

**Role of Thyroid Hormone Receptor Alpha in Susceptibility
and Progression of Osteoarthritis**

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ABSTRACT

Osteoarthritis (OA) is a debilitating joint disease characterised by articular cartilage damage, chondrocyte hypertrophy and subchondral bone changes. Thyroid hormone (TH) regulates chondrocyte differentiation, endochondral ossification and bone maintenance, acting mainly through thyroid hormone receptor alpha ($TR\alpha$). Genetic, pathological and experimental data have implicated a role for TH availability in OA pathogenesis. I hypothesised that inhibition of TH signalling will decrease susceptibility to OA and delay disease progression.

Osteoarthritis was assessed in mice treated with dronedarone (a $TR\alpha$ antagonist), $TR\alpha$ knockouts ($TR\alpha^{0/0}$) or wild type controls 4, 8 and 16 weeks after joint destabilisation surgery (DMM). Overall, articular cartilage damage was not reduced in either mutant or dronedarone treated mice but the studies were underpowered. Subchondral bone mineral content (BMC) in sham operated knees was increased in mutants but reduced with dronedarone treatment. Destabilising surgery increased subchondral BMC and mass but changes were attenuated in mutant and dronedarone treated mice.

Mutant mice lacking iodothyronine deiodinase type 2 (D2KO) that are unable to activate thyroid hormone locally were investigated for OA development with ageing. Mutants had no change in cartilage damage but had increased subchondral BMC.

$TR\alpha$ was further investigated as an osteoporosis drug target. Significantly, dronedarone increased femoral cortical thickness and BMC but did not affect bone strength.

These results suggest a regulatory role for TH in subchondral bone remodelling. The absence of chondroprotection suggest that any potential role of TH in osteoarthritis pathogenesis may be mediated via subchondral bone. Further adequately powered studies with dronedarone are needed to confirm cartilage and bone observations and should include mechanistic studies to explain subchondral bone changes. Further experiments using established models of osteoporosis and more sensitive measures of bone strength should be used to confirm $TR\alpha$ as a novel drug target for osteoporosis.

CONTENTS

ABSTRACT	2
CONTENTS.....	3
LIST OF FIGURES	9
LIST OF TABLES.....	12
ABBREVIATIONS	14
DECLARATION OF ORIGINALITY AND COPYRIGHT DECLARATION.....	18
ACKNOWLEDGEMENTS.....	19
THE THESIS	20
CHAPTER 1 INTRODUCTION	22
1.1 BACKGROUND.....	22
1.1.1 <i>Definition and diagnosis of osteoarthritis.....</i>	<i>22</i>
1.1.2 <i>Epidemiology.....</i>	<i>22</i>
1.1.3 <i>Clinical risk factors and natural history</i>	<i>22</i>
1.1.4 <i>Therapy</i>	<i>23</i>
1.2 NORMAL SYNOVIAL JOINTS	24
1.2.1 <i>Hyaline Articular Cartilage.....</i>	<i>24</i>
1.2.2 <i>Calcified articular cartilage.....</i>	<i>28</i>
1.2.3 <i>Subchondral Bone.....</i>	<i>28</i>
1.2.4 <i>Menisci</i>	<i>29</i>
1.2.5 <i>Ligaments.....</i>	<i>29</i>
1.2.6 <i>Joint capsule and synovium.....</i>	<i>30</i>
1.2.7 <i>Developmental biology of joints.....</i>	<i>30</i>
1.2.8 <i>Embryonic derivation and differentiation of articular and growth plate chondrocytes.....</i>	<i>33</i>
1.3 NORMAL BONE.....	35
1.3.1 <i>Structural and developmental organisation</i>	<i>35</i>
1.4 BONE CELLS.....	38
1.4.1 <i>Osteoblasts.....</i>	<i>38</i>
1.4.2 <i>Osteoclasts</i>	<i>44</i>
1.4.3 <i>Osteocytes.....</i>	<i>48</i>
1.5 EXTRACELLULAR MATRIX OF BONE	51

1.5.1	<i>Mineral</i>	51
1.5.2	<i>Collagen</i>	51
1.6	ENDOCHONDRAL OSSIFICATION AND THE GROWTH PLATE.....	53
1.7	REGULATION OF BONE MODELLING & REMODELLING.....	57
1.7.1	<i>Bone modelling</i>	57
1.7.2	<i>Bone remodelling</i>	57
1.8	PATHOLOGY AND PATHOPHYSIOLOGY OF OSTEOARTHRITIS.....	60
1.8.1	<i>Articular cartilage</i>	60
1.8.2	<i>Subchondral bone remodelling in osteoarthritis</i>	63
1.9	THYROID HORMONE ACTION	67
1.9.1	<i>Introduction</i>	67
1.9.2	<i>Thyroid hormone synthesis</i>	67
1.9.3	<i>The hypothalamic-pituitary thyroid axis</i>	68
1.9.4	<i>Thyroid hormone transporters</i>	70
1.9.5	<i>Thyroid hormone metabolism</i>	70
1.9.6	<i>Thyroid hormone receptors</i>	73
1.10	THYROID HORMONE ACTION IN THE DEVELOPING AND ADULT SKELETON.....	76
1.10.1	<i>Transporters</i>	76
1.10.2	<i>Deiodinases</i>	76
1.10.3	<i>Thyroid hormone receptors</i>	78
1.10.4	<i>Altered systemic thyroid hormone levels and skeletal development</i>	81
1.10.5	<i>Syndromes of resistance to thyroid hormone</i>	81
1.10.6	<i>Skeletal phenotypes of juvenile thyroid hormone receptor knockout mice</i>	82
1.10.7	<i>Thyroid hormone action in osteoblasts</i>	85
1.10.8	<i>Thyroid hormone action in osteoclasts</i>	85
1.10.9	<i>Skeleton remodelling in hypo and hyperthyroidism</i>	86
1.10.10	<i>Skeletal phenotypes of adult thyroid hormone receptor knockout mice</i>	87
1.10.11	<i>The role of aporeceptor</i>	88
1.10.12	<i>Thyroid hormone receptor β and the skeleton</i>	89
1.11	THYROID HORMONE ACTION IN THE JOINT.....	90
1.11.1	<i>Human genetic studies identify thyroid hormone regulation as a modifier of osteoarthritis susceptibility</i>	90
1.11.2	<i>Functional genomic data for DIO2 in osteoarthritis</i>	92
1.11.3	<i>Do patients with thyroid disease develop joint disease?</i>	93
1.11.4	<i>Regulation of Thyroid hormone action in articular cartilage and subchondral bone</i>	94

1.11.5	<i>Effects of thyroid hormone on articular cartilage.....</i>	94
1.11.6	<i>Chondrotoxicity of a thyroid hormone receptor agonist – Eprotirome.....</i>	95
1.11.7	<i>Conclusion.....</i>	95
1.12	EXPERIMENTAL MODELS OF OSTEOARTHRITIS.....	97
1.12.1	<i>Genetic model of skeletal hypothyroidism - TR$\alpha^{0/0}$ mouse.....</i>	97
1.12.2	<i>Dronedarone, an antagonist of Thyroid hormone receptor alpha</i>	99
1.13	EXPERIMENTAL DESIGN.....	102
1.13.1	<i>Hypothesis.....</i>	102
1.13.2	<i>Aims</i>	102
CHAPTER 2	MATERIAL AND METHODS	103
2.1	ANIMALS	103
2.1.1	<i>TR$\alpha^{0/0}$</i>	103
2.1.2	<i>Deiodinase 1 and deiodinase 2 knockout mice</i>	103
2.2	OSTEOARTHRITIS INDUCING SURGERY.....	104
2.3	ENVIRONMENTAL ENRICHMENT DEVICES	105
2.4	CAGE DENSITY	105
2.5	DRONEDARONE ADMINISTRATION	106
2.6	CALCEIN LABELLING.....	106
2.7	SERUM SAMPLES.....	106
2.8	DISSECTION, FIXATION AND STORAGE OF SKELETON	106
2.9	JOINT IMAGING.....	107
2.9.1	<i>Histology.....</i>	107
2.9.2	<i>X-ray microradiography.....</i>	113
2.9.3	<i>Micro computed tomography (microCT) analysis</i>	115
2.9.4	<i>Backscattered electron scanning microscopy (BSE-SEM).....</i>	118
2.10	NON-ARTICULAR BONE IMAGING.....	118
2.10.1	<i>X-ray microradiography.....</i>	118
2.10.2	<i>Micro computed tomography (microCT) analysis.....</i>	120
2.10.3	<i>Confocal microscopy.....</i>	121
2.11	MECHANICAL LOADING AND BONE STRENGTH	124
2.12	STATISTICS	125
2.12.1	<i>Faxitron</i>	125
2.12.2	<i>MicroCT & Histology of knee joint.....</i>	125
2.12.3	<i>MicroCT of whole bone</i>	126
2.12.4	<i>Dronedarone Consumption</i>	126
2.12.5	<i>Body weight</i>	126

CHAPTER 3	VALIDATION OF DESTABILISATION OF MEDIAL MENISCUS AS A MODEL FOR OSTEOARTHRITIS.....	127
3.1	BACKGROUND.....	127
3.2	HISTOLOGICAL VALIDATION OF SURGICAL TECHNIQUE.....	127
3.2.1	<i>Quantitative assessment of cartilage damage following DMM surgery</i>	<i>129</i>
3.3	VALIDATION OF DMM SURGERY USING FAXITRON	131
3.4	VALIDATION OF DMM USING MICRO CT	134
3.5	EFFECT OF CAGE DENSITY ON CARTILAGE DAMAGE.....	135
3.6	DISCUSSION	137
CHAPTER 4	VALIDATION OF VOLUNTARY ORAL DOSING OF DRONEDARONE.	138
4.1	BACKGROUND.....	138
4.2	EFFECTIVE, VOLUNTARY, ORAL DOSING.....	138
4.3	RESULTS.....	140
4.3.1	<i>Effectiveness of dosing regime.....</i>	<i>140</i>
4.3.2	<i>Effect of dosing regimen on body weight.....</i>	<i>140</i>
4.4	DISCUSSION	142
CHAPTER 5	EFFECTS OF TRα INHIBITION AND DELETION ON OSTEOARTHRITIS	143
5.1	BACKGROUND.....	143
5.2	RESULTS.....	143
5.2.1	<i>X-ray microradiography.....</i>	<i>143</i>
5.2.2	<i>MicroCT.....</i>	<i>151</i>
5.2.3	<i>Backscattered scanning electron microscopy (BSE-SEM).....</i>	<i>159</i>
5.2.4	<i>Cartilage histology.....</i>	<i>162</i>
5.3	DISCUSSION	166
5.3.1	<i>TR$\alpha^{0/0}$ mice develop progressive subchondral osteosclerosis with ageing ...</i>	<i>166</i>
5.3.2	<i>BMC is increased in TR$\alpha^{0/0}$ mice but is mineralisation?</i>	<i>166</i>
5.3.3	<i>Methodological considerations in assessing subchondral bone</i>	<i>167</i>
5.3.4	<i>TRα inhibition reduces subchondral BMC and TRα deletion attenuates bone changes following OA-provocation.....</i>	<i>167</i>
5.3.5	<i>Methodological and statistical considerations in articular cartilage evaluation.....</i>	<i>169</i>
5.3.6	<i>Inconsistent chondroprotection with dronedarone</i>	<i>171</i>
CHAPTER 6	SPONTANEOUS OSTEOARTHRITIS IN MICE LACKING TYPE 2 IODOTHYRONINE DEIODINASE	172

6.1	BACKGROUND.....	172
6.2	RESULTS.....	173
6.2.1	<i>Length of articular cartilage.....</i>	<i>173</i>
6.2.2	<i>Articular cartilage is not altered in D2KO mice.....</i>	<i>173</i>
6.2.3	<i>Subchondral bone area is unchanged but bone mineral content is increased in D2KO mice</i>	<i>175</i>
6.3	DISCUSSION	176
CHAPTER 7 EFFECTS OF TRα INHIBITION ON BONE MASS AND STRENGTH.....		179
7.1	BACKGROUND.....	179
7.2	RESULTS.....	180
7.2.1	<i>Structural Parameters</i>	<i>180</i>
7.2.2	<i>Bone mineral content.....</i>	<i>185</i>
7.2.3	<i>Bone formation.....</i>	<i>185</i>
7.2.4	<i>Biomechanical testing.....</i>	<i>188</i>
7.3	DISCUSSION.....	189
7.3.1	<i>Cortical bone mass is increased with TRα inhibition.....</i>	<i>189</i>
7.3.2	<i>Cortex specific increase in bone mineral content by TRα inhibition.....</i>	<i>191</i>
7.3.3	<i>Cortical bone phenotype is likely to be mediated by reduced osteoclastic resorption.....</i>	<i>191</i>
7.3.4	<i>Bone strength is not changed with dronedarone</i>	<i>192</i>
7.3.5	<i>Future experiments and clinical application.....</i>	<i>192</i>
CHAPTER 8 GENERAL DISCUSSION & CONCLUSIONS.....		194
8.1	SUBCHONDRAL BONE CHANGES DUE TO REDUCED THYROID HORMONE SIGNALLING ARE NOT ACCOMPANIED BY ALTERED ARTICULAR CARTILAGE INTEGRITY	194
8.2	INHIBITION OF TR α HAS DIVERGENT EFFECTS ON SUBCHONDRAL BONE AND CORTICAL LONGBONE	197
8.3	DOES INHIBITION OF THYROID HORMONE SIGNALLING DECREASE SUSCEPTIBILITY TO OSTEOARTHRITIS AND DELAY DISEASE PROGRESSION?	198
8.4	FUTURE RESEARCH FOR OSTEOARTHRITIS.....	198
8.4.1	<i>Optimising conditions to assess articular cartilage damage.....</i>	<i>198</i>
8.4.2	<i>Mechanistic studies to investigate subchondral bone changes.....</i>	<i>199</i>
8.4.3	<i>Different models of altered thyroid hormone signalling.....</i>	<i>199</i>
APPENDIX.....		201
REFERENCES.....		215

PUBLICATIONS FROM THESIS	246
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LIST OF FIGURES

Figure 1.1 Normal synovial joint & articular surface	25
Figure 1.2 Extracellular matrix of articular cartilage.....	27
Figure 1.3 Joint Formation	31
Figure 1.4 Transcriptional Control of skeletal cells	34
Figure 1.5 Macroscopic structure of long bones	36
Figure 1.6 Hierarchical structural organisation of bone.....	37
Figure 1.7 Extracellular signals regulating osteoblast differentiation	43
Figure 1.8 Resorbing osteoclast.....	47
Figure 1.9 Type I Collagen synthesis and folding	52
Figure 1.10 Developmental steps during endochondral ossification	54
Figure 1.11 Growth plate regulation by Parathyroid hormone-related protein (PTHrP) and Indian hedgehog (IHH)	56
Figure 1.12 Bone remodelling cycle	59
Figure 1.13 Osteoarthritis pathology	62
Figure 1.14 Subchondral bone changes in osteoarthritis	66
Figure 1.15 Control of circulating thyroid hormone level and target cell thyroid status.....	69
Figure 1.16 Thyroid hormone metabolism by deiodinases	71
Figure 1.17 Thyroid hormone receptor (TR) control of hormone action	75
Figure 1.18 Thyroid hormone action in growth plate cartilage.....	80
Figure 1.19 Thyroid hormone status in pituitary and skeletal cells	84
Figure 1.20 Chemical structure of dronedarone.....	99
Figure 2.1 Diagram of right knee joint	105
Figure 2.2 OARSI pathology scoring	108
Figure 2.3 Atlas of knee histology	110
Figure 2.4 Standardised OA score and erosion index	111
Figure 2.5 Subchondral bone area	112
Figure 2.6 Quantitative digital X-ray microradiography of subchondral bone	114
Figure 2.7 Radiological Atlas of 18-week old male wild type C57Bl/6 mouse knee	116

Figure 2.8 Subchondral bone regions of interest	117
Figure 2.9 Image processing for bone mineral content	118
Figure 2.10 Cortical thickness by X-ray	119
Figure 2.11 Montaged confocal image of a humerus	122
Figure 2.12 Confocal image showing calcein labelling	123
Figure 2.13 Load displacement curves from three-point bend tests of humeri.....	124
Figure 3.1 Histological validation of DMM model.....	128
Figure 3.2 Cartilage damage following knee destabilising surgery (DMM).....	130
Figure 3.3 Knee destabilisation surgery increases subchondral BMC.....	132
Figure 3.4 Subchondral bone mineral content is increased in destabilised knee joints.....	133
Figure 3.5 Subchondral bone assessment with MicroCT	134
Figure 3.6 Relationship between cage density and cartilage damage	136
Figure 4.1 Metal mesh divider used for contemporaneous, voluntary oral dosing of two mice ..	139
Figure 4.2 Voluntary oral dosing	141
Figure 5.1 Comparison of BMC in medial tibial plateau subchondral bone between destabilised and sham operated knees	145
Figure 5.2 Dronedarone treatment reduces subchondral bone mineral content	146
Figure 5.3 Change in bone mineral content with age and surgery in WT and TR $\alpha^{0/0}$ mice	148
Figure 5.4 Subchondral BMC in medial tibial plateau 16 weeks post DMM	149
Figure 5.5 Subchondral bone with dronedarone 16 weeks after surgery	153
Figure 5.6 MicroCT images (coronal view) of subchondral bone with dronedarone 16 weeks after surgery.....	154
Figure 5.7 MicroCT images (sagittal view) of subchondral bone with dronedarone 16 weeks after surgery.....	155
Figure 5.8 MicroCT of subchondral bone in TR $\alpha^{0/0}$ mice.....	157
Figure 5.9 MicroCT images of subchondral bone in TR $\alpha^{0/0}$ mice 8 weeks after knee surgery....	158
Figure 5.10 Scanning Electron Microscopy (SEM) images of tibias in dronedarone treated mice 8 weeks after surgery.....	160
Figure 5.11 Scanning Electron Microscopy (SEM) images of tibias in TR $\alpha^{0/0}$ mice 8 weeks after surgery.....	161
Figure 5.12 Cartilage damage in dronedarone treated mice	163

Figure 5.13 Cartilage damage in TRα^{0/0} mice	165
Figure 6.1 OA pathology and scores in WT, D2KO and D1D2KO mice	174
Figure 6.2 Subchondral bone mineral content in WT, D2KO and D1D2KO mice.....	175
Figure 7.1 Cortical thickness determined by X-ray microradiography.....	181
Figure 7.2 Femoral cortical thickness analysis by microCT	182
Figure 7.3 Images from microCT of whole femurs in mice treated with dronedarone	183
Figure 7.4 Bone mineral content in whole bone following dronedarone treatment.....	186
Figure 7.5 Bone formation with dronedarone.....	187
Figure 7.6 Biomechanical properties in bones treated with dronedarone.....	188

LIST OF TABLES

Table 3.1 OARSI Scores following DMM surgery	129
Table 5.1 Subchondral bone in mice 8 weeks after knee surgery	152
Table 7.1 Trabecular bone assessment with microCT	184
Table 8.1 Status of TR in different experimental models.....	196

ABBREVIATIONS

ACC	Articular chondrocyte
ADAMTS	A disintegrin and metalloproteinase with thrombospondin motifs
ANOVA	Analysis of variance
ATF4	Activating transcription factor 4
BA/TA	Bone area relative to tissue area
BFR	Bone formation rate
BMP	Bone morphogenetic protein
BMD	Bone mineral density
BMU	Basic multicellular unit
BRC	The bone remodelling compartment
BV/TV	Bone volume fraction
CD	Connectivity density
CSLM	Confocal scanning light microscopy
DIO1	Iodothyronine deiodinase enzyme type 1
DIO2	Iodothyronine deiodinase enzyme type 2
DIO3	Iodothyronine deiodinase enzyme type 3
D1D2KO	Iodothyronine deiodinase type 1 and 2 double knockout mice
D2KO	Iodothyronine deiodinase enzyme type 2 knockout mice
D3KO	Iodothyronine deiodinase enzyme type 3 knockout mice
D20	Dronedarone 20mg/kg/day
D60	Dronedarone 60mg/kg/day
DEHAL1	Iodotyrosine dehalogenase 1
DMM	Destabilisation of medial meniscus
DMP-1	Dentin matrix protein 1
DUOX1	Dual oxidase 1
DUOX2	Dual oxidase 2
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
FGF	Fibroblast growth factor
FGFR	Fibroblast growth factor receptor
GAG	Glycosaminoglycan

GDP	Gross domestic product
GDF-5	Growth and differentiation factor-5
GPC	Growth plate chondrocyte
GWAS	Genome wide association studies
HAC	Hyaline articular cartilage
HC	Hypertrophic chondrocytes
HES	Hairy and Enhancer of Split
HEY	HES-related with YRPW motif
HPCL	High performance liquid chromatography
HPT axis	Hypothalamus-pituitary-thyroid axis
IGF-1	Insulin-like growth factor 1
IGFBP	Insulin-like growth factor binding protein
IHH	Indian hedgehog
IL-1β	Interleukin 1 β
JP	Joint progenitor
Km	Michalis constant
LAT	L-type amino acid transporters
M-CSF	Macrophage-colony stimulating factor
MAR	Mineral apposition rate
MCT	Monocarboxylate transporters
MEPE	Matrix extracellular phosphoglycoprotein
MFC	Medial femoral condyle
MicroCT	Micro computed tomography
MIT	3-iodotyrosine (monoiodotyrosine)
DIT	3,5-diiodotyrosine
MMP	Matrix metalloproteinase
mRNA	Messenger ribonucleic acid
MS	Mineralising surface
MTP	Medial tibial plateau
NCoR	Nuclear receptor corepressor
NFκB	Nuclear factor κ B
NICD	Notch intracellular domain
NO	Nitric oxide

OA	Osteoarthritis
OARSI	Osteoarthritis Research Society International
OATP	Organic anion-transporting polypeptide
OB	Osteoblast
OC	Osteoclast
OCP	Osteochondroprogenitor
PGE2	Prostaglandin E2
PHEX	Phosphate regulating gene with homologies to endopeptidases on the X chromosome
PMMA	Polymethyl methacrylate
PTH	Parathyroid hormone
PTHrP	Parathyroid hormone related protein
RANK	Receptor activator of nuclear factor- κ B
RANKL	Receptor activator of nuclear factor- κ B ligand
RBPJκ	Recombination signal-binding protein for Ig of κ region
ROI	Region of interest
Runx2	Runt-related transcription factor 2
rT3	3,3',5'-triiodothyronine
RTHβ	Resistance to thyroid hormone β
RXR	Retinoid X receptor
SB	Subchondral bone
SSC	Skeletogenic stem cells
SMI	Structure model index
SOX	Sex determining region Y-box
T2	3,3'-diiodothyronine
T3	3,5,3'-L-tri-iodothyronine
T4	Thyroxine
Tb.Sp	Trabecular spacing
Tb.Th	Trabecular thickness
TG	Thyroglobulin
TGFβ	transforming growth factor β
<i>THRA/ Thra</i>	Human/Mouse thyroid hormone receptor α gene
<i>THRB/ Thrb</i>	Human/Mouse thyroid hormone receptor β gene

TPO	Thyroid peroxidase
TR	Thyroid hormone receptor
TRacP	Tartrate-resistant acid phosphatase
TRE	Thyroid response element
TRα	Thyroid hormone receptor alpha
TRβ	Thyroid hormone receptor beta
TSH	Thyrotropin/ Thyroid stimulating hormone
TSHR	Thyrotropin/ Thyroid stimulating hormone receptor
V	Vehicle treatment

DECLARATION OF ORIGINALITY AND COPYRIGHT DECLARATION

I declare that to the best of my knowledge, unless otherwise acknowledged, that I performed the experiments, collected and analysed the data presented in this thesis.

This work contains unpublished novel data and provides an original contribution to the knowledge of the scientific fields of thyroid hormone action in the joint.

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THE THESIS

Role of Thyroid Hormones in Susceptibility and Progression of Osteoarthritis

Rationale for investigation

Osteoarthritis is a common, chronic joint disease that causes disability and a high economic burden for which there is no disease modifying treatment. The pathology of osteoarthritis resembles endochondral ossification, in which the biologically active thyroid hormone, T3, plays a critical role in cartilage and bone development. Genetic studies and experimental data support the concept that altered T3 availability may affect osteoarthritis susceptibility.

Hypothesis

I hypothesise that inhibition of thyroid hormone signalling will decrease susceptibility to osteoarthritis and delay disease progression.

To address this hypothesis, I will determine:

1. The response to osteoarthritis-provocation surgery in mice with genetic deletion or pharmacological inhibition of TR α , the main thyroid hormone receptor in skeletal cells.
2. Whether spontaneous osteoarthritis develops in genetically modified mice unable to generate local T3.

Aims

In order to investigate this hypothesis, I will:

1. Validate an established surgical model of osteoarthritis
2. Determine osteoarthritis susceptibility in TR $\alpha^{0/0}$ mice.

3. Determine the effects of pharmacological inhibition of TR α on osteoarthritis development and progression.
4. Compare the spontaneous development of osteoarthritis in D2KO and wild type mice.

CHAPTER 1 INTRODUCTION

1.1 BACKGROUND

1.1.1 Definition and diagnosis of osteoarthritis

Osteoarthritis is a degenerative joint disease involving articular cartilage and its surrounding tissues. The diagnosis may be structural, radiological, clinical or a combination of these (Arden and Nevitt 2006). Structural changes are characterised by focal damage and loss of articular cartilage, subchondral bone remodelling, osteophyte formation, ligamentous laxity, weakening of periarticular muscles and in some cases synovial inflammation (Litwic, Edwards et al. 2013). Symptoms include joint pain, stiffness and loss of function.

1.1.2 Epidemiology

Osteoarthritis may develop in any joint, but most commonly affect the knees, hips, hands, facet joints and feet. The prevalence varies greatly depending on age, sex, geographical area studied and definition used (Litwic, Edwards et al. 2013). Approximately half of patients diagnosed with radiographic evidence of OA are asymptomatic (Hilgsmann, Cooper et al. 2013). Osteoarthritis is the most common joint disease worldwide, with an estimated 10% of men and 18% of women aged ≥ 60 having symptomatic OA (Woolf and Pfleger 2003). Age and obesity are among the strongest and best-established risk factors for OA (Litwic, Edwards et al. 2013). Therefore, the increasing prevalence of obesity and ageing population coupled with the absence of disease modifying treatment has led to increasing health costs attributable to OA. A previous history of joint injury can cause post-traumatic osteoarthritis (PTOA) and this accounts for 12% of all OA cases in the USA (Little and Hunter 2013). Direct costs (hospital admissions, medical examinations, symptom-relieving drug therapy, joint replacement) and indirect costs (loss of productivity) are estimated at between 1%-2.5% of gross domestic product (GDP) for Westernized countries (Hilgsmann, Cooper et al. 2013).

1.1.3 Clinical risk factors and natural history

Risk factors for OA development include obesity, age, female sex, ethnicity, high bone mineral density, sarcopaenia and previous joint injury. The risk of injury progressing

to OA is joint-specific; a larger proportion of OA is attributable to trauma in ankles and knees than hip. The pathogenic role of trauma is evident in the fivefold increased risk of developing knee OA in those with a previous injury, compared to a 1.7-fold relative risk associated with obesity. In addition to direct effects of trauma to the articular surface, damage to supporting structures like ligaments and menisci can predispose to the development of OA. PTOA develops in 50-70% of patients with anterior cruciate ligament ruptures or meniscal damage, 10-15 years after the injury (Lohmander, Englund et al. 2007, Little and Hunter 2013).

Fewer risk factors have been identified for disease progression and they do not correlate well with risk factors for disease development (Litwic, Edwards et al. 2013). Furthermore, these risk factors appear to be joint specific (Arden and Nevitt 2006) and there is poor correlation between structural changes and symptoms. The natural history of the disease is heterogeneous and varies between patients and joints. For example, the evolution of OA in knees is often slow while OA in hands typically develops faster and the rate of disease progression in hip OA is very variable.

1.1.4 Therapy

Current therapeutic options are limited to lifestyle modification, symptom relieving treatment and ultimately joint replacement. There are no licensed disease-modifying drugs available. Therefore, there is an urgent need to further our understanding of the pathogenesis of OA in order to identify novel drug targets that may prevent the development of OA or reduce disease progression.

1.2 NORMAL SYNOVIAL JOINTS

The synovial joint is a specialised organ that permits almost frictionless articulation for movement. It connects two adjacent bones that are covered by a layer of articular cartilage and surrounded by the synovium and fibrous joint capsule. Different tissues within the joint possess unique biomechanical properties derived from distinct extracellular matrices (Poole 2011). These include hyaline articular cartilage, calcified articular cartilage, subchondral bone, synovium and synovial fluid, capsule, ligament and tendons (Figure 1.1). Some joints, such as the knee, have specialist tissues like menisci and patella.

1.2.1 Hyaline Articular Cartilage

Hyaline articular cartilage (HAC) is between 2-4mm thick and covers the articulating ends of bones. Its principal functions are to transmit and dissipate shear and compressive forces, and provide a smooth, lubricated surface for articulation. It is an avascular, aneural tissue with sparsely distributed cells called chondrocytes and has limited capacity for regeneration. Chondrocytes synthesise, secrete and maintain a specialised extracellular matrix (ECM). The ECM is mainly composed of water, collagen and proteoglycans with smaller amounts of non-collagenous proteins and glycoproteins.

Articular cartilage can be subdivided into three zones: superficial (tangential), middle (transition) and deep (Figure 1.1). The superficial (tangential) zone provides 10-20% of articular cartilage thickness and contains tightly packed collagen fibers aligned parallel to the articular surface. This provides tensile strength and protects deeper zones from shear forces. The middle (transition) zone accounts for 40-60% of cartilage volume and contains thicker, obliquely orientated collagen fibrils and proteoglycans that confer resistance to compressive forces. The deep zone has fewer collagen fibrils arranged in perpendicular columns with the highest proteoglycan concentration that provides the greatest resistance to compressive forces. This zone represents 30% of articular cartilage volume (Sophia Fox, Bedi et al. 2009). The tidemark represents the interface between the deep zone and the underlying calcified articular cartilage.

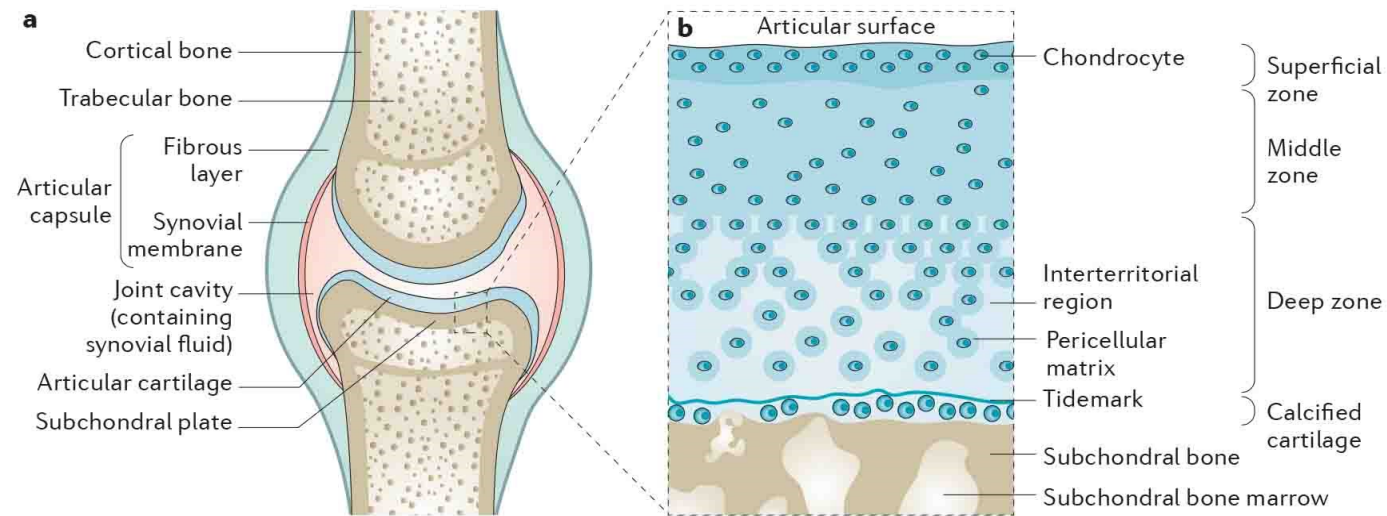


Figure 1.1 Normal synovial joint & articular surface

Figure reproduced from Martel-Pelletier, J et al 2016 with permission.

(A) Two adjacent bones are covered by a thin layer of specialised articular cartilage and encased in a connective tissue capsule lined by synovial membrane.

(B) Section of articular surface. Chondrocytes are the only cells populating hyaline articular cartilage, which is subdivided into superficial, middle and deep zones. Unmineralised cartilage at the surface is separated from calcified cartilage by the tidemark. Subchondral bone lies under articular cartilage and is composed of a cortical bone plate and trabecular bone.

1.2.1.1 Extracellular Matrix of HAC

Type II collagen is the most abundant structural macromolecule and represents 90-95% of the collagen in ECM (Figure 1.2). It forms fibrils and fibres intertwined with hyaluronan and proteoglycan aggregates. Proteoglycans are heavily glycosylated protein monomers and the second largest macromolecule in the ECM. They are made of a central core protein to which glycosaminoglycan (GAG) side chains are covalently linked post-translationally (Kiani, Chen et al. 2002). Articular cartilage proteoglycans include aggrecan, decorin, biglycan and fibromodulin. Most proteoglycans exist as aggregates by the non-covalent association of proteoglycan with hyaluronan and link proteins. Aggrecan is the largest in size, most abundant and functionally most important proteoglycan in articular cartilage. Its numerous negatively charged GAG chains spread out to occupy a large volume in solution. In the cartilage matrix, the negatively charged GAGs attract cations such as Na^+ to create an osmotic gradient. However, the swelling of proteoglycan aggregates is constrained by the collagen fibrillar network. Furthermore, the compression of cartilage brings GAGs in closer proximity, increasing the mutual repulsive forces and adding to the compressive stiffness of cartilage (Mansour 2003). Aggrecan offers great resistance to the flow and redistribution of water, making the tissue poorly permeable. It behaves like a sponge that does not allow fluid to flow through it easily. The frictional resistance to waterflow and osmotic pressure are the basic mechanisms that enable articular cartilage to withstand significant loads (Sophia Fox, Bedi et al. 2009). Thus proteoglycans such as aggrecan are responsible for the compressive stiffness and modulus of cartilage, while collagen fibrils give cartilage its tensile strength (Poole 2011).

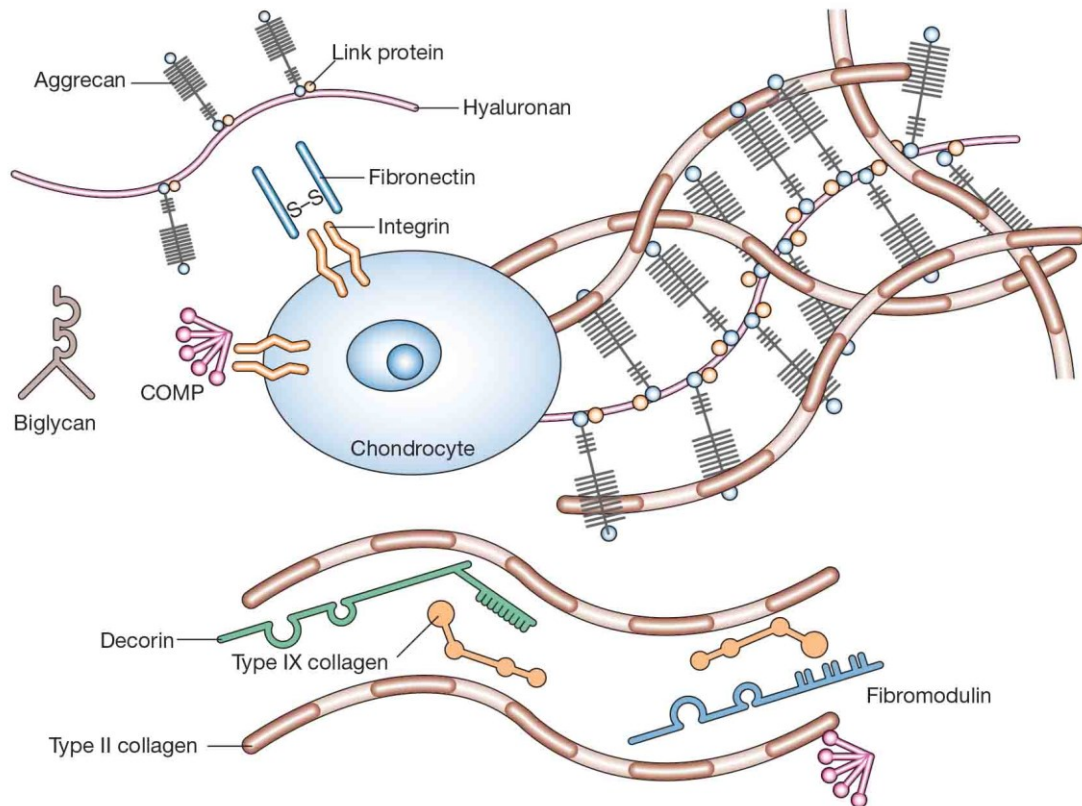


Figure 1.2 Extracellular matrix of articular cartilage

Figure reproduced from (Chen, Rousche et al. 2006) with permission. Three classes of proteins exist in articular cartilage: collagens (mostly type II collagen); proteoglycans (mainly aggrecan); and other noncollagenous proteins (including link protein, fibronectin, cartilage oligomeric matrix protein). The interaction between highly negatively charged cartilage proteoglycans and type II collagen fibrils and constrain from the collagen network is responsible for the compressive and tensile strength of the tissue.

1.2.2 Calcified articular cartilage

The calcified or mineralised articular cartilage represents a transition zone between the unmineralised hyaline articular cartilage above and the subchondral bone below. It helps anchor collagen fibrils to the underlying bone. In contrast to the unmineralised hyaline articular, where chondrocytes are abundant and small, the calcified articular cartilage is characterised by scarce chondrocytes of hypertrophic morphology. These hypertrophic chondrocytes secrete type X collagen fibrils into the extracellular matrix and are similar to hypertrophic chondrocytes of the growth plate (Poole 2011).

1.2.3 Subchondral Bone

Subchondral bone (SB) has two distinct anatomical and physiological compartments. Immediately beneath the calcified articular cartilage lies a 1-3mm thick plate of cortical bone. It is physiologically and mechanically similar to cortical bone in other skeletal locations. Distal to this lies subchondral trabecular bone that is more porous, metabolically active and has a lower volume, density and stiffness than the cortical plate. (Burr and Gallant 2012). The irregular outline of the osteochondral junction, also called the cement line, is able to convert shear stresses into compressive and tensile stresses and SB is better at absorbing shock than the articular cartilage (Imhof, Sulzbacher et al. 2000). There is a dynamic relationship between articular cartilage and underlying subchondral bone that affects their load bearing characteristics and integrity. Loss or damage to articular cartilage increases loads transmitted to subchondral bone. However, increased stiffness of SB can transmit increased loads to articular cartilage and leading to its damage or loss (Goldring and Goldring 2010, Neogi, Nevitt et al. 2010, Li, Yin et al. 2013). In addition to bone shape and architecture, the organisation of its organic phase and mineral composition are important in determining its biomechanical properties. The degree of mineralisation, is in turn related to rate of bone turnover. Slower turnover can lead to longer periods of secondary mineralisation and therefore stiffer bone (Meunier and Boivin 1997, Day, Ding et al. 2001, Goldring and Goldring 2010). The interaction between articular cartilage and subchondral bone is not limited to its biomechanics. It is now clear that there is cross-talk between subchondral bone and cartilage and it should be considered a functional unit (Lories and Luyten 2011, Goldring and Goldring 2016). The calcified cartilage and subchondral bone plates are porous, which allows communication between bone cells and chondrocytes via the

diffusion of soluble factors (Pan, Zhou et al. 2009). There is also evidence of direct cell-cell contact between articular chondrocytes and bone cells (Imhof, Sulzbacher et al. 2000, Lyons, McClure et al. 2006).

1.2.4 Menisci

Menisci are crescent shaped fibrocartilagenous structures found in certain joints, such as the knee, that help distribute and dissipate intra-articular load and maintain stability. Up to 50% of the compressive load is transmitted through the meniscus in extension and this rises to 85% at 90 degrees of flexion. Following meniscectomy, the contact area is reduced by 50-70%, leading to increased peak stresses that are focused on smaller areas of the articulating surfaces (Poole 2011).

1.2.5 Ligaments

Ligament are fibrous connective tissues that connects bones and are essential for joint stability.

1.2.6 Joint capsule and synovium

The synovium is made of a thin layer of cells that cover the joint and serves to ensure an aseptic intra-articular environment and provide lubrication. Type A synovial cells are resident macrophages that endocytose and kill pathogens. Type B synovial cells secrete the joint lubricants hyaluronan and lubricin. Supporting the synovium is a fibrous, neurovascular capsule. Together, the synovium and capsule contribute towards the regenerative capacity of joint by providing a source of mesenchymal stem cells that can differentiate into chondrocytes (Poole 2011).

1.2.7 Developmental biology of joints

Joint formation and morphogenesis involves several key steps (Figure 1.3) that are incompletely understood:

1. Joint site determination.
2. Interzone formation and cell recruitment
3. Joint tissue differentiation
4. Cavitation and joint morphogenesis.

Early embryonic limbs have continuous and uninterrupted cartilage anlagen lacking joints. Unknown upstream regulators determine the location of future joints along the Sox9/Col2/Dcx-expressing cartilage anlagen. The first sign of joint formation is the appearance of compacted and flat cells, called interzone, at each prospective joint. Surgical removal of these cells leads to failure of joint formation (Holder 1977). Genetic cell lineage experiments show that expression of the cartilage marker Col2a1 ceases before the formation of the interzone, which is characterised by expression of markers including the transcription factor growth and differentiation factor-5 (GDF-5) (Hyde, Boot-Handford et al. 2008). Concurrently, cells migrate towards the interzone and activate Tgfr2 expression, a critical regulator of joint formation (Spagnoli, O'Rear et al. 2007). Next, Gdf-5 positive cells, with a Sox9/Col2 history but negative for matrilin-1 expression differentiate into articular chondrocytes under the control of local and long range regulators including Erg and PTHrP. Subsequently, a combination of Gdf-5 lineage cells and Tgfr2 positive cells differentiate to form joint structures including ligaments, menisci in the knee and synovial lining (Koyama, Shibukawa et al. 2008, Decker, Koyama et al. 2015).

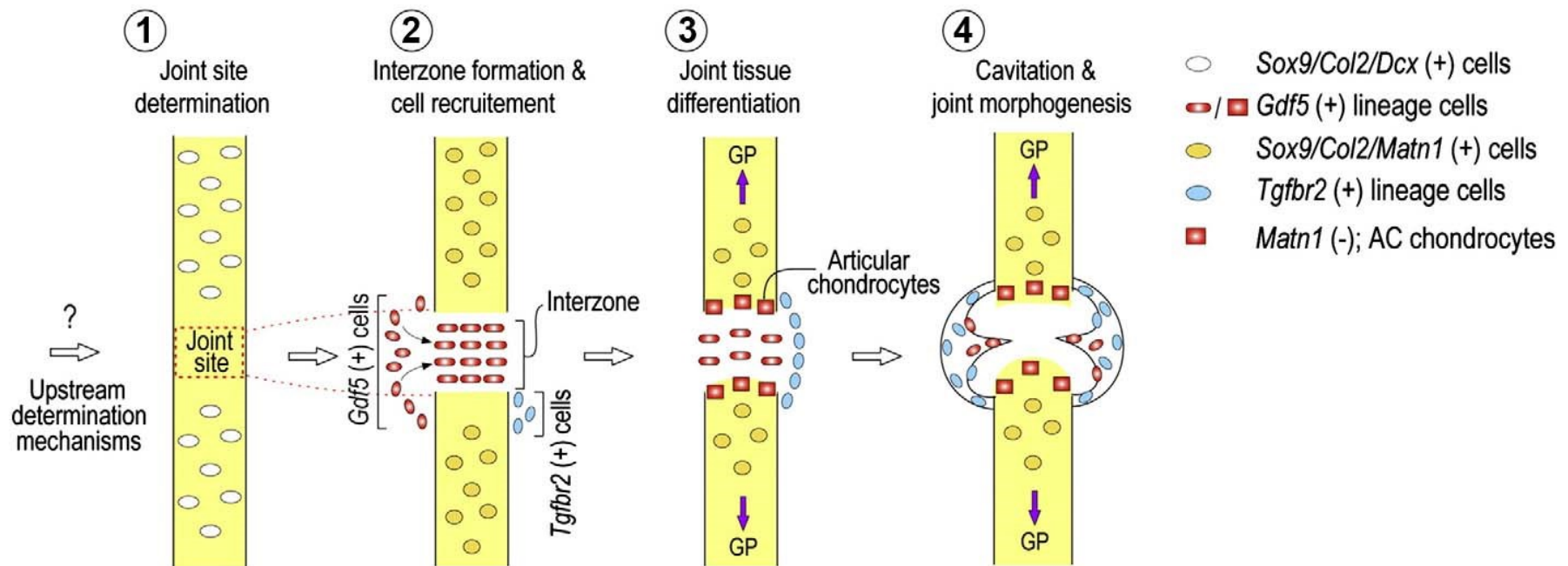


Figure 1.3 Joint Formation

Figure reproduced from (Decker, Koyama et al. 2014) with permission. See text for details.

1.2.7.1 Postnatal articular cartilage growth and organisation

Mature cartilage is a phenotypically complex tissue with quiescent chondrocytes and abundant matrix, organised into distinct zones that determine its biomechanical function. In contrast, at birth articular cartilage has many small chondrocytes, scanty matrix and lacks zonal organisation. How this transition occurs is not fully understood but may involve appositional growth from the surface of the joint. This layer may act as a surface growth plate, providing stem cells that allow the lateral, radial and longitudinal growth of the epiphysis (Archer, Morrison et al. 1994, Hunziker, Kapfinger et al. 2007, Decker, Koyama et al. 2015). Gene array studies in articular cartilage have demonstrated differential gene expression profiles at different developmental stages (young vs mature) and zones (surface, middle and deep)(Mienaltowski, Huang et al. 2008, Amanatullah, Yamane et al. 2014, Lui, Chau et al. 2015). Once articular cartilage acquires its adult size, chondrocytes rarely proliferate and limit their activity to turning over a subset of ECM components, including aggrecan, but not collagens. This limited regenerative capacity is an important factor that contributes to osteoarthritis progression (Heinemeier, Schjerling et al. 2016).

1.2.8 Embryonic derivation and differentiation of articular and growth plate chondrocytes

Mesenchymal progenitor cells or stem cells (MSC) can differentiate into many different cell types (Figure 1.4). Articular chondrocytes and growth plate chondrocytes derive from two different developmental programmes. Articular chondrocytes (ACCs) are derived via a multipotent joint progenitor (JP) stage with potential to form chondrocytes, synovial and other joint cell types (Liu, Samsa et al. 2017). Growth plate chondrocytes (GPCs) are derived through an intermediate, bipotent osteochondroprogenitor (OCPs) stage, with the potential to become either osteoblasts or chondrocytes. The regulation of osteoblast differentiation and bone formation will be discussed in the next chapter.

Chondrocytes are dynamic and undergo multiple steps of maturation in the growth plate. These include alignment into longitudinal columns; the expression of regulatory signals in prehypertrophic chondrocytes (PHCs) that control the proliferation and alignment of columnar GPC; massive expansion of cytoplasmic volume in hypertrophic chondrocytes (HCs) leading to skeletal elongation; remodelling and mineralising cartilage matrix by terminal chondrocytes before undergoing apoptosis. In contrast, ACCs only proliferate and produce extracellular matrix during tissue growth then become quiescent with limited proliferation or matrix turnover in adulthood (Liu, Samsa et al. 2017).

Progenitor cells that give rise to articular chondrocytes and growth plate chondrocytes express Sox-9 and doublecortin (Dcx)(Soeda, Deng et al. 2010). These two types of chondrocytes can be distinguished by their expression history. Expression of Dcx is retained in articular cartilage but lost in growth plate cartilage (Zhang, Cigan et al. 2011). Articular chondrocytes are negative for matrilin-1 but positive for col2 whereas growth plate chondrocytes are positive for both (Hyde, Dover et al. 2007).

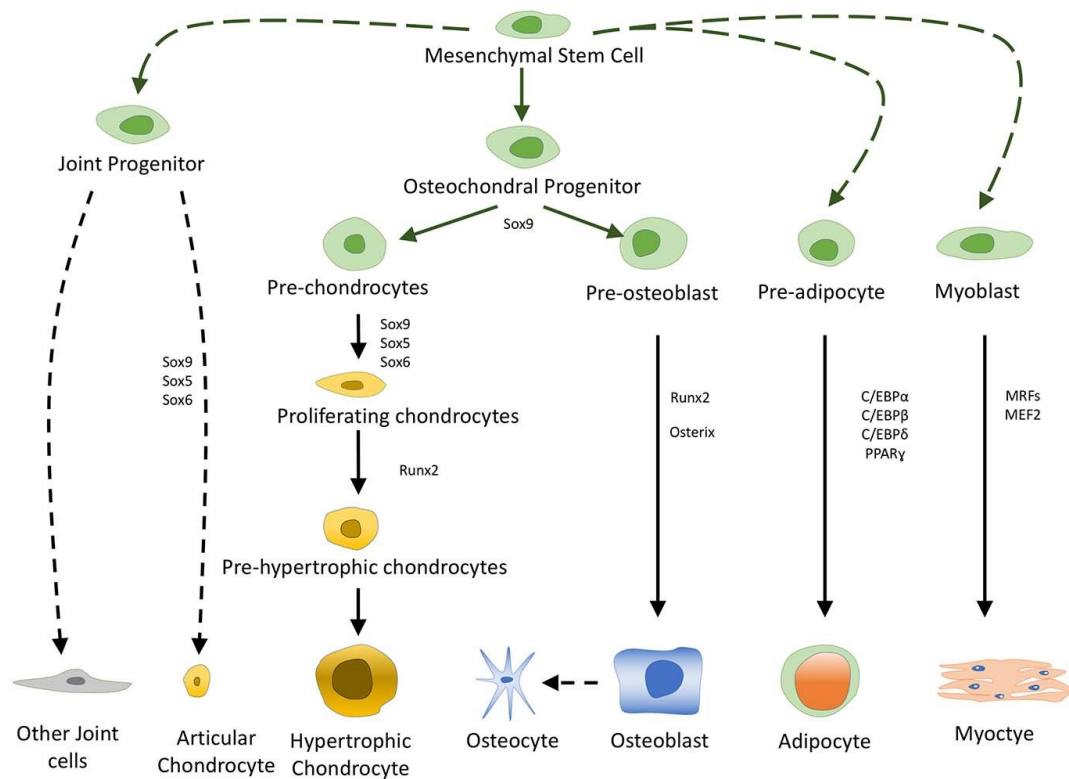


Figure 1.4 Transcriptional Control of skeletal cells

Mesenchymal progenitor cells can differentiate into myocytes, adipocytes, osteoblasts and chondrocytes (see text). Key transcription factors regulating this process are shown. Abbreviations: MEF2, myogenic enhancing factor; MRFs, myogenic regulatory factors (including MyoD, myogenin, myogenic factor 5 and myogenic regulatory factor 4); MEF2, myocyte-enhancer factor 2; C/EBP, CCAAT-enhancer-binding protein; PPAR, peroxisome proliferator-activated receptor; Runx2, runt-related transcription factor 2; Sox, sex determining region Y-box

1.3 NORMAL BONE

An understanding of normal bone physiology is important to interpret the changes in the subchondral bone compartment of joints in ageing and osteoarthritis.

1.3.1 Structural and developmental organisation

Bone can be categorised structurally and developmentally into two types. Flat bones, such as those of the face and skull develop through intramembranous ossification. This involves mesenchymal cells differentiating directly into bone forming osteoblasts. Alternatively, long bones such as the femur are formed on a cartilage scaffold through endochondral ossification. This process is described later in this chapter.

1.3.1.1 Macroscopic organisation

The gross anatomy of a long bone is composed of three regions: the diaphysis, metaphysis and epiphysis (Figure 1.5). The diaphysis is a thick, tubular shaft of dense bone called cortex with a hollow centre. At each end of the diaphysis the structure widens to form a transition zone called the metaphysis. The metaphysis starts at the level of the growth plate where new bone is formed to allow longitudinal growth through a process called endochondral ossification. Beyond the growth plate lies the epiphysis at the end of the bone that is covered by non-mineralised cartilage and can articulate with an adjacent bone to form a joint. The external surface of bone is covered by a thin layer called the periosteum, while the internal surface of the cylinder is called the endosteal surface. In the middle of the diaphysis, bone is thickest and the centre is usually filled with haematopoietic bone marrow. Towards the metaphysis and epiphysis, the cortex becomes thinner and the internal space is filled with a lattice of thin, calcified trabeculae, called trabecular bone (Baron 2008).

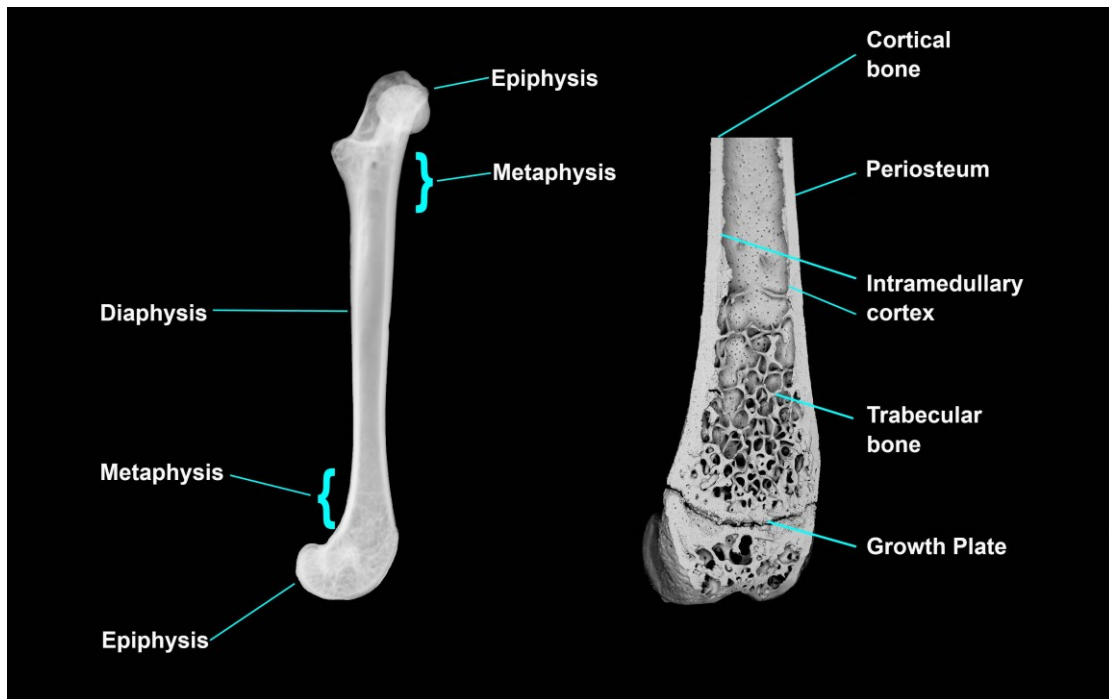


Figure 1.5 Macroscopic structure of long bones

X-ray microradiography (left) and backscattered scanning electron microscopy (BSE-SEM)(right) of femur. BSE-SEM image courtesy of Professor Duncan Bassett.

1.3.1.2 Microscopic organisation

At microscopic level, bone can be divided into woven (primary) bone and lamella (secondary bone). Woven bone is laid down during embryogenesis and during fracture repair. It is composed of osteoid with disorganised collagen fibres arranged randomly. Woven bone is replaced by lamellar bone in the adult skeleton. This consists of highly organised parallel sheets (lamellae) of collagen fibrils and provides increased strength compared to woven bone.

Bone has a hierarchical structural organisation (Figure 1.6). Lamellae wrap in concentric layers around a central Haversian canal to form an osteon or Haversian system. Osteons run parallel to the long axis of bone and are cylinders approximately 200-250 μm in diameter (Rho, Kuhn-Spearing et al. 1998).

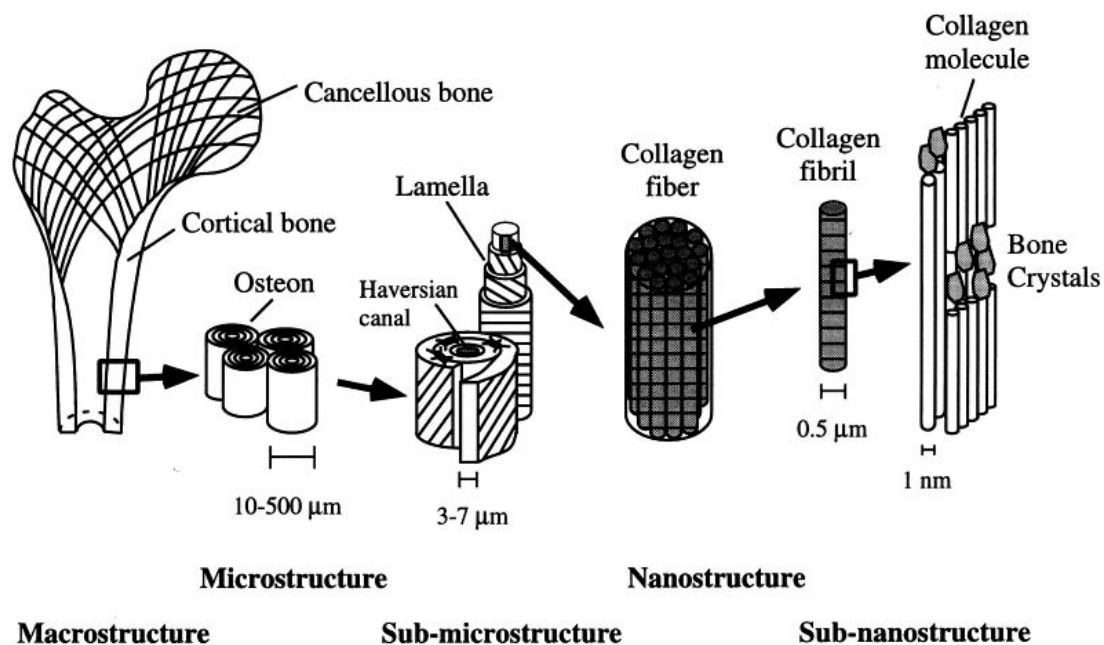


Figure 1.6 Hierarchical structural organisation of bone

Figure reproduced from (Rho, Kuhn-Spearing et al. 1998) with permission.

1.4 BONE CELLS

1.4.1 Osteoblasts

Osteoblasts are bone-forming cells that synthesize and secrete osteoid and are responsible for its subsequent mineralisation. Osteoid is composed of type I collagen, together with minor proteins including proteoglycans, fibronectin, and bone specific proteins such as osteopontin, bone sialoprotein and osteocalcin. Mineralisation is achieved by the action of osteoblast-derived alkaline phosphatase that leads to the precipitation and growth of hydroxyapatite crystals (Crockett, Rogers et al. 2011).

1.4.1.1 *Transcriptional regulation of bone formation*

Mesenchymal progenitor cells differentiate first into pre-osteoblasts that do not secrete mineral matrix and then mature osteoblasts. Several transcription factors are expressed and function at key time points during this process.

Sox 9 is a master transcription factor that determines chondrocyte cell fate. While it is expressed in osteoblast progenitors, mature osteoblasts do not express Sox9 (Akiyama, Kim et al. 2005, Long 2011).

Runx-related transcription factor 2 (Runx2), also known as Cbfa1, Osf2 and AML3 is essential for osteoblast differentiation and hypertrophic differentiation of chondrocytes during both endochondral and intramembranous ossification. It directs pluripotent stem cells to the osteoblast lineage and triggers the expression of major bone matrix protein genes (Komori 2010). Knockout mice have a cartilaginous skeleton without osteoblasts (Komori, Yagi et al. 1997, Otto, Thornell et al. 1997). Haploinsufficiency in humans and mice leads to cleidocranial dysplasia, characterised by hypoplastic clavicles and delayed closure of the fontanelles (Lee, Thirunavukkarasu et al. 1997, Mundlos, Otto et al. 1997). Tight control of osteoblast differentiation is also achieved by numerous nuclear factors that either synergise with Runx2 or antagonise it by reducing Runx2 levels or activity (Long 2011).

Osterix (Osx) is a zinc-finger containing protein that acts downstream of Runx2 and is critical for osteoblastic differentiation during both embryogenesis and postnatal growth. OSX expression is lost in Runx2-null mice and deletion of Osx leads to complete

absence of osteoblasts in mouse embryos (Nakashima, Zhou et al. 2002, Zhou, Zhang et al. 2010).

Activating transcription factor 4 (ATF4) is major regulator of later stages of osteoblast differentiation and important to maintain this phenotype. It also affects OB function including the synthesis of type I collagen through a posttranscriptional mechanism (Yang, Matsuda et al. 2004).

1.4.1.2 Bone regulation by extracellular signals and growth factors

The transcription factors described above are in turn regulated by numerous extracellular signals and growth factors (Figure 1.7). These include Hedgehog, Notch, Wnt, bone morphogenetic proteins and fibroblast growth factor pathways.

Hedgehog signalling is indispensable for osteoblast differentiation in endochondral skeleton but is not required for intramembranous ossification. There are three hedgehog proteins in the mammalian genome. Indian hedgehog (IHH) is expressed by prehypertrophic and early hypertrophic chondrocytes in the endochondral cartilage primordium, where it signals to chondrocytes and adjacent perichondral cells to undergo osteoblastic differentiation. IHH binds to the receptor Patched homologue 1 and then signals through the transmembrane protein Smoothened (SMO) to regulate gene transcription through derepression and activation of GLI family of transcription factors (Long 2011, Alman 2015).

Notch signalling mediates communication between neighbouring cells that are in direct contact and inhibits osteoblast differentiation. After binding to their ligands (Jagged (JAG) or Delta-like (DLL), Notch receptors are proteolytically cleaved by the γ secretase complex. This releases the Notch intracellular domain (NICD) from the cellular membrane. NICD associates with recombination signal-binding protein for Ig of κ region (RBPJ κ) and together they activate downstream target genes. These include Hairy and Enhancer of Split (HES) and HES-related with YRPW motif (HEY) family of transcription factors that lead to inhibition of osteoblast differentiation (Long 2011, Zanotti and Canalis 2016).

Wnt signalling is involved in embryonic skeletal development and postnatal bone accrual. Wnt is a family of glycoproteins that regulate osteoblast differentiation via β -catenin dependent (canonical) or independent (non-canonical) pathways. The predominantly pathway affecting bone cells is the canonical pathway (Baron and Kneissel 2013). In the absence of Wnt ligands, a complex that includes axin, glycogen synthase kinase 3 β (GSK3 β) and adenomatous polyposis coli protein (APC) mediates ubiquitination and proteasomal degradation of β -catenin. When Wnt ligands bind to Frizzled receptor (FZD) and their co-receptor low-density lipoprotein receptor-related protein 5 (LRP5) or LRP6, the axin/ GSK3 β /APC complex is inhibited. This releases β -catenin to enter the nucleus and together with T cell factor/ lymphoid enhancer factor (TCF/LEF) transcription factors stimulate the transcription of target genes. Wnt signalling represses mesenchymal stem cells commitment to the chondrogenic and adipogenic lineages (Day, Guo et al. 2005, Hill, Spater et al. 2005, Kennell and MacDougald 2005) and enhances commitment to and differentiation along the osteoblastic lineage (Kato, Patel et al. 2002, Bennett, Ouyang et al. 2007, Baron and Kneissel 2013). Wnt signalling in osteoblasts and osteocytes also indirectly increases bone mass by increasing the secretion of osteoprotegerin (OPG), that reduces osteoclastic differentiation (Glass, Bialek et al. 2005, Holmen, Zylstra et al. 2005, Kramer, Halleux et al. 2010). This is discussed further in the section on osteoclast. Wnt signalling is modulate by inhibitors including Dickkopf-related 1 (DKK1), sclerostin and secreted frizzled-related protein (Sfrp1). Mutations affecting this regulatory system illustrate its importance in controlling bone mass. Loss of function mutations in LRP5 cause the autosomal recessive osteoporosis-pseudoglioma syndrome (OPPG) that is recapitulated in mice (Gong, Slee et al. 2001, Kato, Patel et al. 2002). Conversely, gain of function mutations that prevent the action of extracellular Wnt inhibitors cause high bone mass syndromes (Boyden, Mao et al. 2002, Ai, Holmen et al. 2005, Ellies, Viviano et al. 2006, Balemans, Piters et al. 2008).

Bone morphogenetic proteins (BMPs) are cytokines belonging to the evolutionarily conserved transforming growth factor superfamily β (TGF β) superfamily. In the canonical pathway, BMPs bind to a tetrameric receptor complex composed of two type 1 and two type 2 serine/threonine kinase receptors. Type 2 receptors then phosphorylate type 1 receptors, which in turn recruits and phosphorylates pathway mediators called

receptor activated SMADS (R-SMADS). The phosphorylated R-SMADS forms a complex with SMAD4, translocate to the nucleus and regulates target gene expression. This pathway is antagonised by secreted ligand inhibitors such as Noggin and follistatin or receptor sequestration by non-signalling ligands such as BMP3 (Daluiski, Engstrand et al. 2001, Salazar, Gamer et al. 2016). Stimulation of BMP-SMAD signalling upregulates expression of Runx2, which interacts with R-SMADS to activate transcription of target genes and promote osteoblast differentiation (Lee, Kim et al. 2000, Nishimura, Hata et al. 2002, Sanchez-Duffhues, Hiepen et al. 2015). BMP2 and BMP4 coexpression is essential for differentiation into mature osteoblasts and controls the transition from Runx2⁺ to Runx2⁺Osx⁺ cells (Bandyopadhyay, Tsuji et al. 2006). Conversely, inhibitors of BMP signalling down-regulate osteoblastogenesis (Wu, Li et al. 2003, Kokabu, Gamer et al. 2012). BMP signalling also regulates osteoblast function at different stages of maturation. Conditional SMAD4 deletion in committed (Osx⁺/Cre) or mature (Osteocalcin⁺/Cre) osteoblasts cause dwarfism with brittle bones and a disorganised matrix due to loss of collagen processing enzymes (Tan, Weng et al. 2007, Salazar, Zarkadis et al. 2013). Similarly, transgenic overexpression of the BMP inhibitor Noggin in mature osteoblasts caused low bone mass during postnatal growth (Devlin, Du et al. 2003), as did conditional deletion of the BMP type 1 receptor in mature osteoblast (Mishina, Starbuck et al. 2004).

BMP signalling interacts with other signalling pathways and is an important regulator of osteoclastic activity. In contrast to the juvenile phenotype, aged mice with osteoblast specific SMAD4 deletion have high bone mass due to reduced osteoclastogenesis, related to increased OPG expression (Tan, Weng et al. 2007). Mice with osteoblast specific deletion of BMP receptor 1A have high bone mass due to increased Wnt signalling from downregulation of Wnt inhibitors Dkk1 and SOST (Kamiya, Ye et al. 2008, Kamiya, Kobayashi et al. 2010).

Fibroblast growth factor (FGF) signalling promotes or inhibits osteoblast proliferation and differentiation depending on cell context. This is determined by the type of FGF and FGF receptor (FGFR) expressed, the stage of cell maturation and microenvironment (proteoglycans and interacting proteins) (Ornitz and Marie 2015). Most FGFs bind to cell surface tyrosine kinase FGF receptors (FGFR), leading to the phosphorylation of signalling proteins including mitogen activated protein kinase

(MAPK), phosphoinositide 3-kinase (PI3K), Signal transducer and activator of transcription 1 (STAT1) and protein kinase C (PKC) (Long 2011).

FGF2 signalling increases cell proliferation in immature osteoblasts, promotes their survival (Debiais, Hott et al. 1998, Agas, Marchetti et al. 2008) and directs stromal cells towards an osteogenic programme (Xiao, Sobue et al. 2010). This expands the pool of osteoblast precursor cells that then differentiate into mature osteoblasts (Montero, Okada et al. 2000). In more mature cells, FGF2 induces the production of osteocalcin and matrix mineralisation (Debiais, Hott et al. 1998) but also induces apoptosis of terminally differentiated osteoblasts (Mansukhani, Bellosta et al. 2000). FGF18 is an important autocrine positive regulator of bone formation (Hamidouche, Fromigue et al. 2010, Jeon, Yun et al. 2012). Mice with genetic deletion of FGF18 have delayed ossification (Liu, Xu et al. 2002, Ohbayashi, Shibayama et al. 2002). FGFR1 has stage specific effects on osteoblast maturation. Signalling in early developmental stages increases osteoblast differentiation without affecting runx2. However, in mature osteoblasts, FGFR1 signalling inhibits their differentiation (Jacob, Smith et al. 2006). In contrast, FGFR2 signalling promotes differentiation of preosteoblasts and bone matrix synthesis in mature osteoblasts (Yu, Xu et al. 2003).

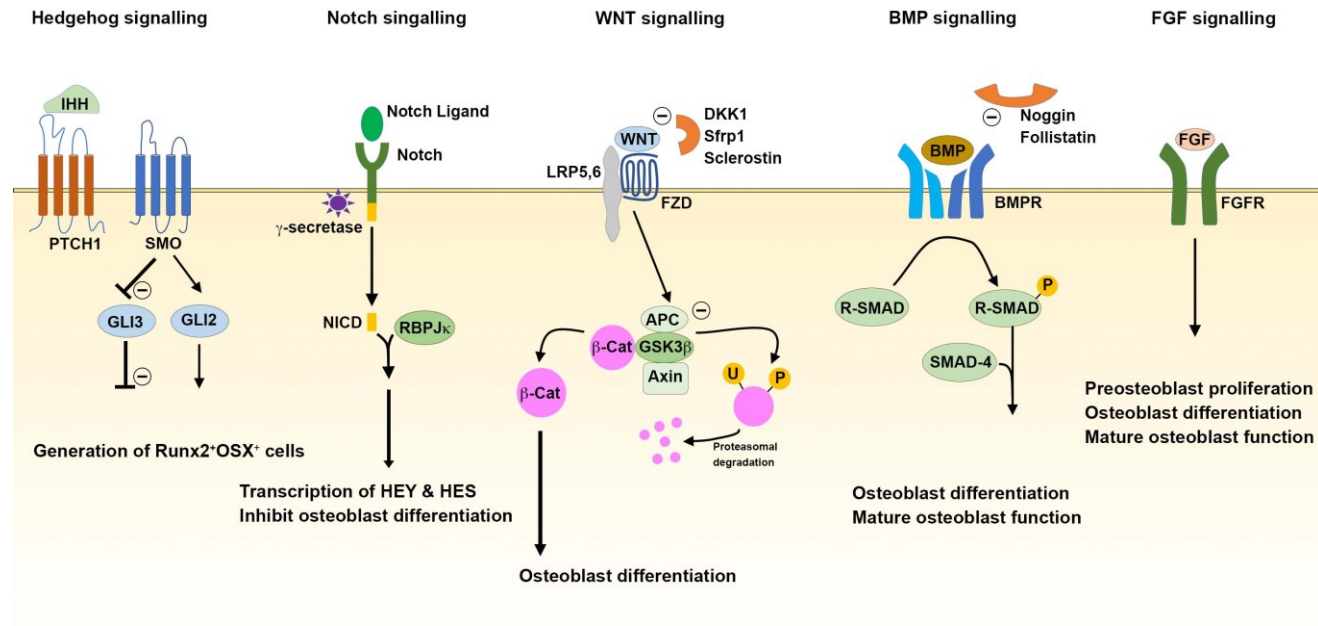


Figure 1.7 Extracellular signals regulating osteoblast differentiation

See text for details. Abbreviations: APC, adenomatous polyposis coli protein; β -cat, β -catenin; BMP, Bone morphogenetic protein; BMPR, Bone morphogenetic protein receptor; DKK1, Dickkopf-related 1; FGF, Fibroblast growth factor; FGFR, Fibroblast growth factor receptor; FZD, Frizzled receptor; GSK3 β , glycogen synthase kinase 3 β ; HES, Hairy and Enhancer of Split; HEY, HES-related with YRPW motif; IHH, Indian Hedgehog; LRP5, low-density lipoprotein receptor-related protein 5; NICD, Notch intracellular domain; P, Phosphate; PTCH1, Patched homologue 1; RBPJ κ , Recombination signal-binding protein for Ig of κ region; R-SMADS, receptor activated SMADS; Sfrp1, secreted frizzled-related protein; SMO, Smoothened; U, ubiquitin

1.4.2 Osteoclasts

Osteoclasts (OC) are the only bone resorptive cell. They are derived from bone marrow mononuclear stem cells and osteoclast differentiation is characterised by fusion of these precursors into giant multinuclear cells (Figure 1.8). Two cytokines, receptor activator of nuclear factor- κ B ligand (RANKL) and macrophage-colony stimulating factor (M-CSF), are essential and sufficient for osteoclastogenesis (Boyle, Simonet et al. 2003). Runx2, a transcription factor critical to osteoblast differentiation, regulates osteoblast production of both RANKL and M-CSF to mediated osteoclastogenesis (Geoffroy, Kneissel et al. 2002, Enomoto, Shiojiri et al. 2003).

