 1995. Interaction of osteopontin with osteoclast integrins. *Ann N Y Acad Sci,* 760**,** 190-200.

HORTON, M. A., TAYLOR, M. L., ARNETT, T. R. & HELFRICH, M. H. 1991. Arg-Gly-Asp (RGD) peptides and the anti-vitronectin receptor antibody 23C6 inhibit dentine resorption and cell spreading by osteoclasts. *Exp Cell Res,* 195**,** 368-75.

HOSONO, S., FARUQI, A. F., DEAN, F. B., DU, Y., SUN, Z., WU, X., DU, J., KINGSMORE, S. F., EGHOLM, M. & LASKEN, R. S. 2003. Unbiased whole-genome amplification directly from clinical samples. *Genome Res,* 13**,** 954-64.

HOTCHIN, N. A. & HALL, A. 1995. The assembly of integrin adhesion complexes requires both extracellular matrix and intracellular rho/rac GTPases. *J Cell Biol,* 131**,** 1857-65.

HOTCHKISS, C. E., STAVISKY, R., NOWAK, J., BROMMAGE, R., LEES, C. J. & KAPLAN, J. 2001. Levormeloxifene prevents increased bone turnover and vertebral bone loss following ovariectomy in cynomolgus monkeys. *Bone,* 29**,** 7-15.

HOUSTON, L. A., GRANT, S. F., REID, D. M. & RALSTON, S. H. 1996. Vitamin D receptor polymorphism, bone mineral density, and osteoporotic vertebral fracture: studies in a UK population. *Bone,* 18**,** 249-52.

HSU, Y. H., NIU, T., TERWEDOW, H. A., XU, X., FENG, Y., LI, Z., BRAIN, J. D., ROSEN, C. J. & LAIRD, N. 2006. Variation in genes involved in the RANKL/RANK/OPG bone remodeling pathway are associated with bone mineral density at different skeletal sites in men. *Hum Genet,* 118**,** 568-77.

HU, Y., BENYA, R. V., CARROLL, R. E. & DIAMOND, A. M. 2005. Allelic loss of the gene for the GPX1 selenium-containing protein is a common event in cancer. *J Nutr,* 135**,** 3021S-3024S.

HUANG, J. C., SAKATA, T., PFLEGER, L. L., BENCSIK, M., HALLORAN, B. P., BIKLE, D. D. & NISSENSON, R. A. 2004. PTH differentially regulates expression of RANKL and OPG. *J Bone Miner Res,* 19**,** 235-44.

HUNT, S. C., ELLISON, R. C., ATWOOD, L. D., PANKOW, J. S., PROVINCE, M. A. & LEPPERT, M. F. 2002. Genome scans for blood pressure and hypertension: the National Heart, Lung, and Blood Institute Family Heart Study. *Hypertension,* 40**,** 1-6.

HUNTER, D. J., DE LANGE, M., ANDREW, T., SNIEDER, H., MACGREGOR, A. J. & SPECTOR, T. D. 2001. Genetic variation in bone mineral density and

calcaneal ultrasound: a study of the influence of menopause using female twins. *Osteoporos Int,* 12**,** 406-11.

HUNTER, D. J., KRAFT, P., JACOBS, K. B., COX, D. G., YEAGER, M., HANKINSON, S. E., WACHOLDER, S., WANG, Z., WELCH, R., HUTCHINSON, A., WANG, J., YU, K., CHATTERJEE, N., ORR, N., WILLETT, W. C., COLDITZ, G. A., ZIEGLER, R. G., BERG, C. D., BUYS, S. S., MCCARTY, C. A., FEIGELSON, H. S., CALLE, E. E., THUN, M. J., HAYES, R. B., TUCKER, M., GERHARD, D. S., FRAUMENI, J. F., JR., HOOVER, R. N., THOMAS, G. & CHANOCK, S. J. 2007. A genome-wide association study identifies alleles in FGFR2 associated with risk of sporadic postmenopausal breast cancer. *Nat Genet,* 39**,** 870-4.

HURST, L. D., PAL, C. & LERCHER, M. J. 2004. The evolutionary dynamics of eukaryotic gene order. *Nat Rev Genet,* 5**,** 299-310.

IGARASHI, M., KAMIYA, N., ITO, K. & TAKAGI, M. 2002. In situ localization and in vitro expression of osteoblast/osteocyte factor 45 mRNA during bone cell differentiation. *Histochem J,* 34**,** 255-63.

IKEDA, K. 2008. Osteocytes in the pathogenesis of osteoporosis. *Geriatr Gerontol Int,* 8**,** 213-7.

ILLUMINA 2004. Golden Gate Assay: Technical Specifications and Operation. *Illumina Technical Bulletin,* Pub. No. 370-2004-006.

ILLUMINA 2007. BeadStudio Normalization Algorithms for Gene Expression Data. *Illumina Technical Bulletin,* Pub. No. 470-2007-005.

ILLUMINA 2008. HumanHT-12 v3 Expression BeadChip. *Illumina Technical Bulletin,* Pub. No. 470-2008-005.

IM, J. A., YU, B. P., JEON, J. Y. & KIM, S. H. 2008. Relationship between osteocalcin and glucose metabolism in postmenopausal women. *Clin Chim Acta,* 396**,** 66-9.

INOUE, K., MIKUNI-TAKAGAKI, Y., OIKAWA, K., ITOH, T., INADA, M., NOGUCHI, T., PARK, J. S., ONODERA, T., KRANE, S. M., NODA, M. & ITOHARA, S. 2006. A crucial role for matrix metalloproteinase 2 in osteocytic canalicular formation and bone metabolism. *J Biol Chem,* 281**,** 33814-24.

INOUE, M., NAMBA, N., CHAPPEL, J., TEITELBAUM, S. L. & ROSS, F. P. 1998. Granulocyte macrophage-colony stimulating factor reciprocally regulates alphav-associated integrins on murine osteoclast precursors. *Mol Endocrinol,* 12**,** 1955-62.

IOANNIDIS, J. P., NG, M. Y., SHAM, P. C., ZINTZARAS, E., LEWIS, C. M., DENG, H. W., ECONS, M. J., KARASIK, D., DEVOTO, M., KAMMERER, C. M., SPECTOR, T., ANDREW, T., CUPPLES, L. A., DUNCAN, E. L., FOROUD, T., KIEL, D. P., KOLLER, D., LANGDAHL, B., MITCHELL, B. D., PEACOCK, M., RECKER, R., SHEN, H., SOL-CHURCH, K., SPOTILA, L. D., UITTERLINDEN, A. G., WILSON, S. G., KUNG, A. W. & RALSTON, S. H. 2007. Meta-analysis of genome-wide scans provides evidence for sex- and site-specific regulation of bone mass. *J Bone Miner Res,* 22**,** 173-83.

IOANNIDIS, J. P., RALSTON, S. H., BENNETT, S. T., BRANDI, M. L., GRINBERG, D., KARASSA, F. B., LANGDAHL, B., VAN MEURS, J. B., MOSEKILDE, L., SCOLLEN, S., ALBAGHA, O. M., BUSTAMANTE, M., CAREY, A. H., DUNNING, A. M., ENJUANES, A., VAN LEEUWEN, J. P., MAVILIA, C., MASI, L., MCGUIGAN, F. E., NOGUES, X., POLS, H.

A., REID, D. M., SCHUIT, S. C., SHERLOCK, R. E. & UITTERLINDEN, A. G. 2004. Differential genetic effects of ESR1 gene polymorphisms on osteoporosis outcomes. *JAMA,* 292**,** 2105-14.

IOF. 2010. *International Osteoporosis Foundation* [Online]. Available: http://www.iofbonehealth.org/newsroom/resources/image-normal-osteoporotic-bone.html [Accessed 27th January 2011].

IOTSOVA, V., CAAMANO, J., LOY, J., YANG, Y., LEWIN, A. & BRAVO, R. 1997. Osteopetrosis in mice lacking NF-kappaB1 and NF-kappaB2. *Nat Med,* 3**,** 1285-9.

JAGUS-SMITH, R. & KAY, J. E. 1976. Messenger ribonucleic acid content of phytohaemagglutinin-treated lymphocytes. *Biochem Soc Trans,* 4**,** 783-5.

JAMES, K., WEITZEL, L. R., ENGELMAN, C. D., ZERBE, G. & NORRIS, J. M. 2003. Genome scan linkage results for longitudinal systolic blood pressure phenotypes in subjects from the Framingham Heart Study. *BMC Genet,* 4 Suppl 1**,** S83.

JAMIESON, S. E., MILLER, E. N., BLACK, G. F., PEACOCK, C. S., CORDELL, H. J., HOWSON, J. M., SHAW, M. A., BURGNER, D., XU, W., LINS-LAINSON, Z., SHAW, J. J., RAMOS, F., SILVEIRA, F. & BLACKWELL, J. M. 2004. Evidence for a cluster of genes on chromosome 17q11-q21 controlling susceptibility to tuberculosis and leprosy in Brazilians. *Genes Immun,* 5**,** 46-57.

JENSEN, B. L. 1990. Somatic development in cleidocranial dysplasia. *Am J Med Genet,* 35**,** 69-74.

JILKA, R. L., WEINSTEIN, R. S., BELLIDO, T., ROBERSON, P., PARFITT, A. M. & MANOLAGAS, S. C. 1999. Increased bone formation by prevention of osteoblast apoptosis with parathyroid hormone. *J Clin Invest,* 104**,** 439-46.

JIMENEZ, M. J., BALBIN, M., LOPEZ, J. M., ALVAREZ, J., KOMORI, T. & LOPEZ-OTIN, C. 1999. Collagenase 3 is a target of Cbfa1, a transcription factor of the runt gene family involved in bone formation. *Mol Cell Biol,* 19**,** 4431-42.

JOAKIMSEN, R. M., FONNEBO, V., MAGNUS, J. H., TOLLAN, A. & SOGAARD, A. J. 1998. The Tromso Study: body height, body mass index and fractures. *Osteoporos Int,* 8**,** 436-42.

JOHNELL, O. & KANIS, J. 2005. Epidemiology of osteoporotic fractures. *Osteoporos Int,* 16 Suppl 2**,** S3-7.

JOHNELL, O. & KANIS, J. A. 2006. An estimate of the worldwide prevalence and disability associated with osteoporotic fractures. *Osteoporos Int,* 17**,** 1726-33.

JOHNS, M. B., JR. & PAULUS-THOMAS, J. E. 1989. Purification of human genomic DNA from whole blood using sodium perchlorate in place of phenol. *Anal Biochem,* 180**,** 276-8.

JOHNSON, M. L., GONG, G., KIMBERLING, W., RECKER, S. M., KIMMEL, D. B. & RECKER, R. B. 1997. Linkage of a gene causing high bone mass to human chromosome 11 (11q12-13). *Am J Hum Genet,* 60**,** 1326-32.

JUPPNER, H., BROWN, E. M. & KRONENBERG, H. M. 1999. Parathyroid Hormone. *In:* FAVUS, M. J. (ed.) *Primer on the Metabolic Bone Diseases and Disorders of Mineral Metabolism - Fourth Edition.* Philidelphia: Lippincott Williams & Wilkins.

KABACK, L. A., SOUNG DO, Y., NAIK, A., SMITH, N., SCHWARZ, E. M., O'KEEFE, R. J. & DRISSI, H. 2008. Osterix/Sp7 regulates mesenchymal stem cell mediated endochondral ossification. *J Cell Physiol,* 214**,** 173-82.

KAMEDA, T., MANO, H., YUASA, T., MORI, Y., MIYAZAWA, K., SHIOKAWA, M., NAKAMARU, Y., HIROI, E., HIURA, K., KAMEDA, A., YANG, N. N., HAKEDA, Y. & KUMEGAWA, M. 1997. Estrogen inhibits bone resorption by directly inducing apoptosis of the bone-resorbing osteoclasts. *J Exp Med,* 186**,** 489-95.

KAMMERER, C. M., SCHNEIDER, J. L., COLE, S. A., HIXSON, J. E., SAMOLLOW, P. B., O'CONNELL, J. R., PEREZ, R., DYER, T. D., ALMASY, L., BLANGERO, J., BAUER, R. L. & MITCHELL, B. D. 2003. Quantitative trait loci on chromosomes 2p, 4p, and 13q influence bone mineral density of the forearm and hip in Mexican Americans. *J Bone Miner Res,* 18**,** 2245-52.

KANAME, T., YANAGI, K., OKAMOTO, N. & NARITOMI, K. 2006. Neurobehavioral disorders in patients with Aarskog-Scott syndrome affected by novel FGD1 mutations. *Am J Med Genet A,* 140**,** 1331-2.

KANEHISA, J. & HEERSCHE, J. N. 1988. Osteoclastic bone resorption: in vitro analysis of the rate of resorption and migration of individual osteoclasts. *Bone,* 9**,** 73-9.

KANIS, J. A. 1996. Maintenance of bone mass. *In:* KANIS, J. A. (ed.) *Textbook of Osteoporosis.* London: Blackwell Science.

KANIS, J. A., JOHANSSON, H., JOHNELL, O., ODEN, A., DE LAET, C., EISMAN, J. A., POLS, H. & TENENHOUSE, A. 2005. Alcohol intake as a risk factor for fracture. *Osteoporos Int,* 16**,** 737-42.

KANIS, J. A., JOHNELL, O., ODEN, A., JOHANSSON, H., DE LAET, C., EISMAN, J. A., FUJIWARA, S., KROGER, H., MCCLOSKEY, E. V., MELLSTROM, D., MELTON, L. J., POLS, H., REEVE, J., SILMAN, A. & TENENHOUSE, A. 2005. Smoking and fracture risk: a meta-analysis. *Osteoporos Int,* 16**,** 155-62.

KANIS, J. A., MELTON, L. J., 3RD, CHRISTIANSEN, C., JOHNSTON, C. C. & KHALTAEV, N. 1994. The diagnosis of osteoporosis. *J Bone Miner Res,* 9**,** 1137-41.

KANIS, J. A., ODEN, A., JOHNELL, O., JOHANSSON, H., DE LAET, C., BROWN, J., BURCKHARDT, P., COOPER, C., CHRISTIANSEN, C., CUMMINGS, S., EISMAN, J. A., FUJIWARA, S., GLUER, C., GOLTZMAN, D., HANS, D., KRIEG, M. A., LA CROIX, A., MCCLOSKEY, E., MELLSTROM, D., MELTON, L. J., 3RD, POLS, H., REEVE, J., SANDERS, K., SCHOTT, A. M., SILMAN, A., TORGERSON, D., VAN STAA, T., WATTS, N. B. & YOSHIMURA, N. 2007. The use of clinical risk factors enhances the performance of BMD in the prediction of hip and osteoporotic fractures in men and women. *Osteoporos Int,* 18**,** 1033-46.

KARASIK, D., CUPPLES, L. A., HANNAN, M. T. & KIEL, D. P. 2003. Age, gender, and body mass effects on quantitative trait loci for bone mineral density: the Framingham Study. *Bone,* 33**,** 308-16.

KARASIK, D., DUPUIS, J., CHO, K., CUPPLES, L. A., ZHOU, Y., KIEL, D. P. & DEMISSIE, S. 2010. Refined QTLs of osteoporosis-related traits by linkage analysis with genome-wide SNPs: Framingham SHARe. *Bone,* 46**,** 1114-21.

KAWAGUCHI, H., MANABE, N., MIYAURA, C., CHIKUDA, H., NAKAMURA, K. & KURO-O, M. 1999. Independent impairment of osteoblast and osteoclast differentiation in klotho mouse exhibiting low-turnover osteopenia. *J Clin Invest,* 104**,** 229-37.

KEEN, R. W., HART, D. J., ARDEN, N. K., DOYLE, D. V. & SPECTOR, T. D. 1999. Family history of appendicular fracture and risk of osteoporosis: a population-based study. *Osteoporos Int,* 10**,** 161-6.

KIEL, D. P., DEMISSIE, S., DUPUIS, J., LUNETTA, K. L., MURABITO, J. M. & KARASIK, D. 2007. Genome-wide association with bone mass and geometry in the Framingham Heart Study. *BMC Med Genet,* 8 Suppl 1**,** S14.

KIM, I. S., OTTO, F., ZABEL, B. & MUNDLOS, S. 1999. Regulation of chondrocyte differentiation by Cbfa1. *Mech Dev,* 80**,** 159-70.

KIM, N., KADONO, Y., TAKAMI, M., LEE, J., LEE, S. H., OKADA, F., KIM, J. H., KOBAYASHI, T., ODGREN, P. R., NAKANO, H., YEH, W. C., LEE, S. K., LORENZO, J. A. & CHOI, Y. 2005. Osteoclast differentiation independent of the TRANCE-RANK-TRAF6 axis. *J Exp Med,* 202**,** 589-95.

KIM, N., TAKAMI, M., RHO, J., JOSIEN, R. & CHOI, Y. 2002. A novel member of the leukocyte receptor complex regulates osteoclast differentiation. *J Exp Med,* 195**,** 201-9.

KIMURA, K., ITO, M., AMANO, M., CHIHARA, K., FUKATA, Y., NAKAFUKU, M., YAMAMORI, B., FENG, J., NAKANO, T., OKAWA, K., IWAMATSU, A. & KAIBUCHI, K. 1996. Regulation of myosin phosphatase by Rho and Rho-associated kinase (Rho-kinase). *Science,* 273**,** 245-8.

KINDBLOM, J. M., OHLSSON, C., LJUNGGREN, O., KARLSSON, M. K., TIVESTEN, A., SMITH, U. & MELLSTROM, D. 2009. Plasma osteocalcin is inversely related to fat mass and plasma glucose in elderly Swedish men. *J Bone Miner Res,* 24**,** 785-91.

KIRKPATRICK, T. J., AU, K. S., MASTROBATTISTA, J. M., MCCREADY, M. E., BULMAN, D. E. & NORTHRUP, H. 2003. Identification of a mutation in the Indian Hedgehog (IHH) gene causing brachydactyly type A1 and evidence for a third locus. *J Med Genet,* 40**,** 42-4.

KISS, C., LI, J., SZELES, A., GIZATULLIN, R. Z., KASHUBA, V. I., LUSHNIKOVA, T., PROTOPOPOV, A. I., KELVE, M., KISS, H., KHOLODNYUK, I. D., IMREH, S., KLEIN, G. & ZABAROVSKY, E. R. 1997. Assignment of the ARHA and GPX1 genes to human chromosome bands 3p21.3 by in situ hybridization and with somatic cell hybrids. *Cytogenet Cell Genet,* 79**,** 228-30.

KITAURA, H., ZHOU, P., KIM, H. J., NOVACK, D. V., ROSS, F. P. & TEITELBAUM, S. L. 2005. M-CSF mediates TNF-induced inflammatory osteolysis. *J Clin Invest,* 115**,** 3418-27.

KLEIN-NULEND, J., NIJWEIDE, P. J. & BURGER, E. H. 2003. Osteocyte and bone structure. *Curr Osteoporos Rep,* 1**,** 5-10.

KLEIN, R. F., MITCHELL, S. R., PHILLIPS, T. J., BELKNAP, J. K. & ORWOLL, E. S. 1998. Quantitative trait loci affecting peak bone mineral density in mice. *J Bone Miner Res,* 13**,** 1648-56.

KNOTHE TATE, M. L. 2003. "Whither flows the fluid in bone?" An osteocyte's perspective. *J Biomech,* 36**,** 1409-24.

KNOTHE TATE, M. L., ADAMSON, J. R., TAMI, A. E. & BAUER, T. W. 2004. The osteocyte. *Int J Biochem Cell Biol,* 36**,** 1-8.

KOBAYASHI, S., INOUE, S., HOSOI, T., OUCHI, Y., SHIRAKI, M. & ORIMO, H. 1996. Association of bone mineral density with polymorphism of the estrogen receptor gene. *J Bone Miner Res,* 11**,** 306-11.

KOBAYASHI, Y., UDAGAWA, N. & TAKAHASHI, N. 2009. Action of RANKL and OPG for osteoclastogenesis. *Crit Rev Eukaryot Gene Expr,* 19**,** 61-72.

KOH, J. M., PARK, B. L., KIM, D. J., KIM, G. S., CHEONG, H. S., KIM, T. H., HONG, J. M., SHIN, H. I., PARK, E. K., KIM, S. Y. & SHIN, H. D. 2007. Identification of novel RANK polymorphisms and their putative association with low BMD among postmenopausal women. *Osteoporos Int,* 18**,** 323-31.

KOMORI, T. 2002. Runx2, a multifunctional transcription factor in skeletal development. *J Cell Biochem,* 87**,** 1-8.

KOMORI, T. 2008. Regulation of bone development and maintenance by Runx2. *Front Biosci,* 13**,** 898-903.

KOMORI, T., YAGI, H., NOMURA, S., YAMAGUCHI, A., SASAKI, K., DEGUCHI, K., SHIMIZU, Y., BRONSON, R. T., GAO, Y. H., INADA, M., SATO, M., OKAMOTO, R., KITAMURA, Y., YOSHIKI, S. & KISHIMOTO, T. 1997. Targeted disruption of Cbfa1 results in a complete lack of bone formation owing to maturational arrest of osteoblasts. *Cell,* 89**,** 755-64.

KONG, Y. Y., FEIGE, U., SAROSI, I., BOLON, B., TAFURI, A., MORONY, S., CAPPARELLI, C., LI, J., ELLIOTT, R., MCCABE, S., WONG, T., CAMPAGNUOLO, G., MORAN, E., BOGOCH, E. R., VAN, G., NGUYEN, L. T., OHASHI, P. S., LACEY, D. L., FISH, E., BOYLE, W. J. & PENNINGER, J. M. 1999. Activated T cells regulate bone loss and joint destruction in adjuvant arthritis through osteoprotegerin ligand. *Nature,* 402**,** 304-9.

KONG, Y. Y., YOSHIDA, H., SAROSI, I., TAN, H. L., TIMMS, E., CAPPARELLI, C., MORONY, S., OLIVEIRA-DOS-SANTOS, A. J., VAN, G., ITIE, A., KHOO, W., WAKEHAM, A., DUNSTAN, C. R., LACEY, D. L., MAK, T. W., BOYLE, W. J. & PENNINGER, J. M. 1999. OPGL is a key regulator of osteoclastogenesis, lymphocyte development and lymph-node organogenesis. *Nature,* 397**,** 315-23.

KOTAKE, S., NANKE, Y., KAWAMOTO, M., YAGO, T., UDAGAWA, N., ICHIKAWA, N., KOBASHIGAWA, T., SAITO, S., MOMOHARA, S., KAMATANI, N. & YAMANAKA, H. 2009. T-cell leukemia translocation-associated gene (TCTA) protein is required for human osteoclastogenesis. *Bone,* 45**,** 627-39.

KOZLOWSKI, K., SILLENCE, D., CORTIS-JONES, R. & OSBORN, R. 1985. Boomerang dysplasia. *Br J Radiol,* 58**,** 369-71.

KRAKOW, D., ROBERTSON, S. P., KING, L. M., MORGAN, T., SEBALD, E. T., BERTOLOTTO, C., WACHSMANN-HOGIU, S., ACUNA, D., SHAPIRO, S. S., TAKAFUTA, T., AFTIMOS, S., KIM, C. A., FIRTH, H., STEINER, C. E., CORMIER-DAIRE, V., SUPERTI-FURGA, A., BONAFE, L., GRAHAM, J. M., JR., GRIX, A., BACINO, C. A., ALLANSON, J., BIALER, M. G., LACHMAN, R. S., RIMOIN, D. L. & COHN, D. H. 2004. Mutations in the gene encoding filamin B disrupt vertebral segmentation, joint formation and skeletogenesis. *Nat Genet,* 36**,** 405-10.

KRALL, E. A. & DAWSON-HUGHES, B. 1993. Heritable and life-style determinants of bone mineral density. *J Bone Miner Res,* 8**,** 1-9.

KRALL, E. A., PARRY, P., LICHTER, J. B. & DAWSON-HUGHES, B. 1995. Vitamin D receptor alleles and rates of bone loss: influences of years since menopause and calcium intake. *J Bone Miner Res,* 10**,** 978-84.

KURO-O, M., MATSUMURA, Y., AIZAWA, H., KAWAGUCHI, H., SUGA, T., UTSUGI, T., OHYAMA, Y., KURABAYASHI, M., KANAME, T., KUME, E., IWASAKI, H., IIDA, A., SHIRAKI-IIDA, T., NISHIKAWA, S., NAGAI,

R. & NABESHIMA, Y. I. 1997. Mutation of the mouse klotho gene leads to a syndrome resembling ageing. *Nature,* 390**,** 45-51.

KUWABARA, K., MATSUMOTO, M., IKEDA, J., HORI, O., OGAWA, S., MAEDA, Y., KITAGAWA, K., IMUTA, N., KINOSHITA, T., STERN, D. M., YANAGI, H. & KAMADA, T. 1996. Purification and characterization of a novel stress protein, the 150-kDa oxygen-regulated protein (ORP150), from cultured rat astrocytes and its expression in ischemic mouse brain. *J Biol Chem,* 271**,** 5025-32.

LACEY, D. L., TIMMS, E., TAN, H. L., KELLEY, M. J., DUNSTAN, C. R., BURGESS, T., ELLIOTT, R., COLOMBERO, A., ELLIOTT, G., SCULLY, S., HSU, H., SULLIVAN, J., HAWKINS, N., DAVY, E., CAPPARELLI, C., ELI, A., QIAN, Y. X., KAUFMAN, S., SAROSI, I., SHALHOUB, V., SENALDI, G., GUO, J., DELANEY, J. & BOYLE, W. J. 1998. Osteoprotegerin ligand is a cytokine that regulates osteoclast differentiation and activation. *Cell,* 93**,** 165-76.

LAIRD, N. M., HORVATH, S. & XU, X. 2000. Implementing a unified approach to family-based tests of association. *Genet Epidemiol,* 19 Suppl 1**,** S36-42.

LAKKAKORPI, P. T., HELFRICH, M. H., HORTON, M. A. & VAANANEN, H. K. 1993. Spatial organization of microfilaments and vitronectin receptor, alpha v beta 3, in osteoclasts. A study using confocal laser scanning microscopy. *J Cell Sci,* 104 (Pt 3)**,** 663-70.

LAKKAKORPI, P. T. & VAANANEN, H. K. 1991. Kinetics of the osteoclast cytoskeleton during the resorption cycle in vitro. *J Bone Miner Res,* 6**,** 817-26.

LAKKAKORPI, P. T. & VAANANEN, H. K. 1996. Cytoskeletal changes in osteoclasts during the resorption cycle. *Microsc Res Tech,* 33**,** 171-81.

LALOR, B. C., FRANCE, M. W., POWELL, D., ADAMS, P. H. & COUNIHAN, T. B. 1986. Bone and mineral metabolism and chronic alcohol abuse. *Q J Med,* 59**,** 497-511.

LAM, J., TAKESHITA, S., BARKER, J. E., KANAGAWA, O., ROSS, F. P. & TEITELBAUM, S. L. 2000. TNF-alpha induces osteoclastogenesis by direct stimulation of macrophages exposed to permissive levels of RANK ligand. *J Clin Invest,* 106**,** 1481-8.

LAMBERT, H. L., EASTELL, R., KARNIK, K., RUSSELL, J. M. & BARKER, M. E. 2008. Calcium supplementation and bone mineral accretion in adolescent girls: an 18-mo randomized controlled trial with 2-y follow-up. *Am J Clin Nutr,* 87**,** 455-62.

LANGER, L. O., JR., GORLIN, R. J., DONNAI, D., HAMEL, B. C. & CLERICUZIO, C. 1994. Spondylocarpotarsal synostosis syndrome (with or without unilateral unsegmented bar). *Am J Med Genet,* 51**,** 1-8.

LAPPALAINEN, P. & DRUBIN, D. G. 1997. Cofilin promotes rapid actin filament turnover in vivo. *Nature,* 388**,** 78-82.

LARSEN, L. J., SCHOTTSTAEDT, E. R. & BOST, F. C. 1950. Multiple congenital dislocations associated with characteristic facial abnormality. *J Pediatr,* 37**,** 574-81.

LEAN, J. M., JAGGER, C. J., CHAMBERS, T. J. & CHOW, J. W. 1995. Increased insulin-like growth factor I mRNA expression in rat osteocytes in response to mechanical stimulation. *Am J Physiol,* 268**,** E318-27.

LEAN, J. M., JAGGER, C. J., KIRSTEIN, B., FULLER, K. & CHAMBERS, T. J. 2005. Hydrogen peroxide is essential for estrogen-deficiency bone loss and osteoclast formation. *Endocrinology,* 146**,** 728-35.

LEE, B., THIRUNAVUKKARASU, K., ZHOU, L., PASTORE, L., BALDINI, A., HECHT, J., GEOFFROY, V., DUCY, P. & KARSENTY, G. 1997. Missense mutations abolishing DNA binding of the osteoblast-specific transcription factor OSF2/CBFA1 in cleidocranial dysplasia. *Nat Genet,* 16**,** 307-10.

LEE, N. K., SOWA, H., HINOI, E., FERRON, M., AHN, J. D., CONFAVREUX, C., DACQUIN, R., MEE, P. J., MCKEE, M. D., JUNG, D. Y., ZHANG, Z., KIM, J. K., MAUVAIS-JARVIS, F., DUCY, P. & KARSENTY, G. 2007. Endocrine regulation of energy metabolism by the skeleton. *Cell,* 130**,** 456-69.

LEE, P. H. & SHATKAY, H. 2008. F-SNP: computationally predicted functional SNPs for disease association studies. *Nucleic Acids Res,* 36**,** D820-4.

LEE, S. K. & LORENZO, J. A. 1999. Parathyroid hormone stimulates TRANCE and inhibits osteoprotegerin messenger ribonucleic acid expression in murine bone marrow cultures: correlation with osteoclast-like cell formation. *Endocrinology,* 140**,** 3552-61.

LEE, Y. H., RHO, Y. H., CHOI, S. J., JI, J. D. & SONG, G. G. 2006. Meta-analysis of genome-wide linkage studies for bone mineral density. *J Hum Genet,* 51**,** 480-6.

LEE, Z. H. & KIM, H. H. 2003. Signal transduction by receptor activator of nuclear factor kappa B in osteoclasts. *Biochem Biophys Res Commun,* 305**,** 211-4.

LEI, S. F., CHEN, Y., XIONG, D. H., LI, L. M. & DENG, H. W. 2006. Ethnic difference in osteoporosis-related phenotypes and its potential underlying genetic determination. *J Musculoskelet Neuronal Interact,* 6**,** 36-46.

LEI, S. F., DENG, F. Y., DVORNYK, V., LIU, M. Y., XIAO, S. M., JIANG, D. K. & DENG, H. W. 2005. The (GT)n polymorphism and haplotype of the COL1A2 gene, but not the (AAAG)n polymorphism of the PTHR1 gene, are associated with bone mineral density in Chinese. *Hum Genet,* 116**,** 200-7.

LEI, X. G., CHENG, W. H. & MCCLUNG, J. P. 2007. Metabolic regulation and function of glutathione peroxidase-1. *Annu Rev Nutr,* 27**,** 41-61.

LEUNG, R., WANG, Y., CUDDY, K., SUN, C., MAGALHAES, J., GRYNPAS, M. & GLOGAUER, M. 2010. Filamin A regulates monocyte migration through Rho small GTPases during osteoclastogenesis. *J Bone Miner Res,* 25**,** 1077-91.

LI, G. H., KUNG, A. W. & HUANG, Q. Y. 2010. Common variants in FLNB/CRTAP, not ARHGEF3 at 3p, are associated with osteoporosis in southern Chinese women. *Osteoporos Int,* 21**,** 1009-20.

LI, J., SAROSI, I., YAN, X. Q., MORONY, S., CAPPARELLI, C., TAN, H. L., MCCABE, S., ELLIOTT, R., SCULLY, S., VAN, G., KAUFMAN, S., JUAN, S. C., SUN, Y., TARPLEY, J., MARTIN, L., CHRISTENSEN, K., MCCABE, J., KOSTENUIK, P., HSU, H., FLETCHER, F., DUNSTAN, C. R., LACEY, D. L. & BOYLE, W. J. 2000. RANK is the intrinsic hematopoietic cell surface receptor that controls osteoclastogenesis and regulation of bone mass and calcium metabolism. *Proc Natl Acad Sci U S A,* 97**,** 1566-71.

LIAN, J. B., STEIN, G. S., CANALIS, E., ROBEY, P. G. & BOSKEY, A. L. 1999. Bone Formation: Osteoblast Lineage Cells, Growth Factors, Matrix Proteins, and the Mineralization Process. *In:* FAVUS, M. J. (ed.) *Primer on the*

Metabolic Bone Diseases and Disorders of Mineral Metabolism. 4th ed. Philidelphia: Lippincott Williams & Wilkins.

LIDEN, M., WILEN, B., LJUNGHALL, S. & MELHUS, H. 1998. Polymorphism at the Sp 1 binding site in the collagen type I alpha 1 gene does not predict bone mineral density in postmenopausal women in sweden. *Calcif Tissue Int,* 63**,** 293-5.

LIM, S. K., PARK, Y. S., PARK, J. M., SONG, Y. D., LEE, E. J., KIM, K. R., LEE, H. C. & HUH, K. B. 1995. Lack of association between vitamin D receptor genotypes and osteoporosis in Koreans. *J Clin Endocrinol Metab,* 80**,** 3677-81.

LINDSAY, R., HART, D. M., AITKEN, J. M., MACDONALD, E. B., ANDERSON, J. B. & CLARKE, A. C. 1976. Long-term prevention of postmenopausal osteoporosis by oestrogen. Evidence for an increased bone mass after delayed onset of oestrogen treatment. *Lancet,* 1**,** 1038-41.

LISIGNOLI, G., TONEGUZZI, S., GRASSI, F., PIACENTINI, A., TSCHON, M., CRISTINO, S., GUALTIERI, G. & FACCHINI, A. 2002. Different chemokines are expressed in human arthritic bone biopsies: IFN-gamma and IL-6 differently modulate IL-8, MCP-1 and rantes production by arthritic osteoblasts. *Cytokine,* 20**,** 231-8.

LITTLE, R. D., CARULLI, J. P., DEL MASTRO, R. G., DUPUIS, J., OSBORNE, M., FOLZ, C., MANNING, S. P., SWAIN, P. M., ZHAO, S. C., EUSTACE, B., LAPPE, M. M., SPITZER, L., ZWEIER, S., BRAUNSCHWEIGER, K., BENCHEKROUN, Y., HU, X., ADAIR, R., CHEE, L., FITZGERALD, M. G., TULIG, C., CARUSO, A., TZELLAS, N., BAWA, A., FRANKLIN, B., MCGUIRE, S., NOGUES, X., GONG, G., ALLEN, K. M., ANISOWICZ, A., MORALES, A. J., LOMEDICO, P. T., RECKER, S. M., VAN EERDEWEGH, P., RECKER, R. R. & JOHNSON, M. L. 2002. A mutation in the LDL receptor-related protein 5 gene results in the autosomal dominant high-bone-mass trait. *Am J Hum Genet,* 70**,** 11-9.

LIU, M., WANG, X., CAI, Z., TANG, Z., CAO, K., LIANG, B., REN, X., LIU, J. Y. & WANG, Q. K. 2006. A novel heterozygous mutation in the Indian hedgehog gene (IHH) is associated with brachydactyly type A1 in a Chinese family. *J Hum Genet,* 51**,** 727-31.

LIU, Z., QIU, L., CHEN, Y. M. & SU, Y. X. 2010. Effect of milk and calcium supplementation on bone density and bone turnover in pregnant Chinese women: a randomized controlled trail. *Arch Gynecol Obstet.*

LOCKER, F. G. 1996. Hormonal regulation of calcium homeostasis. *Nurs Clin North Am,* 31**,** 797-803.

LOMAGA, M. A., YEH, W. C., SAROSI, I., DUNCAN, G. S., FURLONGER, C., HO, A., MORONY, S., CAPPARELLI, C., VAN, G., KAUFMAN, S., VAN DER HEIDEN, A., ITIE, A., WAKEHAM, A., KHOO, W., SASAKI, T., CAO, Z., PENNINGER, J. M., PAIGE, C. J., LACEY, D. L., DUNSTAN, C. R., BOYLE, W. J., GOEDDEL, D. V. & MAK, T. W. 1999. TRAF6 deficiency results in osteopetrosis and defective interleukin-1, CD40, and LPS signaling. *Genes Dev,* 13**,** 1015-24.

LONG, F., CHUNG, U. I., OHBA, S., MCMAHON, J., KRONENBERG, H. M. & MCMAHON, A. P. 2004. Ihh signaling is directly required for the osteoblast lineage in the endochondral skeleton. *Development,* 131**,** 1309-18.

LORENZ-DEPIEREUX, B., BASTEPE, M., BENET-PAGES, A., AMYERE, M., WAGENSTALLER, J., MULLER-BARTH, U., BADENHOOP, K.,

KAISER, S. M., RITTMASTER, R. S., SHLOSSBERG, A. H., OLIVARES, J. L., LORIS, C., RAMOS, F. J., GLORIEUX, F., VIKKULA, M., JUPPNER, H. & STROM, T. M. 2006. DMP1 mutations in autosomal recessive hypophosphatemia implicate a bone matrix protein in the regulation of phosphate homeostasis. *Nat Genet,* 38**,** 1248-50.

LOWIK, C. W., VAN DER PLUIJM, G., BLOYS, H., HOEKMAN, K., BIJVOET, O. L., AARDEN, L. A. & PAPAPOULOS, S. E. 1989. Parathyroid hormone (PTH) and PTH-like protein (PLP) stimulate interleukin-6 production by osteogenic cells: a possible role of interleukin-6 in osteoclastogenesis. *Biochem Biophys Res Commun,* 162**,** 1546-52.

LOWRY, B., MILLER, J. R. & FRASER, F. C. 1971. A new dominant gene mental retardation syndrome. Association with small stature, tapering fingers, characteristic facies, and possible hydrocephalus. *Am J Dis Child,* 121**,** 496-500.

LOZUPONE, E., PALUMBO, C., FAVIA, A., FERRETTI, M., PALAZZINI, S. & CANTATORE, F. P. 1996. Intermittent compressive load stimulates osteogenesis and improves osteocyte viability in bones cultured "in vitro". *Clin Rheumatol,* 15**,** 563-72.

LUXENBURG, C., GEBLINGER, D., KLEIN, E., ANDERSON, K., HANEIN, D., GEIGER, B. & ADDADI, L. 2007. The architecture of the adhesive apparatus of cultured osteoclasts: from podosome formation to sealing zone assembly. *PLoS One,* 2**,** e179.

MACDONALD, B. T., TAMAI, K. & HE, X. 2009. Wnt/beta-catenin signaling: components, mechanisms, and diseases. *Dev Cell,* 17**,** 9-26.

MACDONALD, H. M., MCGUIGAN, F. E., STEWART, A., BLACK, A. J., FRASER, W. D., RALSTON, S. & REID, D. M. 2006. Large-scale population-based study shows no evidence of association between common polymorphism of the VDR gene and BMD in British women. *J Bone Miner Res,* 21**,** 151-62.

MACHUCA-TZILI, L., MONROY-JARAMILLO, N., GONZALEZ-DEL ANGEL, A. & KOFMAN-ALFARO, S. 2002. New mutations in the CBFA1 gene in two Mexican patients with cleidocranial dysplasia. *Clin Genet,* 61**,** 349-53.

MACINTYRE, G., ALFORD, T., XIONG, L., ROULEAU, G. A., TIBBO, P. G. & COX, D. W. 2010. Association of NPAS3 exonic variation with schizophrenia. *Schizophr Res*.

MACINTYRE, I., ZAIDI, M., ALAM, A. S., DATTA, H. K., MOONGA, B. S., LIDBURY, P. S., HECKER, M. & VANE, J. R. 1991. Osteoclastic inhibition: an action of nitric oxide not mediated by cyclic GMP. *Proc Natl Acad Sci U S A,* 88**,** 2936-40.

MACK, C. P., SOMLYO, A. V., HAUTMANN, M., SOMLYO, A. P. & OWENS, G. K. 2001. Smooth muscle differentiation marker gene expression is regulated by RhoA-mediated actin polymerization. *J Biol Chem,* 276**,** 341-7.

MADAULE, P. & AXEL, R. 1985. A novel ras-related gene family. *Cell,* 41**,** 31-40.

MADDALOZZO, G. F., WIDRICK, J. J., CARDINAL, B. J., WINTERS-STONE, K. M., HOFFMAN, M. A. & SNOW, C. M. 2007. The effects of hormone replacement therapy and resistance training on spine bone mineral density in early postmenopausal women. *Bone,* 40**,** 1244-51.

MAEDA, Y., NAKAMURA, E., NGUYEN, M. T., SUVA, L. J., SWAIN, F. L., RAZZAQUE, M. S., MACKEM, S. & LANSKE, B. 2007. Indian Hedgehog

produced by postnatal chondrocytes is essential for maintaining a growth plate and trabecular bone. *Proc Natl Acad Sci U S A,* 104**,** 6382-7.

MAEKAWA, M., ISHIZAKI, T., BOKU, S., WATANABE, N., FUJITA, A., IWAMATSU, A., OBINATA, T., OHASHI, K., MIZUNO, K. & NARUMIYA, S. 1999. Signaling from Rho to the actin cytoskeleton through protein kinases ROCK and LIM-kinase. *Science,* 285**,** 895-8.

MAGI, R., PFEUFER, A., NELIS, M., MONTPETIT, A., METSPALU, A. & REMM, M. 2007. Evaluating the performance of commercial whole-genome marker sets for capturing common genetic variation. *BMC Genomics,* 8**,** 159.