1.4.2.1 *Osteoblasts osteoclast communication and regulation of osteoclastogenesis*

Osteoblast and osteoclasts can communicate through several mechanisms. These cells can be in direct contact, allowing membrane bound ligands and receptor to interact. One example is RANK-RANKL signalling. RANKL is expressed by stromal or osteoblast lineage cells including bone lining cells, osteoblasts and osteocytes (Nakashima, Hayashi et al. 2011, Xiong, Onal et al. 2011, Fumoto, Takeshita et al. 2014). RANKL binds to its receptor RANK on osteoclast precursors to stimulate osteoclastogenesis. This pathway is modulated by a decoy receptor called osteoprotegerin (OPG) that is also secreted by osteoblast lineage cells (Simonet, Lacey et al. 1997). OPG masks RANKL and prevents its binding to RANK. Thus, the balance between RANKL and OPG (RANKL/OPG ratio) is indicative of osteoclastogenic activity in various bone remodelling disease (Matsuo and Irie 2008).

Communication also occurs via diffusible paracrine factors including growth factors, cytokines and chemokines. Chemokines such as monocyte chemoattractant protein-1 (MCP-1, also known as CCL2), are secreted by osteoblasts and recruit osteoclast precursors (Matsuo and Irie 2008, Siddiqui and Partridge 2017). MCP-1 expression is induced by inflammatory cytokines including tumour necrosis factor α (TNF- α) and interleukin 1 β (IL-1 β). RANKL co-operates by inducing MCP-1 receptor expression in OC precursors. Other soluble factors that promote osteoclastogenesis by inducing RANKL expression include parathyroid hormone (PTH), parathyroid hormone related protein (PTHrP), thyroid hormone, 1,25-(OH) $_2$ vitamin D3 and prostaglandin E $_2$.

Several key transcription factors and intracellular signalling pathways that promote osteoclastogenesis are induced by RANK signalling. Nuclear factor of activated T-cells c1 (NFATc1) is a transcription factor essential for osteoclast differentiation (Ishida, Hayashi et al. 2002, Takayanagi, Kim et al. 2002). Nuclear factor κ B (NF κ B) signalling is increased with inflammation and is essential for osteoclast differentiation. Double knockout mice lacking the main Nuclear factor κ B proteins had osteopetrosis and defect of tooth eruption that were rescued by transplantation of wild type haematopoietic precursors (Franzoso, Carlson et al. 1997, Iotsova, Caamano et al. 1997, Boyce, Xing et al. 1999). C-Fos is a member of the AP-1 family of transcription factors acting downstream of RANKL to regulate osteoclastogenesis (Grigoriadis, Wang et al. 1994, Matsuo, Owens et al. 2000).

Eph are tyrosine kinase receptors that act with their ligands (ephrins) to allow bidirectional signalling between osteoclasts and osteoblasts. Activation of EphB receptors by ephrinB ligands is termed forward signalling while activation of ephrinB ligands by EphB receptors is termed reverse signalling. Osteoclasts express ephrinB2 while osteoblast express EphB4. Reverse signalling through ephrinB2 into OC precursors suppresses osteoclast differentiation by blocking the c-Fos-NFATc1 cascade. Forward signalling through EphB4 enhances osteoblastic differentiation. Thus, ephrinB2-EphB4 bidirectional signalling links suppression of osteoclast differentiation with stimulation of bone formation. This may be one mechanism of regulating the transition from bone resorption to formation during the remodelling cycle (Zhao, Irie et al. 2006).

1.4.2.2 Mechanism of bone resorption

Resorption requires the following cellular activities: migration of OC to the resorption surface, its attachment to bone, polarization and formation of new membrane domains, dissolution of hydroxyapatite, degradation of organic matrix, removal of degradation products and finally apoptosis of osteoclasts or return to a non-resorptive state (Vaananen, Zhao et al. 2000).

OC attach to bone targeted for resorption via integrins. The OC becomes polarized to form a sealing zone, ruffled border and functional secretory domain. The sealing zone creates a microenvironment between the OC and the underlying bone matrix. Here, the secretion of HCl by ATP-dependent vacuolar proton pumps and the $2\text{Cl}^-/\text{H}^+$ antiporter (chloride channel 7) through the ruffled border dissolves crystalline hydroxyapatite. After solubilisation of the mineral phase, proteolytic enzymes including matrix metalloproteinases (MMPs) and cathepsin K digest the organic matrix. Degradation products are removed from the resorption lacuna via a transcytotic vesicular pathway to the functional secretory domain for transport into the extracellular space. En route, collagen and other matrix components are further proteolytically degraded by cathepsin K and tartrate-resistant acid phosphatase (TRAcP), an osteoclast specific enzyme (Vaananen, Zhao et al. 2000, Crockett, Rogers et al. 2011).

Small GTPases including Rac, Rho and Rabs regulate osteoclast polarisation and vesicular trafficking necessary for osteoclast function. These require post-translational modification by isoprenylation to enable appropriate intracellular localization and function. Bisphosphonates disrupt key enzymes in the isoprenylation process and are powerful inhibitors of osteoclastic bone resorption (Coxon, Thompson et al. 2006).

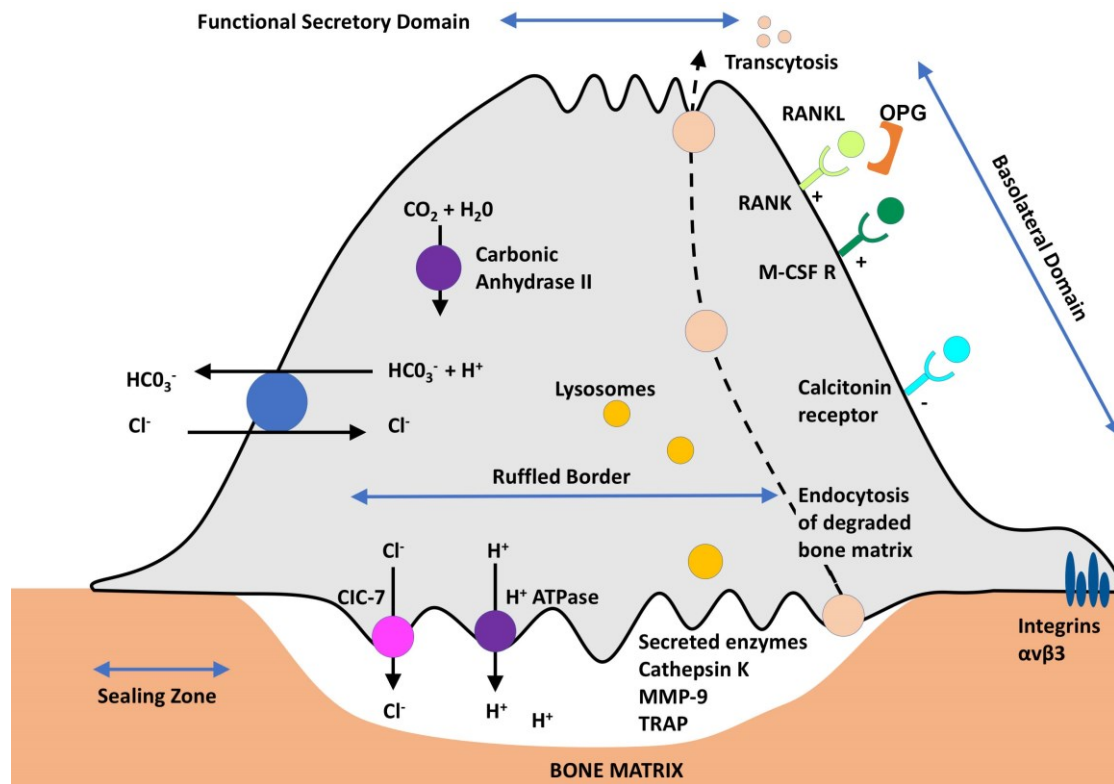


Figure 1.8 Resorbing osteoclast

Osteoclast differentiation and maturation is stimulated by RANKL and M-CSF but inhibited by calcitonin and osteoprotegerin (OPG). Osteoclasts attach to bone surface with integrins, creating a sealing zone that surrounds the ruffled border. Carbonic anhydrase converts CO_2 and water to HCO_3^- and H^+ . HCO_3^- is exchanged out of the cell for Cl^- . H^+ and Cl^- are secreted onto the bone surface to form hydrochloric acid, and together with other proteases resorb bone. Degraded matrix is taken into the osteoclast by endocytosis and exported out of the cell by transcytosis through the functional secretory domain.

1.4.3 Osteocytes

Osteocytes are the most abundant cell type in bone, accounting for 95% of all bone cells. At the end of the bone forming phase, osteoblasts have 3 potential fates: 1) they undergo apoptosis, 2) they become inactive bone-lining cells on the surface of bones, 3) they are embed into bone as osteocytes (Rocheftort, Pallu et al. 2010).

Osteoblasts that become entombed in bone matrix change cell morphology, acquiring a stellate shape with dendritic processes that connect to each other and the bone surface. This enables osteocytes to sense mechanical loads and communicate with other bone cells. Osteocytes reside individually in caves called lacunae while dendritic processes are located within tiny channels called canaliculi. Together they form the lacunocanalicular network.

Osteocyte differentiation is associated with the reduction in certain bone markers (alkaline phosphatase, bone sialoprotein, osteocalcin, collagen I, Runx2), the maintenance of some markers (osteopontin, $\beta 3$ intergrin, E11/gp38 antigen) and the emergence of new markers (CD44, dentrin matrix protein1 (DMP1), matrix extracellular phosphoglycoprotein (MEPE))(Rocheftort, Pallu et al. 2010).

Osteocytes have 2 principle functions: 1) the regulation and co-ordination of bone remodelling, 2) the regulation of mineral and phosphate metabolism. Osteocytes are able to orchestrate bone remodelling because of their diffuse and central location within bones and their ability sense mechanical loads and communicate with other bone cells. These are achieved via the osteocyte and its lacunocanalicular network. Osteocyte can communicate by the paracrine diffusion of nitric oxide (NO) and the transmission of small molecules such as prostaglandin E2 (PGE2) via gap junctions formed by connexin proteins. Furthermore, osteocytes can initiate bone remodelling through apoptosis. Pro-apoptotic signals are observed in osteocytes adjacent to micro-cracks that occur following plastic deformation. Other factors associated with osteocyte apoptosis include oestrogen deficiency, hypoxia, immobilization and glucocorticoid therapy. In contrast, physiological loading and bisphosphonates increase osteocyte viability (Noble and Reeve 2000, Weinstein, Nicholas et al. 2000, Tatsumi, Ishii et al. 2007, Rosen and American Society for Bone and Mineral Research. 2013).

Sclerostin, encoded by *SOST* and produced predominately by osteocytes, inhibits WNT signalling in osteoblast lineage cells and is an important regulator of bone formation. Sclerostin expression is increased during immobilisation (Gaudio, Pennisi et al. 2010) and reduced during mechanical loading in mice (Robling, Niziolek et al. 2008).

Osteocytes are capable of both adding and removing mineral from the perilacunar matrix and canaliculi. In lactating mice, osteocytes express genes used by osteoclasts (TRAcP, cathepsin K) to remodel perilacunar matrix and enlarge lacunae. After weaning, gene expression levels and lacunar size returned to levels seen in virgin mice. This was mediated through PTH1R and does not occur with unloading (Qing, Ardeshipour et al. 2012). Since the surface area of the lacunocanalicular network is several orders of magnitude larger than the bone surface, this enables the osteocyte to contribute to mineral homeostasis and affect the biomechanical properties of bone (Rosen and American Society for Bone and Mineral Research. 2013).

Osteocytes regulates phosphate levels and bone mineralisation through several positive and negative regulators including fibroblast growth factor 23 (FGF23), phosphate-regulating gene with homologies to endopeptidases on the X chromosome (PHEX), dentin matrix protein-1 (DMP-1) and matrix extracellular phosphoglycoprotein (MEPE). Osteocytes are the main source of circulating FGF23, a hormone that causes renal phosphate wasting. FGF23 acts on FGFR with Klotho that converts canonical FGFR1 into a receptor specific for FGF23. In the kidneys, FGF23 decrease the expression of sodium/ phosphate cotransporters needed for phosphate reabsorption. FGF23 also reduces the activation of vitamin D by down-regulating the enzyme 1- α -hydroxylase. This in turn reduces sodium/ phosphate cotransporter expression in the intestine and therefore phosphate absorption (Dallas, Prideaux et al. 2013).

DMP1 is important for osteocyte differentiation, bone mineralisation and is a negative regulator of FGF23. Genetic deletion causes hypophosphataemic rickets (Feng, Ward et al. 2006). PHEX is highly expressed in osteocytes and reduces FGF23 expression (Liu, Guo et al. 2003). Mutations in PHEX cause x-linked hypophosphataemic rickets in humans (Hyp_Consortium 1995). Both DMP-1 and PHEX inhibit FGF23 through a common FGFR signalling pathway (Martin, Liu et al. 2011). MEPE is a potent inhibitor

of mineralisation that increases FGF23 levels by inhibiting PHEX enzymatic activity (Gowen, Petersen et al. 2003, Liu, Rowe et al. 2007).

1.5 EXTRACELLULAR MATRIX OF BONE

The extracellular matrix of bone is composed of mineral, collagen, water, noncollagenous proteins (NCP) and lipids in decreasing proportion. They provide mechanical and metabolic functions.

1.5.1 Mineral

The principal mineral component in bone is a nanocrystalline, highly substituted analogue of the naturally occurring mineral hydroxyapatite ($\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$). Substitutions include carbonate, magnesium, acid phosphate and trace elements. The mechanical functions of bone mineral are to provide rigidity and load bearing strength (Clarke 2008). Its main metabolic function is to act as a reserve for calcium, phosphate, magnesium ions for mineral homeostasis (Boskey and Robey 2013).

1.5.2 Collagen

Type I collagen is the basic building block of bone matrix fibrillary network, making up 90% of the organic matrix. It is a triple helical molecule composed of two identical $\alpha 1$ chains and a structurally similar but genetically different $\alpha 2$ chain (Boskey and Robey 2013). Collagen α chains are synthesised in the endoplasmic reticulum of osteoblasts (Figure 1.9) and contain repeating glycine-X-Y residues that are post-translationally modified by hydroxylation and glycosylation. Then, a multistep process involving the association of three C-propeptide domains, nucleation and propagation forms triple helixes that are secreted from the cell. Extracellular enzymatic cleavage of N- and C-terminals is followed by cross linking of fibrils (Baum and Brodsky 1999). Type I collagen provides a framework for mineral nucleation and growth, modulates signal transduction of bone cells and contributes to the elasticity, flexibility and strength in tension of bone (Clarke 2008, Fang and Holl 2013).

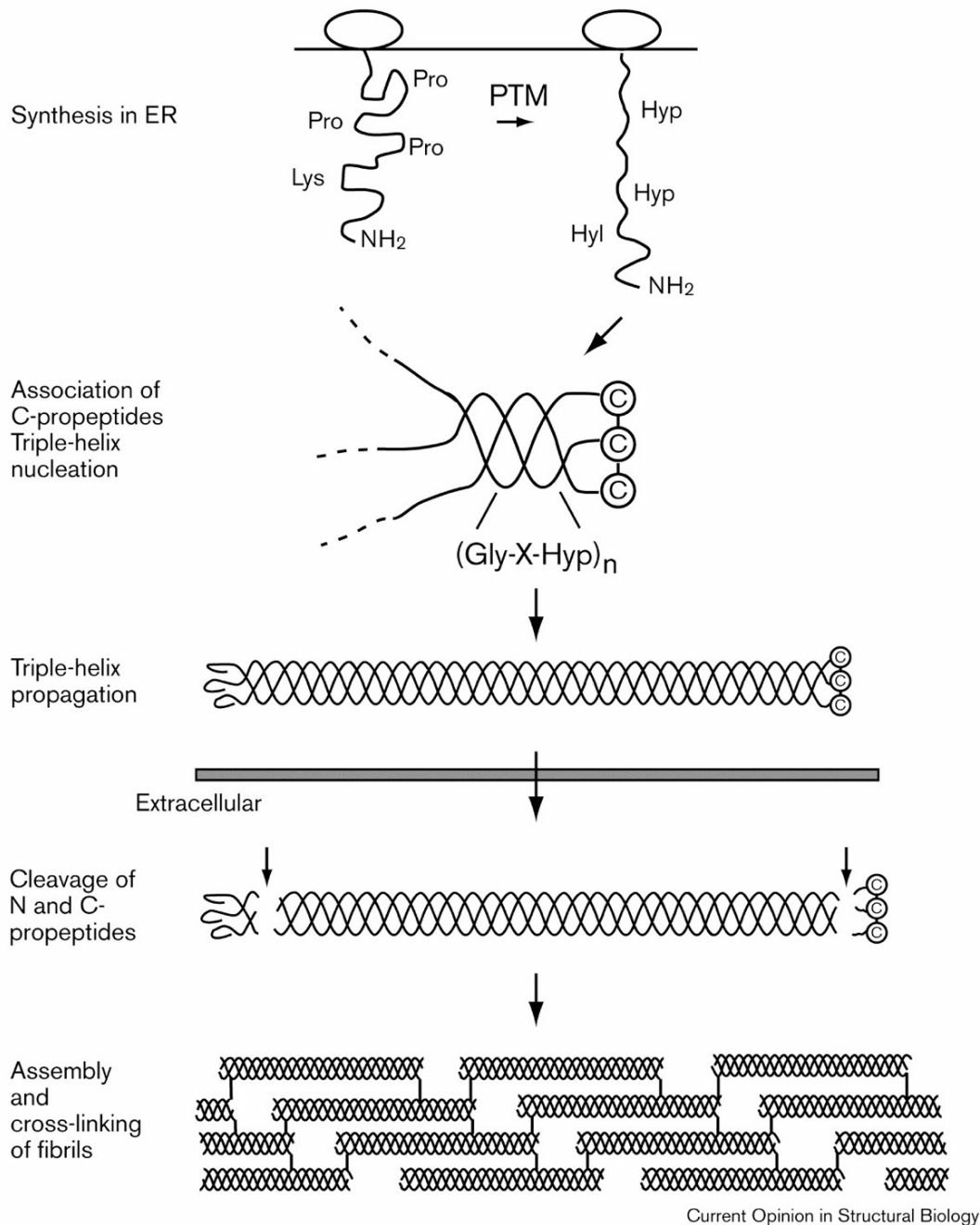


Figure 1.9 Type I Collagen synthesis and folding

Figure reproduced from (Baum and Brodsky 1999) with permission. Collagen α chains are synthesised in endoplasmic reticulum, post-translationally modified and then assembled in triple helixes through nucleation and propagation of chains. These are secreted from osteoblasts, enzymatically cleaved and cross linked to form fibrils. Hyp-Hydroxyproline; Gly-Glycine; Pro-Proline; Hyl-Hydroxylysine, Lys- Lysyl.

1.6 ENDOCHONDRAL OSSIFICATION AND THE GROWTH PLATE

Long bones grow through a process called endochondral ossification, where a cartilage intermediate is replaced by bone. Embryonic mesenchymal cells condense and differentiate into chondrocytes, which secrete a matrix rich in collagen II and aggrecan to form a cartilage model (Figure 1.10). Cells at the border of condensations form a perichondrium. Cartilage enlarges through chondrocyte proliferation and matrix synthesis. Subsequently, chondrocytes at the centre of the cartilage stop proliferating, undergo hypertrophic differentiation, secrete type X collagen and mineralise the surrounding matrix and eventually undergo apoptosis. Hypertrophic chondrocytes regulate bone growth through several actions. Adjacent perichondral cells are directed to become osteoblasts and form a bone collar in the midshaft (diaphysis); chondroclasts are recruited to digest cartilage matrix; vascular growth factors promote vascular invasion and the recruitment of osteoblasts to deposit bone matrix on the cartilage scaffold (Kronenberg 2003).

Secondary ossification centres form at the ends of long bones and are separated from the primary ossification centre by the epiphyseal growth plate. The growth plate is a collection of chondrocytes organized into discrete zones. The reserve zone contains progenitor cells that are recruited into the proliferative zone, where cells are organized into discrete columns. Differentiation begins in the pre-hypertrophic zone, with 10-fold volume expansion of chondrocytes in the hypertrophic zone and expression of collagen X before eventually undergoing apoptosis. This tightly regulated sequence of chondrocyte proliferation, hypertrophic differentiation and apoptosis mediates longitudinal bone growth. In humans, fusion of epiphyseal growth plates occurs after pubertal growth when final adult height is attained.

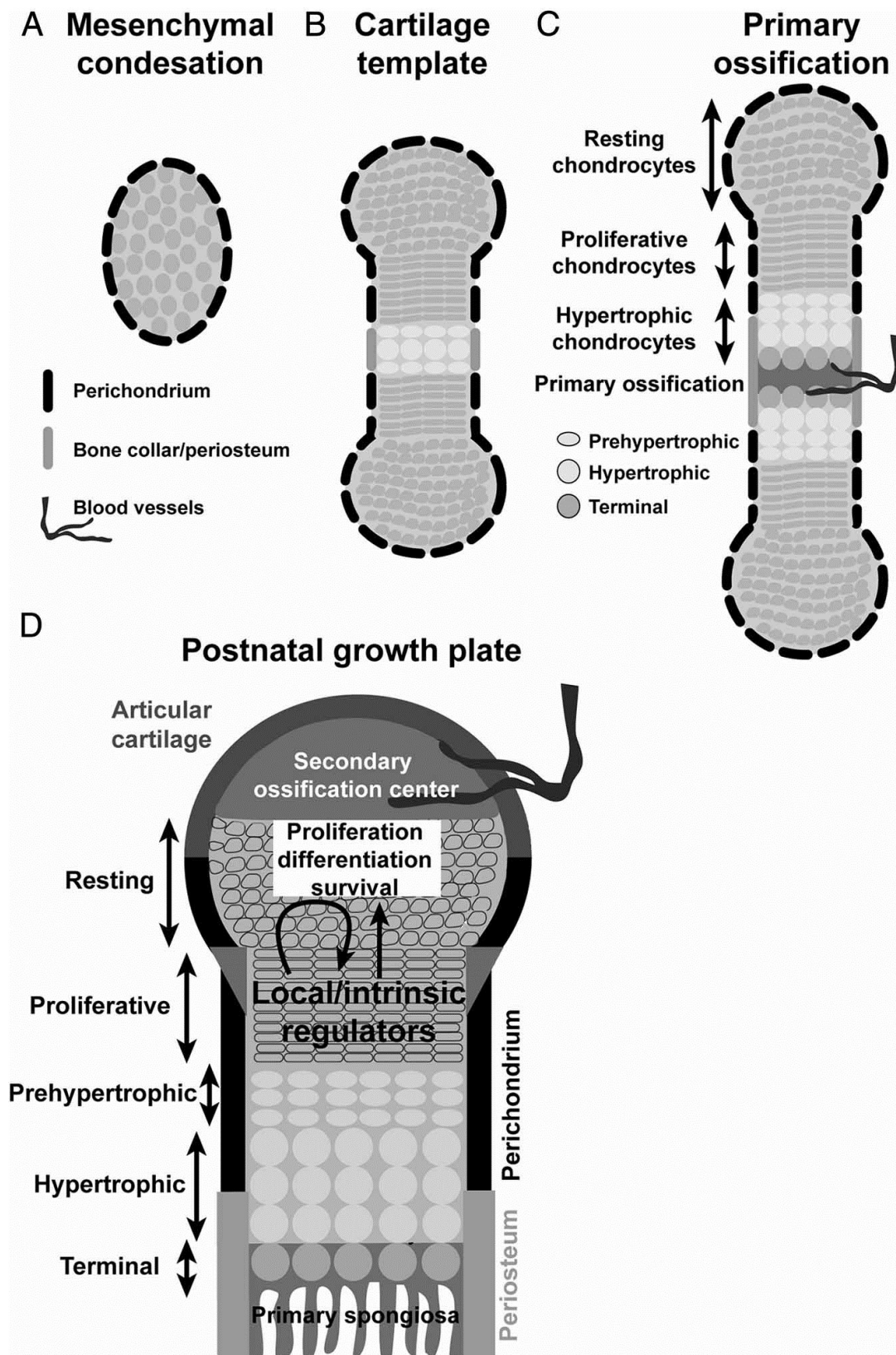


Figure 1.10 Developmental steps during endochondral ossification

Adapted from (Rosello-Diez and Joyner 2015) with permission. See text for details.

Several hormones and growth factors regulate the pace of chondrocyte differentiation and maturation. Indian hedgehog (IHH) and parathyroid hormone related peptide (PTHrP) are critical regulators that participate in a negative feedback loop to coordinate chondrocyte maturation in the growth plate (Figure 1.11). PTHrP is secreted by perichondral cells and proliferating chondrocytes to inhibit differentiation, maintain chondrocytes in the proliferating phase and inhibit the production of IHH. IHH is produced by prehypertrophic and early hypertrophic chondrocytes that are sufficiently far from a source of PTHrP. IHH diffuses towards the articular end of the growth plate, where through unknown mechanisms, activates the expression of PTHrP. This negative feedback loop can be modified by interaction with other signalling molecules including BMPs and FGFs.

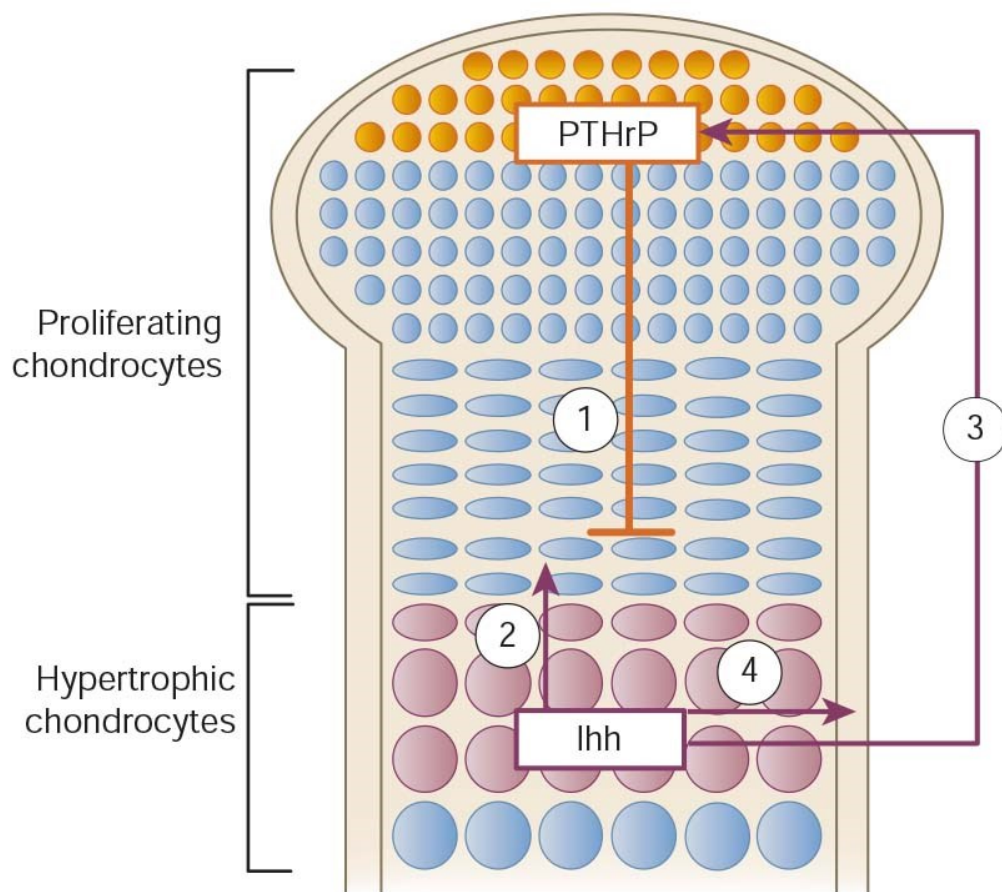


Figure 1.11 Growth plate regulation by Parathyroid hormone-related protein (PTHrP) and Indian hedgehog (Ihh)

Figure reproduced from (Kronenberg 2003) with permission. 1) proliferating chondrocytes secrete PTHrP that prevents differentiation and keeps cells in a proliferating state. 2) Chondrocytes far enough from PTHrP secrete Ihh that accelerates proliferation and 3) feeds back to chondrocytes at the articular ends to produce PTHrP and inhibit Ihh production. 4) It also acts on perichondral cells to encourage osteoblast differentiation.

1.7 REGULATION OF BONE MODELLING & REMODELLING

Bone formation and resorption is a tightly regulated process in growth and adulthood. Modelling is the process by which bones change shape in response to physiological influences or mechanical forces. Bone formation and resorption are not tightly coupled in time or space and it occurs primarily during growth. Bone remodelling is the process by which bone is continually renewed to maintain strength and mineral homeostasis. Here formation and resorption is tightly coupled and it occurs as part of adult bone maintenance (Clarke 2008).

1.7.1 Bone modelling

Longitudinal bone growth occurs by endochondral ossification while the diameter is increased by deposition of bone under the periosteum. In order to change shape or axis, bone resorption by osteoclasts and deposition by osteoblast must occur independently. An example is the resorption around the metaphysis that maintains a narrower diaphysis. This process of bone growth in one area and resorption in another is called modelling.

Bone deposition occurs on the periosteal surface and resorption occurs on the endosteal surface to maintain the medullary cavity. Thus, cortical thickness represents the difference between bone deposition and resorption on these two surfaces and gives an indication of osteoclast function.

1.7.2 Bone remodelling

Bone remodelling is performed by a functional and anatomic structure called the basic multicellular unit (BMU). This consists of the bone lining cells, osteocytes, osteoblasts and osteoclasts. There are four distinct phases in the remodelling process (Figure 1.12) (Feng and McDonald 2011):

1. Initiation of remodelling
2. Bone resorption and recruitment of osteoprogenitors
3. Osteoblasts differentiation and function (osteoid synthesis)
4. Mineralisation of osteoid

Apoptotic osteocytes direct the initiation of remodelling to the targeted bone site following microdamage (Verborgt, Gibson et al. 2000, Hedgecock, Hadi et al. 2007). Apoptosis of osteocytes may release osteoclastogenic factors or lead to a loss of inhibitory signals that suppress osteoclastogenesis. Nitric oxide and TGF- β have been proposed as possible anti-osteoclastogenic molecules (Heino, Hentunen et al. 2002, Tan, de Vries et al. 2007). An enclosed vascular compartment called the bone remodelling compartment (BRC) forms with bone lining cells creating a roof under which osteoclast precursors are recruited either through the bone lining cell monolayer or directly from bone marrow. The recruitment of osteoclast precursors, their differentiation and activation occurs in response to M-CSF and RANKL and other key chemokines, co-stimulatory molecules and transcription factors described earlier in the section on the regulation of osteoclastogenesis. Osteoclastic resorption lasts about 3 weeks in humans.

Mesenchymal stem cells or osteoprogenitors are recruited to the BRC in preparation for the next phase of remodelling. In the transition phase, coupling between osteoclasts and osteoblasts coordinates the switch from bone resorption to bone formation as the main activity. Coupling factors may include liberated growth factors from the resorbed matrix including TGF- β and insulin-like growth factor I (IGF-I). Other regulatory mechanisms include bidirectional signalling through Eph-Ephrin interactions described earlier (Kular, Tickner et al. 2012). The result of this coupling is that osteoclasts stop resorbing bone and undergo apoptosis, while osteoprogenitors differentiate into mature osteoblasts that synthesize osteoid.

Newly synthesized osteoid is mineralised before the remodelling cycle is complete. This final terminal phase is much slower and last about 3 months from the start of bone formation. Mature osteoblasts either become buried inside new bone to become osteocytes, or undergo apoptosis. Osteocyte derived sclerostin is likely to be an important regulator of this termination phase by inhibiting osteoblastic bone formation (van Bezooijen, Roelen et al. 2004, Matsuo and Irie 2008).

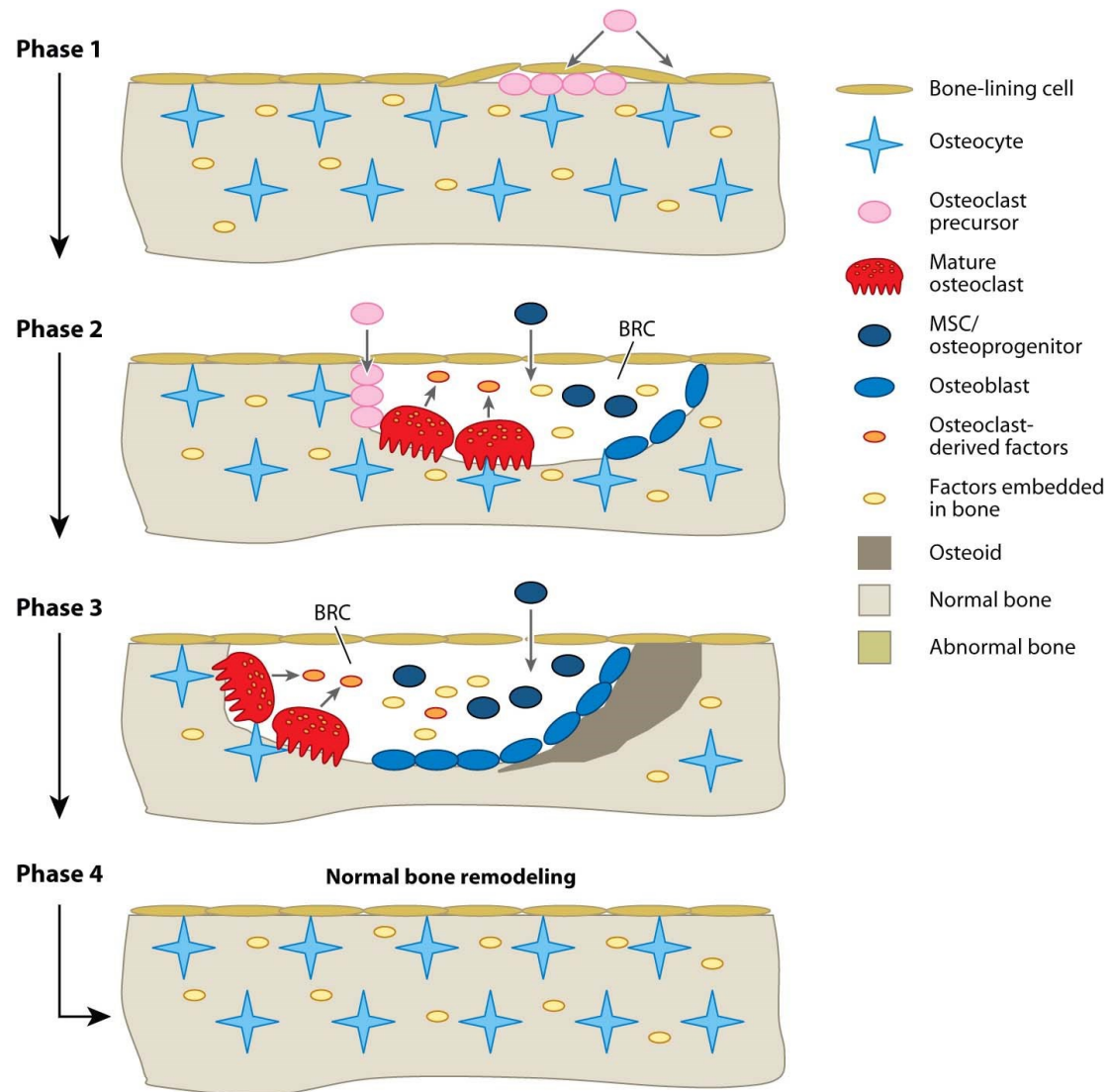


Figure 1.12 Bone remodelling cycle

Figure reproduced from (Feng and McDonald 2011) with permission. Phases of remodelling cycle: (1) Activation of bone remodelling at a specific site. (2) Bone resorption and recruitment of mesenchymal stem cells and osteoprogenitors. (3) Osteoblast differentiation and secretion of osteoid. (4) mineralisation of osteoid and completion of remodelling.

1.8 PATHOLOGY AND PATHOPHYSIOLOGY OF OSTEOARTHRITIS

Osteoarthritis (OA) is a disease process that affects all joint tissues, leading to joint dysfunction (Lotz 2012). Pathological features seen in OA joints reflect the cellular response to injury. Changes include degradation of the articular cartilage, thickening of the subchondral bone, formation of osteophytes, variable degrees of inflammation of the synovium, degeneration of ligaments and, in the knee, the menisci, and hypertrophy of the joint capsule (Loeser, Goldring et al. 2012).

1.8.1 Articular cartilage

Articular cartilage changes have been the best studied. Initially there is loss of cartilage proteoglycan through increased expression and activity of degrading matrix metalloproteinase enzymes (mainly MMP-13) (Little, Barai et al. 2009) and aggrecanases (a disintegrin and metalloproteinase with thrombospondin motifs, ADAMTS) (Glasson, Askew et al. 2005). This is detected as decreased staining of the matrix with metachromatic stains such as safranin O. The articular cartilage appears roughened due to the presence of fibrillations, fissures and cartilage erosion (Figure 1.13). Fibrillations are surface discontinuities without matrix loss parallel/tangential to the surface in the superficial cartilage layer. In time fibrillations extend, arch down, and become vertical cracks, now termed fissures, extending in a simple or branching manner into deeper cartilage zones. Erosions appear as cartilage tissue is lost as a result of both mechanical and proteolytic activity. Initially confined to the surface, cartilage erosion eventually extend more deeply in the absence or presence of fissures until there is full depth cartilage loss and exposure of the underlying bone (Salter, Buckwalter et al. 2011).

In normal cartilage, chondrocytes are quiescent and do not undergo terminal differentiation. However, in response to injury and changes in the matrix, chondrocytes undergo hypertrophic differentiation and express markers (including Collagen X, MMP-13) characteristic of the hypertrophic chondrocytes of the growth plate in a process that resembles endochondral ossification (Dreier 2010). This latter process is required for maturation of osteophytes that are characteristic of osteoarthritis (van Osch, van der Kraan et al. 1996, van der Kraan and van den Berg 2007). Alternatively, chondrocytes either proliferate, forming clusters with increased production of matrix

protein and degrading enzymes, or undergo cell death by apoptosis or necrosis (Salter, Buckwalter et al. 2011, Loeser, Goldring et al. 2012). The switch towards chondrocyte hypertrophy is accompanied by calcification of the extracellular matrix and advancement of the tidemark between calcified and non-calcified cartilage (Lories and Luyten 2011). Histologically this is seen as multiple tidemarks. Calcification of the articular cartilage also increases with age and severity of OA (Mitsuyama, Healey et al. 2007, Fuerst, Bertrand et al. 2009)

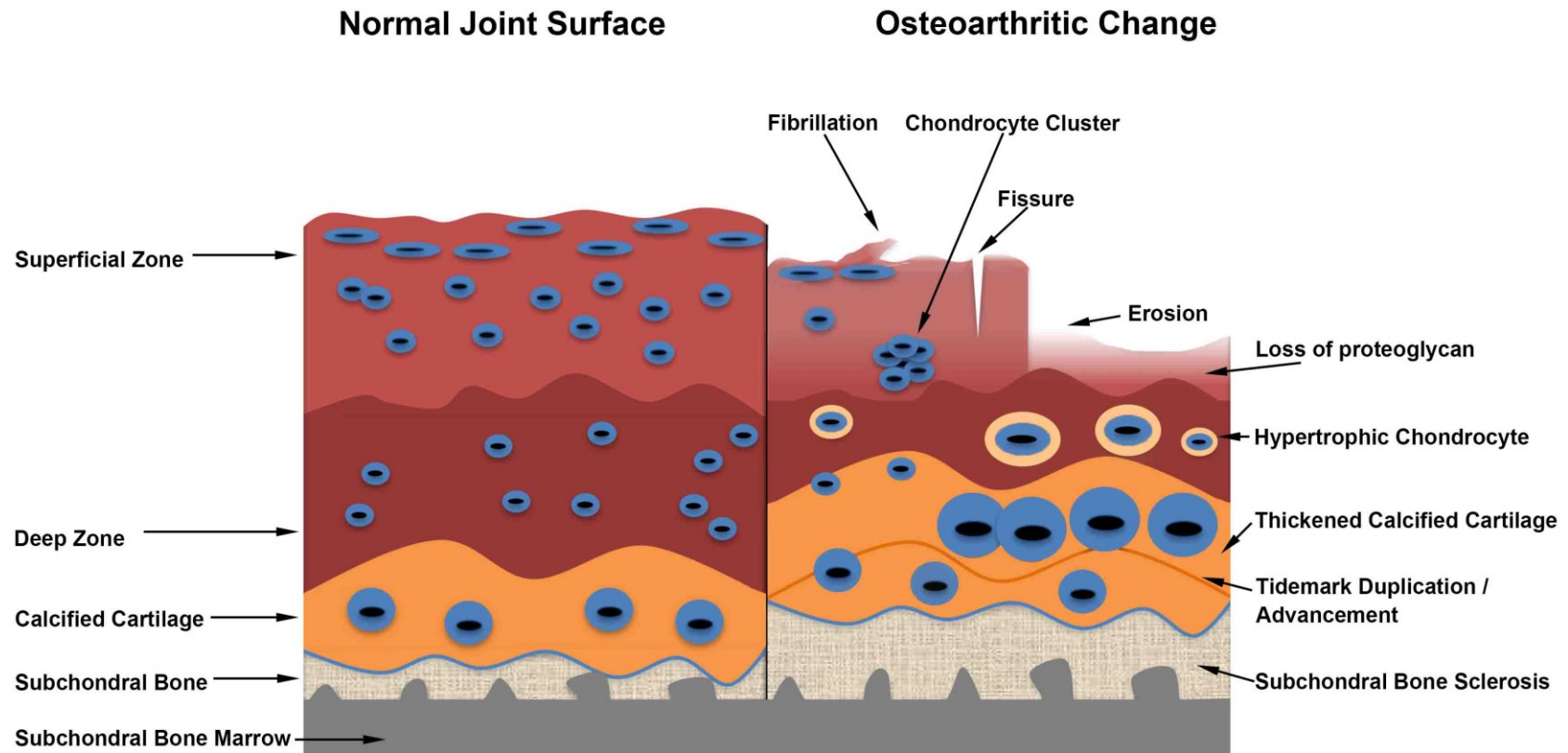


Figure 1.13 Osteoarthritis pathology

See text for details

1.8.2 Subchondral bone remodelling in osteoarthritis

Established osteoarthritis is characterised by subchondral plate sclerosis and increase in BMD. The thickness of subchondral bone plate is inversely correlated with the integrity and thickness of overlying articular cartilage. (Aho, Finnila et al. 2017). Subchondral bone changes in OA (Figure 1.14) can be divided into early and late phases. The early phase is characterised by increased bone turnover and an initial loss of bone. Late phase changes are characterised by a progressive increase in cortical plate density due to a net balance in favour of bone formation. Our understanding of pathophysiology is informed by experiments that demonstrate subchondral bone changes in this temporal sequence. Furthermore, interventions that target different locations in subchondral bone or different time points of the remodelling process have been informative.

Animal models of OA have shown that early subchondral bone plate thinning or increased osteoclastic activity is associated with cartilage damage. Furthermore, the areas of cartilage damage co-localise with subchondral bone changes (Snijkers, Intema et al. 2008, Bellido, Lugo et al. 2010, Bertuglia, Lacourt et al. 2016). Dynamic histomorphometry demonstrated that in early experimental OA, subchondral bone mineral apposition rate is increased three to fivefold (Benske, Schunke et al. 1988). Human studies have shown similar relationships between subchondral bone loss and cartilage damage, as well as increased bone resorption markers in subjects with progressive OA (Bettica, Cline et al. 2002, Reichenbach, Guermazi et al. 2008, Neogi, Felson et al. 2009).

In late phase OA subchondral bone resorption reduces without a reduction in formation (Kumarasinghe, Perilli et al. 2010) due to uncoupling of osteoblastic and osteoclastic activity (Kuliwaba, Findlay et al. 2000). This results in net bone formation, leading to loss of trabecular bone that is replaced with sclerotic subchondral plate. Radin proposed in 1970 that subchondral bone changes preceded cartilage damage (Radin, Paul et al. 1970). The hypothesis was that stiff, sclerotic subchondral bone increased loads transmitted to the articular cartilage and increased susceptibility to damage. However, several groups have demonstrated that in late stage OA, subchondral bone volume is increased but the tissue is less mineralised, presumably due to increased bone turnover and the deposition of osteoid that has not had time to fully mineralise (Li and

Aspden 1997, Day, Ding et al. 2001, Coats, Zioupos et al. 2003, Cox, van Donkelaar et al. 2012). Thus, a new model is that sclerotic subchondral bone is less stiff and might increase articular cartilage deformation, local stresses and lead to cartilage loss (Karsdal, Leeming et al. 2008, Burr and Gallant 2012).

Joint injury may occur with an excessive load to a normal joint, for example in obesity; alternatively, a normal load may be transmitted through a structurally abnormal joint, for example following trauma to the knee (Tanamas, Hanna et al. 2009, Little and Hunter 2013). This is supported by the reproducible cartilage and bone changes in joint destabilisation models of OA (Vincent, Williams et al. 2012). Osteophytes may be an adaptive response to stabilise joint and surgical removal increases joint instability (Pottenger, Phillips et al. 1990). Osteophyte formation involves chondrocyte hypertrophy and endochondral ossification, with TGF β and BMPs acting as key regulators (van der Kraan and van den Berg 2007). Together, these observations point to the importance of mechanical loads in osteoarthritis pathogenesis.

While biomechanical properties of subchondral bone are clearly important, its alteration does not always result in OA. High bone mass patients such as those with gain of function LRP5 mutations are not at increased risk of OA (Boyden, Mao et al. 2002). Sheep with metal rods inserted into the subchondral plate develop sclerosis early but do not develop cartilage damage after 5 years. Finally, two guinea pig strains with different subchondral bone characteristics illustrate this point. Dunkin-Hartley guinea pigs have increased bone turnover and develop cartilage damage, while strain 13 that has 6-7 times thicker subchondral bone plate at baseline but a slower increase does not develop OA (Huebner, Hanes et al. 2002, Burr and Gallant 2012).

These studies suggest the rate of change in subchondral bone density and plate thickness may be more important in determining the risk of developing cartilage damage. Mechanisms that lead to initiation or accelerated subchondral bone remodelling may include microcracks in the subchondral plate that release RANKL and downregulate OPG (Bentolila, Boyce et al. 1998, Verborgt, Gibson et al. 2000, Nakashima, Hayashi et al. 2011, Kennedy, Herman et al. 2012); vascular invasion mediated by angiogenic factors (Ludin, Sela et al. 2013) and inflammatory mediators in the synovium (Hugle and Geurts 2017). Therapies targeting subchondral bone remodelling have shown

promise in animal models of OA (Hayami, Pickarski et al. 2004, Kadri, Ea et al. 2008, Kadri, Funck-Brentano et al. 2010, Bellido, Lugo et al. 2011, Sondergaard, Catala-Lehnen et al. 2012). However, this has not been reproduced in human clinical trials with anti-resorptive agents (Castaneda, Roman-Blas et al. 2012). Timing with early intervention is likely to be important since in late-stage OA, bone resorption is already suppressed but bone formation is not adequately targeted.

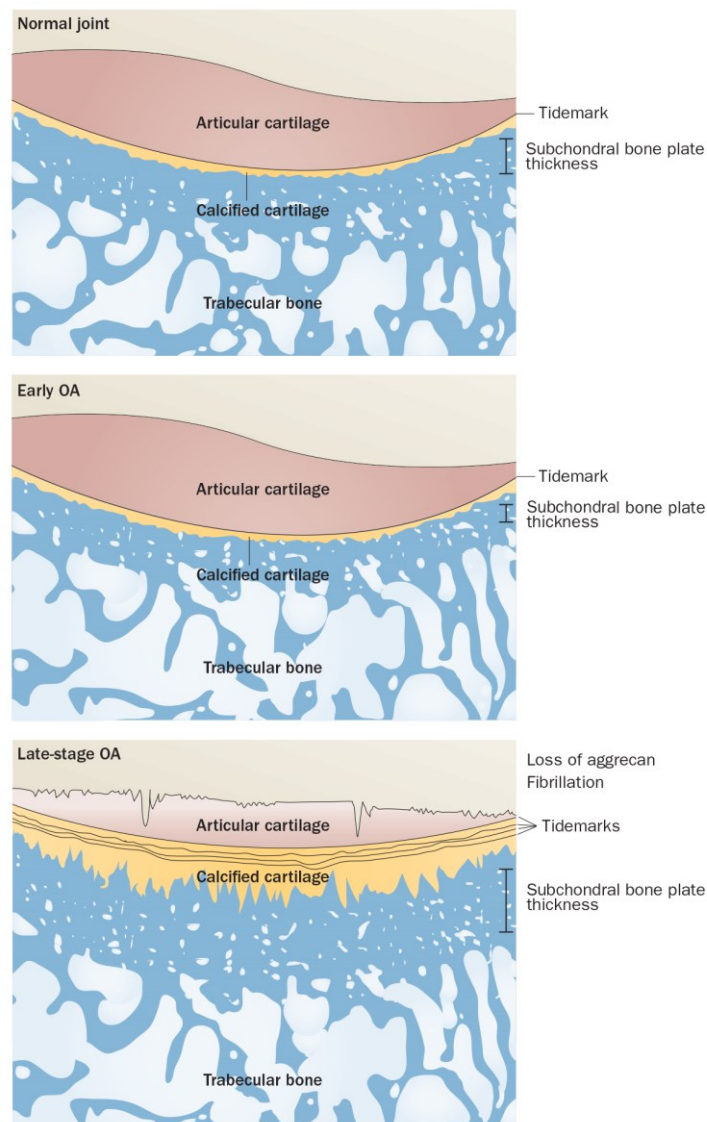


Figure 1.14 Subchondral bone changes in osteoarthritis

Reproduced from (Burr and Gallant 2012) with permission . Beneath articular cartilage lies mineralised tissue that consists of calcified articular cartilage, dense subchondral bone plate and porous trabecular bone. In early OA, subchondral plate becomes thinner due to increased remodelling. Trabecular bone is also lost as trabecular plates become thinner and more rod like. In later OA, subchondral plate thickens but trabecular bone remains osteopaenic. Calcified cartilage advances into a receding hyaline articular cartilage, leaving multiple mineralising fronts seen as tidemarks. Thus, the mineralised plate (made of both cortical plate and calcified cartilage) is seen as subchondral sclerosis. At the same time, non-mineralised tissue becomes thinner and loses aggrecan (paler colour figure), develops fibrillations and is unable to repair.

1.9 THYROID HORMONE ACTION

1.9.1 Introduction

This section will describe thyroid hormone action, including synthesis, feedback regulation in the circulation, transport into cells, metabolism and action mediated via its nuclear receptor. The next section will review our current understanding of thyroid hormone action in the developing and adult skeleton, focussing on bone cells and growth plate chondrocytes. Finally, our current understanding of thyroid hormone action in the joints is reviewed, including evidence that implicates a potential pathophysiological role for thyroid hormone in osteoarthritis.

1.9.2 Thyroid hormone synthesis

Thyroid hormones are composed of iodinated tyrosine moieties joined by an ether link. Thyroid hormones are synthesised, stored and secreted by follicular cells in the thyroid gland under the control of thyrotropin (TSH). This process involves iodine trapping, where iodide is actively transported by the sodium-iodide symporter across the basolateral membrane of follicular cells. Iodide is transported across the apical surface into the follicular lumen, or colloid, before organification. This process is incompletely understood but the membrane glycoprotein Pendrin may play a role.

A scaffold protein containing many tyrosine residues called thyroglobulin (TG) is synthesised in follicular cells. The enzyme thyroid peroxidase (TPO) catalyses the oxidation of iodide to iodine and subsequent attachment of iodine to tyrosine residues. This process is called organification and forms the hormonally inactive iodotyrosines 3-iodotyrosine (monoiodotyrosine, MIT) and 3,5-diiodotyrosine (diiodotyrosine, DIT). Hydrogen peroxide (H_2O_2) is necessary for the oxidation process and this is generated by enzymes dual oxidase 1 (DUOX1) and dual oxidase 2 (DUOX2). Finally, TPO catalyses the coupling of iodotyrosines residues to form iodothyronines. The coupling of two DIT residues forms 3,5,3',5'-L-tetraiodothyronine (thyroxine, T4), while the coupling of MIT and DIT forms 3,5,3'-L-triiodothyronine (T3) within a thyroglobulin molecule.

The iodothyronines are liberated from TG in the colloid when secretion is required (Taurog 2000). The thyroid is unique amongst endocrine glands because of the large

quantities of hormone it stores and the low rate of turnover (Salvatore, Davies et al. 2011). TSH stimulates the resorption of thyroglobulin into follicular cells by endocytosis. This fuses with lysosomes containing proteases and thyroglobulin is subsequently hydrolysed and released from T4, T3, DIT and MIT. Uncoupled MIT and DIT are deiodinated in the follicular cell by iodotyrosine dehalogenase 1 (DEHAL1) and the free tyrosine and iodide are recycled (Moreno and Visser 2010). The release of T4 and T3 into the circulation is incompletely understood. It may involve active transport by the monocarboxylate transporter-8 (MCT8) (Di Cosmo, Liao et al. 2010) or fusion of thyroid hormone containing vesicles into the basolateral membrane.

1.9.3 The hypothalamic-pituitary thyroid axis

Circulating thyroid hormone levels are regulated by a negative feedback loop mediated by the hypothalamic-pituitary-thyroid (HPT) axis (Figure 1.15). The hypothalamus secretes thyrotropin-releasing hormone (TRH) that stimulates pituitary thyrotrophs to secrete thyroid-stimulating hormone (thyrotropin, TSH). TSH acts on thyroid follicular cells to stimulate the synthesis and secretion of the thyroid hormones 3,5,3',5'-tetraiodothyronine (thyroxine, T4) and 3,5,3'-triiodothyronine (T3). Thyroid hormones bind to thyroid hormone nuclear receptors in the hypothalamus and pituitary to inhibit TRH and TSH production and secretion and complete the negative feedback loop (Waung, Bassett et al. 2012).

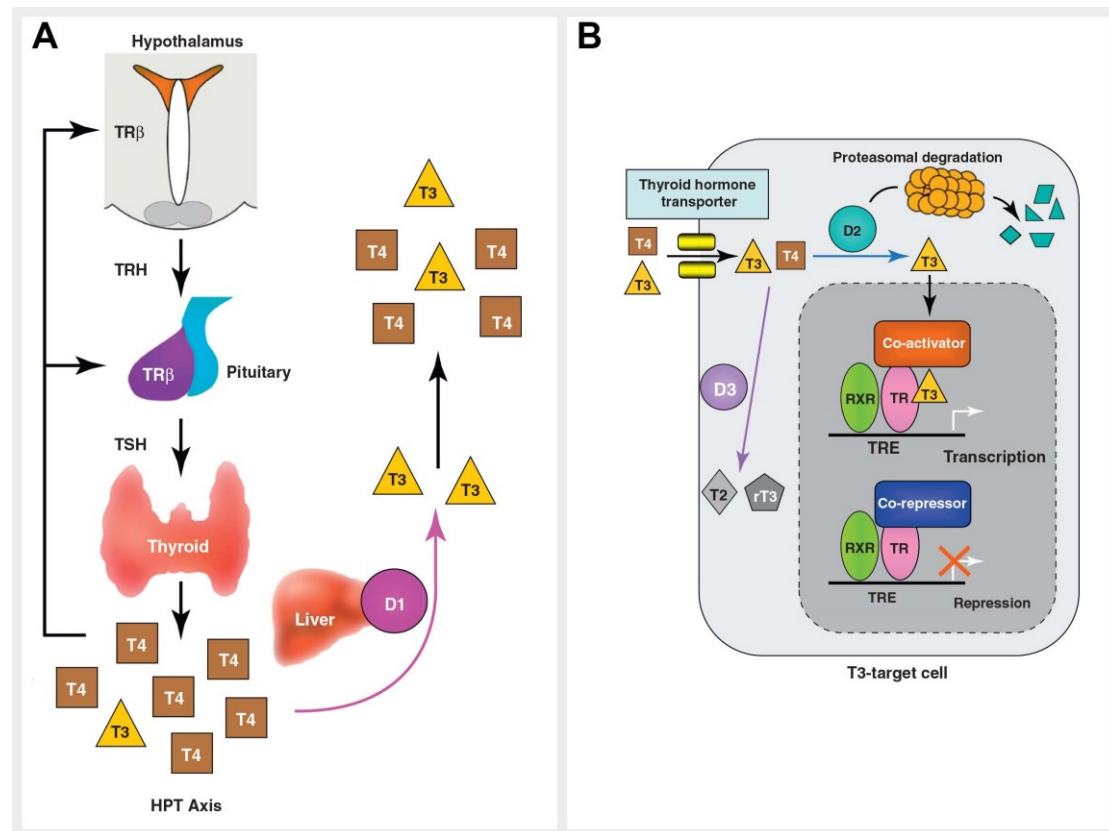


Figure 1.15 Control of circulating thyroid hormone level and target cell thyroid status

Reproduced from (Waung, Bassett et al. 2012) with permission. A) The hypothalamus pituitary thyroid axis controls circulating thyroid hormone levels. TRH stimulates pituitary TSH secretion, which stimulates the thyroid to secrete T4 and some T3. Feedback inhibition on the hypothalamus and pituitary occurs via TR β . DIO1 converts T4 to T3 in the liver, contributing significantly to the pool of circulating T3.

B) T4 and T3 enter target cells via specific membrane transporters such as MCT8. T4 is either activated to T3 by DIO2 enzyme, or deactivated to rT3 by DIO3. T3 either enters the nucleus or is inactivated to T2 by DIO3. DIO2 is rapidly degraded by ubiquitination and this is regulated by the availability of T4. Once inside the nucleus, T3 binds to TR to promote transcription of target genes.

1.9.4 Thyroid hormone transporters

Thyroid hormone enters target cells via specific membrane transporters. These including the monocarboxylate transporters (MCT)8 , MCT10, various members of the organic anion-transporting polypeptide (OATP) family, and the non-specific amino acid transporters including L-type amino acid transporters (LAT1, LAT2) (Visser, Friesema et al. 2011). The importance of thyroid hormone transporters is best illustrated by Allan-Herndon-Dudley syndrome due to mutation of MCT8 on the X chromosome. Affected males have elevated T3 concentrations with reduced T4 and inappropriately normal or modestly elevated TSH. Severe, psychomotor retardation presents early and is thought to represent a failure of T3 entry into neurons and subsequent abnormal T3 action and metabolism (Friesema, Jansen et al. 2006).

1.9.5 Thyroid hormone metabolism

Although circulating levels of T4 are approximately 5-fold higher than T3, T4 binds to the nuclear TR with 10-15-fold lower affinity for the nuclear TR compared to T3 (Oppenheimer, Schwartz et al. 1974, Salvatore, Davies et al. 2011, Abdalla and Bianco 2014). Therefore, T4 is a prohormone that necessitates conversion to the biologically active T3.

There are three selenoenzymes called iodothyronine deiodinases that regulate the activation and deactivation of thyroid hormones (Figure 1.15 & Figure 1.16). Activation of T4 involves the removal of iodine atom from the outer ring (5'-deiodination) to form T3. This is efficiently achieved by type 2 deiodinase (DIO2). Conversely, type 3 deiodinase (DIO3) irreversibly inactivates T3 and prevents the activation of T4 by removing an iodine atom from the inner ring (5-deiodination) to generate 3,3', 5'-triiodothyronine (reverse T3, rT3) and 3,3'diiodothyronine (T2), respectively. Type 1 deiodinase (DIO1) is capable of both outer and inner ring deiodination in equimolar portions to generate T3, rT3 and T2 depending on substrate availability.

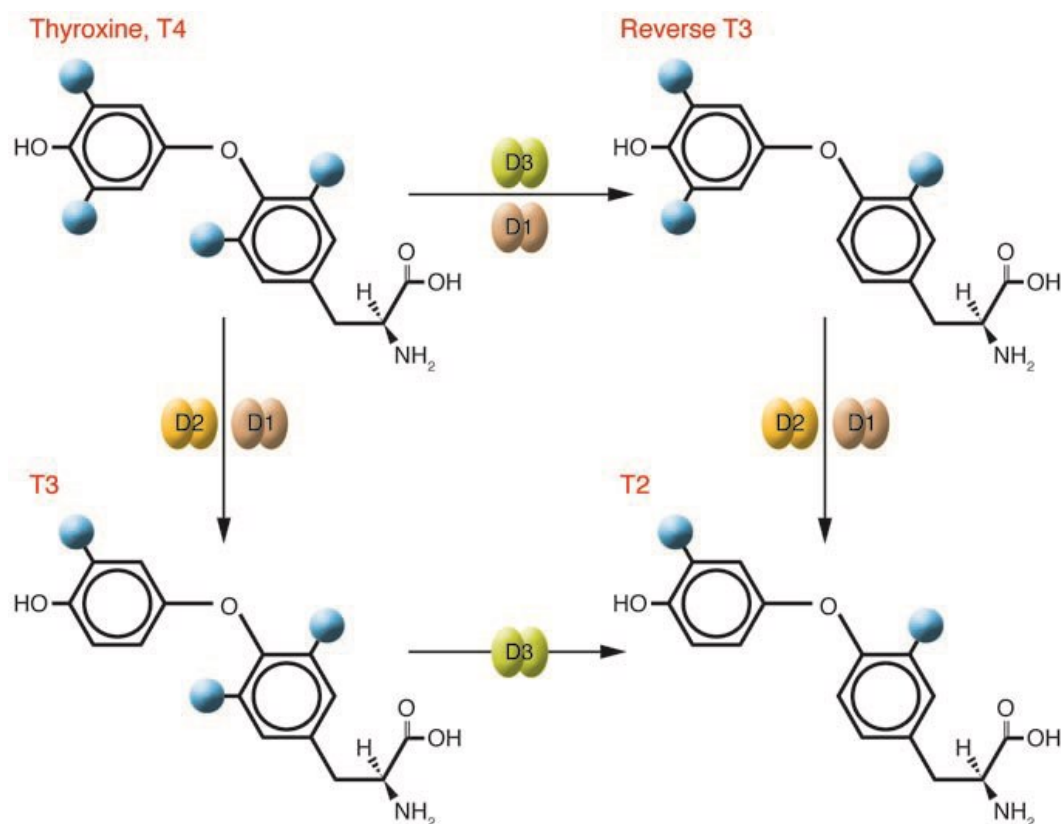


Figure 1.16 Thyroid hormone metabolism by deiodinases

Reproduced from (Bianco and Kim 2006) with permission. Deiodinases remove iodine moieties (blue circles) from the phenolic (outer ring) or tyrosyl (inner rings) of iodothyronines. Prohormone T4 can be activated to T3 by outer ring deiodination through DIO1 or DIO2 action. DIO3 inactivates T3 or prevents the activation of T4 by inner ring deiodination. T3 or rT3 can be further monodeiodinated to inactive metabolite T2.

1.9.5.1 *Deiodinase 1*

In contrast to DIO2 and DIO3, type 1 deiodinase (DIO1) is able to catalyse both inner and outer ring deiodination on a variety of different iodothyronines, including those that have undergone sulfate conjugation on the hydroxyl group on the outer ring. DIO1 is primarily expressed in the thyroid, liver and kidney and is a major source of circulating T3. Its precise physiological role is uncertain as DIO1 knockout mice have normal circulating T3 levels. A principal role as a scavenging enzyme has been proposed (St Germain, Galton et al. 2009). DIO1 is relatively inefficient with a Michaelis constant (K_m) in the micromolar range compared to both DIO2 and DIO3 that have a K_m in the nanomolar range. Furthermore, it catalyses the deiodination of rT3 (600-fold) and sulphated iodothyronines with much greater efficiencies than the 5'-deiodination of T4.

1.9.5.2 *Deiodinase 2*

DIO2 is expressed in skeletal muscle and contributes to circulating T3 levels. However, its subcellular localisation within the endoplasmic reticulum suggests that its main role is to ensure adequate intracellular T3. Thyroid status controls DIO2 activity at both pre- and post-translational levels (Burmeister, Pachucki et al. 1997). DIO2 protein has a short half-life of 40 minutes, and is rapidly ubiquitinated and degraded by the proteasome. This process is accelerated by substrate availability (Figure 1.15), providing a rapid post-translational mechanism of regulating DIO2 activity (Gereben, Zavacki et al. 2008). Reactivation of DIO2 can occur via deubiquitination (Curcio-Morelli, Zavacki et al. 2003). DIO2 has important functions in the development of many tissues including sensory organs (Ng, Goodyear et al. 2004), skeleton (reviewed later), regulation of hypothalamic pituitary thyroid axis by generating local T3 (Schneider, Fiering et al. 2001, Christoffolete, Ribeiro et al. 2006) and adaptive thermogenesis (de Jesus, Carvalho et al. 2001, Arrojo and Bianco 2011).

1.9.5.3 *Deiodinase 3*

In contrast to DIO2, the role of DIO3 is to prevent excessive tissue exposure to T3. An example is the high placental DIO3 activity that protects the developing fetus from excessive T3 and permit cell proliferation (Mortimer, Galligan et al. 1996). After birth, DIO3 activity declines rapidly while DIO2 expression increases in T3-responsive tissues to trigger cell differentiation and organ maturation. Thus, the expression of DIO2 and

DIO3 are frequently regulated reciprocally and in a tissue-specific and temporal pattern. Therefore, the balance of DIO2 and DIO3 activities determines the availability of T3 to bind to TR.

Induction of DIO3 activity may influence the healing or regenerative process in numerous tissues. DIO3 induction has been observed in animal models of tissue injury including partial hepatectomy (Kester, Toussaint et al. 2009), cardiac hypoxia, hypertrophy and failure (Wassen, Schiel et al. 2002, Simonides, Mulcahey et al. 2008), local inflammation and bacterial infection (Boelen, Kwakkel et al. 2005, Boelen, Boorsma et al. 2008) and nerve injury (Li, Le Goascogne et al. 2001). DIO3 expression is positively regulated by TR α 1 (Barca-Mayo, Liao et al. 2011).

1.9.6 Thyroid hormone receptors

The major actions of thyroid hormone are mediated by the binding of T3 to thyroid hormone receptors (TRs), ligand-dependent transcription factors, in the nucleus. There are two TR genes, *THRA* and *THRB*. *THRA* encodes a functional receptor TR α 1, and two variants that do not bind to T3 (TR α 2, TR α 3). Transcription from an internal promoter in intron 7 give rise to truncated TRs (TR $\Delta\alpha$ 1 and TR $\Delta\alpha$ 2) that retain T3-binding domain but lack amino-terminal A/B and DNA domains. They act as dominant negative antagonists *in vitro* but their physiological role is unclear (Chassande, Fraichard et al. 1997). *THRB* encodes two functional receptors, TR β 1 and TR β 2. The roles of other transcripts (TR β 3 and TR $\Delta\beta$ 3) are unclear (Williams 2000, Harvey, Bassett et al. 2007).

The expression pattern of TR α 1 and TR β 1 may change during development and in adulthood. TR α 1 is constitutively expressed during embryonic development, while TR β 1 is expressed in later stages (Cheng, Leonard et al. 2010). Although both TR α 1 and TR β 1 are widely expressed, the relative amounts is tissue-specific. Predominance of one isoform over the other may lead T3 target tissue to display TR-isoform specificity. Studies of genetically modified mice with deletions or mutations of *Thra* and *Thrb* have shown that T3 actions are mediated predominantly by TR α in the brain, heart, skeleton and gastrointestinal tract; TR β 1 mediates T3 action in the liver and lung. Both TR α 1 and TR β 1 mediate the actions of T3 in skeletal muscle and adipose tissue

that lack TR-isoform specificity. (Brent 2012). TR β 2 has a restricted expression in the brain, retina and inner ear where it regulates the timing of onset of hearing and colour vision. Its expression in the hypothalamus and pituitary is responsible for mediating feedback regulation in the hypothalamus-pituitary-thyroid (HPT) axis.

TR are localised in the nucleus and bind thyroid response elements (TREs) in the promoter of T3-target genes (Figure 1.17). TRs exist as homodimers or heterodimers with retinoic X receptors (RXRs). In positive regulated genes, unliganded TRs, also called aporeceptor, bind corepressor proteins such as nuclear corepressor molecules (NCoR) and silencing mediator for retinoid and TR (SMRT) that recruit other complexes including histone deacetylase. Deacetylation of histones maintains chromatin in a compact, repressed state. This leads to transcriptional repression by silencing of the target gene. In the presence of T3, hormone binding to the TR releases co-repressors and recruits co-activators such as steroid receptor co-activator-1 (SRC1) and histone acetylase. This leads to an uncoiled, open chromatin arrangement that permits the transcriptional machinery and RNA polymerase to access the T3 target gene promoter and increase gene expression (Bernal 2007, Flamant, Gauthier et al. 2007).

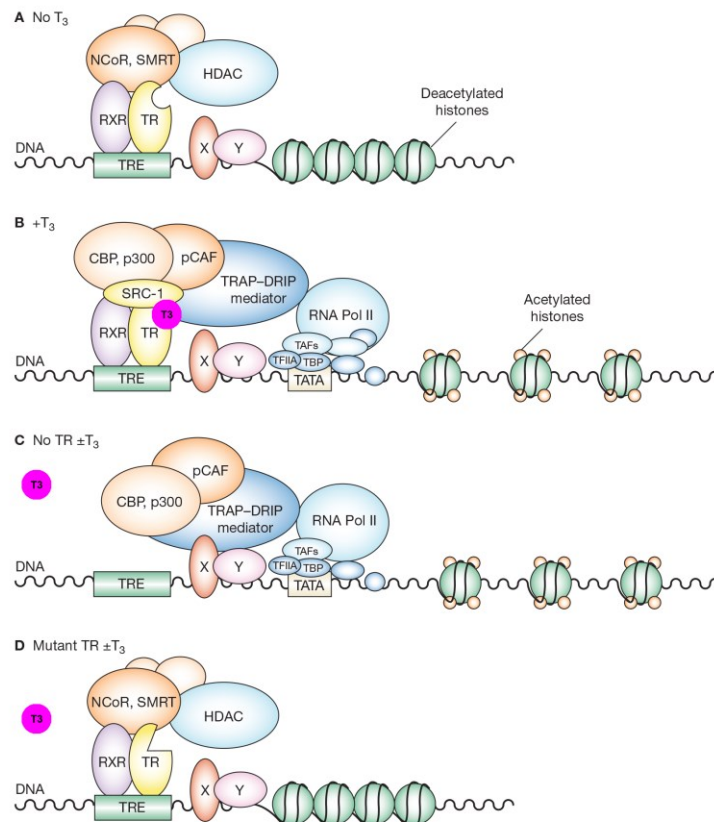


Figure 1.17 Thyroid hormone receptor (TR) control of hormone action

Adapted from Bernal et al 2007 with permission. TR exists usually as a heterodimer with RXR or homodimers and bind to TRE in target genes. In positively regulated genes:

(A) In the absence of T₃, the unliganded receptor binds corepressors (e.g. NCoR, SMRT) and histone deacetylases that maintain the chromatin in a compact, repressed state.