MAHR, S., BURMESTER, G. R., HILKE, D., GOBEL, U., GRUTZKAU, A., HAUPL, T., HAUSCHILD, M., KOCZAN, D., KRENN, V., NEIDEL, J., PERKA, C., RADBRUCH, A., THIESEN, H. J. & MULLER, B. 2006. Cis- and trans-acting gene regulation is associated with osteoarthritis. *Am J Hum Genet,* 78**,** 793-803.

MALLUCHE, H. H., HENRY, H., MEYER-SABELLAK, W., SHERMAN, D., MASSRY, S. G. & NORMAN, A. W. 1980. Effects and interactions of 24R,25(OH)2D3 and 1,25(OH)2D3 on bone. *Am J Physiol,* 238**,** E494-8.

MANNSTADT, M., JUPPNER, H. & GARDELLA, T. J. 1999. Receptors for PTH and PTHrP: their biological importance and functional properties. *Am J Physiol,* 277**,** F665-75.

MANOLAGAS, S. C. 2000. Birth and death of bone cells: basic regulatory mechanisms and implications for the pathogenesis and treatment of osteoporosis. *Endocr Rev,* 21**,** 115-37.

MANOLAGAS, S. C. & JILKA, R. L. 1992. Cytokines, hematopoiesis, osteoclastogenesis, and estrogens. *Calcif Tissue Int,* 50**,** 199-202.

MARCHISIO, P. C., CIRILLO, D., NALDINI, L., PRIMAVERA, M. V., TETI, A. & ZAMBONIN-ZALLONE, A. 1984. Cell-substratum interaction of cultured avian osteoclasts is mediated by specific adhesion structures. *J Cell Biol,* 99**,** 1696-705.

MARIE, P. J. 2008. Transcription factors controlling osteoblastogenesis. *Arch Biochem Biophys,* 473**,** 98-105.

MARKS, S. C., JR. & LANE, P. W. 1976. Osteopetrosis, a new recessive skeletal mutation on chromosome 12 of the mouse. *J Hered,* 67**,** 11-18.

MAROTEAUX, P., SPRANGER, J., STANESCU, V., LE MAREC, B., PFEIFFER, R. A., BEIGHTON, P. & MATTEI, J. F. 1982. Atelosteogenesis. *Am J Med Genet,* 13**,** 15-25.

MARSHALL, D., JOHNELL, O. & WEDEL, H. 1996. Meta-analysis of how well measures of bone mineral density predict occurrence of osteoporotic fractures. *Bmj,* 312**,** 1254-9.

MARTIGNETTI, J. A., AQEEL, A. A., SEWAIRI, W. A., BOUMAH, C. E., KAMBOURIS, M., MAYOUF, S. A., SHETH, K. V., EID, W. A., DOWLING, O., HARRIS, J., GLUCKSMAN, M. J., BAHABRI, S., MEYER, B. F. & DESNICK, R. J. 2001. Mutation of the matrix metalloproteinase 2 gene (MMP2) causes a multicentric osteolysis and arthritis syndrome. *Nat Genet,* 28**,** 261-5.

MARTYN-ST JAMES, M. & CARROLL, S. 2006. High-intensity resistance training and postmenopausal bone loss: a meta-analysis. *Osteoporos Int,* 17**,** 1225-40.

MCBEATH, R., PIRONE, D. M., NELSON, C. M., BHADRIRAJU, K. & CHEN, C. S. 2004. Cell shape, cytoskeletal tension, and RhoA regulate stem cell lineage commitment. *Dev Cell,* 6**,** 483-95.

MCCREADY, M. E., SWEENEY, E., FRYER, A. E., DONNAI, D., BAIG, A., RACACHO, L., WARMAN, M. L., HUNTER, A. G. & BULMAN, D. E. 2002. A novel mutation in the IHH gene causes brachydactyly type A1: a 95-year-old mystery resolved. *Hum Genet,* 111**,** 368-75.

MCHUGH, K. P., HODIVALA-DILKE, K., ZHENG, M. H., NAMBA, N., LAM, J., NOVACK, D., FENG, X., ROSS, F. P., HYNES, R. O. & TEITELBAUM, S. L. 2000. Mice lacking beta3 integrins are osteosclerotic because of dysfunctional osteoclasts. *J Clin Invest,* 105**,** 433-40.

MCSHEEHY, P. M. & CHAMBERS, T. J. 1986. Osteoblastic cells mediate osteoclastic responsiveness to parathyroid hormone. *Endocrinology,* 118**,** 824-8.

MEIKLE, M. C., BORD, S., HEMBRY, R. M., COMPSTON, J., CROUCHER, P. I. & REYNOLDS, J. J. 1992. Human osteoblasts in culture synthesize collagenase and other matrix metalloproteinases in response to osteotropic hormones and cytokines. *J Cell Sci,* 103 (Pt 4)**,** 1093-9.

MELTON, L. J., 3RD 1995. How many women have osteoporosis now? *J Bone Miner Res,* 10**,** 175-7.

MERCURI, E., MESSINA, S., BRUNO, C., MORA, M., PEGORARO, E., COMI, G. P., D'AMICO, A., AIELLO, C., BIANCHERI, R., BERARDINELLI, A., BOFFI, P., CASSANDRINI, D., LAVERDA, A., MOGGIO, M., MORANDI, L., MORONI, I., PANE, M., PEZZANI, R., PICHIECCHIO, A., PINI, A., MINETTI, C., MONGINI, T., MOTTARELLI, E., RICCI, E., RUGGIERI, A., SAREDI, S., SCUDERI, C., TESSA, A., TOSCANO, A., TORTORELLA, G., TREVISAN, C. P., UGGETTI, C., VASCO, G., SANTORELLI, F. M. & BERTINI, E. 2009. Congenital muscular dystrophies with defective glycosylation of dystroglycan: a population study. *Neurology,* 72**,** 1802-9.

MESSEGUER, X., ESCUDERO, R., FARRE, D., NUNEZ, O., MARTINEZ, J. & ALBA, M. M. 2002. PROMO: detection of known transcription regulatory elements using species-tailored searches. *Bioinformatics,* 18**,** 333-4.

MEYER, B. H., FREULER, F., GUERINI, D. & SIEHLER, S. 2008. Reversible translocation of p115-RhoGEF by G(12/13)-coupled receptors. *J Cell Biochem,* 104**,** 1660-70.

MEYERS, V. E., ZAYZAFOON, M., DOUGLAS, J. T. & MCDONALD, J. M. 2005. RhoA and cytoskeletal disruption mediate reduced osteoblastogenesis and enhanced adipogenesis of human mesenchymal stem cells in modeled microgravity. *J Bone Miner Res,* 20**,** 1858-66.

MICHAELSSON, K., MELHUS, H., FERM, H., AHLBOM, A. & PEDERSEN, N. L. 2005. Genetic liability to fractures in the elderly. *Arch Intern Med,* 165**,** 1825-30.

MIGNONE, F., GISSI, C., LIUNI, S. & PESOLE, G. 2002. Untranslated regions of mRNAs. *Genome Biol,* 3**,** REVIEWS0004.

MITCHELL, P. J. & TJIAN, R. 1989. Transcriptional regulation in mammalian cells by sequence-specific DNA binding proteins. *Science,* 245**,** 371-8.

MITTER, D., KRAKOW, D., FARRINGTON-ROCK, C. & MEINECKE, P. 2008. Expanded clinical spectrum of spondylocarpotarsal synostosis syndrome and possible manifestation in a heterozygous father. *Am J Med Genet A,* 146A**,** 779-83.

MIYAUCHI, A., ALVAREZ, J., GREENFIELD, E. M., TETI, A., GRANO, M., COLUCCI, S., ZAMBONIN-ZALLONE, A., ROSS, F. P., TEITELBAUM,

S. L., CHERESH, D. & ET AL. 1991. Recognition of osteopontin and related peptides by an alpha v beta 3 integrin stimulates immediate cell signals in osteoclasts. *J Biol Chem,* 266**,** 20369-74.

MIZUGUCHI, T., FURUTA, I., WATANABE, Y., TSUKAMOTO, K., TOMITA, H., TSUJIHATA, M., OHTA, T., KISHINO, T., MATSUMOTO, N., MINAKAMI, H., NIIKAWA, N. & YOSHIURA, K. 2004. LRP5, low-density-lipoprotein-receptor-related protein 5, is a determinant for bone mineral density. *J Hum Genet,* 49**,** 80-6. Epub 2004 Jan 15.

MIZUNO, A., AMIZUKA, N., IRIE, K., MURAKAMI, A., FUJISE, N., KANNO, T., SATO, Y., NAKAGAWA, N., YASUDA, H., MOCHIZUKI, S., GOMIBUCHI, T., YANO, K., SHIMA, N., WASHIDA, N., TSUDA, E., MORINAGA, T., HIGASHIO, K. & OZAWA, H. 1998. Severe osteoporosis in mice lacking osteoclastogenesis inhibitory factor/osteoprotegerin. *Biochem Biophys Res Commun,* 247**,** 610-5.

MLAKAR, S. J., OSREDKAR, J., PREZELJ, J. & MARC, J. 2010. The antioxidant enzyme GPX1 gene polymorphisms are associated with low BMD and increased bone turnover markers. *Dis Markers,* 29**,** 71-80.

MOCHIZUKI, H., HAKEDA, Y., WAKATSUKI, N., USUI, N., AKASHI, S., SATO, T., TANAKA, K. & KUMEGAWA, M. 1992. Insulin-like growth factor-I supports formation and activation of osteoclasts. *Endocrinology,* 131**,** 1075-80.

MOCK, B., BLACKWELL, J., HILGERS, J., POTTER, M. & NACY, C. 1993. Genetic control of Leishmania major infection in congenic, recombinant inbred and F2 populations of mice. *Eur J Immunogenet,* 20**,** 335-48.

MOESTER, M. J., PAPAPOULOS, S. E., LOWIK, C. W. & VAN BEZOOIJEN, R. L. 2010. Sclerostin: Current Knowledge and Future Perspectives. *Calcif Tissue Int*.

MOFFETT, S. P., ZMUDA, J. M., CAULEY, J. A., STONE, K. L., NEVITT, M. C., ENSRUD, K. E., HILLIER, T. A., HOCHBERG, M. C., JOSLYN, G., MORIN, P. & CUMMINGS, S. R. 2004. Association of the G-174C variant in the interleukin-6 promoter region with bone loss and fracture risk in older women. *J Bone Miner Res,* 19**,** 1612-8.

MOON, S. Y. & ZHENG, Y. 2003. Rho GTPase-activating proteins in cell regulation. *Trends Cell Biol,* 13**,** 13-22.

MORAVA, E., KARTESZI, J., WEISENBACH, J., CALIEBE, A., MUNDLOS, S. & MEHES, K. 2002. Cleidocranial dysplasia with decreased bone density and biochemical findings of hypophosphatasia. *Eur J Pediatr,* 161**,** 619-22.

MORIN, S., TSANG, J. F. & LESLIE, W. D. 2009. Weight and body mass index predict bone mineral density and fractures in women aged 40 to 59 years. *Osteoporos Int,* 20**,** 363-70.

MORINAGA, T., NAKAGAWA, N., YASUDA, H., TSUDA, E. & HIGASHIO, K. 1998. Cloning and characterization of the gene encoding human osteoprotegerin/osteoclastogenesis-inhibitory factor. *Eur J Biochem,* 254**,** 685-91.

MORRIS, S. W., VALENTINE, M. B., SHAPIRO, D. N., SUBLETT, J. E., DEAVEN, L. L., FOUST, J. T., ROBERTS, W. M., CERRETTI, D. P. & LOOK, A. T. 1991. Reassignment of the human CSF1 gene to chromosome 1p13-p21. *Blood,* 78**,** 2013-20.

MORRISON, N. A., QI, J. C., TOKITA, A., KELLY, P. J., CROFTS, L., NGUYEN, T. V., SAMBROOK, P. N. & EISMAN, J. A. 1994. Prediction of bone density from vitamin D receptor alleles. *Nature,* 367**,** 284-7.

MORTIER, G. R., KRAMER, P. P., GIEDION, A. & BEEMER, F. A. 2003. Acrocapitofemoral dysplasia: an autosomal recessive skeletal dysplasia with cone shaped epiphyses in the hands and hips. *J Med Genet,* 40**,** 201-7.

MOSEKILDE, L., EBBESEN, E. N., TORNVIG, L. & THOMSEN, J. S. 2000. Trabecular bone structure and strength - remodelling and repair. *J Musculoskelet Neuronal Interact,* 1**,** 25-30.

MUHLRAD, D., DECKER, C. J. & PARKER, R. 1995. Turnover mechanisms of the stable yeast PGK1 mRNA. *Mol Cell Biol,* 15**,** 2145-56.

MULLENDER, M. G., VAN DER MEER, D. D., HUISKES, R. & LIPS, P. 1996. Osteocyte density changes in aging and osteoporosis. *Bone,* 18**,** 109-13.

MULLIGAN, M. K., PONOMAREV, I., HITZEMANN, R. J., BELKNAP, J. K., TABAKOFF, B., HARRIS, R. A., CRABBE, J. C., BLEDNOV, Y. A., GRAHAME, N. J., PHILLIPS, T. J., FINN, D. A., HOFFMAN, P. L., IYER, V. R., KOOB, G. F. & BERGESON, S. E. 2006. Toward understanding the genetics of alcohol drinking through transcriptome meta-analysis. *Proc Natl Acad Sci U S A,* 103**,** 6368-73.

MUNDLOS, S., OTTO, F., MUNDLOS, C., MULLIKEN, J. B., AYLSWORTH, A. S., ALBRIGHT, S., LINDHOUT, D., COLE, W. G., HENN, W., KNOLL, J. H., OWEN, M. J., MERTELSMANN, R., ZABEL, B. U. & OLSEN, B. R. 1997. Mutations involving the transcription factor CBFA1 cause cleidocranial dysplasia. *Cell,* 89**,** 773-9.

MUNDY, G. 1999. Bone Remodeling. *In:* FAVUS, M. J. (ed.) *Primer on the Metabolic Bone Diseases and Disorders of Mineral Metabolism.* 4th ed. Philadelphia: Lippincott Williams & Wilkins.

MURRAY, R. E., MCGUIGAN, F., GRANT, S. F., REID, D. M. & RALSTON, S. H. 1997. Polymorphisms of the interleukin-6 gene are associated with bone mineral density. *Bone,* 21**,** 89-92.

MYKHAYLYK, O., ZELPHATI, O., ROSENECKER, J. & PLANK, C. 2008. siRNA delivery by magnetofection. *Curr Opin Mol Ther,* 10**,** 493-505.

NAITO, A., AZUMA, S., TANAKA, S., MIYAZAKI, T., TAKAKI, S., TAKATSU, K., NAKAO, K., NAKAMURA, K., KATSUKI, M., YAMAMOTO, T. & INOUE, J. 1999. Severe osteopetrosis, defective interleukin-1 signalling and lymph node organogenesis in TRAF6-deficient mice. *Genes Cells,* 4**,** 353-62.

NAKAGAWA, N., KINOSAKI, M., YAMAGUCHI, K., SHIMA, N., YASUDA, H., YANO, K., MORINAGA, T. & HIGASHIO, K. 1998. RANK is the essential signaling receptor for osteoclast differentiation factor in osteoclastogenesis. *Biochem Biophys Res Commun,* 253**,** 395-400.

NAKAJIMA, T., OTA, N., SHIRAI, Y., HATA, A., YOSHIDA, H., SUZUKI, T., HOSOI, T., ORIMO, H. & EMI, M. 1999. Ethnic difference in contribution of Sp1 site variation of COLIA1 gene in genetic predisposition to osteoporosis. *Calcif Tissue Int,* 65**,** 352-3.

NAKASHIMA, K., ZHOU, X., KUNKEL, G., ZHANG, Z., DENG, J. M., BEHRINGER, R. R. & DE CROMBRUGGHE, B. 2002. The novel zinc finger-containing transcription factor osterix is required for osteoblast differentiation and bone formation. *Cell,* 108**,** 17-29.

NAMPEI, A., HASHIMOTO, J., HAYASHIDA, K., TSUBOI, H., SHI, K., TSUJI, I., MIYASHITA, H., YAMADA, T., MATSUKAWA, N., MATSUMOTO,

M., MORIMOTO, S., OGIHARA, T., OCHI, T. & YOSHIKAWA, H. 2004. Matrix extracellular phosphoglycoprotein (MEPE) is highly expressed in osteocytes in human bone. *J Bone Miner Metab,* 22**,** 176-84.

NANAO, K., TAKADA, G., TAKAHASHI, E., SEKI, N., KOMATSU, Y., OKAMURA-IKEDA, K., MOTOKAWA, Y. & HAYASAKA, K. 1994. Structure and chromosomal localization of the aminomethyltransferase gene (AMT). *Genomics,* 19**,** 27-30.

NARAYANAN, K., RAMACHANDRAN, A., HAO, J., HE, G., PARK, K. W., CHO, M. & GEORGE, A. 2003. Dual functional roles of dentin matrix protein 1. Implications in biomineralization and gene transcription by activation of intracellular Ca2+ store. *J Biol Chem,* 278**,** 17500-8.

NARUMIYA, S. & MORII, N. 1993. rho gene products, botulinum C3 exoenzyme and cell adhesion. *Cell Signal,* 5**,** 9-19.

NARUMIYA, S., TANJI, M. & ISHIZAKI, T. 2009. Rho signaling, ROCK and mDia1, in transformation, metastasis and invasion. *Cancer Metastasis Rev,* 28**,** 65-76.

NELSON, D. A., BARONDESS, D. A., HENDRIX, S. L. & BECK, T. J. 2000. Cross-sectional geometry, bone strength, and bone mass in the proximal femur in black and white postmenopausal women. *J Bone Miner Res,* 15**,** 1992-7.

NEMOTO, Y., NAMBA, T., KOZAKI, S. & NARUMIYA, S. 1991. Clostridium botulinum C3 ADP-ribosyltransferase gene. Cloning, sequencing, and expression of a functional protein in Escherichia coli. *J Biol Chem,* 266**,** 19312-9.

NETTER, F. H. 1987. *Musculoskeletal system: anatomy, physiology, and metabolic disorders,* Summit, Ciba-Geigy Corporation

NG, K. W., ROMAS, E., DONNAN, L. & FINDLAY, D. M. 1997. Bone biology. *Baillieres Clin Endocrinol Metab,* 11**,** 1-22.

NILSSON, B. E. 1970. Spinal osteoporosis and femoral neck fracture. *Clin Orthop Relat Res,* 68**,** 93-5.

NISHIMURA, G., HORIUCHI, T., KIM, O. H. & SASAMOTO, Y. 1997. Atypical skeletal changes in otopalatodigital syndrome type II: phenotypic overlap among otopalatodigital syndrome type II, boomerang dysplasia, atelosteogenesis type I and type III, and lethal male phenotype of Melnick-Needles syndrome. *Am J Med Genet,* 73**,** 132-8.

NISHIO, Y., DONG, Y., PARIS, M., O'KEEFE, R. J., SCHWARZ, E. M. & DRISSI, H. 2006. Runx2-mediated regulation of the zinc finger Osterix/Sp7 gene. *Gene,* 372**,** 62-70.

NOBES, C. D. & HALL, A. 1995. Rho, rac, and cdc42 GTPases regulate the assembly of multimolecular focal complexes associated with actin stress fibers, lamellipodia, and filopodia. *Cell,* 81**,** 53-62.

NOBLE, B. S. 2008. The osteocyte lineage. *Arch Biochem Biophys,* 473**,** 106-11.

NOBLE, B. S., PEET, N., STEVENS, H. Y., BRABBS, A., MOSLEY, J. R., REILLY, G. C., REEVE, J., SKERRY, T. M. & LANYON, L. E. 2003. Mechanical loading: biphasic osteocyte survival and targeting of osteoclasts for bone destruction in rat cortical bone. *Am J Physiol Cell Physiol,* 284**,** C934-43.

O'BRIEN, E. A., WILLIAMS, J. H. & MARSHALL, M. J. 2000. Osteoprotegerin ligand regulates osteoclast adherence to the bone surface in mouse calvaria. *Biochem Biophys Res Commun,* 274**,** 281-90.

ODA, T. & MAEDA, Y. 2010. Multiple Conformations of F-actin. *Structure,* 18**,** 761-7.

OGURA, Y., BONEN, D. K., INOHARA, N., NICOLAE, D. L., CHEN, F. F., RAMOS, R., BRITTON, H., MORAN, T., KARALIUSKAS, R., DUERR, R. H., ACHKAR, J. P., BRANT, S. R., BAYLESS, T. M., KIRSCHNER, B. S., HANAUER, S. B., NUNEZ, G. & CHO, J. H. 2001. A frameshift mutation in NOD2 associated with susceptibility to Crohn's disease. *Nature,* 411**,** 603-6.