(B) In the presence of T₃, the hormone binds TR, releasing corepressors and recruiting co-activators (e.g. CBP, p300, pCAF) and other mediators (e.g. TRAP and DRIP), which facilitate access of transcriptional machinery to the promoter.

(C) In the absence of TR (e.g. TR $\alpha^{0/0}$), basal transcriptional repression is lost and transcription may occur through the action of other transcription factors. The presence or absence of T₃ is irrelevant.

(D) In the presence of a mutant receptor that blocks T₃ binding with intact TRE binding, basal repression occurs.

CBP, CREB-binding protein; DRIP, vitamin D receptor-interacting protein; HDAC, histone deacetylase; NCoR, nuclear receptor corepressor; pCAF, mammalian homolog of the yeast transcriptional activator GCN5; RNA Pol II, RNA polymerase II; RXR, 9-cis retinoic acid receptor; SRC-1, steroid receptor coactivator 1; SMRT, silencing mediator for retinoic and thyroid receptor; TAF, transcription initiation factor; TBP, TATA box binding protein; TFIIA, transcription initiation factor IIA; TR, thyroid hormone receptor; TRE, thyroid hormone response element; TRAP, thyroid-hormone-receptor-associated protein.

1.10 THYROID HORMONE ACTION IN THE DEVELOPING AND ADULT SKELETON

1.10.1 Transporters

MCT10 is the most widely expressed TH-specific transporter in the growth plate with levels 46-fold higher than MCT8 (Abe, Namba et al. 2012). Its expression in bone cells has not been reported. MCT8 is widely expressed in chondrocytes, osteoblasts and osteoclasts (Williams, Robson et al. 2008). However, MCT8, MCT10 and double knockout mice do not have growth retardation (Muller, Mayerl et al. 2014). Expression of the less specific LAT1 and LAT2 transporters has been identified in murine femurs (Capelo, Beber et al. 2009) but the relatively TH-specific OAT1P1c transporter is not expressed in cartilage or bone (Williams, Robson et al. 2008, Capelo, Beber et al. 2009, Abe, Namba et al. 2012, Williams 2013). The expression of TH transporter in articular cartilage has not been reported.

1.10.2 Deiodinases

Metabolism of TH to regulate the local availability of T3 has an important role in skeletal physiology.

1.10.2.1 Deiodinase 1

DIO1 is not expressed in cartilage or bone cells in primary culture (Williams, Robson et al. 2008) or immortalised cell lines (LeBron, Pekary et al. 1989, Dreher, Schutze et al. 1998, Gouveia, Christoffolete et al. 2005). Furthermore, neither C3H/HeJ inbred mice that have 90% reduction in hepatic DIO1 activity (Berry, Grieco et al. 1993), nor DIO1 knockout mice (Schoenmakers, Pigmans et al. 1993) exhibit any growth or skeletal abnormalities. Therefore, DIO1 has no role in the skeleton in development or adulthood (Waung, Bassett et al. 2012).

1.10.2.2 Deiodinase 2

An important role of DIO2 in endochondral ossification was identified in the developing chick skeleton. DIO2 expression was restricted to the perichondrium adjacent to the growth plate. DIO2 activity was inhibited by hedgehog morphogens by stimulating ubiquitin-mediated degradation of the enzyme and resulted in increased PTHrP expression. This observation implies an important role for DIO2-mediated regulation of T3 action in the perichondrium in the IHH-PTHrP feedback loop. IHH-

induced DIO2 ubiquitination causes local hypothyroidism, induces PTHrP expression and inhibits chondrocyte differentiation. This is consistent with the delayed endochondral ossification and growth restriction observed in hypothyroidism (Dentice, Bandyopadhyay et al. 2005, Waung, Bassett et al. 2012).

DIO2 activity is regulated post-translationally, with substrate (T4) dependent ubiquitination and degradation. Therefore, hypothyroidism in T3-target tissue increases DIO2 activity while activity is inhibited in thyrotoxicosis. Thus, DIO2 mRNA expression and enzyme activity are not always concordant. Accordingly, the study of DIO2 expression and activity in the skeletal tissue has yielded conflicting results (Miura, Tanaka et al. 2002, Gouveia, Christoffolete et al. 2005, Morimura, Tsunekawa et al. 2005, Capelo, Beber et al. 2008). The function of DIO2 in different skeletal cell populations has been clarified with a highly specific and sensitive high performance liquid chromatography (HPLC)-based DIO2 assay. DIO2 activity was only demonstrated in mature primary osteoblasts and was absent from cultures of primary chondrocytes and osteoclasts (Williams, Robson et al. 2008). Consistent with this, DIO2 is expressed in the developing limb at embryonic day 14.5 (E14.5) and increases until E18.5, with marked increase in postnatal development that coincides with maximal longitudinal growth (Capelo, Beber et al. 2008).

DIO2 knockout mice (D2KO) have pituitary resistance to thyroid hormones with 2-fold elevation in TSH, 1.4-fold elevated T4 but normal circulating T3 levels (Schneider, Fiering et al. 2001). They have a transient reduction in growth plate height but normal linear growth and cortical bone thickness (Bassett, Boyde et al. 2010). Therefore, the functional role of DIO2 in perichondrium is not essential for normal postnatal growth (Dentice, Bandyopadhyay et al. 2005). In contrast, bones from adult D2KO mice are brittle and more mineralised despite normal microarchitecture. An osteoblast-specific defect results in 50% reduction in bone formation, a prolonged remodelling cycle and extended period of secondary mineralisation (Bassett, Boyde et al. 2010). The importance of skeletal euthyroidism is illustrated by the adverse consequences of perturbations in either direction. Thyrotoxicosis is associated with high turnover, osteoporosis and increased risk of insufficiency fracture; hypothyroidism is associated with low turnover and increased fracture risk (Vestergaard and Mosekilde 2002, Vestergaard, Rejnmark et al. 2005). DIO2 appears to perform an important homeostatic

function by regulating osteoblastic T3 availability and ensuring optimum mineralisation and strength (Bassett, Boyde et al. 2010).

1.10.2.3 Deiodinase 3

DIO3 expression and activity was detected in cultures of primary growth plates, osteoblasts and osteoclasts. However, enzyme activity was only detected in 3-week old weaning mice but not from older animals (Williams, Robson et al. 2008). This suggests that DIO3 may limit thyroid hormone action in chondrocytes to restrict longitudinal growth prior to weaning. This hypothesis is supported by high levels of DIO3 expression in embryonic skeleton and the reciprocal change in DIO2 activity during embryonic and postnatal growth. Accordingly, systemic hypothyroidism in early embryonic development has no effect on skeletal development (Capelo, Beber et al. 2008).

DIO3 knockout mice (D3KO) have abnormal hypothalamic-pituitary-thyroid axis maturation and consequent alteration in systemic thyroid status. These mice exhibit neonatal thyrotoxicosis followed by central hypothyroidism. They exhibit persistent growth retardation as a result of multiple systemic abnormalities that confound interpretation of the skeletal phenotype and the role of DIO3 in skeletal maturation (Hernandez, Martinez et al. 2006, Hernandez, Martinez et al. 2007).

1.10.3 Thyroid hormone receptors

Thyroid hormone receptor $\alpha 1$ (TR $\alpha 1$) and $\beta 1$ (TR $\beta 1$) are both expressed in the skeleton, although quantitative RT-PCR studies have shown that TR $\alpha 1$ is expressed at more than 10-fold greater levels than TR $\beta 1$ (O'Shea, Harvey et al. 2003, Bookout, Jeong et al. 2006). Both receptors are expressed in growth plate chondrocytes, bone marrow stromal cells and osteoblasts. However, it is unclear if TRs are expressed in osteocytes or osteoclasts (Williams 2013).

TR $\alpha 1$ and TR $\beta 1$ are expressed in the reserve zone (RZ) and proliferating zone (PZ) of growth plate cartilage (Robson, Siebler et al. 2000). T3 stimulates clonal expansion of chondrocyte progenitor cells in the resting zone and inhibits subsequent cell proliferation. T3 promotes hypertrophic differentiation, inducing the expression of markers such as collagen X and alkaline phosphatase (Figure 1.18). This is

accompanied by chondrocyte volume expansion and eventual apoptosis (Ishikawa, Genge et al. 1998, Ballock, Zhou et al. 2000, Robson, Siebler et al. 2000, Williams 2013). T3 regulates the pace of chondrocyte proliferation and differentiation during endochondral differentiation by interacting with the negative feedback loop involving PTHrP, IHH and other paracrine factors including IGF1, Wnt, bone morphogenetic proteins (BMPs) and FGFs (Stevens, Hasserjian et al. 2000, Dentice, Bandyopadhyay et al. 2005, Wojcicka, Bassett et al. 2013). T3 further regulates the expression of genes involved in cartilage matrix synthesis, mineralisation and degradation (Bassett, Swinhoe et al. 2006). Degrading enzymes include aggrecanase-2 (a disintegrin and metalloproteinase with thrombospondin motifs 1, ADAMTS5) and matrix metalloproteinase-13 (MMP-13) (Pereira, Jorgetti et al. 1999, Himeno, Enomoto et al. 2002, Makihiro, Yan et al. 2003). In summary, thyroid hormone is critical for the coordinated programme of endochondral ossification by regulating chondrocyte maturation, cartilage matrix synthesis, mineralisation and degradation.

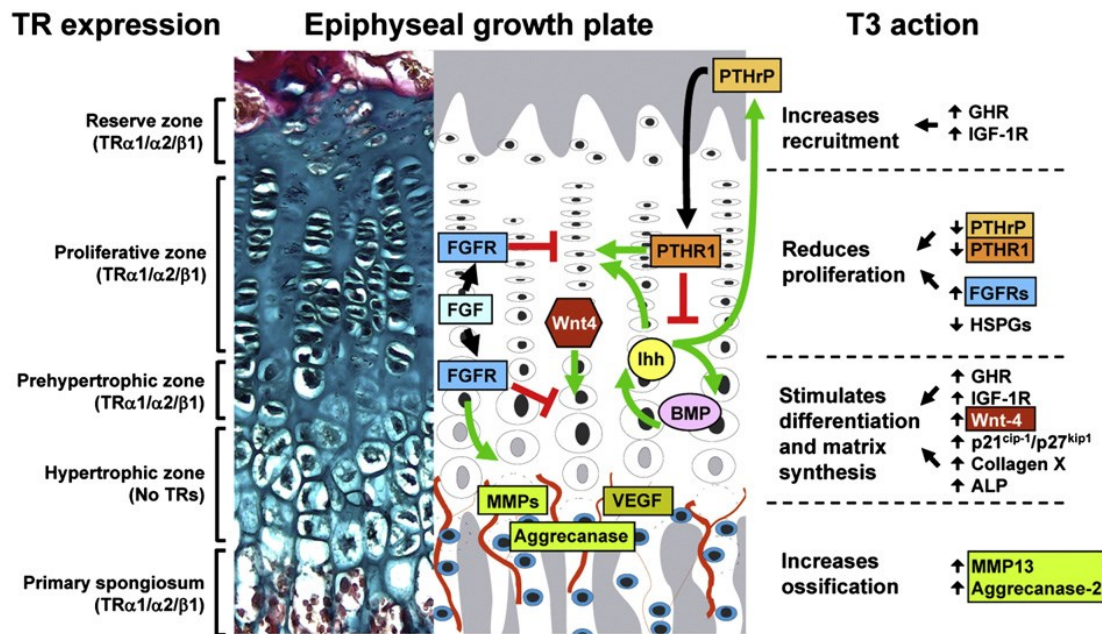


Figure 1.18 Thyroid hormone action in growth plate cartilage

Reproduced with permission from Wojcicka et al 2013. Left shows histological section of mouse growth plate with expression of TR α and TR β . Different zones of growth plate with expression of TR. Chondrocytes from the reserve zone undergo clonal expansion to form columns of cells in the proliferative zone that secrete type II collagen. Pre-hypertrophic chondrocytes undergo terminal differentiation to enlarge, secrete type X collagen then undergo apoptosis. T3 actions are shown on the right and described in the text.

1.10.4 Altered systemic thyroid hormone levels and skeletal development

Euthyroid status is critical for normal skeletal growth and maturation. Juvenile hypothyroidism results growth retardation, delayed bone age and short stature. Dramatic catch up growth can be achieved with thyroid hormone replacement therapy even after prolonged hypothyroidism. However, full, predicted height may not be achieved, especially in patients whose hypothyroidism is longstanding (Rivkees, Bode et al. 1988, Boersma, Otten et al. 1996).

Childhood thyrotoxicosis causes accelerated bone maturation but can result in short stature due to premature fusion of the epiphyseal growth plates. Severe cases can cause craniosynostosis due to premature fusion of cranial sutures and fontanelles (Segni, Leonardi et al. 1999). Similarly, advanced bone aged has been reported in congenital thyrotoxicosis due to activating mutations of the TSH receptor (TSHR) (Supornsilchai, Sahakitrungruang et al. 2009).

1.10.5 Syndromes of resistance to thyroid hormone

Resistance to thyroid hormone (RTH β) is an autosomal condition caused by mutations in *THRB* that result in the expression of dominant negative TR β proteins. It is characterised by elevated circulating levels of thyroid hormone with elevated or non-suppressed thyrotropin levels due to pituitary resistance. The skeletal phenotype is variable and confounded by the effects of treatment and the heterogeneity of TR β mutations that have different functional properties and activities (Dumitrescu and Refetoff 2013).

A new syndrome called Resistance to thyroid hormone α (RTH α) due to dominant negative mutations in *THRA* has been described. To date, 15 *THRA* mutations in 11 separate codons affecting 29 individuals from 16 families have been described. Nine mutations affect TR α 1 only and six result in the expression of mutant proteins of both TR α 1 and TR α 2 (Bochukova, Schoenmakers et al. 2012, van Mullem, van Heerebeek et al. 2012, Moran, Schoenmakers et al. 2013, van Mullem, Chrysis et al. 2013, Moran, Agostini et al. 2014, Espiard, Savagner et al. 2015, Tylki-Szymanska, Acuna-Hidalgo et al. 2015, Yuen, Thiruvahindrapuram et al. 2015, Demir, van Gucht et al. 2016, van Gucht, Meima et al. 2016, Moran, Agostini et al. 2017, Williams and Bassett 2018).

The mutant receptor exerts dominant negative effect on intact TR α 1 and all amino acid changes described affect the ligand binding domain of receptors. The biochemical signature of RTH α is normal serum TSH with normal/low T4, high/normal T3 resulting in reduced fT4:fT3 ratio (Bochukova, Schoenmakers et al. 2012, Tylki-Szymanska, Acuna-Hidalgo et al. 2015). The skeletal phenotype of patients with RTH α reflects impaired thyroid hormone action in cartilage and bone. Children have skeletal dysplasia with variable features including delayed bone age, disproportionately short lower limbs with relatively long torso, delayed closure of fontanelles, epiphyseal dysgenesis, acetabular hypoplasia, congenital hip dislocation, delayed tooth eruption, macrocephaly, broad faces with short, flat nasal bridges and hypertelorism. Adults have variable skeletal changes including skull vault thickening, increased cortical bone mass, hearing impairment due to otosclerosis. Other common features of RTH α patients are constipation and anaemia, reflecting an important role of TR α in gut motility and erythropoiesis.

Overall, patients with mutations affecting both TR α 1 and TR α 2 proteins are similar to patients with mutations restricted to TR α 1, with no added phenotype attributable to the presence of mutant TR α 2 (Moran, Agostini et al. 2014, Demir, van Gucht et al. 2016, van Gucht, Meima et al. 2016). A notable exception is a patient with a more profound bone abnormalities including a long neck, clavicular and 12th rib agenesis, humeroradial synostosis and syndactyly who had a N359Y mutation affecting both TR α 1 and TR α 2 (Espiard, Savagner et al. 2015). This raise the possibility of an unrecognised role of TR α 2 in skeletal development (Williams and Bassett 2018).

1.10.6 Skeletal phenotypes of juvenile thyroid hormone receptor knockout mice

The skeletal phenotype observed in patients with TR α mutations is entirely consistent with current understanding of TH action in the skeleton gained through the study of mice harbouring mutation in thyroid hormone receptors (Figure 1.19). Mice lacking all TR α (TR α ^{0/0}) (Gauthier, Plateroti et al. 2001, Bassett, O'Shea et al. 2007) or with dominant negative mutations (TR α 1^{PV/+}, TR α 1^{R384C/+}) (Kaneshige, Suzuki et al. 2001, Tinnikov, Nordstrom et al. 2002, O'Shea, Bassett et al. 2005, Bassett, Nordstrom et al. 2007) display skeletal hypothyroidism with normal circulating TH levels. Juveniles display post-natal growth retardation with delayed endochondral ossification,

characterised by reduced growth plate chondrocyte maturation and mineralisation. Mice with dominant negative mutation have a more pronounced phenotype. $TR\alpha1^{R384C/+}$ mice have transient growth delay with delayed endochondral and intramembranous ossification (Bassett, Nordstrom et al. 2007). The $TR\alpha1^{PV/+}$ mutant has the most severe phenotype, with persistent post-natal growth restriction due to impaired chondrocyte differentiation and endochondral ossification. They also show reduced mineralisation, cortical thickness and impaired intramembranous ossification (O'Shea, Bassett et al. 2005). Analysis of T3-target genes in these mutants showed reduced expression of fibroblast growth factor receptors (FGFR) 1 and 3 (Stevens, Harvey et al. 2003, Barnard, Williams et al. 2005, O'Shea, Bassett et al. 2005, Bassett, Nordstrom et al. 2007, Bassett, O'Shea et al. 2007).

Mice lacking $TR\beta$ ($TR\beta^{-/-}$) have pituitary resistance to TH due to loss of feedback inhibition mediated by $TR\beta$. They have elevated circulating TSH and T4 levels (Forrest, Hanebuth et al. 1996, Gauthier, Plateroti et al. 2001). However, $TR\alpha$ receptors are intact and they thus exhibit skeletal thyrotoxicosis with increased expression of the T3-target genes FGFR1 and FGFR3 (O'Shea, Harvey et al. 2003, Bassett, Nordstrom et al. 2007, Bassett, O'Shea et al. 2007). They display advanced endochondral ossification and increased mineralisation. Chondrocyte differentiation is accelerated and the longitudinal growth impaired due to premature growth plate quiescence (Bassett, Nordstrom et al. 2007, Bassett, O'Shea et al. 2007). Mice with a dominant negative mutation of $TR\beta$ ($TR\beta^{PV/PV}$) have 15-fold elevation in T4 and >400-fold elevation in TSH levels (Kaneshige, Kaneshige et al. 2000). The skeletal phenotype is more severe, with accelerated intrauterine growth, advanced endochondral ossification with persistent growth retardation due to growth plate quiescence. Bones are more mineralised. Intramembranous ossification is similarly advanced leading to craniosynostosis (O'Shea, Harvey et al. 2003, O'Shea, Bassett et al. 2005, Bassett, Nordstrom et al. 2007). In summary, these studies of juvenile mice with TR mutations demonstrate that the actions of T3 in the skeleton are anabolic and mediated principally by $TR\alpha$ during growth (Bassett and Williams 2009).

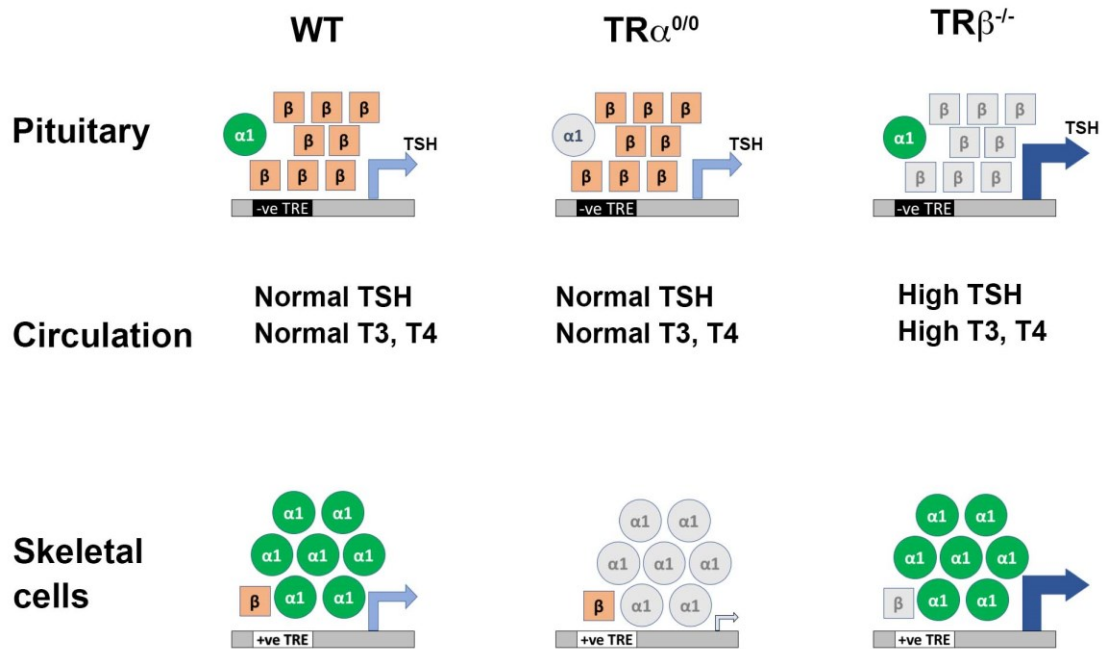


Figure 1.19 Thyroid hormone status in pituitary and skeletal cells

In WT mice, circulating hormone inhibits TSH transcription via $TR\beta$ in the pituitary, thus maintaining normal TH levels. In skeletal cells, T3 acts predominantly on $TR\alpha$ to positively regulate transcription of target genes.

In $TR\alpha^{0/0}$ mice, absent $TR\alpha$ (grey) does not affect pituitary function or circulating TH levels. In skeletal cells, transcription of T3 target gene is impaired because it is mainly mediated by $TR\alpha$ that is absent, leading to skeletal hypothyroidism.

In $TR\beta^{-/-}$ mice, feedback inhibition of TSH is impaired because $TR\beta$ is absent (grey). This leads to elevated TSH and TH levels. In skeletal cells, increased T3 availability acts on normal $TR\alpha$ to increase target gene transcription and results in skeletal thyrotoxicosis.

1.10.7 Thyroid hormone action in osteoblasts

Thyroid hormone receptor mRNA and proteins are expressed by primary osteoblasts and immortalised cells (Bland, Sammons et al. 1997, Abu, Horner et al. 2000). Studies are contradictory but suggest that T3 stimulates osteoblastic activity (Kim and Mohan 2013, Williams 2013). T3 increases the expression of osteoblast differentiation markers including osteocalcin (Varga, Rumpler et al. 1997, Gouveia, Schultz et al. 2001), collagen I (Varga, Rumpler et al. 2010) and alkaline phosphatase (Sato, Han et al. 1987, Banovac and Koren 2000). T3 enhances bone remodelling by stimulating the expression of matrix degrading metalloproteinases MMP-9 and MMP-13 (Pereira, Jorgetti et al. 1999). T3 also regulates other signalling pathways involved in osteoblastic activity. T3 increases osteoblastic FGFR1 expression and stimulates FGF-mediated MAPK signalling (Stevens, Harvey et al. 2003). T3 induces IGF-1 transcription and regulates its binding proteins IGFBP2 and IGFBP4 (Huang, Golden et al. 2000, Milne, Quail et al. 2001, Xing, Govoni et al. 2012) and interacts with Wnt/ β -catenin signalling (O'Shea, Kim et al. 2012). Thus, T3 stimulates osteoblast activity both directly and indirectly leading to osteoblast proliferation, differentiation, bone matrix synthesis, modelling / remodelling and mineralisation (Bassett and Williams 2016).

1.10.8 Thyroid hormone action in osteoclasts

Although TR α and TR β mRNA is expressed in osteoclasts, current antibodies are of low affinity and lack the sensitivity to detect endogenous TRs (Abu, Horner et al. 2000, Kim and Mohan 2013). TH stimulates bone resorption in organ culture but it is unclear whether the T3 acts directly on osteoclasts.

Early co-culture experiments showed that T3 stimulated osteoclastic resorption in the presence of osteoblasts but not in their absence (Allain, Chambers et al. 1992, Britto, Fenton et al. 1994). However, other primary culture experiments have yielded contradictory results (Mundy, Shapiro et al. 1976, Kanatani, Sugimoto et al. 2004). A proposed mechanism is that T3 may regulate osteoclastogenesis through its effects on RANKL and its decoy receptor osteoprotegerin (OPG) (Varga, Spitzer et al. 2004). However, this view has been challenged by studies that showing an effect of T3 on osteoclastogenesis that is independent of RANKL (Kanatani, Sugimoto et al. 2004,

Saraiva, Teixeira et al. 2008). Therefore, at present it is well established that thyrotoxicosis increases osteoclast numbers and activity leading to bone resorption, but the mechanism remains unclear.

1.10.9 Skeleton remodelling in hypo and hyperthyroidism

Thyroid hormones regulate the initiation and duration of bone remodelling cycles. Hypothyroidism is associated with fewer remodelling cycles of longer duration. This leads to a positive balance between resorption and formation for each cycle and a prolonged period of secondary mineralisation (Eriksen, Mosekilde et al. 1986). Conversely, thyrotoxicosis is associated with increased frequency of remodelling. The duration of resorption is maintained but the duration of bone formation is reduced by a third. This uncoupling of osteoblasts and osteoclast activity leads to a 10% net loss of mineralised bone per remodelling cycle (Bassett and Williams 2003). Bone loss is more marked in cortical bone (Greenspan and Greenspan 1999).

Population studies have shown that hypothyroidism is associated with a two- to three-fold increased risk of fracture, with the greatest risk around the time of diagnosis (Vestergaard and Mosekilde 2002). The risk persists for 10 years after diagnosis (Vestergaard, Rejnmark et al. 2005). Subclinical hypothyroidism is characterised by an elevation in TSH with circulating thyroid hormones levels that are at the lower limit of the normal range. A recent meta-analysis has not shown increased fracture risk among patients with endogenous subclinical hypothyroidism (Blum, Bauer et al. 2015). However, a large observational study has shown increased risk of fracture in patients with elevated TSH who were on levothyroxine treatment (Flynn, Bonellie et al. 2010).

Untreated thyrotoxicosis is an established risk factor for osteoporosis and fragility fractures. Treatment of thyrotoxicosis leads to normalisation of bone turnover markers and BMD but an increased risk of fracture persists for up to 5 years following treatment (Vestergaard and Mosekilde 2003, Vestergaard, Rejnmark et al. 2005). Subclinical hyperthyroidism is defined as suppressed TSH in the context of normal circulating thyroid hormone levels. This may be endogenous or related to supraphysiological doses of levothyroxine treatment. Large population studies have investigated the risk of fracture and bone loss in subclinical hyperthyroidism. There is an increased risk of fracture in endogenous subclinical hyperthyroidism (Blum, Bauer et al. 2015) and those

taking levothyroxine (Flynn, Bonellie et al. 2010). The risk of fracture is highest with lower TSH values and longer duration of TSH suppression (Abrahamsen, Jorgensen et al. 2014, Blum, Bauer et al. 2015) and is observed most consistently in post-menopausal women (Nicholls, Brassill et al. 2012) and the elderly. Furthermore, high free T4 levels within normal range are associated with lower BMD (van Rijn, Pop et al. 2014) and increased risk of fracture independent of TSH levels (Murphy, Gluer et al. 2010). The treatment of differentiated thyroid cancer (DTC) includes exogenous suppression of TSH with supraphysiological doses of levothyroxine. Studies investigating the effects on bone are heterogenous and have not investigated the risk of fracture (Williams 2014). However, postmenopausal women with DTC treated with TSH-suppressive doses of levothyroxine have reduced BMD and increased risk of osteoporosis (Sugitani and Fujimoto 2011, Wang, Smith et al. 2015). Therefore, guidelines recommend that patients with TSH suppression below 0.05mIU/L for >5 years should be considered to have a 1 standard deviation reduction in measured BMD (Perros, Boelaert et al. 2014).

1.10.10 Skeletal phenotypes of adult thyroid hormone receptor knockout mice

The skeletal phenotypes of juvenile mice with deletions or dominant negative mutations of TR α and TR β demonstrated that T3 is anabolic during growth. Juvenile TR $\alpha^{0/0}$ mice have growth delay and reduced mineralisation due to skeletal hypothyroidism. Remarkably, adult TR $\alpha^{0/0}$ mice have increased bone mass or osteosclerosis. They have increased calcified cartilage due to impaired remodelling and have lower osteoclast numbers and activity (Bassett, O'Shea et al. 2007). TR $\alpha^{1R384C/+}$ mice have a dominant negative mutation and more pronounced phenotype compared to TR $\alpha^{0/0}$. Thus, adult TR $\alpha^{1R384C/+}$ mice have osteosclerosis with progressively increasing trabecular thickness and connectivity. The persistence of calcified cartilage and reduced osteoclastic remodelling leads to increase bone mineralisation (Bassett and Williams 2009). In contrast, while juvenile TR $\beta^{-/-}$ mice have advanced ossification due to skeletal thyrotoxicosis, adults develop progressive osteoporosis. Two TR $\beta^{-/-}$ strains from different backgrounds showed increased osteoclast numbers and activity. Trabecular mineralisation was reduced in both strains but cortical mineralisation only in one strain (Bassett, O'Shea et al. 2007, Bassett and Williams 2009). Thus, mice with skeletal hypothyroidism in adulthood have increased bone mass and reduced bone remodelling while mice with skeletal thyrotoxicosis have osteoporosis and reduced

mineralisation. This shows that the actions of T3 in the adult skeleton are catabolic, mediated by TR α and in direct contrast to its anabolic actions during growth.

1.10.11 The role of aporeceptor

The importance of unliganded TRs in the skeleton is illustrated by studies in athyreotic mice. Pax8 is an essential transcription factor for thyroid gland development. Pax8 null (Pax8^{-/-}) mice have undetectable levels of T4 and T3 with 1900-fold TSH levels (Mansouri, Chowdhury et al. 1998, Pasca di Magliano, Di Lauro et al. 2000). These mice have severely delayed endochondral ossification, impaired linear growth and do not survive beyond weaning.

In contrast, Mutant mice lacking all TR (TR α ^{0/0}TR β ^{-/-}) can survive beyond weaning and have a less severe skeletal phenotype than Pax8^{-/-} mice. This implies that unliganded receptors are more detrimental to skeletal development than the absence of TR. Deletion of TR β (Pax8^{-/-}TR β ^{-/-}) does not improve the skeletal phenotype but deletion of TR α (Pax8^{-/-}TR α ^{0/0}) leads to partial rescue (Flamant, Poguët et al. 2002). This suggests that TR α mediates the majority of the effects from unliganded TR and is the main determinant of skeletal phenotype. This interpretation is supported by the findings in mice with single mutations of TR α or TR β described earlier. However, Pax8^{-/-}TR α 1^{-/-} mutants that still express non-T3 binding forms, TR α 2 and TR $\Delta\alpha$ 2, are not rescued from the Pax8^{-/-} phenotype. This implies a more complex regulatory mechanism that may involve TR α 2 and an unliganded TR isoform (Mittag, Friedrichsen et al. 2005).

1.10.12 Thyroid hormone receptor β and the skeleton

Strong evidence establishes TR α as the main mediator of skeletal thyroid hormone action. Nevertheless, TR β has important actions in bone and cartilage. T3 treatment reduces proliferation but increases differentiation in primary chondrocyte culture but this is abolished in TR $\beta^{-/-}$ mice (Rabier, Williams et al. 2006). Treatment with GC-1, a TR β 1 specific receptor agonist promotes chondrocyte differentiation and ossification (Freitas, Capelo et al. 2005). TR β 1 is critical for the formation of epiphyseal bone by increasing the expression of IHH (Xing, Cheng et al. 2014, Aghajanian, Xing et al. 2017). Compared to TR $\alpha^{0/0}$ mutants, mice lacking all TR (TR $\alpha^{0/0}$ TR $\beta^{-/-}$) exhibit a more profound phenotype with delayed bone maturation that may reflect partial compensation by TR β in TR $\alpha^{0/0}$ mutants, or result from reduced GH/IGF-1 levels (Gauthier, Plateroti et al. 2001).

1.11 THYROID HORMONE ACTION IN THE JOINT

There are several types of evidence implicating thyroid hormone action in OA disease susceptibility: human genetic studies, functional genomic studies designed to determine causality, biological plausibility based on *in vitro* and *in vivo* effects of thyroid hormone or TR agonists on skeletal cells.

1.11.1 Human genetic studies identify thyroid hormone regulation as a modifier of osteoarthritis susceptibility

Genetic susceptibility for the development of structural OA ranges from 40-65% depending on the joint site (Spector, Cicuttini et al. 1996, Kraus, Jordan et al. 2007, Valdes and Spector 2011, Tsezou 2014). Genetic association studies aim to identify which genetic variants at which genetic locations (loci) influence the risk of developing disease in unrelated individuals. However, this is challenging in OA because it is a highly polygenic, complex disease with potentially thousands of loci associated with disease risk, each conferring small risk. Furthermore, this is complicated by the heterogeneity of the definition of phenotype (e.g. sex, ethnicity, joint(s) affected, symptomatic or radiological diagnosis, spontaneous or trauma associated). This has led to efforts to standardise and better phenotype OA with the aim of stratifying disease into more homogenous genetic and environmental subsets and increase power of genetic studies (Kerkhof, Meulenbelt et al. 2011, Meulenbelt, Kraus et al. 2011).

DIO2 was first identified as a disease susceptibility gene using a linkage study. This type of study is based on the cosegregation of a disease trait of interest with one or more genetic markers to identify disease loci at specific regions of the genome. The Genetics Osteoarthritis and Progression (GARP) study had 179 siblings pairs and 4 trios affected predominantly by symptomatic and radiographic OA at multiple sites (Meulenbelt, Kloppenburg et al. 2007). A non-synonymous coding variant of deiodinase 2 (rs225014; Thr92Ala) was identified as a disease susceptibility locus. Confirmation studies in European and Asian populations showed that the haplotype C-C, consisting of the minor allele of SNP rs225014 and the common allele (rs12885300) was associated with increased risk of hip osteoarthritis in women (Meulenbelt, Min et al. 2008). These finding was reinforced using an allele-sharing statistical approach that uses extra data (family history) from ungenotyped relatives (Callegaro, Meulenbelt et

al. 2010). One way risk variants may increase OA susceptibility is by altering hip morphology. This was investigated in *DIO2* since thyroid hormone is a critical regulator of skeletal development. *DIO2* polymorphisms were not more commonly associated with four types of abnormal hip morphology that predisposes to OA. However, amongst subjects with those four hip shapes, those with the rs12885300 *DIO2* polymorphism had increased risk of developing OA (Waarsing, Kloppenburg et al. 2011). Subsequently, genome wide association studies (GWAS) have been the main method of identifying new risk loci in a hypothesis free manner. One of the statistical challenges of GWAS is that of multiple testing, necessitating a strict threshold for the definition of genome-wide significance ($P < 5 \times 10^{-8}$), extremely large sample sizes or very large genetic effects (such as odds ratio of > 2.0) that are uncommon for common variants in a complex and prevalent disease like OA (Valdes and Spector 2011). A meta-analysis of 4 European populations with 5384 individuals identified a possible protective variant in the minor allele of *DIO3* rs945006. This was seen all joints but most significant in cases with knee and/or hip OA with an odds ratio of 0.81 (Meulenbelt, Bos et al. 2011). Since *DIO2* rs225014 is not associated with any change in circulating TH and TSH level (Peeters, van Toor et al. 2003, Appelhof, Peeters et al. 2005, Mentuccia, Thomas et al. 2005, Peeters, van den Beld et al. 2005, de Jong, Peeters et al. 2007, Gumieniak, Perlstein et al. 2007, Panicker, Cluett et al. 2008, Guerra, Sapio et al. 2013, Roef, Taes et al. 2013, Medici, Visser et al. 2015), these studies suggest that the regulation of thyroid hormone availability in the joint may affect susceptibility to osteoarthritis. Complementing this hypothesis is the identification of nuclear receptor coactivator 3 (*NCOA3*) as novel variant that reached genome-wide significance (Evangelou, Kerkhof et al. 2013) in a meta-analysis of GWAS with more than 78000 participants investigating Hip OA. *NCOA3* is a nuclear receptor coactivator that could affect transcriptional regulation of T3, although other mechanism independent of T3 may be responsible for modifying disease risk. Although *DIO2* has been one of the few robust signals from human genetic studies of OA (Meulenbelt, Kraus et al. 2011), the relationship between this haplotype and OA was not identified in the Rotterdam Study population (Kerkhof, Lories et al. 2010) and some subsequent meta-analyses (Evangelou, Kerkhof et al. 2013, Rodriguez-Fontenla, Calaza et al. 2014).

The functional implication of *DIO2* rs225014 polymorphisms is uncertain. Initial transfection studies showed no difference in protein half-life, enzyme kinetics or its sensitivity to substrate mediated inhibition. However, the same study showed that in skeletal muscle and thyroid tissue biopsies, *DIO2* enzyme velocity was reduced by half in subjects homozygous for the Ala risk allele compared to wild type or heterozygous subjects (Canani, Capp et al. 2005). However, it has been suggested this finding may result from a methodological artefact (Larsen 2009). Most recently, studies in patients before and after thyroidectomy showed reduced T3 levels post-operatively in subjects with the Ala allele. Remarkably, protein stability was increased with Ala allele in transfection studies but showed reduced activity in functional assays of *DIO2*. However, enzyme activity was not measured (Castagna, Dentice et al. 2017).

In summary, human genetic studies have identified the regulation of thyroid hormone availability as potential modifier of OA risk through statistical methods. The challenge is determining if these signals are of biological relevance and causally associated with OA.

1.11.2 Functional genomic data for *DIO2* in osteoarthritis

Functional genomic studies (genomic, transcriptomics, epigenomic and proteomic data) can help to bridge the gap between genetic evidence and molecular mechanisms underlying OA (Ramos and Meulenbelt 2015). Numerous studies have shown increase *DIO2* mRNA and protein expression in cartilage and subchondral bone tissue affected by OA compared to normal tissue (Ijiri, Zerbini et al. 2008, Karlsson, Dehne et al. 2010, Bos, Bovee et al. 2012, Chou, Lee et al. 2013, Chou, Wu et al. 2013, Nagase, Nagasawa et al. 2013). Furthermore, increase expression of the risk allele from rs225014 relative to the wild type allele (allelic imbalance) was observed from OA cartilage from heterozygous patients. Epigenetic modification may underlie this observation. A CpG site 2031 base pairs proximal to the *DIO2* transcription start site showed increased methylation in OA tissue compared to normal tissue and was positively associated with increased *DIO2* expression (Bos, Bovee et al. 2012, Bomer, den Hollander et al. 2015). These observations link increased *DIO2* expression to OA pathology but do not address causality.

Cell and animal models of OA have been used to further probe for molecular mechanisms that might explain how altered thyroid hormone availability might affect OA risk. Lentiviral overexpression of *DIO2* in an *in vitro* chondrogenesis study showed reduced expression of genes associated with a quiescent articular cartilage (*COL2A*, *ACAN* that encode collagen 2 and aggrecan, respectively) and increased expression of hypertrophic markers and cartilage degrading enzymes (*RUNX2*, *ADAMTS*, *MMP13*, *EPAS1*) compared to control (Bomer, den Hollander et al. 2015). Finally, *in vivo* studies of transgenic rats overexpressing human *DIO2* showed increased susceptibility to OA from surgical destabilisation of the knee joint (Nagase, Nagasawa et al. 2013). Conversely, D2KO mice were protected from cartilage damage and synovitis in a forced exercise model of OA (Bomer, Cornelis et al. 2016). Taken together, there is some evidence to suggest that reducing T3 availability may confer protection in cell and animal models of OA.

1.11.3 Do patients with thyroid disease develop joint disease?

Osteoarthritis pathology usually develops over many years and is progressive. In contrast, the symptoms of hypothyroidism and hyperthyroidism are generally easily recognised; treatment is started promptly; and patients do not experience sustained periods with deranged thyroid function. Accordingly, a population study has shown a lack of association between thyroid status and osteoarthritis (Chaisson, McAlindon et al. 1996, Hellevik, Johnsen et al. 2017). There are, however, historical case reports and series documenting the presence of arthropathy in patients with primary hypothyroidism. Characteristically patients reported joint stiffness and swelling that resolved upon treatment with levothyroxine (Bland and Frymoyer 1970, Dorwart and Schumacher 1975, Gerster, Quadri et al. 1985, Golding 1993, Gillan, Scofield et al. 2002). Therefore, the arthropathy associated with the onset of hypothyroidism appears distinct from osteoarthritis. These observations are consistent with the fact that patient with polymorphism of *DIO2* who are at increased risk of OA are systemically euthyroid. This suggests that it is the local availability of T3 in joints, rather than circulating levels that is critical in determining OA risk.

1.11.4 Regulation of Thyroid hormone action in articular cartilage and subchondral bone

Most research investigating the role of thyroid hormone in the skeleton has not focused on the joint. Therefore, it is not known how accurately TH action in growth plate chondrocytes reflects its action in articular chondrocytes. Equally, action of thyroid hormone in subchondral bone, a separate functional unit to diaphyseal and metaphyseal bone, has not been well investigated. Data concerning the key regulators of thyroid hormone action specific to articular cartilage and subchondral bone are outlined below.

The expression of thyroid hormone transporters in articular cartilage or subchondral bone is not reported. DIO1 is not expressed in articular cartilage (Rosenthal, Heinkel et al. 2003, Chou, Lee et al. 2013, Nagase, Nagasawa et al. 2013) or bone and appears to have no role in skeletal physiology (Waung, Bassett et al. 2012). Type 2 and 3 deiodinases are both expressed in articular cartilage and a whole genome microarray of subchondral bone has demonstrated increased expression of *DIO2* in osteoarthritis (Chou, Wu et al. 2013). Although local production of T3 in articular chondrocyte culture has been reported, the activities of DIO2 and DIO3 in articular cartilage or subchondral bone has not been reported. Finally, functional thyroid hormone receptors have been reported in articular cartilage (Rosenthal and Henry 1999, Bateman, Rowley et al. 2013).

1.11.5 Effects of thyroid hormone on articular cartilage

Early studies in rabbits showed that thyroid hormones increased collagen II and proteoglycan synthesis as well as articular chondrocyte proliferation (Hendricson, Havdrup et al. 1982, Glade, Kanwar et al. 1994). Primary articular chondrocyte cultures have consistently demonstrated that thyroid hormones promote hypertrophic differentiation. This is characterised by increased expression of collagen X, MMP-13, alkaline phosphatase activity and cartilage mineralisation (Rosenthal and Henry 1999, Rosenthal, Heinkel et al. 2003, Jiang, Leong et al. 2008, Randau, Schildberg et al. 2013, Lee, Gegg et al. 2015).

The effects of T3 appear to be specific to different zonal populations of articular chondrocytes. T3 induced hypertrophic differentiation and mineralisation in deep zone bovine articular chondrocytes. This was prevented by co-culture with surface zone chondrocytes or medium from this population. Alternatively, treatment with PTHrP

also prevented deep zone chondrocytes from undergoing T3-induced hypertrophic differentiation. Therefore, surface zone chondrocytes are able to produce a factor (possibly PTHrP) that prevents deep zone chondrocytes from undergoing hypertrophic differentiation. This regulatory mechanism appears similar to the PTHrP/IHH feedback loop seen in the growth plate (Jiang, Leong et al. 2008). Perichondrial DIO2 plays a key role in regulating PTHrP in the developing chick skeleton and was described earlier (Dentice, Bandyopadhyay et al. 2005) (see 1.10.2.2). It is unclear if DIO2 has a role in regulating PTHrP and hypertrophic differentiation of articular chondrocytes. Finally, T3 treatment stimulates the activity of transglutaminases, protein cross-linking enzymes that promote chondrocyte hypertrophy and mineralisation (Johnson, van Etten et al. 2003, Rosenthal, Heinkel et al. 2003).

1.11.6 Chondrotoxicity of a thyroid hormone receptor agonist – Eprotirome

Thyroid hormones have favourable effects in lowering plasma cholesterol (via TR β in the liver) but its use as a therapy for hypercholesterolaemia is limited by its unfavourable effects on other organs, such as the heart (tachycardia, arrhythmia, heart failure) and bone (osteoporosis and fracture). Eprotirome was developed as a highly liver selective, weakly TR β selective thyroid hormone agonist to treat hypercholesterolaemia (Lammel Lindemann and Webb 2016). Results from phase 1 and phase 2 studies were very promising, showing reduction in low density cholesterol without adverse effect on the heart, liver and pituitary (Berkenstam, Kristensen et al. 2008, Ladenson, Kristensen et al. 2010). Unexpectedly, long-term toxicity studies showed cartilage damage in dogs treated for over one year. This led to the premature termination of a phase 3 clinical trial and cessation of drug development (Sjouke, Langslet et al. 2014). Details regarding the observed canine chondrotoxicity are not published. Eprotirome, unlike thyroid hormones, is not subject to metabolism by deiodinases. This raises the possibility that increased signalling through TR may be chondrotoxic.

1.11.7 Conclusion

In summary, articular chondrocytes express thyroid hormone receptors and T3 stimulates hypertrophic differentiation and matrix degrading enzymes in a process that resembles endochondral ossification and is characteristic of osteoarthritis. Human

genetic studies have identified variants of deiodinase 2 and 3 that are associated with increased risk of OA. Human and animal studies have shown increased DIO2 expression in OA tissue. Animal models with deletion or transgenic expression of *Dio2* have shown protection from, or increased susceptibility to OA, respectively. Finally, a thyroid hormone agonist has been shown to cause cartilage damage. Taken together, these studies suggest that the regulation of thyroid hormone availability may affect susceptibility to osteoarthritis. Current evidence suggests that increased availability of T3 may have a detrimental role for the maintenance of joint integrity. Since thyroid hormone receptor alpha is the predominant final mediator of thyroid hormone action in the skeleton, it represents a logical target to investigate the role of thyroid hormone signalling in osteoarthritis pathogenesis.

1.12 EXPERIMENTAL MODELS OF OSTEOARTHRITIS

The complexity of OA pathophysiology requires *in vivo* studies. The ease of genetic modification, rapid onset of disease and low cost are advantages of a murine model. Certain strains (e.g. STR/ort) have a predisposition to spontaneous osteoarthritis but the onset is often slow and variable (Mason, Chambers et al. 2001, Vincent, Williams et al. 2012). Destabilisation of knee joints offers a predictable onset that mimics a common human injury. Intra-articular injection of collagenases and monoiodoacetate are technically simple, but are associated with a greater inflammatory component that may not reflect OA pathophysiology (Little and Zaki 2012). Various surgical knee destabilisation models have been developed, with increasing instability determining the severity and site of pathology (Kamekura, Hoshi et al. 2005, Glasson, Blanchet et al. 2007). Of these, the destabilisation of medial meniscus (DMM) model has been widely adopted among leading researchers. It offers a mild-moderate, reproducible osteoarthritis-like pathology focused on the medial compartment, especially the medial tibial plateau (Glasson, Blanchet et al. 2007, Little and Zaki 2012, Vincent, Williams et al. 2012). It is highly penetrant and has been validated in genetically modified animals. There are important sex and strain differences in the susceptibility to osteoarthritis (Glasson, Blanchet et al. 2006). Female mice are protected from OA while androgens increase male susceptibility with 100% disease penetrance (Ma, Blanchet et al. 2007, Vincent, Williams et al. 2012). The 129/SvEv strain is more susceptible than C57Bl/6 to DMM induced OA; although many groups have used the latter strain to good effect when studying genetically modified mice (Yang, Kim et al. 2010, Sondergaard, Catala-Lehnen et al. 2012).

1.12.1 Genetic model of skeletal hypothyroidism - TR α ^{0/0} mouse

Detailed studies of mutant mice suggests that the action of T3 in skeletal cells are mainly mediated by TR α 1 (Bassett and Williams 2016). The TR α ^{0/0} mouse lacks all TR α transcripts (TR α 1 and TR α 2 encoded by the TR α gene). Furthermore, it lacks transcripts from an internal promoter (TR $\Delta\alpha$ 1 and TR $\Delta\alpha$ 2) that antagonise transcriptional activation by T3 (Chassande, Fraichard et al. 1997). Mice are generally euthyroid, with normal thyroxine levels at P21 and P154 and only slightly lower levels at P56. However, T3 and TSH levels are the same as wild type mice (Gauthier, Chassande et al. 1999, Macchia, Takeuchi et al. 2001, Bassett, O'Shea et al. 2007).

Skeletal T3 target genes show reduced expression in mutant mice. Thus, the observed skeletal effects in $TR\alpha^{0/0}$ are due to skeletal hypothyroidism rather than systemic effects.

$TR\alpha^{0/0}$ have delayed postnatal longitudinal growth but catch up in early adulthood. Mutants are the same size as wild type at E17.5 and P1. They have shorter tibias and tails between P21 and P56 but there is no difference by P70. Juvenile mutants have reduced tibia cortical thickness but there is no difference after P21. Juvenile mutants have a wide growth plate with impaired recruitment of chondrocyte from the resting zone and delayed chondrocyte maturation. Adults have reduced bone turnover due to reduced osteoclastic resorption but normal bone formation. Although bone mass is increased, bone mineralisation is normal in adult mutant mice. Severe thyrotoxicosis in mutant $TR\alpha^{0/0}$ mice induced bone loss, indicating that $TR\beta$ was able to partially compensate for the lack of $TR\alpha$ in the presence of hormone excess (Bassett, O'Shea et al. 2007). In summary, $TR\alpha^{0/0}$ mice in early adulthood do not have any persistent growth deficit but displaying ongoing skeletal hypothyroidism and reduced bone turnover. $TR\alpha$ mediates this action but the function of $TR\beta$ is preserved. Therefore, the $TR\alpha^{0/0}$ mouse represents a good genetic model for investigating the effects of reduced thyroid hormone action in the joint. The joint phenotype of this mouse has not been reported.

1.12.2 Dronedarone, an antagonist of Thyroid hormone receptor alpha

1.12.2.1 Background

Amiodarone is an iodine-rich benzofuran derivate used extensively to treat resistant tachyarrhythmias (Figure 1.20). However, both amiodarone and its metabolite desethylamiodarone (DEA) can cause adverse effects in many organ including thyroid, lung, liver, cornea, skin and peripheral nerves (Barez-Lopez, Bosch-Garcia et al. 2014). Dronedarone (Multaq, Sanofi) was developed as a non-iodinated alternative to amiodarone to avoid toxicity to the thyroid gland. A methane sulphonyl group was added to decreases lipophilicity and thereby reduce half-life and tissue accumulation. Similar to amiodarone, dronedarone is a Vaughan-Williams class III anti-arrhythmic drug that has multi-channel blocking effects by inhibiting the K^+ , Na^+ and Ca^{2+} currents in isolated cardiomyocytes. It also exhibits anti-adrenergic properties (Varro, Takacs et al. 2001, Gautier, Guillemare et al. 2003, Patel, Yan et al. 2009, Sullivan and Olshansky 2010).

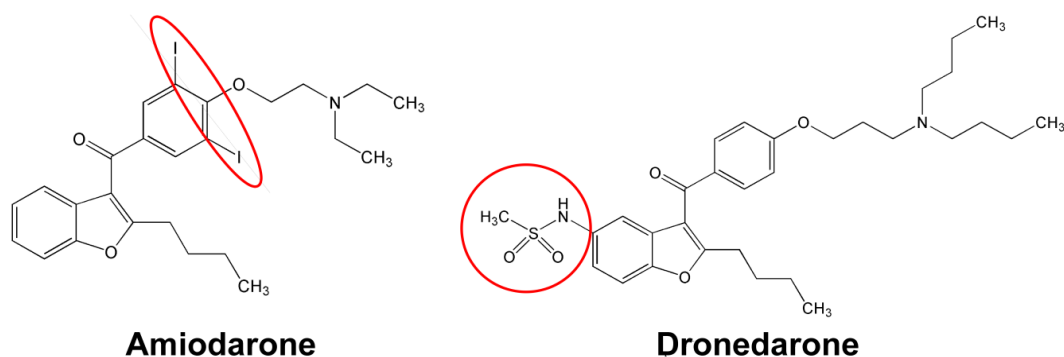


Figure 1.20 Chemical structure of dronedarone

Figure shows iodine moieties in amiodarone (circled, left) have been removed in dronedarone. A methane-sulphonyl group (circled, right) is added in dronedarone to decrease lipophilicity.

Amiodarone, dronedarone and thyroid hormone signalling

In addition to its effects on the thyroid gland, amiodarone affects extra thyroidal metabolism of thyroid hormones. It decreases 5' deiodination of T4 into T3 in the liver by inhibiting cellular T4 uptake (Van Beeren, Jong et al. 2003). The main metabolite of amiodarone, desethylamiodarone (DEA), inhibits T3 binding to its nuclear receptors. Amiodarone treatment causes dose dependent inhibition of T3 target gene expression (Van Beeren, Jong et al. 2003). It competitively inhibits T3 binding to TR α 1 (van Beeren, Bakker et al. 1995), but inhibits TR β 1 non-competitively (Bakker, van Beeren et al. 1994).

The effects of Dronedarone and its active metabolite, debutyldronedarone (DBDron), on thyroid hormone receptors alpha and beta were investigated *in vitro* and *in vivo*.

1.12.2.2 Evidence that Dronedarone is a TR alpha selective antagonist

1.12.2.2.1 In Vitro evidence: T3 binding assay

The binding of radiolabelled T3 in the presence of DEA, dronedarone and DBDron was measured in *Escherichia coli* expressing chicken α 1 and human β 1 thyroid hormone receptors. Dronedarone was a weak but 100% selective inhibitor of TR α , while its first pass hepatic metabolite is a potent TR-antagonist with 5-fold selectivity for TR α over TR β . The nature of DBDron inhibition on T3 binding to TR α 1 was competitive while the inhibitory effect on TR β 1 was too low to perform kinetic studies (Van Beeren, Jong et al. 2003).

1.12.2.2.2 In Vivo evidence: Effects on thyroid hormone levels

The transcription of TSH is negatively regulated by TR β 2 receptors. Animal studies have consistently shown that dronedarone does not affect serum thyroid hormone levels (Pantos, Mourouzis et al. 2002, Van Beeren, Jong et al. 2003, Pantos, Mourouzis et al. 2005, Stoykov, van Beeren et al. 2007, van Beeren, Kwakkel et al. 2012, Mourouzis, Kostakou et al. 2013). Importantly, dronedarone has no effect on TSH, which is negatively regulated in the pituitary by TR β 2 receptors. This is in direct contrast to amiodarone, which increases T4 and TSH, providing *in vivo* evidence that it acts as a non-selective antagonist of TR receptors (Van Beeren, Jong et al. 2003).

1.12.2.2.3 Effects on T3 target genes in TR α and TR β responsive tissue.

T3 regulates cardiac function by influencing cardiac gene expression, the sensitivity of the sympathetic nervous system and through peripheral vascular effects. Hyperthyroidism increases myocardial contractility and has marked chronotropic (electrical impulse generation) and dromotropic (conduction) effects. Clinically this can be observed as increase heart rate and reduced QTc interval. Studies using thyroid hormone receptor knockout mice have demonstrated that TR α is the main receptor that mediates the actions T3 in the heart (Wikstrom, Johansson et al. 1998, Gloss, Trost et al. 2001). TR α target genes that are positively regulated by T3 include sarcoplasmic reticulum Ca²⁺ATPase (SERCa2), alpha myosin heavy chain (α -MHC) and hyperpolarization-activated cyclic nucleotide-gated current gene 2 (HCN2)(Kahaly and Dillmann 2005). Dronedarone has been shown to reduce α -MHC expression, reduce heart rate and prolong QTc (Van Beeren, Jong et al. 2003, Pantos, Mourouzis et al. 2005, Stoykov, van Beeren et al. 2007).

The liver and pituitary are predominantly TR β target tissues. Liver DIO1 and low density lipoprotein receptor (LDLr) expression is positively regulated by T3 via TR β 1. TSH secretion is inhibited by TR β 2 receptors in the hypothalamus and pituitary to maintain negative feedback regulation of the hypothalamic pituitary thyroid axis. Dronedarone treatment has no effect on liver DIO1 activity; LDL or TSH levels (Van Beeren, Jong et al. 2003, Stoykov, van Beeren et al. 2007, van Beeren, Kwakkel et al. 2012).

Taken together, there is *in vitro* and *in vivo* evidence that dronedarone acts as a selective antagonist of TR α 1.

1.13 EXPERIMENTAL DESIGN

1.13.1 Hypothesis

I hypothesize that:

1. Inhibition of thyroid hormone signalling will decrease susceptibility to osteoarthritis and delay disease progression.
2. Pharmacological inhibition of TR α will increase bone mass and strength.

1.13.2 Aims

I aim to:

1. Compare the development and progression of osteoarthritis in TR $\alpha^{0/0}$ and wild type mice using a surgical model.
2. Determine the effects of pharmacological inhibition of TR α on osteoarthritis development and progression.
3. Compare the spontaneous development of osteoarthritis in D2KO, D1D2KO and wild type mice.
4. Determine the effects of pharmacological inhibition of TR α on bone mass and strength in adult mice.

CHAPTER 2 MATERIAL AND METHODS

2.1 ANIMALS

TR α ^{0/0} mice were housed in the registered Central Biomedical Services (CBS) facility at Imperial College. Studies were conducted in accordance with the Animals (Scientific Procedures) Act 1986 and recommendations of the Weatherall report, and were approved by the Imperial College Ethical Review Committee. All animals were weaned at 21 days after birth, fed ad libitum and housed with 12-hour light and dark cycles.

2.1.1 TR α ^{0/0}

TR α ^{0/0} mice were originally derived in a mixed C57BL/6 and 129Sv genetic background (Gauthier, Plateroti et al. 2001, Bassett, O'Shea et al. 2007). They were backcrossed onto C57BL/6N for more than 20 generations. TR α ^{0/0} mutant and wild type colonies were maintained. Wild type mice were used as controls for experiments to investigate the effects of deletion of TR α on osteoarthritis.

2.1.2 Deiodinase 1 and deiodinase 2 knockout mice

Deiodinase 1 knockout (D1KO) or deiodinase 2 knockout (D2KO) mice were generated after targeted disruption of *Dio1* or *Dio2* in 129/SvJ embryonic stem cells. Mice were bred and maintained as homozygous colonies in a C57BL/6 background at Dartmouth Medical School animal facility using protocols approved by the Institutional Review Board (Schneider, Fiering et al. 2001, Schneider, Fiering et al. 2006). D1KO and D2KO mice were crossed to obtain double heterozygous F1 progeny, which were used to generate a colony of D1D2KO homozygous double mutants (Galton, Schneider et al. 2009, Bassett, Boyde et al. 2010). Mice were housed under conditions of controlled lighting and temperature and sacrificed at 16 weeks of age. Both D2KO and D1D2KO mice have pituitary resistance to thyroid hormone but normal circulating T3 concentration. D2KO mice have a 1.4 -fold increase in T4 levels and 2-fold increase in TSH (Schneider, Fiering et al. 2001). Double knockouts have 2-fold increase in T4 and 2.6-fold increase TSH (Galton, Schneider et al. 2009). Bone samples were shipped to Imperial College London for phenotyping studies in which nine wild type (WT), five D2KO, and three D1D2KO male mice were used for detailed histological analysis of

the joints. Histological sections from four wild type, four D2KO, and three D1D2KO mice were prepared at directly comparable levels for subchondral bone analysis.

2.2 OSTEOARTHRITIS INDUCING SURGERY

10-week-old C57BL/6 wild type or TR $\alpha^{0/0}$ mice had osteoarthritis inducing destabilisation of medial meniscus (DMM) surgery performed at 10 weeks of age. A post-doctoral fellow in a neighbouring research group within Imperial College who had extensive experience performing DMM surgery and teaching this technique trained the author, who performed all knee surgery reported in this thesis. All surgery was performed in accordance with the Animals (Scientific Procedures) Act 1986.

Mice were weighed prior to surgery and anaesthetised using vaporised isoflurane. Surgery was conducted using aseptic technique. A longitudinal incision was made over the distal patella to proximal tibial plateau. The joint capsule was incised with a 5mm, 15 degree microknife (world precision instruments). Blunt dissection exposed the medial meniscus and the medial menisco-tibial ligament (MMTL) was transected (Figure 2.1). The MMTL anchors the medial meniscus to the tibial plateau. DMM surgery leads to medial displacement of the medial meniscus, causing weight bearing to occur over a smaller area and increased mechanical stress. Surgical notes recorded the ease of transection of MMTL and subsequent degree of mobility of medial meniscus. The capsule was closed with a single 6/0 Vicryl absorbable suture (Ethicon). Skin was closed with 4/0 monofilament sutures (Ethicon). For sham surgery, capsule was blunt dissected to expose the medial meniscus but the MMTL was not cut. Buprenorphine 0.09mg/kg was administered subcutaneously post operatively and mice were ambulant within 15 minutes (Glasson, Blanchet et al. 2007).

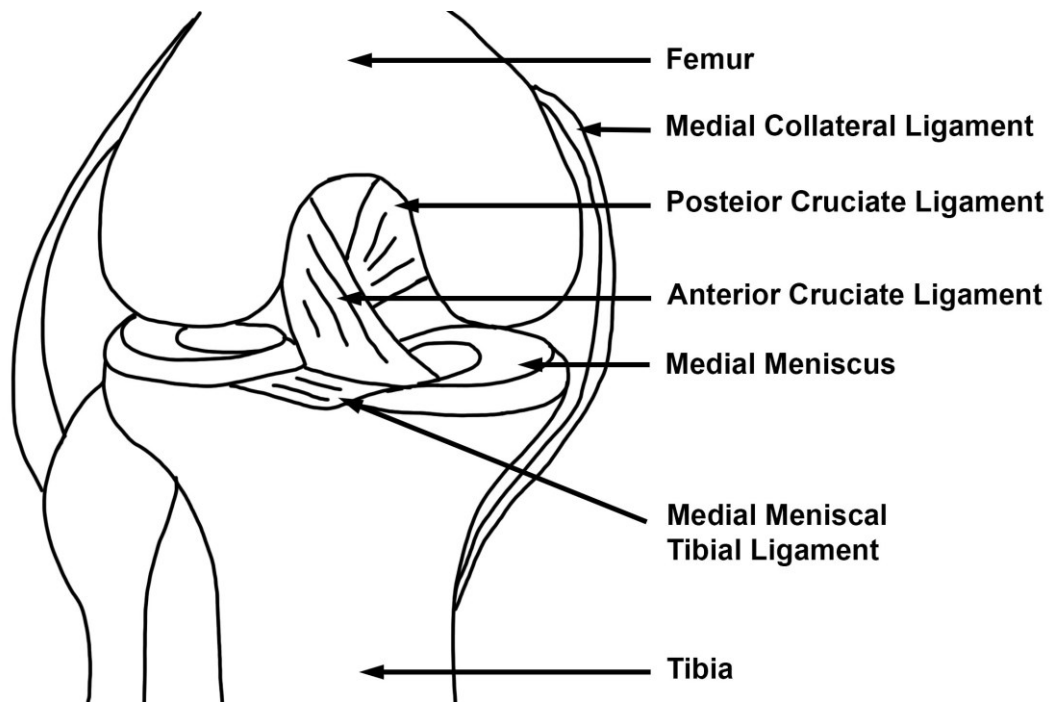


Figure 2.1 Diagram of right knee joint

2.3 ENVIRONMENTAL ENRICHMENT DEVICES

The development of osteoarthritis following DMM surgery depends on the activity level of the animal. Environmental enrichment devices have been shown to affect osteoarthritis severity (Salvarrey-Strati, Watson et al. 2008). Since animals involved in early experiments were caged with a tongue depressor stick as the only enrichment device, this was maintained for all experiments to avoid the risk of confounding results.

2.4 CAGE DENSITY

Personal communication with an investigator experienced in the DMM model of osteoarthritis raised concerns over the influence of cage density on osteoarthritis development. The hypothesis was that animals caged in isolation or in small groups would be less physically active than animals caged in larger group. This was investigated during post hoc analysis using wild type control mice from both the dronedarone and the $TR\alpha^{0/0}$ experiments. Cartilage damage scores in animals housed with different numbers of littermates was compared in animals sacrificed 4-, 8- and 16-weeks following DMM surgery.

2.5 DRONEDARONE ADMINISTRATION

Wild type control mice were treated with vehicle or dronedarone at 20mg/kg/day or 60mg/kg/day to investigate the effects of pharmacological inhibition of TR α on osteoarthritis. Dronedarone was dissolved in 0.6% methylcellulose and mixed with 0.25g of a coconut flavoured jam in a plastic weighing dish. This was administered daily starting one week prior to knee surgery and continued until sacrifice. A score of 0 (completely uneaten) to 10 (consumed entirely) was recorded after each administered dose. The development and validation of this method of drug administration is described in chapter 4.

2.6 CALCEIN LABELLING

All operated mice were given two injections of calcein at 2.5mg/kg (Sigma CO875) via the tail vein 4 days and 1 day prior to sacrifice.

2.7 SERUM SAMPLES

Mice were fasted overnight prior to sacrifice and blood obtained by cardiac puncture (1ml syringe, 25G needle, TERUSS). Blood samples were incubated at room temperature for 30 minutes. Coagulated samples were spun at 12 000 rpm for 10 minutes at 4°C. Serum supernatant was collected and stored at -80°C.

2.8 DISSECTION, FIXATION AND STORAGE OF SKELETON

Lower limb samples were fixed in 10% neutral buffered formalin overnight and stored in 70% ethanol. Soft tissue was removed but integrity of the knee joint capsule and patella was preserved. Upper limbs and vertebrae used for biomechanical analysis were stored directly in 70% ethanol. All samples were stored at 4°C.

2.9 JOINT IMAGING

2.9.1 Histology

Knees were decalcified in 10% Ethylenediaminetetraacetic acid (EDTA), pH 7.4 for 10-21 days. Knee joints were embedded in paraffin and 5µm-thick coronal sections were cut at 80µm intervals throughout the joint. Slides were stained for cartilage proteoglycan using Safranin-O Fast Green according to Osteoarthritis Research Society International (OARSI) recommendations (Glasson, Chambers et al. 2010) and imaged using a Leica MZ75 binocular microscope (Leica AG, Heerbrugg, Switzerland), KL1500 light source, and DFC320 digital camera.

Effective destabilisation surgery was confirmed by a blinded observer. Medial displacement of the medial meniscus was confirmed using histology and reviewing surgical notes. Where inadequate destabilisation surgery occurred (medial meniscus not displaced histologically and surgical records did not document clear medial displacement post MMTL transection), both DMM and sham operated knee joints were excluded from all analyses (histology, X-ray, microCT). Articular cartilage pathology was scored by a blinded observer using the Osteoarthritis Research Society International (OARSI) histopathology scale for murine OA (Glasson, Chambers et al. 2010) (Figure 2.2). A score of 0 represents normal cartilage; 0.5 represents loss of Safranin-O staining without structural changes; 1 represents small fibrillations without loss of cartilage; and 2 represents vertical clefts down to the layer immediately below the superficial layer and some loss of surface lamina. Vertical clefts or erosions to the calcified cartilage extending to < 25%, 25– 50%, 50–75%, or > 75% of the articular surface are assigned scores of 3, 4, 5, or 6 respectively. Using this system, the severity and extent of osteoarthritis is expressed as a maximum score for each joint and as a cumulative score over the whole joint (Glasson, Chambers et al. 2010).

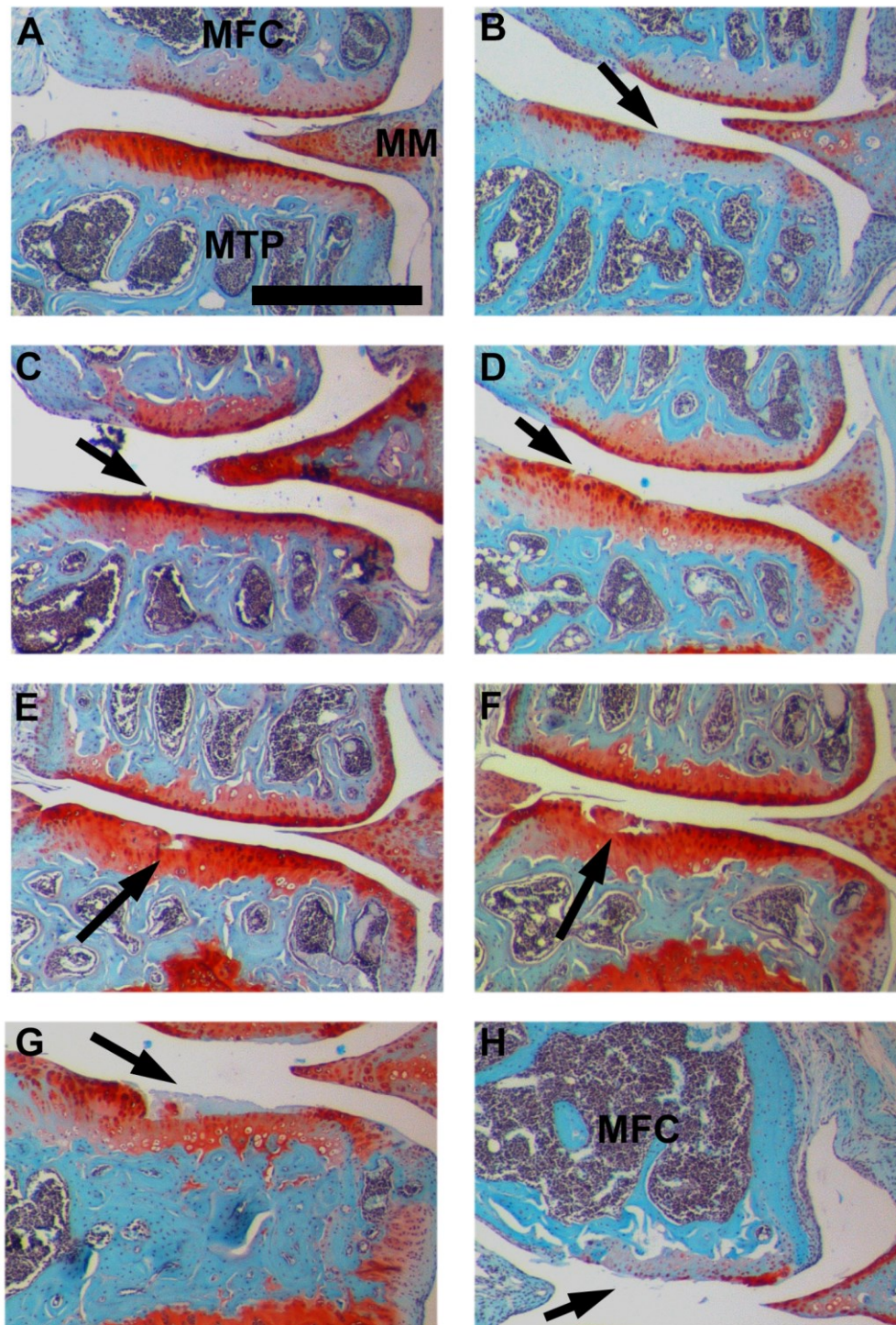


Figure 2.2 OARSI pathology scoring

Safranin-O photomicrographs showing medial femoral condyle (MFC), medial meniscus (MM) and medial tibial plateau (MTP) displaying the range of OA severity scores. Areas of cartilage damage indicated by arrows. All scores refer to MTP except H where only MFC is displayed. (A) 0; (B) 0.5; (C) 1; (D) 2; (E) 3; (F) 4; (G) 5; (H) 6. Scale bar = 0.5mm.

The anterior and posterior limits of the joint were determined using anatomical landmarks. For the anterior limit of the joint, entry of significant amounts of synovial tissue with convergence of medial and lateral borders of tibial plateau and loss of cartilage. The landmarks for the posterior limits of the joint are the appearance of the flattened tibial plateau (Glasson, Chambers et al. 2010). The midpoint between the joint limits was chosen as representing the anterior-posterior centre of the joint. 3 levels anterior and 3 levels posterior to the centre were included for analysis to ensure 7 levels were consistently used for the assessment of osteoarthritis severity.

There was more variation in the orientation of paraffin embedded knee joints in the spontaneous osteoarthritis experiments (WT, D2KO, D1D2KO). This resulted in significant variation in the number of histological levels obtained for scoring OA pathology between individual animals. To address this, a standardised OA score was determined (Waung, Bassett et al. 2015). A 16-week-old male C57Bl/6 wild type mouse was used to create a reference histological atlas. Coronal section 5 μ m thick sectioned at 80 μ m intervals yielded 16 levels, of which 9 passed through the joint cavity (Figure 2.3). The total articulating surface was determined for the histological reference atlas and each experimental animal (Figure 2.4). A standardized OA score for each experimental animal was defined as: cumulative OA score from all histological sections $\times$ (reference articular surface/experimental animal articular surface).

Additionally, a validated erosion index was also determined to assess the extent of cartilage lesions as a measure of OA progression (Hoegh-Andersen, Tanko et al. 2004, Sondergaard, Catala-Lehnen et al. 2012): the length of eroded articular cartilage was divided by the total length of articular cartilage surface and expressed as a percentage (Figure 2.4). Articular cartilage cellularity was also calculated and expressed as the mean number of chondrocytes per mm².

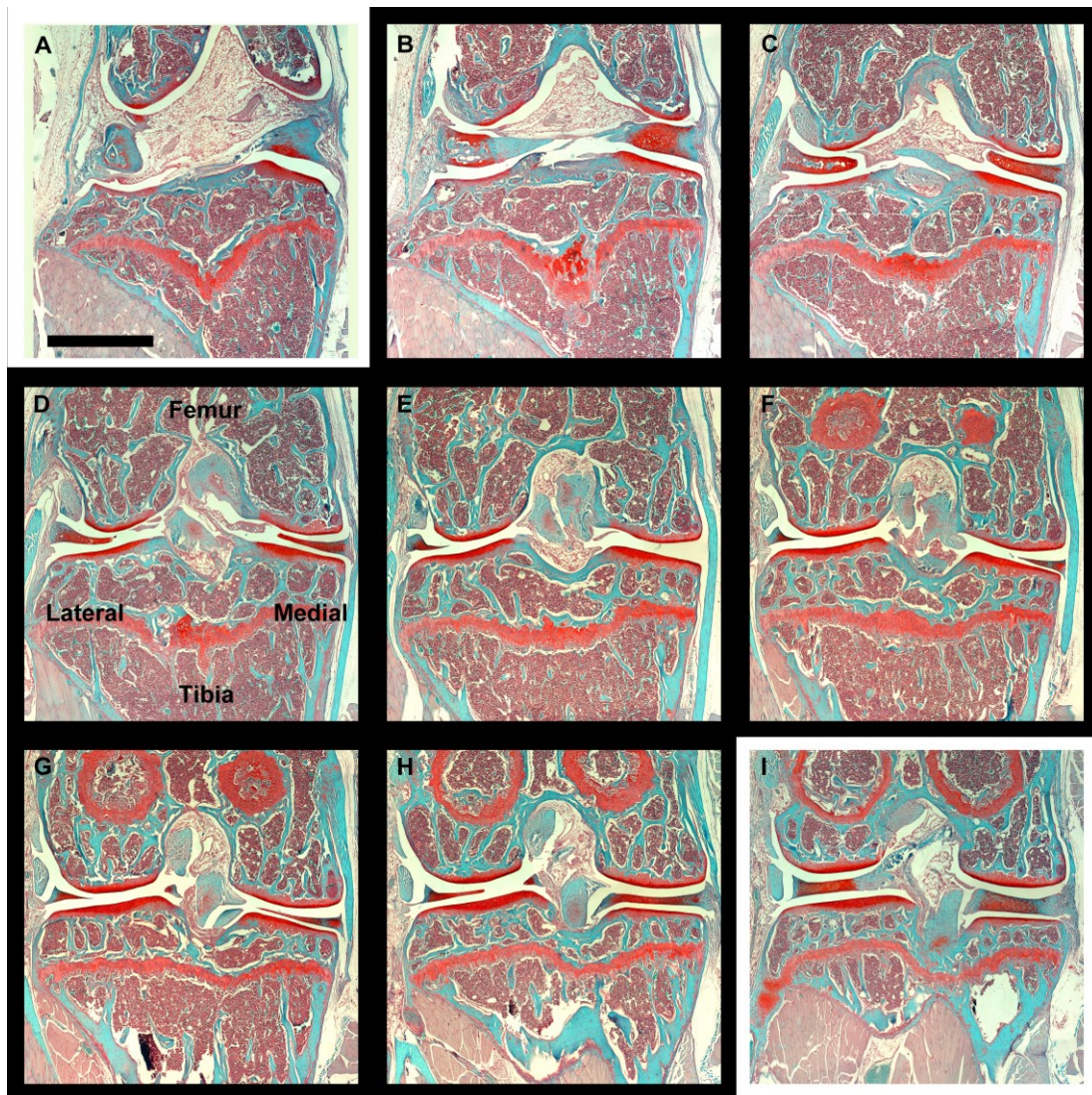


Figure 2.3 Atlas of knee histology

Coronal sections are 80µm apart anterior (A) to posterior (I). Level B-H (with black borders) represent the 7 most central sections used for scoring. The articular surface is measured for all levels (A-I) and used to determine the standardised OA score.

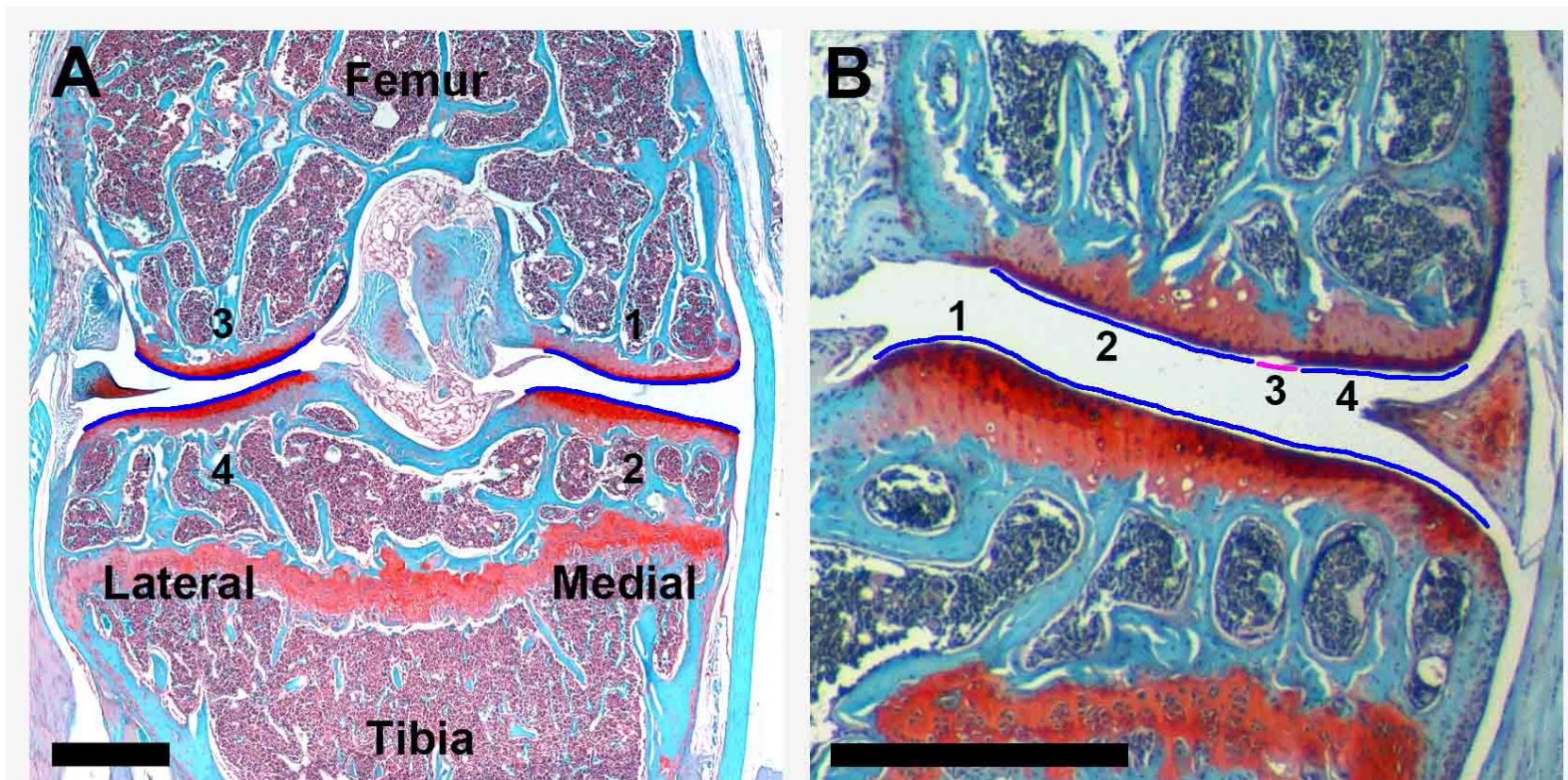


Figure 2.4 Standardised OA score and erosion index

Figure reproduced from Waung et al 2015 with permission. (A) Right knee mid-joint coronal section from 16-week-old male C57Bl/6 mouse stained with Safranin-O Fast-Green. Numbered lines (1-4) outline articular surfaces. Scale bar = 0.5mm. (B) Erosion index is calculated by dividing eroded surface (3) by total articular surface (1+2+3+4). Scale bar = 0.5mm

2.9.1.1 Histological analysis of subchondral bone area

Histological sections were imaged in 8-bit Tiff format. The subchondral bone region of interest (ROI) between the articular cartilage and epiphyseal growth plate of the tibia was contoured using ImageJ (<http://rsb.info.nih.gov/ij/>), and the subchondral bone area relative to tissue area (BA/TA) determined (Figure 2.5).

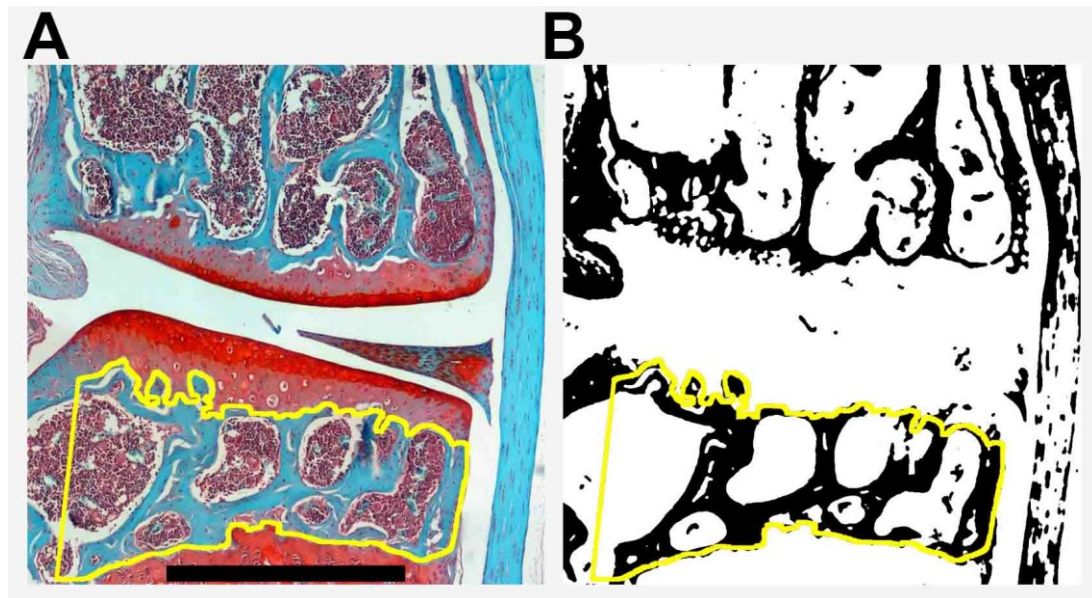


Figure 2.5 Subchondral bone area

Figure reproduced from Waung et al 2015 with permission. (A) Subchondral bone region of interest enclosed by yellow line. Scale bar = 0.5 mm. (B) Articular cartilage and marrow space after thresholding in Image J to allow the subchondral bone area in the region of interest to be determined automatically.

2.9.2 X-ray microradiography

Knee joints were flexed to 105° and held in position within a 37mm diameter plastic ring using a synthetic adhesive. This maintained the knee joint 12 mm above the mount with tibial growth plate and subchondral bone in a vertical plane. The patella was used as a landmark to confirm correct orientation of the joint (Figure 2.6). Anterior-posterior projection X-ray images were recorded at 10 µm pixel resolution using a Faxitron MX-20 variable kV point projection X-ray source and digital image system (Qados, Cross Technologies plc, Berkshire, UK) operating at 26 kV for 15 s and 5× magnification. Images were calibrated by taking an X-ray of a digital micrometer. The relative mineral content of subchondral bone was determined by comparison between experimental groups. Each image contained standards (1mm diameter steel, aluminium and polyester wires). 16-bit (2368 × 2340) DICOM images were converted to 8-bit TIFF using ImageJ (<http://rsb.info.nih.gov/ij/>). The greyscale histogram was stretched between polyester (grey level 0) and steel (grey level 255) standards. Increasing gradations of mineralisation density were represented by a pseudocolour scheme of 16 equal intervals (Waung, Maynard et al. 2014). The medial tibial plateau (MTP) is the site of maximal articular cartilage damage and subchondral bone change in the DMM model of osteoarthritis. Therefore, a 1000µm × 250µm area of MTP subchondral bone was selected as the region of interest. Individual ROI were montaged according to treatment groups.

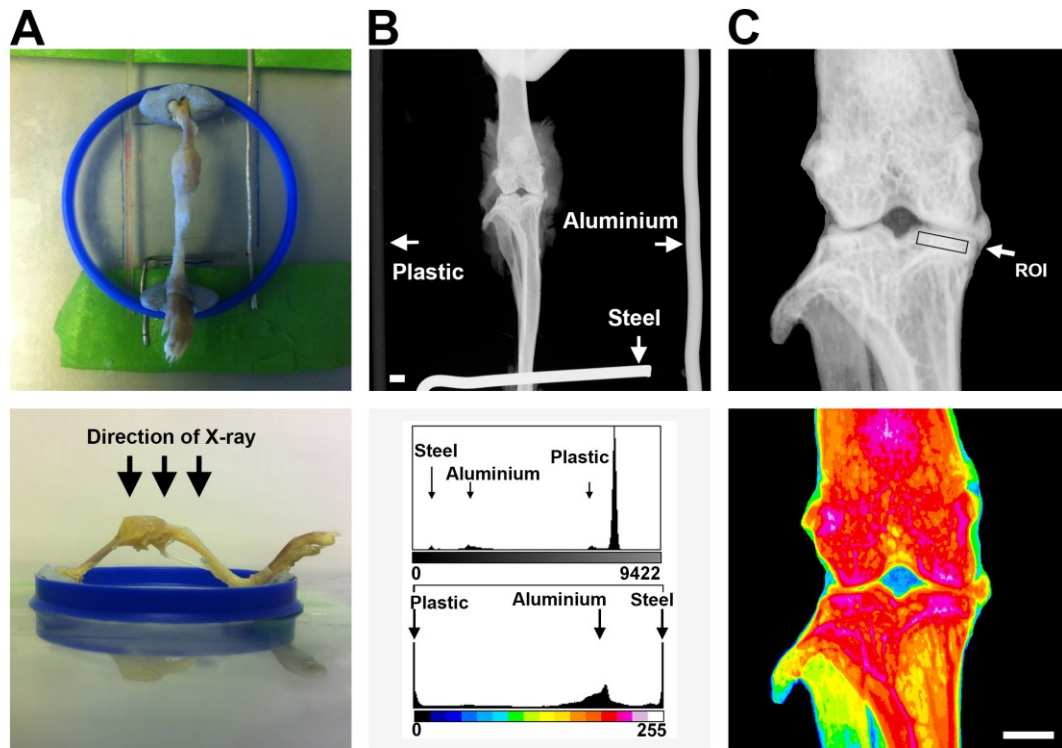


Figure 2.6 Quantitative digital X-ray microradiography of subchondral bone

Figure reproduced from Waung et al 2014 with permission. (A) Knees are held in position using a plastic ring and synthetic adhesive. The standards are seen inside the ring on the top panel. The patella is used as a gross morphological landmark for positioning. The bottom panel shows the tibial plateau and subchondral bone are in a vertical plane and the direction of X-rays. (B) Top panel shows a Faxitron microradiograph with location of standards as indicated. The patella is located in the mid-sagittal plane. White bar = 1000µm. Histogram from upper panel shows is from the original 16-bit DICOM image. The large peak on the right represents the background. The lower panel shows a histogram of the image after stretching between plastic and steel and conversion to 8-bit TIFF format. A 16-colour look up table is used to pseudocolour the image according to relative mineralisation. (C) Top panel shows a grey scale image of the right knee joint. The medial tibial plateau subchondral bone is selected as a region of interest for quantitative analysis. Lower panel shows the pseudocoloured TIFF image with lower BMC represented by green and yellow while higher BMC is represented by red, purple and white. Scale bar = 1000µm.

2.9.3 Micro computed tomography (microCT) analysis

Intact knees joints were scanned with Scanco CT 40 microCT scanner (Scanco Medical AG, Brüttisellen, Switzerland) at 70kV and 200 μ A and 5 μ m voxel size. A Gaussian filter removed noise and reconstructed images were segmented using a global high-level threshold of 300 to distinguish calcified tissues from non-calcified tissues. In order to study subchondral bone, the epiphysis of the tibia was chosen as the region of interest. A radiological atlas (Figure 2.7) was produced to determine the region of subchondral bone to investigate with the DMM model of osteoarthritis.

A 0.5mm mediolateral width x 1mm ventrodorsal length region between the non-calcified articular cartilage proximally and growth plate distally was defined as the region of interest for subchondral bone of the medial tibial plateau (Figure 2.8) (Botter, Glasson et al. 2009). This area includes the calcified articular cartilage, subchondral bone plate and subchondral trabecular bone. The centre of this ROI was defined in 3 planes in this order: 1) The midpoint anterior-posteriorly of the tibia. 2) the mid axial level between the tibial growth plate and the proximal end of the medial tibial plateau. 3) The mid sagittal point of the MTP.

The following bone morphometric parameters were calculated: bone volume fraction (BV/TV), which is the ratio of bone volume divided by tissue volume, trabecular thickness (Tb.Th., in mm), trabecular spacing (Tb.Sp., in mm), connectivity density (CD), which calculates the number of trabecular connections per unit volume, structure model index (SMI), indicating whether trabeculae have a rod-like (value near 3) or plate-like (value near 0) shape, apparent density and material density.

Different genotype (wild type vs $TR\alpha^{0/0}$) and treatment (dronedarone vs vehicle) may affect baseline characteristics in subchondral bones prior to DMM surgery. Therefore, in addition to determining absolute bone mass and mineralisation in each joint, the relative increase in subchondral bone density between DMM-operated and sham-operated knee for each animal was determined.

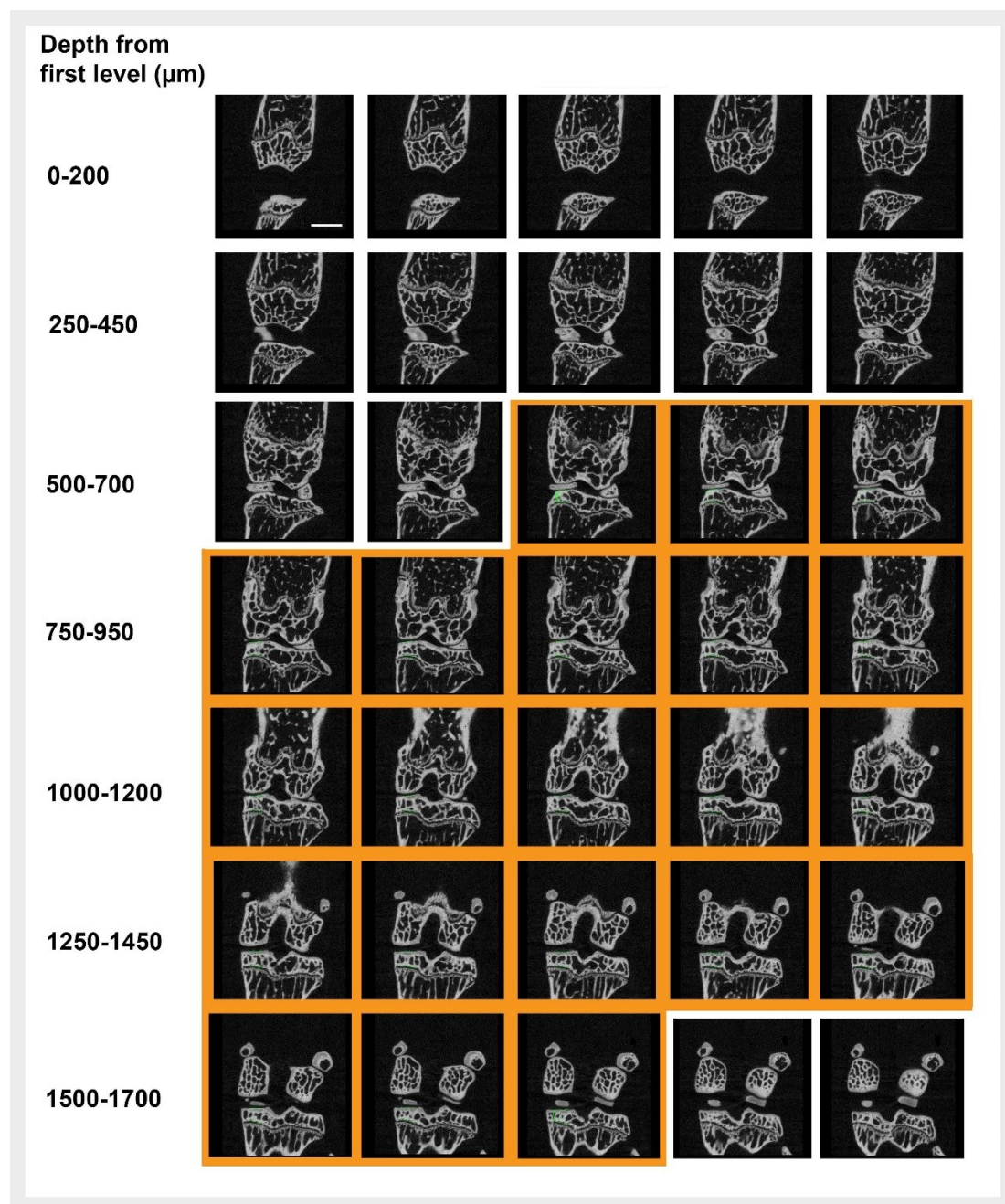


Figure 2.7 Radiological Atlas of 18-week old male wild type C57Bl/6 mouse knee

Coronal sections from anterior (top left panel) to posterior (bottom right panel) of left knee. Femur is at the top of image and tibia at the bottom. Each level is $5\mu\text{m}$ thick with a $50\mu\text{m}$ interval between images. Levels at the centre of the joint were chosen as ROI ($1000\mu\text{m}$ depth) have orange border.

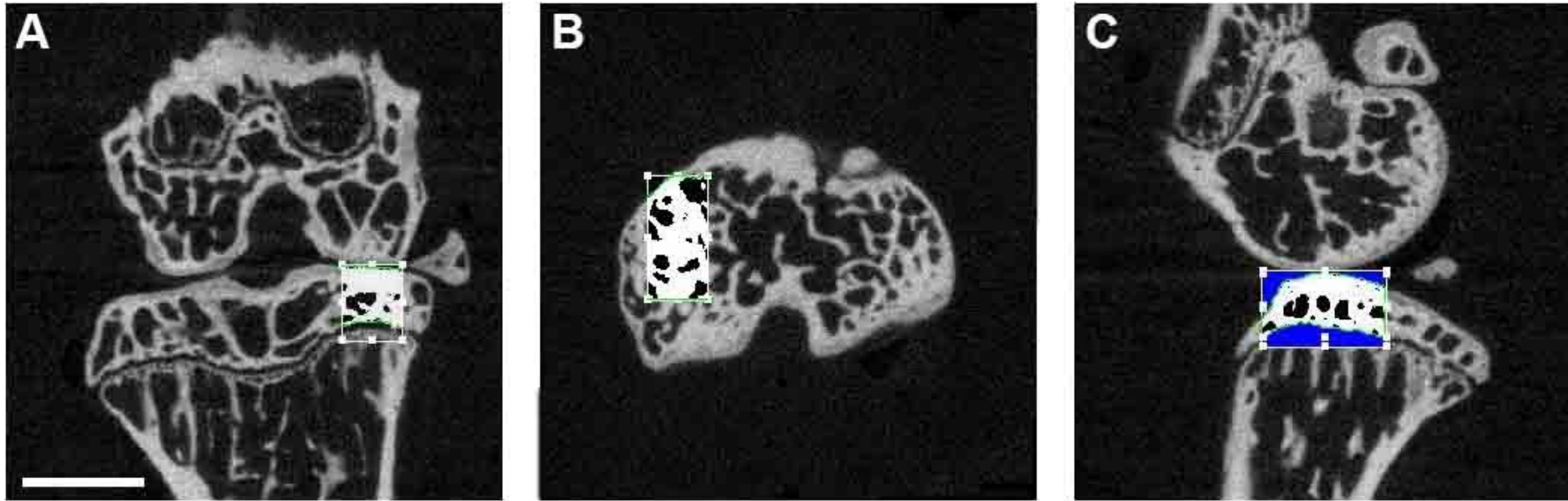


Figure 2.8 Subchondral bone regions of interest

(A) Mid coronal section of right knee joint. Medial tibial plateau ROI shown by box with green contours at top (junction between mineralized tissue and non-mineralised articular cartilage) and bottom (tibial growth plate). After thresholding, mineralised tissue appears white and is included for quantitative analysis. Scale bar = 1000 μ m. (B) Mid axial section of medial tibial plateau subchondral bone. (C) Mid Sagittal section of medial tibial plateau subchondral bone. Note murine knee joints are normally flexed but were imaged in the extension because of size constraints of the intact knee joint.

2.9.4 Backscattered electron scanning microscopy (BSE-SEM)

Knee joints were embedded in Polymethyl methacrylate (PMMA), cut in coronal plane and polished to an optically flat finish. Surfaces were coated with carbon and analysed using backscattered electrons at 20kV, 0.5nA with a working distance of 17mm and a sample-to-detector distance of 11mm (Boyde and Firth 2005, Bassett, Boyde et al. 2010). Samples were mounted and imaged by Professor Alan Boyde and analysed by the author.

2.10 NON-ARTICULAR BONE IMAGING.

2.10.1 X-ray microradiography

Intact lower limbs and proximal tail vertebrae digital X-ray images were recorded and processed (converted, stretched and pseudocoloured) using the same settings as for knee joints (Figure 2.9). The whole femur and 2 contiguous proximal tail vertebrae were selected as ROI. Each was digitally cleaned and a montage made using samples from the same treatment group. Femurs and tail vertebrae were analysed for bone mineral content using the same methods and statistics as for subchondral bone.



Figure 2.9 Image processing for bone mineral content

(A) Faxitron DICOM image showing lower limb, tail vertebrae and standards. (B) Image stretched between steel and polyester. (C) Pseudocoloured image showing gradations of mineral densities divided into 16 equal intervals

Cortical bone thickness and diameter were measured at the mid diaphysis in mediolateral projection position (Figure 2.10). A 100-pixel wide grey box was superimposed over the mid-diaphysis using Photoshop CSI 5 software. 5 measurements between periosteal surfaces and 5 measurements between endosteal surfaces at equal intervals along this box were used to calculate mean cortical thickness for each bone.

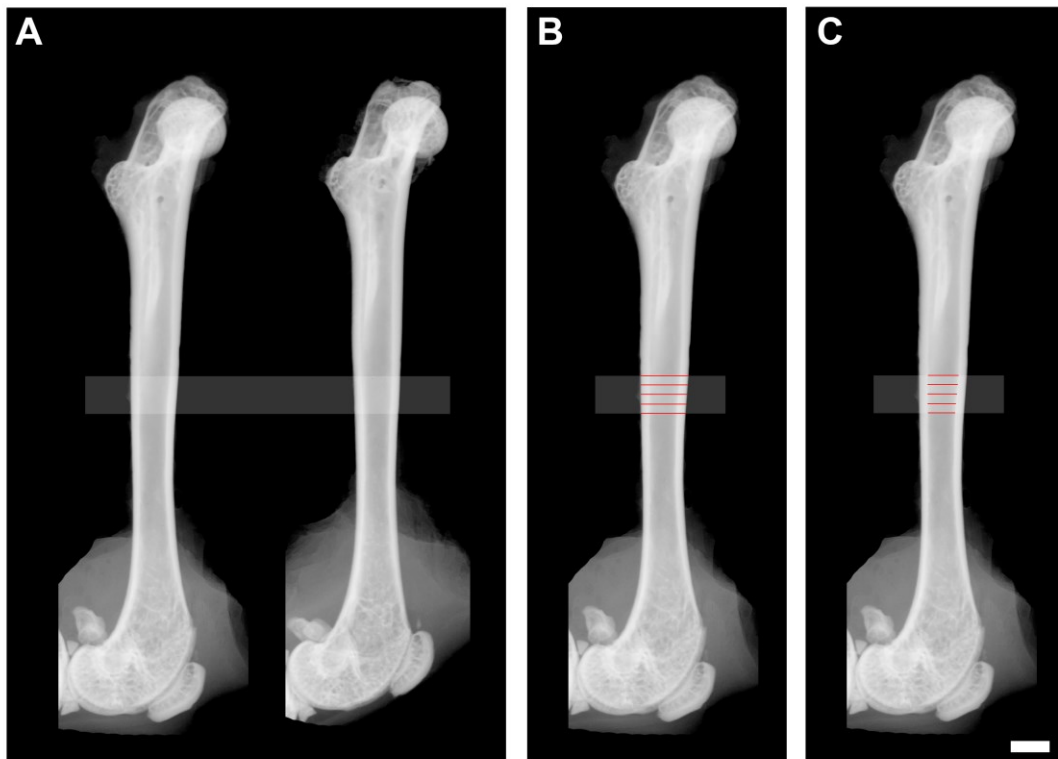


Figure 2.10 Cortical thickness by X-ray

(A) Cortical thickness was measured in the mid diaphysis of femurs shown by the grey box. (B) 5 measurements were made between the external cortical surfaces. (C) 5 measurements between internal cortical surfaces (marrow diameter). The average cortical thickness was determined by subtracting C from B and dividing by 2.

2.10.2 Micro computed tomography (microCT) analysis

The left (sham-operated) proximal tibia was used to investigate the effects of dronedarone on non-articular bone. A volume of 1mm^3 of trabecular bone was selected as the region of interest, 0.2 mm distal to the proximal tibial growth plate. The following parameters were determined. Trabecular bone volume as a proportion of tissue volume (BV/TV), trabecular separation (Tb.Sp), trabecular number (Tb.N), trabecular number (Tb.N) , connectivity density (CD), which calculates the number of trabecular connections per unit volume, structure model index (SMI), indicating whether trabeculae have a rod-like (value near 3) or plate-like (value near 0) shape, apparent density and material density.

The whole left femur was imaged at $10\mu\text{m}$ resolution to assess the effect of dronedarone on cortical bone. A 1.5mm long section of femoral cortex, 56% of the distance between proximal femur and the tip of femoral condyles was analysed to determine cortical thickness, apparent mineral density and material density. This region was chosen because the femur is most cylindrical at this point and there is no trabecular bone.

2.10.3 Confocal microscopy

Calcein double labelled humeri were examined with confocal scanning laser microscopy (CSLM) to determine the proportion of bone surface undergoing active bone formation (Doubé, Firth et al. 2007). Humeri were embedded in Polymethyl methacrylate (PMMA), cut in midline longitudinal section and polished to an optically flat finish. Samples were imaged with Leica SP5 reflection confocal microscope using 488 nm excitation and 10x/1.0 and 40x/1.25 objectives. Tissue layers immediately deep to the block surface were visualised to ensure that only fluorescently labelled bone forming surfaces that lie in orthogonal planes were analysed. Montages of overlapping CSLM fields were constructed for each bone using Photoshop CSI 5 software and ImageJ. Two-dimensional parameters of bone formation were determined according to the American Society for Bone and Mineral Research (ASBMR) system of nomenclature (Dempster, Compston et al. 2013).

Trabecular bone was quantified in a 1mm² area below the humeral head growth plate. Each humerus had 1mm lengths of cortex imaged on the anterior and posterior surface at 1mm distance from the growth plate (Figure 2.11). The average of both cortical surfaces is reported. ImageJ software was used to measure bone parameters. Bone surface (BS), the length of single and double labelled bone was measured. The perpendicular lines between the midpoints of double labelled bone was measured at multiple points along the length of the labels. Mineralising surface (MS) was determined by adding the length of double labelled surfaces to half the length of single labels. MS/BS was calculated by dividing the total MS by total BS. Mineral apposition rate (MAR) was the mean separation between double labels. Bone formation rate (BFR) was calculated as the product of MAR and MS/BS (Figure 2.12) (Bassett, Boyde et al. 2010, Dempster, Compston et al. 2013).

Samples were embedded and sectioned by Mo Arora at QMUL. Polishing was done by both Mo Arora and the author. Confocal microscopy, imaging, digital manipulation, measurement of parameters and analysis was performed by the author.

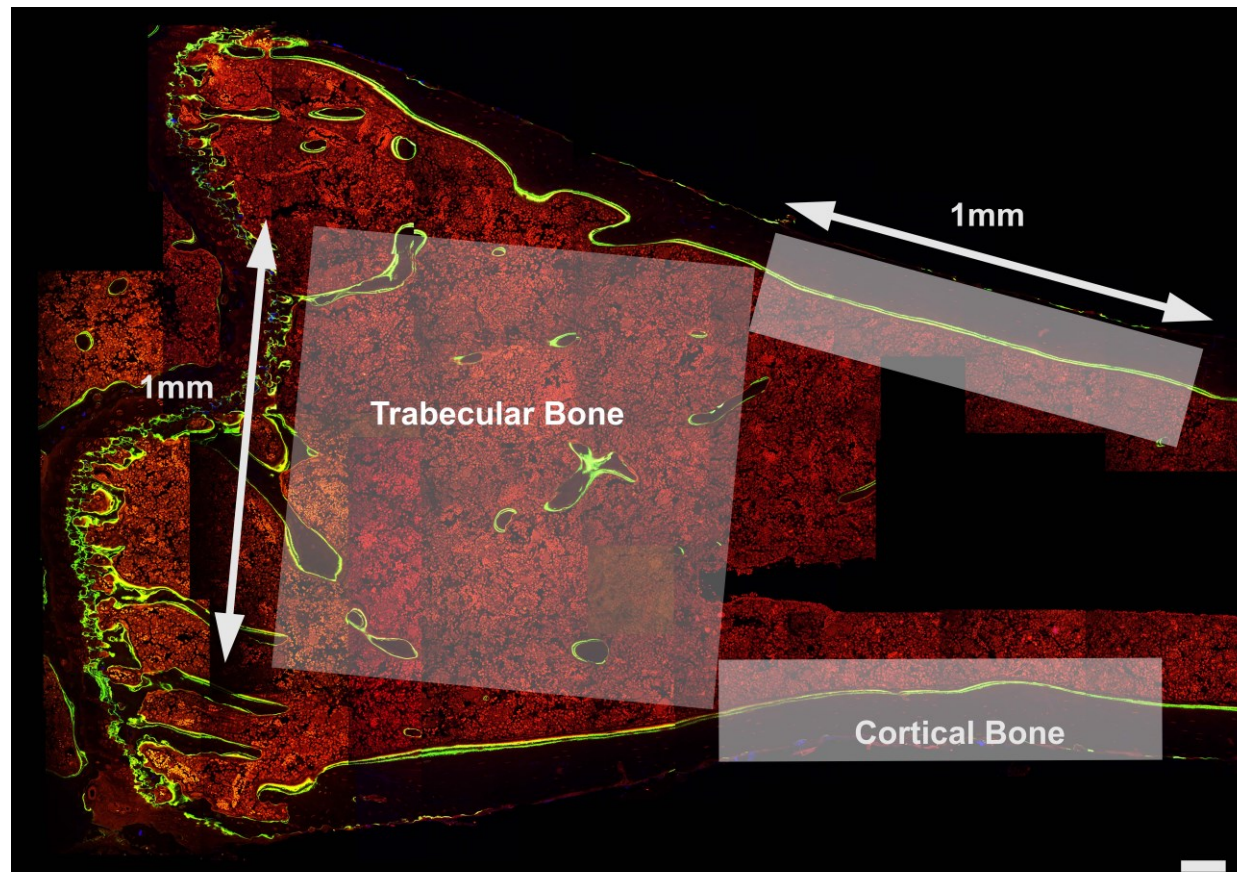


Figure 2.11 Montaged confocal image of a humerus

Bright green calcein labels are clearly seen. Trabecular bone is quantified in a 1mm^2 area below the humeral head growth plate. 1mm lengths of cortical bone was measured 1mm from the growth plate. Scale bar = $100\mu\text{m}$

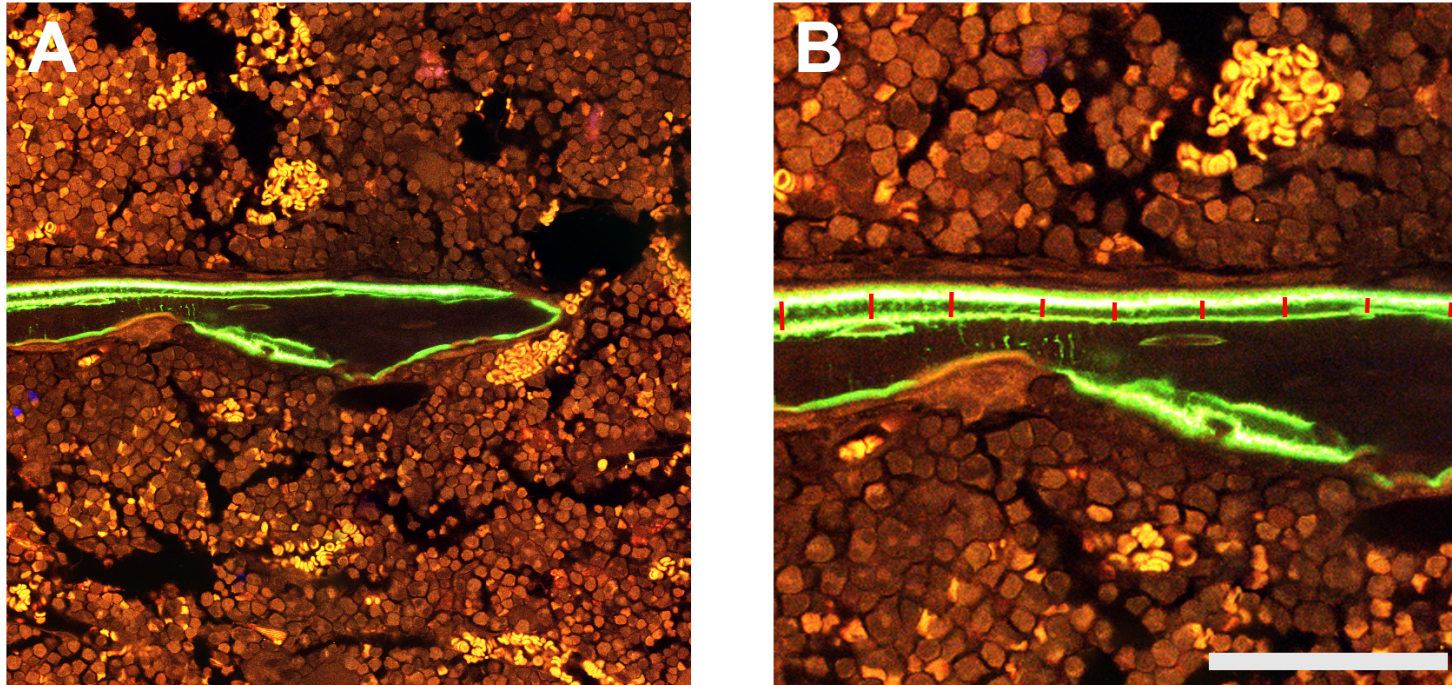


Figure 2.12 Confocal image showing calcein labelling

Confocal image of trabeculae (A). Bone is dark and bordered by green calcein labels. Bone marrow are cellular areas and appear orange. High magnification view (B) showing single and double labels. Mineral apposition rate (MAR) is the perpendicular distance (red lines) between the midpoints of the double labels. Scale bar = 100 μ m

2.11 MECHANICAL LOADING AND BONE STRENGTH

Destructive three-point bend tests were performed on humeri using an Instron 5543 materials testing load frame (Instron, UK). Bones were positioned horizontally and load was applied vertically to the mid-shaft with a constant rate of displacement of 0.03mm/second until fracture. Load displacement curves were generated in Excel and the stiffness, yield, maximum and fracture loads determined (Figure 2.13). The load displacement curve can be divided into 3 phases. First, a linear elastic deformation ending at the yield point. Stiffness is the gradient of this line. Second, a plastic deformation phase including maximum load that is the greatest force that can be applied through the bone. Finally fracture load represent the point of bone failure. During the plastic deformation phase, non-propagating microfractures absorb energy, therefore reducing damage at the point of fracture (Esapa, Bassett et al. 2012).

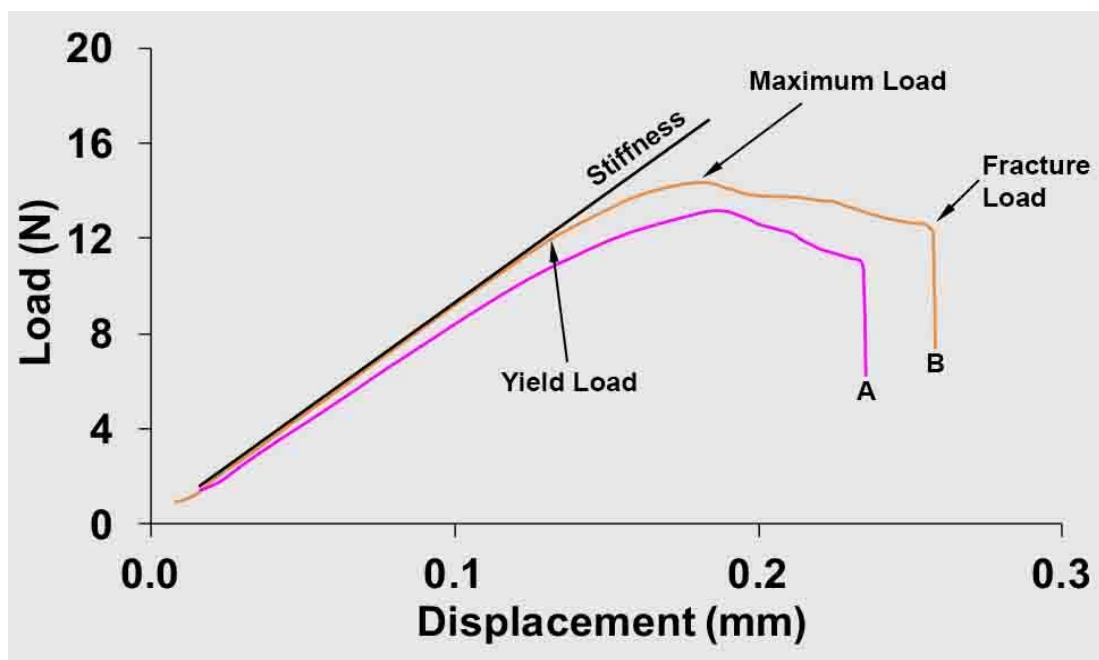


Figure 2.13 Load displacement curves from three-point bend tests of humeri

Plastic deformation occurs until the yield load is reached. The gradient represents stiffness, with stiffer bones having steeper lines. The maximum and fracture load occur during plastic deformation. Bone A is less stiff, has a lower yield, maximum and fracture load compared to bone B.

2.12 STATISTICS

The software used for statistical analysis was GraphPad Prism 6.0.

2.12.1 Faxitron

The Kolmogorov-Smirnov test was used to compare grey level distributions (cumulative frequencies) from the montages ROI (subchondral bone or whole bone (femur or vertebrae)) between experimental groups (Demidenko 2004). P values for the D statistic in 1024 pixel datasets were $D \geq 6.01$, $P < 0.05$; $D \geq 7.20$, $P < 0.01$; and $D \geq 8.62$, $P < 0.001$ (Bassett, Gogakos et al. 2012). Previous experience in our group shows whole femur BMC follows a Gaussian distribution. This was confirmed in medial tibial plateaux subchondral bone with the D'Agostino & Pearson omnibus normality test. Paired Student's t -test was used to compare median grey levels for sham and DMM-operated knees in mice of the same genotype or given the same drug treatment. Unpaired t -test (wild type vs $TR\alpha^{0/0}$) or ANOVA (vehicle vs dronedarone low dose and high dose) followed by Tukey's post hoc test to compare ipsilateral joints. Results are reported as mean and standard error of mean (SEM). $P < 0.05$ was considered statistically significant.

2.12.2 MicroCT & Histology of knee joint

The D'Agostino & Pearson omnibus normality test was applied to mice sacrificed 16 week following surgery to confirm if the data followed a Gaussian distribution. All sham-operated knees passed the normality test but for DMM-operated knees only mice treated with high dose dronedarone passed the normality test. Therefore, all analysis was done using non-parametric tests.

Non-parametric Wilcoxon signed rank test was used to compare sham and DMM-operated knees in mice of the same genotype or given the same drug treatment. Non-parametric Mann Whitney U/Kruskall Wallis test was used to compare the effects of genotype or drug treatment in spontaneous osteoarthritis or in corresponding DMM-operated or sham-operated knees (Botter, Glasson et al. 2009). Comparisons between WT and mutant mice for mean standardized OA score, erosion index, and subchondral bone area were analysed by unpaired Student's t -test. Results are reported as median and standard deviation (SD). $P < 0.05$ was considered statistically significant.

2.12.3 MicroCT of whole bone

Extensive previous experience from Prof. Williams' and Prof. Bassett's group in skeletal phenotyping whole bones of mutant mice showed data followed a Gaussian distribution. Therefore, ANOVA followed by Tukey's post hoc test was used to compare the effects of dronedarone treatment on whole bone (proximal tibia, femoral cortex). Results are reported as mean $\pm$ standard error of mean (SEM). $P < 0.05$ was considered statistically significant.

2.12.4 Dronedarone Consumption

One-way ANOVA with Tukey's multiple comparison correction. $P < 0.05$ was considered significant.

2.12.5 Body weight

Multiple t -tests were performed (one for age compared) with correction for multiple comparisons using the Holm-Sidak method. $P < 0.05$ was considered significant.

CHAPTER 3 VALIDATION OF DESTABILISATION OF MEDIAL MENISCUS AS A MODEL FOR OSTEOARTHRITIS

3.1 BACKGROUND

The DMM model is technically demanding and the importance of validating surgical technique prior to interpreting experimental data has been highlighted (Glasson and Little 2011). Thus, wild type control mice from the dronedarone and TR $\alpha^{0/0}$ experiments were analysed, separately and then together. These mice had DMM surgery in the right knee and sham surgery in the left knee and were sacrificed at 4, 8 or 16 weeks after surgery.

3.2 HISTOLOGICAL VALIDATION OF SURGICAL TECHNIQUE

Effective knee destabilising surgery was confirmed histologically (Figure 3.1). The medial meniscus was displaced medially. Cartilage damage was focused on the medial joint compartment and especially the medial tibial plateau. This was accompanied by subchondral bone sclerosis and frequently osteophytes. In contrast the sham operated knee has no osteoarthritis related pathology and the medial meniscus was not displaced.

Mice where inadequate destabilisation surgery occurred were excluded from histological and all other types of analyses. This occurred in 2 mice in the dronedarone experiments and 6 mice in the TR $\alpha^{0/0}$ experiments.

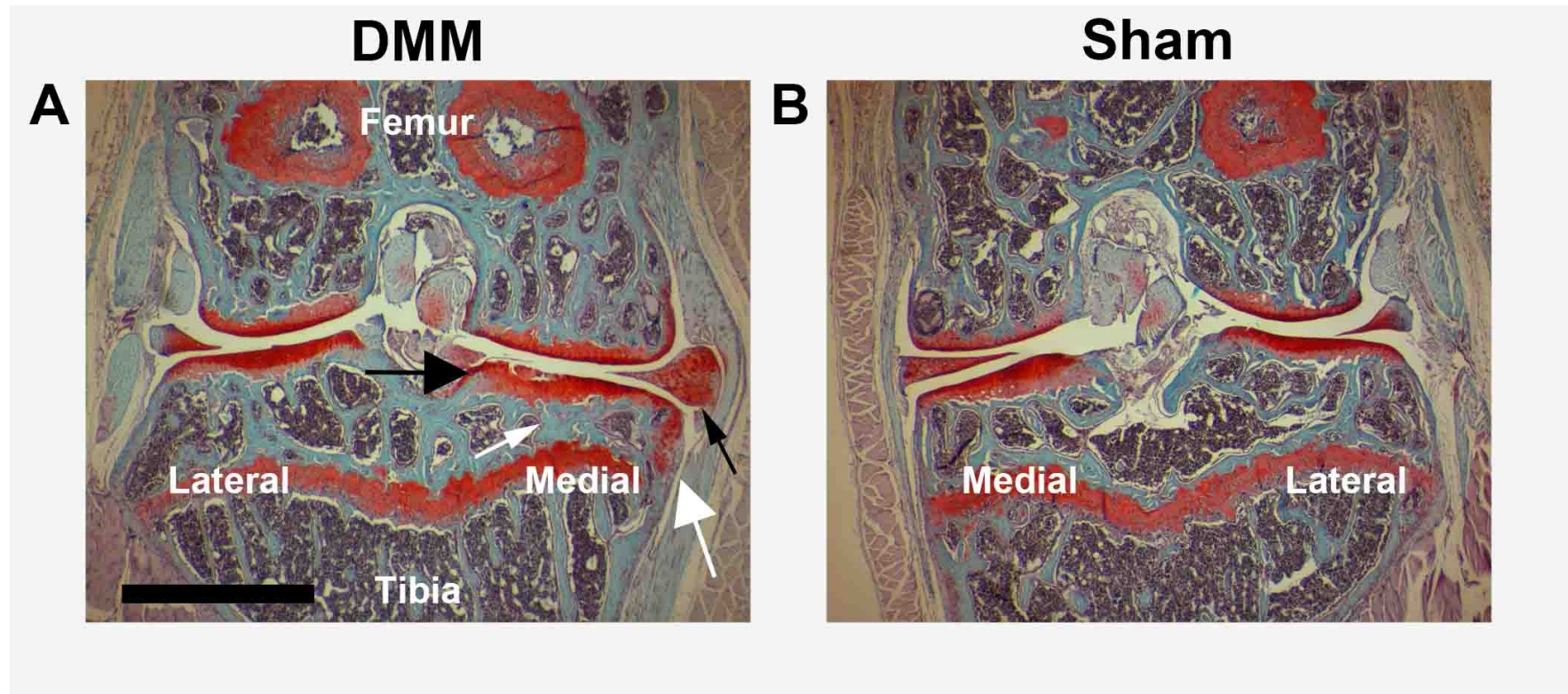


Figure 3.1 Histological validation of DMM model

(A) Right knee 8 weeks following destabilising surgery. Small black arrow shows medial meniscus displaced medially. Wide black arrow shows damage to articular cartilage focused on medial meniscus. Small white arrow shows subchondral bone sclerosis. Wide white arrow shows osteophyte. Scale bar = 1mm. (B) left knee 8 weeks after sham surgery showing no features of osteoarthritis.

3.2.1 Quantitative assessment of cartilage damage following DMM surgery

Maximal and summed OARSI scores were calculated for each DMM operated joint and its contralateral sham operated control. Control mice from dronedarone experiments or TR $\alpha^{0/0}$ experiments were analysed separately. Subsequently, these genetically identical control mice were combined to provide greater statistical power. For both experiments, maximal and summed scores of knee joints that had DMM surgery were generally higher than contralateral sham operated controls. However, there was no statistically significant difference in mice sacrificed at any time point after surgery. In both experiments, there was a single mouse that had a higher summed OA score in the sham operated knee compared to the destabilised knee. In both cases, histological assessment confirmed medial displacement of the medial meniscus consistent with adequate destabilisation surgery. Groups sizes were small at each time point for all experiments (n=3-5).

When dronedarone and TR $\alpha^{0/0}$ experiments were combined (Table 3.1), groups sizes of 7-8 were available for analysis at 4, 8 and 16-weeks post-surgery. Knee joints that had destabilising surgery had significantly higher cartilage damage scores at all time points (Figure 3.2).

Weeks post DMM	Sample number (pairs)	Max Score (Median; IQR)		P Value	Summed Score (Median; IQR)		P Value
		Sham	DMM		Sham	DMM	
4	8	1.0; 0-2.0	2.5; 2.0-3.8	0.063	1.0; 0-3.0	11.3; 6.5-15.9	0.016*
8	7	1.0; 0-1.0	4.0; 2.0-4.0	0.016*	1.0; 0-3.0	16.0; 8.5-29.0	0.016*
16	8	1.5; 0.1-2.0	2.5; 2.0-4.8	0.016*	2.5; 0.2-6.0	15.3; 5.8-26.5	0.016*

Table 3.1 OARSI Scores following DMM surgery

Maximal and summed OARSI score for cartilage damage in control mice sacrificed 4, 8 and 16 weeks after surgery. Wilcoxon paired t test. * $P < 0.05$.

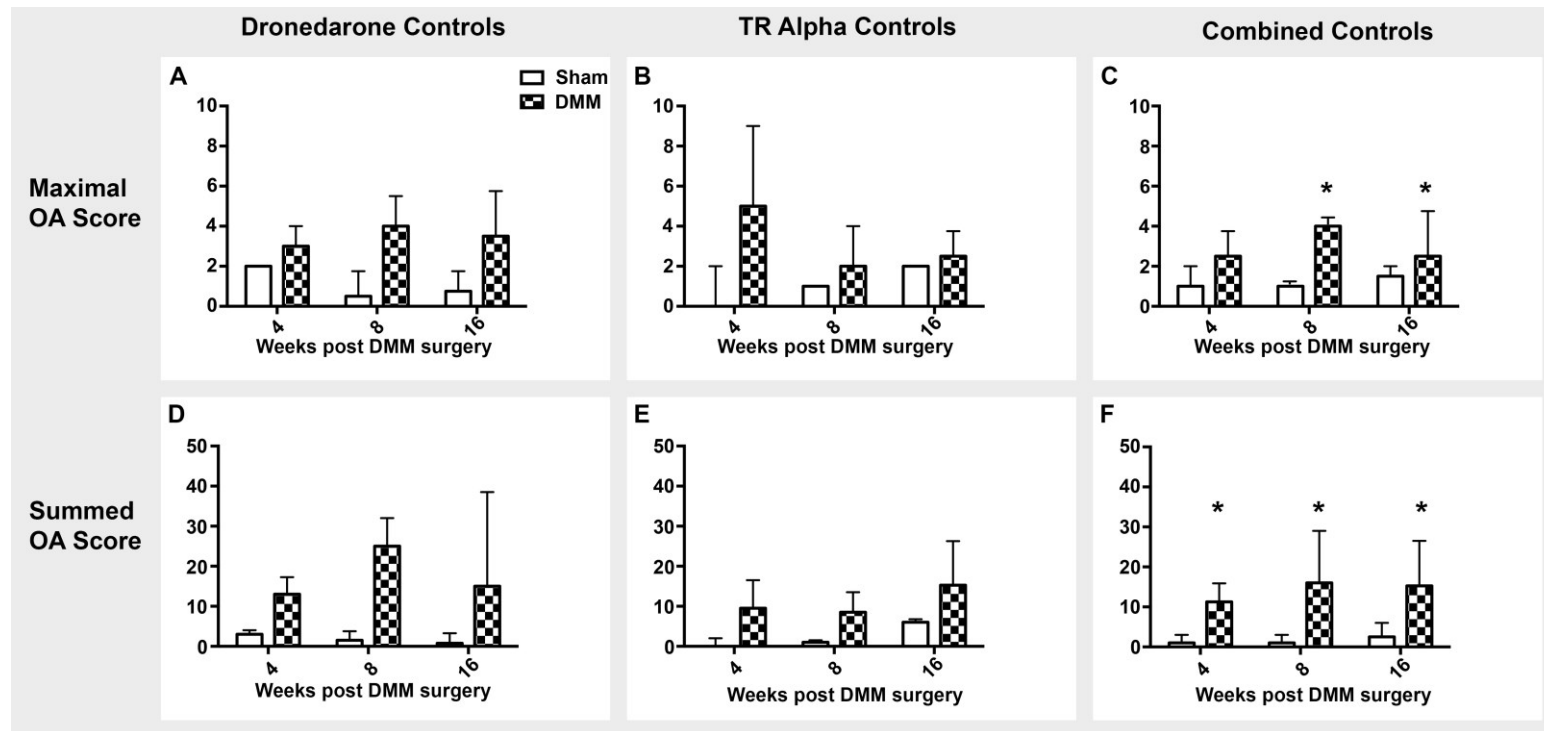


Figure 3.2 Cartilage damage following knee destabilising surgery (DMM)

Maximal OA scores (top row) and summed OA scores (bottom row). (A) and (D) Control mice from the dronedarone experiment. Destabilising surgery did not significantly increase OA scores. (B) and (E) Control mice from $TR\alpha^{0/0}$ experiment. Destabilising surgery did not significantly increase OA scores. (C) and (F) Control mice combined from left and middle columns. Cartilage damage was increased in mice sacrificed 4, 8, and 16 weeks after knee destabilising surgery. * $P < 0.05$ compared to sham

3.3 VALIDATION OF DMM SURGERY USING FAXITRON

X-ray microradiography was used to quantify changes in the medial tibial plateaux subchondral bone following destabilisation surgery. Genetically identical control mice from dronedarone and TR $\alpha^{0/0}$ experiments were analysed together to increase statistical power. Destabilisation surgery resulted in significantly higher BMC compared to the contralateral sham operated knee (Figure 3.3). This was evident in mice sacrificed 4, 8, and 16 weeks post-surgery.

In order to compare the effects of surgery over time, median grey levels for MTP subchondral bone were determined for each joint (Figure 3.4). BMC increased in sham operated knees between mice sacrificed 4 and 8 weeks (aged 14 and 18 weeks) after surgery. There was no further increase in BMC in the sham operated knee joints of 26-week-old mice.

In contrast, destabilised knee joints had greater subchondral BMC compared to the contralateral sham operated knee at all time points. Furthermore, the difference between destabilised and sham operated knee was greatest in mice sacrificed 16 weeks following surgery. Thus, destabilisation surgery caused a progressive increase in BMC.

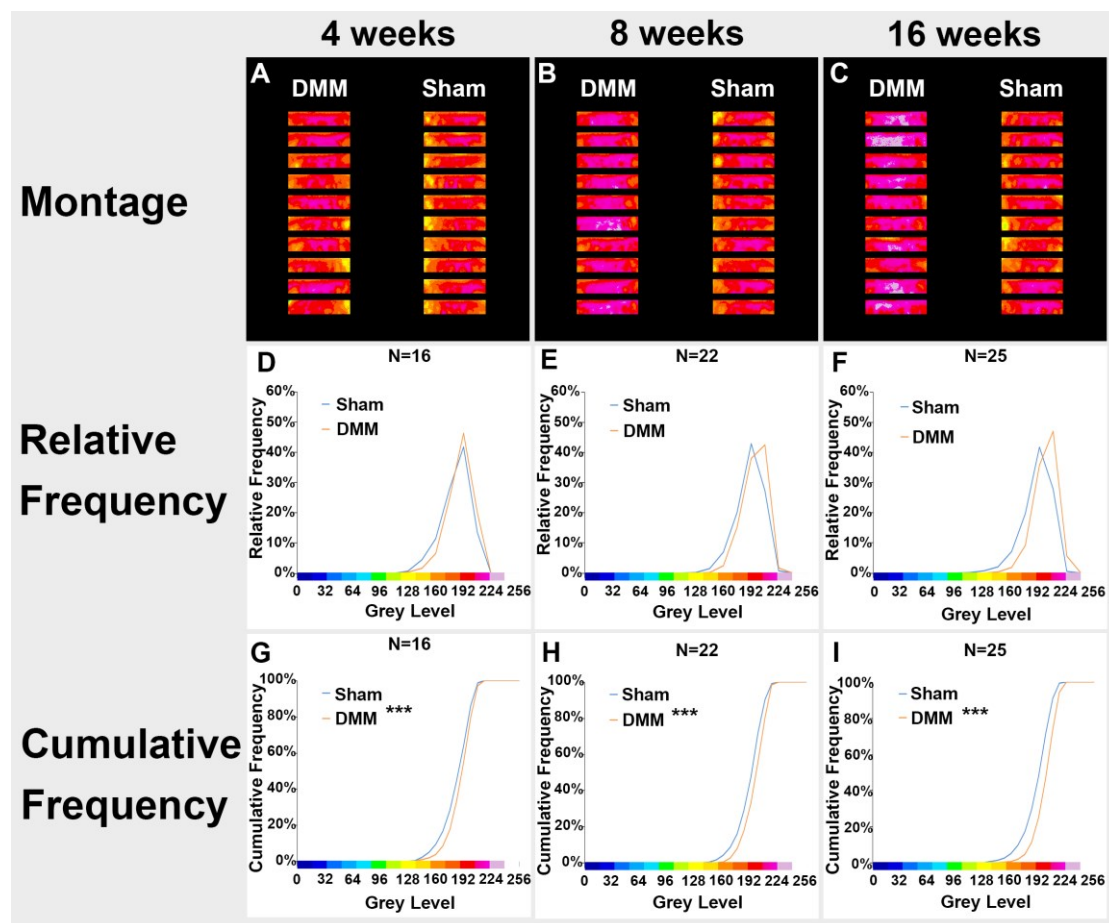


Figure 3.3 Knee destabilisation surgery increases subchondral BMC

Montages (A-C) of medial tibial plateaux subchondral bone from destabilised and contralateral sham operated knee 4, 8 and 16 weeks following surgery. Relative (D-F) and cumulative (G-I) frequency distributions. The separation between sham (blue) and DMM (orange) lines increases with longer interval after surgery. *** $P < 0.001$ Kolmogorov Smirnov.

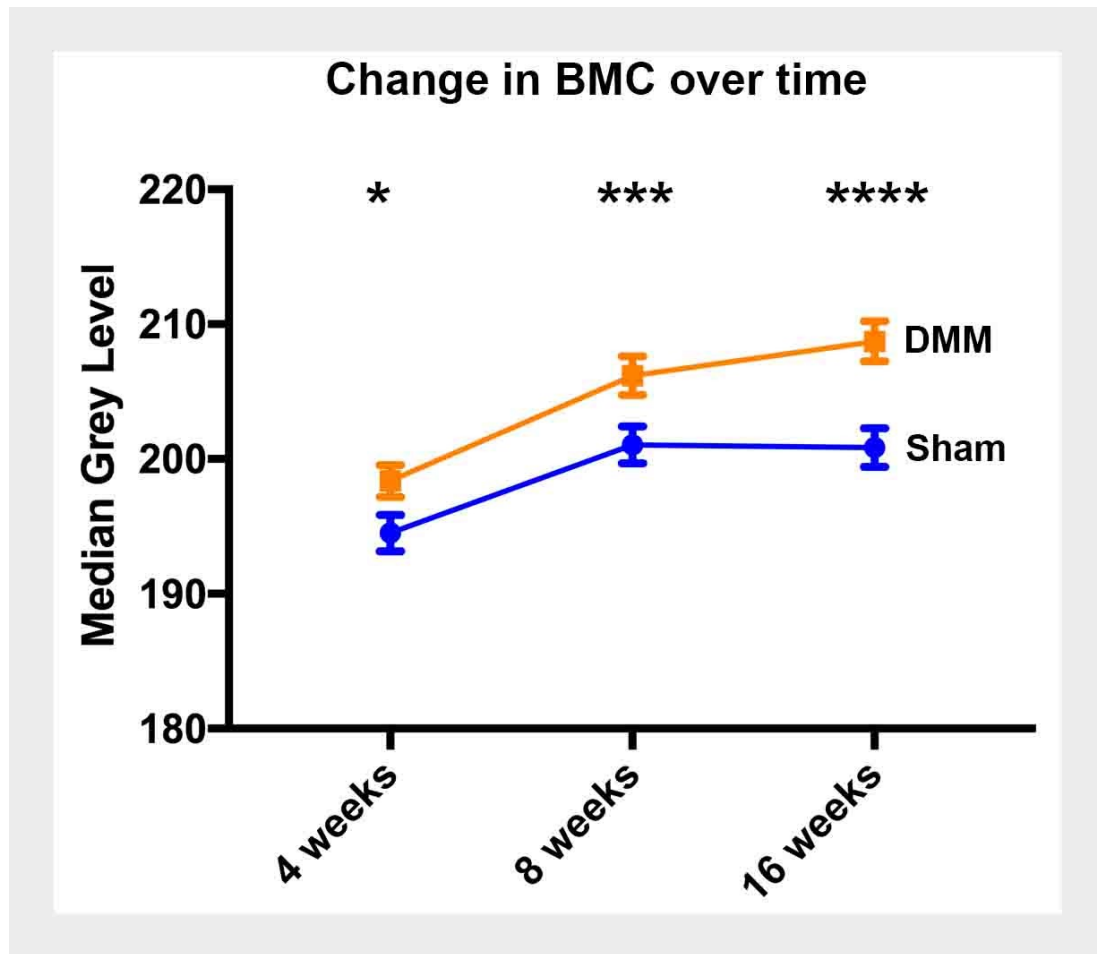


Figure 3.4 Subchondral bone mineral content is increased in destabilised knee joints.

The effect is greatest with the longest duration of destabilisation. Mean + SEM. Paired t-test. * $P < 0.05$, *** $P < 0.001$, **** $P < 0.0001$.

3.4 VALIDATION OF DMM USING MICRO CT

Subchondral bone changes were further characterised with MicroCT in a subset of animals. Again, genetically identical control mice from dronedarone and $TR\alpha^{0/0}$ experiments were combined to increase statistical power. Destabilisation surgery resulted in significantly higher bone mass, apparent density and material density compared to the contralateral sham operated knee (Figure 3.5). This was evident in mice sacrificed 8 and 16 weeks post surgery.

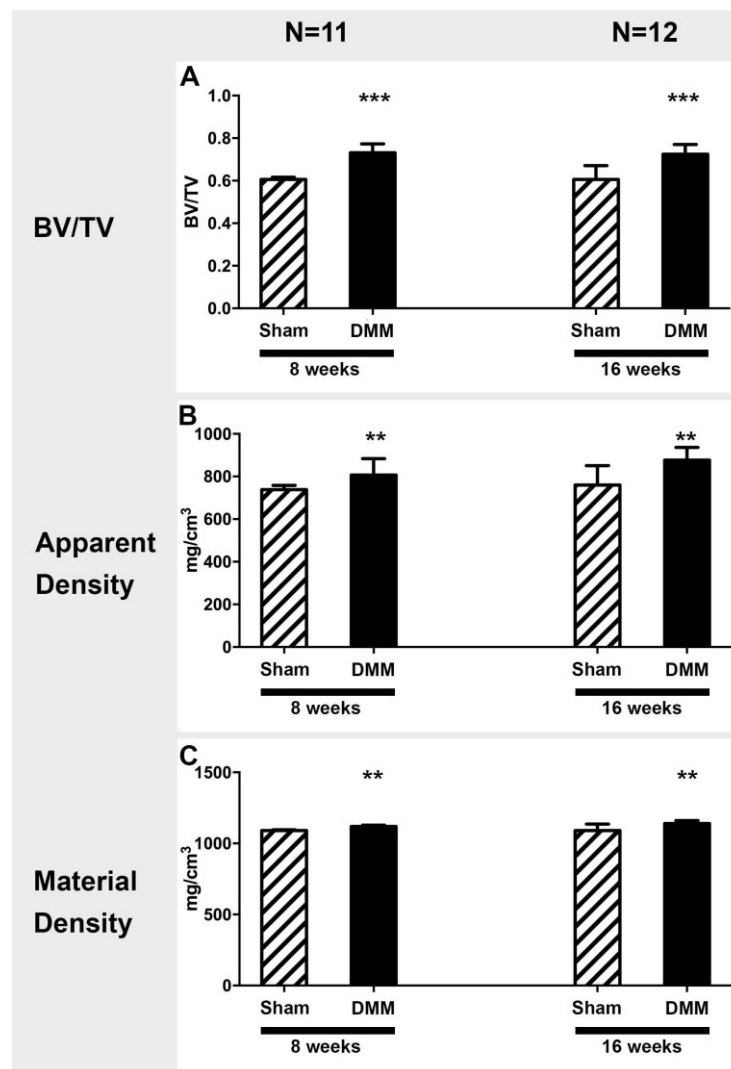


Figure 3.5 Subchondral bone assessment with MicroCT

Knee joints of mice sacrificed 8 weeks (left) and 16 weeks (right) after sham (patterned) and DMM (filled) surgery. ** P < 0.01, *** P < 0.001

3.5 EFFECT OF CAGE DENSITY ON CARTILAGE DAMAGE

The effects of cage density on osteoarthritis was investigated in control animals by comparing cartilage damage scores with the total number of animals that were housed in that cage. There were insufficient matched animals to permit two-way ANOVA analysis. No relationship between cage density and cartilage damage scores was evident (Figure 3.6).

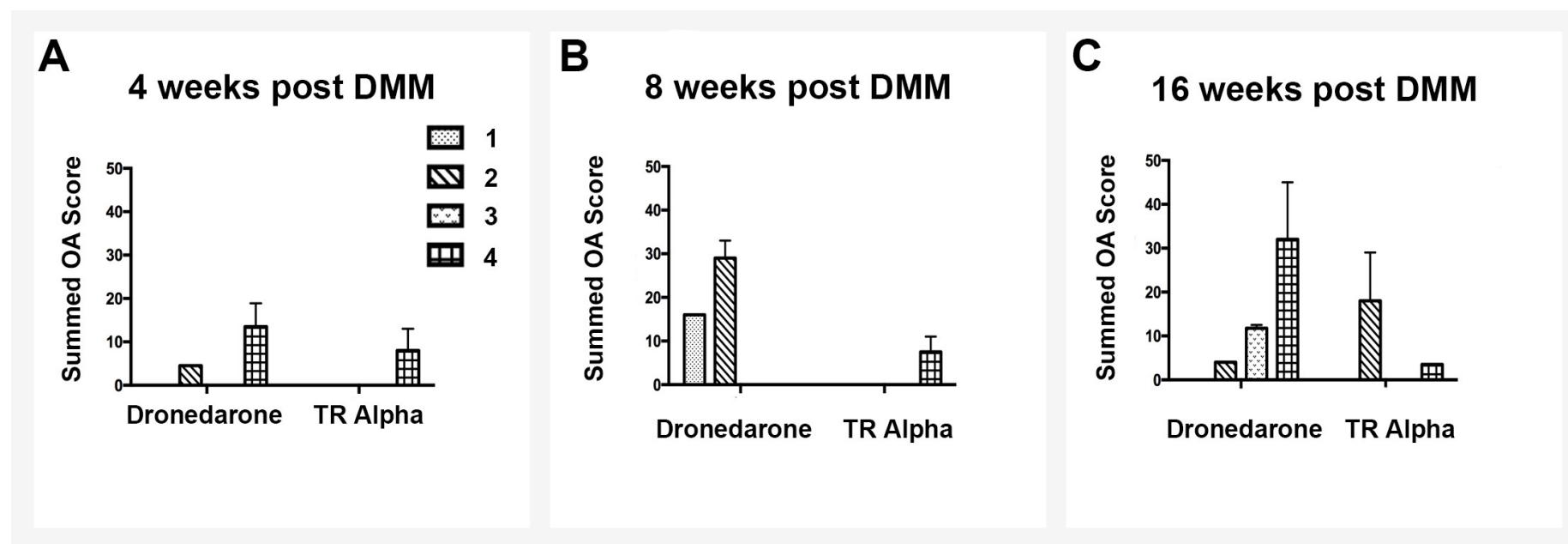


Figure 3.6 Relationship between cage density and cartilage damage

Summed OA scores (A) 4 weeks, (B) 8 weeks and (C) 16 weeks after knee destabilising surgery. Each panel shows control mice from dronedarone experiment (left) and TR $\alpha^{0/0}$ experiment (right) and the cage density (1-4 mice / cage).