OLOFSSON, B. 1999. Rho guanine dissociation inhibitors: pivotal molecules in cellular signalling. *Cell Signal,* 11**,** 545-54.

OLSEN, B. R. 1999. Bone Morphogenesis and Embryologic Development. *In:* FAVUS, M. J. (ed.) *Primer on the Metabolic Bone Diseases and Disorders of Mineral Metabolism.* 4th ed. Philidelphia: Lippincott Williams & Wilkins.

ORRICO, A., GALLI, L., BUONI, S., HAYEK, G., LUCHETTI, A., LORENZINI, S., ZAPPELLA, M., POMPONI, M. G. & SORRENTINO, V. 2005. Attention-deficit/hyperactivity disorder (ADHD) and variable clinical expression of Aarskog-Scott syndrome due to a novel FGD1 gene mutation (R408Q). *Am J Med Genet A,* 135**,** 99-102.

ORRICO, A., GALLI, L., CAVALIERE, M. L., GARAVELLI, L., FRYNS, J. P., CRUSHELL, E., RINALDI, M. M., MEDEIRA, A. & SORRENTINO, V. 2004. Phenotypic and molecular characterisation of the Aarskog-Scott syndrome: a survey of the clinical variability in light of FGD1 mutation analysis in 46 patients. *Eur J Hum Genet,* 12**,** 16-23.

ORRICO, A., GALLI, L., FAIVRE, L., CLAYTON-SMITH, J., AZZARELLO-BURRI, S. M., HERTZ, J. M., JACQUEMONT, S., TAURISANO, R., ARROYO CARRERA, I., TARANTINO, E., DEVRIENDT, K., MELIS, D., THELLE, T., MEINHARDT, U. & SORRENTINO, V. 2010. Aarskog-Scott syndrome: Clinical update and report of nine novel mutations of the FGD1 gene. *Am J Med Genet A.*

ORRICO, A., GALLI, L., FALCIANI, M., BRACCI, M., CAVALIERE, M. L., RINALDI, M. M., MUSACCHIO, A. & SORRENTINO, V. 2000. A mutation in the pleckstrin homology (PH) domain of the FGD1 gene in an Italian family with faciogenital dysplasia (Aarskog-Scott syndrome). *FEBS Lett,* 478**,** 216-20.

ORRICO, A., GALLI, L., OBREGON, M. G., DE CASTRO PEREZ, M. F., FALCIANI, M. & SORRENTINO, V. 2007. Unusually severe expression of craniofacial features in Aarskog-Scott syndrome due to a novel truncating mutation of the FGD1 gene. *Am J Med Genet A,* 143**,** 58-63.

OSBOURN, A. E. & FIELD, B. 2009. Operons. *Cell Mol Life Sci,* 66**,** 3755-75.

OTTO, F., THORNELL, A. P., CROMPTON, T., DENZEL, A., GILMOUR, K. C., ROSEWELL, I. R., STAMP, G. W., BEDDINGTON, R. S., MUNDLOS, S., OLSEN, B. R., SELBY, P. B. & OWEN, M. J. 1997. Cbfa1, a candidate gene for cleidocranial dysplasia syndrome, is essential for osteoblast differentiation and bone development. *Cell,* 89**,** 765-71.

OWENS, G. K. 1995. Regulation of differentiation of vascular smooth muscle cells. *Physiol Rev,* 75**,** 487-517.

OZAWA, K., KUWABARA, K., TAMATANI, M., TAKATSUJI, K., TSUKAMOTO, Y., KANEDA, S., YANAGI, H., STERN, D. M., EGUCHI, Y., TSUJIMOTO, Y., OGAWA, S. & TOHYAMA, M. 1999. 150-kDa oxygen-regulated protein (ORP150) suppresses hypoxia-induced apoptotic cell death. *J Biol Chem,* 274**,** 6397-404.

PAGANI, F., FRANCUCCI, C. M. & MORO, L. 2005. Markers of bone turnover: biochemical and clinical perspectives. *J Endocrinol Invest,* 28**,** 8-13.

PANTALONI, D. & CARLIER, M. F. 1993. How profilin promotes actin filament assembly in the presence of thymosin beta 4. *Cell,* 75**,** 1007-14.

PARFITT, A. M. 1976. The actions of parathyroid hormone on bone: relation to bone remodeling and turnover, calcium homeostasis, and metabolic bone diseases. II. PTH and bone cells: bone turnover and plasma calcium regulation. *Metabolism,* 25**,** 909-55.

PASCO, J. A., SANDERS, K. M., HOEKSTRA, F. M., HENRY, M. J., NICHOLSON, G. C. & KOTOWICZ, M. A. 2005. The human cost of fracture. *Osteoporos Int,* 16**,** 2046-52.

PASTERIS, N. G., CADLE, A., LOGIE, L. J., PORTEOUS, M. E., SCHWARTZ, C. E., STEVENSON, R. E., GLOVER, T. W., WILROY, R. S. & GORSKI, J. L. 1994. Isolation and characterization of the faciogenital dysplasia (Aarskog-Scott syndrome) gene: a putative Rho/Rac guanine nucleotide exchange factor. *Cell,* 79**,** 669-78.

PATERSON, H. F., SELF, A. J., GARRETT, M. D., JUST, I., AKTORIES, K. & HALL, A. 1990. Microinjection of recombinant p21rho induces rapid changes in cell morphology. *J Cell Biol,* 111**,** 1001-7.

PEACOCK, M., KOLLER, D. L., FISHBURN, T., KRISHNAN, S., LAI, D., HUI, S., JOHNSTON, C. C., FOROUD, T. & ECONS, M. J. 2005. Sex-specific and non-sex-specific quantitative trait loci contribute to normal variation in bone mineral density in men. *J Clin Endocrinol Metab,* 90**,** 3060-6.

PECK, J. W., OBERST, M., BOUKER, K. B., BOWDEN, E. & BURBELO, P. D. 2002. The RhoA-binding protein, rhophilin-2, regulates actin cytoskeleton organization. *J Biol Chem,* 277**,** 43924-32.

PEROLA, M., KAINULAINEN, K., PAJUKANTA, P., TERWILLIGER, J. D., HIEKKALINNA, T., ELLONEN, P., KAPRIO, J., KOSKENVUO, M., KONTULA, K. & PELTONEN, L. 2000. Genome-wide scan of predisposing loci for increased diastolic blood pressure in Finnish siblings. *J Hypertens,* 18**,** 1579-85.

PETTWAY, G. J., MEGANCK, J. A., KOH, A. J., KELLER, E. T., GOLDSTEIN, S. A. & MCCAULEY, L. K. 2008. Parathyroid hormone mediates bone growth through the regulation of osteoblast proliferation and differentiation. *Bone,* 42**,** 806-18.

PIMM, J., MCQUILLIN, A., THIRUMALAI, S., LAWRENCE, J., QUESTED, D., BASS, N., LAMB, G., MOOREY, H., DATTA, S. R., KALSI, G., BADACSONYI, A., KELLY, K., MORGAN, J., PUNUKOLLU, B., CURTIS, D. & GURLING, H. 2005. The Epsin 4 gene on chromosome 5q, which encodes the clathrin-associated protein enthoprotin, is involved in the genetic susceptibility to schizophrenia. *Am J Hum Genet,* 76**,** 902-7.

PITERS, E., BOUDIN, E. & VAN HUL, W. 2008. Wnt signaling: a win for bone. *Arch Biochem Biophys,* 473**,** 112-6.

PITSILLIDES, A. A., RAWLINSON, S. C., SUSWILLO, R. F., BOURRIN, S., ZAMAN, G. & LANYON, L. E. 1995. Mechanical strain-induced NO production by bone cells: a possible role in adaptive bone (re)modeling? *FASEB J,* 9**,** 1614-22.

PITTAS, A. G., HARRIS, S. S., ELIADES, M., STARK, P. & DAWSON-HUGHES, B. 2009. Association between serum osteocalcin and markers of metabolic phenotype. *J Clin Endocrinol Metab,* 94**,** 827-32.

PITTS, T. O. & VAN THIEL, D. H. 1986. Disorders of divalent ions and vitamin D metabolism in chronic alcoholism. *Recent Dev Alcohol,* 4**,** 357-77.

POCOCK, N. A., EISMAN, J. A., HOPPER, J. L., YEATES, M. G., SAMBROOK, P. N. & EBERL, S. 1987. Genetic determinants of bone mass in adults. A twin study. *J Clin Invest,* 80**,** 706-10.

POLLARD, T. D. 2007. Regulation of actin filament assembly by Arp2/3 complex and formins. *Annu Rev Biophys Biomol Struct,* 36**,** 451-77.

POOLE, K. E. & REEVE, J. 2005. Parathyroid hormone - a bone anabolic and catabolic agent. *Curr Opin Pharmacol,* 5**,** 612-7.

POOLE, K. E., VAN BEZOOIJEN, R. L., LOVERIDGE, N., HAMERSMA, H., PAPAPOULOS, S. E., LOWIK, C. W. & REEVE, J. 2005. Sclerostin is a delayed secreted product of osteocytes that inhibits bone formation. *FASEB J,* 19**,** 1842-4.

POPOWICZ, G. M., SCHLEICHER, M., NOEGEL, A. A. & HOLAK, T. A. 2006. Filamins: promiscuous organizers of the cytoskeleton. *Trends Biochem Sci,* 31**,** 411-9.

PRINCE, R. L. & DICK, I. 1997. Oestrogen effects on calcium membrane transport: a new view of the inter-relationship between oestrogen deficiency and age-related osteoporosis. *Osteoporos Int,* 7 Suppl 3**,** S150-4.

PROVENZANO, M. & MOCELLIN, S. 2007. Complementary techniques: validation of gene expression data by quantitative real time PCR. *Adv Exp Med Biol,* 593**,** 66-73.

PURCELL, S., CHERNY, S. S. & SHAM, P. C. 2003. Genetic Power Calculator: design of linkage and association genetic mapping studies of complex traits. *Bioinformatics,* 19**,** 149-50.

QIAGEN. 2010. *AllStars Negative Controls* [Online]. Available: https://www1.qiagen.com/Products/GeneSilencing/AllStarRNAiControls/AllStarsNegativeControls.aspx [Accessed 30th April 2010].

RADEFF, J. M., NAGY, Z. & STERN, P. H. 2004. Rho and Rho kinase are involved in parathyroid hormone-stimulated protein kinase C alpha translocation and IL-6 promoter activity in osteoblastic cells. *J Bone Miner Res,* 19**,** 1882-91.

RALSTON, S. H. 2003. Genetic determinants of susceptibility to osteoporosis. *Curr Opin Pharmacol,* 3**,** 286-90.

RALSTON, S. H., GALWEY, N., MACKAY, I., ALBAGHA, O. M., CARDON, L., COMPSTON, J. E., COOPER, C., DUNCAN, E., KEEN, R., LANGDAHL, B., MCLELLAN, A., O'RIORDAN, J., POLS, H. A., REID, D. M., UITTERLINDEN, A. G., WASS, J. & BENNETT, S. T. 2005. Loci for regulation of bone mineral density in men and women identified by genome wide linkage scan: the FAMOS study. *Hum Mol Genet,* 14**,** 943-51.

RALSTON, S. H. & UITTERLINDEN, A. G. 2010. Genetics of Osteoporosis. *Endocr Rev*.

RALSTON, S. H., UITTERLINDEN, A. G., BRANDI, M. L., BALCELLS, S., LANGDAHL, B. L., LIPS, P., LORENC, R., OBERMAYER-PIETSCH, B., SCOLLEN, S., BUSTAMANTE, M., HUSTED, L. B., CAREY, A. H., DIEZ-PEREZ, A., DUNNING, A. M., FALCHETTI, A., KARCZMAREWICZ, E., KRUK, M., VAN LEEUWEN, J. P., VAN MEURS, J. B., MANGION, J., MCGUIGAN, F. E., MELLIBOVSKY, L., DEL MONTE, F., POLS, H. A., REEVE, J., REID, D. M., RENNER, W., RIVADENEIRA, F., VAN SCHOOR, N. M., SHERLOCK, R. E. & IOANNIDIS, J. P. 2006. Large-scale evidence for the effect of the COLIA1

Sp1 polymorphism on osteoporosis outcomes: the GENOMOS study. *PLoS Med,* 3**,** e90.

RAUCH, F. 2005. Bone growth in length and width: the Yin and Yang of bone stability. *J Musculoskelet Neuronal Interact,* 5**,** 194-201.

REGINSTER, J. Y. & BURLET, N. 2006. Osteoporosis: a still increasing prevalence. *Bone,* 38**,** S4-9.

REID, I. R., BOLLAND, M. J. & GREY, A. 2008. Effect of calcium supplementation on hip fractures. *Osteoporos Int,* 19**,** 1119-23.

REIJNDERS, C. M., BRAVENBOER, N., TROMP, A. M., BLANKENSTEIN, M. A. & LIPS, P. 2007. Effect of mechanical loading on insulin-like growth factor-I gene expression in rat tibia. *J Endocrinol,* 192**,** 131-40.

RENDINA, D., DE FILIPPO, G., TAUCHMANOVA, L., INSABATO, L., MUSCARIELLO, R., GIANFRANCESCO, F., ESPOSITO, T., CIOFFI, M., COLAO, A., STRAZZULLO, P. & MOSSETTI, G. 2009. Bone turnover and the osteoprotegerin-RANKL pathway in tumor-induced osteomalacia: a longitudinal study of five cases. *Calcif Tissue Int,* 85**,** 293-300.

RESZKA, A. A. & RODAN, G. A. 2004. Nitrogen-containing bisphosphonate mechanism of action. *Mini Rev Med Chem,* 4**,** 711-9.

RIBOT, C., TREMOLLIERES, F. & POUILLES, J. M. 1994. The effect of obesity on postmenopausal bone loss and the risk of osteoporosis. *Adv Nutr Res,* 9**,** 257-71.

RIBOT, C., TREMOLLIERES, F., POUILLES, J. M., BONNEU, M., GERMAIN, F. & LOUVET, J. P. 1987. Obesity and postmenopausal bone loss: the influence of obesity on vertebral density and bone turnover in postmenopausal women. *Bone,* 8**,** 327-31.

RICHARDS, J. B., KAVVOURA, F. K., RIVADENEIRA, F., STYRKARSDOTTIR, U., ESTRADA, K., HALLDORSSON, B. V., HSU, Y. H., ZILLIKENS, M. C., WILSON, S. G., MULLIN, B. H., AMIN, N., AULCHENKO, Y. S., CUPPLES, L. A., DELOUKAS, P., DEMISSIE, S., HOFMAN, A., KONG, A., KARASIK, D., VAN MEURS, J. B., OOSTRA, B. A., POLS, H. A., SIGURDSSON, G., THORSTEINSDOTTIR, U., SORANZO, N., WILLIAMS, F. M., ZHOU, Y., RALSTON, S. H., THORLEIFSSON, G., VAN DUIJN, C. M., KIEL, D. P., STEFANSSON, K., UITTERLINDEN, A. G., IOANNIDIS, J. P. & SPECTOR, T. D. 2009. Collaborative meta-analysis: associations of 150 candidate genes with osteoporosis and osteoporotic fracture. *Ann Intern Med,* 151**,** 528-37.

RICHARDS, J. B., RIVADENEIRA, F., INOUYE, M., PASTINEN, T. M., SORANZO, N., WILSON, S. G., ANDREW, T., FALCHI, M., GWILLIAM, R., AHMADI, K. R., VALDES, A. M., ARP, P., WHITTAKER, P., VERLAAN, D. J., JHAMAI, M., KUMANDURI, V., MOORHOUSE, M., VAN MEURS, J. B., HOFMAN, A., POLS, H. A., HART, D., ZHAI, G., KATO, B. S., MULLIN, B. H., ZHANG, F., DELOUKAS, P., UITTERLINDEN, A. G. & SPECTOR, T. D. 2008. Bone mineral density, osteoporosis, and osteoporotic fractures: a genome-wide association study. *Lancet,* 371**,** 1505-12.

RIDLEY, A. J. & HALL, A. 1992. The small GTP-binding protein rho regulates the assembly of focal adhesions and actin stress fibers in response to growth factors. *Cell,* 70**,** 389-99.

RIENTO, K. & RIDLEY, A. J. 2003. Rocks: multifunctional kinases in cell behaviour. *Nat Rev Mol Cell Biol,* 4**,** 446-56.

RIGGS, B. L., KHOSLA, S. & MELTON, L. J., 3RD 1998. A unitary model for involutional osteoporosis: estrogen deficiency causes both type I and type II osteoporosis in postmenopausal women and contributes to bone loss in aging men. *J Bone Miner Res,* 13**,** 763-73.

RIGGS, B. L. & MELTON, L. J., 3RD 1995. The worldwide problem of osteoporosis: insights afforded by epidemiology. *Bone,* 17**,** 505S-511S.

RIGGS, B. L., NGUYEN, T. V., MELTON, L. J., 3RD, MORRISON, N. A., O'FALLON, W. M., KELLY, P. J., EGAN, K. S., SAMBROOK, P. N., MUHS, J. M. & EISMAN, J. A. 1995. The contribution of vitamin D receptor gene alleles to the determination of bone mineral density in normal and osteoporotic women. *J Bone Miner Res,* 10**,** 991-6.

RINGNER, M. & KROGH, M. 2005. Folding free energies of 5'-UTRs impact post-transcriptional regulation on a genomic scale in yeast. *PLoS Comput Biol,* 1**,** e72.

RIVADENEIRA, F., STYRKARSDOTTIR, U., ESTRADA, K., HALLDORSSON, B. V., HSU, Y. H., RICHARDS, J. B., ZILLIKENS, M. C., KAVVOURA, F. K., AMIN, N., AULCHENKO, Y. S., CUPPLES, L. A., DELOUKAS, P., DEMISSIE, S., GRUNDBERG, E., HOFMAN, A., KONG, A., KARASIK, D., VAN MEURS, J. B., OOSTRA, B., PASTINEN, T., POLS, H. A., SIGURDSSON, G., SORANZO, N., THORLEIFSSON, G., THORSTEINSDOTTIR, U., WILLIAMS, F. M., WILSON, S. G., ZHOU, Y., RALSTON, S. H., VAN DUIJN, C. M., SPECTOR, T., KIEL, D. P., STEFANSSON, K., IOANNIDIS, J. P. & UITTERLINDEN, A. G. 2009. Twenty bone-mineral-density loci identified by large-scale meta-analysis of genome-wide association studies. *Nat Genet,* 41**,** 1199-206.

ROBERTS, M., MOCK, B. A. & BLACKWELL, J. M. 1993. Mapping of genes controlling Leishmania major infection in CXS recombinant inbred mice. *Eur J Immunogenet,* 20**,** 349-62.

RODAN, G. A. 1992. Introduction to bone biology. *Bone,* 13 Suppl 1**,** S3-6.

RODAN, G. A. & MARTIN, T. J. 1981. Role of osteoblasts in hormonal control of bone resorption--a hypothesis. *Calcif Tissue Int,* 33**,** 349-51.

ROSS, F. P. 2000. RANKing the importance of measles virus in Paget's disease. *J Clin Invest,* 105**,** 555-8.

ROSS, F. P., CHAPPEL, J., ALVAREZ, J. I., SANDER, D., BUTLER, W. T., FARACH-CARSON, M. C., MINTZ, K. A., ROBEY, P. G., TEITELBAUM, S. L. & CHERESH, D. A. 1993. Interactions between the bone matrix proteins osteopontin and bone sialoprotein and the osteoclast integrin alpha v beta 3 potentiate bone resorption. *J Biol Chem,* 268**,** 9901-7.

ROSS, P., HALL, L., SMIRNOV, I. & HAFF, L. 1998. High level multiplex genotyping by MALDI-TOF mass spectrometry. *Nat Biotechnol,* 16**,** 1347-51.

ROSSMAN, K. L., DER, C. J. & SONDEK, J. 2005. GEF means go: turning on RHO GTPases with guanine nucleotide-exchange factors. *Nat Rev Mol Cell Biol,* 6**,** 167-80.

ROZEN, S. & SKALETSKY, H. 2000. Primer3 on the WWW for general users and for biologist programmers. *Methods Mol Biol,* 132**,** 365-86.

SALMEN, T., HEIKKINEN, A. M., MAHONEN, A., KROGER, H., KOMULAINEN, M., SAARIKOSKI, S., HONKANEN, R. & MAENPAA, P. H. 2000. Early postmenopausal bone loss is associated with PvuII estrogen

receptor gene polymorphism in Finnish women: effect of hormone replacement therapy. *J Bone Miner Res,* 15**,** 315-21.

SANO, M., INOUE, S., HOSOI, T., OUCHI, Y., EMI, M., SHIRAKI, M. & ORIMO, H. 1995. Association of estrogen receptor dinucleotide repeat polymorphism with osteoporosis. *Biochem Biophys Res Commun,* 217**,** 378-83.

SANTAMA, N., ER, C. P., ONG, L. L. & YU, H. 2004. Distribution and functions of kinectin isoforms. *J Cell Sci,* 117**,** 4537-49.