3.6 DISCUSSION

Adequate knee destabilising surgery was confirmed histologically with medial displacement of the medial meniscus. This caused focal cartilage damage in the medial compartment as has been described in the literature (Glasson, Blanchet et al. 2007). Cartilage damage was increased 4, 8 and 16-weeks following DMM surgery compared to the contralateral sham operated limb. Therefore, the surgical technique of the operator and this model of osteoarthritis is validated for each time point. However, despite adequate surgery, some mice did not develop arthritis in the operated knee. Furthermore, when group sizes were small, the observed difference was not consistently significant. Control mice from dronedarone experiments and $TR\alpha^{0/0}$ experiments were combined to address this problem. These mice were genetically identical wild type C57Bl/6 mice. The dronedarone experiments were conducted earlier than the $TR\alpha^{0/0}$ experiments and it has been reported that improvement in surgical technique can lead to decreasing OA scores over time in the same operator (Glasson, Blanchet et al. 2007). Indeed, control mice from the latter, $TR\alpha^{0/0}$ experiments did have lower cartilage damage scores than control mice from the dronedarone experiments. Nevertheless, these combined groups were larger and thus had the power to validate this experimental model. Therefore, cartilage damage following DMM-surgery can be variable and large numbers of mice are required to have the power to observe a statistically significant difference. There was no relationship between cage density and cartilage damage but sample sizes were too small to perform statistical analyses.

Quantitative changes in subchondral bone mineral content were assessed with X-ray microradiography. BMC increased in sham operated knees joints between 14 and 18-week-old mice with no cartilage damage, but there was no further increase in older mice. Therefore, BMC increases as part of ageing but there is a plateau in BMC. In contrast, destabilised knees with osteoarthritis confirmed histologically demonstrated a progressive increase in BMC compared to the contralateral control knee. Furthermore, microcomputed tomography showed increased subchondral bone mass, apparent density and material density in destabilised joints. These studies demonstrate that X-ray microradiography and microCT are sensitive techniques to assess the changes in subchondral bone associated with osteoarthritis and further confirms the validity of this surgical model to analyse the effect of $TR\alpha$ inhibition or deletion.

CHAPTER 4 VALIDATION OF VOLUNTARY ORAL DOSING OF DRONEDARONE

4.1 BACKGROUND

Dronedarone has been administered to rodents (Pantos, Mourouzis et al. 2002, Pantos, Mourouzis et al. 2005, Stoykov, van Beeren et al. 2007) by once daily oral gavage in published studies but these have all been of short duration. Oral gavage is labour intensive, stressful to animals and may lead to respiratory interference, stomach distension and development of granulation tissue in the oropharynx following repeated dosing. Therefore, a method for voluntary oral administration of dronedarone was developed.

4.2 EFFECTIVE, VOLUNTARY, ORAL DOSING

A method of voluntary oral administration of drugs dissolved in a flavoured jelly has been described (Zhang 2011). Dronedarone is insoluble in water but was dissolved in 0.6% methylcellulose, added to a flavoured gelatine solution and made into 250µl portions. Multiple trials showed that the mice consistently ate vehicle jelly but did not eat the dronedarone-containing jelly. This occurred irrespective of the type and intensity of flavouring, fasting status or prior training with vehicle jelly.

A variety of palatable substances were then tested to determine which could be used to induce mice to consume the entire fixed dose of dronedarone reliably. Kaya, a coconut flavoured jam popular in Southeast Asia, proved to be an effective vehicle for dronedarone. Individual dosing was achieved by weighing 0.25g of jam and mixing dronedarone 20 or 60mg/kg in a disposable polystyrene weighing dish. Controls were given jam only once daily and the drug treated mice were given dronedarone-jam mix at the same frequency. Treatment started one week prior to knee surgery and continued until sacrifice. Mice were trained by having a portion of jam with their caged littermates for two days, followed by a single day of individual feeding with jam prior to the start of dronedarone administration. Overnight fasting was not required prior to the first presentation of jam.

Published studies of voluntary oral administration of drugs to mice have involved individually caged animals. However, individual caging is expensive and inefficient. To address this, mice caged in groups in XJ NexGen cages with wire lids (Allentown) were temporarily separated by a metal mesh wire placed transversely in the cage. An individual mouse was given the drug-jam dose in one compartment while its littermates were kept in the other compartment. Once the individual mouse had consumed its drug dose, the process was repeated with another littermate. Mice caged as pairs were given the drug contemporaneously after the cage had been divided. (Figure 4.1). A score of 0 (completely uneaten) to 10 (consumed entirely) was recorded after each administered dose. Mice were only caged with littermates receiving the same treatment.



Figure 4.1 Metal mesh divider used for contemporaneous, voluntary oral dosing of two mice

4.3 RESULTS

4.3.1 Effectiveness of dosing regime

The time taken to feed a group of mice was on average less than 2 minutes per mouse. Each treatment group received over 2700 individual vehicle or drug doses. All groups consistently consumed vehicle or drug mixture (Figure 4.2) but mice given the high dose dronedarone consumed significantly less (Vehicle 9.91 (0.78), dronedarone 20mg/kg/day 9.92 (0.81), dronedarone 60mg/kg/day 9.72 (1.02); mean (standard deviation) consumption score, ANOVA, $P < 0.05$, $n=2718-2817$). However, the high dose dronedarone group still consumed over 97% of the administered dose. Therefore, the difference is not biologically significant and the results validate this novel drug delivery method.

4.3.2 Effect of dosing regimen on body weight

All mice in the dronedarone experiment were weighed weekly. There was progressive increase in body weight and no difference between treatment groups (Figure 4.2). A random selection of WT control mice from the $TR\alpha^{0/0}$ experiment were weighed regularly to study the effects of daily jam on body weight. These mice also gained weight progressively with ageing. However, control mice from the dronedarone experiment were significantly heavier at multiple time point including before the start of jam administration.

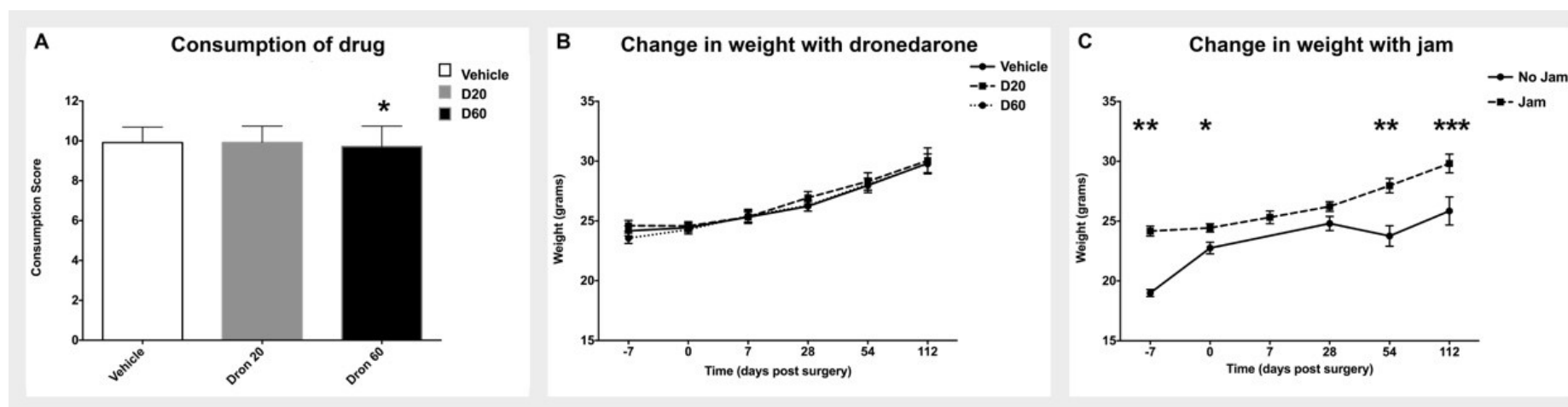


Figure 4.2 Voluntary oral dosing

(A) mice consistently consumed dronedarone administered in Jam. Mice treated with high dose dronedarone consumed significantly less than those given low dose dronedarone or vehicle only. * $P < 0.05$ (B) The dose of dronedarone had no effect on body weight. (C) Mice fed jam had significantly higher body weight at multiple time points compared to control mice that did not receive jam. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

4.4 DISCUSSION

A novel method for delivering dronedarone was developed that avoided the need for daily oral gavage. Experimental animals voluntarily consumed dronedarone administered in a small amount of jam. Although mice administered high dose dronedarone consumed significantly less of the drug than the other groups, the difference was not biologically significant as more than 97% of the administered drug was delivered. Therefore, dronedarone was delivered effectively through a non-invasive route.

Mice fed jam were significantly heavier than those that never received jam. However, this difference was observed prior to the onset of treatment and the difference in weight did not become more pronounced over time. Therefore, it is unclear whether the difference in weight is attributable to jam. Other factors that may have contributed to a difference in weight may include variable severity of osteoarthritis that may have affected physical activity.

Dividing cages temporarily with a mesh wire allowed individual, monitored drug dosing for mice caged in groups. Mice caged in pairs could be dosed contemporaneously, therefore improving the efficiency of drug dosing. The ability to give fixed drug doses to mice without the need for them to be caged individually is a significant development. Housing mice in isolation may not be desirable for some experiments. Furthermore, the method reported in this chapter could lead to a substantial cost saving compared to individual cages, especially when applied to studies of long duration. In summary, dronedarone was delivered effectively using a novel, non-invasive, efficient method that allowed animals to be caged in groups.

CHAPTER 5 EFFECTS OF TR α INHIBITION AND DELETION ON OSTEOARTHRITIS

5.1 BACKGROUND

Thyroid hormone receptor alpha is the dominant nuclear receptor expressed in articular cartilage and bone. It is the final mediator of thyroid hormone action and therefore a logical pharmacological target. To study the effects of thyroid hormone signalling via TR α on osteoarthritis, control mice were compared to mice treated with a selective inhibitor of TR α , dronedarone, or genetically modified TR $\alpha^{0/0}$ mice that lacked all TR α . All control mice were genetically identical C57Bl6 wild types, but each set of experiments had its own controls. DMM surgery was performed at 10 weeks of age, when mice have reached skeletal maturity. The consequences of DMM surgery were determined using the same analytical techniques for both sets of experiments and therefore are presented in the same chapter. For each analytical technique, data from dronedarone experiments will be presented first, followed by data from TR $\alpha^{0/0}$ mice.

5.2 RESULTS

5.2.1 X-ray microradiography

5.2.1.1 *Dronedarone*

5.2.1.1.1 *BMC increases with age*

The subchondral BMC in sham operated knees increased between control mice aged 14 weeks and 18 weeks, sacrificed 4 and 8 weeks after surgery respectively. There was no further increase seen in 26-week-old mice sacrificed 16 weeks following surgery (Figure 5.1J). Results from vehicle treated controls described here were combined with WT controls (from genetic deletion of TR α experiments) for the analysis in chapter 3 (Figure 3.4) and the results are consistent.

5.2.1.1.2 *BMC increases following destabilisation surgery*

Destabilisation of medial meniscus (DMM) surgery increased medial tibial plateau (MTP) subchondral bone mineral content (BMC) compared to sham surgery. This effect was seen at 4, 8, and 16 weeks following surgery. BMC increased progressively with the duration of knee destabilisation following surgery (Figure 5.1J). As DMM surgery has already been demonstrated to lead to articular cartilage damage and subchondral sclerosis in chapter 3, increased subchondral BMC can be considered a marker of subchondral OA pathology.

5.2.1.1.3 *Dronedarone treatment lowers subchondral BMC*

Dronedarone treated mice had lower subchondral BMC compared to vehicle treated mice. This was seen in both DMM-operated and sham-operated knee joints. No clear dose response was observed. Although a difference between dronedarone treatment and vehicle treatment was observed at all time points (Figure 5.2), this was most significant in mice treated with high dose dronedarone and sacrificed 4 weeks post-surgery. A large variability in BMC was noted within each treatment group.

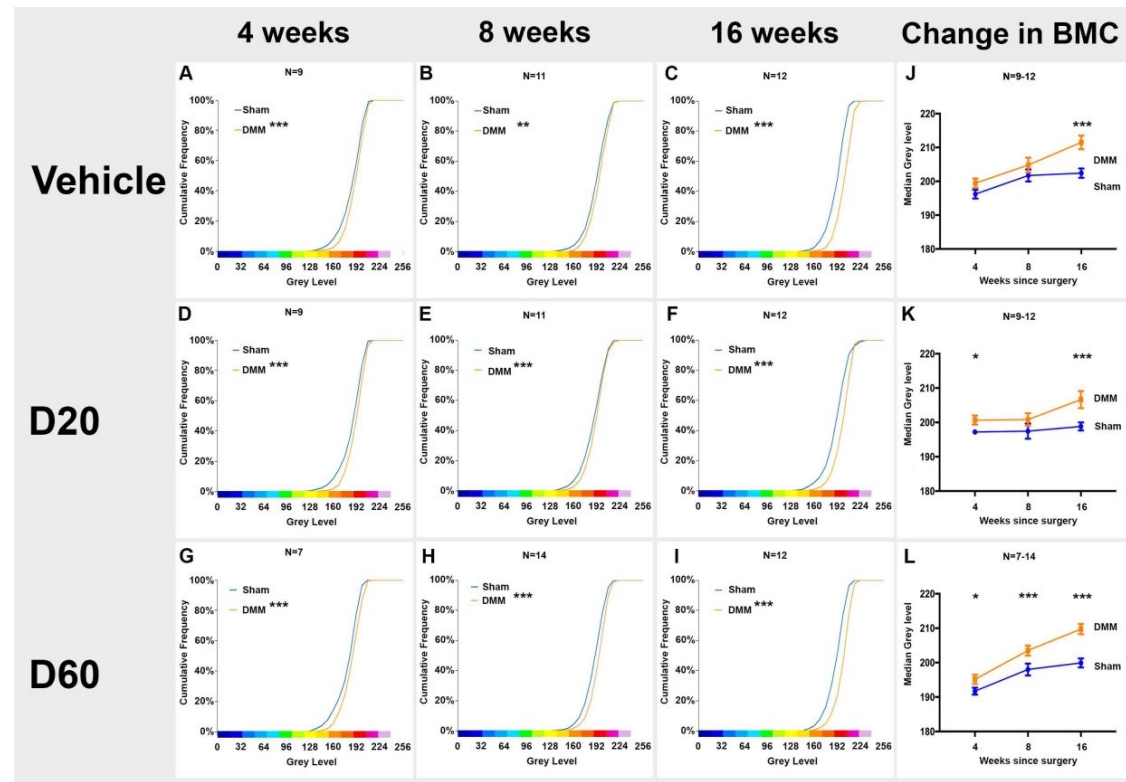


Figure 5.1 Comparison of BMC in medial tibial plateau subchondral bone between destabilised and sham operated knees

Cumulative frequency distributions of BMC from sham operated (blue) and destabilised (orange) knee joints from vehicle (A, B, C), low dose dronedarone (D, E, F) and high dose dronedarone (G, H, I) treated mice. Changes to BMC over time are shown in J, K, L. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ Kolmogorov Smirnov. The distance between blue and orange lines increases with longer interval after surgery (compare A, B and C).

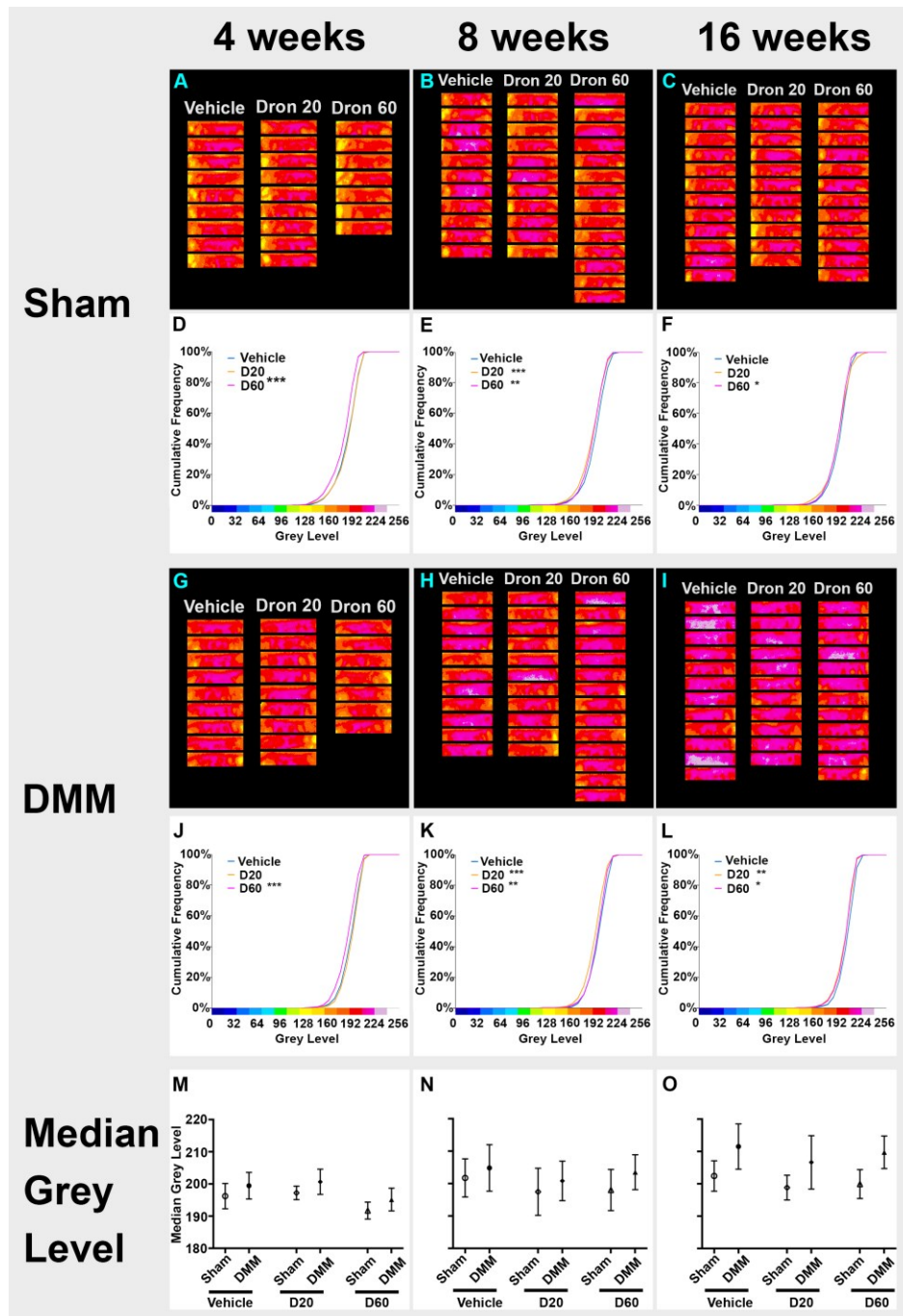


Figure 5.2 Dronedarone treatment reduces subchondral bone mineral content

Montages of MTP from sham operated (A-C) and destabilised (G-I) knee joints. Cumulative frequency distributions of sham operated (D-F) and destabilised knee joints comparing mice treated with vehicle (blue), low dose 20mg/kg/day dronedarone (orange) and high dose 60mg/kg/day dronedarone (pink). Median grey levels (mean and SD) from knee joints 4 (M), 8 (N) and 16 weeks (O) after surgery. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ Kolmogorov Smirnov. Note dronedarone treated mice had lower BMC (line shifted to left).

5.2.1.2 $TR\alpha^{0/0}$

5.2.1.2.1 $TR\alpha^{0/0}$ mice have higher BMC that increases progressively

In wild type mice, subchondral BMC was higher in mice sacrificed 8 or 16 weeks post sham surgery compared to younger mice. There was no further increase in subchondral BMC in mice sacrificed 16 weeks post-surgery (Figure 5.3). This was consistent with control mice from dronedarone experiments.

Knees from sham operated $TR\alpha^{0/0}$ mice had significantly higher subchondral BMC compared to wild types at all times points (Figure 5.3 & Figure 5.4). In contrast to WT mice, subchondral BMC continued to increase with time and was greatest in 26-week-old mutant mice. Thus, subchondral BMC increased with ageing in both genotypes but reached a plateau in WT mice only. Subchondral BMC continued to increase in ageing mutants (Figure 5.3).

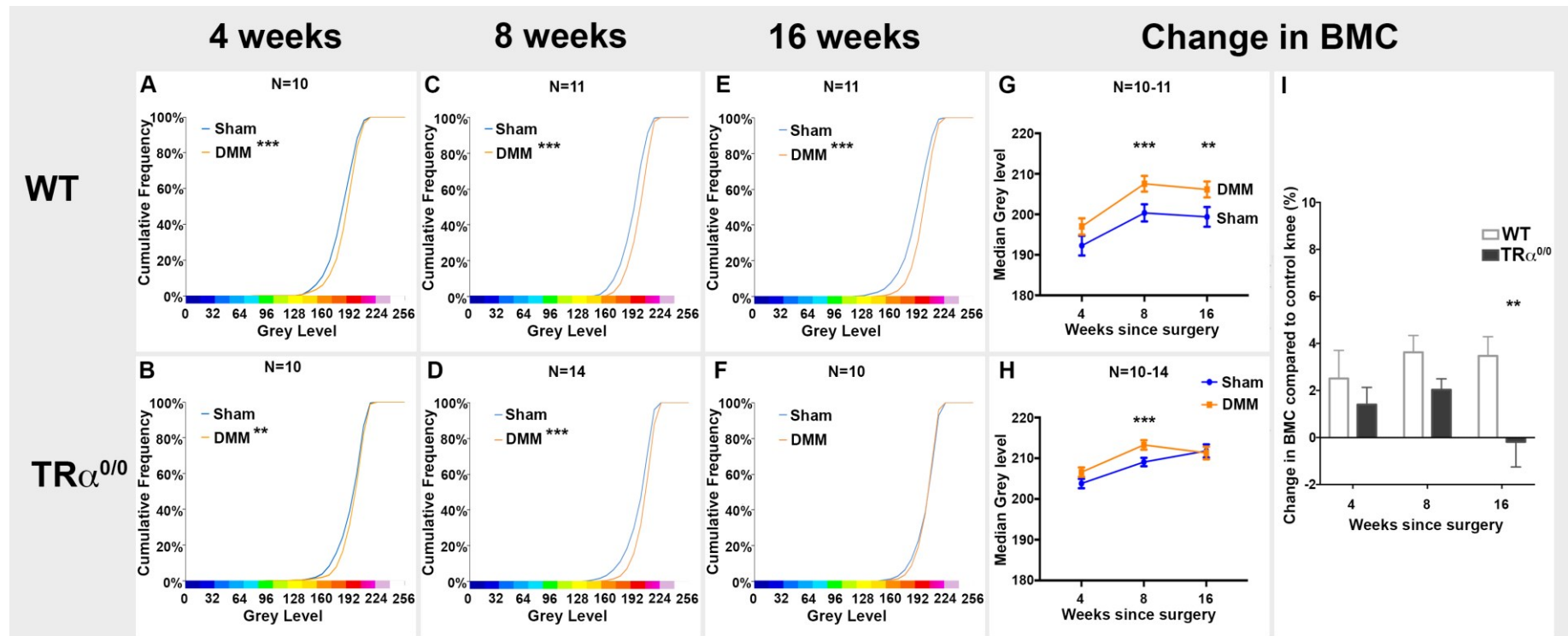


Figure 5.3 Change in bone mineral content with age and surgery in WT and TR $\alpha^{0/0}$ mice

Cumulative frequency distributions of medial tibial plateau subchondral bone 4 weeks (A) and (B), 8 weeks (C) and (D), 16 weeks (E) and (F) following knee destabilisation (orange) and sham (blue) surgery. Top and bottom rows represent WT and TR $\alpha^{0/0}$ mice, respectively. BMC increases with age in sham operated knees in mutant mice (blue line in H). There is no further increase in BMC between WT mice sacrificed 8 or 16 weeks post-surgery (blue line in G). DMM surgery increases BMC compared to control knees in both genotypes at 4 and 8 weeks. The magnitude of increase in BMC from destabilising surgery is attenuated in mutant mice (I). * $P < 0.1$, ** $P < 0.01$, *** $P < 0.001$; Kolmogorov Smirnov (A-F), paired student's t test (G-H), unpaired student t-test (I).

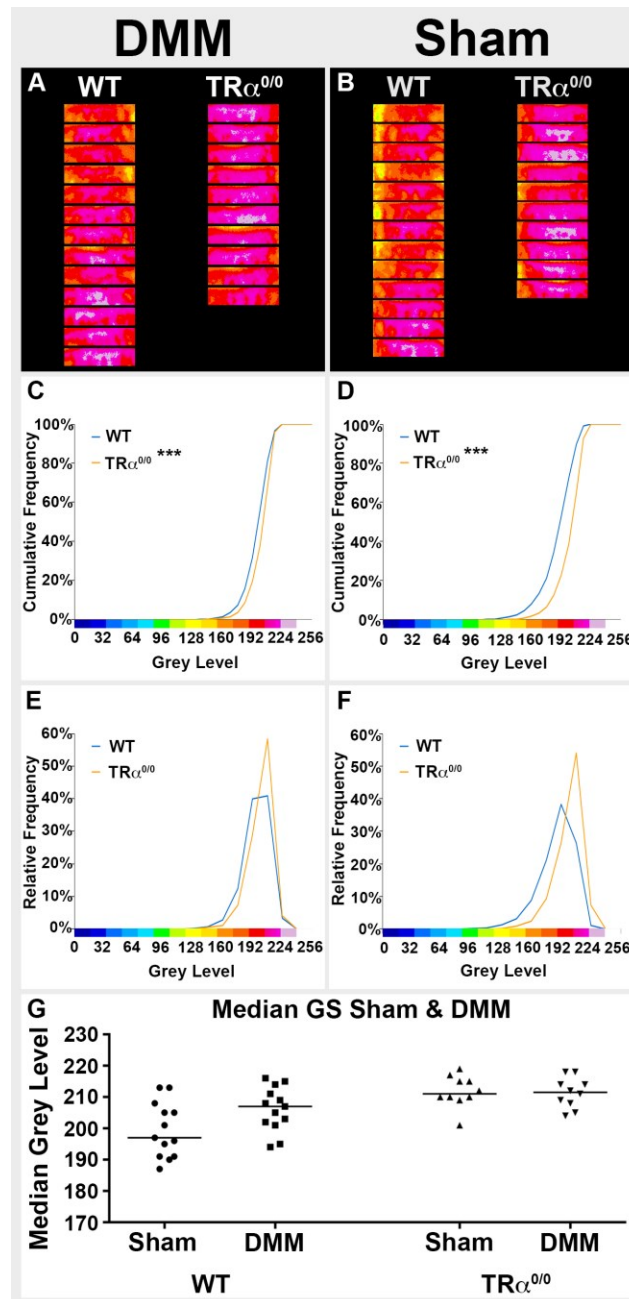


Figure 5.4 Subchondral BMC in medial tibial plateau 16 weeks post DMM

Knee joints 16 weeks after knee destabilising surgery (left column) and sham surgery (right column). (A) and (B) Montages of medial tibial plateau subchondral bone regions of interest. (C) and (D) Cumulative frequency distributions showing significantly increased BMC in both DMM and sham operated knees of TR $\alpha^{0/0}$ mice compared to WT controls. *** $P < 0.001$ Kolmogorov Smirnov. (E) and (F) Relative frequency distributions. (G) Median grey level are higher in both DMM and sham operated knees of TR $\alpha^{0/0}$ mice compared to WT controls. DMM surgery increases median grey level in WT but not TR $\alpha^{0/0}$ mice.

5.2.1.2.2 *The increase in subchondral BMC following destabilising surgery is attenuated in TR α ^{0/0} mice*

Similar to sham operated knees, BMC from the contralateral destabilised knees were higher in mutant mice than WT controls. However, a direct comparison between destabilised knees of TR α ^{0/0} and WT mice must be interpreted with an understanding of their different baseline values. Therefore, the relative increase in BMC between the destabilised knee and its contralateral control within the same animal may be an informative way of comparing the two genotypes. In wild type mice, BMC was significantly increased in the destabilised knee compared to the contralateral control knee (figure 5.3 & 5.4). This was observed at all time points investigated. In contrast, the increase in BMC observed following surgery was attenuated in mutant mice. This was seen at all time points but was most significant in mice sacrificed 16 weeks post surgery (figure 5.4). In these older mutant mice, there was no difference in BMC between sham and destabilised knees. Therefore, TR α ^{0/0} mutants have higher absolute BMC than WT controls. However, the increase in BMC associated with osteoarthritis inducing surgery is reduced.

5.2.2 MicroCT

5.2.2.1 Dronedarone

5.2.2.1.1 Subchondral bone volume is not affected by treatment with dronedarone for 9 weeks

Subchondral bone was analysed in mice 8 and 16 weeks after knee surgery. In mice 8-weeks post-surgery, there was no difference in subchondral bone volume or density between control and dronedarone-treated mice for either sham-operated or DMM-operated knees (Table 5.1). DMM surgery did not significantly increase bone volume in any treatment groups. Dronedarone treatment did not affect the relative increase in bone volume and density as a result of surgery.

5.2.2.1.2 Subchondral bone changes following destabilising surgery are attenuated by 17 weeks dronedarone treatment.

Subchondral bone volume and mineralisation increased 16 weeks following DMM surgery in all treatment groups (Figure 5.5, Figure 5.6 and Figure 5.7). There was no difference in absolute subchondral bone volume between control and dronedarone-treated mice. However, the relative increase in bone mass and density between destabilised joint and its contralateral control in the same animal was significantly reduced in mice treated with dronedarone.

	Vehicle (Median, SD)			D20 (Median, SD)			D60 (Median, SD)		
Sample size	4			4			5		
	Sham	DMM	Relative increase with DMM (%)	Sham	DMM	Relative increase with DMM (%)	Sham	DMM	Relative increase with DMM (%)
BV/TV (%)	57.6, 0.072	68.5, 0.089	7.1, 7.5	56.0, 0.066	61.9, 0.086	9.9, 5.9	54.6, 0.115	70.5, 0.087	11.1, 10.8
Tb.Th (mm)	0.073, 0.011	0.096, 0.020	31.8, 8.7	0.068, 0.009	0.080, 0.016	12.9, 12.2	0.072, 0.017	0.092, 0.022	20.0, 19.4
Tb.Sp (mm)	0.112, 0.008	0.102, 0.015	-5.6, 12.9	0.108, 0.009	0.103, 0.014	10.5, 11.6	0.121, 0.014	0.111, 0.007	-7.2, 9.1
CD	566.3, 493.5	298.4, 250.8	-12.0, 36.9	523.5, 97.4	465.7, 149.2	2.4, 17.4	500.2, 185.3	278.6, 181.0	-35.3, 23.7
SMI	-2.67, 1.30	-3.09, 1.29	-5.3, 55.2	-1.68, 0.70	-2.15, 1.30	20.4, 63.4	-2.25, 1.48	-4.51, 1.02	57.9, 39.3
Apparent Density	699, 74	825, 95	11.2, 6.9	695, 73	765, 98	8.0, 4.3	666, 118	841, 96	11.0, 9.3
Material Density	1074, 20	1122, 31	4.0, 1.6	1079, 23	1113, 29	3.0, 0.6	1071, 28	1128, 31	3.0, 1.6

Table 5.1 Subchondral bone in mice 8 weeks after knee surgery

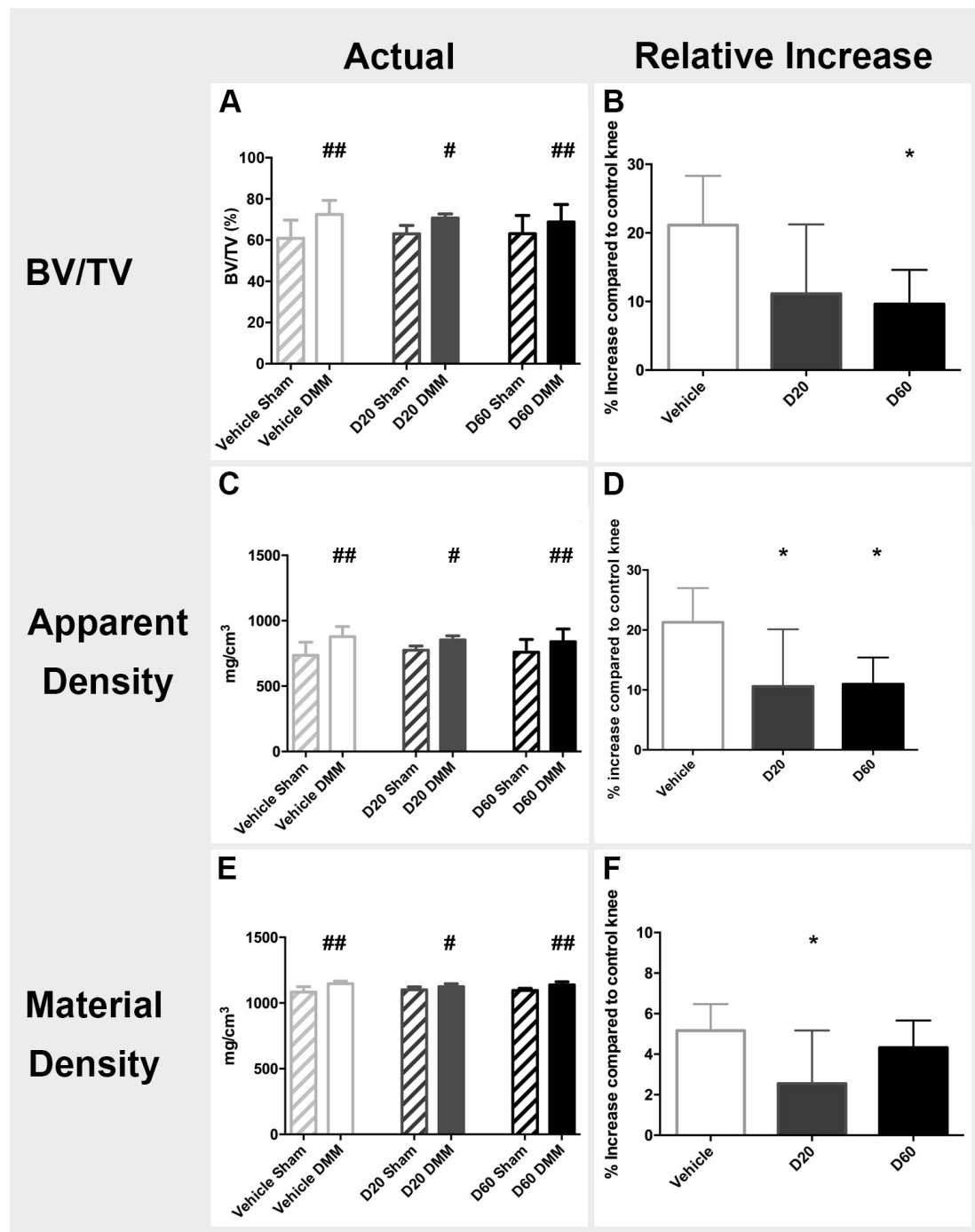


Figure 5.5 Subchondral bone with dronedarone 16 weeks after surgery

(A) Bone mass (C) apparent density and (E) material density are increased following DMM surgery in all treatment groups. There is no difference between treatment groups. # $P < 0.05$; ## $P < 0.01$ DMM vs sham in same mouse.

Dronedarone treatment attenuates the relative increase in (B) bone mass (D) apparent density and (F) material density in the destabilized joint compared its contralateral control joint * $P < 0.05$.

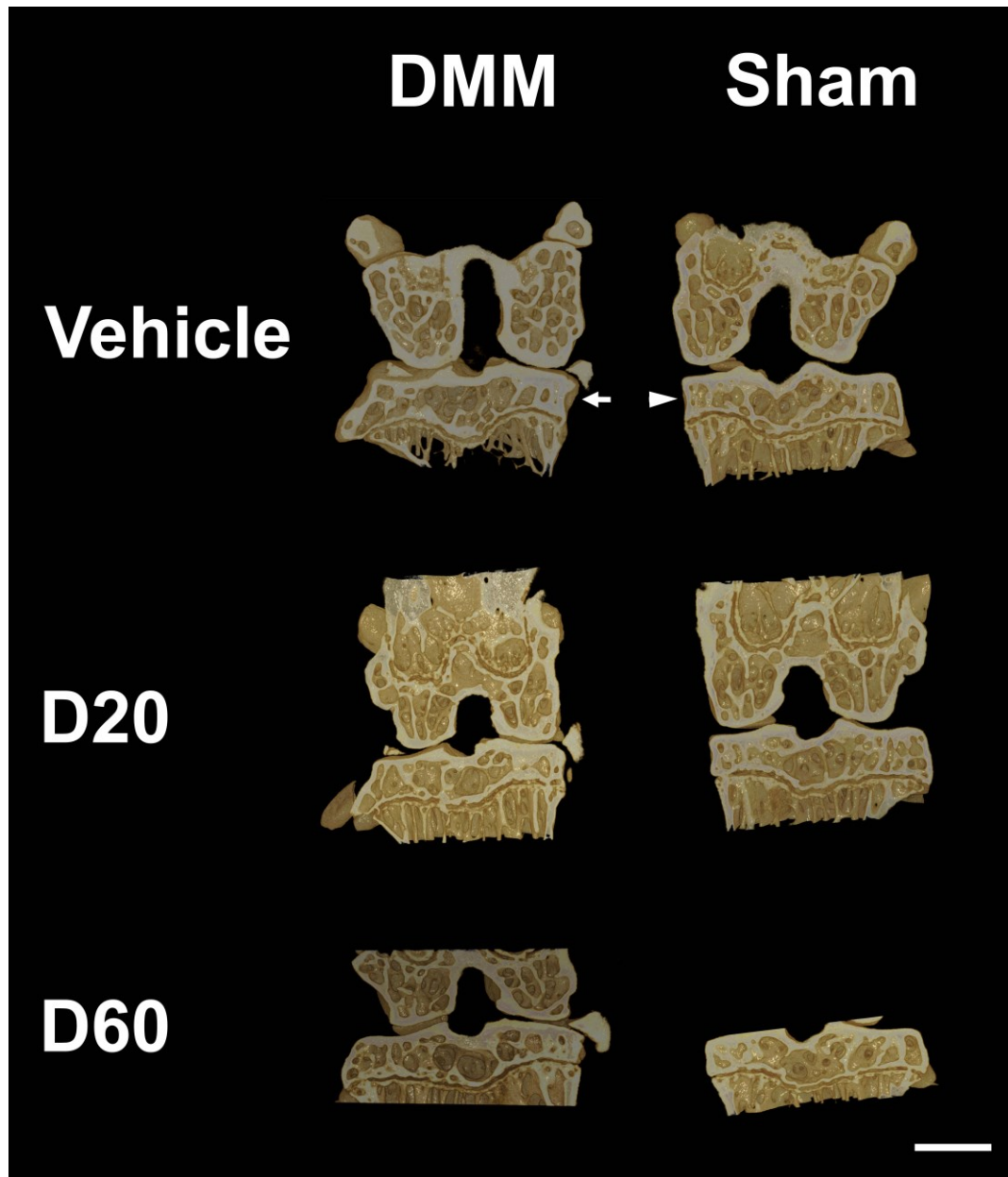


Figure 5.6 MicroCT images (coronal view) of subchondral bone with dronedarone 16 weeks after surgery

Note increased MTP subchondral bone in DMM joint (arrow) compared to sham operated joint (arrow head).

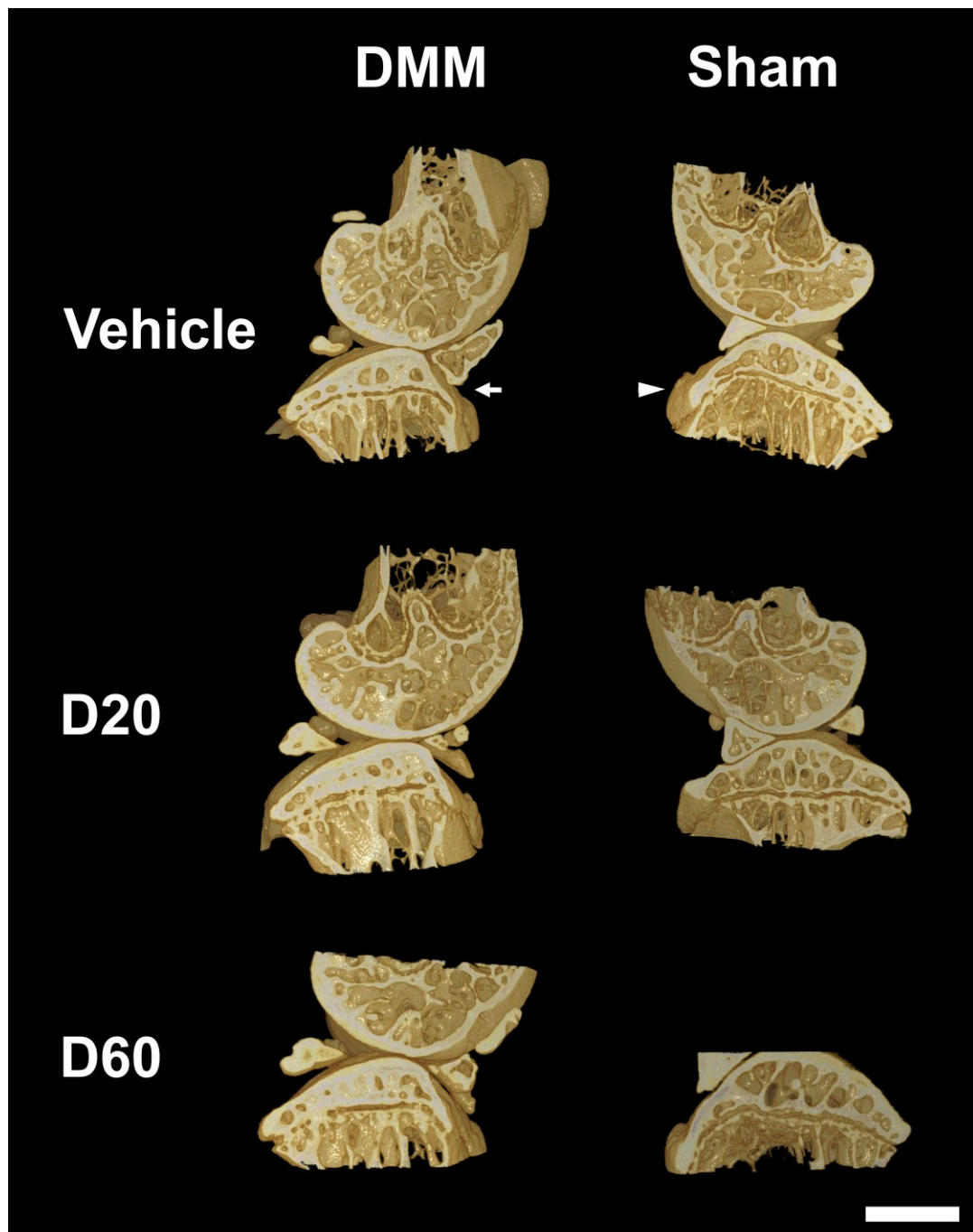


Figure 5.7 MicroCT images (sagittal view) of subchondral bone with dronedarone 16 weeks after surgery

Note increased tibial plateau subchondral bone in DMM joint (arrow) compared to sham operated joint (arrow head).

5.2.2.2 $TR\alpha^{0/0}$

5.2.2.2.1 $TR\alpha^{0/0}$ mice have higher subchondral bone mass and mineralisation

Subchondral bone was analysed in mice 4, 8 and 16 weeks after knee surgery (Figure 5.8). $TR\alpha^{0/0}$ mice had higher bone mass and mineralisation compared to WT mice in control knees 8 week after sham operation. There was no difference in the control knees of WT and $TR\alpha^{0/0}$ mice 4 and 16 weeks after surgery.

5.2.2.2.2 Subchondral bone changes following destabilisation surgery are attenuated in $TR\alpha^{0/0}$ mice

In wild type mice, DMM operated knees had higher subchondral bone mass, apparent density and material density compared with sham operated knees 8 weeks post surgery. There was no significant difference between sham and DMM operated knees 4 and 16 weeks after surgery, although sample sizes were smaller. This contrast with the observation in chapter 3 that DMM operated knees had higher subchondral bone mass and density compared to control joints 16 weeks after surgery (Figure 3.5) when analysed with larger pooled samples of WT mice.

In $TR\alpha^{0/0}$ mice, destabilised knees also had higher bone mass and mineralisation compared to sham operated knees 8 weeks after surgery. There was no difference between DMM operated and control knee at 4 and 16 weeks.

In WT mice, the DMM operated knee had 28% higher BV/TV compared to its control knee. In contrast, the bone mass of DMM operated knee in $TR\alpha^{0/0}$ mice was only 3% higher than its control knee. Therefore, the magnitude of increase in bone mass in destabilised joints compared to their contralateral control knees was attenuated in mutant mice compared to wildtype (Figure 5.8 and Figure 5.9).

In summary, mutant mice had higher subchondral bone mass and mineralisation compared to WT mice 8 weeks following surgery. Destabilising surgery increased bone mass and mineralisation in both genotypes but the magnitude of increase was much lower in mutant mice.

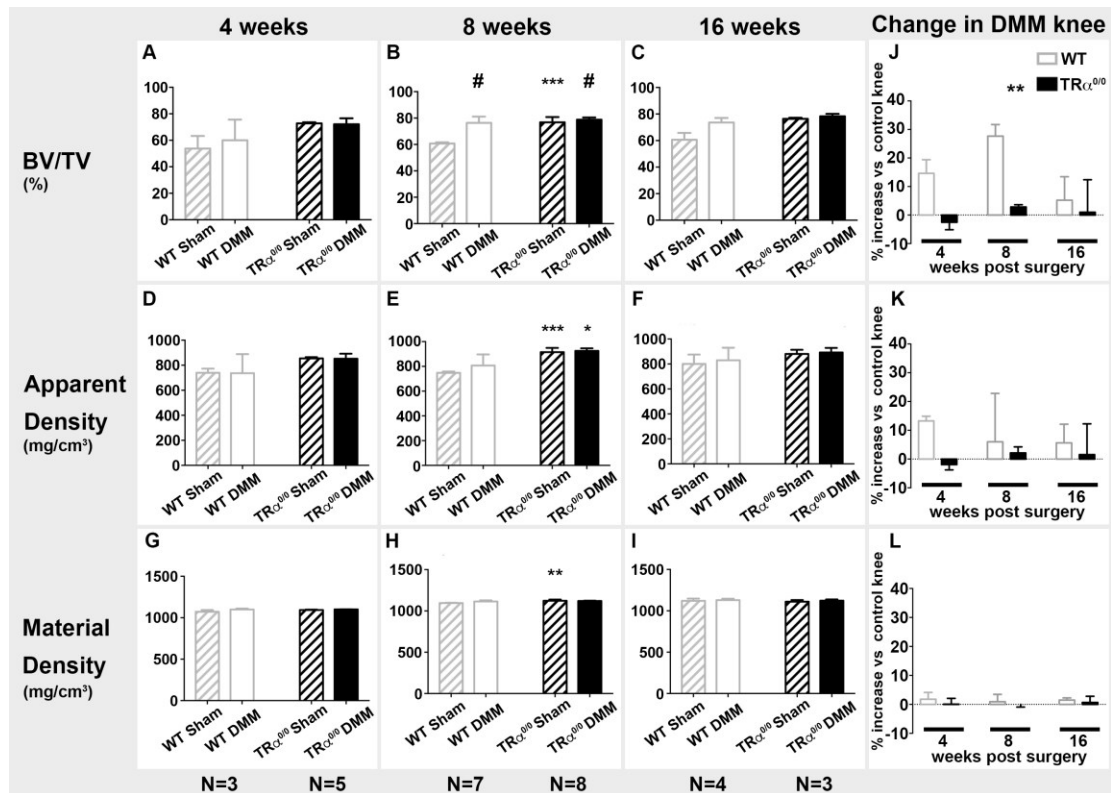


Figure 5.8 MicroCT of subchondral bone in TRα^{0/0} mice

(A-C) bone mass (D-F) apparent density and (G-I) material density are higher in TRα^{0/0} compared to wild type mice 8 weeks after sham surgery. Destabilisation surgery increases bone mass compared to contralateral sham controls in wild type and mutant mice 8 weeks following surgery. The magnitude of increase in bone mass (J) but not apparent density (K) or material density (L) in destabilised knees compared to their contralateral control is greater in wild type compared to mutant mice. ** $P < 0.01$, *** TRα^{0/0} vs wild type in the corresponding joint. $P < 0.001$ # $P < 0.05$ DMM vs sham knee in same mouse.

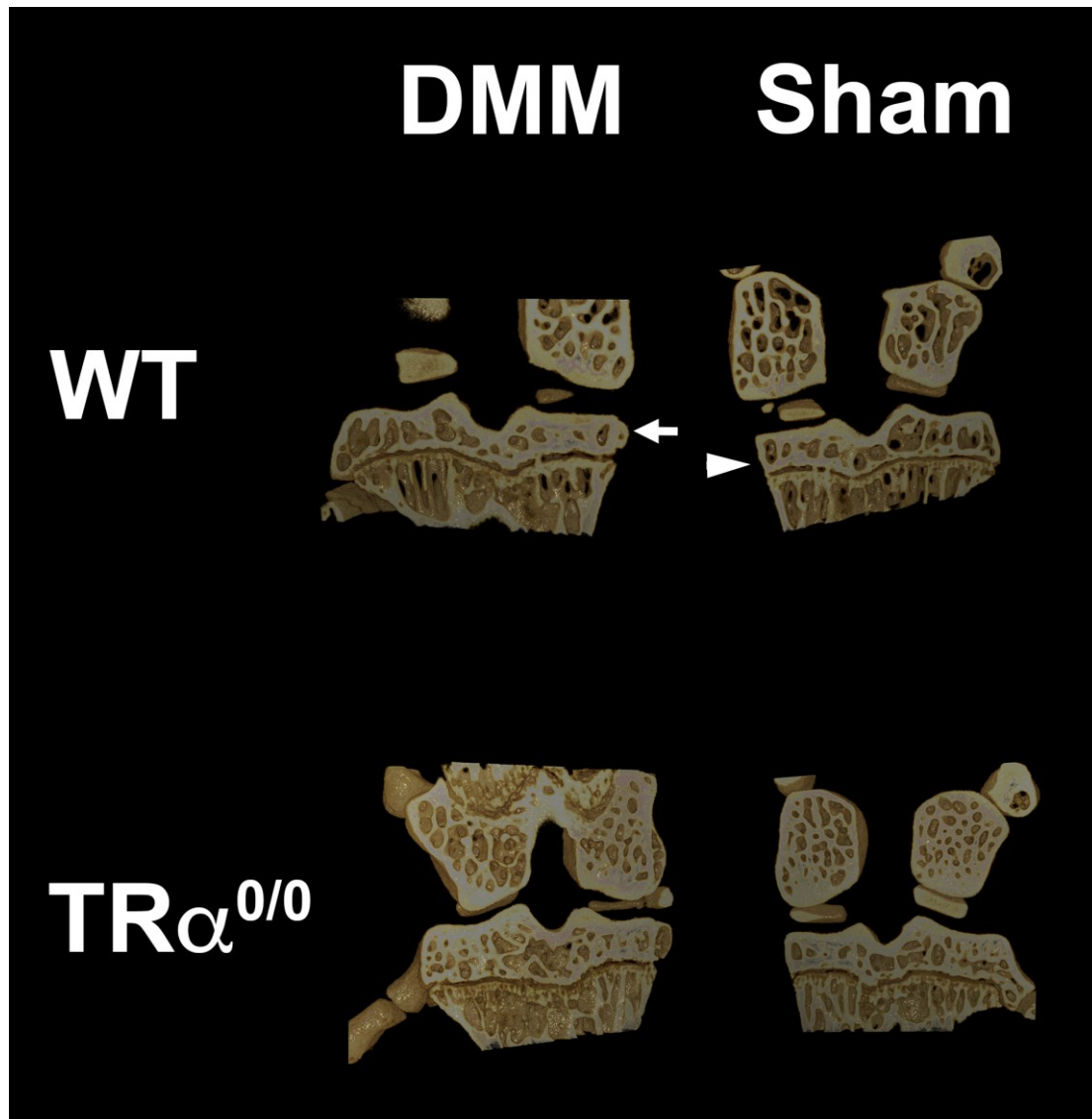


Figure 5.9 MicroCT images of subchondral bone in TR $\alpha^{0/0}$ mice 8 weeks after knee surgery

TR $\alpha^{0/0}$ mice have increased subchondral bone mass in both medial and lateral tibial plateaux compared to wild type mice. Destabilising surgery is associated with increased subchondral bone mass in the MTP in both wild type (arrow) and mutant mice compared to sham operated joint (arrow head). The magnitude of change is smaller in mutant mice

5.2.3 Backscattered scanning electron microscopy (BSE-SEM)

Scanning electron microscopy was used to give a further qualitative assessment of subchondral bone 8 weeks after surgery.

5.2.3.1 *Dronedarone*

There was no clear qualitative difference between vehicle treated and dronedarone treated tibias (Figure 5.10). Unfortunately, the medial tibial plateau of DMM operated joint treated with high dose dronedarone was damaged during sample preparation.

5.2.3.2 *TR α ^{0/0}*

TR α ^{0/0} mice had higher subchondral and metaphyseal trabecular and cortical bone mass (Figure 5.11). Destabilisation surgery increased MTP subchondral bone mass in WT mice. The difference between sham operated and destabilised knees in mutant mice was much smaller

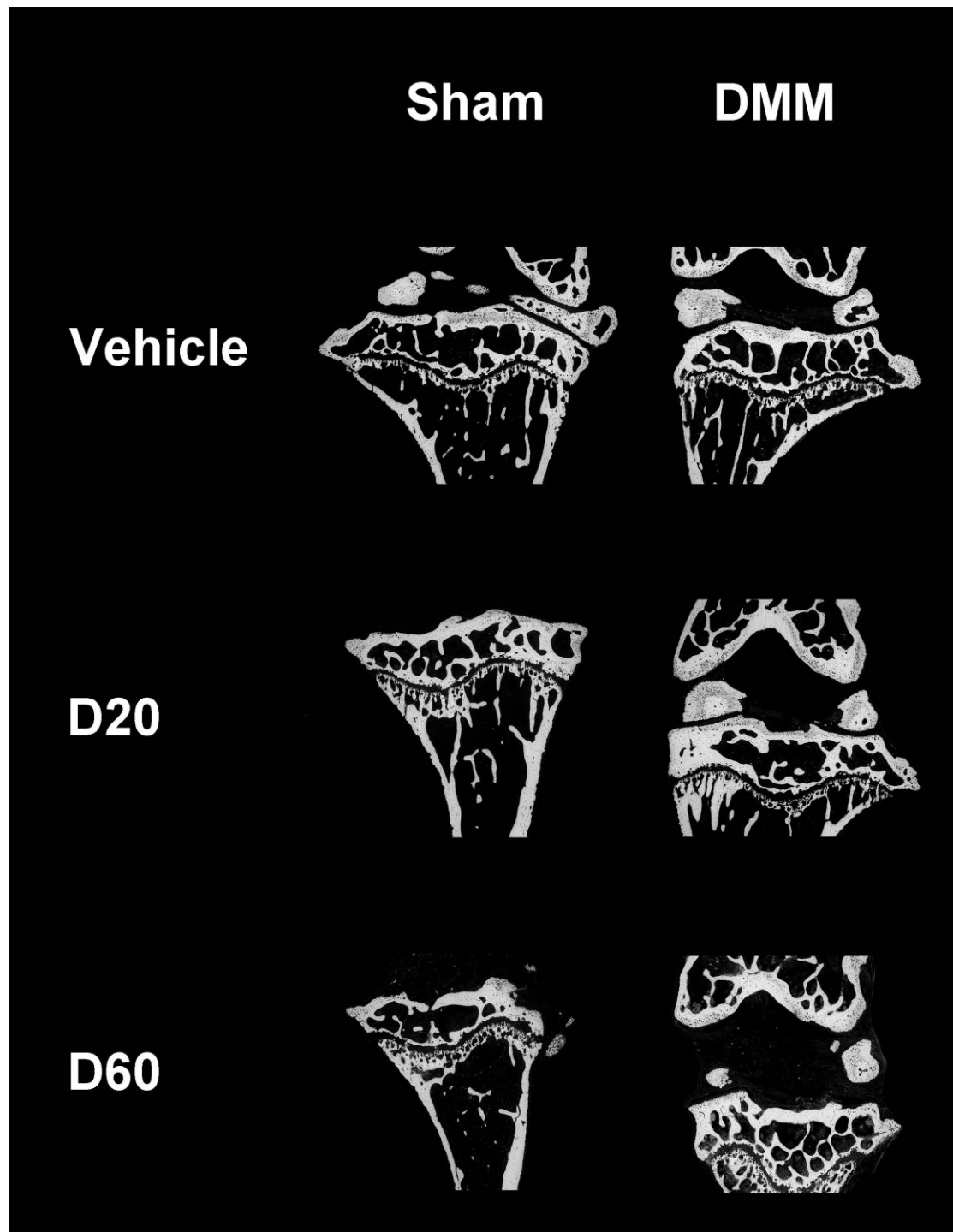


Figure 5.10 Scanning Electron Microscopy (SEM) images of tibias in dronedarone treated mice 8 weeks after surgery

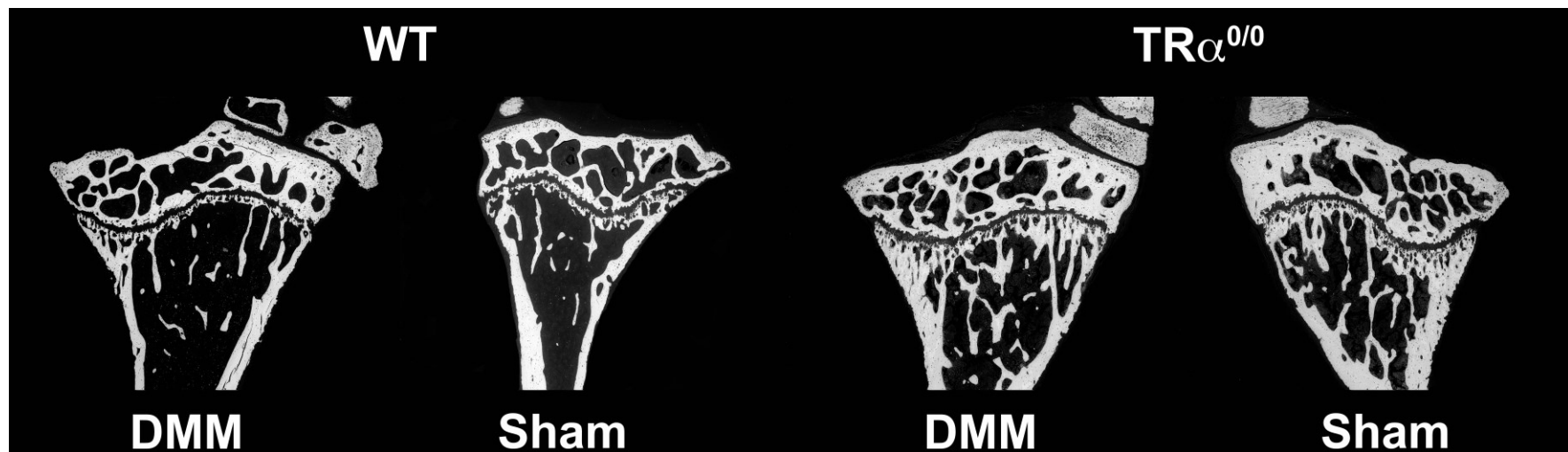


Figure 5.11 Scanning Electron Microscopy (SEM) images of tibias in TR $\alpha^{0/0}$ mice 8 weeks after surgery

5.2.4 Cartilage histology

5.2.4.1 Dronedarone

5.2.4.1.1 Inconsistent evidence of chondroprotection with dronedarone

The effect of dronedarone treatment on osteoarthritis was observed by comparing destabilised joints in mice treated with low dose (20mg/kg/day, D20), high dose (60mg/kg/day, D60) or vehicle (V). Histology was performed in mice 4, 8 and 16-weeks after DMM surgery. There was a large variability in scores and there was no significant difference in cartilage damage scores between destabilised and sham operated knees (Figure 5.12). This included control animals given vehicle only.

There was no difference between treatment groups in cartilage damage scores of destabilised joints 4 or 16 weeks after surgery. However, 8 weeks after surgery mice treated with low dose dronedarone had lower maximal and summed OA scores.

5.2.4.2 $TR\alpha^{0/0}$

5.2.4.2.1 *Articular cartilage is not affected by $TR\alpha$ deletion*

Articular cartilage damage was assessed with histology at 4, 8, and 16 weeks after DMM surgery (Figure 5.13). Although destabilised knees consistently had higher pathology scores than the contralateral sham operated knee, this difference was not statistically significant in either wild type or mutant mice. There was no difference between wild type and $TR\alpha^{0/0}$ mice at any time point. At 8 weeks post surgery, there were two mutants that were outliers with much higher cartilage damage scores.

Mutant mice demonstrated greater subchondral bone mass compared to wild type mice in both sham and destabilised knee joints on qualitative assessment

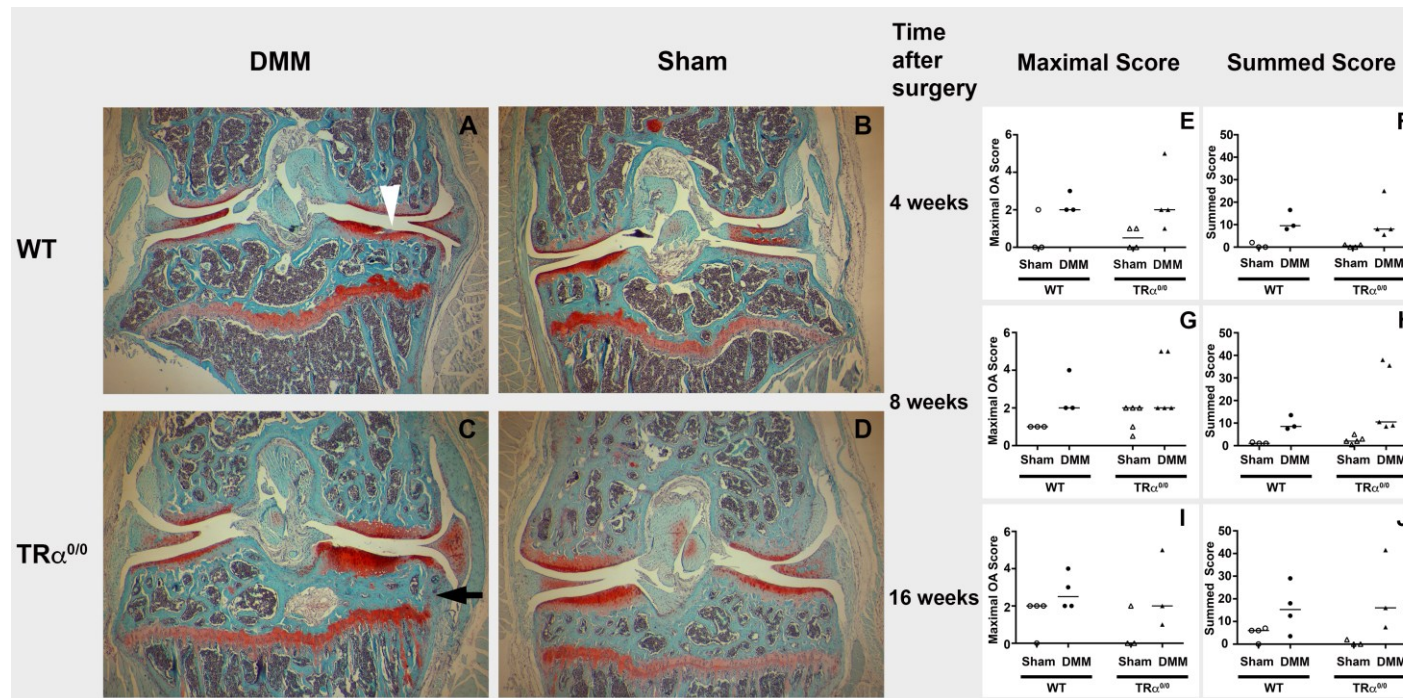


Figure 5.13 Cartilage damage in TR $\alpha^{0/0}$ mice

(A-D) Representative samples 8 weeks after knee surgery. Wild type (A-B) and TR $\alpha^{0/0}$ (C-D) mice. Destabilised knee joints are on the left (A, C) and sham operated joints on the right (B, D). Note greater MTP subchondral bone mass in mutant mice. This is most evident in the medial tibial plateau of destabilised knees (black arrow). Note area of articular cartilage damage (white arrow head).

Maximal (E, G, I) and summed (F, H, J) cartilage damage scores in mice 4 (top), 8 (middle) and 16 weeks (bottom) after surgery. None of results were significantly different.

5.3 DISCUSSION

The results in this chapter report for the first time the effects of inhibition or deletion of TR α on osteoarthritis susceptibility and examine the role of thyroid hormone on subchondral bone, a critical component of the joint.

5.3.1 TR $\alpha^{0/0}$ mice develop progressive subchondral osteosclerosis with ageing

Normal ageing is associated with increased mineralisation of subchondral bone (Grynpas, Alpert et al. 1991). In my experiments, sham-operated joints of wild type control mice provided an insight into the effects of ageing on subchondral bone. Bone mass and mineral content increased with age but reached a plateau at 18 weeks of age. Therefore, normal ageing in mice is associated with increased subchondral bone mass and BMC. By comparison, mutant TR $\alpha^{0/0}$ mice had much higher bone mass and BMC that continued to increase beyond 18 weeks. Thus, adult mutants have subchondral bone sclerosis that matches the phenotype of whole long bones (Bassett, O'Shea et al. 2007). My experiments did not investigate the mechanism underlying subchondral osteosclerosis in TR $\alpha^{0/0}$ mice, however, Bassett's study of long bones demonstrated reduced osteoclastic activity. The subchondral bone phenotype of juvenile TR $\alpha^{0/0}$ mice is not known so it is unclear if subchondral osteosclerosis is constitutive or acquired and if so at what age. These developmental differences are likely to influence the biomechanical properties of the joint and necessitate methodological considerations that are discussed later.

5.3.2 BMC is increased in TR $\alpha^{0/0}$ mice but is mineralisation?

BMC is a product of bone volume and mineralisation. Therefore, the increase in subchondral BMC observed in TR $\alpha^{0/0}$ mice may be due to increases in subchondral bone volume, mineralisation, or both. Previous studies showed that P154 TR $\alpha^{0/0}$ mice have normal mineralisation but increase bone volume in femurs (Bassett, O'Shea et al. 2007). In contrast, a recent study showed increased distal femur trabecular bone mass as well as increase micro-mineralisation of the proximal tibia and lumbar vertebrae in P112 TR $\alpha^{0/0}$ mice (Cheung 2014). Therefore, further studies with qBSE-SEM are required to determine the mineralisation in subchondral bone in TR $\alpha^{0/0}$ mice.

5.3.3 Methodological considerations in assessing subchondral bone

DMM surgery induces osteoarthritis characterised by cartilage degradation and subchondral bone sclerosis. A simple comparison between drug or vehicle treated animals in ipsilateral destabilised knee joints is an established method of determining the effect of such an intervention on subchondral bone remodelling related to OA. This method was therefore applied to study the effects of TR α inhibition with dronedarone in adulthood.

However, as discussed in the last section, subchondral bone mass and mineral content of TR $\alpha^{0/0}$ mutants are much higher than in wild type mice. Therefore, a direct comparison of ipsilateral destabilised joints between mutant and wild type animals will incorrectly imply greater subchondral bone changes in the mutant due to OA. The contralateral sham-operated joint can be used as an internal control for DMM surgery for each animal to allow the relative change (attributable to OA-induction) to be measured. This means two genotypes with different baseline characteristics can be compared. Therefore, the relative change in subchondral bone between sham and DMM-operated joints in each genotype was used to assess the effects of TR α deletion. However, contralateral (unoperated) limb SCB cortical thickness and OA development increases following mechanical loading, including from DMM surgery, thus limiting its effectiveness as an internal control (Loeser, Olex et al. 2012, Fang and Beier 2014, Poulet, de Souza et al. 2015, Das Neves Borges, Vincent et al. 2017).

5.3.4 TR α inhibition reduces subchondral BMC and TR α deletion attenuates bone changes following OA-provocation

Dronedarone treatment reduced subchondral BMC for both sham-operated and destabilised joints. However, the relative increase in BMC between destabilised and contralateral control joints was not affected. It is unclear if the lower BMC is due to reduction in bone volume, mineralisation or both. Using microCT, there was no difference in subchondral bone volume between treatment groups but the relative increase in bone mass in destabilised joints compared to their contralateral control was halved with dronedarone.

In contrast to WT mice, there was no difference in BMC between sham operated and destabilised knees in TR $\alpha^{0/0}$ mice sacrificed 16 weeks after surgery. Furthermore,

mutant mice had relatively little increase in BMC between destabilized joints and sham operated joints at earlier time points when compared to control mice. In addition, the increase in bone mass with destabilisation surgery was attenuated in mutant $TR\alpha^{0/0}$ compared to WT mice (3% vs 28%). Thus, $TR\alpha$ inhibition reduces BMC and the relative increase in bone mass with OA induction. $TR\alpha$ deletion attenuates the relative increase in both BMC and bone mass with OA induction.

Subchondral bone remodelling in osteoarthritis involves an initial resorptive phase, followed by a decrease in bone resorption without a reduction in bone formation, leading to sclerosis (Burr and Gallant 2012, Nakasa, Ishikawa et al. 2016). My experiments show that subchondral bone sclerosis and increase in BMC due to OA-provocation appear to be mitigated by both inhibition and deletion of $TR\alpha$. Therefore, $TR\alpha$ may regulate early bone resorption, subsequent bone formation or both processes during OA pathogenesis. Osteoclastic activity was not assessed and the protocol for dynamic histomorphometry demonstrated calcein double labels in intact long bone but not in subchondral bone (results not shown). Additional experiments aimed at assessing the early changes after DMM surgery are required to determine the role of $TR\alpha$ on initial subchondral bone resorption.

Although both $TR\alpha^{0/0}$ and dronedarone treated mice have reduced signalling through $TR\alpha$, the former have high while the latter have low subchondral bone volume and mineral content. Mechanistically the main differences relate to the degree of signalling through $TR\alpha$ and the duration of this disruption. Mutant mice have no $TR\alpha$ and therefore no signalling through this receptor throughout life. Dronedarone treated mice have unliganded $TR\alpha$ and therefore repression of positively regulated TR target genes. However, this disruption only occurs after skeletal maturity is reached and upon OA-induction. I, therefore, propose the following hypothesis to explain the subchondral bone phenotypes of $TR\alpha^{0/0}$ and dronedarone treated mice.

Firstly, osteosclerosis in $TR\alpha^{0/0}$ mice may be the result of lifelong reduced subchondral bone osteoclastic activity in that predates OA-inducing or sham surgery. In contrast, bone turnover is normal in dronedarone treated animals until the time of surgery as the drug is only administered when skeletal maturity is attained. Second, absent signalling

through TR α leads to skeletal hypothyroidism and a low bone turnover state through reduced osteoclastic activity. This in turn limits subchondral bone remodelling and subsequent sclerosis in destabilised joints. The concept that accelerated subchondral bone turnover may be deleterious and reduced turnover protective is supported by several observations. Osteoprotegerin is a decoy receptor for RANKL and reduces osteoclastic activity. OPG knockout mice have reduced subchondral bone and develop accelerated articular cartilage damage. Treatment with OPG reduced cartilage damage and subchondral bone changes following knee destabilisation surgery (Shimizu, Asou et al. 2007, Kadri, Ea et al. 2008). Cathepsin K is highly expressed by osteoclasts and is critical for bone resorption. Cathepsin K knockout mice have high bone mass but are protected from subchondral bone changes and cartilage damage following surgical destabilisation of the knee. Rabbits treated with a cathepsin K inhibitor were protected from OA in a surgical instability model (Hayami, Zhuo et al. 2012). Conversely, mice over-expressing cathepsin K develop spontaneous articular cartilage degeneration and subchondral bone degradation (Morko, Kiviranta et al. 2005). Finally, rats and rabbits treated with the osteoclast inhibitor alendronate were protected from subchondral bone remodelling and cartilage damage due to joint destabilisation (Hayami, Pickarski et al. 2004, Hayami, Zhuo et al. 2012).

5.3.5 Methodological and statistical considerations in articular cartilage evaluation

The destabilisation of medial meniscus model was validated in chapter 3. Although articular cartilage damage was greater in destabilised joints compared to sham operated controls at 4, 8 and 16 weeks post knee surgery, the difference was not statistically significant in any single dronedarone or TR $\alpha^{0/0}$ experiment.

There are important differences in methodology used to assess subchondral bone and articular cartilage that had a major effect on the power to detect an effect of treatment in articular cartilage. Subchondral bone was investigated using X-ray microradiography and microCT. X-ray microradiography had coefficients of variation of 2-3%. MicroCT had coefficients of variation of 11-13% for BV/TV, 10-12% for apparent density and 2-3% for material density. In contrast, histology had coefficients of variation of 31-91% for summed OA scores. This was higher than published studies and may reflect changes in surgical technique of the operator over time. Furthermore,

samples sizes for X-ray microradiography (n=7-14) and microCT (n=4-8) were larger than those for histology (n=3-5) as the former are non-destructive techniques. The combined effect is that all histology experiments were underpowered.

I will illustrate this point by comparing my data with Glasson's study validating the DMM technique (Glasson, Blanchet et al. 2007). Glasson's paper showed mean summed OA score of 35.7 with standard deviation 3.8 assessed 8 weeks after DMM surgery. The calculated coefficient of variation was 10.6%. Based on this data, in order to demonstrate a 20% reduction in OARSI score with an intervention, with 80% power and a significance level of 0.05, the sample size needs to be 5. I pooled data from all my experiments using WT control animals sacrificed 8 weeks after DMM. The mean summed OA score was 18.36 with standard deviation of 9.8. Calculated coefficient of variation was 53.4%. Therefore, cartilage damage in my control group was much lower than expected and the data was more variable. Using a sample size of 3-4 (as in my experiments) to detect a 20% reduction in OA summed score in TR $\alpha^{0/0}$ or dronedarone treated mice with a significance level of 0.05 meant the experiment only had 8% power. Alternatively, samples sizes of 108 would be required to detect a 20% difference in OA summed score with a significance level of 0.05 and 80% power. This sample size is too large for the experiments to be feasible. Therefore, a strategy would be needed to reduce the variance (better surgical technique, histological preparation and increased animal activity) before repeating these experiments.

What useful interpretations can be made in the context of such underpowered experiments with regards to cartilage damage? Underpowered studies increase the probability of type II statistical errors, namely the failure to detect a genuine treatment effect or failure to reject the null hypothesis when it is false. However, underpowered studies also increase the risk of false positive results and therefore type I statistical error, rejecting the null hypothesis when it is true (Christley 2010). The probability of false positive results is inversely related to the pre-test probability of the null hypothesis being false. This means if there is strong biological plausibility underlying the hypothesis, the likelihood of false positive results is lower.

5.3.6 Inconsistent chondroprotection with dronedarone

There was no difference in cartilage damage between $TR\alpha^{0/0}$ and wild type mice. Dronedarone treatment did not affect articular cartilage 4 and 16 weeks after OA-inducing surgery. However, treatment with low dose dronedarone significantly reduced cartilage damage 8 weeks after destabilising surgery compared to control mice. A similar (but not significant) trend was seen with high dose dronedarone. This occurred despite these methodological and statistical limitations described above. Importantly, chondroprotection was accompanied by an attenuation in osteoarthritis subchondral bone changes. Thus, the observed articular cartilage protection with dronedarone may be biologically significant. What factors might explain chondroprotection with dronedarone but lack of chondroprotection in $TR\alpha^{0/0}$ mice? Mutant mice have higher subchondral BMC and mass, which in many genetically modified animal models is associated with greater cartilage damage (van der Weyden, Wei et al. 2006, Wu, Kim et al. 2008). Similarly, genetically modified mice with lower subchondral bone mass and mineral content often have less articular cartilage damage (Botter, Glasson et al. 2009, Huesa, Ortiz et al. 2015, Bradley, Carpio et al. 2016). Alternatively, the deletion of $TR\alpha$ results in loss of transcriptional activity of positively regulated T3 target genes. However, pharmacological blockade results in unliganded $TR\alpha$ and increased repression of positively regulated T3 target genes. This may have greater inhibitory effects on chondrocyte hypertrophy and matrix proteases and thus result in chondroprotection. However, given the mixed results and underpowered studies, no firm conclusions should be drawn regarding the effects of $TR\alpha$ deletion or inhibition on articular cartilage integrity.

CHAPTER 6 SPONTANEOUS OSTEOARTHRITIS IN MICE LACKING TYPE 2 IODOTHYRONINE DEIODINASE

6.1 BACKGROUND

Genetic studies identified altered regulation of thyroid hormone availability as novel determinant of osteoarthritis susceptibility, with research focused on type 2 iodothyronine deiodinase. The functional consequences of the Ala risk allele of rs225014 remains controversial. Nevertheless, increased DIO2 mRNA and protein expression is increased in cartilage affected by osteoarthritis in humans (Karlsson, Dehne et al. 2010, Bos, Bovee et al. 2012). In rat articular cartilage, the level of DIO2 mRNA expression was also found to be similar to brown adipose tissue (Nagase, Nagasawa et al. 2013). However, the activity of DIO2 in articular cartilage has not been measured in this or other studies and it remains unclear if there is increased or decreased T3 signalling in osteoarthritic tissue.

In osteoarthritis, articular chondrocytes escape from maturational arrest and undergo terminal hypertrophic differentiation in a process resembling endochondral ossification. T3 promotes cartilage maturation, is critical for endochondral ossification and increases the activities of cartilage degrading enzymes in the growth plate (Pereira, Jorgetti et al. 1999, Himeno, Enomoto et al. 2002, Makihiro, Yan et al. 2003, Waung, Bassett et al. 2012, Chen-An, Andreassen et al. 2013). Transgenic rats over-expressing human DIO2 have increased susceptibility to joint instability induced OA (Nagase, Nagasawa et al. 2013). Therefore, current evidence suggests that increased T3 availability in the joint may be deleterious. I hypothesize that restricted T3 availability may be protective against osteoarthritis and tested this by determining the joint phenotype in DIO2 knock out mice (D2KO) lacking the ability to generate local T3.

6.2 RESULTS

Mice lacking type 2 deiodinase aged 16 weeks

6.2.1 Length of articular cartilage

The measured complete articular cartilage surface in the reference joint was 27.4 mm, and this length was used to calculate standardized OA scores. In sections from experimental samples, the measured articular cartilage surface was: WT 5.0 ± 1.2 , $n=9$; D2KO 6.0 ± 1.1 , $n=5$; D1D2KO 10.99 ± 2.99 , $n=3$ (mm $\pm$ SEM; ANOVA; not significant).

6.2.2 Articular cartilage is not altered in D2KO mice

There were no differences in maximum and standardized OA scores or erosion indices between WT and D2KO mice (Figure 6.1). The maximum OA scores were: WT 3 (0–3), $n=9$; D2KO 4 (0.5–4), $n=5$ (maximum score (range); Mann–Whitney U-test; not significant). The standardized OA scores were: WT 7.8 ± 2.1 , $n=9$; D2KO 10.9 ± 3.6 , $n=5$ (mean standardized score $\pm$ SEM; Student's *t*-test; not significant). The erosion indices were WT 2.7 ± 1.4 , $n=9$; D2KO 6.1 ± 2.7 , $n=5$ (mean % $\pm$ SEM; Student's *t*-test; not significant). There were also no differences in these parameters in D1D2KO mice: maximum OA score (0–2), $n=3$; standardized OA score 9.6 ± 5.4 , $n=3$; erosion index 5.5 ± 3.3 , $n=3$ (Mann–Whitney U-test and Student's *t*-tests vs. WT or D2KO mice; not significant).

Articular cartilage cellularity also did not differ between WT and D2KO mice: WT 1412 ± 82 , $n=9$; D2KO 1705 ± 161 , $n=5$ (mean number of chondrocytes per $\text{mm}^2 \pm$ SEM; Student's *t*-test; not significant). Articular cartilage cellularity also did not differ in D1D2KO mice: 1493 ± 251 , $n=3$ (Student's *t*-test vs. WT or D2KO mice; not significant).

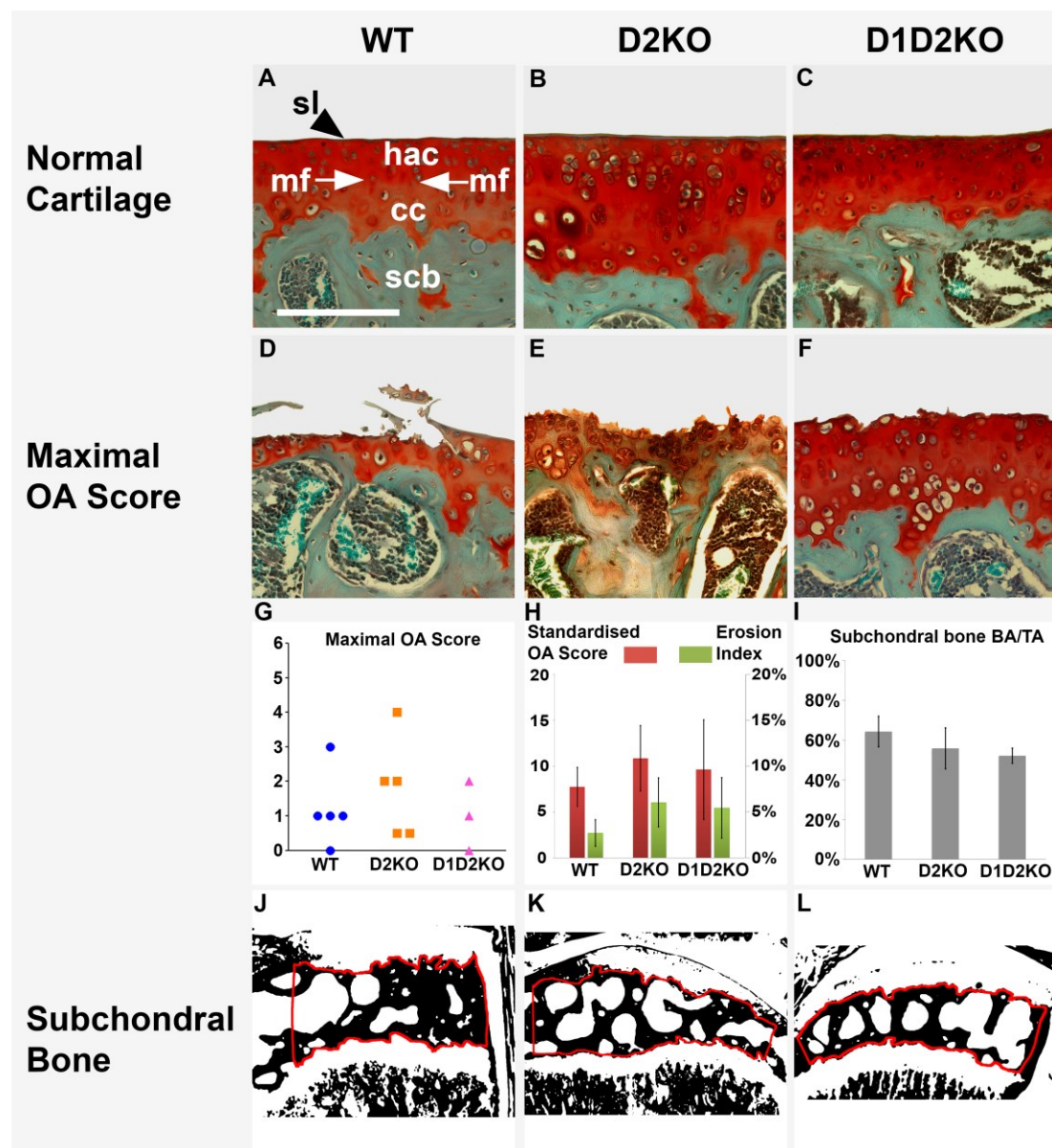


Figure 6.1 OA pathology and scores in WT, D2KO and D1D2KO mice

Modified from (Waung, Bassett et al. 2015) with permission. (A-C) High-powered views showing normal articular cartilage with an OARSI Score of zero in WT and mutant mice. Scale bar = 50 μ m. sl, surface lamina; hac, hyaline articular cartilage; mf, mineralizing front; cc, calcified cartilage; scb, subchondral bone. (D-F) Maximum OA scores for WT (3 = vertical clefts or erosions to the calcified cartilage extending to < 25% of articular surface), D2KO (4 = vertical clefts or erosions to the calcified cartilage extending to 25–50% of articular surface) and D1D2KO mice (2 = vertical clefts down to the layer immediately below the superficial layer and some loss of surface lamina). (G) Graphs showing maximum OA scores in WT, D2KO and D1D2KO mice. (H) measures of cartilage damage (mean standardized OA score in red bars, mean erosion index in green bars). (I) Mean subchondral bone area (BA/TA). Error bars represent standard error of the mean, with no significant differences among genotypes for all parameters. (J-L) Subchondral bone regions of interest in WT and mutant mice

6.2.3 Subchondral bone area is unchanged but bone mineral content is increased in D2KO mice

The subchondral bone area did not differ between WT and D2KO mice: WT $64 \pm 7.6\%$, $n=4$; D2KO $56 \pm 10.3\%$, $n=4$ (mean BA/TA; Student's *t*-test; not significant; Figure 6.2) and was also not different in D1D2KO mice: $52 \pm 3.8\%$, $n=3$ (Student's *t*-test vs. WT or D2KO mice; not significant). Despite this, subchondral bone mineral content was markedly increased in D2KO mice ($n=4$) compared to WT ($n=5$), Kolmogorov–Smirnov test ($p < 0.001$; Figure 6.2), and a similar increase was evident in D1D2KO mice ($n = 2$; $p < 0.001$).

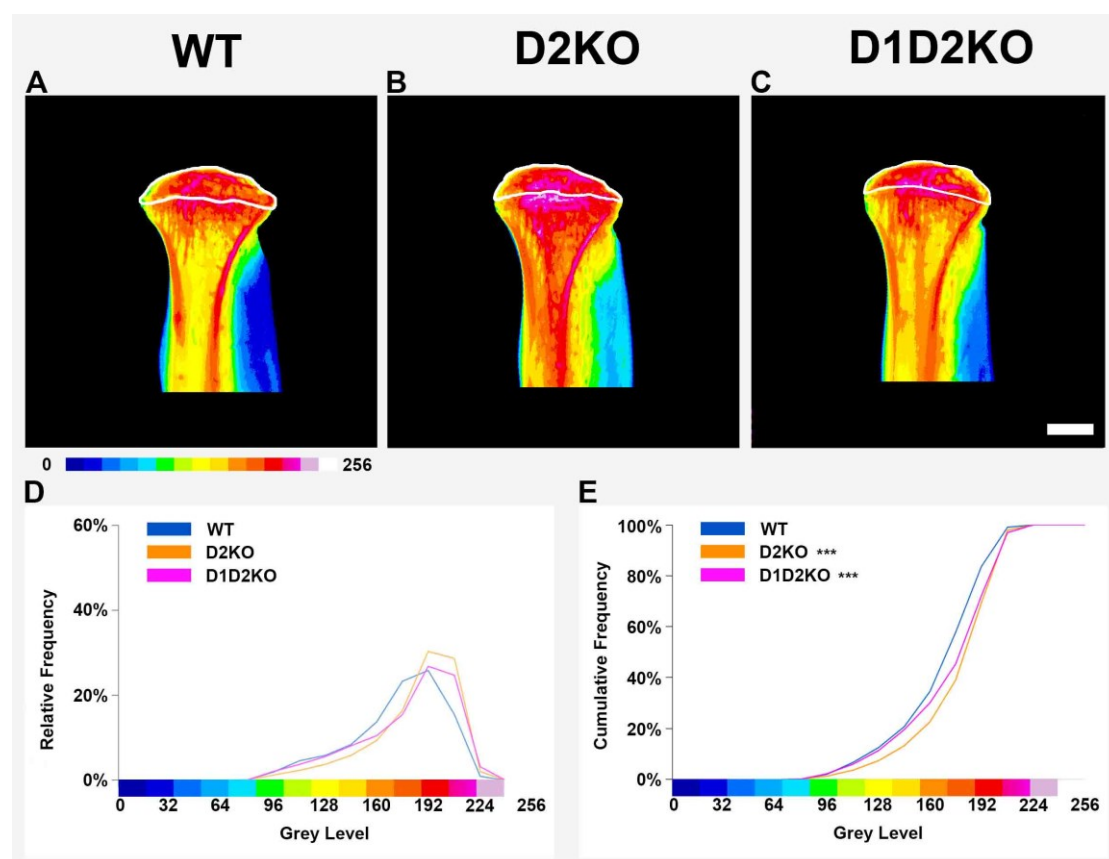


Figure 6.2 Subchondral bone mineral content in WT, D2KO and D1D2KO mice.

(A-C) Pseudocoloured images of proximal tibias from WT and mice in which blue-green is low BMC, and pink-white is high BMC. Subchondral bone region of interest is indicated within the white boundary. (B) Relative and cumulative frequency histograms demonstrating increased subchondral bone mineral content in mutant mice. Kolmogorov–Smirnov test; *** $p < 0.001$ (WT $n = 5$, D2KO $n=4$, D1D2KO $n=2$).

6.3 DISCUSSION

These studies showed that 16-week-old male D2KO mice, that have normal circulating T3 levels but are unable to generate T3 in target tissues, have increased subchondral bone mineral content but normal articular cartilage and no features of OA. Double knockout mice further lacking DIO1 had the same phenotype as D2KO, supporting the view that DIO1 has no physiological role in the skeleton.

D2KO mice do not have a developmental phenotype but have an osteoblast specific hypothyroidism that results in a generalised increase in bone mass and mineralisation in adult mice. The functional consequence is brittle bones with increased susceptibility to fracture (Bassett, Boyde et al. 2010). My experiments show that these mutants also have increased mineral content in subchondral bone but no defect in articular cartilage.

OA pathogenesis is complex and it is unclear if the observed subchondral bone abnormality in D2KO mice is functionally significant. Primary defects of the articular cartilage can lead to subchondral bone remodelling and sclerosis, hallmarks of OA. Equally, primary defects of the underlying subchondral bone may lead to articular cartilage damage and secondary OA. It is unclear if these changes occur concurrently or if one precedes the other and in which order (Burr and Schaffler 1997, Burr and Gallant 2012, van der Kraan and van den Berg 2012). It is tempting to speculate that increased mineralisation and stiffness in subchondral bone would increase mechanical forces on articular cartilage and predispose to OA. This is supported by the observation that rabbits treated with parathyroid hormone have increased subchondral bone mineral density but worsened articular cartilage integrity (Orth, Cucchiari et al. 2014). However, cathepsin K knockout mice have increased subchondral bone mineralisation but are protected from OA (Hayami, Zhuo et al. 2012). Since the observed abnormality in D2KO mice subchondral bone occurred in the absence of articular cartilage damage, this suggests that any effects of DIO2 deletion on OA pathogenesis are likely to be mediated via a primary defect in subchondral bone osteoblasts.

Limitations of my experiments are that sample sizes were small and cartilage damage in controls was modest, making it harder to detect any protective effect in D2KO. Bomer et al used a forced exercise model with larger sample sizes to show that similarly aged D2KO mice were protected from articular cartilage damage and synovial

hyperplasia (Bomer, Cornelis et al. 2016). However, the pathology was mild and it is unclear if small difference in cartilage damage between wild type and D2KO is biologically significant. Nevertheless, the protection observed in D2KO mice supports a number of studies implicating a role for DIO2 in osteoarthritis pathogenesis. Dio2 mRNA (Nagase, Nagasawa et al. 2013) and protein (Bos, Bovee et al. 2012) expression is increased in human articular cartilage from joints with end-stage OA. Additionally, transgenic rats overexpressing Dio2 in chondrocytes had increased susceptibility to OA following surgical destabilisation of the knee (Nagase, Nagasawa et al. 2013). However, none of these studies have demonstrated a causal relationship between increased DIO2 expression and OA susceptibility because DIO2 activity was not measured. This is important because studies in growth plate chondrocytes have shown that measurable DIO2 mRNA expression does not necessarily correlate with detectable enzyme activity (Williams, Robson et al. 2008). Furthermore, in a previous study, transgenic mice overexpressing DIO2 in the heart surprisingly demonstrated only mild thyrotoxic changes in cardiomyocytes (Carvalho-Bianco, Kim et al. 2004), probably because the overexpressed enzyme was rapidly degraded by its substrate (T4). It is the lability of DIO2 and its rapid degradation to create a local feedback loops that makes measuring DIO2 activity so challenging. Another difficulty is that current antibodies that detect DIO2 by immunohistochemistry or western blotting are insufficiently sensitive or reliable. Thus, it is currently not known if functional DIO2 is expressed in articular cartilage and studies showing increased DIO2 expression in osteoarthritis do not show causality.

An alternative explanation for the increased expression of DIO2 in OA cartilage is that it represents a secondary response to joint damage. There is growing evidence that inflammation cytokines produced by synovial macrophages plays an important role in OA (Bondeson, Wainwright et al. 2006, Kraus, McDaniel et al. 2016). In animal models of both acute and chronic inflammation, macrophages in the liver had increased Dio2 expression and activity (Kwakkel, Surovtseva et al. 2014). Thus, the increased DIO2 expression observed in end stage human OA may represent the results of the activation of pro-inflammatory pathways including NFkB, which are known to stimulate DIO2 expression (Roman-Blas and Jimenez 2006, Zeold, Doleschall et al. 2006). Consistent with this, SiRNA mediated inhibition of DIO2 mRNA expression in primary human chondrocytes resulted in reduced expression of liver X receptor α

(LXR α); increased expression of IL-1 β and IL-1 β induced COX2 gene expression (Cheng, Bolognesi et al. 2012). This led the authors to proposed an anti-inflammatory and protective role for DIO2 in chondrocytes.

In summary, D2KO mice have increase subchondral bone mineral content but no articular cartilage defect in a model of spontaneous osteoarthritis. This suggests that altered subchondral bone properties do not always predispose to OA development. OA is more likely to be due to an initial abnormality of cartilage or dependent on the magnitude of subchondral bone remodelling. This study did not measure change to subchondral bone over time. D2KO mice have osteoblast specific hypothyroidism with low turnover and it is tempting to speculate that reduced subchondral bone remodelling may be a mechanism that confers protection against OA seen in the forced exercise model (Bomer, Cornelis et al. 2016). Further studies with OA-provocation models such as DMM-surgery should be performed to confirm if they are protected from OA and include analysis of the subchondral bone before and after DMM-surgery.

CHAPTER 7 EFFECTS OF TR α INHIBITION ON BONE MASS AND STRENGTH

7.1 BACKGROUND

Thyroid hormone is catabolic in adult skeleton and untreated thyrotoxicosis is an established cause of secondary osteoporosis. TR α is the predominant receptor expressed in bones and previous work by our group showed that adult TR $\alpha^{0/0}$ mice develop osteosclerosis. Therefore, I hypothesised that TR α inhibition would increase bone mass and strength. This was investigated in adult mice treated with dronedarone from 9 weeks of age until sacrifice 17 weeks later. Caudal vertebrae and long bones from the sham operated limb were used to investigate trabecular and cortical bone changes. Humeri were used to investigate changes to mechanical properties

7.2 RESULTS

7.2.1 Structural Parameters

7.2.1.1 *Femoral cortical bone*

Cortical bone diameter reflects periosteal apposition of bone on the external cortical surface. Cortical thickness or width is determined by the net balance between external cortical periosteal apposition by osteoblasts and internal cortical resorption by osteoclasts.

Cortical bone was assessed in the mid femoral diaphysis with X-ray microradiography (Figure 7.1). There was no difference between dronedarone and vehicle treatment in periosteal and intra medullary cortical diameter or cortical thickness.

Mid diaphysis cortical bone in the femur was further assessed with micro CT (Figure 7.2 and Figure 7.3). Cortical thickness was increased by 7.3% with high dose dronedarone. Cortical thickness was more modestly increased by 6.0% with low dose dronedarone compared to vehicle treated controls, however, this was not statistically significant.

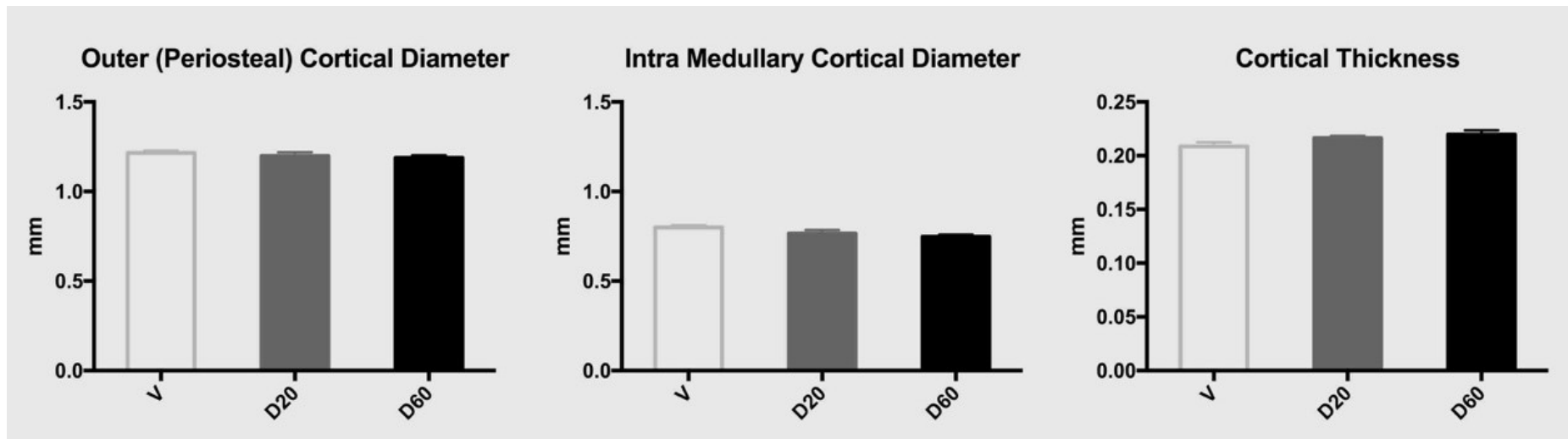


Figure 7.1 Cortical thickness determined by X-ray microradiography

Outer (periosteal), Inner (intramedullary) cortical diameters and cortical thickness of mid femur determined by X-ray microradiography (n=12). ANOVA and Tukey's post hoc test. There was no difference compared to vehicle treatment in any parameter.

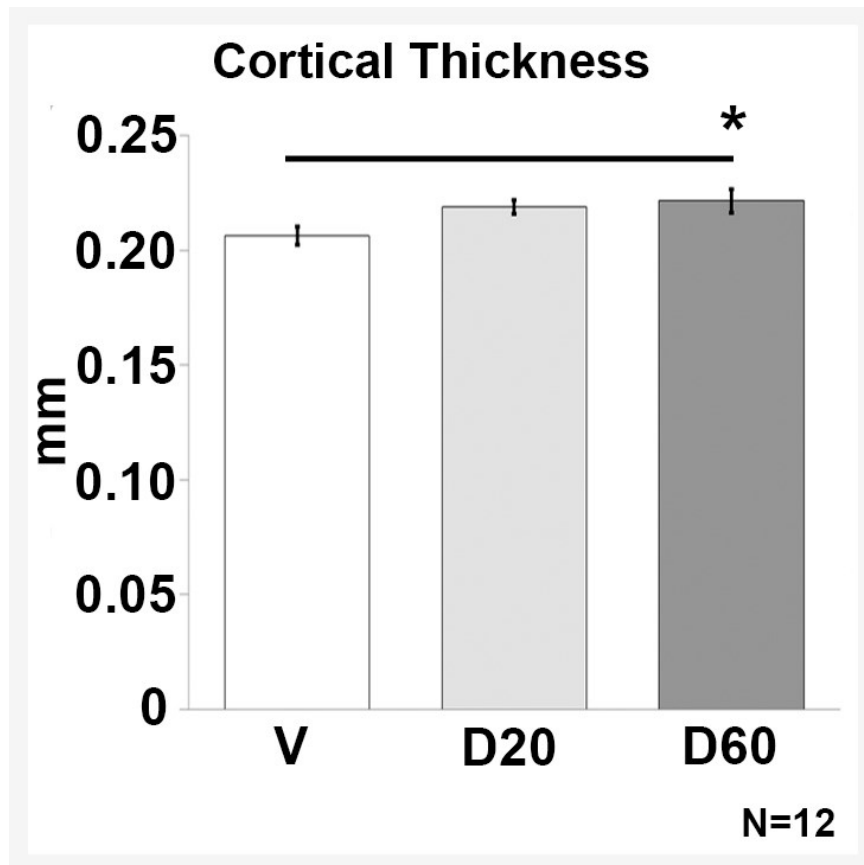


Figure 7.2 Femoral cortical thickness analysis by microCT

Quantitative analysis of cortical thickness (Ct.Th) determined by micro CT analysis of mid femur (n=12). ANOVA and Tukey's post hoc test. * $P < 0.05$ compared to vehicle treatment.



Figure 7.3 Images from microCT of whole femurs in mice treated with dronedarone

Representative images of whole femurs in mice treated with dronedarone for 17 weeks. Scale bar = 1mm

7.2.1.2 Tibial trabecular bone

Trabecular bone distal to the growth plate of the proximal tibia was measured by Micro CT (Table 7.1). There was no difference in any measured parameter with dronedarone treatment compared to vehicle treatment.

	Vehicle	Dronedarone 20mg/kg/day	Dronedarone 60mg/kg/day
BV/TV (%)	12.7±0.9	14.1±0.9	12.81±1.5
Tb.Th (mm)	0.05±0.001	0.04±0.0004	0.05±0.001
Tb.Sp (mm)	0.29±0.009	0.26±0.008	0.28±0.011
Tb.N (mm⁻¹)	3.56±0.10	3.97±0.13	3.71±0.15
Conn.D (mm⁻³)	38.9±5.7	55.0±6.7	47.8±9.2
SMI	2.85±0.14	2.44±0.11	2.48±0.15

Table 7.1 Trabecular bone assessment with microCT

Quantitative analysis of bone volume (BV/TV), Trabecular thickness (Tb.Th), trabecular spacing (Tb.Sp), trabecular number (Tb.N), connectivity density (Conn.D) and structure model index (SMI) determined by micro CT analysis of proximal tibia (n=12). ANOVA and Tukey's post hoc test. None were significant compared to vehicle treatment.

7.2.2 Bone mineral content

The effects of dronedarone on BMC were assessed by X-ray microradiography (Figure 7.4). Dronedarone had no effect on bone mineral content in caudal vertebrae. In femurs, the median grey level was 183, 185 and 187 for mice treated with vehicle, low dose and high dose dronedarone, respectively. The BMC was significantly increased with high dose dronedarone compared to untreated controls. Mice treated with low dose dronedarone had BMC that was intermediate between untreated and high dose dronedarone.

7.2.3 Bone formation

Bone formation in humeral cortex was assessed with dynamic histomorphometry (Figure 7.5). There was no difference in mineralising surface (MS/BS), mineral apposition rate (MAR) or bone formation rate (BFR) between treatment groups.

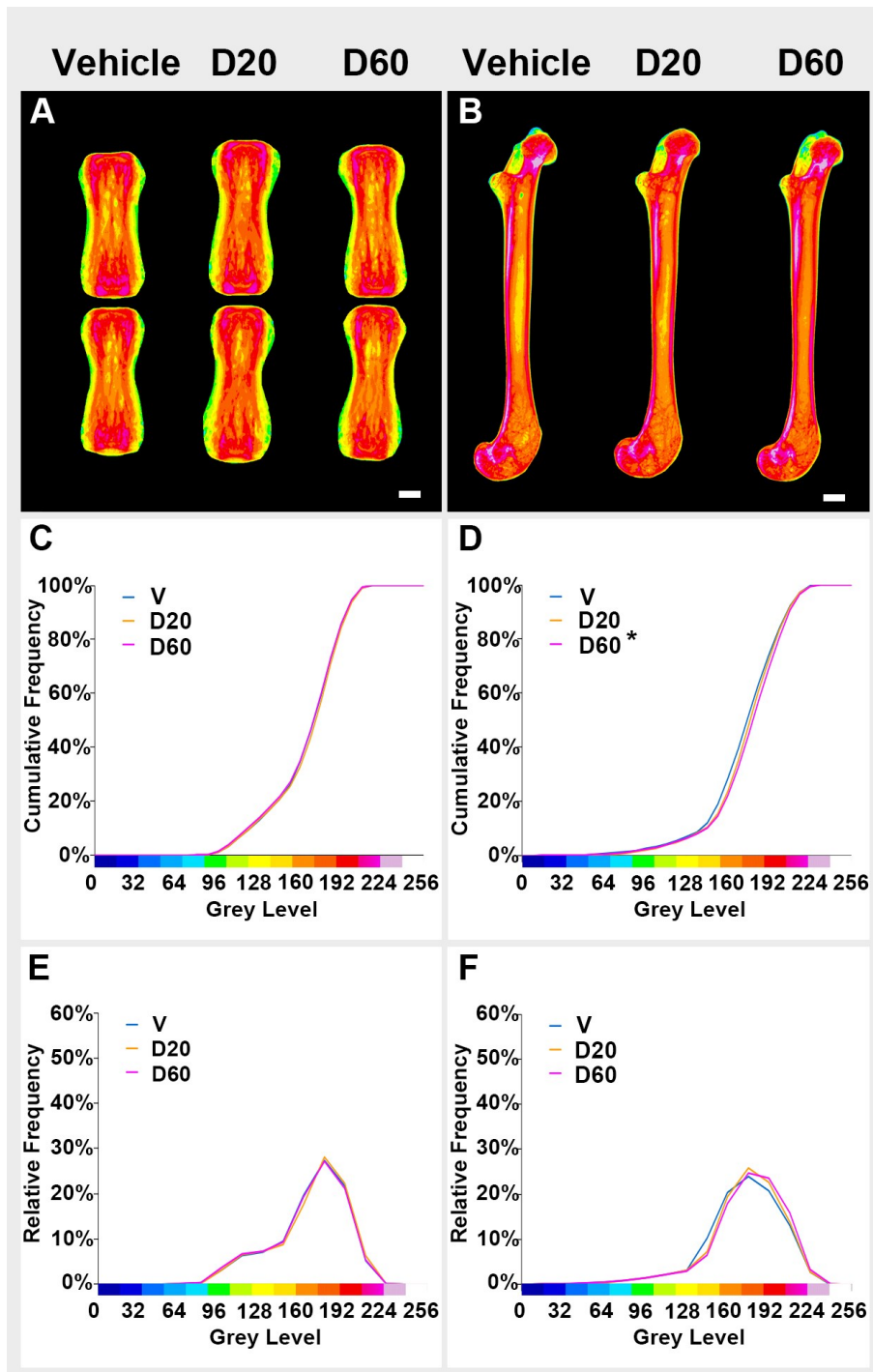


Figure 7.4 Bone mineral content in whole bone following dronedarone treatment

Montage of representative samples showing BMC in vertebrae (A) and femurs (B) treated for 17 weeks with dronedarone. Cumulative (C, D) and relative (E, F) frequency histograms showing no difference in vertebral BMC but a dose dependent increase in femoral BMC with dronedarone treatment. Kolmogorov–Smirnov test; * $p < 0.05$ vs vehicle

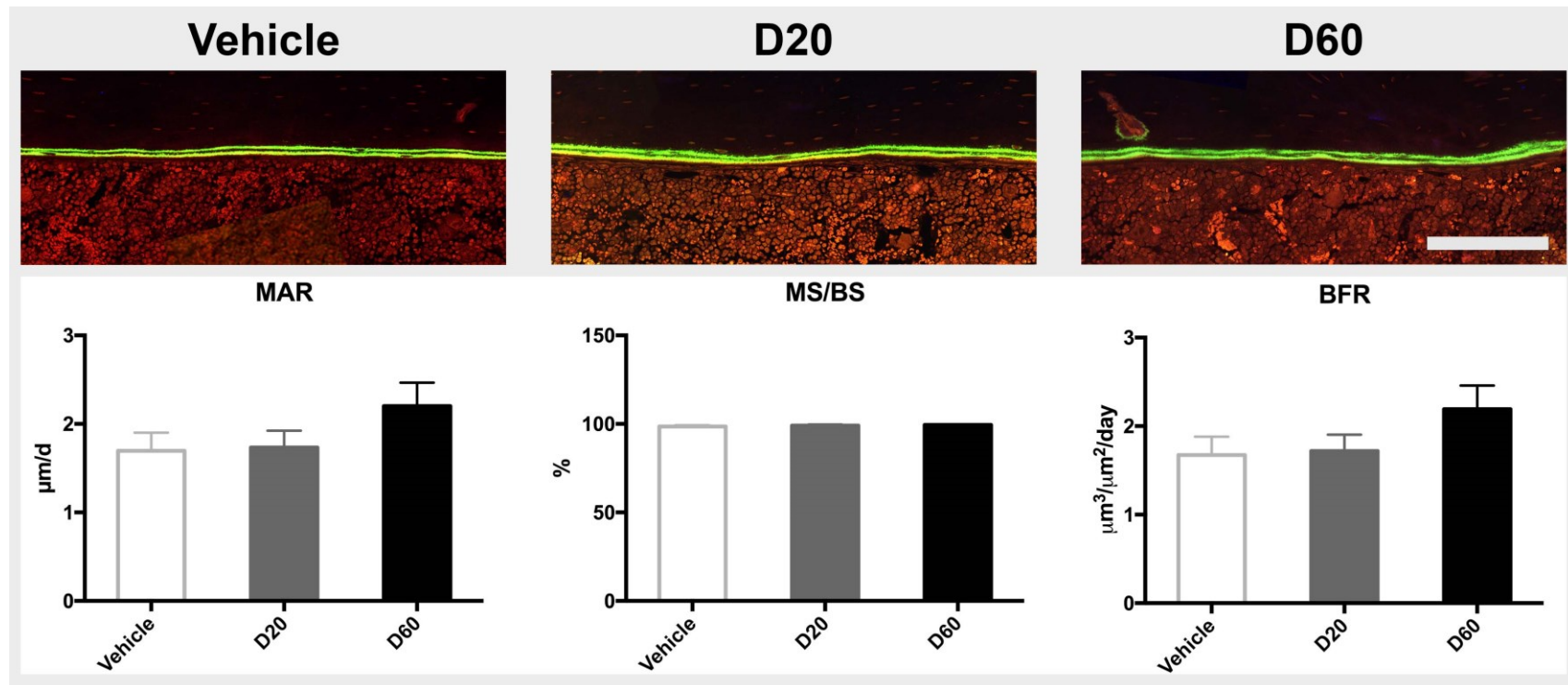


Figure 7.5 Bone formation with dronedarone

Bone formation in 14-week-old mice. Confocal scanning laser microscopy showing double calcein labelling in humeral cortex. Data is mean $\pm$ SEM. N=3-4 per group. ANOVA and Tukey's multiple comparison post hoc test. Not significant versus vehicle treatment. Scale Bar = 100µm.

7.2.4 Biomechanical testing

Dronedarone treatment had no effect on mechanical properties of humeri assessed with fracture analysis (Figure 7.6). There was no difference in yield load, maximum load, fracture load or stiffness.

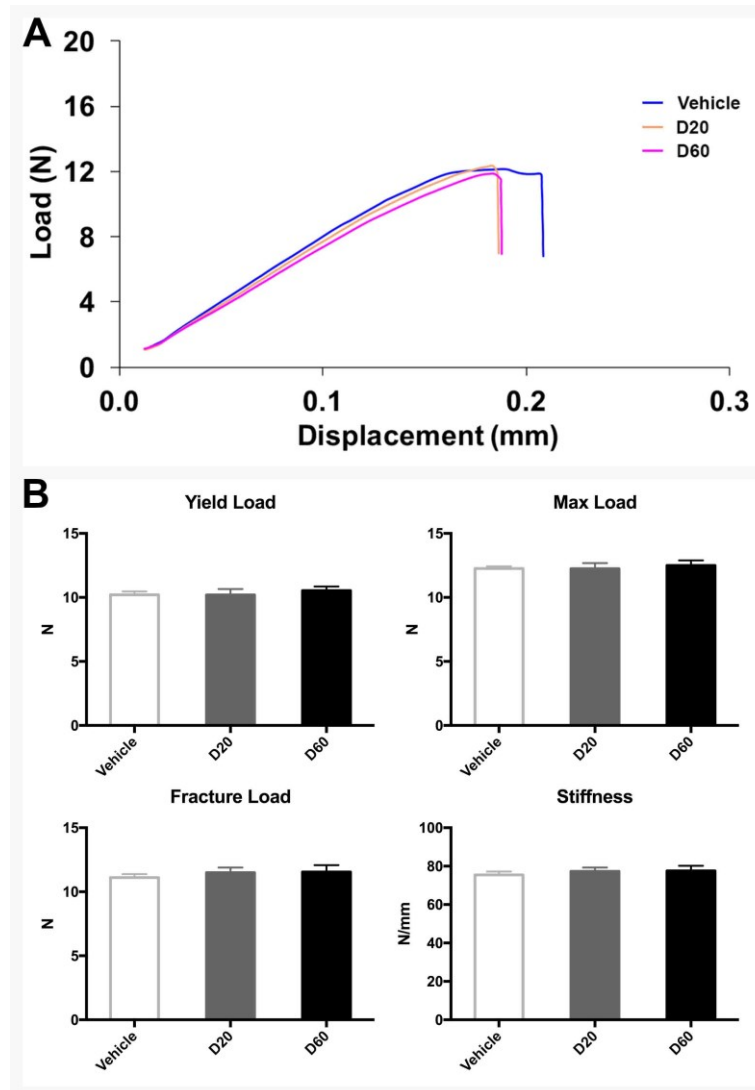


Figure 7.6 Biomechanical properties in bones treated with dronedarone

Load displacement curves of representative samples (A) and graphs (B) show yield load, maximum load, fracture load and stiffness in humeri of P208 mice treated for 17 weeks with dronedarone. Data expressed as mean $\pm$ SEM, $n=11-12$ and analysed by ANOVA and Tukey's multiple comparisons post hoc. Not significant compared to vehicle treatment.

7.3 DISCUSSION

Thyroid hormones are anabolic during growth and catabolic in adulthood. Therefore, the observed skeletal phenotype depends on the timing, duration and severity of thyroid hormone manipulation in experimental animals. The role of TR α has been studied in genetically modified mice with mutations in TR α and TR β . However, in these animals, it is difficult to dissect out the role of TR α in adult bone maintenance from its developmental effects. This chapter described the effects of selective inhibition of TR α in adult wild type mice. Therefore, the developmental effects of TR α inhibition were avoided and the skeletal phenotype observed reflects the role of TR α on adult bone maintenance.

7.3.1 Cortical bone mass is increased with TR α inhibition

X-ray microradiography demonstrated a non-significant trend towards greater femoral cortical thickness with dronedarone treatment. However, when analysed using the more sensitive microCT, a dose dependent increase in cortical thickness was observed. In contrast, dronedarone had no effect on tibial trabecular bone. Although the skeletal effects of dronedarone treatment have not been published, several studies have investigated the skeletal effects of hypothyroidism in adult mice. Adult (P84) female 129Sv mice rendered hypothyroid for 3 or 5 weeks had increased trabecular bone in the distal femur compared to thyrotoxic animals, but were not compared to euthyroid controls. However, there was no difference in cortical bone parameters between hypothyroid and thyrotoxic animals (Monfoulet, Rabier et al. 2011). More recently, hypothyroidism was induced in adult (P84) male C57BL/6 mice for 4 weeks and compared to euthyroid controls. Vertebral body bone volume was increased by 35% in hypothyroid animals. Trabecular bone in the distal femur showed similar changes but the results were not presented. In contrast, there was no change in cortical bone in the distal femur of hypothyroid animals (Tsourdi, Rijntjes et al. 2015). Previous work by Dr. M Cheung (thesis page 119-122)(Cheung 2014) in Prof. Williams and Prof. Bassett's laboratory compared the skeletal phenotype of adult (P70) female C57BL/6 mice rendered hypothyroid or thyrotoxic for 6 weeks with euthyroid controls. Hypothyroid animals had 43% greater trabecular bone mass and increased trabecular thickness in distal femurs measured with microCT. However, despite a reported pixel resolution of 4.3 μ m, these results were reported with an increased Tb.Th but no

difference in Tb.N or Tb.Sp. These results are incongruous and therefore the accuracy of this data is unclear. Mid shaft femoral cortex was thicker in hypothyroid animals compared to controls but the difference was not significant. However, this was measured with the less sensitive X-ray microradiography only (Cheung 2014). In summary, the results in this chapter are discordant with published work on hypothyroid animals where trabecular bone is increased but no cortical phenotype was observed.

It is important when comparing the skeletal phenotypes of dronedarone treatment and experimental hypothyroidism to consider the biological differences of these treatments. Tsourdi, Cheung and Monfoulet's experiments induced profound hypothyroidism, with measured T4 levels less than 15% of euthyroid controls. This would result in most TR α and TR β being unliganded (apoTR) and exerting strong transcriptional repression of T3 target genes. By comparison, dronedarone would compete with TH for TR α only and thus some TR α , and most TR β , would be liganded with T3. TR β is known to partially compensate for a lack of TR α in the presence of thyroid hormone excess (Bassett, O'Shea et al. 2007). It is plausible that liganded TR β may attenuate the effects of unliganded TR α .

It is worth considering whether methodological differences may have contributed to the absence of a cortical bone phenotype in hypothyroid mice. Cheung's experiments assessed mid diaphyseal femoral cortical thickness using X-ray microradiography only. Although not statistically significant, cortex was thickest in hypothyroid mice and thinnest in thyrotoxic animals, with euthyroid controls in between. Of note, dronedarone treatment resulted in a similar dose dependent trend of increased thickness that was not significant when assessed by X-ray microradiography but was significant using micro CT. The region of interest for femoral cortical bone was also different in Monfoulet and Tsourdi's studies. They assessed cortical bone in the femoral metaphysis where there is greater variability in bone shape compared to the mid diaphysis, where dronedarone treated bones were assessed.

Dronedarone did not affect trabecular bone, despite a positive effect on cortical thickness. This is in direct contrast to the gain in trabecular bone seen in hypothyroid adult mice. Several additional methodological differences including sex, age of mice,

duration of treatment and region of interest assessed should also be considered. Cheung and Monfoulet's experiment were in female mice while dronedarone experiments were in older male mice that had sham knee surgery in the assessed tibia. Dronedarone experiments assessed trabecular bone in proximal tibia while the hypothyroidism studies assessed vertebrae or distal femur. Proximal tibia may lack sufficient sensitivity to detect a difference or be confounded by the effects of (sham) knee surgery to a greater extent than femoral cortical bone. Alternatively, inhibition of TR α in adulthood may have predominant action on cortical bone compared to trabecular bone.

7.3.2 Cortex specific increase in bone mineral content by TR α inhibition

Dronedarone treatment resulted in a dose dependent increase in BMC in the femur only. There was no difference in BMC in caudal vertebrae. Whole femur BMC reflects a predominantly cortical bone, while vertebrae represent predominantly trabecular bone. Therefore, these results suggest that inhibition of TR α has a predominant effect on cortical bone over trabecular bone. Cheung's experiments showed that hypothyroid mice also demonstrated a site-specific increase in BMC restricted to the femur but not in vertebrae. In contrast, when bone mineral density was assessed with micro CT, Tsourdi showed increased trabecular BMD but no change in cortical BMD. BMC is a product of bone volume and mineralisation. Therefore, the increased BMC in dronedarone treated femurs may be due to increases in cortical bone volume alone, an increase in bone mineralisation or both. To address this, bone micro-mineralisation density can be quantified by qBSE-SEM but was not performed due to time constraints. However, Cheung showed that in hypothyroid wild types and untreated TR $\alpha^{0/0}$ mice, bone micro-mineralisation was increased in trabecular bone of vertebrae and proximal tibia and cortices of tibia compared to euthyroid wild type controls. Therefore, it is likely that TR α inhibition with dronedarone increases BMC through a combination of increased bone volume and micromineralisation. However, this requires confirmation with qBSE-SEM.

7.3.3 Cortical bone phenotype is likely to be mediated by reduced osteoclastic resorption.

The cortices of humeri were investigated to determine if increased bone mass was due to increased bone formation. Bone formation was assessed in young (P98) mice that had been treated for 5 weeks with dronedarone. Bone formation was not increased

following dronedarone treatment. This may be due to insufficient sensitivity of the bone formation assay, or by deduction, reduced osteoclastic resorption must mediate the effects of dronedarone on cortical bone mass. However, this was not measured in my experiments. Studies of hypothyroid mice have, however, shown that CTX, a bone resorption marker, was reduced compared to euthyroid controls or at the lower limit of the assay in hypothyroid and $TR\alpha^{0/0}$ mice. Furthermore, osteoclast number and/or surface were reduced in hypothyroid animals (Cheung 2014, Tsourdi, Rijntjes et al. 2015). Taken together, dronedarone is likely to increase femoral cortical thickness through reduced osteoclastic resorption.

7.3.4 Bone strength is not changed with dronedarone

Mechanical testing in different models of skeletal hypothyroidism have yielded variable results. Cheung (page 137 of thesis) reported no changes in mechanical properties in the tibias of $TR\alpha^{0/0}$ mice or wild type mice rendered hypothyroid for 6 weeks compared to euthyroid controls (Cheung 2014). In contrast, the femurs of 16 weeks old D2KO mice were brittle compared to wild type mice (Bassett, Boyde et al. 2010). Wild type specimens dissipated more energy from the point of maximal load to fracture while mutants sustained a sudden fracture almost immediately after maximum load. Since dronedarone treatment leads to increased cortical thickness and bone mineral content in femurs, one might expect these bones to be stiffer. However, fracture analysis showed that dronedarone treatment did not affect bone strength in humeri. This may be because D2KO mice have severe osteoblast specific hypothyroidism due to failure to generate intracellular T3 and therefore increased presence of ApoTR. Additionally, dronedarone treatment only began in adulthood while the phenotype of D2KO includes development effects. Methodological differences may also be important. Review of load displacement curves shows that most vehicle treated and dronedarone treated humeri sustained fractures shortly after maximal load was reached. Therefore, humeri may be less sensitive than femurs in detecting subtle changes in bone mechanical properties associated with hypothyroidism or dronedarone.

7.3.5 Future experiments and clinical application

Adult male C57BL/6 mice are not an optimal experimental model to explore the effects of $TR\alpha$ inhibition on bone mass and strength. These experiments were conceived to

add value to the experiments investigating the effects of dronedarone in osteoarthritis. Dronedarone treatment did not affect mechanical properties of humeri but did increase cortical thickness and mineral content in the femurs only, suggesting a site-specific effect in cortical bone. Therefore, despite its limitations in design, these experiments serve as a proof of principle that TR α may be a novel drug target for osteoporosis.

Future experiments can be designed with a greater sensitivity to detect a bone protective effect of dronedarone and have direct, translational value to clinical practice. Post-menopausal osteoporosis is a major clinical problem and ovariectomy in WT female mice is an established experimental model. It could be used to test the hypothesis that dronedarone delays bone loss with ovariectomy. This will be discussed further in the next chapter. Alternatively, adult female mice could be treated with thyroid hormone to induce thyrotoxicosis and secondary osteoporosis. This is analogous to therapeutic, long-term supraphysiological dosing of levothyroxine in patients treated for differentiated thyroid cancer (DTC). The goal of therapy is to suppress TSH (via TR β in the pituitary) to reduce the risk of DTC recurrence. However, treatment can be associated with toxicity to TR α tissues such as bone and heart and associated with bone loss, fractures and atrial fibrillation. If dronedarone demonstrated a bone protective effect, it could potentially open up a new therapeutic indication for a drug already licensed to treat atrial fibrillation.

CHAPTER 8 GENERAL DISCUSSION & CONCLUSIONS

This thesis has examined the effects of reduced signalling through TR in the joint using different models of hypothyroidism. Several themes emerge which will be discussed.

8.1 SUBCHONDRAL BONE CHANGES DUE TO REDUCED THYROID HORMONE SIGNALLING ARE NOT ACCOMPANIED BY ALTERED ARTICULAR CARTILAGE INTEGRITY

Bone mineralisation during remodelling involves a two-step process: primary mineralisation on the calcification front, followed by a slower secondary mineralisation that includes increasing the amount and/or size of mineral crystals. The biological determinant of mineralisation is the rate of bone turnover (Grynpas 1993, Boivin and Meunier 2002), with low turnover leading to increased mineralisation and high turnover reducing mineralisation. Hypothyroidism causes low bone turnover and therefore increased secondary mineralisation. The observed subchondral bone phenotype in $TR\alpha^{0/0}$ and D2KO appear consistent with this model.

$TR\alpha^{0/0}$ and D2KO mice were used as models of reduced thyroid hormone signalling in the skeleton, however, a direct comparison is impossible as different models of osteoarthritis were used. OA was studied in 4, 8 and 16 weeks after joint destabilising surgery performed in 10 week old $TR\alpha^{0/0}$ mice. Spontaneous or aged associated OA was studied in 26 week old D2KO mice. Nevertheless, both mutants are characterised by increased subchondral bone mineral content and are largely concordant with their previously reported long bone phenotypes. D2KO have normal bone volume but increased mineralisation (Bassett, Boyde et al. 2010), while $TR\alpha^{0/0}$ have increased bone volume and conflicting reports of increased mineralisation (Bassett, O'Shea et al. 2007, Cheung 2014).

The differences in thyroid hormone signalling between $TR\alpha^{0/0}$ and D2KO are summarised in Table 8.1. $TR\alpha$ and $TR\beta$ are both expressed in chondrocytes and osteoblasts but the constitutive expression is much higher for $TR\alpha$ than $TR\beta$ (Robson, Siebler et al. 2000, O'Shea, Harvey et al. 2003, Bookout, Jeong et al. 2006, Beber, Capelo et al. 2009, Xing, Aghajanian et al. 2016). D2KO have unliganded $TR\alpha$ and

TR β that are restricted to adult osteoblasts because of the restricted cellular activity of the enzyme. TR $\alpha^{0/0}$ have no signalling through TR α because the receptor is absent but TR β is liganded. However, loss of signalling through TR α may reduce chondrocyte TR β 1 expression (Xing, Aghajanian et al. 2016), resulting in lower signalling through TR β 1 as well. The clear subchondral bone phenotype in both D2KO and TR $\alpha^{0/0}$ mice suggest that subchondral bone formation and mineralisation is mainly regulated by TR α , either directly or indirectly. Neither mutant had any change in articular cartilage integrity compared to their wild type controls. Methodological limitations have been discussed in previous chapters and may account for the reduced power to detect an articular cartilage phenotype. However, the clear subchondral bone phenotype in the absence of alterations to cartilage integrity suggests that any effect of altered T3 signalling on OA pathogenesis is likely to be mediated via primary effects on subchondral bone. Future studies investigating the role of thyroid hormone in OA should include detailed analysis of the subchondral bone compartment

		WT / Control		Dronedarone		TR α ^{0/0}		D2KO	
		TR α	TR β	TR α	TR β	TR α	TR β	TR α	TR β
Development	Osteoblast expression & Ligand status	+++ Liganded	+ Liganded	+++ Liganded	+ Liganded	Absent	+ Liganded	+++ Unliganded	+ Unliganded
	Chondrocyte expression & Ligand status	+++ Liganded	+ Liganded	+++ Liganded	+ Liganded	Absent	+ /- Liganded	+++ Liganded	+ Liganded
Adulthood (+/- post DMM)	Osteoblast expression & Ligand status	+++ Liganded	+ Liganded	+++ Reduced T3 signalling	+ Liganded	Absent	+ Liganded	+++ Unliganded	+ Unliganded
	Chondrocyte expression & Ligand status	+++ Liganded	+ Liganded	+++ Reduced T3 signalling	+ Liganded	Absent	+ Liganded	+++ Unliganded	+ Liganded

Table 8.1 Status of TR in different experimental models

Signalling through TR α and TR β in the different experimental models hypothyroidism used in this thesis. Relative constitutive expression (reduced-; low +; high +++) of TR isoform and degree of T3 signalling in different skeletal cells are shown.

8.2 INHIBITION OF TR α HAS DIVERGENT EFFECTS ON SUBCHONDRAL BONE AND CORTICAL LONGBONE

Dronedarone treatment in adulthood increased cortical thickness and bone mineral content but did not affect caudal vertebrae that is composed mainly of trabecular bone. This confirmed the hypothesis that TR α inhibition would increase bone mass and is a proof of concept that TR α is a potential drug target for osteoporosis. The mechanism underlying these changes is unclear as there was no difference in osteoblastic bone formation but osteoclastic activity was not assessed. However, they are compatible with reported models of skeletal hypothyroidism with reduced bone turnover in wild type mice rendered hypothyroid (Cheung 2014) and untreated TR $\alpha^{0/0}$ mice (Bassett, O'Shea et al. 2007).

Both TR $\alpha^{0/0}$ and D2KO mice have global deletions and it is impossible to separate the subchondral bone effects due to OA from developmental effects. This limitation was addressed in WT mice by using dronedarone treatment in adulthood upon OA-induction. Surprisingly, dronedarone caused an opposite effect in subchondral bone compared to cortical long bone, with reduction in BMC. Furthermore, dronedarone attenuated the increase in subchondral bone mass and mineralisation that accompanies OA-inducing surgery. This suggests that TR α is important for normal subchondral bone remodelling with ageing and is involved in pathological remodelling that occurs with osteoarthritis. Future experiments should investigate the mechanism underlying these subchondral bone changes.

The divergent, but potentially beneficial effects seen in femoral cortex and tibial subchondral bone makes dronedarone a promising drug for further investigation. In particular, inhibition of TR α may be a therapeutic target for postmenopausal osteoporosis. Surprisingly, Cheung showed that TR α signalling was osteoprotective because TR $\alpha^{0/0}$ mice were particularly susceptible to ovariectomy induced bone loss (Cheung 2014). This raises the possibility that thyroid hormone action in regulating adult bone maintenance is sexually dimorphic (Van Vliet, Larroque et al. 2003). Dronedarone treatment in a WT female following ovariectomy would be a useful tool to investigate the molecular mechanisms underlying these observations and would avoid the confounding effects of higher baseline bone mass associated with TR $\alpha^{0/0}$

mutants. Future experiments with dronedarone to investigate osteoporosis should be adequately powered, include bone turnover markers, qBSE-SEM to assess micromineralisation, histomorphometry to determine osteoclast activity, and fracture testing with femurs that might be more sensitive than humeri in demonstrating differences in biomechanical properties.

8.3 DOES INHIBITION OF THYROID HORMONE SIGNALLING DECREASE SUSCEPTIBILITY TO OSTEOARTHRITIS AND DELAY DISEASE PROGRESSION?

Osteoarthritis should be considered a disease of all components of the joint. It is well established OA is accompanied by subchondral bone changes, and extensive experimental evidence that demonstrates alteration to subchondral bone can be either protective or deleterious to articular cartilage. My experiments have shown clear changes in subchondral bone in response to treatment with dronedarone and in genetically altered mice with altered thyroid hormone signalling. This is significant and adds to the growing evidence implicating a role for thyroid hormone in OA pathogenesis. Histological assessment of articular cartilage damage, however, remains the gold standard for investigating osteoarthritis in experimental animal models. In this regard, irrespective of methodological limitations, my experiments have not demonstrated consistent protection in articular cartilage in different models of reduced thyroid hormone signalling. Therefore, the null hypothesis cannot be rejected based on my experiments.

8.4 FUTURE RESEARCH FOR OSTEOARTHRITIS

The experiments in this thesis contribute to the emerging studies on the role of thyroid hormone in osteoarthritis development. My hypothesis, that reducing thyroid hormone signalling would prevent osteoarthritis onset and progression, remains valid but unproven. The experimental design with joint assessment 4, 8 and 16 weeks after destabilisation was chosen to offer the best chance of demonstrating either a protective or deleterious effect from reduced thyroid hormone signalling.

8.4.1 Optimising conditions to assess articular cartilage damage.

Future studies should focus resources on demonstrating the effects of thyroid hormone on articular cartilage. Surgical technique would have to be refined to achieve less

variability to permit adequately powered studies with feasible numbers of animals that remained cost effective. There should be careful consideration of how to optimise condition for assessing articular cartilage damage. I would recommend prioritising 8-weeks of joint instability as the only time point to investigate. This would allow larger sample sizes for histological assessment, the possibility of dropout (rejecting inadequate DMM surgery). Higher cartilage damage scores can be achieved by providing environmental enrichment devices that would increase ambulation (Salvarrey-Strati, Watson et al. 2008).

8.4.2 Mechanistic studies to investigate subchondral bone changes

Studies investigating the role thyroid hormone in OA pathogenesis should include detailed subchondral bone analysis to investigate underlying mechanism. My thesis describes a novel, rapid, non-destructive method of assessing subchondral bone mineral content. It would be informative to have baseline values in 10-week-old animals prior to knee surgery to better understand subchondral bone change related to OA-induction and developmental effects in the genetically modified mice. Mechanistic studies should include micromineralisation in subchondral bone with qBSE-SEM at 8 weeks and a very early changes (< 1 week after DMM-surgery) to assess bone histomorphometry (Nakasa, Ishikawa et al. 2016).

8.4.3 Different models of altered thyroid hormone signalling

The chondroprotection observed with dronedarone treatment in mice sacrificed 8 weeks after surgery should be confirmed with further experiments. Thyroid hormone could be administered to investigate if it worsens OA. Another group could be given both thyroid hormone and dronedarone to see if it rescues the phenotype. In addition to understanding OA pathogenesis, these experiments would add value to investigating dronedarone's potential as a bone protective agent for osteoporosis. These experiments are easily translatable to human disease as it models the therapeutic TSH-suppression with supraphysiological doses of levothyroxine in patient treated for differentiated thyroid cancer who are at risk of bone loss. If cartilage protection is confirmed with dronedarone, *in vitro* studies to investigate its mechanism of action could include osteoblast and osteoclast cultures as well as transfection studies to demonstrate TR receptor specificity.

Another model to investigate increased skeletal thyroid hormone signalling is to study aged TR β ^{-/-} mice. These mice have systemic thyrotoxicosis due to pituitary resistance but normal TR α . The long bone phenotype is osteoporosis and reduced BMC. If TR α were key to subchondral bone remodelling, the hypothesis would be that they would have increase subchondral bone remodelling as seen with OA pathogenesis. This experiment would also have clinical translation as this is the molecular defect that underlies the human condition resistance to thyroid hormone receptor β (RTH β).

The developmental effects of genetic modification can confound OA phenotyping. The Cre-*loxP* system can be used to achieve temporal and tissue specific control of gene function. For example, mice harbouring the floxed construct for TR α can be crossed with tamoxifen-inducible aggrecan-cre mice (Henry, Jang et al. 2009). The progeny can be used to achieve deletion of TR α in articular chondrocytes upon administration of tamoxifen at the time of OA-inducing surgery.

Further work is required to understand the relationship between thyroid hormone and osteoarthritis pathogenesis in the search of potential disease modifying therapies.

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REFERENCES

- Abdalla, S. M. and A. C. Bianco (2014). "Defending plasma T3 is a biological priority." Clin Endocrinol (Oxf) **81**(5): 633-641.
- Abe, S., N. Namba, M. Abe, M. Fujiwara, T. Aikawa, M. Kogo and K. Ozono (2012). "Monocarboxylate transporter 10 functions as a thyroid hormone transporter in chondrocytes." Endocrinology **153**(8): 4049-4058.
- Abrahamsen, B., H. L. Jorgensen, A. S. Laulund, M. Nybo, T. H. Brix and L. Hegedus (2014). "Low serum thyrotropin level and duration of suppression as a predictor of major osteoporotic fractures-the OPENTHYRO register cohort." J Bone Miner Res **29**(9): 2040-2050.
- Abu, E. O., A. Horner, A. Teti, V. K. Chatterjee and J. E. Compston (2000). "The localization of thyroid hormone receptor mRNAs in human bone." Thyroid **10**(4): 287-293.
- Agas, D., L. Marchetti, G. Menghi, S. Materazzi, G. Materazzi, M. Capacchietti, M. M. Hurley and M. G. Sabbieti (2008). "Anti-apoptotic Bcl-2 enhancing requires FGF-2/FGF receptor 1 binding in mouse osteoblasts." J Cell Physiol **214**(1): 145-152.
- Aghajanian, P., W. Xing, S. Cheng and S. Mohan (2017). "Epiphyseal bone formation occurs via thyroid hormone regulation of chondrocyte to osteoblast transdifferentiation." Sci Rep **7**(1): 10432.
- Aho, O. M., M. Finnila, J. Thevenot, S. Saarakkala and P. Lehenkari (2017). "Subchondral bone histology and grading in osteoarthritis." PLoS One **12**(3): e0173726.
- Ai, M., S. L. Holmen, W. Van Hul, B. O. Williams and M. L. Warman (2005). "Reduced affinity to and inhibition by DKK1 form a common mechanism by which high bone mass-associated missense mutations in LRP5 affect canonical Wnt signaling." Mol Cell Biol **25**(12): 4946-4955.
- Akiyama, H., J. E. Kim, K. Nakashima, G. Balmes, N. Iwai, J. M. Deng, Z. Zhang, J. F. Martin, R. R. Behringer, T. Nakamura and B. de Crombrughe (2005). "Osteochondroprogenitor cells are derived from Sox9 expressing precursors." Proc Natl Acad Sci U S A **102**(41): 14665-14670.
- Allain, T. J., T. J. Chambers, A. M. Flanagan and A. M. McGregor (1992). "Triiodothyronine stimulates rat osteoclastic bone resorption by an indirect effect." J Endocrinol **133**(3): 327-331.
- Alman, B. A. (2015). "The role of hedgehog signalling in skeletal health and disease." Nat Rev Rheumatol **11**(9): 552-560.
- Amanatullah, D. F., S. Yamane and A. H. Reddi (2014). "Distinct patterns of gene expression in the superficial, middle and deep zones of bovine articular cartilage." J Tissue Eng Regen Med **8**(7): 505-514.
- Appelhof, B. C., R. P. Peeters, W. M. Wiersinga, T. J. Visser, E. M. Wekking, J. Huyser, A. H. Schene, J. G. Tijssen, W. J. Hoogendijk and E. Fliers (2005). "Polymorphisms in type 2 deiodinase are not associated with well-being, neurocognitive functioning, and preference for combined thyroxine/3,5,3'-triiodothyronine therapy." J Clin Endocrinol Metab **90**(11): 6296-6299.
- Archer, C. W., H. Morrison and A. A. Pitsillides (1994). "Cellular aspects of the development of diarthrodial joints and articular cartilage." J Anat **184** (Pt 3): 447-456.
- Arden, N. and M. C. Nevitt (2006). "Osteoarthritis: epidemiology." Best Pract Res Clin Rheumatol **20**(1): 3-25.

Arrojo, E. D. R. and A. C. Bianco (2011). "Type 2 deiodinase at the crossroads of thyroid hormone action." *Int J Biochem Cell Biol* **43**(10): 1432-1441.

Bakker, O., H. C. van Beeren and W. M. Wiersinga (1994). "Desethylamiodarone is a noncompetitive inhibitor of the binding of thyroid hormone to the thyroid hormone beta 1-receptor protein." *Endocrinology* **134**(4): 1665-1670.

Balemans, W., E. Piters, E. Cleiren, M. Ai, L. Van Wesenbeeck, M. L. Warman and W. Van Hul (2008). "The binding between sclerostin and LRP5 is altered by DKK1 and by high-bone mass LRP5 mutations." *Calcif Tissue Int* **82**(6): 445-453.

Ballock, R. T., X. Zhou, L. M. Mink, D. H. Chen, B. C. Mita and M. C. Stewart (2000). "Expression of cyclin-dependent kinase inhibitors in epiphyseal chondrocytes induced to terminally differentiate with thyroid hormone." *Endocrinology* **141**(12): 4552-4557.

Bandyopadhyay, A., K. Tsuji, K. Cox, B. D. Harfe, V. Rosen and C. J. Tabin (2006). "Genetic analysis of the roles of BMP2, BMP4, and BMP7 in limb patterning and skeletogenesis." *PLoS Genet* **2**(12): e216.

Banovac, K. and E. Koren (2000). "Triiodothyronine stimulates the release of membrane-bound alkaline phosphatase in osteoblastic cells." *Calcif Tissue Int* **67**(6): 460-465.

Barca-Mayo, O., X. H. Liao, M. Alonso, C. Di Cosmo, A. Hernandez, S. Refetoff and R. E. Weiss (2011). "Thyroid hormone receptor alpha and regulation of type 3 deiodinase." *Mol Endocrinol* **25**(4): 575-583.

Barez-Lopez, S., D. Bosch-Garcia, D. Gomez-Andres, I. Pulido-Valdeolivas, A. Montero-Pedrazuela, M. J. Obregon and A. Guadano-Ferraz (2014). "Abnormal motor phenotype at adult stages in mice lacking type 2 deiodinase." *PLoS One* **9**(8): e103857.

Barnard, J. C., A. J. Williams, B. Rabier, O. Chassande, J. Samarut, S. Y. Cheng, J. H. Bassett and G. R. Williams (2005). "Thyroid hormones regulate fibroblast growth factor receptor signaling during chondrogenesis." *Endocrinology* **146**(12): 5568-5580.

Baron, R. (2008). "Anatomy and Ultrastructure of Bone – Histogenesis, Growth and Remodeling." *Endotext*.

Baron, R. and M. Kneissel (2013). "WNT signaling in bone homeostasis and disease: from human mutations to treatments." *Nat Med* **19**(2): 179-192.

Bassett, J. H., A. Boyde, P. G. Howell, R. H. Bassett, T. M. Galliford, M. Archanco, H. Evans, M. A. Lawson, P. Croucher, D. L. St Germain, V. A. Galton and G. R. Williams (2010). "Optimal bone strength and mineralization requires the type 2 iodothyronine deiodinase in osteoblasts." *Proc Natl Acad Sci U S A* **107**(16): 7604-7609.

Bassett, J. H., A. Gogakos, J. K. White, H. Evans, R. M. Jacques, A. H. van der Spek, P. Sanger Mouse Genetics, R. Ramirez-Solis, E. Ryder, D. Sunter, A. Boyde, M. J. Campbell, P. I. Croucher and G. R. Williams (2012). "Rapid-throughput skeletal phenotyping of 100 knockout mice identifies 9 new genes that determine bone strength." *PLoS Genet* **8**(8): e1002858.

Bassett, J. H., K. Nordstrom, A. Boyde, P. G. Howell, S. Kelly, B. Vennstrom and G. R. Williams (2007). "Thyroid status during skeletal development determines adult bone structure and mineralization." *Mol Endocrinol* **21**(8): 1893-1904.

Bassett, J. H., P. J. O'Shea, S. Srisankantharajah, B. Rabier, A. Boyde, P. G. Howell, R. E. Weiss, J. P. Roux, L. Malaval, P. Clement-Lacroix, J. Samarut, O. Chassande and G. R. Williams (2007). "Thyroid hormone excess rather than thyrotropin deficiency induces osteoporosis in hyperthyroidism." *Molecular endocrinology* **21**(5): 1095-1107.

Bassett, J. H., R. Swinhoe, O. Chassande, J. Samarut and G. R. Williams (2006). "Thyroid hormone regulates heparan sulfate proteoglycan expression in the growth plate." *Endocrinology* **147**(1): 295-305.

Bassett, J. H. and G. R. Williams (2003). "The molecular actions of thyroid hormone in bone." Trends Endocrinol Metab **14**(8): 356-364.

Bassett, J. H. and G. R. Williams (2009). "The skeletal phenotypes of TRalpha and TRbeta mutant mice." J Mol Endocrinol **42**(4): 269-282.

Bassett, J. H. and G. R. Williams (2016). "Role of Thyroid Hormones in Skeletal Development and Bone Maintenance." Endocr Rev **37**(2): 135-187.