SANTORI, C., CECCANTI, M., DIACINTI, D., ATTILIA, M. L., TOPPO, L., D'ERASMO, E., ROMAGNOLI, E., MASCIA, M. L., CIPRIANI, C., PRASTARO, A., CARNEVALE, V. & MINISOLA, S. 2008. Skeletal turnover, bone mineral density, and fractures in male chronic abusers of alcohol. *J Endocrinol Invest,* 31**,** 321-6.

SARATHCHANDRA, P., POPE, F. M. & ALI, S. Y. 1996. An ultrastructural and immunogold localization study of proteoglycans associated with the osteocytes of fetal bone in osteogenesis imperfecta. *Calcif Tissue Int,* 58**,** 435-42.

SARKISIAN, M. R., BARTLEY, C. M. & RAKIC, P. 2008. Trouble making the first move: interpreting arrested neuronal migration in the cerebral cortex. *Trends Neurosci,* 31**,** 54-61.

SATAKE, W., NAKABAYASHI, Y., MIZUTA, I., HIROTA, Y., ITO, C., KUBO, M., KAWAGUCHI, T., TSUNODA, T., WATANABE, M., TAKEDA, A., TOMIYAMA, H., NAKASHIMA, K., HASEGAWA, K., OBATA, F., YOSHIKAWA, T., KAWAKAMI, H., SAKODA, S., YAMAMOTO, M., HATTORI, N., MURATA, M., NAKAMURA, Y. & TODA, T. 2009. Genome-wide association study identifies common variants at four loci as genetic risk factors for Parkinson's disease. *Nat Genet,* 41**,** 1303-7.

SATO, H., TAKINO, T., OKADA, Y., CAO, J., SHINAGAWA, A., YAMAMOTO, E. & SEIKI, M. 1994. A matrix metalloproteinase expressed on the surface of invasive tumour cells. *Nature,* 370**,** 61-5.

SATO, T., WATANABE, K., MASUHARA, M., HADA, N. & HAKEDA, Y. 2007. Production of IL-7 is increased in ovariectomized mice, but not RANKL mRNA expression by osteoblasts/stromal cells in bone, and IL-7 enhances generation of osteoclast precursors in vitro. *J Bone Miner Metab,* 25**,** 19-27.

SAVILLE, P. D. 1965. CHANGES IN BONE MASS WITH AGE AND ALCOHOLISM. *J Bone Joint Surg Am,* 47**,** 492-9.

SAWYER, G. M., CLARK, A. R., ROBERTSON, S. P. & SUTHERLAND-SMITH, A. J. 2009. Disease-associated substitutions in the filamin B actin binding domain confer enhanced actin binding affinity in the absence of major structural disturbance: Insights from the crystal structures of filamin B actin binding domains. *J Mol Biol,* 390**,** 1030-47.

SCHALL, T. J., BACON, K., TOY, K. J. & GOEDDEL, D. V. 1990. Selective attraction of monocytes and T lymphocytes of the memory phenotype by cytokine RANTES. *Nature,* 347**,** 669-71.

SCHINDLER, A. E., EBERT, A. & FRIEDRICH, E. 1972. Conversion of androstenedione to estrone by human tissue. *J Clin Endocrinol Metab,* 35**,** 627-30.

SCHLESINGER, P. H., BLAIR, H. C., TEITELBAUM, S. L. & EDWARDS, J. C. 1997. Characterization of the osteoclast ruffled border chloride channel and its role in bone resorption. *J Biol Chem,* 272**,** 18636-43.

SCHMIDT, A. & HALL, A. 2002. Guanine nucleotide exchange factors for Rho GTPases: turning on the switch. *Genes Dev,* 16**,** 1587-609.

SCHMITTGEN, T. D. & LIVAK, K. J. 2008. Analyzing real-time PCR data by the comparative C(T) method. *Nat Protoc,* 3**,** 1101-8.

SCHNEEWEIS, L. A., WILLARD, D. & MILLA, M. E. 2005. Functional dissection of osteoprotegerin and its interaction with receptor activator of NF-kappaB ligand. *J Biol Chem,* 280**,** 41155-64.

SCHULTZ, C., LANGER, L. O., LAXOVA, R. & PAULI, R. M. 1999. Atelosteogenesis type III: long term survival, prenatal diagnosis, and evidence for dominant transmission. *Am J Med Genet,* 83**,** 28-42.

SCHWARTZ, C. E., GILLESSEN-KAESBACH, G., MAY, M., CAPPA, M., GORSKI, J., STEINDL, K. & NERI, G. 2000. Two novel mutations confirm FGD1 is responsible for the Aarskog syndrome. *Eur J Hum Genet,* 8**,** 869-74.

SCILLITANI, A., JANG, C., WONG, B. Y., HENDY, G. N. & COLE, D. E. 2006. A functional polymorphism in the PTHR1 promoter region is associated with adult height and BMD measured at the femoral neck in a large cohort of young caucasian women. *Hum Genet,* 119**,** 416-21.

SCOTT, C. I. 1971. Unusual facies, joint hypermobility, genital anomaly and short stature: a new dysmorphic syndrome. *Birth Defects Orig Artic Ser,* 7**,** 240-6.

SEEMAN, E., HOPPER, J. L., YOUNG, N. R., FORMICA, C., GOSS, P. & TSALAMANDRIS, C. 1996. Do genetic factors explain associations between muscle strength, lean mass, and bone density? A twin study. *Am J Physiol,* 270**,** E320-7.

SEKINE, A., FUJIWARA, M. & NARUMIYA, S. 1989. Asparagine residue in the rho gene product is the modification site for botulinum ADP-ribosyltransferase. *J Biol Chem,* 264**,** 8602-5.

SERNBO, I. & JOHNELL, O. 1993. Consequences of a hip fracture: a prospective study over 1 year. *Osteoporos Int,* 3**,** 148-53.

SHALEV, S. A., CHERVINSKI, E., WEINER, E., MAZOR, G., FRIEZ, M. J. & SCHWARTZ, C. E. 2006. Clinical variation of Aarskog syndrome in a large family with 2189delA in the FGD1 gene. *Am J Med Genet A,* 140**,** 162-5.

SHAMAH, S. M., LIN, M. Z., GOLDBERG, J. L., ESTRACH, S., SAHIN, M., HU, L., BAZALAKOVA, M., NEVE, R. L., CORFAS, G., DEBANT, A. & GREENBERG, M. E. 2001. EphA receptors regulate growth cone dynamics through the novel guanine nucleotide exchange factor ephexin. *Cell,* 105**,** 233-44.

SHEN, H., LONG, J. R., XIONG, D. H., LIU, Y. J., LIU, Y. Z., XIAO, P., ZHAO, L. J., DVORNYK, V., ZHANG, Y. Y., ROCHA-SANCHEZ, S., LIU, P. Y., LI, J. L. & DENG, H. W. 2005. Mapping quantitative trait loci for cross-sectional geometry at the femoral neck. *J Bone Miner Res,* 20**,** 1973-82.

SIEHLER, S. 2009. Regulation of RhoGEF proteins by G12/13-coupled receptors. *Br J Pharmacol,* 158**,** 41-9.

SILVE, C. & JUPPNER, H. 2006. Ollier disease. *Orphanet J Rare Dis,* 1**,** 37.

SIMON-SANCHEZ, J., SCHULTE, C., BRAS, J. M., SHARMA, M., GIBBS, J. R., BERG, D., PAISAN-RUIZ, C., LICHTNER, P., SCHOLZ, S. W., HERNANDEZ, D. G., KRUGER, R., FEDEROFF, M., KLEIN, C., GOATE, A., PERLMUTTER, J., BONIN, M., NALLS, M. A., ILLIG, T., GIEGER, C., HOULDEN, H., STEFFENS, M., OKUN, M. S., RACETTE, B. A., COOKSON, M. R., FOOTE, K. D., FERNANDEZ, H. H., TRAYNOR, B. J., SCHREIBER, S., AREPALLI, S., ZONOZI, R., GWINN, K., VAN DER

BRUG, M., LOPEZ, G., CHANOCK, S. J., SCHATZKIN, A., PARK, Y., HOLLENBECK, A., GAO, J., HUANG, X., WOOD, N. W., LORENZ, D., DEUSCHL, G., CHEN, H., RIESS, O., HARDY, J. A., SINGLETON, A. B. & GASSER, T. 2009. Genome-wide association study reveals genetic risk underlying Parkinson's disease. *Nat Genet,* 41**,** 1308-12.

SIMONET, W. S., LACEY, D. L., DUNSTAN, C. R., KELLEY, M., CHANG, M. S., LUTHY, R., NGUYEN, H. Q., WOODEN, S., BENNETT, L., BOONE, T., SHIMAMOTO, G., DEROSE, M., ELLIOTT, R., COLOMBERO, A., TAN, H. L., TRAIL, G., SULLIVAN, J., DAVY, E., BUCAY, N., RENSHAW-GEGG, L., HUGHES, T. M., HILL, D., PATTISON, W., CAMPBELL, P., SANDER, S., VAN, G., TARPLEY, J., DERBY, P., LEE, R. & BOYLE, W. J. 1997. Osteoprotegerin: a novel secreted protein involved in the regulation of bone density. *Cell,* 89**,** 309-19.

SINGER, V. L., JONES, L. J., YUE, S. T. & HAUGLAND, R. P. 1997. Characterization of PicoGreen reagent and development of a fluorescence-based solution assay for double-stranded DNA quantitation. *Anal Biochem,* 249**,** 228-38.

SIPOS, W., PIETSCHMANN, P., RAUNER, M., KERSCHAN-SCHINDL, K. & PATSCH, J. 2009. Pathophysiology of osteoporosis. *Wien Med Wochenschr,* 159**,** 230-4.

SLIVA, K. & SCHNIERLE, B. S. 2010. Selective gene silencing by viral delivery of short hairpin RNA. *Virol J,* 7**,** 248.

SMALL, J., ROTTNER, K., HAHNE, P. & ANDERSON, K. I. 1999. Visualising the actin cytoskeleton. *Microsc Res Tech,* 47**,** 3-17.

SMALL, J. V., STRADAL, T., VIGNAL, E. & ROTTNER, K. 2002. The lamellipodium: where motility begins. *Trends Cell Biol,* 12**,** 112-20.

SMITH, D. M., NANCE, W. E., KANG, K. W., CHRISTIAN, J. C. & JOHNSTON, C. C., JR. 1973. Genetic factors in determining bone mass. *J Clin Invest,* 52**,** 2800-8.

SOBACCHI, C., FRATTINI, A., GUERRINI, M. M., ABINUN, M., PANGRAZIO, A., SUSANI, L., BREDIUS, R., MANCINI, G., CANT, A., BISHOP, N., GRABOWSKI, P., DEL FATTORE, A., MESSINA, C., ERRIGO, G., COXON, F. P., SCOTT, D. I., TETI, A., ROGERS, M. J., VEZZONI, P., VILLA, A. & HELFRICH, M. H. 2007. Osteoclast-poor human osteopetrosis due to mutations in the gene encoding RANKL. *Nat Genet,* 39**,** 960-2.

SPEER, M. Y., LI, X., HIREMATH, P. G. & GIACHELLI, C. M. 2010. Runx2/Cbfa1, but not loss of myocardin, is required for smooth muscle cell lineage reprogramming toward osteochondrogenesis. *J Cell Biochem,* 110**,** 935-47.

ST-JACQUES, B., HAMMERSCHMIDT, M. & MCMAHON, A. P. 1999. Indian hedgehog signaling regulates proliferation and differentiation of chondrocytes and is essential for bone formation. *Genes Dev,* 13**,** 2072-86.

STARK, Z. & SAVARIRAYAN, R. 2009. Osteopetrosis. *Orphanet J Rare Dis,* 4**,** 5.

STEINER, C., EHTESHAM, N., TAYLOR, K. D., SEBALD, E., CANTOR, R., KING, L. M., GUO, X., HANG, T., HU, M. S., CUI, J. R., FRIEDMAN, B., NORATO, D., ALLANSON, J., HONEYWELL, C., METTLER, G., FIELD, F., LACHMAN, R., COHN, D. H. & KRAKOW, D. 2004. A locus for spondylocarpotarsal synostosis syndrome at chromosome 3p14. *J Med Genet,* 41**,** 266-9.

STOSSEL, T. P. 1989. From signal to pseudopod. How cells control cytoplasmic actin assembly. *J Biol Chem,* 264**,** 18261-4.

STOSSEL, T. P., CONDEELIS, J., COOLEY, L., HARTWIG, J. H., NOEGEL, A., SCHLEICHER, M. & SHAPIRO, S. S. 2001. Filamins as integrators of cell mechanics and signalling. *Nat Rev Mol Cell Biol,* 2**,** 138-45.

STRANSKY, M. & RYSAVA, L. 2009. Nutrition as prevention and treatment of osteoporosis. *Physiol Res,* 58 Suppl 1**,** S7-S11.

STROH, T., ERBEN, U., KUHL, A. A., ZEITZ, M. & SIEGMUND, B. 2010. Combined pulse electroporation--a novel strategy for highly efficient transfection of human and mouse cells. *PLoS One,* 5**,** e9488.

STYRKARSDOTTIR, U., HALLDORSSON, B. V., GRETARSDOTTIR, S., GUDBJARTSSON, D. F., WALTERS, G. B., INGVARSSON, T., JONSDOTTIR, T., SAEMUNDSDOTTIR, J., CENTER, J. R., NGUYEN, T. V., BAGGER, Y., GULCHER, J. R., EISMAN, J. A., CHRISTIANSEN, C., SIGURDSSON, G., KONG, A., THORSTEINSDOTTIR, U. & STEFANSSON, K. 2008. Multiple genetic loci for bone mineral density and fractures. *N Engl J Med,* 358**,** 2355-65.

STYRKARSDOTTIR, U., HALLDORSSON, B. V., GRETARSDOTTIR, S., GUDBJARTSSON, D. F., WALTERS, G. B., INGVARSSON, T., JONSDOTTIR, T., SAEMUNDSDOTTIR, J., SNORRADOTTIR, S., CENTER, J. R., NGUYEN, T. V., ALEXANDERSEN, P., GULCHER, J. R., EISMAN, J. A., CHRISTIANSEN, C., SIGURDSSON, G., KONG, A., THORSTEINSDOTTIR, U. & STEFANSSON, K. 2009. New sequence variants associated with bone mineral density. *Nat Genet,* 41**,** 15-7.

SUGA, T., KURABAYASHI, M., SANDO, Y., OHYAMA, Y., MAENO, T., MAENO, Y., AIZAWA, H., MATSUMURA, Y., KUWAKI, T., KURO, O. M., NABESHIMA, Y. & NAGAI, R. 2000. Disruption of the klotho gene causes pulmonary emphysema in mice. Defect in maintenance of pulmonary integrity during postnatal life. *Am J Respir Cell Mol Biol,* 22**,** 26-33.

SUN, Y. Q., MCLEOD, K. J. & RUBIN, C. T. 1995. Mechanically induced periosteal bone formation is paralleled by the upregulation of collagen type one mRNA in osteocytes as measured by in situ reverse transcript-polymerase chain reaction. *Calcif Tissue Int,* 57**,** 456-62.

SUN, Z. F., JIANG, H., YE, Z. Q., JIA, B., ZHANG, X. L. & ZHANG, K. Q. 2009. Expression of Rho GDIalpha in rat osteoblasts intermittently exposed to parathyroid hormone in vitro and in vivo. *Acta Pharmacol Sin,* 30**,** 1001-7.

SUZUKI, H., AMIZUKA, N., ODA, K., LI, M., YOSHIE, H., OHSHIMA, H., NODA, M. & MAEDA, T. 2005. Histological evidence of the altered distribution of osteocytes and bone matrix synthesis in klotho-deficient mice. *Arch Histol Cytol,* 68**,** 371-81.

SZULC, P., CHAPUY, M. C., MEUNIER, P. J. & DELMAS, P. D. 1993. Serum undercarboxylated osteocalcin is a marker of the risk of hip fracture in elderly women. *J Clin Invest,* 91**,** 1769-74.

TAES, Y., LAPAUW, B., VANBILLEMONT, G., BOGAERT, V., DE BACQUER, D., GOEMAERE, S., ZMIERCZAK, H. & KAUFMAN, J. M. 2009. Early Smoking is Associated with Peak Bone Mass and Prevalent Fractures in Young Healthy Men. *J Bone Miner Res*.

TAKAHASHI, N., UDAGAWA, N., AKATSU, T., TANAKA, H., ISOGAI, Y. & SUDA, T. 1991. Deficiency of osteoclasts in osteopetrotic mice is due to a

defect in the local microenvironment provided by osteoblastic cells. *Endocrinology,* 128**,** 1792-6.

TAKE, I., KOBAYASHI, Y., YAMAMOTO, Y., TSUBOI, H., OCHI, T., UEMATSU, S., OKAFUJI, N., KURIHARA, S., UDAGAWA, N. & TAKAHASHI, N. 2005. Prostaglandin E2 strongly inhibits human osteoclast formation. *Endocrinology,* 146**,** 5204-14.

TAMAKI, J., IKI, M., SATO, Y., KAJITA, E., KAGAMIMORI, S., KAGAWA, Y. & YONESHIMA, H. 2009. Smoking among premenopausal women is associated with increased risk of low bone status: the JPOS Study. *J Bone Miner Metab*.

TANG, B. M., ESLICK, G. D., NOWSON, C., SMITH, C. & BENSOUSSAN, A. 2007. Use of calcium or calcium in combination with vitamin D supplementation to prevent fractures and bone loss in people aged 50 years and older: a meta-analysis. *Lancet,* 370**,** 657-66.

TATSUMI, S., ISHII, K., AMIZUKA, N., LI, M., KOBAYASHI, T., KOHNO, K., ITO, M., TAKESHITA, S. & IKEDA, K. 2007. Targeted ablation of osteocytes induces osteoporosis with defective mechanotransduction. *Cell Metab,* 5**,** 464-75.

TEITELBAUM, S. L. 2000. Bone resorption by osteoclasts. *Science,* 289**,** 1504-8.

TEITELBAUM, S. L. 2006. Osteoclasts; culprits in inflammatory osteolysis. *Arthritis Res Ther,* 8**,** 201.

TEITELBAUM, S. L. 2007. Osteoclasts: what do they do and how do they do it? *Am J Pathol,* 170**,** 427-35.

TEIXEIRA, C. C., AGOSTON, H. & BEIER, F. 2008. Nitric oxide, C-type natriuretic peptide and cGMP as regulators of endochondral ossification. *Dev Biol,* 319**,** 171-8.

TENNE, M., MCGUIGAN, F., JANSSON, L., GERDHEM, P., OBRANT, K. J., LUTHMAN, H. & AKESSON, K. 2008. Genetic variation in the PTH pathway and bone phenotypes in elderly women: evaluation of PTH, PTHLH, PTHR1 and PTHR2 genes. *Bone,* 42**,** 719-27.

THIESEN, S., KUBART, S., ROPERS, H. H. & NOTHWANG, H. G. 2000. Isolation of two novel human RhoGEFs, ARHGEF3 and ARHGEF4, in 3p13-21 and 2q22. *Biochem Biophys Res Commun,* 273**,** 364-9.

THOMAS, G., JACOBS, K. B., YEAGER, M., KRAFT, P., WACHOLDER, S., ORR, N., YU, K., CHATTERJEE, N., WELCH, R., HUTCHINSON, A., CRENSHAW, A., CANCEL-TASSIN, G., STAATS, B. J., WANG, Z., GONZALEZ-BOSQUET, J., FANG, J., DENG, X., BERNDT, S. I., CALLE, E. E., FEIGELSON, H. S., THUN, M. J., RODRIGUEZ, C., ALBANES, D., VIRTAMO, J., WEINSTEIN, S., SCHUMACHER, F. R., GIOVANNUCCI, E., WILLETT, W. C., CUSSENOT, O., VALERI, A., ANDRIOLE, G. L., CRAWFORD, E. D., TUCKER, M., GERHARD, D. S., FRAUMENI, J. F., JR., HOOVER, R., HAYES, R. B., HUNTER, D. J. & CHANOCK, S. J. 2008. Multiple loci identified in a genome-wide association study of prostate cancer. *Nat Genet,* 40**,** 310-5.

THOMPSON, D. D., SEEDOR, J. G., QUARTUCCIO, H., SOLOMON, H., FIORAVANTI, C., DAVIDSON, J., KLEIN, H., JACKSON, R., CLAIR, J., FRANKENFIELD, D. & ET AL. 1992. The bisphosphonate, alendronate, prevents bone loss in ovariectomized baboons. *J Bone Miner Res,* 7**,** 951-60.

TOBIAS, J. H., COOK, D. G., CHAMBERS, T. J. & DALZELL, N. 1994. A comparison of bone mineral density between Caucasian, Asian and Afro-Caribbean women. *Clin Sci (Lond),* 87**,** 587-91.