Bateman, J. F., L. Rowley, D. Belluoccio, B. Chan, K. Bell, A. J. Fosang and C. B. Little (2013). "Transcriptomics of wild-type mice and mice lacking ADAMTS-5 activity identifies genes involved in osteoarthritis initiation and cartilage destruction." Arthritis Rheum **65**(6): 1547-1560.

Baum, J. and B. Brodsky (1999). "Folding of peptide models of collagen and misfolding in disease." Curr Opin Struct Biol **9**(1): 122-128.

Beber, E. H., L. P. Capelo, T. L. Fonseca, C. C. Costa, C. F. Lotfi, T. S. Scanlan and C. H. Gouveia (2009). "The thyroid hormone receptor (TR) beta-selective agonist GC-1 inhibits proliferation but induces differentiation and TR beta mRNA expression in mouse and rat osteoblast-like cells." Calcif Tissue Int **84**(4): 324-333.

Bellido, M., L. Lugo, J. A. Roman-Blas, S. Castaneda, J. R. Caeiro, S. Dapia, E. Calvo, R. Largo and G. Herrero-Beaumont (2010). "Subchondral bone microstructural damage by increased remodelling aggravates experimental osteoarthritis preceded by osteoporosis." Arthritis Res Ther **12**(4): R152.

Bellido, M., L. Lugo, J. A. Roman-Blas, S. Castaneda, E. Calvo, R. Largo and G. Herrero-Beaumont (2011). "Improving subchondral bone integrity reduces progression of cartilage damage in experimental osteoarthritis preceded by osteoporosis." Osteoarthritis Cartilage **19**(10): 1228-1236.

Bennett, C. N., H. Ouyang, Y. L. Ma, Q. Zeng, I. Gerin, K. M. Sousa, T. F. Lane, V. Krishnan, K. D. Hankenson and O. A. MacDougald (2007). "Wnt10b increases postnatal bone formation by enhancing osteoblast differentiation." J Bone Miner Res **22**(12): 1924-1932.

Benske, J., M. Schunke and B. Tillmann (1988). "Subchondral bone formation in arthrosis. Polychrome labeling studies in mice." Acta Orthop Scand **59**(5): 536-541.

Bentolila, V., T. M. Boyce, D. P. Fyhrie, R. Drumb, T. M. Skerry and M. B. Schaffler (1998). "Intracortical remodeling in adult rat long bones after fatigue loading." Bone **23**(3): 275-281.

Berkenstam, A., J. Kristensen, K. Mellstrom, B. Carlsson, J. Malm, S. Rehnmark, N. Garg, C. M. Andersson, M. Rudling, F. Sjoberg, B. Angelin and J. D. Baxter (2008). "The thyroid hormone mimetic compound KB2115 lowers plasma LDL cholesterol and stimulates bile acid synthesis without cardiac effects in humans." Proc Natl Acad Sci U S A **105**(2): 663-667.

Bernal, J. (2007). "Thyroid hormone receptors in brain development and function." Nat Clin Pract Endocrinol Metab **3**(3): 249-259.

Berry, M. J., D. Grieco, B. A. Taylor, A. L. Maia, J. D. Kieffer, W. Beamer, E. Glover, A. Poland and P. R. Larsen (1993). "Physiological and genetic analyses of inbred mouse strains with a type I iodothyronine 5' deiodinase deficiency." J Clin Invest **92**(3): 1517-1528.

Bertuglia, A., M. Lacourt, C. Girard, G. Beauchamp, H. Richard and S. Laverty (2016). "Osteoclasts are recruited to the subchondral bone in naturally occurring post-traumatic equine carpal osteoarthritis and may contribute to cartilage degradation." Osteoarthritis Cartilage **24**(3): 555-566.

Bettica, P., G. Cline, D. J. Hart, J. Meyer and T. D. Spector (2002). "Evidence for increased bone resorption in patients with progressive knee osteoarthritis: longitudinal results from the Chingford study." *Arthritis Rheum* **46**(12): 3178-3184.

Bianco, A. C. and B. W. Kim (2006). "Deiodinases: implications of the local control of thyroid hormone action." *J Clin Invest* **116**(10): 2571-2579.

Bland, J. H. and J. W. Frymoyer (1970). "Rheumatic syndromes of myxedema." *N Engl J Med* **282**(21): 1171-1174.

Bland, R., R. L. Sammons, M. C. Sheppard and G. R. Williams (1997). "Thyroid hormone, vitamin D and retinoid receptor expression and signalling in primary cultures of rat osteoblastic and immortalised osteosarcoma cells." *J Endocrinol* **154**(1): 63-74.

Blum, M. R., D. C. Bauer, T. H. Collet, H. A. Fink, A. R. Cappola, B. R. da Costa, C. D. Wirth, R. P. Peeters, B. O. Asvold, W. P. den Elzen, R. N. Luben, M. Imaizumi, A. P. Bremner, A. Gogakos, R. Eastell, P. M. Kearney, E. S. Strotmeyer, E. R. Wallace, M. Hoff, G. Ceresini, F. Rivadeneira, A. G. Uitterlinden, D. J. Stott, R. G. Westendorp, K. T. Khaw, A. Langhammer, L. Ferrucci, J. Gussekloo, G. R. Williams, J. P. Walsh, P. Juni, D. Aujesky, N. Rodondi and C. Thyroid Studies (2015). "Subclinical thyroid dysfunction and fracture risk: a meta-analysis." *JAMA* **313**(20): 2055-2065.

Bochukova, E., N. Schoenmakers, M. Agostini, E. Schoenmakers, O. Rajanayagam, J. M. Keogh, E. Henning, J. Reinemund, E. Gevers, M. Sarri, K. Downes, A. Offiah, A. Albanese, D. Halsall, J. W. Schwabe, M. Bain, K. Lindley, F. Muntoni, F. Vargha-Khadem, M. Dattani, I. S. Farooqi, M. Gurnell and K. Chatterjee (2012). "A mutation in the thyroid hormone receptor alpha gene." *N Engl J Med* **366**(3): 243-249.

Boelen, A., J. Boorsma, J. Kwakkel, C. W. Wieland, R. Renckens, T. J. Visser, E. Fliers and W. M. Wiersinga (2008). "Type 3 deiodinase is highly expressed in infiltrating neutrophilic granulocytes in response to acute bacterial infection." *Thyroid* **18**(10): 1095-1103.

Boelen, A., J. Kwakkel, A. Alkemade, R. Renckens, E. Kaptein, G. Kuiper, W. M. Wiersinga and T. J. Visser (2005). "Induction of type 3 deiodinase activity in inflammatory cells of mice with chronic local inflammation." *Endocrinology* **146**(12): 5128-5134.

Boersma, B., B. J. Otten, G. B. Stoeltinga and J. M. Wit (1996). "Catch-up growth after prolonged hypothyroidism." *Eur J Pediatr* **155**(5): 362-367.

Boivin, G. and P. J. Meunier (2002). "Changes in bone remodeling rate influence the degree of mineralization of bone." *Connect Tissue Res* **43**(2-3): 535-537.

Bomer, N., F. M. Cornelis, Y. F. Ramos, W. den Hollander, L. Storms, R. van der Breggen, N. Lakenberg, P. E. Slagboom, I. Meulenbelt and R. J. Lories (2016). "The effect of forced exercise on knee joints in Dio2^{-/-} mice: type II iodothyronine deiodinase-deficient mice are less prone to develop OA-like cartilage damage upon excessive mechanical stress." *Ann Rheum Dis* **75**(3): 571-577.

Bomer, N., W. den Hollander, Y. F. Ramos, S. D. Bos, R. van der Breggen, N. Lakenberg, B. A. Pepers, A. E. van Eeden, A. Darvishan, E. W. Tobi, B. J. Duijnisveld, E. B. van den Akker, B. T. Heijmans, W. M. van Roon-Mom, F. J. Verbeek, G. J. van Osch, R. G. Nelissen, P. E. Slagboom and I. Meulenbelt (2015). "Underlying molecular mechanisms of DIO2 susceptibility in symptomatic osteoarthritis." *Ann Rheum Dis* **74**(8): 1571-1579.

Bondeson, J., S. D. Wainwright, S. Lauder, N. Amos and C. E. Hughes (2006). "The role of synovial macrophages and macrophage-produced cytokines in driving aggrecanases, matrix metalloproteinases, and other destructive and inflammatory responses in osteoarthritis." *Arthritis Res Ther* **8**(6): R187.

Bookout, A. L., Y. Jeong, M. Downes, R. T. Yu, R. M. Evans and D. J. Mangelsdorf (2006). "Anatomical profiling of nuclear receptor expression reveals a hierarchical transcriptional network." Cell **126**(4): 789-799.

Bos, S. D., J. V. Bovee, B. J. Duijnisveld, E. V. Raine, W. J. van Dalen, Y. F. Ramos, R. van der Breggen, R. G. Nelissen, P. E. Slagboom, J. Loughlin and I. Meulenbelt (2012). "Increased type II deiodinase protein in OA-affected cartilage and allelic imbalance of OA risk polymorphism rs225014 at DIO2 in human OA joint tissues." Ann Rheum Dis **71**(7): 1254-1258.

Boskey, A. L. and P. G. Robey (2013). The Composition of Bone. Primer of the Metabolic Bone Diseases and Disorders of Mineral Metabolism. C. J. Rosen, Wiley-Blackwell: 49-58.

Botter, S. M., S. S. Glasson, B. Hopkins, S. Clockaerts, H. Weinans, J. P. van Leeuwen and G. J. van Osch (2009). "ADAMTS5^{-/-} mice have less subchondral bone changes after induction of osteoarthritis through surgical instability: implications for a link between cartilage and subchondral bone changes." Osteoarthritis Cartilage **17**(5): 636-645.

Boyce, B. F., L. Xing, G. Franzoso and U. Siebenlist (1999). "Required and nonessential functions of nuclear factor-kappa B in bone cells." Bone **25**(1): 137-139.

Boyde, A. and E. C. Firth (2005). "Musculoskeletal responses of 2-year-old Thoroughbred horses to early training. 8. Quantitative back-scattered electron scanning electron microscopy and confocal fluorescence microscopy of the epiphysis of the third metacarpal bone." N Z Vet J **53**(2): 123-132.

Boyden, L. M., J. Mao, J. Belsky, L. Mitzner, A. Farhi, M. A. Mitnick, D. Wu, K. Insogna and R. P. Lifton (2002). "High bone density due to a mutation in LDL-receptor-related protein 5." N Engl J Med **346**(20): 1513-1521.

Boyle, W. J., W. S. Simonet and D. L. Lacey (2003). "Osteoclast differentiation and activation." Nature **423**(6937): 337-342.

Bradley, E. W., L. R. Carpio, M. E. McGee-Lawrence, C. Castillejo Becerra, D. F. Amanatullah, L. E. Ta, M. Otero, M. B. Goldring, S. Kakar and J. J. Westendorf (2016). "Phlpp1 facilitates post-traumatic osteoarthritis and is induced by inflammation and promoter demethylation in human osteoarthritis." Osteoarthritis Cartilage **24**(6): 1021-1028.

Brent, G. A. (2012). "Mechanisms of thyroid hormone action." J Clin Invest **122**(9): 3035-3043.

Britto, J. M., A. J. Fenton, W. R. Holloway and G. C. Nicholson (1994). "Osteoblasts mediate thyroid hormone stimulation of osteoclastic bone resorption." Endocrinology **134**(1): 169-176.

Burmeister, L. A., J. Pachucki and D. L. St Germain (1997). "Thyroid hormones inhibit type 2 iodothyronine deiodinase in the rat cerebral cortex by both pre- and posttranslational mechanisms." Endocrinology **138**(12): 5231-5237.

Burr, D. B. and M. A. Gallant (2012). "Bone remodelling in osteoarthritis." Nat Rev Rheumatol **8**(11): 665-673.

Burr, D. B. and M. B. Schaffler (1997). "The involvement of subchondral mineralized tissues in osteoarthrosis: quantitative microscopic evidence." Microsc Res Tech **37**(4): 343-357.

Callegaro, A., I. Meulenbelt, M. Kloppenburg, P. E. Slagboom and J. J. Houwing-Duistermaat (2010). "Allele-sharing statistics using information on family history." Ann Hum Genet **74**(6): 547-554.

Canani, L. H., C. Capp, J. M. Dora, E. L. Meyer, M. S. Wagner, J. W. Harney, P. R. Larsen, J. L. Gross, A. C. Bianco and A. L. Maia (2005). "The type 2 deiodinase A/G

(Thr92Ala) polymorphism is associated with decreased enzyme velocity and increased insulin resistance in patients with type 2 diabetes mellitus." J Clin Endocrinol Metab **90**(6): 3472-3478.

Capelo, L. P., E. H. Beber, T. L. Fonseca and C. H. Gouveia (2009). "The monocarboxylate transporter 8 and L-type amino acid transporters 1 and 2 are expressed in mouse skeletons and in osteoblastic MC3T3-E1 cells." Thyroid **19**(2): 171-180.

Capelo, L. P., E. H. Beber, S. A. Huang, T. M. Zorn, A. C. Bianco and C. H. Gouveia (2008). "Deiodinase-mediated thyroid hormone inactivation minimizes thyroid hormone signaling in the early development of fetal skeleton." Bone **43**(5): 921-930.

Carvalho-Bianco, S. D., B. W. Kim, J. X. Zhang, J. W. Harney, R. S. Ribeiro, B. Gereben, A. C. Bianco, U. Mende and P. R. Larsen (2004). "Chronic cardiac-specific thyrotoxicosis increases myocardial beta-adrenergic responsiveness." Mol Endocrinol **18**(7): 1840-1849.

Castagna, M. G., M. Dentice, S. Cantara, R. Ambrosio, F. Maino, T. Porcelli, C. Marzocchi, C. Garbi, F. Pacini and D. Salvatore (2017). "DIO2 Thr92Ala Reduces Deiodinase-2 Activity and Serum-T3 Levels in Thyroid-Deficient Patients." J Clin Endocrinol Metab **102**(5): 1623-1630.

Castaneda, S., J. A. Roman-Blas, R. Largo and G. Herrero-Beaumont (2012). "Subchondral bone as a key target for osteoarthritis treatment." Biochem Pharmacol **83**(3): 315-323.

Chaisson, C. E., T. E. McAlindon, D. T. Felson, A. Naimark, P. W. Wilson and C. T. Sawin (1996). "Lack of association between thyroid status and chondrocalcinosis or osteoarthritis: the Framingham Osteoarthritis Study." J Rheumatol **23**(4): 711-715.

Chassande, O., A. Fraichard, K. Gauthier, F. Flamant, C. Legrand, P. Savatier, V. Laudet and J. Samarut (1997). "Identification of transcripts initiated from an internal promoter in the c-erbA alpha locus that encode inhibitors of retinoic acid receptor-alpha and triiodothyronine receptor activities." Mol Endocrinol **11**(9): 1278-1290.

Chen, F. H., K. T. Rousche and R. S. Tuan (2006). "Technology Insight: adult stem cells in cartilage regeneration and tissue engineering." Nat Clin Pract Rheumatol **2**(7): 373-382.

Chen-An, P., K. V. Andreassen, K. Henriksen, M. A. Karsdal and A. C. Bay-Jensen (2013). "Investigation of chondrocyte hypertrophy and cartilage calcification in a full-depth articular cartilage explants model." Rheumatology international **33**(2): 401-411.

Cheng, A. W., M. Bolognesi and V. B. Kraus (2012). "DIO2 modifies inflammatory responses in chondrocytes." Osteoarthritis Cartilage **20**(5): 440-445.

Cheng, S. Y., J. L. Leonard and P. J. Davis (2010). "Molecular aspects of thyroid hormone actions." Endocr Rev **31**(2): 139-170.

Cheung, M. S. (2014). Oestrogen and Thyroid Hormone Interactions in the Regulation of Bone Mass Thesis for Doctor of Philosophy (PhD), Imperial College London.

Chou, C. H., C. H. Lee, L. S. Lu, I. W. Song, H. P. Chuang, S. Y. Kuo, J. Y. Wu, Y. T. Chen, V. B. Kraus, C. C. Wu and M. T. Lee (2013). "Direct assessment of articular cartilage and underlying subchondral bone reveals a progressive gene expression change in human osteoarthritic knees." Osteoarthritis Cartilage **21**(3): 450-461.

Chou, C. H., C. C. Wu, I. W. Song, H. P. Chuang, L. S. Lu, J. H. Chang, S. Y. Kuo, C. H. Lee, J. Y. Wu, Y. T. Chen, V. B. Kraus and M. T. Lee (2013). "Genome-wide expression profiles of subchondral bone in osteoarthritis." Arthritis Res Ther **15**(6): R190.

Christley, R. (2010). "Power and Error: Increased Risk of False Positive Results in Underpowered Studies." The Open Epidemiology Journal **3**: 16-19.

Christoffolete, M. A., R. Ribeiro, P. Singru, C. Fekete, W. S. da Silva, D. F. Gordon, S. A. Huang, A. Crescenzi, J. W. Harney, E. C. Ridgway, P. R. Larsen, R. M. Lechan and A. C. Bianco (2006). "Atypical expression of type 2 iodothyronine deiodinase in thyrotrophs explains the thyroxine-mediated pituitary thyrotropin feedback mechanism." *Endocrinology* **147**(4): 1735-1743.

Clarke, B. (2008). "Normal bone anatomy and physiology." *Clin J Am Soc Nephrol* **3 Suppl 3**: S131-139.

Coats, A. M., P. Zioupos and R. M. Aspden (2003). "Material properties of subchondral bone from patients with osteoporosis or osteoarthritis by microindentation testing and electron probe microanalysis." *Calcif Tissue Int* **73**(1): 66-71.

Cox, L. G., C. C. van Donkelaar, B. van Rietbergen, P. J. Emans and K. Ito (2012). "Decreased bone tissue mineralization can partly explain subchondral sclerosis observed in osteoarthritis." *Bone* **50**(5): 1152-1161.

Coxon, F. P., K. Thompson and M. J. Rogers (2006). "Recent advances in understanding the mechanism of action of bisphosphonates." *Curr Opin Pharmacol* **6**(3): 307-312.

Crockett, J. C., M. J. Rogers, F. P. Coxon, L. J. Hocking and M. H. Helfrich (2011). "Bone remodelling at a glance." *J Cell Sci* **124**(Pt 7): 991-998.

Curcio-Morelli, C., A. M. Zavacki, M. Christoffolete, B. Gereben, B. C. de Freitas, J. W. Harney, Z. Li, G. Wu and A. C. Bianco (2003). "Deubiquitination of type 2 iodothyronine deiodinase by von Hippel-Lindau protein-interacting deubiquitinating enzymes regulates thyroid hormone activation." *J Clin Invest* **112**(2): 189-196.

Dallas, S. L., M. Prideaux and L. F. Bonewald (2013). "The osteocyte: an endocrine cell ... and more." *Endocr Rev* **34**(5): 658-690.

Daluiski, A., T. Engstrand, M. E. Bahamonde, L. W. Gamer, E. Agius, S. L. Stevenson, K. Cox, V. Rosen and K. M. Lyons (2001). "Bone morphogenetic protein-3 is a negative regulator of bone density." *Nat Genet* **27**(1): 84-88.

Das Neves Borges, P., T. L. Vincent and M. Marenzana (2017). "Automated assessment of bone changes in cross-sectional micro-CT studies of murine experimental osteoarthritis." *PLoS One* **12**(3): e0174294.

Day, J. S., M. Ding, J. C. van der Linden, I. Hvid, D. R. Sumner and H. Weinans (2001). "A decreased subchondral trabecular bone tissue elastic modulus is associated with pre-arthritis cartilage damage." *J Orthop Res* **19**(5): 914-918.

Day, T. F., X. Guo, L. Garrett-Beal and Y. Yang (2005). "Wnt/beta-catenin signaling in mesenchymal progenitors controls osteoblast and chondrocyte differentiation during vertebrate skeletogenesis." *Dev Cell* **8**(5): 739-750.

de Jesus, L. A., S. D. Carvalho, M. O. Ribeiro, M. Schneider, S. W. Kim, J. W. Harney, P. R. Larsen and A. C. Bianco (2001). "The type 2 iodothyronine deiodinase is essential for adaptive thermogenesis in brown adipose tissue." *J Clin Invest* **108**(9): 1379-1385.

de Jong, F. J., R. P. Peeters, T. den Heijer, W. M. van der Deure, A. Hofman, A. G. Uitterlinden, T. J. Visser and M. M. Breteler (2007). "The association of polymorphisms in the type 1 and 2 deiodinase genes with circulating thyroid hormone parameters and atrophy of the medial temporal lobe." *J Clin Endocrinol Metab* **92**(2): 636-640.

Debiais, F., M. Hott, A. M. Graulet and P. J. Marie (1998). "The effects of fibroblast growth factor-2 on human neonatal calvaria osteoblastic cells are differentiation stage specific." *J Bone Miner Res* **13**(4): 645-654.

Decker, R. S., E. Koyama and M. Pacifici (2014). "Genesis and morphogenesis of limb synovial joints and articular cartilage." *Matrix Biol* **39**: 5-10.

Decker, R. S., E. Koyama and M. Pacifici (2015). "Articular Cartilage: Structural and Developmental Intricacies and Questions." Curr Osteoporos Rep **13**(6): 407-414.

Demidenko, E. (2004). Kolmogorov-Smirnov Test for Image Comparison. Computational Science and Its Applications – ICCSA 2004. ICCSA 2004. Lecture Notes in Computer Science. A. Lagana, M. Gavrilova, K. V et al. Berlin, Heidelberg, Springer **3046**.

Demir, K., A. L. van Gucht, M. Buyukinan, G. Catli, Y. Ayhan, V. N. Bas, B. Dundar, B. Ozkan, M. E. Meima, W. E. Visser, R. P. Peeters and T. J. Visser (2016). "Diverse Genotypes and Phenotypes of Three Novel Thyroid Hormone Receptor-alpha Mutations." J Clin Endocrinol Metab **101**(8): 2945-2954.

Dempster, D. W., J. E. Compston, M. K. Drezner, F. H. Glorieux, J. A. Kanis, H. Malluche, P. J. Meunier, S. M. Ott, R. R. Recker and A. M. Parfitt (2013). "Standardized nomenclature, symbols, and units for bone histomorphometry: a 2012 update of the report of the ASBMR Histomorphometry Nomenclature Committee." J Bone Miner Res **28**(1): 2-17.

Dentice, M., A. Bandyopadhyay, B. Gereben, I. Callebaut, M. A. Christoffolete, B. W. Kim, S. Nissim, J. P. Mornon, A. M. Zavacki, A. Zeold, L. P. Capelo, C. Curcio-Morelli, R. Ribeiro, J. W. Harney, C. J. Tabin and A. C. Bianco (2005). "The Hedgehog-inducible ubiquitin ligase subunit WSB-1 modulates thyroid hormone activation and PTHrP secretion in the developing growth plate." Nat Cell Biol **7**(7): 698-705.

Devlin, R. D., Z. Du, R. C. Pereira, R. B. Kimble, A. N. Economides, V. Jorgetti and E. Canalis (2003). "Skeletal overexpression of noggin results in osteopenia and reduced bone formation." Endocrinology **144**(5): 1972-1978.

Di Cosmo, C., X. H. Liao, A. M. Dumitrescu, N. J. Philp, R. E. Weiss and S. Refetoff (2010). "Mice deficient in MCT8 reveal a mechanism regulating thyroid hormone secretion." J Clin Invest **120**(9): 3377-3388.

Dorwart, B. B. and H. R. Schumacher (1975). "Joint effusions, chondrocalcinosis and other rheumatic manifestations in hypothyroidism. A clinicopathologic study." Am J Med **59**(6): 780-790.

Doube, M., E. C. Firth and A. Boyde (2007). "Variations in articular calcified cartilage by site and exercise in the 18-month-old equine distal metacarpal condyle." Osteoarthritis Cartilage **15**(11): 1283-1292.

Dreher, I., N. Schutze, A. Baur, K. Hesse, D. Schneider, J. Kohrle and F. Jakob (1998). "Selenoproteins are expressed in fetal human osteoblast-like cells." Biochem Biophys Res Commun **245**(1): 101-107.

Dreier, R. (2010). "Hypertrophic differentiation of chondrocytes in osteoarthritis: the developmental aspect of degenerative joint disorders." Arthritis Res Ther **12**(5): 216.

Dumitrescu, A. M. and S. Refetoff (2013). "The syndromes of reduced sensitivity to thyroid hormone." Biochim Biophys Acta **1830**(7): 3987-4003.

Ellies, D. L., B. Viviano, J. McCarthy, J. P. Rey, N. Itasaki, S. Saunders and R. Krumlauf (2006). "Bone density ligand, Sclerostin, directly interacts with LRP5 but not LRP5G171V to modulate Wnt activity." J Bone Miner Res **21**(11): 1738-1749.

Enomoto, H., S. Shiojiri, K. Hoshi, T. Furuichi, R. Fukuyama, C. A. Yoshida, N. Kanatani, R. Nakamura, A. Mizuno, A. Zanna, K. Yano, H. Yasuda, K. Higashio, K. Takada and T. Komori (2003). "Induction of osteoclast differentiation by Runx2 through receptor activator of nuclear factor-kappa B ligand (RANKL) and osteoprotegerin regulation and partial rescue of osteoclastogenesis in Runx2^{-/-} mice by RANKL transgene." J Biol Chem **278**(26): 23971-23977.

Eriksen, E. F., L. Mosekilde and F. Melsen (1986). "Kinetics of trabecular bone resorption and formation in hypothyroidism: evidence for a positive balance per remodeling cycle." Bone **7**(2): 101-108.

Esapa, C. T., J. H. Bassett, H. Evans, P. I. Croucher, G. R. Williams and R. V. Thakker (2012). "Bone Mineral Content and Density." Curr Protoc Mouse Biol **2**(4): 365-400.

Espiard, S., F. Savagner, F. Flamant, V. Vlaeminck-Guillem, R. Guyot, M. Munier, M. d'Herbomez, W. Bourguet, G. Pinto, C. Rose, P. Rodien and J. L. Wemeau (2015). "A Novel Mutation in THRA Gene Associated With an Atypical Phenotype of Resistance to Thyroid Hormone." J Clin Endocrinol Metab **100**(8): 2841-2848.

Evangelou, E., H. J. Kerkhof, U. Styrkarsdottir, E. E. Ntzani, S. D. Bos, T. Esko, D. S. Evans, S. Metrustry, K. Panoutsopoulou, Y. F. Ramos, G. Thorleifsson, K. K. Tsilidis, N. Arden, N. Aslam, N. Bellamy, F. Birrell, F. J. Blanco, A. Carr, K. Chapman, A. G. Day-Williams, P. Deloukas, M. Doherty, G. Engstrom, H. T. Helgadottir, A. Hofman, T. Ingvarsson, H. Jonsson, A. Keis, J. C. Keurentjes, M. Kloppenburg, P. A. Lind, A. McCaskie, N. G. Martin, L. Milani, G. W. Montgomery, R. G. Nelissen, M. C. Nevitt, P. M. Nilsson, W. E. Ollier, N. Parimi, A. Rai, S. H. Ralston, M. R. Reed, J. A. Riancho, F. Rivadeneira, C. Rodriguez-Fontenla, L. Southam, U. Thorsteinsdottir, A. Tsezou, G. A. Wallis, J. M. Wilkinson, A. Gonzalez, N. E. Lane, L. S. Lohmander, J. Loughlin, A. Metspalu, A. G. Uitterlinden, I. Jonsdottir, K. Stefansson, P. E. Slagboom, E. Zeggini, I. Meulenbelt, J. P. Ioannidis, T. D. Spector, J. B. van Meurs and A. M. Valdes (2013). "A meta-analysis of genome-wide association studies identifies novel variants associated with osteoarthritis of the hip." Ann Rheum Dis.

Fang, H. and F. Beier (2014). "Mouse models of osteoarthritis: modelling risk factors and assessing outcomes." Nat Rev Rheumatol **10**(7): 413-421.

Fang, M. and M. M. Holl (2013). "Variation in type I collagen fibril nanomorphology: the significance and origin." Bonekey Rep **2**: 394.

Feng, J. Q., L. M. Ward, S. Liu, Y. Lu, Y. Xie, B. Yuan, X. Yu, F. Rauch, S. I. Davis, S. Zhang, H. Rios, M. K. Drezner, L. D. Quarles, L. F. Bonewald and K. E. White (2006). "Loss of DMP1 causes rickets and osteomalacia and identifies a role for osteocytes in mineral metabolism." Nat Genet **38**(11): 1310-1315.

Feng, X. and J. M. McDonald (2011). "Disorders of bone remodeling." Annu Rev Pathol **6**: 121-145.

Flamant, F., K. Gauthier and J. Samarut (2007). "Thyroid hormones signaling is getting more complex: STORMs are coming." Mol Endocrinol **21**(2): 321-333.

Flamant, F., A. L. Pogue, M. Plateroti, O. Chassande, K. Gauthier, N. Streichenberger, A. Mansouri and J. Samarut (2002). "Congenital hypothyroid Pax8(-/-) mutant mice can be rescued by inactivating the TRalpha gene." Mol Endocrinol **16**(1): 24-32.

Flynn, R. W., S. R. Bonellie, R. T. Jung, T. M. MacDonald, A. D. Morris and G. P. Leese (2010). "Serum thyroid-stimulating hormone concentration and morbidity from cardiovascular disease and fractures in patients on long-term thyroxine therapy." J Clin Endocrinol Metab **95**(1): 186-193.

Forrest, D., E. Hanebuth, R. J. Smeyne, N. Everds, C. L. Stewart, J. M. Wehner and T. Curran (1996). "Recessive resistance to thyroid hormone in mice lacking thyroid hormone receptor beta: evidence for tissue-specific modulation of receptor function." EMBO J **15**(12): 3006-3015.

Franzoso, G., L. Carlson, L. Xing, L. Poljak, E. W. Shores, K. D. Brown, A. Leonardi, T. Tran, B. F. Boyce and U. Siebenlist (1997). "Requirement for NF-kappaB in osteoclast and B-cell development." Genes Dev **11**(24): 3482-3496.

Freitas, F. R., L. P. Capelo, P. J. O'Shea, V. Jorgetti, A. S. Moriscot, T. S. Scanlan, G. R. Williams, T. M. Zorn and C. H. Gouveia (2005). "The thyroid hormone receptor

beta-specific agonist GC-1 selectively affects the bone development of hypothyroid rats." J Bone Miner Res **20**(2): 294-304.

Friesema, E. C., J. Jansen, H. Heuer, M. Trajkovic, K. Bauer and T. J. Visser (2006). "Mechanisms of disease: psychomotor retardation and high T3 levels caused by mutations in monocarboxylate transporter 8." Nat Clin Pract Endocrinol Metab **2**(9): 512-523.

Fuerst, M., J. Bertrand, L. Lammers, R. Dreier, F. Echtermeyer, Y. Nitschke, F. Rutsch, F. K. Schafer, O. Niggemeyer, J. Steinhagen, C. H. Lohmann, T. Pap and W. Ruther (2009). "Calcification of articular cartilage in human osteoarthritis." Arthritis Rheum **60**(9): 2694-2703.

Fumoto, T., S. Takeshita, M. Ito and K. Ikeda (2014). "Physiological functions of osteoblast lineage and T cell-derived RANKL in bone homeostasis." J Bone Miner Res **29**(4): 830-842.

Galton, V. A., M. J. Schneider, A. S. Clark and D. L. St Germain (2009). "Life without thyroxine to 3,5,3'-triiodothyronine conversion: studies in mice devoid of the 5'-deiodinases." Endocrinology **150**(6): 2957-2963.

Gaudio, A., P. Pennisi, C. Bratengeier, V. Torrisi, B. Lindner, R. A. Mangiafico, I. Pulvirenti, G. Hawa, G. Tringali and C. E. Fiore (2010). "Increased sclerostin serum levels associated with bone formation and resorption markers in patients with immobilization-induced bone loss." J Clin Endocrinol Metab **95**(5): 2248-2253.

Gauthier, K., O. Chassande, M. Plateroti, J. P. Roux, C. Legrand, B. Pain, B. Rousset, R. Weiss, J. Trouillas and J. Samarut (1999). "Different functions for the thyroid hormone receptors TRalpha and TRbeta in the control of thyroid hormone production and post-natal development." EMBO J **18**(3): 623-631.

Gauthier, K., M. Plateroti, C. B. Harvey, G. R. Williams, R. E. Weiss, S. Refetoff, J. F. Willott, V. Sundin, J. P. Roux, L. Malaval, M. Hara, J. Samarut and O. Chassande (2001). "Genetic analysis reveals different functions for the products of the thyroid hormone receptor alpha locus." Mol Cell Biol **21**(14): 4748-4760.

Gautier, P., E. Guillemare, A. Marion, J. P. Bertrand, Y. Tourneur and D. Nisato (2003). "Electrophysiologic characterization of dronedarone in guinea pig ventricular cells." Journal of cardiovascular pharmacology **41**(2): 191-202.

Geoffroy, V., M. Kneissel, B. Fournier, A. Boyde and P. Matthias (2002). "High bone resorption in adult aging transgenic mice overexpressing cbfa1/runx2 in cells of the osteoblastic lineage." Mol Cell Biol **22**(17): 6222-6233.

Gereben, B., A. M. Zavacki, S. Ribich, B. W. Kim, S. A. Huang, W. S. Simonides, A. Zeold and A. C. Bianco (2008). "Cellular and molecular basis of deiodinase-regulated thyroid hormone signaling." Endocr Rev **29**(7): 898-938.

Gerster, J. C., P. Quadri and Y. Saudan (1985). "Hypothyroidism presenting as destructive arthropathy of the fingers." Postgrad Med J **61**(712): 157-159.

Gillan, M. M., R. H. Scofield and J. B. Harley (2002). "Hashimoto's thyroiditis presenting as bilateral knee arthropathy." J Okla State Med Assoc **95**(5): 323-325.

Glade, M. J., Y. S. Kanwar and P. H. Stern (1994). "Insulin and thyroid hormones stimulate matrix metabolism in primary cultures of articular chondrocytes from young rabbits independently and in combination." Connect Tissue Res **31**(1): 37-44.

Glass, D. A., 2nd, P. Bialek, J. D. Ahn, M. Starbuck, M. S. Patel, H. Clevers, M. M. Taketo, F. Long, A. P. McMahon, R. A. Lang and G. Karsenty (2005). "Canonical Wnt signaling in differentiated osteoblasts controls osteoclast differentiation." Dev Cell **8**(5): 751-764.

Glasson, S. and C. Little (2011). "The recent paper "Multimodal imaging demonstrates concomitant changes in bone and cartilage after destabilization of the medial meniscus

and increased joint laxity". Osteoarthritis Cartilage **19**(8): 1076-1077; author reply 1078.

Glasson, S. S., R. Askew, B. Sheppard, B. Carito, T. Blanchet, H. L. Ma, C. R. Flannery, D. Peluso, K. Kanki, Z. Yang, M. K. Majumdar and E. A. Morris (2005). "Deletion of active ADAMTS5 prevents cartilage degradation in a murine model of osteoarthritis." Nature **434**(7033): 644-648.

Glasson, S. S., T. Blanchet, B. Hopkins, M. Ma, D. Peluso, M. Rivera-Bermudez and E. A. Morris (2006). "A24 MURINE SURGICALLY-INDUCED OSTEOARTHRITIS SEX AND STRAIN DEPENDENT." Osteoarthritis and Cartilage **14**: S29.

Glasson, S. S., T. J. Blanchet and E. A. Morris (2007). "The surgical destabilization of the medial meniscus (DMM) model of osteoarthritis in the 129/SvEv mouse." Osteoarthritis and cartilage / OARS, Osteoarthritis Research Society **15**(9): 1061-1069.

Glasson, S. S., M. G. Chambers, W. B. Van Den Berg and C. B. Little (2010). "The OARSI histopathology initiative - recommendations for histological assessments of osteoarthritis in the mouse." Osteoarthritis Cartilage **18 Suppl 3**: S17-23.

Gloss, B., S. Trost, W. Bluhm, E. Swanson, R. Clark, R. Winkfein, K. Janzen, W. Giles, O. Chassande, J. Samarut and W. Dillmann (2001). "Cardiac ion channel expression and contractile function in mice with deletion of thyroid hormone receptor alpha or beta." Endocrinology **142**(2): 544-550.

Golding, D. N. (1993). "Rheumatism and the thyroid." J R Soc Med **86**(3): 130-132.

Goldring, M. B. and S. R. Goldring (2010). "Articular cartilage and subchondral bone in the pathogenesis of osteoarthritis." Ann N Y Acad Sci **1192**: 230-237.

Goldring, S. R. and M. B. Goldring (2016). "Changes in the osteochondral unit during osteoarthritis: structure, function and cartilage-bone crosstalk." Nat Rev Rheumatol **12**(11): 632-644.

Gong, Y., R. B. Slee, N. Fukai, G. Rawadi, S. Roman-Roman, A. M. Reginato, H. Wang, T. Cundy, F. H. Glorieux, D. Lev, M. Zacharin, K. Oexle, J. Marcelino, W. Suwairi, S. Heeger, G. Sabatakos, S. Apte, W. N. Adkins, J. Allgrove, M. Arslan-Kirchner, J. A. Batch, P. Beighton, G. C. Black, R. G. Boles, L. M. Boon, C. Borrone, H. G. Brunner, G. F. Carle, B. Dallapiccola, A. De Paepe, B. Floege, M. L. Halfhide, B. Hall, R. C. Hennekam, T. Hirose, A. Jans, H. Juppner, C. A. Kim, K. Keppler-Noreuil, A. Kohlschuetter, D. LaCombe, M. Lambert, E. Lemyre, T. Letteboer, L. Peltonen, R. S. Ramesar, M. Romanengo, H. Somer, E. Steichen-Gersdorf, B. Steinmann, B. Sullivan, A. Superti-Furga, W. Swoboda, M. J. van den Boogaard, W. Van Hul, M. Vikkula, M. Votruba, B. Zabel, T. Garcia, R. Baron, B. R. Olsen, M. L. Warman and G. Osteoporosis-Pseudoglioma Syndrome Collaborative (2001). "LDL receptor-related protein 5 (LRP5) affects bone accrual and eye development." Cell **107**(4): 513-523.

Gouveia, C. H., M. A. Christoffolete, C. R. Zaitune, J. M. Dora, J. W. Harney, A. L. Maia and A. C. Bianco (2005). "Type 2 iodothyronine selenodeiodinase is expressed throughout the mouse skeleton and in the MC3T3-E1 mouse osteoblastic cell line during differentiation." Endocrinology **146**(1): 195-200.

Gouveia, C. H., J. J. Schultz, A. C. Bianco and G. A. Brent (2001). "Thyroid hormone stimulation of osteocalcin gene expression in ROS 17/2.8 cells is mediated by transcriptional and post-transcriptional mechanisms." J Endocrinol **170**(3): 667-675.

Gowen, L. C., D. N. Petersen, A. L. Mansolf, H. Qi, J. L. Stock, G. T. Tkalecivic, H. A. Simmons, D. T. Crawford, K. L. Chidsey-Frink, H. Z. Ke, J. D. McNeish and T. A. Brown (2003). "Targeted disruption of the osteoblast/osteocyte factor 45 gene (OF45) results in increased bone formation and bone mass." J Biol Chem **278**(3): 1998-2007.

Greenspan, S. L. and F. S. Greenspan (1999). "The effect of thyroid hormone on skeletal integrity." Ann Intern Med **130**(9): 750-758.

Grigoriadis, A. E., Z. Q. Wang, M. G. Cecchini, W. Hofstetter, R. Felix, H. A. Fleisch and E. F. Wagner (1994). "c-Fos: a key regulator of osteoclast-macrophage lineage determination and bone remodeling." Science **266**(5184): 443-448.

Grynepas, M. (1993). "Age and disease-related changes in the mineral of bone." Calcif Tissue Int **53 Suppl 1**: S57-64.

Grynepas, M. D., B. Alpert, I. Katz, I. Lieberman and K. P. Pritzker (1991). "Subchondral bone in osteoarthritis." Calcif Tissue Int **49**(1): 20-26.

Guerra, A., M. R. Sapio, M. Carrano, V. Di Stasi, A. Volpe, A. Murino, G. Izzo and M. Vitale (2013). "Prevalence of Dio2(T92A) polymorphism and its association with thyroid autoimmunity." J Endocrinol Invest **36**(5): 303-306.

Gumieniak, O., T. S. Perlstein, J. S. Williams, P. N. Hopkins, N. J. Brown, B. A. Raby and G. H. Williams (2007). "Ala92 type 2 deiodinase allele increases risk for the development of hypertension." Hypertension **49**(3): 461-466.

Hamidouche, Z., O. Fromigue, U. Nuber, P. Vaudin, J. C. Pages, R. Ebert, F. Jakob, H. Miraoui and P. J. Marie (2010). "Autocrine fibroblast growth factor 18 mediates dexamethasone-induced osteogenic differentiation of murine mesenchymal stem cells." J Cell Physiol **224**(2): 509-515.

Harvey, C. B., J. H. Bassett, P. Maruvada, P. M. Yen and G. R. Williams (2007). "The rat thyroid hormone receptor (TR) Deltabeta3 displays cell-, TR isoform-, and thyroid hormone response element-specific actions." Endocrinology **148**(4): 1764-1773.

Hayami, T., M. Pickarski, G. A. Wesolowski, J. McLane, A. Bone, J. Destefano, G. A. Rodan and L. T. Duong (2004). "The role of subchondral bone remodeling in osteoarthritis: reduction of cartilage degeneration and prevention of osteophyte formation by alendronate in the rat anterior cruciate ligament transection model." Arthritis Rheum **50**(4): 1193-1206.

Hayami, T., Y. Zhuo, G. A. Wesolowski, M. Pickarski and T. Duong le (2012). "Inhibition of cathepsin K reduces cartilage degeneration in the anterior cruciate ligament transection rabbit and murine models of osteoarthritis." Bone **50**(6): 1250-1259.

Hedgecock, N. L., T. Hadi, A. A. Chen, S. B. Curtiss, R. B. Martin and S. J. Hazelwood (2007). "Quantitative regional associations between remodeling, modeling, and osteocyte apoptosis and density in rabbit tibial midshafts." Bone **40**(3): 627-637.

Heinemeier, K. M., P. Schjerling, J. Heinemeier, M. B. Moller, M. R. Krogsgaard, T. Grum-Schwensen, M. M. Petersen and M. Kjaer (2016). "Radiocarbon dating reveals minimal collagen turnover in both healthy and osteoarthritic human cartilage." Sci Transl Med **8**(346): 346ra390.

Heino, T. J., T. A. Hentunen and H. K. Vaananen (2002). "Osteocytes inhibit osteoclastic bone resorption through transforming growth factor-beta: enhancement by estrogen." J Cell Biochem **85**(1): 185-197.

Hellevik, A. I., M. B. Johnsen, A. Langhammer, A. M. Fenstad, O. Furnes, K. Storheim, J. A. Zwart, G. Flugsrud and L. Nordsletten (2017). "Incidence of total hip or knee replacement due to osteoarthritis in relation to thyroid function: a prospective cohort study (The Nord-Trondelag Health Study)." BMC Musculoskelet Disord **18**(1): 201.

Hendricson, A. S., T. Havdrup and H. Telhag (1982). "The effect of growth hormone and thyroxine on adult joint cartilage." Clin Orthop Relat Res(162): 270-275.

Henry, S. P., C. W. Jang, J. M. Deng, Z. Zhang, R. R. Behringer and B. de Crombrughe (2009). "Generation of aggrecan-CreERT2 knockin mice for inducible Cre activity in adult cartilage." Genesis **47**(12): 805-814.

Hernandez, A., M. E. Martinez, S. Fiering, V. A. Galton and D. St Germain (2006). "Type 3 deiodinase is critical for the maturation and function of the thyroid axis." J Clin Invest **116**(2): 476-484.

Hernandez, A., M. E. Martinez, X. H. Liao, J. Van Sande, S. Refetoff, V. A. Galton and D. L. St Germain (2007). "Type 3 deiodinase deficiency results in functional abnormalities at multiple levels of the thyroid axis." Endocrinology **148**(12): 5680-5687.

Hilgsmann, M., C. Cooper, N. Arden, M. Boers, J. C. Branco, M. Luisa Brandi, O. Bruyere, F. Guillemin, M. C. Hochberg, D. J. Hunter, J. A. Kanis, T. K. Kvien, A. Laslop, J. P. Pelletier, D. Pinto, S. Reiter-Niesert, R. Rizzoli, L. C. Rovati, J. L. Severens, S. Silverman, Y. Tsouderos, P. Tugwell and J. Y. Reginster (2013). "Health economics in the field of osteoarthritis: an expert's consensus paper from the European Society for Clinical and Economic Aspects of Osteoporosis and Osteoarthritis (ESCEO)." Semin Arthritis Rheum **43**(3): 303-313.

Hill, T. P., D. Spater, M. M. Taketo, W. Birchmeier and C. Hartmann (2005). "Canonical Wnt/beta-catenin signaling prevents osteoblasts from differentiating into chondrocytes." Dev Cell **8**(5): 727-738.

Himeno, M., H. Enomoto, W. Liu, K. Ishizeki, S. Nomura, Y. Kitamura and T. Komori (2002). "Impaired vascular invasion of Cbfa1-deficient cartilage engrafted in the spleen." J Bone Miner Res **17**(7): 1297-1305.

Hoegh-Andersen, P., L. B. Tanko, T. L. Andersen, C. V. Lundberg, J. A. Mo, A. M. Heegaard, J. M. Delaisse and S. Christgau (2004). "Ovariectomized rats as a model of postmenopausal osteoarthritis: validation and application." Arthritis Res Ther **6**(2): R169-180.

Holder, N. (1977). "An experimental investigation into the early development of the chick elbow joint." J Embryol Exp Morphol **39**: 115-127.

Holmen, S. L., C. R. Zylstra, A. Mukherjee, R. E. Sigler, M. C. Faugere, M. L. Bouxsein, L. Deng, T. L. Clemens and B. O. Williams (2005). "Essential role of beta-catenin in postnatal bone acquisition." J Biol Chem **280**(22): 21162-21168.

Huang, B. K., L. A. Golden, G. Tarjan, L. D. Madison and P. H. Stern (2000). "Insulin-like growth factor I production is essential for anabolic effects of thyroid hormone in osteoblasts." J Bone Miner Res **15**(2): 188-197.

Huebner, J. L., M. A. Hanes, B. Beekman, J. M. TeKoppele and V. B. Kraus (2002). "A comparative analysis of bone and cartilage metabolism in two strains of guinea-pig with varying degrees of naturally occurring osteoarthritis." Osteoarthritis Cartilage **10**(10): 758-767.

Huesa, C., A. C. Ortiz, L. Dunning, L. McGavin, L. Bennett, K. McIntosh, A. Crilly, M. Kurowska-Stolarska, R. Plevin, R. J. van 't Hof, A. D. Rowan, I. B. McInnes, C. S. Goodyear, J. C. Lockhart and W. R. Ferrell (2015). "Proteinase-activated receptor 2 modulates OA-related pain, cartilage and bone pathology." Ann Rheum Dis.

Hugle, T. and J. Geurts (2017). "What drives osteoarthritis?-synovial versus subchondral bone pathology." Rheumatology (Oxford) **56**(9): 1461-1471.

Hunziker, E. B., E. Kapfinger and J. Geiss (2007). "The structural architecture of adult mammalian articular cartilage evolves by a synchronized process of tissue resorption and neoformation during postnatal development." Osteoarthritis Cartilage **15**(4): 403-413.

Hyde, G., R. P. Boot-Handford and G. A. Wallis (2008). "Col2a1 lineage tracing reveals that the meniscus of the knee joint has a complex cellular origin." J Anat **213**(5): 531-538.

Hyde, G., S. Dover, A. Aszodi, G. A. Wallis and R. P. Boot-Handford (2007). "Lineage tracing using matrilin-1 gene expression reveals that articular chondrocytes exist as the joint interzone forms." Dev Biol **304**(2): 825-833.

Hyp_Consortium (1995). "A gene (PEX) with homologies to endopeptidases is mutated in patients with X-linked hypophosphatemic rickets. The HYP Consortium." Nat Genet **11**(2): 130-136.

Ijiri, K., L. F. Zerbini, H. Peng, H. H. Otu, K. Tsuchimochi, M. Otero, C. Dragomir, N. Walsh, B. E. Bierbaum, D. Mattingly, G. van Flandern, S. Komiya, T. Aigner, T. A. Libermann and M. B. Goldring (2008). "Differential expression of GADD45beta in normal and osteoarthritic cartilage: potential role in homeostasis of articular chondrocytes." Arthritis Rheum **58**(7): 2075-2087.

Imhof, H., I. Sulzbacher, S. Grampp, C. Czerny, S. Youssefzadeh and F. Kainberger (2000). "Subchondral bone and cartilage disease: a rediscovered functional unit." Invest Radiol **35**(10): 581-588.

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

Ishida, N., K. Hayashi, M. Hoshijima, T. Ogawa, S. Koga, Y. Miyatake, M. Kumegawa, T. Kimura and T. Takeya (2002). "Large scale gene expression analysis of osteoclastogenesis in vitro and elucidation of NFAT2 as a key regulator." J Biol Chem **277**(43): 41147-41156.

Ishikawa, Y., B. R. Genge, R. E. Wuthier and L. N. Wu (1998). "Thyroid hormone inhibits growth and stimulates terminal differentiation of epiphyseal growth plate chondrocytes." J Bone Miner Res **13**(9): 1398-1411.

Jacob, A. L., C. Smith, J. Partanen and D. M. Ornitz (2006). "Fibroblast growth factor receptor 1 signaling in the osteo-chondrogenic cell lineage regulates sequential steps of osteoblast maturation." Dev Biol **296**(2): 315-328.

Jeon, E., Y. R. Yun, W. Kang, S. Lee, Y. H. Koh, H. W. Kim, C. K. Suh and J. H. Jang (2012). "Investigating the role of FGF18 in the cultivation and osteogenic differentiation of mesenchymal stem cells." PLoS One **7**(8): e43982.

Jiang, J., N. L. Leong, J. C. Mung, C. Hidaka and H. H. Lu (2008). "Interaction between zonal populations of articular chondrocytes suppresses chondrocyte mineralization and this process is mediated by PTHrP." Osteoarthritis Cartilage **16**(1): 70-82.

Johnson, K. A., D. van Etten, N. Nanda, R. M. Graham and R. A. Terkeltaub (2003). "Distinct transglutaminase 2-independent and transglutaminase 2-dependent pathways mediate articular chondrocyte hypertrophy." J Biol Chem **278**(21): 18824-18832.

Kadri, A., H. K. Ea, C. Bazille, D. Hannouche, F. Liote and M. E. Cohen-Solal (2008). "Osteoprotegerin inhibits cartilage degradation through an effect on trabecular bone in murine experimental osteoarthritis." Arthritis Rheum **58**(8): 2379-2386.

Kadri, A., T. Funck-Brentano, H. Lin, H. K. Ea, D. Hannouche, C. Marty, F. Liote, V. Geoffroy and M. E. Cohen-Solal (2010). "Inhibition of bone resorption blunts osteoarthritis in mice with high bone remodelling." Ann Rheum Dis **69**(8): 1533-1538.

Kahaly, G. J. and W. H. Dillmann (2005). "Thyroid hormone action in the heart." Endocr Rev **26**(5): 704-728.

Kamekura, S., K. Hoshi, T. Shimoaka, U. Chung, H. Chikuda, T. Yamada, M. Uchida, N. Ogata, A. Seichi, K. Nakamura and H. Kawaguchi (2005). "Osteoarthritis development in novel experimental mouse models induced by knee joint instability." Osteoarthritis Cartilage **13**(7): 632-641.

Kamiya, N., T. Kobayashi, Y. Mochida, P. B. Yu, M. Yamauchi, H. M. Kronenberg and Y. Mishina (2010). "Wnt inhibitors Dkk1 and Sost are downstream targets of BMP

signaling through the type IA receptor (BMPRIA) in osteoblasts." J Bone Miner Res **25**(2): 200-210.

Kamiya, N., L. Ye, T. Kobayashi, Y. Mochida, M. Yamauchi, H. M. Kronenberg, J. Q. Feng and Y. Mishina (2008). "BMP signaling negatively regulates bone mass through sclerostin by inhibiting the canonical Wnt pathway." Development **135**(22): 3801-3811.

Kanatani, M., T. Sugimoto, H. Sowa, T. Kobayashi, M. Kanzawa and K. Chihara (2004). "Thyroid hormone stimulates osteoclast differentiation by a mechanism independent of RANKL-RANK interaction." J Cell Physiol **201**(1): 17-25.

Kaneshige, M., K. Kaneshige, X. Zhu, A. Dace, L. Garrett, T. A. Carter, R. Kazlauskaitė, D. G. Pankratz, A. Wynshaw-Boris, S. Refetoff, B. Weintraub, M. C. Willingham, C. Barlow and S. Cheng (2000). "Mice with a targeted mutation in the thyroid hormone beta receptor gene exhibit impaired growth and resistance to thyroid hormone." Proc Natl Acad Sci U S A **97**(24): 13209-13214.

Kaneshige, M., H. Suzuki, K. Kaneshige, J. Cheng, H. Wimbrow, C. Barlow, M. C. Willingham and S. Cheng (2001). "A targeted dominant negative mutation of the thyroid hormone alpha 1 receptor causes increased mortality, infertility, and dwarfism in mice." Proc Natl Acad Sci U S A **98**(26): 15095-15100.

Karlsson, C., T. Dehne, A. Lindahl, M. Brittberg, A. Pruss, M. Sittinger and J. Ringe (2010). "Genome-wide expression profiling reveals new candidate genes associated with osteoarthritis." Osteoarthritis Cartilage **18**(4): 581-592.

Karsdal, M. A., D. J. Leeming, E. B. Dam, K. Henriksen, P. Alexandersen, P. Pastoureau, R. D. Altman and C. Christiansen (2008). "Should subchondral bone turnover be targeted when treating osteoarthritis?" Osteoarthritis Cartilage **16**(6): 638-646.

Kato, M., M. S. Patel, R. Levasseur, I. Lobov, B. H. Chang, D. A. Glass, 2nd, C. Hartmann, L. Li, T. H. Hwang, C. F. Brayton, R. A. Lang, G. Karsenty and L. Chan (2002). "Cbfal-independent decrease in osteoblast proliferation, osteopenia, and persistent embryonic eye vascularization in mice deficient in Lrp5, a Wnt coreceptor." J Cell Biol **157**(2): 303-314.

Kennedy, O. D., B. C. Herman, D. M. Laudier, R. J. Majeska, H. B. Sun and M. B. Schaffler (2012). "Activation of resorption in fatigue-loaded bone involves both apoptosis and active pro-osteoclastogenic signaling by distinct osteocyte populations." Bone **50**(5): 1115-1122.

Kennell, J. A. and O. A. MacDougald (2005). "Wnt signaling inhibits adipogenesis through beta-catenin-dependent and -independent mechanisms." J Biol Chem **280**(25): 24004-24010.

Kerkhof, H. J., R. J. Lories, I. Meulenbelt, I. Jonsdottir, A. M. Valdes, P. Arp, T. Ingvarsson, M. Jhamai, H. Jonsson, L. Stolk, G. Thorleifsson, G. Zhai, F. Zhang, Y. Zhu, R. van der Breggen, A. Carr, M. Doherty, S. Doherty, D. T. Felson, A. Gonzalez, B. V. Halldorsson, D. J. Hart, V. B. Hauksson, A. Hofman, J. P. Ioannidis, M. Kloppenburg, N. E. Lane, J. Loughlin, F. P. Luyten, M. C. Nevitt, N. Parimi, H. A. Pols, F. Rivadeneira, E. P. Slagboom, U. Styrkarsdottir, A. Tsezou, T. van de Putte, J. Zmuda, T. D. Spector, K. Stefansson, A. G. Uitterlinden and J. B. van Meurs (2010). "A genome-wide association study identifies an osteoarthritis susceptibility locus on chromosome 7q22." Arthritis Rheum **62**(2): 499-510.

Kerkhof, H. J., I. Meulenbelt, T. Akune, N. K. Arden, A. Aromaa, S. M. Bierma-Zeinstra, A. Carr, C. Cooper, J. Dai, M. Doherty, S. A. Doherty, D. Felson, A. Gonzalez, A. Gordon, A. Harilainen, D. J. Hart, V. B. Hauksson, M. Heliovaara, A. Hofman, S. Ikegawa, T. Ingvarsson, Q. Jiang, H. Jonsson, I. Jonsdottir, H. Kawaguchi,

M. Kloppenburg, U. M. Kujala, N. E. Lane, P. Leino-Arjas, L. S. Lohmander, F. P. Luyten, K. N. Malizos, M. Nakajima, M. C. Nevitt, H. A. Pols, F. Rivadeneira, D. Shi, E. Slagboom, T. D. Spector, K. Stefansson, A. Sudo, A. Tamm, A. E. Tamm, A. Tsezou, A. Uchida, A. G. Uitterlinden, J. M. Wilkinson, N. Yoshimura, A. M. Valdes and J. B. van Meurs (2011). "Recommendations for standardization and phenotype definitions in genetic studies of osteoarthritis: the TREAT-OA consortium." Osteoarthritis Cartilage **19**(3): 254-264.

Kester, M. H., M. J. Toussaint, C. A. Punt, R. Matondo, A. M. Aarnio, V. M. Darras, M. E. Everts, A. de Bruin and T. J. Visser (2009). "Large induction of type III deiodinase expression after partial hepatectomy in the regenerating mouse and rat liver." Endocrinology **150**(1): 540-545.

Kiani, C., L. Chen, Y. J. Wu, A. J. Yee and B. B. Yang (2002). "Structure and function of aggrecan." Cell Res **12**(1): 19-32.

Kim, H. Y. and S. Mohan (2013). "Role and Mechanisms of Actions of Thyroid Hormone on the Skeletal Development." Bone Res **1**(2): 146-161.

Kokabu, S., L. Gamer, K. Cox, J. Lowery, K. Tsuji, R. Raz, A. Economides, T. Katagiri and V. Rosen (2012). "BMP3 suppresses osteoblast differentiation of bone marrow stromal cells via interaction with Acvr2b." Mol Endocrinol **26**(1): 87-94.

Komori, T. (2010). "Regulation of bone development and extracellular matrix protein genes by RUNX2." Cell Tissue Res **339**(1): 189-195.

Komori, T., H. Yagi, S. Nomura, A. Yamaguchi, K. Sasaki, K. Deguchi, Y. Shimizu, R. T. Bronson, Y. H. Gao, M. Inada, M. Sato, R. Okamoto, Y. Kitamura, S. Yoshiki and T. Kishimoto (1997). "Targeted disruption of Cbfa1 results in a complete lack of bone formation owing to maturational arrest of osteoblasts." Cell **89**(5): 755-764.

Koyama, E., Y. Shibukawa, M. Nagayama, H. Sugito, B. Young, T. Yuasa, T. Okabe, T. Ochiai, N. Kamiya, R. B. Rountree, D. M. Kingsley, M. Iwamoto, M. Enomoto-Iwamoto and M. Pacifici (2008). "A distinct cohort of progenitor cells participates in synovial joint and articular cartilage formation during mouse limb skeletogenesis." Dev Biol **316**(1): 62-73.

Kramer, I., C. Halleux, H. Keller, M. Pegurri, J. H. Gooi, P. B. Weber, J. Q. Feng, L. F. Bonewald and M. Kneissel (2010). "Osteocyte Wnt/beta-catenin signaling is required for normal bone homeostasis." Mol Cell Biol **30**(12): 3071-3085.

Kraus, V. B., J. M. Jordan, M. Doherty, A. G. Wilson, R. Moskowitz, M. Hochberg, R. Loeser, M. Hooper, J. B. Renner, M. M. Crane, P. Hastie, S. Sundseth and U. Atif (2007). "The Genetics of Generalized Osteoarthritis (GOGO) study: study design and evaluation of osteoarthritis phenotypes." Osteoarthritis Cartilage **15**(2): 120-127.

Kraus, V. B., G. McDaniel, J. L. Huebner, T. V. Stabler, C. F. Pieper, S. W. Shipes, N. A. Petry, P. S. Low, J. Shen, T. A. McNearney and P. Mitchell (2016). "Direct in vivo evidence of activated macrophages in human osteoarthritis." Osteoarthritis Cartilage **24**(9): 1613-1621.

Kronenberg, H. M. (2003). "Developmental regulation of the growth plate." Nature **423**(6937): 332-336.

Kular, J., J. Tickner, S. M. Chim and J. Xu (2012). "An overview of the regulation of bone remodelling at the cellular level." Clin Biochem **45**(12): 863-873.

Kuliwaba, J. S., D. M. Findlay, G. J. Atkins, M. R. Forwood and N. L. Fazzalari (2000). "Enhanced expression of osteocalcin mRNA in human osteoarthritic trabecular bone of the proximal femur is associated with decreased expression of interleukin-6 and interleukin-11 mRNA." J Bone Miner Res **15**(2): 332-341.

Kumarasinghe, D. D., E. Perilli, H. Tsangari, L. Truong, J. S. Kuliwaba, B. Hopwood, G. J. Atkins and N. L. Fazzalari (2010). "Critical molecular regulators,

histomorphometric indices and their correlations in the trabecular bone in primary hip osteoarthritis." Osteoarthritis Cartilage **18**(10): 1337-1344.

Kwakkel, J., O. V. Surovtseva, E. M. de Vries, J. Stap, E. Fliers and A. Boelen (2014). "A novel role for the thyroid hormone-activating enzyme type 2 deiodinase in the inflammatory response of macrophages." Endocrinology **155**(7): 2725-2734.

Ladenson, P. W., J. D. Kristensen, E. C. Ridgway, A. G. Olsson, B. Carlsson, I. Klein, J. D. Baxter and B. Angelin (2010). "Use of the thyroid hormone analogue eprotirome in statin-treated dyslipidemia." N Engl J Med **362**(10): 906-916.

Lammel Lindemann, J. and P. Webb (2016). "Sobetirome: the past, present and questions about the future." Expert Opin Ther Targets **20**(2): 145-149.

Larsen, P. R. (2009). "Type 2 iodothyronine deiodinase in human skeletal muscle: new insights into its physiological role and regulation." J Clin Endocrinol Metab **94**(6): 1893-1895.

LeBron, B. A., A. E. Pekary, C. Mirell, T. J. Hahn and J. M. Hershman (1989). "Thyroid hormone 5'-deiodinase activity, nuclear binding, and effects on mitogenesis in UMR-106 osteoblastic osteosarcoma cells." J Bone Miner Res **4**(2): 173-178.

Lee, B., K. Thirunavukkarasu, L. Zhou, L. Pastore, A. Baldini, J. Hecht, V. Geoffroy, P. Ducy and G. Karsenty (1997). "Missense mutations abolishing DNA binding of the osteoblast-specific transcription factor OSF2/CBFA1 in cleidocranial dysplasia." Nat Genet **16**(3): 307-310.

Lee, J. K., C. A. Gegg, J. C. Hu, A. H. Reddi and K. A. Athanasiou (2015). "Thyroid hormones enhance the biomechanical functionality of scaffold-free neocartilage." Arthritis Res Ther **17**: 28.

Lee, K. S., H. J. Kim, Q. L. Li, X. Z. Chi, C. Ueta, T. Komori, J. M. Wozney, E. G. Kim, J. Y. Choi, H. M. Ryoo and S. C. Bae (2000). "Runx2 is a common target of transforming growth factor beta1 and bone morphogenetic protein 2, and cooperation between Runx2 and Smad5 induces osteoblast-specific gene expression in the pluripotent mesenchymal precursor cell line C2C12." Mol Cell Biol **20**(23): 8783-8792.

Li, B. and R. M. Aspden (1997). "Mechanical and material properties of the subchondral bone plate from the femoral head of patients with osteoarthritis or osteoporosis." Ann Rheum Dis **56**(4): 247-254.

Li, G., J. Yin, J. Gao, T. S. Cheng, N. J. Pavlos, C. Zhang and M. H. Zheng (2013). "Subchondral bone in osteoarthritis: insight into risk factors and microstructural changes." Arthritis Res Ther **15**(6): 223.

Li, W. W., C. Le Goascogne, M. Rmauge, M. Schumacher, M. Pierre and F. Courtin (2001). "Induction of type 3 iodothyronine deiodinase by nerve injury in the rat peripheral nervous system." Endocrinology **142**(12): 5190-5197.

Little, C. B., A. Barai, D. Burkhardt, S. M. Smith, A. J. Fosang, Z. Werb, M. Shah and E. W. Thompson (2009). "Matrix metalloproteinase 13-deficient mice are resistant to osteoarthritic cartilage erosion but not chondrocyte hypertrophy or osteophyte development." Arthritis Rheum **60**(12): 3723-3733.

Little, C. B. and D. J. Hunter (2013). "Post-traumatic osteoarthritis: from mouse models to clinical trials." Nat Rev Rheumatol **9**(8): 485-497.

Little, C. B. and S. Zaki (2012). "What constitutes an "animal model of osteoarthritis"-the need for consensus?" Osteoarthritis Cartilage **20**(4): 261-267.

Litwic, A., M. H. Edwards, E. M. Dennison and C. Cooper (2013). "Epidemiology and burden of osteoarthritis." Br Med Bull **105**: 185-199.

Liu, C. F., W. E. Samsa, G. Zhou and V. Lefebvre (2017). "Transcriptional control of chondrocyte specification and differentiation." Semin Cell Dev Biol **62**: 34-49.

Liu, S., R. Guo, L. G. Simpson, Z. S. Xiao, C. E. Burnham and L. D. Quarles (2003). "Regulation of fibroblastic growth factor 23 expression but not degradation by PHEX." J Biol Chem **278**(39): 37419-37426.

Liu, S., P. S. Rowe, L. Vierthaler, J. Zhou and L. D. Quarles (2007). "Phosphorylated acidic serine-aspartate-rich MEPE-associated motif peptide from matrix extracellular phosphoglycoprotein inhibits phosphate regulating gene with homologies to endopeptidases on the X-chromosome enzyme activity." J Endocrinol **192**(1): 261-267.

Liu, Z., J. Xu, J. S. Colvin and D. M. Ornitz (2002). "Coordination of chondrogenesis and osteogenesis by fibroblast growth factor 18." Genes Dev **16**(7): 859-869.

Loeser, R. F., S. R. Goldring, C. R. Scanzello and M. B. Goldring (2012). "Osteoarthritis: a disease of the joint as an organ." Arthritis Rheum **64**(6): 1697-1707.

Loeser, R. F., A. L. Olex, M. A. McNulty, C. S. Carlson, M. F. Callahan, C. M. Ferguson, J. Chou, X. Leng and J. S. Fetrow (2012). "Microarray analysis reveals age-related differences in gene expression during the development of osteoarthritis in mice." Arthritis Rheum **64**(3): 705-717.

Lohmander, L. S., P. M. Englund, L. L. Dahl and E. M. Roos (2007). "The long-term consequence of anterior cruciate ligament and meniscus injuries: osteoarthritis." Am J Sports Med **35**(10): 1756-1769.

Long, F. (2011). "Building strong bones: molecular regulation of the osteoblast lineage." Nat Rev Mol Cell Biol **13**(1): 27-38.

Lories, R. J. and F. P. Luyten (2011). "The bone-cartilage unit in osteoarthritis." Nat Rev Rheumatol **7**(1): 43-49.

Lotz, M. (2012). "Osteoarthritis year 2011 in review: biology." Osteoarthritis Cartilage **20**(3): 192-196.

Ludin, A., J. J. Sela, A. Schroeder, Y. Samuni, D. W. Nitzan and G. Amir (2013). "Injection of vascular endothelial growth factor into knee joints induces osteoarthritis in mice." Osteoarthritis Cartilage **21**(3): 491-497.

Lui, J. C., M. Chau, W. Chen, C. S. Cheung, J. Hanson, J. Rodriguez-Canales, O. Nilsson and J. Baron (2015). "Spatial regulation of gene expression during growth of articular cartilage in juvenile mice." Pediatr Res **77**(3): 406-415.

Lyons, T. J., S. F. McClure, R. W. Stoddart and J. McClure (2006). "The normal human chondro-osseous junctional region: evidence for contact of uncalcified cartilage with subchondral bone and marrow spaces." BMC Musculoskelet Disord **7**: 52.

Ma, H. L., T. J. Blanchet, D. Peluso, B. Hopkins, E. A. Morris and S. S. Glasson (2007). "Osteoarthritis severity is sex dependent in a surgical mouse model." Osteoarthritis Cartilage **15**(6): 695-700.

Macchia, P. E., Y. Takeuchi, T. Kawai, K. Cua, K. Gauthier, O. Chassande, H. Seo, Y. Hayashi, J. Samarut, Y. Murata, R. E. Weiss and S. Refetoff (2001). "Increased sensitivity to thyroid hormone in mice with complete deficiency of thyroid hormone receptor alpha." Proc Natl Acad Sci U S A **98**(1): 349-354.

Makihira, S., W. Yan, H. Murakami, M. Furukawa, T. Kawai, H. Nikawa, E. Yoshida, T. Hamada, Y. Okada and Y. Kato (2003). "Thyroid hormone enhances aggrecanase-2/ADAM-TS5 expression and proteoglycan degradation in growth plate cartilage." Endocrinology **144**(6): 2480-2488.

Mansour, J. M. (2003). Biomechanics of cartilage.

Mansouri, A., K. Chowdhury and P. Gruss (1998). "Follicular cells of the thyroid gland require Pax8 gene function." Nat Genet **19**(1): 87-90.

Mansukhani, A., P. Bellosta, M. Sahni and C. Basilico (2000). "Signaling by fibroblast growth factors (FGF) and fibroblast growth factor receptor 2 (FGFR2)-activating

mutations blocks mineralization and induces apoptosis in osteoblasts." J Cell Biol **149**(6): 1297-1308.

Martin, A., S. Liu, V. David, H. Li, A. Karydis, J. Q. Feng and L. D. Quarles (2011). "Bone proteins PHEX and DMP1 regulate fibroblastic growth factor Fgf23 expression in osteocytes through a common pathway involving FGF receptor (FGFR) signaling." FASEB J **25**(8): 2551-2562.

Mason, R. M., M. G. Chambers, J. Flannelly, J. D. Gaffen, J. Dudhia and M. T. Bayliss (2001). "The STR/ort mouse and its use as a model of osteoarthritis." Osteoarthritis Cartilage **9**(2): 85-91.

Matsuo, K. and N. Irie (2008). "Osteoclast-osteoblast communication." Arch Biochem Biophys **473**(2): 201-209.

Matsuo, K., J. M. Owens, M. Tonko, C. Elliott, T. J. Chambers and E. F. Wagner (2000). "Fosl1 is a transcriptional target of c-Fos during osteoclast differentiation." Nat Genet **24**(2): 184-187.

Medici, M., W. E. Visser, T. J. Visser and R. P. Peeters (2015). "Genetic determination of the hypothalamic-pituitary-thyroid axis: where do we stand?" Endocr Rev **36**(2): 214-244.

Mentuccia, D., M. J. Thomas, G. Coppotelli, L. J. Reinhart, B. D. Mitchell, A. R. Shuldiner and F. S. Celi (2005). "The Thr92Ala deiodinase type 2 (DIO2) variant is not associated with type 2 diabetes or indices of insulin resistance in the old order of Amish." Thyroid **15**(11): 1223-1227.

Meulenbelt, I., S. D. Bos, K. Chapman, R. van der Breggen, J. J. Houwing-Duistermaat, D. Kremer, M. Kloppenburg, A. Carr, A. Tsezou, A. Gonzalez, J. Loughlin and P. E. Slagboom (2011). "Meta-analyses of genes modulating intracellular T3 bio-availability reveal a possible role for the DIO3 gene in osteoarthritis susceptibility." Ann Rheum Dis **70**(1): 164-167.

Meulenbelt, I., M. Kloppenburg, H. M. Kroon, J. J. Houwing-Duistermaat, P. Garnero, M. P. Hellio-Le Graverand, J. DeGroot and P. E. Slagboom (2007). "Clusters of biochemical markers are associated with radiographic subtypes of osteoarthritis (OA) in subject with familial OA at multiple sites. The GARP study." Osteoarthritis Cartilage **15**(4): 379-385.

Meulenbelt, I., V. B. Kraus, L. J. Sandell and J. Loughlin (2011). "Summary of the OA biomarkers workshop 2010 - genetics and genomics: new targets in OA." Osteoarthritis Cartilage **19**(9): 1091-1094.