TOKITA, A., MATSUMOTO, H., MORRISON, N. A., TAWA, T., MIURA, Y., FUKAMAUCHI, K., MITSUHASHI, N., IRIMOTO, M., YAMAMORI, S., MIURA, M., WATANABE, T., KUWABARA, Y., YABUTA, K. & EISMAN, J. A. 1996. Vitamin D receptor alleles, bone mineral density and turnover in premenopausal Japanese women. *J Bone Miner Res,* 11**,** 1003-9.

TONJES, A., KORIATH, M., SCHLEINITZ, D., DIETRICH, K., BOTTCHER, Y., RAYNER, N. W., ALMGREN, P., ENIGK, B., RICHTER, O., ROHM, S., FISCHER-ROSINSKY, A., PFEIFFER, A., HOFFMANN, K., KROHN, K., AUST, G., SPRANGER, J., GROOP, L., BLUHER, M., KOVACS, P. & STUMVOLL, M. 2009. Genetic variation in GPR133 is associated with height: genome wide association study in the self-contained population of Sorbs. *Hum Mol Genet,* 18**,** 4662-8.

TOONE, J. R., APPLEGARTH, D. A., COULTER-MACKIE, M. B. & JAMES, E. R. 2001. Identification of the first reported splice site mutation (IVS7-1G-->A) in the aminomethyltransferase (T-protein) gene (AMT) of the glycine cleavage complex in 3 unrelated families with nonketotic hyperglycinemia. *Hum Mutat,* 17**,** 76.

TOTOKI, Y., TOYODA, A., TAKEDA, T., SAKAKI, Y., TANAKA, A., YOKOYAMA, S., OHARA, O., NAGASE, T. & KIKUNO, R. F. 2007. *Homo sapiens protein coding cDNA* [Online]. Kisarazu. Available: http://www.ncbi.nlm.nih.gov/nuccore/62088901 [Accessed].

TOYOSAWA, S., SHINTANI, S., FUJIWARA, T., OOSHIMA, T., SATO, A., IJUHIN, N. & KOMORI, T. 2001. Dentin matrix protein 1 is predominantly expressed in chicken and rat osteocytes but not in osteoblasts. *J Bone Miner Res,* 16**,** 2017-26.

TRINKLEIN, N. D., ALDRED, S. J., SALDANHA, A. J. & MYERS, R. M. 2003. Identification and functional analysis of human transcriptional promoters. *Genome Res,* 13**,** 308-12.

TSAI, K. S., HSU, S. H., CHENG, W. C., CHEN, C. K., CHIENG, P. U. & PAN, W. H. 1996. Bone mineral density and bone markers in relation to vitamin D receptor gene polymorphisms in Chinese men and women. *Bone,* 19**,** 513-8.

TSUKAMOTO, K., YOSHIDA, H., WATANABE, S., SUZUKI, T., MIYAO, M., HOSOI, T., ORIMO, H. & EMI, M. 1999. Association of radial bone mineral density with CA repeat polymorphism at the interleukin 6 locus in postmenopausal Japanese women. *J Hum Genet,* 44**,** 148-51.

UDAGAWA, N., TAKAHASHI, N., AKATSU, T., TANAKA, H., SASAKI, T., NISHIHARA, T., KOGA, T., MARTIN, T. J. & SUDA, T. 1990. Origin of osteoclasts: mature monocytes and macrophages are capable of differentiating into osteoclasts under a suitable microenvironment prepared by bone marrow-derived stromal cells. *Proc Natl Acad Sci U S A,* 87**,** 7260-4.

UEHATA, M., ISHIZAKI, T., SATOH, H., ONO, T., KAWAHARA, T., MORISHITA, T., TAMAKAWA, H., YAMAGAMI, K., INUI, J., MAEKAWA, M. & NARUMIYA, S. 1997. Calcium sensitization of smooth muscle mediated by a Rho-associated protein kinase in hypertension. *Nature,* 389**,** 990-4.

UITTERLINDEN, A. G., ARP, P. P., PAEPER, B. W., CHARMLEY, P., PROLL, S., RIVADENEIRA, F., FANG, Y., VAN MEURS, J. B., BRITSCHGI, T.

B., LATHAM, J. A., SCHATZMAN, R. C., POLS, H. A. & BRUNKOW, M. E. 2004. Polymorphisms in the sclerosteosis/van Buchem disease gene (SOST) region are associated with bone-mineral density in elderly whites. *Am J Hum Genet,* 75**,** 1032-45.

UITTERLINDEN, A. G., RALSTON, S. H., BRANDI, M. L., CAREY, A. H., GRINBERG, D., LANGDAHL, B. L., LIPS, P., LORENC, R., OBERMAYER-PIETSCH, B., REEVE, J., REID, D. M., AMEDEI, A., BASSITI, A., BUSTAMANTE, M., HUSTED, L. B., DIEZ-PEREZ, A., DOBNIG, H., DUNNING, A. M., ENJUANES, A., FAHRLEITNER-PAMMER, A., FANG, Y., KARCZMAREWICZ, E., KRUK, M., VAN LEEUWEN, J. P., MAVILIA, C., VAN MEURS, J. B., MANGION, J., MCGUIGAN, F. E., POLS, H. A., RENNER, W., RIVADENEIRA, F., VAN SCHOOR, N. M., SCOLLEN, S., SHERLOCK, R. E. & IOANNIDIS, J. P. 2006. The association between common vitamin D receptor gene variations and osteoporosis: a participant-level meta-analysis. *Ann Intern Med,* 145**,** 255-64.

UTSUGI, T., OHNO, T., OHYAMA, Y., UCHIYAMA, T., SAITO, Y., MATSUMURA, Y., AIZAWA, H., ITOH, H., KURABAYASHI, M., KAWAZU, S., TOMONO, S., OKA, Y., SUGA, T., KURO-O, M., NABESHIMA, Y. & NAGAI, R. 2000. Decreased insulin production and increased insulin sensitivity in the klotho mutant mouse, a novel animal model for human aging. *Metabolism,* 49**,** 1118-23.

VAANANEN, H. K., ZHAO, H., MULARI, M. & HALLEEN, J. M. 2000. The cell biology of osteoclast function. *J Cell Sci,* 113 (Pt 3)**,** 377-81.

VALENCIA, A., CHARDIN, P., WITTINGHOFER, A. & SANDER, C. 1991. The ras protein family: evolutionary tree and role of conserved amino acids. *Biochemistry,* 30**,** 4637-48.

VAN BEZOOIJEN, R. L., PAPAPOULOS, S. E. & LOWIK, C. W. 2005. Bone morphogenetic proteins and their antagonists: the sclerostin paradigm. *J Endocrinol Invest,* 28**,** 15-7.

VAN DER FLIER, A. & SONNENBERG, A. 2001. Structural and functional aspects of filamins. *Biochim Biophys Acta,* 1538**,** 99-117.

VAN EERDEWEGH, P., LITTLE, R. D., DUPUIS, J., DEL MASTRO, R. G., FALLS, K., SIMON, J., TORREY, D., PANDIT, S., MCKENNY, J., BRAUNSCHWEIGER, K., WALSH, A., LIU, Z., HAYWARD, B., FOLZ, C., MANNING, S. P., BAWA, A., SARACINO, L., THACKSTON, M., BENCHEKROUN, Y., CAPPARELL, N., WANG, M., ADAIR, R., FENG, Y., DUBOIS, J., FITZGERALD, M. G., HUANG, H., GIBSON, R., ALLEN, K. M., PEDAN, A., DANZIG, M. R., UMLAND, S. P., EGAN, R. W., CUSS, F. M., RORKE, S., CLOUGH, J. B., HOLLOWAY, J. W., HOLGATE, S. T. & KEITH, T. P. 2002. Association of the ADAM33 gene with asthma and bronchial hyperresponsiveness. *Nature,* 418**,** 426-30.

VAN MEURS, J. B., TRIKALINOS, T. A., RALSTON, S. H., BALCELLS, S., BRANDI, M. L., BRIXEN, K., KIEL, D. P., LANGDAHL, B. L., LIPS, P., LJUNGGREN, O., LORENC, R., OBERMAYER-PIETSCH, B., OHLSSON, C., PETTERSSON, U., REID, D. M., ROUSSEAU, F., SCOLLEN, S., VAN HUL, W., AGUEDA, L., AKESSON, K., BENEVOLENSKAYA, L. I., FERRARI, S. L., HALLMANS, G., HOFMAN, A., HUSTED, L. B., KRUK, M., KAPTOGE, S., KARASIK, D., KARLSSON, M. K., LORENTZON, M., MASI, L., MCGUIGAN, F. E., MELLSTROM, D., MOSEKILDE, L.,

NOGUES, X., POLS, H. A., REEVE, J., RENNER, W., RIVADENEIRA, F., VAN SCHOOR, N. M., WEBER, K., IOANNIDIS, J. P. & UITTERLINDEN, A. G. 2008. Large-scale analysis of association between LRP5 and LRP6 variants and osteoporosis. *JAMA,* 299**,** 1277-90.

VANSTEELANDT, S., DEMEO, D. L., LASKY-SU, J., SMOLLER, J. W., MURPHY, A. J., MCQUEEN, M., SCHNEITER, K., CELEDON, J. C., WEISS, S. T., SILVERMAN, E. K. & LANGE, C. 2008. Testing and estimating gene-environment interactions in family-based association studies. *Biometrics,* 64**,** 458-67.

VAUGHAN, T., PASCO, J. A., KOTOWICZ, M. A., NICHOLSON, G. C. & MORRISON, N. A. 2002. Alleles of RUNX2/CBFA1 gene are associated with differences in bone mineral density and risk of fracture. *J Bone Miner Res,* 17**,** 1527-34.

VERBORGT, O., GIBSON, G. J. & SCHAFFLER, M. B. 2000. Loss of osteocyte integrity in association with microdamage and bone remodeling after fatigue in vivo. *J Bone Miner Res,* 15**,** 60-7.

VIITANEN, A., KARKKAINEN, M., LAITINEN, K., LAMBERG-ALLARDT, C., KAINULAINEN, K., RASANEN, L., VIIKARI, J., VALIMAKI, M. J. & KONTULA, K. 1996. Common polymorphism of the vitamin D receptor gene is associated with variation of peak bone mass in young finns. *Calcif Tissue Int,* 59**,** 231-4.

VILARINO-GUELL, C., MILES, L. J., DUNCAN, E. L., RALSTON, S. H., COMPSTON, J. E., COOPER, C., LANGDAHL, B. L., MACLELLAND, A., POLS, H. A., REID, D. M., UITTERLINDEN, A. G., STEER, C. D., TOBIAS, J. H., WASS, J. A. & BROWN, M. A. 2007. PTHR1 polymorphisms influence BMD variation through effects on the growing skeleton. *Calcif Tissue Int,* 81**,** 270-8.

VOLIN, M. V., SHAH, M. R., TOKUHIRA, M., HAINES, G. K., WOODS, J. M. & KOCH, A. E. 1998. RANTES expression and contribution to monocyte chemotaxis in arthritis. *Clin Immunol Immunopathol,* 89**,** 44-53.

VORTKAMP, A., LEE, K., LANSKE, B., SEGRE, G. V., KRONENBERG, H. M. & TABIN, C. J. 1996. Regulation of rate of cartilage differentiation by Indian hedgehog and PTH-related protein. *Science,* 273**,** 613-22.

VUJIC, M., HALLSTENSSON, K., WAHLSTROM, J., LUNDBERG, A., LANGMAACK, C. & MARTINSON, T. 1995. Localization of a gene for autosomal dominant Larsen syndrome to chromosome region 3p21.1-14.1 in the proximity of, but distinct from, the COL7A1 locus. *Am J Hum Genet,* 57**,** 1104-13.

WAGNER, E. R., ZHU, G., ZHANG, B. Q., LUO, Q., SHI, Q., HUANG, E., GAO, Y., GAO, J. L., KIM, S. H., RASTEGAR, F., YANG, K., HE, B. C., CHEN, L., ZUO, G. W., BI, Y., SU, Y., LUO, J., LUO, X., HUANG, J., DENG, Z. L., REID, R. R., LUU, H. H., HAYDON, R. C. & HE, T. C. 2010. The Therapeutic Potential of the Wnt Signaling Pathway in Bone Disorders. *Curr Mol Pharmacol*.

WAGNER, H., MELHUS, H., PEDERSEN, N. L. & MICHAELSSON, K. 2009. Heritability of impaired balance: a nationwide cohort study in twins. *Osteoporos Int,* 20**,** 577-83.

WAIN, H. M., BRUFORD, E. A., LOVERING, R. C., LUSH, M. J., WRIGHT, M. W. & POVEY, S. 2002. Guidelines for human gene nomenclature. *Genomics,* 79**,** 464-70.

WALKER, D. G. 1975a. Bone resorption restored in osteopetrotic mice by transplants of normal bone marrow and spleen cells. *Science,* 190**,** 784-5.

WALKER, D. G. 1975b. Control of bone resorption by hematopoietic tissue. The induction and reversal of congenital osteopetrosis in mice through use of bone marrow and splenic transplants. *J Exp Med,* 142**,** 651-63.

WALKER, D. G. 1975c. Spleen cells transmit osteopetrosis in mice. *Science,* 190**,** 785-7.

WANG, J. & STERN, P. H. 2010. Osteoclastogenic activity and RANKL expression are inhibited in osteoblastic cells expressing constitutively active Galpha(12) or constitutively active RhoA. *J Cell Biochem*.

WANG, L., YANG, L., BURNS, K., KUAN, C. Y. & ZHENG, Y. 2005a. Cdc42GAP regulates c-Jun N-terminal kinase (JNK)-mediated apoptosis and cell number during mammalian perinatal growth. *Proc Natl Acad Sci U S A,* 102**,** 13484-9.

WANG, L., YANG, L., DEBIDDA, M., WITTE, D. & ZHENG, Y. 2007. Cdc42 GTPase-activating protein deficiency promotes genomic instability and premature aging-like phenotypes. *Proc Natl Acad Sci U S A,* 104**,** 1248-53.

WANG, Y. H., LIU, Y., BUHL, K. & ROWE, D. W. 2005b. Comparison of the action of transient and continuous PTH on primary osteoblast cultures expressing differentiation stage-specific GFP. *J Bone Miner Res,* 20**,** 5-14.

WARD, K. D. & KLESGES, R. C. 2001. A meta-analysis of the effects of cigarette smoking on bone mineral density. *Calcif Tissue Int,* 68**,** 259-70.

WASNICH, R. 1993. Bone mass measurement: prediction of risk. *Am J Med,* 95**,** 6S-10S.

WATANABE, N., MADAULE, P., REID, T., ISHIZAKI, T., WATANABE, G., KAKIZUKA, A., SAITO, Y., NAKAO, K., JOCKUSCH, B. M. & NARUMIYA, S. 1997. p140mDia, a mammalian homolog of Drosophila diaphanous, is a target protein for Rho small GTPase and is a ligand for profilin. *EMBO J,* 16**,** 3044-56.

WEICHETOVA, M., STEPAN, J. J., MICHALSKA, D., HAAS, T., POLS, H. A. & UITTERLINDEN, A. G. 2000. COLIA1 polymorphism contributes to bone mineral density to assess prevalent wrist fractures. *Bone,* 26**,** 287-90.

WEIHING, R. R. 1988. Actin-binding and dimerization domains of HeLa cell filamin. *Biochemistry,* 27**,** 1865-9.

WEINREB, M., SHINAR, D. & RODAN, G. A. 1990. Different pattern of alkaline phosphatase, osteopontin, and osteocalcin expression in developing rat bone visualized by in situ hybridization. *J Bone Miner Res,* 5**,** 831-42.

WEITZMANN, M. N., CENCI, S., RIFAS, L., BROWN, C. & PACIFICI, R. 2000. Interleukin-7 stimulates osteoclast formation by up-regulating the T-cell production of soluble osteoclastogenic cytokines. *Blood,* 96**,** 1873-8.

WEITZMANN, M. N. & PACIFICI, R. 2005. The role of T lymphocytes in bone metabolism. *Immunol Rev,* 208**,** 154-68.

WHYTE, M. P., OBRECHT, S. E., FINNEGAN, P. M., JONES, J. L., PODGORNIK, M. N., MCALISTER, W. H. & MUMM, S. 2002. Osteoprotegerin deficiency and juvenile Paget's disease. *N Engl J Med,* 347**,** 175-84.

WIELAND, T. & FAULSTICH, H. 1978. Amatoxins, phallotoxins, phallolysin, and antamanide: the biologically active components of poisonous Amanita mushrooms. *CRC Crit Rev Biochem,* 5**,** 185-260.

WIGG, K. G., FENG, Y., CROSBIE, J., TANNOCK, R., KENNEDY, J. L., ICKOWICZ, A., MALONE, M., SCHACHAR, R. & BARR, C. L. 2008. Association of ADHD and the Protogenin gene in the chromosome 15q21.3 reading disabilities linkage region. *Genes Brain Behav,* 7**,** 877-86.

WILKINS, T. D. & LYERLY, D. M. 1996. Clostridium difficile toxins attack Rho. *Trends Microbiol,* 4**,** 49-51.

WILLIAMS, F. M. & SPECTOR, T. D. 2007. The genetics of osteoporosis. *Acta Reumatol Port,* 32**,** 231-40.

WILLIG, R., LUUKINEN, H. & JALOVAARA, P. 2003. Factors related to occurrence of hip fracture during a fall on the hip. *Public Health,* 117**,** 25-30.

WILSON, S. G., JONES, M. R., MULLIN, B. H., DICK, I. M., RICHARDS, J. B., PASTINEN, T. M., GRUNDBERG, E., LJUNGGREN, O., SURDULESCU, G. L., DUDBRIDGE, F., ELLIOTT, K. S., CERVINO, A. C., SPECTOR, T. D. & PRINCE, R. L. 2009. Common Sequence Variation in FLNB Regulates Bone Structure in Women in the General Population and FLNB mRNA Expression in Osteoblasts in Vitro. *J Bone Miner Res,* 24**,** 1989-1997.

WILSON, S. G., REED, P. W., BANSAL, A., CHIANO, M., LINDERSSON, M., LANGDOWN, M., PRINCE, R. L., THOMPSON, D., THOMPSON, E., BAILEY, M., KLEYN, P. W., SAMBROOK, P., SHI, M. M. & SPECTOR, T. D. 2003. Comparison of genome screens for two independent cohorts provides replication of suggestive linkage of bone mineral density to 3p21 and 1p36. *Am J Hum Genet,* 72**,** 144-55.

WINGATE, L. 1984. The epidemiology of osteoporosis. *J Med,* 15**,** 245-66.

WINKLER, D. G., SUTHERLAND, M. K., GEOGHEGAN, J. C., YU, C., HAYES, T., SKONIER, J. E., SHPEKTOR, D., JONAS, M., KOVACEVICH, B. R., STAEHLING-HAMPTON, K., APPLEBY, M., BRUNKOW, M. E. & LATHAM, J. A. 2003. Osteocyte control of bone formation via sclerostin, a novel BMP antagonist. *EMBO J,* 22**,** 6267-76.

WINTERS-STONE, K. M. & SNOW, C. M. 2006. Site-specific response of bone to exercise in premenopausal women. *Bone,* 39**,** 1203-9.

WINZENBERG, T. M., SHAW, K., FRYER, J. & JONES, G. 2006. Calcium supplementation for improving bone mineral density in children. *Cochrane Database Syst Rev***,** CD005119.

WISE, C. A., PARIS, M., MORAR, B., WANG, W., KALAYDJIEVA, L. & BITTLES, A. H. 2003. A standard protocol for single nucleotide primer extension in the human genome using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. *Rapid Commun Mass Spectrom,* 17**,** 1195-202.

WRIGHT, K. M. & FRIEDLAND, J. S. 2002. Differential regulation of chemokine secretion in tuberculous and staphylococcal osteomyelitis. *J Bone Miner Res,* 17**,** 1680-90.

WULF, E., DEBOBEN, A., BAUTZ, F. A., FAULSTICH, H. & WIELAND, T. 1979. Fluorescent phallotoxin, a tool for the visualization of cellular actin. *Proc Natl Acad Sci U S A,* 76**,** 4498-502.

WYNNE, F., DRUMMOND, F. J., DALY, M., BROWN, M., SHANAHAN, F., MOLLOY, M. G. & QUANE, K. A. 2003. Suggestive linkage of 2p22-25 and 11q12-13 with low bone mineral density at the lumbar spine in the Irish population. *Calcif Tissue Int,* 72**,** 651-8.

XIAO, P., SHEN, H., GUO, Y. F., XIONG, D. H., LIU, Y. Z., LIU, Y. J., ZHAO, L. J., LONG, J. R., GUO, Y., RECKER, R. R. & DENG, H. W. 2006. Genomic

regions identified for BMD in a large sample including epistatic interactions and gender-specific effects. *J Bone Miner Res,* 21**,** 1536-44.

XIONG, D. H., LIU, X. G., GUO, Y. F., TAN, L. J., WANG, L., SHA, B. Y., TANG, Z. H., PAN, F., YANG, T. L., CHEN, X. D., LEI, S. F., YERGES, L. M., ZHU, X. Z., WHEELER, V. W., PATRICK, A. L., BUNKER, C. H., GUO, Y., YAN, H., PEI, Y. F., ZHANG, Y. P., LEVY, S., PAPASIAN, C. J., XIAO, P., LUNDBERG, Y. W., RECKER, R. R., LIU, Y. Z., LIU, Y. J., ZMUDA, J. M. & DENG, H. W. 2009. Genome-wide association and follow-up replication studies identified ADAMTS18 and TGFBR3 as bone mass candidate genes in different ethnic groups. *Am J Hum Genet,* 84**,** 388-98.

XIONG, D. H., SHEN, H., XIAO, P., GUO, Y. F., LONG, J. R., ZHAO, L. J., LIU, Y. Z., DENG, H. Y., LI, J. L., RECKER, R. R. & DENG, H. W. 2006a. Genome-wide scan identified QTLs underlying femoral neck cross-sectional geometry that are novel studied risk factors of osteoporosis. *J Bone Miner Res,* 21**,** 424-37.

XIONG, D. H., SHEN, H., ZHAO, L. J., XIAO, P., YANG, T. L., GUO, Y., WANG, W., GUO, Y. F., LIU, Y. J., RECKER, R. R. & DENG, H. W. 2006b. Robust and comprehensive analysis of 20 osteoporosis candidate genes by very high-density single-nucleotide polymorphism screen among 405 white nuclear families identified significant association and gene-gene interaction. *J Bone Miner Res,* 21**,** 1678-95.

XU, W., XIE, Z., CHUNG, D. W. & DAVIE, E. W. 1998. A novel human actin-binding protein homologue that binds to platelet glycoprotein Ibalpha. *Blood,* 92**,** 1268-76.

XU, X. H., DONG, S. S., GUO, Y., YANG, T. L., LEI, S. F., PAPASIAN, C. J., ZHAO, M. & DENG, H. W. 2010. Molecular Genetic Studies of Gene Identification for Osteoporosis: The 2009 Update. *Endocr Rev*.

YAMADA, Y., MIYAUCHI, A., GOTO, J., TAKAGI, Y., OKUIZUMI, H., KANEMATSU, M., HASE, M., TAKAI, H., HARADA, A. & IKEDA, K. 1998. Association of a polymorphism of the transforming growth factor-beta1 gene with genetic susceptibility to osteoporosis in postmenopausal Japanese women. *J Bone Miner Res,* 13**,** 1569-76.

YANG, T. L., CHEN, X. D., GUO, Y., LEI, S. F., WANG, J. T., ZHOU, Q., PAN, F., CHEN, Y., ZHANG, Z. X., DONG, S. S., XU, X. H., YAN, H., LIU, X., QIU, C., ZHU, X. Z., CHEN, T., LI, M., ZHANG, H., ZHANG, L., DREES, B. M., HAMILTON, J. J., PAPASIAN, C. J., RECKER, R. R., SONG, X. P., CHENG, J. & DENG, H. W. 2008. Genome-wide copy-number-variation study identified a susceptibility gene, UGT2B17, for osteoporosis. *Am J Hum Genet,* 83**,** 663-74.

YANG, X. & KARSENTY, G. 2004. ATF4, the osteoblast accumulation of which is determined post-translationally, can induce osteoblast-specific gene expression in non-osteoblastic cells. *J Biol Chem,* 279**,** 47109-14.

YANG, X., MATSUDA, K., BIALEK, P., JACQUOT, S., MASUOKA, H. C., SCHINKE, T., LI, L., BRANCORSINI, S., SASSONE-CORSI, P., TOWNES, T. M., HANAUER, A. & KARSENTY, G. 2004. ATF4 is a substrate of RSK2 and an essential regulator of osteoblast biology; implication for Coffin-Lowry Syndrome. *Cell,* 117**,** 387-98.

YANO, S., MENTAVERRI, R., KANUPARTHI, D., BANDYOPADHYAY, S., RIVERA, A., BROWN, E. M. & CHATTOPADHYAY, N. 2005. Functional expression of beta-chemokine receptors in osteoblasts: role of regulated upon

activation, normal T cell expressed and secreted (RANTES) in osteoblasts and regulation of its secretion by osteoblasts and osteoclasts. *Endocrinology,* 146**,** 2324-35.

YAO, Z., LI, P., ZHANG, Q., SCHWARZ, E. M., KENG, P., ARBINI, A., BOYCE, B. F. & XING, L. 2006. Tumor necrosis factor-alpha increases circulating osteoclast precursor numbers by promoting their proliferation and differentiation in the bone marrow through up-regulation of c-Fms expression. *J Biol Chem,* 281**,** 11846-55.

YASUDA, H., SHIMA, N., NAKAGAWA, N., YAMAGUCHI, K., KINOSAKI, M., MOCHIZUKI, S., TOMOYASU, A., YANO, K., GOTO, M., MURAKAMI, A., TSUDA, E., MORINAGA, T., HIGASHIO, K., UDAGAWA, N., TAKAHASHI, N. & SUDA, T. 1998. Osteoclast differentiation factor is a ligand for osteoprotegerin/osteoclastogenesis-inhibitory factor and is identical to TRANCE/RANKL. *Proc Natl Acad Sci U S A,* 95**,** 3597-602.

YE, H., ARRON, J. R., LAMOTHE, B., CIRILLI, M., KOBAYASHI, T., SHEVDE, N. K., SEGAL, D., DZIVENU, O. K., VOLOGODSKAIA, M., YIM, M., DU, K., SINGH, S., PIKE, J. W., DARNAY, B. G., CHOI, Y. & WU, H. 2002. Distinct molecular mechanism for initiating TRAF6 signalling. *Nature,* 418**,** 443-7.

YERAMIAN, P., CHARDIN, P., MADAULE, P. & TAVITIAN, A. 1987. Nucleotide sequence of human rho cDNA clone 12. *Nucleic Acids Res,* 15**,** 1869.

YOSHIDA, H., HAYASHI, S., KUNISADA, T., OGAWA, M., NISHIKAWA, S., OKAMURA, H., SUDO, T. & SHULTZ, L. D. 1990. The murine mutation osteopetrosis is in the coding region of the macrophage colony stimulating factor gene. *Nature,* 345**,** 442-4.

YU, S., FRANCESCHI, R. T., LUO, M., FAN, J., JIANG, D., CAO, H., KWON, T. G., LAI, Y., ZHANG, J., PATRENE, K., HANKENSON, K., ROODMAN, G. D. & XIAO, G. 2009. Critical role of activating transcription factor 4 in the anabolic actions of parathyroid hormone in bone. *PLoS One,* 4**,** e7583.

YU, S., FRANCESCHI, R. T., LUO, M., ZHANG, X., JIANG, D., LAI, Y., JIANG, Y., ZHANG, J. & XIAO, G. 2008. Parathyroid hormone increases activating transcription factor 4 expression and activity in osteoblasts: requirement for osteocalcin gene expression. *Endocrinology,* 149**,** 1960-8.

YUAN, Y. Y., KOSTENUIK, P. J., OMINSKY, M. S., MORONY, S., ADAMU, S., SIMIONESCU, D. T., BASALYGA, D. M., ASUNCION, F. J. & BATEMAN, T. A. 2008. Skeletal deterioration induced by RANKL infusion: a model for high-turnover bone disease. *Osteoporos Int,* 19**,** 625-35.

ZEBBOUDJ, A. F., SHIN, V. & BOSTROM, K. 2003. Matrix GLA protein and BMP-2 regulate osteoinduction in calcifying vascular cells. *J Cell Biochem,* 90**,** 756-65.

ZHAI, G., ANDREW, T., KATO, B. S., BLAKE, G. M. & SPECTOR, T. D. 2009. Genetic and environmental determinants on bone loss in postmenopausal Caucasian women: a 14-year longitudinal twin study. *Osteoporos Int,* 20**,** 949-53.

ZHANG, D., UDAGAWA, N., NAKAMURA, I., MURAKAMI, H., SAITO, S., YAMASAKI, K., SHIBASAKI, Y., MORII, N., NARUMIYA, S., TAKAHASHI, N. & ET AL. 1995. The small GTP-binding protein, rho p21, is involved in bone resorption by regulating cytoskeletal organization in osteoclasts. *J Cell Sci,* 108 (Pt 6)**,** 2285-92.

ZHANG, X., YU, S., GALSON, D. L., LUO, M., FAN, J., ZHANG, J., GUAN, Y. & XIAO, G. 2008. Activating transcription factor 4 is critical for proliferation and survival in primary bone marrow stromal cells and calvarial osteoblasts. *J Cell Biochem,* 105**,** 885-95.

ZHANG, X. J., HUANG, W., YANG, S., SUN, L. D., ZHANG, F. Y., ZHU, Q. X., ZHANG, F. R., ZHANG, C., DU, W. H., PU, X. M., LI, H., XIAO, F. L., WANG, Z. X., CUI, Y., HAO, F., ZHENG, J., YANG, X. Q., CHENG, H., HE, C. D., LIU, X. M., XU, L. M., ZHENG, H. F., ZHANG, S. M., ZHANG, J. Z., WANG, H. Y., CHENG, Y. L., JI, B. H., FANG, Q. Y., LI, Y. Z., ZHOU, F. S., HAN, J. W., QUAN, C., CHEN, B., LIU, J. L., LIN, D., FAN, L., ZHANG, A. P., LIU, S. X., YANG, C. J., WANG, P. G., ZHOU, W. M., LIN, G. S., WU, W. D., FAN, X., GAO, M., YANG, B. Q., LU, W. S., ZHANG, Z., ZHU, K. J., SHEN, S. K., LI, M., ZHANG, X. Y., CAO, T. T., REN, W., ZHANG, X., HE, J., TANG, X. F., LU, S., YANG, J. Q., ZHANG, L., WANG, D. N., YUAN, F., YIN, X. Y., HUANG, H. J., WANG, H. F., LIN, X. Y. & LIU, J. J. 2009. Psoriasis genome-wide association study identifies susceptibility variants within LCE gene cluster at 1q21. *Nat Genet,* 41**,** 205-10.

ZHANG, Y. Y., LIU, P. Y., LU, Y., XIAO, P., LIU, Y. J., LONG, J. R., SHEN, H., ZHAO, L. J., ELZE, L., RECKER, R. R. & DENG, H. W. 2006. Tests of linkage and association of PTH/PTHrP receptor type 1 gene with bone mineral density and height in Caucasians. *J Bone Miner Metab,* 24**,** 36-41.

ZHAO, B., SCHLESIGER, C., MASUCCI, M. G. & LINDSTEN, K. 2009. The ubiquitin specific protease 4 (USP4) is a new player in the Wnt signalling pathway. *J Cell Mol Med,* 13**,** 1886-95.

ZHAO, F., XUAN, Z., LIU, L. & ZHANG, M. Q. 2005a. TRED: a Transcriptional Regulatory Element Database and a platform for in silico gene regulation studies. *Nucleic Acids Res,* 33**,** D103-7.

ZHAO, H. Y., LIU, J. M., NING, G., ZHAO, Y. J., ZHANG, L. Z., SUN, L. H., XU, M. Y., UITTERLINDEN, A. G. & CHEN, J. L. 2005b. The influence of Lys3Asn polymorphism in the osteoprotegerin gene on bone mineral density in Chinese postmenopausal women. *Osteoporos Int,* 16**,** 1519-24.

ZHAO, H., ROSS, F. P. & TEITELBAUM, S. L. 2005. Unoccupied alpha(v)beta3 integrin regulates osteoclast apoptosis by transmitting a positive death signal. *Mol Endocrinol,* 19**,** 771-80.

ZHAO, S., ZHANG, Y. K., HARRIS, S., AHUJA, S. S. & BONEWALD, L. F. 2002. MLO-Y4 osteocyte-like cells support osteoclast formation and activation. *J Bone Miner Res,* 17**,** 2068-79.

ZHAO, W., BYRNE, M. H., WANG, Y. & KRANE, S. M. 2000. Osteocyte and osteoblast apoptosis and excessive bone deposition accompany failure of collagenase cleavage of collagen. *J Clin Invest,* 106**,** 941-9.

ZHAO, X. H., LASCHINGER, C., ARORA, P., SZASZI, K., KAPUS, A. & MCCULLOCH, C. A. 2007. Force activates smooth muscle alpha-actin promoter activity through the Rho signaling pathway. *J Cell Sci,* 120**,** 1801-9.

ZHENG, Y., FISCHER, D. J., SANTOS, M. F., TIGYI, G., PASTERIS, N. G., GORSKI, J. L. & XU, Y. 1996. The faciogenital dysplasia gene product FGD1 functions as a Cdc42Hs-specific guanine-nucleotide exchange factor. *J Biol Chem,* 271**,** 33169-72.

ZHOU, G., CHEN, Y., ZHOU, L., THIRUNAVUKKARASU, K., HECHT, J., CHITAYAT, D., GELB, B. D., PIRINEN, S., BERRY, S. A., GREENBERG,

C. R., KARSENTY, G. & LEE, B. 1999. CBFA1 mutation analysis and functional correlation with phenotypic variability in cleidocranial dysplasia. *Hum Mol Genet,* 8**,** 2311-6.

ZHOU, X., TIAN, F., SANDZEN, J., CAO, R., FLABERG, E., SZEKELY, L., CAO, Y., OHLSSON, C., BERGO, M. O., BOREN, J. & AKYUREK, L. M. 2007. Filamin B deficiency in mice results in skeletal malformations and impaired microvascular development. *Proc Natl Acad Sci U S A,* 104**,** 3919-24.

ZHU, K., DEVINE, A., DICK, I. M., WILSON, S. G. & PRINCE, R. L. 2008. Effects of calcium and vitamin D supplementation on hip bone mineral density and calcium-related analytes in elderly ambulatory Australian women: a five-year randomized controlled trial. *J Clin Endocrinol Metab,* 93**,** 743-9.

ZIROS, P. G., GIL, A. P., GEORGAKOPOULOS, T., HABEOS, I., KLETSAS, D., BASDRA, E. K. & PAPAVASSILIOU, A. G. 2002. The bone-specific transcriptional regulator Cbfa1 is a target of mechanical signals in osteoblastic cells. *J Biol Chem,* 277**,** 23934-41.

ZOU, W. & TEITELBAUM, S. L. 2010. Integrins, growth factors, and the osteoclast cytoskeleton. *Ann N Y Acad Sci,* 1192**,** 27-31.

Appendix I

Buffers

1X Tris EDTA (TE) buffer

Item	**Quantity**
1M Tris-HCl (pH 8.0)	10 ml
0.25 M EDTA	400 µl
Distilled-deionised water	990 ml

Sterilise by autoclaving

1X phosphate buffered saline (PBS)

Item	**Quantity**
Sodium Chloride (NaCl)	8 g
Potassium Chloride (KCl)	0.2 g
Sodium Phosphate Dibasic (Na_2HPO_4)	1.44 g
Potassium Phosphate (KH_2PO_4)	0.24 g
Distilled-deionised water	Make up to 1 L

Adjust pH to 7.4

Sterilise by autoclaving

TRAP stain buffer (500 ml)

Item	**Quantity**
Sodium Acetate	6.8 g
Sodium Tartrate Dihydrate	5.8 g
Glacial Acetic Acid	1.1 ml
Distilled-deionised water	Make up to 500 ml

Adjust pH to 5.0

TRAP stain solution (500 ml)

Item	**Quantity**
Naphthol AS-MX Phosphate Disodium Salt	50 mg
2-Ethoxyethanol	2.5 ml
Fast Red Violet LB Salt	300 mg
TRAP Stain Buffer	Make up to 500 ml

Mix well and remove precipitate using 0.8 µm filter

Appendix II

Primers

Oligonucleotide primer sequences used in MALDI-ToF mass spectrometry SNP genotyping

SNP	Primer sequences
rs7646054	Forward: 5'-GCCAGAAACGATTCCATGAT-3'
	Reverse: 5'-GCATTGTCACCTGAAGCAGA-3'
	Genotyping: 5'-TGAATCTCCCAAGATGA-3'
rs1566487	Forward: 5'-GCCTCATGCCACTCTGAAAT-3'
	Reverse: 5'-GGTACAGATGCTTGGCCACT-3'
	Genotyping: 5'-TTTGGGGCCCATAACTCAGGAAA-3'
rs6803697	Forward: 5'-TGGCTCAATGACCATAACCA-3'
	Reverse: 5'-CCTCGGGCTGTACTTCTTGA-3'
	Genotyping: 5'-AAATTACAGGATTCTAACT-3'
rs12632941	Forward: 5'-TCCTTTCCCAGCCAGTAGAA-3'
	Reverse: 5'-CCACTCCAGTCTGAGCAACA-3'
	Genotyping: 5'-AGAGAGACTGTGTCACA-3'
rs4681928	Forward: 5'-TGATGGCTAACAAGCAGCAG-3'
	Reverse: 5'-ATTTGGCAGCCCTCTATCCT-3'
	Genotyping: 5'-ACTCTGTCATTCAACAAAC-3'
rs1344142	Forward: 5'-TCCATCTTGTCATGGTCCAG-3'
	Reverse: 5'-CTTGATTCTGGGAGCCAGTC-3'
	Genotyping: 5'-TTGTCATGGTCCAGCCAGGCA-3'
rs17080528	Forward: 5'-GGGAGACAGGTTGGAGTGAG-3'
	Reverse: 5'-GCCACACTCTCTGGTTAGGC-3'
	Genotyping: 5'-TTCTCCAGCCAGGATCAGG-3'
rs17595772	Forward: 5'-GGGCCCTGTGTCCATATAAA-3'
	Reverse: 5'-GAGCCACGATGATTGCTCTT-3'
	Genotyping: 5'-CAGAGGGTGTTAATTCAGCCT-3'

Custom designed oligonucleotide primer sequences used in quantitative real-time PCR

Target	**Primer sequences**
NM_001128616	Forward: 5’-CGTGAATTTGGAATCCTGCT-3’
	Reverse: 5’-AATGCTGGAAAGCTTGGAGA-3’
ACTA2	Forward: 5’-GCAACACGAAGCTCATTGTAGA-3’
	Reverse: 5’-CTGACCCTGAAGTACCCGATAG-3’

Appendix III

Microarray candidate gene lists

Osteoblast-specific	Osteoclast-specific	
AHSG	ACP5	PRMT3
ALPL	ADORA1	RAC2
AMBN	AKAP5	RELB
AMELY	ARG1	RPL17
BGLAP	ATP5C1	RPS20
BMP1	ATP6V0D1	SNCA
BMP2	ATP6V1E1	TCIRG1
BMP3	BIRC3	THBS1
BMP4	CALCR	THBS2
BMP5	CALM1	TM7SF4
BMP6	CASP10	TNF
CDH11	CCL5	TNFRSF11A
COL10A1	CCR2	TRAF6
COL11A1	CD44	TSC2
COL12A1	CD70	XRCC1
COL1A1	CDH5	ZNF33A
COL1A2	CDK7	
COL2A1	CLCN7	
COMP	CSF2RA	
DMP1	CSF3R	
DSPP	CTSK	
ENAM	DLG1	
ESR1	DNAJB14	
FGFR1	DTX1	
GDF10	EGR1	
GNL3	EMILIN2	
IBSP	ERO1L	
MINPP1	ERP29	
MSX1	FMNL3	
PDGFRB	FUBP1	
PHEX	GABPA	
PTHLH	GBF1	
PTHR1	GSR	
RUNX2	HLA-C	
SOST	IL5	
SOX9	ILF3	
SP7	KCNA2	
SPP1	LHX1	
STATH	LTA	
TFIP11	MEFV	
TNFRSF11B	MMP9	
TNFSF11	MYL6	
TP53	NFATC1	
TUFT1	OAZ1	
TWIST1	OSCAR	
	PCTK1	

RHOA signalling pathway			
ACTA1	GNA12	MYH14	PKN1
ACTA2	GNAI1	MYH2	PLD1
ACTB	GNAI2	MYH3	PPAP2B
ACTC1	GNAI3	MYH4	PPP1R12A
ACTG1	IGF1	MYH6	PPP1R12B
ACTG2	IGF1R	MYH7	PTK2
ACTR2	IGF2	MYH7B	PTK2B
ACTR3	ITGA1	MYH8	RDX
ANGPTL2	ITGA10	MYH9	RHOA
ARHGAP1	ITGA11	MYL1	RHOB
ARHGAP22	ITGA2	MYL2	RHPN1
ARHGAP4	ITGA2B	MYL3	RHPN2
ARHGAP5	ITGA3	MYL4	ROCK1
ARHGDIA	ITGA4	MYL5	ROCK2
ARHGDIB	ITGA5	MYL7	RTKN
ARHGEF1	ITGA6	MYL9	SLC9A1
ARHGEF10	ITGA7	MYLK	TLN1
ARHGEF11	ITGA8	MYO10	VIL2
ARHGEF12	ITGA9	MYO15A	WASF1
ARHGEF15	ITGAD	MYO18B	
ARHGEF16	ITGAE	MYO1A	
ARHGEF2	ITGAL	MYO1B	
ARHGEF3	ITGAM	MYO1C	
ARHGEF4	ITGAV	MYO1D	
ARHGEF5	ITGAX	MYO1E	
ARHGEF6	ITGB1	MYO1F	
ARHGEF7	ITGB2	MYO3A	
ARHGEF9	ITGB3	MYO5A	
ARPC2	ITGB4	MYO5B	
ARPC3	ITGB5	MYO5C	
BAIAP2	ITGB6	MYO6	
CDC42EP1	ITGB7	MYO7A	
CDC42EP2	ITGB8	MYO7B	
CDC42EP3	KTN1	MYO9A	
CDC42EP4	LIMK1	MYO9B	
CDC42EP5	LIMK2	NET1	
CFL1	LPAR1	NGEF	
CFL2	MCF2L	PFN1	
CHN1	MGRN1	PFN2	
CHN2	MRLC2	PIP4K2B	
CIT	MSN	PIP4K2C	
EDG4	MYBPH	PIP5K1A	
EDG7	MYH1	PIP5K1B	
EPHA1	MYH10	PIP5K1C	
GDI1	MYH11	PIP5K2A	
GNA11	MYH13	PIP5K2B	

ELSEVIER LICENSE
TERMS AND CONDITIONS

Jul 17, 2011

This is a License Agreement between Benjamin Mullin ("You") and Elsevier ("Elsevier") provided by Copyright Clearance Center ("CCC"). The license consists of your order details, the terms and conditions provided by Elsevier, and the payment terms and conditions.

All payments must be made in full to CCC. For payment instructions, please see information listed at the bottom of this form.

Supplier	Elsevier Limited The Boulevard,Langford Lane Kidlington,Oxford,OX5 1GB,UK
Registered Company Number	1982084
Customer name	Benjamin Mullin
Customer address	1st Floor C Block Nedlands, other 6009
License number	2601091042363
License date	Feb 02, 2011
Licensed content publisher	Elsevier
Licensed content publication	The American Journal of Human Genetics
Licensed content title	Identification of a Role for the *ARHGEF3* Gene in Postmenopausal Osteoporosis
Licensed content author	Ben H. Mullin, Richard L. Prince, Ian M. Dick, Deborah J. Hart, Tim D. Spector, Frank Dudbridge, Scott G. Wilson
Licensed content date	6 June 2008
Licensed content volume number	82
Licensed content issue number	6
Number of pages	8
Start Page	1262
End Page	1269
Type of Use	reuse in a thesis/dissertation
Intended publisher of new work	other
Portion	full article
Format	both print and electronic
Are you the author of this Elsevier article?	Yes
Will you be translating?	No
Order reference number	
Title of your thesis/dissertation	Analysis of Candidate Genes Within the 3p14-p22 Region of the Human Genome for Association With Bone Mineral Density

	Phenotypes
Expected completion date	Feb 2011
Estimated size (number of pages)	150
Elsevier VAT number	GB 494 6272 12
Permissions price	0.00 USD
Value added tax 0.0%	0.00 USD / GBP
Total	0.00 USD
Terms and Conditions	

INTRODUCTION

1. The publisher for this copyrighted material is Elsevier. By clicking "accept" in connection with completing this licensing transaction, you agree that the following terms and conditions apply to this transaction (along with the Billing and Payment terms and conditions established by Copyright Clearance Center, Inc. ("CCC"), at the time that you opened your Rightslink account and that are available at any time at http://myaccount.copyright.com).

GENERAL TERMS

2. Elsevier hereby grants you permission to reproduce the aforementioned material subject to the terms and conditions indicated.

3. Acknowledgement: If any part of the material to be used (for example, figures) has appeared in our publication with credit or acknowledgement to another source, permission must also be sought from that source. If such permission is not obtained then that material may not be included in your publication/copies. Suitable acknowledgement to the source must be made, either as a footnote or in a reference list at the end of your publication, as follows:

"Reprinted from Publication title, Vol /edition number, Author(s), Title of article / title of chapter, Pages No., Copyright (Year), with permission from Elsevier [OR APPLICABLE SOCIETY COPYRIGHT OWNER]." Also Lancet special credit - "Reprinted from The Lancet, Vol. number, Author(s), Title of article, Pages No., Copyright (Year), with permission from Elsevier."

4. Reproduction of this material is confined to the purpose and/or media for which permission is hereby given.

5. Altering/Modifying Material: Not Permitted. However figures and illustrations may be altered/adapted minimally to serve your work. Any other abbreviations, additions, deletions and/or any other alterations shall be made only with prior written authorization of Elsevier Ltd. (Please contact Elsevier at permissions@elsevier.com)

6. If the permission fee for the requested use of our material is waived in this instance, please be advised that your future requests for Elsevier materials may attract a fee.

7. Reservation of Rights: Publisher reserves all rights not specifically granted in the combination of (i) the license details provided by you and accepted in the course of this licensing transaction, (ii) these terms and conditions and (iii) CCC's Billing and Payment terms and conditions.

8. License Contingent Upon Payment: While you may exercise the rights licensed immediately upon issuance of the license at the end of the licensing process for the transaction, provided that you have disclosed complete and accurate details of your proposed use, no license is finally effective unless and until full payment is received from you (either by publisher or by CCC) as provided in CCC's Billing and Payment terms and conditions. If full payment is not received on a timely basis, then any license preliminarily granted shall be deemed automatically revoked and shall be void as if never granted. Further, in the event that you breach any of these terms and conditions or any of CCC's Billing and Payment terms and conditions, the license is automatically revoked and shall be void as if never granted. Use of materials as described in a revoked license, as well as any use of the materials beyond the scope of an unrevoked license, may constitute copyright infringement and publisher reserves the right to take any and all action to protect its copyright in the materials.

9. Warranties: Publisher makes no representations or warranties with respect to the licensed material.

10. Indemnity: You hereby indemnify and agree to hold harmless publisher and CCC, and their respective officers, directors, employees and agents, from and against any and all claims arising out of your use of the licensed material other than as specifically authorized pursuant to this license.

11. No Transfer of License: This license is personal to you and may not be sublicensed, assigned, or transferred by you to any other person without publisher's written permission.

12. No Amendment Except in Writing: This license may not be amended except in a writing signed by both parties (or, in the case of publisher, by CCC on publisher's behalf).

13. Objection to Contrary Terms: Publisher hereby objects to any terms contained in any purchase order, acknowledgment, check endorsement or other writing prepared by you, which terms are inconsistent with these terms and conditions or CCC's Billing and Payment terms and conditions. These terms and conditions, together with CCC's Billing and Payment terms and conditions (which are incorporated herein), comprise the entire agreement between you and publisher (and CCC) concerning this licensing transaction. In the event of any conflict between your obligations established by these terms and conditions and those established by CCC's Billing and Payment terms and conditions, these terms and conditions shall control.

14. Revocation: Elsevier or Copyright Clearance Center may deny the permissions described in this License at their sole discretion, for any reason or no reason, with a full refund payable to you. Notice of such denial will be made using the contact information provided by you. Failure to receive such notice will not alter or invalidate the denial. In no event will Elsevier or Copyright Clearance Center be responsible or liable for any costs, expenses or damage incurred by you as a result of a denial of your permission request, other than a refund of the amount(s) paid by you to Elsevier and/or Copyright Clearance Center for denied permissions.

LIMITED LICENSE

The following terms and conditions apply only to specific license types:

15. **Translation**: This permission is granted for non-exclusive world **English** rights only unless your license was granted for translation rights. If you licensed translation rights you may only translate this content into the languages you requested. A professional translator

must perform all translations and reproduce the content word for word preserving the integrity of the article. If this license is to re-use 1 or 2 figures then permission is granted for non-exclusive world rights in all languages.

16. **Website**: The following terms and conditions apply to electronic reserve and author websites:
Electronic reserve: If licensed material is to be posted to website, the web site is to be password-protected and made available only to bona fide students registered on a relevant course if:
This license was made in connection with a course,
This permission is granted for 1 year only. You may obtain a license for future website posting,
All content posted to the web site must maintain the copyright information line on the bottom of each image,
A hyper-text must be included to the Homepage of the journal from which you are licensing at http://www.sciencedirect.com/science/journal/xxxxx or the Elsevier homepage for books at http://www.elsevier.com , and
Central Storage: This license does not include permission for a scanned version of the material to be stored in a central repository such as that provided by Heron/XanEdu.

17. **Author website** for journals with the following additional clauses:

All content posted to the web site must maintain the copyright information line on the bottom of each image, and
he permission granted is limited to the personal version of your paper. You are not allowed to download and post the published electronic version of your article (whether PDF or HTML, proof or final version), nor may you scan the printed edition to create an electronic version,
A hyper-text must be included to the Homepage of the journal from which you are licensing at http://www.sciencedirect.com/science/journal/xxxxx , As part of our normal production process, you will receive an e-mail notice when your article appears on Elsevier's online service ScienceDirect (www.sciencedirect.com). That e-mail will include the article's Digital Object Identifier (DOI). This number provides the electronic link to the published article and should be included in the posting of your personal version. We ask that you wait until you receive this e-mail and have the DOI to do any posting.
Central Storage: This license does not include permission for a scanned version of the material to be stored in a central repository such as that provided by Heron/XanEdu.

18. **Author website** for books with the following additional clauses:
Authors are permitted to place a brief summary of their work online only.
A hyper-text must be included to the Elsevier homepage at http://www.elsevier.com

All content posted to the web site must maintain the copyright information line on the bottom of each image
You are not allowed to download and post the published electronic version of your chapter, nor may you scan the printed edition to create an electronic version.
Central Storage: This license does not include permission for a scanned version of the material to be stored in a central repository such as that provided by Heron/XanEdu.

19. **Website** (regular and for author): A hyper-text must be included to the Homepage of the journal from which you are licensing at
http://www.sciencedirect.com/science/journal/xxxxx. or for books to the Elsevier homepage

at http://www.elsevier.com

20. **Thesis/Dissertation**: If your license is for use in a thesis/dissertation your thesis may be submitted to your institution in either print or electronic form. Should your thesis be published commercially, please reapply for permission. These requirements include permission for the Library and Archives of Canada to supply single copies, on demand, of the complete thesis and include permission for UMI to supply single copies, on demand, of the complete thesis. Should your thesis be published commercially, please reapply for permission.

21. **Other Conditions**:

v1.6

Gratis licenses (referencing $0 in the Total field) are free. Please retain this printable license for your reference. No payment is required.

If you would like to pay for this license now, please remit this license along with your payment made payable to "COPYRIGHT CLEARANCE CENTER" otherwise you will be invoiced within 48 hours of the license date. Payment should be in the form of a check or money order referencing your account number and this invoice number RLNK10924785.
Once you receive your invoice for this order, you may pay your invoice by credit card. Please follow instructions provided at that time.

Make Payment To:
Copyright Clearance Center
Dept 001
P.O. Box 843006
Boston, MA 02284-3006

For suggestions or comments regarding this order, contact Rightslink Customer Support: customercare@copyright.com or +1-877-622-5543 (toll free in the US) or +1-978-646-2777.

ELSEVIER LICENSE
TERMS AND CONDITIONS

Jul 17, 2011

This is a License Agreement between Benjamin Mullin ("You") and Elsevier ("Elsevier") provided by Copyright Clearance Center ("CCC"). The license consists of your order details, the terms and conditions provided by Elsevier, and the payment terms and conditions.

All payments must be made in full to CCC. For payment instructions, please see information listed at the bottom of this form.

Supplier	Elsevier Limited The Boulevard,Langford Lane Kidlington,Oxford,OX5 1GB,UK
Registered Company Number	1982084
Customer name	Benjamin Mullin
Customer address	1st Floor C Block Nedlands, other 6009
License number	2592380626889
License date	Jan 19, 2011
Licensed content publisher	Elsevier
Licensed content publication	Bone
Licensed content title	Further genetic evidence suggesting a role for the RhoGTPase-RhoGEF pathway in osteoporosis
Licensed content author	Ben H. Mullin, Richard L. Prince, Cyril Mamotte, Tim D. Spector, Deborah J. Hart, Frank Dudbridge, Scott G. Wilson
Licensed content date	August 2009
Licensed content volume number	45
Licensed content issue number	2
Number of pages	5
Start Page	387
End Page	391
Type of Use	reuse in a thesis/dissertation
Portion	full article
Format	both print and electronic
Are you the author of this Elsevier article?	Yes
Will you be translating?	No
Order reference number	
Title of your thesis/dissertation	Analysis of Candidate Genes Within the 3p14-p22 Region of the Human Genome for Association With Bone Mineral Density Phenotypes
Expected completion date	Feb 2011

Estimated size (number of pages)	150
Elsevier VAT number	GB 494 6272 12
Permissions price	0.00 USD
Value added tax 0.0%	0.0 USD / 0.0 GBP
Total	0.00 USD
Terms and Conditions	

INTRODUCTION

1. The publisher for this copyrighted material is Elsevier. By clicking "accept" in connection with completing this licensing transaction, you agree that the following terms and conditions apply to this transaction (along with the Billing and Payment terms and conditions established by Copyright Clearance Center, Inc. ("CCC"), at the time that you opened your Rightslink account and that are available at any time at http://myaccount.copyright.com).

GENERAL TERMS

2. Elsevier hereby grants you permission to reproduce the aforementioned material subject to the terms and conditions indicated.

3. Acknowledgement: If any part of the material to be used (for example, figures) has appeared in our publication with credit or acknowledgement to another source, permission must also be sought from that source. If such permission is not obtained then that material may not be included in your publication/copies. Suitable acknowledgement to the source must be made, either as a footnote or in a reference list at the end of your publication, as follows:

"Reprinted from Publication title, Vol /edition number, Author(s), Title of article / title of chapter, Pages No., Copyright (Year), with permission from Elsevier [OR APPLICABLE SOCIETY COPYRIGHT OWNER]." Also Lancet special credit - "Reprinted from The Lancet, Vol. number, Author(s), Title of article, Pages No., Copyright (Year), with permission from Elsevier."

4. Reproduction of this material is confined to the purpose and/or media for which permission is hereby given.

5. Altering/Modifying Material: Not Permitted. However figures and illustrations may be altered/adapted minimally to serve your work. Any other abbreviations, additions, deletions and/or any other alterations shall be made only with prior written authorization of Elsevier Ltd. (Please contact Elsevier at permissions@elsevier.com)

6. If the permission fee for the requested use of our material is waived in this instance, please be advised that your future requests for Elsevier materials may attract a fee.

7. Reservation of Rights: Publisher reserves all rights not specifically granted in the combination of (i) the license details provided by you and accepted in the course of this licensing transaction, (ii) these terms and conditions and (iii) CCC's Billing and Payment terms and conditions.

8. License Contingent Upon Payment: While you may exercise the rights licensed immediately upon issuance of the license at the end of the licensing process for the

transaction, provided that you have disclosed complete and accurate details of your proposed use, no license is finally effective unless and until full payment is received from you (either by publisher or by CCC) as provided in CCC's Billing and Payment terms and conditions. If full payment is not received on a timely basis, then any license preliminarily granted shall be deemed automatically revoked and shall be void as if never granted. Further, in the event that you breach any of these terms and conditions or any of CCC's Billing and Payment terms and conditions, the license is automatically revoked and shall be void as if never granted. Use of materials as described in a revoked license, as well as any use of the materials beyond the scope of an unrevoked license, may constitute copyright infringement and publisher reserves the right to take any and all action to protect its copyright in the materials.

9. Warranties: Publisher makes no representations or warranties with respect to the licensed material.

10. Indemnity: You hereby indemnify and agree to hold harmless publisher and CCC, and their respective officers, directors, employees and agents, from and against any and all claims arising out of your use of the licensed material other than as specifically authorized pursuant to this license.

11. No Transfer of License: This license is personal to you and may not be sublicensed, assigned, or transferred by you to any other person without publisher's written permission.

12. No Amendment Except in Writing: This license may not be amended except in a writing signed by both parties (or, in the case of publisher, by CCC on publisher's behalf).

13. Objection to Contrary Terms: Publisher hereby objects to any terms contained in any purchase order, acknowledgment, check endorsement or other writing prepared by you, which terms are inconsistent with these terms and conditions or CCC's Billing and Payment terms and conditions. These terms and conditions, together with CCC's Billing and Payment terms and conditions (which are incorporated herein), comprise the entire agreement between you and publisher (and CCC) concerning this licensing transaction. In the event of any conflict between your obligations established by these terms and conditions and those established by CCC's Billing and Payment terms and conditions, these terms and conditions shall control.

14. Revocation: Elsevier or Copyright Clearance Center may deny the permissions described in this License at their sole discretion, for any reason or no reason, with a full refund payable to you. Notice of such denial will be made using the contact information provided by you. Failure to receive such notice will not alter or invalidate the denial. In no event will Elsevier or Copyright Clearance Center be responsible or liable for any costs, expenses or damage incurred by you as a result of a denial of your permission request, other than a refund of the amount(s) paid by you to Elsevier and/or Copyright Clearance Center for denied permissions.

LIMITED LICENSE

The following terms and conditions apply only to specific license types:

15. **Translation**: This permission is granted for non-exclusive world **English** rights only unless your license was granted for translation rights. If you licensed translation rights you may only translate this content into the languages you requested. A professional translator must perform all translations and reproduce the content word for word preserving the integrity of the article. If this license is to re-use 1 or 2 figures then permission is granted for

non-exclusive world rights in all languages.

16. **Website**: The following terms and conditions apply to electronic reserve and author websites:
Electronic reserve: If licensed material is to be posted to website, the web site is to be password-protected and made available only to bona fide students registered on a relevant course if:
This license was made in connection with a course,
This permission is granted for 1 year only. You may obtain a license for future website posting,
All content posted to the web site must maintain the copyright information line on the bottom of each image,
A hyper-text must be included to the Homepage of the journal from which you are licensing at http://www.sciencedirect.com/science/journal/xxxxx or the Elsevier homepage for books at http://www.elsevier.com , and
Central Storage: This license does not include permission for a scanned version of the material to be stored in a central repository such as that provided by Heron/XanEdu.

17. **Author website** for journals with the following additional clauses:

All content posted to the web site must maintain the copyright information line on the bottom of each image, and
he permission granted is limited to the personal version of your paper. You are not allowed to download and post the published electronic version of your article (whether PDF or HTML, proof or final version), nor may you scan the printed edition to create an electronic version,
A hyper-text must be included to the Homepage of the journal from which you are licensing at http://www.sciencedirect.com/science/journal/xxxxx , As part of our normal production process, you will receive an e-mail notice when your article appears on Elsevier's online service ScienceDirect (www.sciencedirect.com). That e-mail will include the article's Digital Object Identifier (DOI). This number provides the electronic link to the published article and should be included in the posting of your personal version. We ask that you wait until you receive this e-mail and have the DOI to do any posting.
Central Storage: This license does not include permission for a scanned version of the material to be stored in a central repository such as that provided by Heron/XanEdu.

18. **Author website** for books with the following additional clauses:
Authors are permitted to place a brief summary of their work online only.
A hyper-text must be included to the Elsevier homepage at http://www.elsevier.com

All content posted to the web site must maintain the copyright information line on the bottom of each image
You are not allowed to download and post the published electronic version of your chapter, nor may you scan the printed edition to create an electronic version.
Central Storage: This license does not include permission for a scanned version of the material to be stored in a central repository such as that provided by Heron/XanEdu.

19. **Website** (regular and for author): A hyper-text must be included to the Homepage of the journal from which you are licensing at
http://www.sciencedirect.com/science/journal/xxxxx. or for books to the Elsevier homepage at http://www.elsevier.com

20. **Thesis/Dissertation**: If your license is for use in a thesis/dissertation your thesis may be submitted to your institution in either print or electronic form. Should your thesis be published commercially, please reapply for permission. These requirements include permission for the Library and Archives of Canada to supply single copies, on demand, of the complete thesis and include permission for UMI to supply single copies, on demand, of the complete thesis. Should your thesis be published commercially, please reapply for permission.

21. **Other Conditions**:

v1.6

Gratis licenses (referencing $0 in the Total field) are free. Please retain this printable license for your reference. No payment is required.

If you would like to pay for this license now, please remit this license along with your payment made payable to "COPYRIGHT CLEARANCE CENTER" otherwise you will be invoiced within 48 hours of the license date. Payment should be in the form of a check or money order referencing your account number and this invoice number RLNK10915980.
Once you receive your invoice for this order, you may pay your invoice by credit card. Please follow instructions provided at that time.

Make Payment To:
Copyright Clearance Center
Dept 001
P.O. Box 843006
Boston, MA 02284-3006

For suggestions or comments regarding this order, contact Rightslink Customer Support: customercare@copyright.com or +1-877-622-5543 (toll free in the US) or +1-978-646-2777.