Meulenbelt, I., J. L. Min, S. Bos, N. Riyazi, J. J. Houwing-Duistermaat, H. J. van der Wijk, H. M. Kroon, M. Nakajima, S. Ikegawa, A. G. Uitterlinden, J. B. van Meurs, W. M. van der Deure, T. J. Visser, A. B. Seymour, N. Lakenberg, R. van der Breggen, D. Kremer, C. M. van Duijn, M. Kloppenburg, J. Loughlin and P. E. Slagboom (2008). "Identification of DIO2 as a new susceptibility locus for symptomatic osteoarthritis." Hum Mol Genet **17**(12): 1867-1875.

Meunier, P. J. and G. Boivin (1997). "Bone mineral density reflects bone mass but also the degree of mineralization of bone: therapeutic implications." Bone **21**(5): 373-377.

Mienaltowski, M. J., L. Huang, A. J. Stromberg and J. N. MacLeod (2008). "Differential gene expression associated with postnatal equine articular cartilage maturation." BMC Musculoskelet Disord **9**: 149.

Milne, M., J. M. Quail, C. J. Rosen and D. T. Baran (2001). "Insulin-like growth factor binding proteins in femoral and vertebral bone marrow stromal cells: expression and regulation by thyroid hormone and dexamethasone." J Cell Biochem **81**(2): 229-240.

Mishina, Y., M. W. Starbuck, M. A. Gentile, T. Fukuda, V. Kasparcova, J. G. Sedor, M. C. Hanks, M. Amling, G. J. Pinero, S. Harada and R. R. Behringer (2004). "Bone

morphogenetic protein type IA receptor signaling regulates postnatal osteoblast function and bone remodeling." *J Biol Chem* **279**(26): 27560-27566.

Mitsuyama, H., R. M. Healey, R. A. Terkeltaub, R. D. Coutts and D. Amiel (2007). "Calcification of human articular knee cartilage is primarily an effect of aging rather than osteoarthritis." *Osteoarthritis Cartilage* **15**(5): 559-565.

Mittag, J., S. Friedrichsen, H. Heuer, S. Polsfuss, T. J. Visser and K. Bauer (2005). "Athyroid Pax8^{-/-} mice cannot be rescued by the inactivation of thyroid hormone receptor alpha1." *Endocrinology* **146**(7): 3179-3184.

Miura, M., K. Tanaka, Y. Komatsu, M. Suda, A. Yasoda, Y. Sakuma, A. Ozasa and K. Nakao (2002). "Thyroid hormones promote chondrocyte differentiation in mouse ATDC5 cells and stimulate endochondral ossification in fetal mouse tibias through iodothyronine deiodinases in the growth plate." *J Bone Miner Res* **17**(3): 443-454.

Monfoulet, L. E., B. Rabier, R. Dacquin, A. Anginot, J. Photsavang, P. Jurdic, L. Vico, L. Malaval and O. Chassande (2011). "Thyroid hormone receptor beta mediates thyroid hormone effects on bone remodeling and bone mass." *J Bone Miner Res* **26**(9): 2036-2044.

Montero, A., Y. Okada, M. Tomita, M. Ito, H. Tsurukami, T. Nakamura, T. Doetschman, J. D. Coffin and M. M. Hurley (2000). "Disruption of the fibroblast growth factor-2 gene results in decreased bone mass and bone formation." *J Clin Invest* **105**(8): 1085-1093.

Moran, C., M. Agostini, A. McGowan, E. Schoenmakers, L. Fairall, G. Lyons, O. Rajanayagam, L. Watson, A. Offiah, J. Barton, S. Price, J. Schwabe and K. Chatterjee (2017). "Contrasting Phenotypes in Resistance to Thyroid Hormone Alpha Correlate with Divergent Properties of Thyroid Hormone Receptor alpha1 Mutant Proteins." *Thyroid* **27**(7): 973-982.

Moran, C., M. Agostini, W. E. Visser, E. Schoenmakers, N. Schoenmakers, A. C. Offiah, K. Poole, O. Rajanayagam, G. Lyons, D. Halsall, M. Gurnell, D. Chrysis, A. Efthymiadou, C. Buchanan, S. Aylwin and K. K. Chatterjee (2014). "Resistance to thyroid hormone caused by a mutation in thyroid hormone receptor (TR)alpha1 and TRalpha2: clinical, biochemical, and genetic analyses of three related patients." *Lancet Diabetes Endocrinol* **2**(8): 619-626.

Moran, C., N. Schoenmakers, M. Agostini, E. Schoenmakers, A. Offiah, A. Kydd, G. Kahaly, S. Mohr-Kahaly, O. Rajanayagam, G. Lyons, N. Wareham, D. Halsall, M. Dattani, S. Hughes, M. Gurnell, S. M. Park and K. Chatterjee (2013). "An adult female with resistance to thyroid hormone mediated by defective thyroid hormone receptor alpha." *J Clin Endocrinol Metab* **98**(11): 4254-4261.

Moreno, J. C. and T. J. Visser (2010). "Genetics and phenomics of hypothyroidism and goiter due to iodothyronine deiodinase (DEHAL1) gene mutations." *Mol Cell Endocrinol* **322**(1-2): 91-98.

Morimura, T., K. Tsunekawa, T. Kasahara, K. Seki, T. Ogiwara, M. Mori and M. Murakami (2005). "Expression of type 2 iodothyronine deiodinase in human osteoblast is stimulated by thyrotropin." *Endocrinology* **146**(4): 2077-2084.

Morko, J., R. Kiviranta, K. Joronen, A. M. Saamanen, E. Vuorio and H. Salminen-Mankonen (2005). "Spontaneous development of synovitis and cartilage degeneration in transgenic mice overexpressing cathepsin K." *Arthritis Rheum* **52**(12): 3713-3717.

Mortimer, R. H., J. P. Galligan, G. R. Cannell, R. S. Addison and M. S. Roberts (1996). "Maternal to fetal thyroxine transmission in the human term placenta is limited by inner ring deiodination." *J Clin Endocrinol Metab* **81**(6): 2247-2249.

Mourouzis, I., E. Kostakou, G. Galanopoulos, P. Mantzouratou and C. Pantos (2013). "Inhibition of thyroid hormone receptor alpha1 impairs post-ischemic cardiac

performance after myocardial infarction in mice." Molecular and cellular biochemistry **379**(1-2): 97-105.

Muller, J., S. Mayerl, T. J. Visser, V. M. Darras, A. Boelen, L. Frappart, L. Mariotta, F. Verrey and H. Heuer (2014). "Tissue-specific alterations in thyroid hormone homeostasis in combined Mct10 and Mct8 deficiency." Endocrinology **155**(1): 315-325.

Mundlos, S., F. Otto, C. Mundlos, J. B. Mulliken, A. S. Aylsworth, S. Albright, D. Lindhout, W. G. Cole, W. Henn, J. H. Knoll, M. J. Owen, R. Mertelsmann, B. U. Zabel and B. R. Olsen (1997). "Mutations involving the transcription factor CBFA1 cause cleidocranial dysplasia." Cell **89**(5): 773-779.

Mundy, G. R., J. L. Shapiro, J. G. Bandelin, E. M. Canalis and L. G. Raisz (1976). "Direct stimulation of bone resorption by thyroid hormones." J Clin Invest **58**(3): 529-534.

Murphy, E., C. C. Gluer, D. M. Reid, D. Felsenberg, C. Roux, R. Eastell and G. R. Williams (2010). "Thyroid function within the upper normal range is associated with reduced bone mineral density and an increased risk of nonvertebral fractures in healthy euthyroid postmenopausal women." J Clin Endocrinol Metab **95**(7): 3173-3181.

Nagase, H., Y. Nagasawa, Y. Tachida, S. Sakakibara, J. Okutsu, N. Suematsu, S. Arita and K. Shimada (2013). "Deiodinase 2 upregulation demonstrated in osteoarthritis patients cartilage causes cartilage destruction in tissue-specific transgenic rats." Osteoarthritis Cartilage **21**(3): 514-523.

Nakasa, T., M. Ishikawa, T. Takada, S. Miyaki and M. Ochi (2016). "Attenuation of cartilage degeneration by calcitonin gene-related peptide receptor antagonist via inhibition of subchondral bone sclerosis in osteoarthritis mice." J Orthop Res **34**(7): 1177-1184.

Nakashima, K., X. Zhou, G. Kunkel, Z. Zhang, J. M. Deng, R. R. Behringer and B. de Crombrughe (2002). "The novel zinc finger-containing transcription factor osterix is required for osteoblast differentiation and bone formation." Cell **108**(1): 17-29.

Nakashima, T., M. Hayashi, T. Fukunaga, K. Kurata, M. Oh-Hora, J. Q. Feng, L. F. Bonewald, T. Kodama, A. Wutz, E. F. Wagner, J. M. Penninger and H. Takayanagi (2011). "Evidence for osteocyte regulation of bone homeostasis through RANKL expression." Nat Med **17**(10): 1231-1234.

Neogi, T., D. Felson, J. Niu, J. Lynch, M. Nevitt, A. Guermazi, F. Roemer, C. E. Lewis, B. Wallace and Y. Zhang (2009). "Cartilage loss occurs in the same subregions as subchondral bone attrition: a within-knee subregion-matched approach from the Multicenter Osteoarthritis Study." Arthritis Rheum **61**(11): 1539-1544.

Neogi, T., M. Nevitt, J. Niu, L. Sharma, F. Roemer, A. Guermazi, C. E. Lewis, J. Torner, K. Javaid and D. Felson (2010). "Subchondral bone attrition may be a reflection of compartment-specific mechanical load: the MOST Study." Ann Rheum Dis **69**(5): 841-844.

Ng, L., R. J. Goodyear, C. A. Woods, M. J. Schneider, E. Diamond, G. P. Richardson, M. W. Kelley, D. L. Germain, V. A. Galton and D. Forrest (2004). "Hearing loss and retarded cochlear development in mice lacking type 2 iodothyronine deiodinase." Proc Natl Acad Sci U S A **101**(10): 3474-3479.

Nicholls, J. J., M. J. Brassill, G. R. Williams and J. H. Bassett (2012). "The skeletal consequences of thyrotoxicosis." J Endocrinol **213**(3): 209-221.

Nishimura, R., K. Hata, S. E. Harris, F. Ikeda and T. Yoneda (2002). "Core-binding factor alpha 1 (Cbfa1) induces osteoblastic differentiation of C2C12 cells without interactions with Smad1 and Smad5." Bone **31**(2): 303-312.

Noble, B. S. and J. Reeve (2000). "Osteocyte function, osteocyte death and bone fracture resistance." Mol Cell Endocrinol **159**(1-2): 7-13.

O'Shea, P. J., J. H. Bassett, S. Sriskantharajah, H. Ying, S. Y. Cheng and G. R. Williams (2005). "Contrasting skeletal phenotypes in mice with an identical mutation targeted to thyroid hormone receptor alpha1 or beta." Mol Endocrinol **19**(12): 3045-3059.

O'Shea, P. J., C. B. Harvey, H. Suzuki, M. Kaneshige, K. Kaneshige, S. Y. Cheng and G. R. Williams (2003). "A thyrotoxic skeletal phenotype of advanced bone formation in mice with resistance to thyroid hormone." Mol Endocrinol **17**(7): 1410-1424.

O'Shea, P. J., D. W. Kim, J. G. Logan, S. Davis, R. L. Walker, P. S. Meltzer, S. Y. Cheng and G. R. Williams (2012). "Advanced bone formation in mice with a dominant-negative mutation in the thyroid hormone receptor beta gene due to activation of Wnt/beta-catenin protein signaling." J Biol Chem **287**(21): 17812-17822.

Ohbayashi, N., M. Shibayama, Y. Kurotaki, M. Imanishi, T. Fujimori, N. Itoh and S. Takada (2002). "FGF18 is required for normal cell proliferation and differentiation during osteogenesis and chondrogenesis." Genes Dev **16**(7): 870-879.

Oppenheimer, J. H., H. L. Schwartz, D. Koerner and M. I. Surks (1974). "Limited binding capacity sites for L-triiodothyronine in rat liver nuclei. Nuclear-cytoplasmic interrelation, binding constants, and cross-reactivity with L-thyroxine." J Clin Invest **53**(3): 768-777.

Ornitz, D. M. and P. J. Marie (2015). "Fibroblast growth factor signaling in skeletal development and disease." Genes Dev **29**(14): 1463-1486.

Orth, P., M. Cucchiari, S. Wagenpfeil, M. D. Menger and H. Madry (2014). "PTH [1-34]-induced alterations of the subchondral bone provoke early osteoarthritis." Osteoarthritis Cartilage **22**(6): 813-821.

Otto, F., A. P. Thornell, T. Crompton, A. Denzel, K. C. Gilmour, I. R. Rosewell, G. W. Stamp, R. S. Beddington, S. Mundlos, B. R. Olsen, P. B. Selby and M. J. Owen (1997). "Cbfa1, a candidate gene for cleidocranial dysplasia syndrome, is essential for osteoblast differentiation and bone development." Cell **89**(5): 765-771.

Pan, J., X. Zhou, W. Li, J. E. Novotny, S. B. Doty and L. Wang (2009). "In situ measurement of transport between subchondral bone and articular cartilage." J Orthop Res **27**(10): 1347-1352.

Panicker, V., C. Cluett, B. Shields, A. Murray, K. S. Parnell, J. R. Perry, M. N. Weedon, A. Singleton, D. Hernandez, J. Evans, C. Durant, L. Ferrucci, D. Melzer, P. Saravanan, T. J. Visser, G. Ceresini, A. T. Hattersley, B. Vaidya, C. M. Dayan and T. M. Frayling (2008). "A common variation in deiodinase 1 gene DIO1 is associated with the relative levels of free thyroxine and triiodothyronine." J Clin Endocrinol Metab **93**(8): 3075-3081.

Pantos, C., I. Mourouzis, M. Delbruyere, V. Malliopoulou, S. Tzeis, D. D. Cokkinos, N. Nikitas, H. Carageorgiou, D. Varonos, D. Cokkinos and D. Nisato (2002). "Effects of dronedarone and amiodarone on plasma thyroid hormones and on the basal and postischemic performance of the isolated rat heart." Eur J Pharmacol **444**(3): 191-196.

Pantos, C., I. Mourouzis, V. Malliopoulou, I. Paizis, S. Tzeis, P. Moraitis, K. Sfakianoudis, D. D. Varonos and D. V. Cokkinos (2005). "Dronedarone administration prevents body weight gain and increases tolerance of the heart to ischemic stress: a possible involvement of thyroid hormone receptor alpha1." Thyroid **15**(1): 16-23.

Pasca di Magliano, M., R. Di Lauro and M. Zannini (2000). "Pax8 has a key role in thyroid cell differentiation." Proc Natl Acad Sci U S A **97**(24): 13144-13149.

Patel, C., G. X. Yan and P. R. Kowey (2009). "Dronedarone." Circulation **120**(7): 636-644.

Peeters, R. P., A. W. van den Beld, H. Attalki, H. Toor, Y. B. de Rijke, G. G. Kuiper, S. W. Lamberts, J. A. Janssen, A. G. Uitterlinden and T. J. Visser (2005). "A new polymorphism in the type II deiodinase gene is associated with circulating thyroid hormone parameters." Am J Physiol Endocrinol Metab **289**(1): E75-81.

Peeters, R. P., H. van Toor, W. Klootwijk, Y. B. de Rijke, G. G. Kuiper, A. G. Uitterlinden and T. J. Visser (2003). "Polymorphisms in thyroid hormone pathway genes are associated with plasma TSH and iodothyronine levels in healthy subjects." J Clin Endocrinol Metab **88**(6): 2880-2888.

Pereira, R. C., V. Jorgetti and E. Canalis (1999). "Triiodothyronine induces collagenase-3 and gelatinase B expression in murine osteoblasts." Am J Physiol **277**(3 Pt 1): E496-504.

Perros, P., K. Boelaert, S. Colley, C. Evans, R. M. Evans, G. Gerrard Ba, J. Gilbert, B. Harrison, S. J. Johnson, T. E. Giles, L. Moss, V. Lewington, K. Newbold, J. Taylor, R. V. Thakker, J. Watkinson, G. R. Williams and A. British Thyroid (2014). "Guidelines for the management of thyroid cancer." Clin Endocrinol (Oxf) **81 Suppl 1**: 1-122.

Poole, K. (2011). The Normal Synovial Joint. Osteoarthritis Research Society International (OARSI) Primer.

Pottenger, L. A., F. M. Phillips and L. F. Draganich (1990). "The effect of marginal osteophytes on reduction of varus-valgus instability in osteoarthritic knees." Arthritis Rheum **33**(6): 853-858.

Poulet, B., R. de Souza, A. V. Kent, L. Saxon, O. Barker, A. Wilson, Y. M. Chang, M. Cake and A. A. Pitsillides (2015). "Intermittent applied mechanical loading induces subchondral bone thickening that may be intensified locally by contiguous articular cartilage lesions." Osteoarthritis Cartilage **23**(6): 940-948.

Qing, H., L. Ardeshipour, P. D. Pajevic, V. Dusevich, K. Jahn, S. Kato, J. Wysolmerski and L. F. Bonewald (2012). "Demonstration of osteocytic perilacunar/canalicular remodeling in mice during lactation." J Bone Miner Res **27**(5): 1018-1029.

Rabier, B., A. J. Williams, F. Mallein-Gerin, G. R. Williams and O. Chassande (2006). "Thyroid hormone-stimulated differentiation of primary rib chondrocytes in vitro requires thyroid hormone receptor beta." J Endocrinol **191**(1): 221-228.

Radin, E. L., I. L. Paul and M. J. Tolkoff (1970). "Subchondral bone changes in patients with early degenerative joint disease." Arthritis Rheum **13**(4): 400-405.

Ramos, Y. F. and I. Meulenbelt (2015). "Implementation of Functional Genomics for Bench-to-Bedside Transition in Osteoarthritis." Curr Rheumatol Rep **17**(8): 53.

Randau, T. M., F. A. Schildberg, M. Alini, M. D. Wimmer, M. Haddouti el, S. Gravius, K. Ito and M. J. Stoddart (2013). "The effect of dexamethasone and triiodothyronine on terminal differentiation of primary bovine chondrocytes and chondrogenically differentiated mesenchymal stem cells." PLoS One **8**(8): e72973.

Reichenbach, S., A. Guermazi, J. Niu, T. Neogi, D. J. Hunter, F. W. Roemer, C. E. McLennan, G. Hernandez-Molina and D. T. Felson (2008). "Prevalence of bone attrition on knee radiographs and MRI in a community-based cohort." Osteoarthritis Cartilage **16**(9): 1005-1010.

Rho, J. Y., L. Kuhn-Spearing and P. Zioupos (1998). "Mechanical properties and the hierarchical structure of bone." Med Eng Phys **20**(2): 92-102.

Rivkees, S. A., H. H. Bode and J. D. Crawford (1988). "Long-term growth in juvenile acquired hypothyroidism: the failure to achieve normal adult stature." N Engl J Med **318**(10): 599-602.

Robling, A. G., P. J. Niziolek, L. A. Baldrige, K. W. Condon, M. R. Allen, I. Alam, S. M. Mantila, J. Gluhak-Heinrich, T. M. Bellido, S. E. Harris and C. H. Turner (2008).

"Mechanical stimulation of bone in vivo reduces osteocyte expression of Sost/sclerostin." J Biol Chem **283**(9): 5866-5875.

Robson, H., T. Siebler, D. A. Stevens, S. M. Shalet and G. R. Williams (2000). "Thyroid hormone acts directly on growth plate chondrocytes to promote hypertrophic differentiation and inhibit clonal expansion and cell proliferation." Endocrinology **141**(10): 3887-3897.

Rocheftort, G. Y., S. Pallu and C. L. Benhamou (2010). "Osteocyte: the unrecognized side of bone tissue." Osteoporos Int **21**(9): 1457-1469.

Rodriguez-Fontenla, C., M. Calaza, E. Evangelou, A. M. Valdes, N. Arden, F. J. Blanco, A. Carr, K. Chapman, P. Deloukas, M. Doherty, T. Esko, C. M. Garces Aleta, J. J. Gomez-Reino Carnota, H. Helgadottir, A. Hofman, I. Jonsdottir, H. J. Kerkhof, M. Kloppenburg, A. McCaskie, E. E. Ntzani, W. E. Ollier, N. Oreiro, K. Panoutsopoulou, S. H. Ralston, Y. F. Ramos, J. A. Riancho, F. Rivadeneira, P. E. Slagboom, U. Styrkarsdottir, U. Thorsteinsdottir, G. Thorleifsson, A. Tsezou, A. G. Uitterlinden, G. A. Wallis, J. M. Wilkinson, G. Zhai, Y. Zhu, O. C. arc, D. T. Felson, J. P. Ioannidis, J. Loughlin, A. Metspalu, I. Meulenbelt, K. Stefansson, J. B. van Meurs, E. Zeggini, T. D. Spector and A. Gonzalez (2014). "Assessment of osteoarthritis candidate genes in a meta-analysis of nine genome-wide association studies." Arthritis Rheumatol **66**(4): 940-949.

Roef, G., Y. Taes, K. Toye, S. Goemaere, T. Fiers, A. Verstraete and J. M. Kaufman (2013). "Heredity and lifestyle in the determination of between-subject variation in thyroid hormone levels in euthyroid men." Eur J Endocrinol **169**(6): 835-844.

Roman-Blas, J. A. and S. A. Jimenez (2006). "NF-kappaB as a potential therapeutic target in osteoarthritis and rheumatoid arthritis." Osteoarthritis Cartilage **14**(9): 839-848.

Rosello-Diez, A. and A. L. Joyner (2015). "Regulation of Long Bone Growth in Vertebrates; It Is Time to Catch Up." Endocr Rev **36**(6): 646-680.

Rosen, C. J. and American Society for Bone and Mineral Research. (2013). Primer on the metabolic bone diseases and disorders of mineral metabolism. Ames, Iowa, Wiley-Blackwell.

Rosenthal, A. K., D. Heinkel and C. M. Gohr (2003). "Thyroxine stimulates transglutaminase activity in articular chondrocytes." Osteoarthritis Cartilage **11**(6): 463-470.

Rosenthal, A. K. and L. A. Henry (1999). "Thyroid hormones induce features of the hypertrophic phenotype and stimulate correlates of CPPD crystal formation in articular chondrocytes." J Rheumatol **26**(2): 395-401.

Salazar, V. S., L. W. Gamer and V. Rosen (2016). "BMP signalling in skeletal development, disease and repair." Nat Rev Endocrinol **12**(4): 203-221.

Salazar, V. S., N. Zarkadis, L. Huang, J. Norris, S. K. Grimston, G. Mbalaviele and R. Civitelli (2013). "Embryonic ablation of osteoblast Smad4 interrupts matrix synthesis in response to canonical Wnt signaling and causes an osteogenesis-imperfecta-like phenotype." J Cell Sci **126**(Pt 21): 4974-4984.

Salter, D., J. Buckwalter and L. Sandell (2011). Pathology of Osteoarthritis. Osteoarthritis Research Society International (OARSI) Primer: 1-15.

Salvarrey-Strati, A., L. Watson, T. Blanchet, N. Lu and S. S. Glasson (2008). "The influence of enrichment devices on development of osteoarthritis in a surgically induced murine model." ILAR J **49**(4): 23-30.

Salvatore, D., T. F. Davies, S. Martin-Jean, H. I. D and P. R. Larsen (2011). "Thyroid physiology and diagnostic evaluation of patients with thyroid disorders." Williams Textbook of Endocrinology 12th Edition.

Sanchez-Duffhues, G., C. Hiepen, P. Knaus and P. Ten Dijke (2015). "Bone morphogenetic protein signaling in bone homeostasis." Bone **80**: 43-59.

Saraiva, P. P., S. S. Teixeira, C. R. Padovani and C. R. Nogueira (2008). "Triiodothyronine (T3) does not induce Rankl expression in rat Ros 17/2.8 cells." Arq Bras Endocrinol Metabol **52**(1): 109-113.

Sato, K., D. C. Han, Y. Fujii, T. Tsushima and K. Shizume (1987). "Thyroid hormone stimulates alkaline phosphatase activity in cultured rat osteoblastic cells (ROS 17/2.8) through 3,5,3'-triiodo-L-thyronine nuclear receptors." Endocrinology **120**(5): 1873-1881.

Schneider, M. J., S. N. Fiering, S. E. Pallud, A. F. Parlow, D. L. St Germain and V. A. Galton (2001). "Targeted disruption of the type 2 selenodeiodinase gene (DIO2) results in a phenotype of pituitary resistance to T4." Mol Endocrinol **15**(12): 2137-2148.

Schneider, M. J., S. N. Fiering, B. Thai, S. Y. Wu, E. St Germain, A. F. Parlow, D. L. St Germain and V. A. Galton (2006). "Targeted disruption of the type 1 selenodeiodinase gene (Dio1) results in marked changes in thyroid hormone economy in mice." Endocrinology **147**(1): 580-589.

Schoenmakers, C. H., I. G. Pigmans, A. Poland and T. J. Visser (1993). "Impairment of the selenoenzyme type I iodothyronine deiodinase in C3H/He mice." Endocrinology **132**(1): 357-361.

Segni, M., E. Leonardi, B. Mazzoncin, I. Pucarelli and A. M. Pasquino (1999). "Special features of Graves' disease in early childhood." Thyroid **9**(9): 871-877.

Shimizu, S., Y. Asou, S. Itoh, U. I. Chung, H. Kawaguchi, K. Shinomiya and T. Muneta (2007). "Prevention of cartilage destruction with intraarticular osteoclastogenesis inhibitory factor/osteoprotegerin in a murine model of osteoarthritis." Arthritis Rheum **56**(10): 3358-3365.

Siddiqui, J. A. and N. C. Partridge (2017). "CCL2/Monocyte Chemoattractant Protein 1 and Parathyroid Hormone Action on Bone." Front Endocrinol (Lausanne) **8**: 49.

Simonet, W. S., D. L. Lacey, C. R. Dunstan, M. Kelley, M. S. Chang, R. Luthy, H. Q. Nguyen, S. Wooden, L. Bennett, T. Boone, G. Shimamoto, M. DeRose, R. Elliott, A. Colombero, H. L. Tan, G. Trail, J. Sullivan, E. Davy, N. Bucay, L. Renshaw-Gegg, T. M. Hughes, D. Hill, W. Pattison, P. Campbell, S. Sander, G. Van, J. Tarpley, P. Derby, R. Lee and W. J. Boyle (1997). "Osteoprotegerin: a novel secreted protein involved in the regulation of bone density." Cell **89**(2): 309-319.

Simonides, W. S., M. A. Mulcahey, E. M. Redout, A. Muller, M. J. Zuidwijk, T. J. Visser, F. W. Wassen, A. Crescenzi, W. S. da-Silva, J. Harney, F. B. Engel, M. J. Obregon, P. R. Larsen, A. C. Bianco and S. A. Huang (2008). "Hypoxia-inducible factor induces local thyroid hormone inactivation during hypoxic-ischemic disease in rats." J Clin Invest **118**(3): 975-983.

Sjouke, B., G. Langslet, R. Ceska, S. J. Nicholls, S. E. Nissen, M. Ohlander, P. W. Ladenson, A. G. Olsson, G. K. Hovingh and J. J. Kastelein (2014). "Eprotirome in patients with familial hypercholesterolaemia (the AKKA trial): a randomised, double-blind, placebo-controlled phase 3 study." Lancet Diabetes Endocrinol **2**(6): 455-463.

Sniekers, Y. H., F. Intema, F. P. Lafeber, G. J. van Osch, J. P. van Leeuwen, H. Weinans and S. C. Mastbergen (2008). "A role for subchondral bone changes in the process of osteoarthritis; a micro-CT study of two canine models." BMC Musculoskelet Disord **9**: 20.

Soeda, T., J. M. Deng, B. de Crombrughe, R. R. Behringer, T. Nakamura and H. Akiyama (2010). "Sox9-expressing precursors are the cellular origin of the cruciate ligament of the knee joint and the limb tendons." Genesis **48**(11): 635-644.

Sondergaard, B. C., P. Catala-Lehnen, A. K. Huebner, A. C. Bay-Jensen, T. Schinke, K. Henriksen, S. Schilling, M. Haberland, R. H. Nielsen, M. Amling and M. A. Karsdal (2012). "Mice over-expressing salmon calcitonin have strongly attenuated osteoarthritic histopathological changes after destabilization of the medial meniscus." Osteoarthritis Cartilage **20**(2): 136-143.

Sophia Fox, A. J., A. Bedi and S. A. Rodeo (2009). "The basic science of articular cartilage: structure, composition, and function." Sports Health **1**(6): 461-468.

Spagnoli, A., L. O'Rear, R. L. Chandler, F. Granero-Molto, D. P. Mortlock, A. E. Gorska, J. A. Weis, L. Longobardi, A. Chytil, K. Shimer and H. L. Moses (2007). "TGF-beta signaling is essential for joint morphogenesis." J Cell Biol **177**(6): 1105-1117.

Spector, T. D., F. Cicuttini, J. Baker, J. Loughlin and D. Hart (1996). "Genetic influences on osteoarthritis in women: a twin study." BMJ **312**(7036): 940-943.

St Germain, D. L., V. A. Galton and A. Hernandez (2009). "Minireview: Defining the roles of the iodothyronine deiodinases: current concepts and challenges." Endocrinology **150**(3): 1097-1107.

Stevens, D. A., C. B. Harvey, A. J. Scott, P. J. O'Shea, J. C. Barnard, A. J. Williams, G. Brady, J. Samarut, O. Chassande and G. R. Williams (2003). "Thyroid hormone activates fibroblast growth factor receptor-1 in bone." Mol Endocrinol **17**(9): 1751-1766.

Stevens, D. A., R. P. Hasserjian, H. Robson, T. Siebler, S. M. Shalet and G. R. Williams (2000). "Thyroid hormones regulate hypertrophic chondrocyte differentiation and expression of parathyroid hormone-related peptide and its receptor during endochondral bone formation." J Bone Miner Res **15**(12): 2431-2442.

Stoykov, I., H. C. van Beeren, A. F. Moorman, V. M. Christoffels, W. M. Wiersinga and O. Bakker (2007). "Effect of amiodarone and dronedarone administration in rats on thyroid hormone-dependent gene expression in different cardiac components." European journal of endocrinology / European Federation of Endocrine Societies **156**(6): 695-702.

Sugitani, I. and Y. Fujimoto (2011). "Effect of postoperative thyrotropin suppressive therapy on bone mineral density in patients with papillary thyroid carcinoma: a prospective controlled study." Surgery **150**(6): 1250-1257.

Sullivan, R. M. and B. Olshansky (2010). "Dronedarone: evidence supporting its therapeutic use in the treatment of atrial fibrillation." Core Evid **5**: 49-59.

Supornsilchai, V., T. Sahakitrungruang, N. Wongjitrat, S. Wacharasindhu, K. Suphapeetiporn and V. Shotelersuk (2009). "Expanding clinical spectrum of non-autoimmune hyperthyroidism due to an activating germline mutation, p.M453T, in the thyrotropin receptor gene." Clin Endocrinol (Oxf) **70**(4): 623-628.

Takayanagi, H., S. Kim, T. Koga, H. Nishina, M. Isshiki, H. Yoshida, A. Saiura, M. Isobe, T. Yokochi, J. Inoue, E. F. Wagner, T. W. Mak, T. Kodama and T. Taniguchi (2002). "Induction and activation of the transcription factor NFATc1 (NFAT2) integrate RANKL signaling in terminal differentiation of osteoclasts." Dev Cell **3**(6): 889-901.

Tan, S. D., T. J. de Vries, A. M. Kuijpers-Jagtman, C. M. Semeins, V. Everts and J. Klein-Nulend (2007). "Osteocytes subjected to fluid flow inhibit osteoclast formation and bone resorption." Bone **41**(5): 745-751.

Tan, X., T. Weng, J. Zhang, J. Wang, W. Li, H. Wan, Y. Lan, X. Cheng, N. Hou, H. Liu, J. Ding, F. Lin, R. Yang, X. Gao, D. Chen and X. Yang (2007). "Smad4 is required for maintaining normal murine postnatal bone homeostasis." J Cell Sci **120**(Pt 13): 2162-2170.

Tanamas, S., F. S. Hanna, F. M. Cicuttini, A. E. Wluka, P. Berry and D. M. Urquhart (2009). "Does knee malalignment increase the risk of development and progression of knee osteoarthritis? A systematic review." *Arthritis Rheum* **61**(4): 459-467.

Tatsumi, S., K. Ishii, N. Amizuka, M. Li, T. Kobayashi, K. Kohno, M. Ito, S. Takeshita and K. Ikeda (2007). "Targeted ablation of osteocytes induces osteoporosis with defective mechanotransduction." *Cell Metab* **5**(6): 464-475.

Taurog, A. (2000). "Hormone synthesis: Thyroid iodine metabolism, in Werner & Ingbar's The Thyroid."

Tinnikov, A., K. Nordstrom, P. Thoren, J. M. Kindblom, S. Malin, B. Rozell, M. Adams, O. Rajanayagam, S. Pettersson, C. Ohlsson, K. Chatterjee and B. Vennstrom (2002). "Retardation of post-natal development caused by a negatively acting thyroid hormone receptor alpha1." *EMBO J* **21**(19): 5079-5087.

Tsezou, A. (2014). "Osteoarthritis year in review 2014: genetics and genomics." *Osteoarthritis Cartilage* **22**(12): 2017-2024.

Tsourd, E., E. Rijntjes, J. Kohrle, L. C. Hofbauer and M. Rauner (2015). "Hyperthyroidism and Hypothyroidism in Male Mice and Their Effects on Bone Mass, Bone Turnover, and the Wnt Inhibitors Sclerostin and Dickkopf-1." *Endocrinology* **156**(10): 3517-3527.

Tylki-Szymanska, A., R. Acuna-Hidalgo, M. Krajewska-Walasek, A. Lecka-Ambroziak, M. Steehouwer, C. Gilissen, H. G. Brunner, A. Jurecka, A. Rozdzyńska-Swiatkowska, A. Hoischen and K. H. Chrzanowska (2015). "Thyroid hormone resistance syndrome due to mutations in the thyroid hormone receptor alpha gene (THRA)." *J Med Genet* **52**(5): 312-316.

Vaananen, H. K., H. Zhao, M. Mulari and J. M. Halleen (2000). "The cell biology of osteoclast function." *J Cell Sci* **113** (Pt 3): 377-381.

Valdes, A. M. and T. D. Spector (2011). "Genetic epidemiology of hip and knee osteoarthritis." *Nat Rev Rheumatol* **7**(1): 23-32.

van Beeren, H. C., O. Bakker and W. M. Wiersinga (1995). "Desethylamiodarone is a competitive inhibitor of the binding of thyroid hormone to the thyroid hormone alpha 1-receptor protein." *Molecular and cellular endocrinology* **112**(1): 15-19.

Van Beeren, H. C., W. M. Jong, E. Kaptein, T. J. Visser, O. Bakker and W. M. Wiersinga (2003). "Dronerone acts as a selective inhibitor of 3,5,3'-triiodothyronine binding to thyroid hormone receptor-alpha1: in vitro and in vivo evidence." *Endocrinology* **144**(2): 552-558.

van Beeren, H. C., J. Kwakkel, M. T. Ackermans, W. M. Wiersinga, E. Fliers and A. Boelen (2012). "Action of specific thyroid hormone receptor alpha(1) and beta(1) antagonists in the central and peripheral regulation of thyroid hormone metabolism in the rat." *Thyroid* **22**(12): 1275-1282.

van Bezooijen, R. L., B. A. Roelen, A. Visser, L. van der Wee-Pals, E. de Wilt, M. Karperien, H. Hamersma, S. E. Papapoulos, P. ten Dijke and C. W. Lowik (2004). "Sclerostin is an osteocyte-expressed negative regulator of bone formation, but not a classical BMP antagonist." *J Exp Med* **199**(6): 805-814.

van der Kraan, P. M. and W. B. van den Berg (2007). "Osteophytes: relevance and biology." *Osteoarthritis Cartilage* **15**(3): 237-244.

van der Kraan, P. M. and W. B. van den Berg (2012). "Chondrocyte hypertrophy and osteoarthritis: role in initiation and progression of cartilage degeneration?" *Osteoarthritis Cartilage* **20**(3): 223-232.

van der Weyden, L., L. Wei, J. Luo, X. Yang, D. E. Birk, D. J. Adams, A. Bradley and Q. Chen (2006). "Functional knockout of the matrilin-3 gene causes premature

chondrocyte maturation to hypertrophy and increases bone mineral density and osteoarthritis." Am J Pathol **169**(2): 515-527.

van Gucht, A. L., M. E. Meima, N. Zwaveling-Soonawala, W. E. Visser, E. Fliers, J. M. Wennink, C. Henny, T. J. Visser, R. P. Peeters and A. S. van Trotsenburg (2016). "Resistance to Thyroid Hormone Alpha in an 18-Month-Old Girl: Clinical, Therapeutic, and Molecular Characteristics." Thyroid **26**(3): 338-346.

van Mullem, A., R. van Heerebeek, D. Chrysis, E. Visser, M. Medici, M. Andrikoula, A. Tsatsoulis, R. Peeters and T. J. Visser (2012). "Clinical phenotype and mutant TRalpha1." N Engl J Med **366**(15): 1451-1453.

van Mullem, A. A., D. Chrysis, A. Eythimiadou, E. Chroni, A. Tsatsoulis, Y. B. de Rijke, W. E. Visser, T. J. Visser and R. P. Peeters (2013). "Clinical phenotype of a new type of thyroid hormone resistance caused by a mutation of the TRalpha1 receptor: consequences of LT4 treatment." J Clin Endocrinol Metab **98**(7): 3029-3038.

van Osch, G. J., P. M. van der Kraan, A. A. van Valburg and W. B. van den Berg (1996). "The relation between cartilage damage and osteophyte size in a murine model for osteoarthritis in the knee." Rheumatol Int **16**(3): 115-119.

van Rijn, L. E., V. J. Pop and G. R. Williams (2014). "Low bone mineral density is related to high physiological levels of free thyroxine in peri-menopausal women." Eur J Endocrinol **170**(3): 461-468.

Van Vliet, G., B. Larroque, L. Bubuteishvili, K. Supernant, J. Leger and I. E. Association of Francaise pour le Depistage et la Prevention des Handicaps de (2003). "Sex-specific impact of congenital hypothyroidism due to thyroid dysgenesis on skeletal maturation in term newborns." J Clin Endocrinol Metab **88**(5): 2009-2013.

Varga, F., M. Rumpler, E. Luegmayr, N. Fratzl-Zelman, H. Glantschnig and K. Klaushofer (1997). "Triiodothyronine, a regulator of osteoblastic differentiation: depression of histone H4, attenuation of c-fos/c-jun, and induction of osteocalcin expression." Calcif Tissue Int **61**(5): 404-411.

Varga, F., M. Rumpler, R. Zoehrer, C. Turecek, S. Spitzer, R. Thaler, E. P. Paschalis and K. Klaushofer (2010). "T3 affects expression of collagen I and collagen cross-linking in bone cell cultures." Biochem Biophys Res Commun **402**(2): 180-185.

Varga, F., S. Spitzer and K. Klaushofer (2004). "Triiodothyronine (T3) and 1,25-dihydroxyvitamin D3 (1,25D3) inversely regulate OPG gene expression in dependence of the osteoblastic phenotype." Calcif Tissue Int **74**(4): 382-387.

Varro, A., J. Takacs, M. Nemeth, O. Hala, L. Virag, N. Iost, B. Balati, M. Agoston, A. Vereckei, G. Pastor, M. Delbruyere, P. Gautier, D. Nisato and J. G. Papp (2001). "Electrophysiological effects of dronedarone (SR 33589), a noniodinated amiodarone derivative in the canine heart: comparison with amiodarone." British journal of pharmacology **133**(5): 625-634.

Verborgt, O., G. J. Gibson and M. B. Schaffler (2000). "Loss of osteocyte integrity in association with microdamage and bone remodeling after fatigue in vivo." J Bone Miner Res **15**(1): 60-67.

Vestergaard, P. and L. Mosekilde (2002). "Fractures in patients with hyperthyroidism and hypothyroidism: a nationwide follow-up study in 16,249 patients." Thyroid **12**(5): 411-419.

Vestergaard, P. and L. Mosekilde (2003). "Hyperthyroidism, bone mineral, and fracture risk--a meta-analysis." Thyroid **13**(6): 585-593.

Vestergaard, P., L. Rejnmark and L. Mosekilde (2005). "Influence of hyper- and hypothyroidism, and the effects of treatment with antithyroid drugs and levothyroxine on fracture risk." Calcif Tissue Int **77**(3): 139-144.

Vincent, T. L., R. O. Williams, R. Maciewicz, A. Silman and P. Garside (2012). "Mapping pathogenesis of arthritis through small animal models." Rheumatology (Oxford) **51**(11): 1931-1941.

Visser, W. E., E. C. Friesema and T. J. Visser (2011). "Minireview: thyroid hormone transporters: the knowns and the unknowns." Mol Endocrinol **25**(1): 1-14.

Waarsing, J. H., M. Kloppenburg, P. E. Slagboom, H. M. Kroon, J. J. Houwing-Duistermaat, H. Weinans and I. Meulenbelt (2011). "Osteoarthritis susceptibility genes influence the association between hip morphology and osteoarthritis." Arthritis Rheum **63**(5): 1349-1354.

Wang, L. Y., A. W. Smith, F. L. Palmer, R. M. Tuttle, A. Mahrous, I. J. Nixon, S. G. Patel, I. Ganly, J. A. Fagin and L. Boucai (2015). "Thyrotropin suppression increases the risk of osteoporosis without decreasing recurrence in ATA low- and intermediate-risk patients with differentiated thyroid carcinoma." Thyroid **25**(3): 300-307.

Wassen, F. W., A. E. Schiel, G. G. Kuiper, E. Kaptein, O. Bakker, T. J. Visser and W. S. Simonides (2002). "Induction of thyroid hormone-degrading deiodinase in cardiac hypertrophy and failure." Endocrinology **143**(7): 2812-2815.

Waung, J. A., J. H. Bassett and G. R. Williams (2012). "Thyroid hormone metabolism in skeletal development and adult bone maintenance." Trends Endocrinol Metab **23**(4): 155-162.

Waung, J. A., J. H. Bassett and G. R. Williams (2015). "Adult mice lacking the type 2 iodothyronine deiodinase have increased subchondral bone but normal articular cartilage." Thyroid **25**(3): 269-277.

Waung, J. A., S. A. Maynard, S. Gopal, A. Gogakos, J. G. Logan, G. R. Williams and J. H. Bassett (2014). "Quantitative X-ray microradiography for high-throughput phenotyping of osteoarthritis in mice." Osteoarthritis Cartilage.

Weinstein, R. S., R. W. Nicholas and S. C. Manolagas (2000). "Apoptosis of osteocytes in glucocorticoid-induced osteonecrosis of the hip." J Clin Endocrinol Metab **85**(8): 2907-2912.

Wikstrom, L., C. Johansson, C. Salto, C. Barlow, A. Campos Barros, F. Baas, D. Forrest, P. Thoren and B. Vennstrom (1998). "Abnormal heart rate and body temperature in mice lacking thyroid hormone receptor alpha 1." EMBO J **17**(2): 455-461.

Williams, A. J., H. Robson, M. H. Kester, J. P. van Leeuwen, S. M. Shalet, T. J. Visser and G. R. Williams (2008). "Iodothyronine deiodinase enzyme activities in bone." Bone **43**(1): 126-134.

Williams, G. R. (2000). "Cloning and characterization of two novel thyroid hormone receptor beta isoforms." Mol Cell Biol **20**(22): 8329-8342.

Williams, G. R. (2013). "Thyroid hormone actions in cartilage and bone." Eur Thyroid J **2**(1): 3-13.

Williams, G. R. (2014). "Is prophylactic anti-resorptive therapy required in thyroid cancer patients receiving TSH-suppressive treatment with thyroxine?" J Endocrinol Invest **37**(8): 775-779.

Williams, G. R. and J. H. D. Bassett (2018). "Thyroid diseases and bone health." J Endocrinol Invest **41**(1): 99-109.

Wojcicka, A., J. H. Bassett and G. R. Williams (2013). "Mechanisms of action of thyroid hormones in the skeleton." Biochim Biophys Acta **1830**(7): 3979-3986.

Woolf, A. D. and B. Pfleger (2003). "Burden of major musculoskeletal conditions." Bull World Health Organ **81**(9): 646-656.

Wu, Q., K. O. Kim, E. R. Sampson, D. Chen, H. Awad, T. O'Brien, J. E. Puzas, H. Drissi, E. M. Schwarz, R. J. O'Keefe, M. J. Zuscik and R. N. Rosier (2008). "Induction

of an osteoarthritis-like phenotype and degradation of phosphorylated Smad3 by Smurf2 in transgenic mice." *Arthritis Rheum* **58**(10): 3132-3144.

Wu, X. B., Y. Li, A. Schneider, W. Yu, G. Rajendren, J. Iqbal, M. Yamamoto, M. Alam, L. J. Brunet, H. C. Blair, M. Zaidi and E. Abe (2003). "Impaired osteoblastic differentiation, reduced bone formation, and severe osteoporosis in noggin-overexpressing mice." *J Clin Invest* **112**(6): 924-934.

Xiao, L., T. Sobue, A. Esliger, M. S. Kronenberg, J. D. Coffin, T. Doetschman and M. M. Hurley (2010). "Disruption of the Fgf2 gene activates the adipogenic and suppresses the osteogenic program in mesenchymal marrow stromal stem cells." *Bone* **47**(2): 360-370.

Xing, W., P. Aghajanian, H. Goodluck, C. Kesavan, S. Cheng, S. Pourteymoor, H. Watt, C. Alarcon and S. Mohan (2016). "Thyroid hormone receptor-beta1 signaling is critically involved in regulating secondary ossification via promoting transcription of the Ihh gene in the epiphysis." *Am J Physiol Endocrinol Metab* **310**(10): E846-854.

Xing, W., S. Cheng, J. Wergedal and S. Mohan (2014). "Epiphyseal chondrocyte secondary ossification centers require thyroid hormone activation of Indian hedgehog and osterix signaling." *J Bone Miner Res* **29**(10): 2262-2275.

Xing, W., K. E. Govoni, L. R. Donahue, C. Kesavan, J. Wergedal, C. Long, J. H. Bassett, A. Gogakos, A. Wojcicka, G. R. Williams and S. Mohan (2012). "Genetic evidence that thyroid hormone is indispensable for prepubertal insulin-like growth factor-I expression and bone acquisition in mice." *J Bone Miner Res* **27**(5): 1067-1079.

Xiong, J., M. Onal, R. L. Jilka, R. S. Weinstein, S. C. Manolagas and C. A. O'Brien (2011). "Matrix-embedded cells control osteoclast formation." *Nat Med* **17**(10): 1235-1241.

Yang, S., J. Kim, J. H. Ryu, H. Oh, C. H. Chun, B. J. Kim, B. H. Min and J. S. Chun (2010). "Hypoxia-inducible factor-2alpha is a catabolic regulator of osteoarthritic cartilage destruction." *Nat Med* **16**(6): 687-693.

Yang, X., K. Matsuda, P. Bialek, S. Jacquot, H. C. Masuoka, T. Schinke, L. Li, S. Brancorsini, P. Sassone-Corsi, T. M. Townes, A. Hanauer and G. Karsenty (2004). "ATF4 is a substrate of RSK2 and an essential regulator of osteoblast biology; implication for Coffin-Lowry Syndrome." *Cell* **117**(3): 387-398.

Yu, K., J. Xu, Z. Liu, D. Sasic, J. Shao, E. N. Olson, D. A. Towler and D. M. Ornitz (2003). "Conditional inactivation of FGF receptor 2 reveals an essential role for FGF signaling in the regulation of osteoblast function and bone growth." *Development* **130**(13): 3063-3074.

Yuen, R. K., B. Thiruvahindrapuram, D. Merico, S. Walker, K. Tammimies, N. Hoang, C. Chrysler, T. Nalpathamkalam, G. Pellicchia, Y. Liu, M. J. Gazzellone, L. D'Abate, E. Deneault, J. L. Howe, R. S. Liu, A. Thompson, M. Zarrei, M. Uddin, C. R. Marshall, R. H. Ring, L. Zwaigenbaum, P. N. Ray, R. Weksberg, M. T. Carter, B. A. Fernandez, W. Roberts, P. Szatmari and S. W. Scherer (2015). "Whole-genome sequencing of quartet families with autism spectrum disorder." *Nat Med* **21**(2): 185-191.

Zanotti, S. and E. Canalis (2016). "Notch Signaling and the Skeleton." *Endocr Rev* **37**(3): 223-253.

Zeold, A., M. Doleschall, M. C. Haffner, L. P. Capelo, J. Menyhart, Z. Liposits, W. S. da Silva, A. C. Bianco, I. Kacsokovics, C. Fekete and B. Gereben (2006). "Characterization of the nuclear factor-kappa B responsiveness of the human dio2 gene." *Endocrinology* **147**(9): 4419-4429.

Zhang, L. (2011). "Voluntary oral administration of drugs in mice." *Protocol Exchange*.

Zhang, Q., A. D. Cigan, L. Marrero, C. Lopreore, S. Liu, D. Ge, F. H. Savoie and Z. You (2011). "Expression of doublecortin reveals articular chondrocyte lineage in mouse embryonic limbs." Genesis **49**(2): 75-82.

Zhao, C., N. Irie, Y. Takada, K. Shimoda, T. Miyamoto, T. Nishiwaki, T. Suda and K. Matsuo (2006). "Bidirectional ephrinB2-EphB4 signaling controls bone homeostasis." Cell Metab **4**(2): 111-121.

Zhou, X., Z. Zhang, J. Q. Feng, V. M. Dusevich, K. Sinha, H. Zhang, B. G. Darnay and B. de Crombrughe (2010). "Multiple functions of Osterix are required for bone growth and homeostasis in postnatal mice." Proc Natl Acad Sci U S A **107**(29): 12919-12924.

PUBLICATIONS FROM THESIS

Papers

Waung, J. A., J. H. Bassett and G. R. Williams (2012). "Thyroid hormone metabolism in skeletal development and adult bone maintenance." Trends Endocrinol Metab 23(4): 155-162.

Waung, J. A., S. A. Maynard, S. Gopal, A. Gogakos, J. G. Logan, G. R. Williams and J. H. Bassett (2014). "Quantitative X-ray microradiography for high-throughput phenotyping of osteoarthritis in mice." Osteoarthritis Cartilage.

Waung, J. A., J. H. Bassett and G. R. Williams (2015). "Adult mice lacking the type 2 iodothyronine deiodinase have increased subchondral bone but normal articular cartilage." Thyroid 25(3): 269-277.

Abstracts, Oral Presentations

Department of Medicine Young Scientist Day, London, UK, 2012

Jelly or Jam? Refinement of a protocol for voluntary oral dosing in mice

Julian A Waung, J H Duncan Bassett & Graham R Williams

Poster Presentation

10th International workshop on resistance to thyroid hormone & thyroid hormone action, Quebec, Canada, 2012

&

82nd Meeting of the American Thyroid Association, Quebec, Canada, 2012

Osteoarthritis in mice lacking 5' deiodinase activity

Julian A Waung, Ann Sandison, Donald L. St. Germain, Valerie Anne Galton, JH

Duncan Bassett, Graham R Williams

Poster Presentation

British Thyroid Association 61st Annual Meeting, London, 2012

Osteoarthritis in mice lacking 5' deiodinase activity

Julian A Waung, Ann Sandison, Donald L. St. Germain, Valerie Anne Galton, JH Duncan Bassett, Graham R Williams

Oral Presentation

Society for Endocrinology BES 2013, Harrogate, United Kingdom

Local regulation of T3 availability in susceptibility to osteoarthritis

Julian A Waung, Ann Sandison, Donald L. St. Germain, Valerie Anne Galton, JH Duncan Bassett, Graham R Williams

Poster Presentation

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Society for Endocrinology BES 2013, Harrogate, United Kingdom

High throughput detection of early joint pathology in mouse models of osteoarthritis

AE Draghici, **JA Waung**, JHD Bassett, GR Williams

Poster Presentation

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Society for Endocrinology BES 2014, Liverpool, United Kingdom

Treatment with a TR α 1 antagonist increases bone mineral content

Julian A Waung, J H Duncan Bassett & Graham R Williams

Poster Presentation

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Arthritis Research UK Fellows Meeting 2014, Loughborough, United Kingdom

Role of thyroid hormone receptor alpha in osteoarthritis susceptibility and prevention

Julian A Waung, J H Duncan Bassett & Graham R Williams

Oral Presentation

Thyroid hormone metabolism in skeletal development and adult bone maintenance

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Metabolism of thyroid hormones by the type 2 and type 3 iodothyronine deiodinases (D2, D3) in T3-responsive target cells is a sophisticated mechanism that helps to maintain local T3 concentrations and facilitates T3 action in a cell-specific manner that is independent of circulating thyroid hormone concentrations. Recent findings have demonstrated an essential physiological role for the thyroid hormone-activating enzyme D2 in the optimization of bone mineralization and strength. Emerging population studies have also identified the genes encoding D2 and the thyroid hormone-inactivating enzyme D3 as susceptibility loci for osteoarthritis. These new data reveal an essential role for the local control of T3 availability in osteoblasts and chondrocytes during maintenance and repair of bone and cartilage.

Physiological regulation of thyroid hormone action

The hypothalamic–pituitary–thyroid axis

Circulating thyroid hormone levels are regulated by a classic negative feedback loop mediated by the hypothalamic–pituitary–thyroid (HPT) axis (Figure 1) [1]. Thyrotropin-releasing hormone (TRH) is secreted from the hypothalamus and stimulates anterior pituitary thyrotrophs to release thyroid-stimulating hormone (thyrotropin, TSH). TSH acts on thyroid follicular cells to stimulate synthesis and secretion of the thyroid hormones 3,5,3',5'-L-tetraiodothyronine (thyroxine, T4) and 3,5,3'-L-triiodothyronine (T3). The thyroid hormones act via the nuclear thyroid hormone receptor β (TR β) in the hypothalamus and pituitary to inhibit TRH production and secretion [2,3] and thus complete a negative feedback loop that maintains a physiological inverse relationship between T4 and T3, and TSH. The thyroid gland mainly secretes T4, which is converted to T3 by the type 1 deiodinase (D1) predominantly in the liver, which thereby increases T3 in the peripheral circulation.

Thyroid hormone transport

Thyroid hormones enter target cells via specific membrane transporter proteins [4], which include the monocarboxylate transporters MCT8 and MCT10 and the sodium independent organic anion transporter protein-1C1 (OATP1C1), as well as non-specific amino acid transporters including the L-type amino acid transporters 1 and 2

(LAT1, LAT2). The best-characterized specific and active transporter MCT8 is expressed widely and its importance has been demonstrated by the finding that inactivating mutations of *MCT8* result in the Allan–Herndon–Dudley syndrome (see Glossary) [5,6]. Affected boys have elevated T3 concentrations with reduced T4 and inappropriately normal or modestly elevated TSH. These changes are accompanied by a severe, X-linked, psychomotor retardation syndrome that presents in early childhood and is thought to result from defective neuronal T3 entry causing abnormal T3 action and metabolism [7]. There is global developmental delay, including poor communication skills, no speech development, poor head control, mental retardation and varying degrees of truncal hypotonia, athetosis and motor deficiency, which suggest a critical role for MCT8 and T3 action in neurological development.

Thyroid hormone metabolism

The major actions of thyroid hormones are mediated in the nucleus by T3, but non-genomic actions occurring at the cell membrane or in the cytoplasm are mediated by either T4 or T3 [8] and the availability of these ligands is regulated by the activities of three iodothyronine deiodinases (D1, D2 and D3) [9,10]. The predominant circulating thyroid hormone is the pro-hormone T4, which can be converted to the biologically more potent hormone T3 by

Glossary

- Chondrocyte:** cartilage-forming cell.
- Craniosynostosis:** premature fusion of the cranial sutures.
- Endochondral ossification:** process by which bone forms on a cartilage scaffold.
- Euthyroidism:** normal physiological thyroid status.
- Histomorphometry:** histological method to quantify bone formation and resorption.
- Hyperthyroidism:** thyroid hormone excess.
- Hypothyroidism:** thyroid hormone deficiency.
- Intramembranous ossification:** process by which bone forms directly within condensations of mesenchyme.
- Osteoblast:** bone-forming and -mineralizing cell.
- Osteoclast:** bone-resorbing cell.
- Osteocyte:** osteoblast-derived cell embedded within bone that acts as a sensor of mechanical stress and bone microdamage.
- Periarticular perichondrium:** sheath of cells encapsulating cartilage adjacent to the developing joint.
- Perichondrium:** sheath of cells encapsulating cartilage at the growth plate.
- Subclinical hyperthyroidism:** condition in which thyroid hormone concentrations are within the normal reference range but the level of TSH is suppressed.
- Thyrotoxicosis:** disease in which thyroid hormone production is increased.

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Review

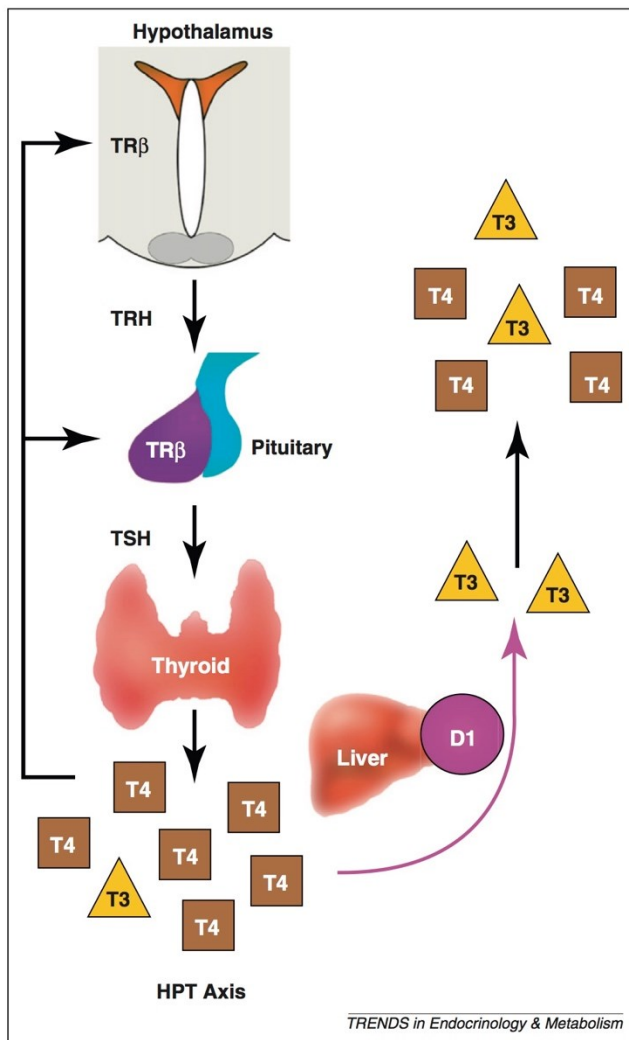


Figure 1. Control of systemic thyroid status. Circulating thyroid status is maintained by the hypothalamic-pituitary-thyroid (HPT) axis. TRH stimulates release of TSH from the anterior pituitary. TSH in turn stimulates synthesis and secretion of T4 and T3, which bind to and activate TR, resulting in feedback inhibition of TRH production and TSH secretion. D1 converts T4 to T3 in the liver, contributing significantly to the pool of circulating T3.

removal of a 5'-iodine atom. This reaction is catalyzed by D1 or D2, whereas D3 irreversibly removes a 5-iodine atom from either T4 or T3 to generate the inactive metabolites 3,3',5'-L-triiodothyronine (reverse T3, rT3) and 3,3'-diiodothyronine (T2), respectively [9,10]. D1 is expressed mainly in the thyroid gland, liver and kidney, where it converts T4 to T3 and thus contributes significantly to the pool of circulating T3, but its precise physiological role is uncertain because serum T3 concentrations remain normal in D1 knockout mice [11]. By contrast, D2 and D3 are expressed in T3-target cells, including the central nervous system, cochlea, retina, heart and skeleton [12,13], and their relative activities are thought to control local tissue availability of the active hormone (Figure 2). During fetal development, high levels of D3 activity in the placenta, uterus and fetal tissues protect developing tissues from exposure to inappropriate levels of T3 and facilitate cell proliferation. At birth, levels of D3 decline rapidly and expression of D2 in T3-target tissues increases markedly to

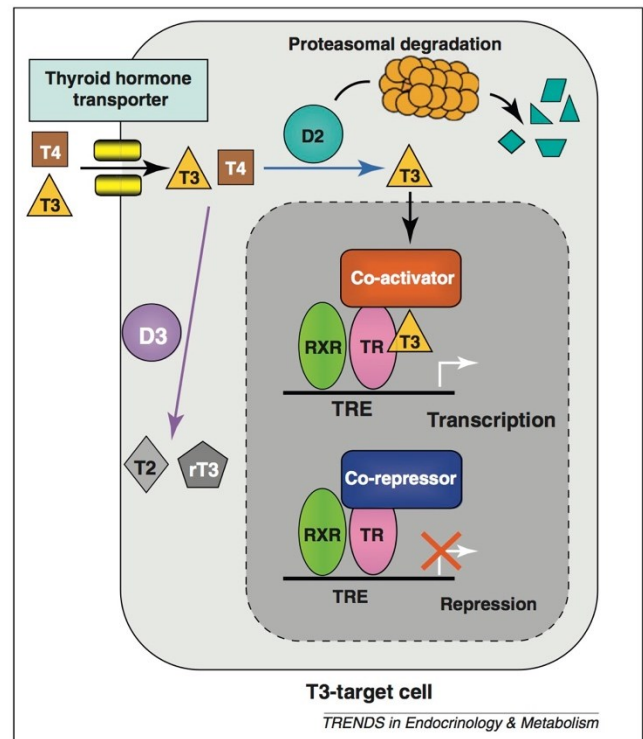


Figure 2. Control of local thyroid status. T4 and T3 enter target cells via specific cell membrane transporters such as MCT8. T4 is either activated to T3 via D2 or inactivated to rT3 by D3. T3 either enters the nucleus or is inactivated to T2 by D3. D2 is subject to rapid T4-dependent ubiquitination and proteasomal degradation, which serves to optimize the intracellular concentration of T3. TRs form heterodimers with RXR and bind to T3 response elements (TREs) located in the promoter regions of T3 target genes. In the absence of T3, the RXR-TR heterodimer recruits co-repressor proteins to inhibit target gene transcription. After binding T3, the liganded receptor complex recruits co-activator proteins, which results in activation of gene transcription.

trigger cell differentiation and organ maturation during normal postnatal development [9,10,13].

Thyroid hormone receptors

The nuclear thyroid hormone receptors α and β (TR α , TR β) act as T3-inducible transcription factors that regulate target gene expression and mediate target tissue responses to T3 [14]. The *THRA* gene encodes the functional receptor TR α 1, which is constitutively expressed in most tissues, as well as TR α 2, a non-T3 binding variant of unknown function. The *THRB* gene encodes two functional proteins, TR β 1 and TR β 2. TR β 1 is widely expressed, whereas TR β 2 is restricted to the hypothalamus and pituitary, where it regulates feedback control of the HPT axis by thyroid hormones [3,15] (Figure 1). Although TR α 1 and TR β 1 are widely expressed, their relative levels of expression vary in a temporospatial manner, especially in the central nervous system, where TR β expression increases markedly after birth but expression of TR α remains relatively constant [16]. Furthermore, target tissues including bone and heart mainly express TR α 1; others such as the liver express predominantly TR β 1, whereas some tissues including skeletal muscle express both isoforms. In the nucleus, TRs form heterodimers with retinoid X receptors (RXR) and bind T3 response elements (TREs) in target gene promoters to regulate gene transcription. Co-repressor proteins are recruited to the

Review

RXR-TR heterodimer in the absence of T3 and inhibit target gene expression. T3 binding displaces the co-repressor, allowing co-activator proteins to interact with the RXR-TR heterodimer and activate gene transcription in a hormone-dependent manner (Figure 2) [14].

Skeletal development

Bone development occurs via two distinct mechanisms. Intramembranous ossification is the process by which mesenchyme progenitors differentiate into bone-forming osteoblasts, which secrete and mineralize an osteoid matrix to form the flat bones of the face and skull. By contrast, endochondral ossification is the process by which long bones form on a cartilage scaffold [17]. Mesenchyme precursor cells condense and differentiate into chondrocytes, which secrete matrix proteins to form a cartilage template. At the primary ossification center, a coordinated program of chondrocyte proliferation, hypertrophic differentiation and apoptosis leads to mineralization of cartilage (Figure 3). Subsequently, vascular invasion and migration of osteoblasts results in replacement of mineralized cartilage with trabecular bone. Concomitantly, peripheral mesenchyme precursors located in the perichondrium differentiate into osteoblasts and form a collar of cortical bone. In addition, secondary ossification centers form within cartilage at the ends of long bones and remain separated from the primary ossification center by the epiphyseal growth plates. Chondrocyte proliferation, hypertrophic differentiation and apoptosis continue in the growth plates until adulthood and mediate longitudinal growth. The process of endochondral ossification is tightly regulated by a local feedback loop

involving the morphogen Indian hedgehog (Ihh) and parathyroid hormone-related peptide (PTHrP) [17,18]. Ihh is expressed by pre-hypertrophic chondrocytes in the growth plate and acts on perichondrial cells to induce expression of PTHrP. Subsequently, PTHrP feeds back on proliferating chondrocytes to limit further hypertrophic differentiation and control the rate of linear growth (Figure 3).

Thyroid hormone action and skeletal development

Euthyroid status is essential for normal skeletal development. Hypothyroidism in children results in growth arrest and delayed bone maturation [19,20]. Treatment with thyroxine results in a period of rapid catch-up growth, although full predicted final height may not be achieved, particularly in children in whom the diagnosis and treatment of hypothyroidism are delayed. Juvenile thyrotoxicosis accelerates growth and advances bone age, but results in short stature due to premature fusion of the growth plates. In severe cases, hyperthyroidism during early childhood may also result in craniosynostosis due to premature fusion of the sutures of the skull [21]. These clinical observations demonstrate the exquisite sensitivity of the developing skeleton to thyroid hormones.

Thyroid hormone action in bone is mediated principally via TR α , which is expressed in the skeleton at >10-fold higher levels than TR β [22,23]. Skeletal responses to thyroid hormones are incompletely characterized but involve the Ihh-PTHrP feedback loop [24], growth hormone, insulin-like growth factor 1 [25] and fibroblast growth factor receptor signaling pathways [26,27] and the Wnt- β -catenin pathway [28].

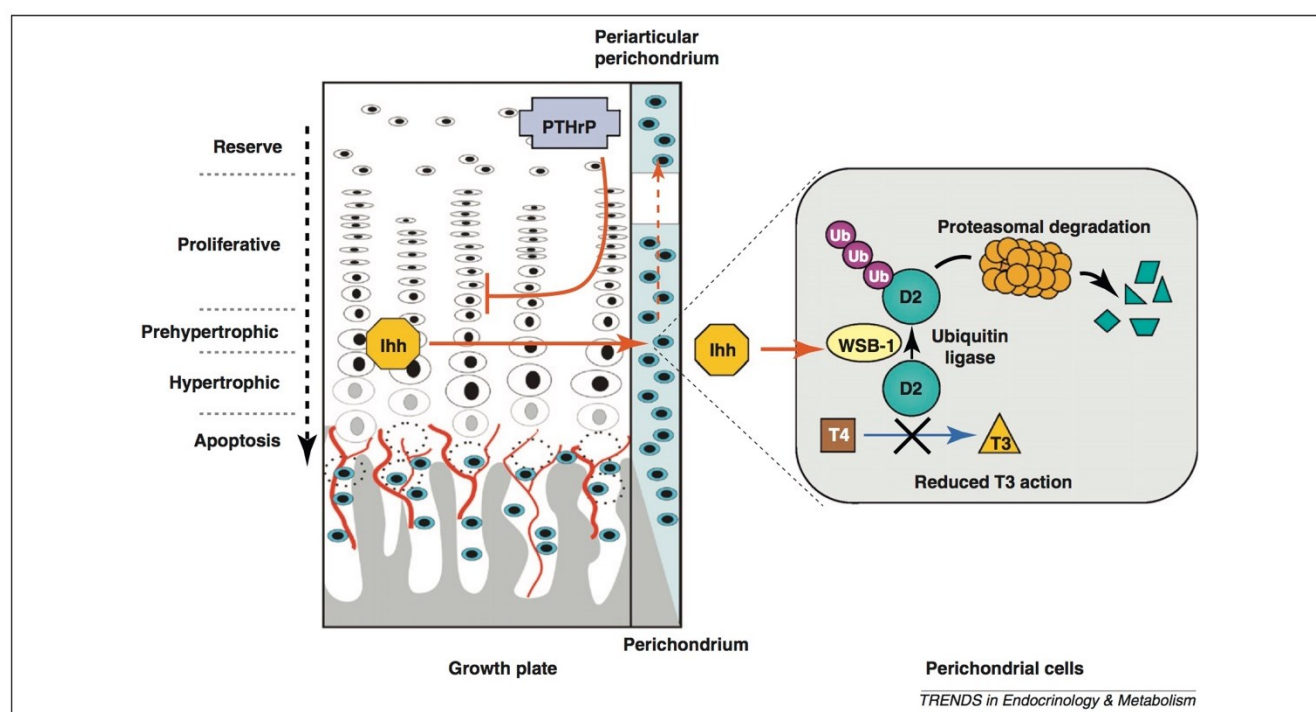


Figure 3. Control of D2 activity in endochondral ossification. Growth plate chondrocytes are organized in discrete zones. The reserve zone contains progenitor cells that are recruited into the proliferative zone, in which discrete columns of proliferating cells organize. Chondrocyte differentiation is initiated in the pre-hypertrophic zone; maturing chondrocytes undergo 10-fold volume expansion in the hypertrophic zone and finally undergo apoptosis. The pace of chondrocyte proliferation and differentiation is regulated by a local paracrine feedback loop involving PTHrP and Ihh. Ihh acts on perichondrial cells to induce expression of the WSB1 component of the E3 ubiquitin ligase enzyme complex, which mediates ubiquitination of D2, targeting it for degradation by the proteasome. Degradation of D2 prevents conversion of T4 to T3, and thus reduces the intracellular T3 concentration and inhibits target gene expression.

Review

Mice with deletion or mutation of TR α are systemically euthyroid [29] but display the phenotypic characteristics of juvenile hypothyroidism, including impaired endochondral ossification with reduced bone mineral deposition and delayed growth [23,25,30–32]. By contrast, mice with deletion or mutation of TR β have elevated circulating thyroid hormone concentrations due to disruption of the HPT axis [29]. Juvenile mice with deletion or mutation of TR β display the characteristic effects of hyperthyroidism on bone and have advanced ossification with increased bone mineral deposition, but display short stature due to accelerated growth-plate maturation [23,31]. These features are consistent with the hypothesis that elevated circulating thyroid hormone levels in TR β mutant mice result in an increased skeletal response to T3 mediated by TR α expressed in bone [33].

Thyroid hormones and adult bone maintenance

Adult bone structure and strength are maintained by a continuous process of bone turnover and repair in the bone remodeling cycle (Figure 4). Osteoclasts are recruited by osteocytes to sites of microdamage or increased mechanical strain and initiate a period of bone resorption. The resulting defects are repaired by osteoblasts, which synthesize, secrete and mineralize osteoid, produce new bone and maintain structural integrity. Thus, balanced coupling of bone resorption and bone formation is essential to maintain the architecture, mineralization and strength of bone [34,35].

Hypothyroidism in adults results in reduced bone turnover with impaired osteoclastic bone resorption and osteoblastic bone formation. Increased duration of the bone remodeling cycle results in a prolonged period of secondary mineralization [36,37]. Accordingly, large population

studies have identified an increased risk of fracture in individuals with hypothyroidism [38,39]. Adult thyrotoxicosis is an established cause of high-bone-turnover osteoporosis, which results from a net increase in bone resorption. Thus, hyperthyroidism is associated with reduced bone mineral density and an increased susceptibility to fragility fracture [37–39]. In addition, studies have also demonstrated an increased risk of fragility fracture in post-menopausal women with subclinical hyperthyroidism, defined by suppressed TSH levels in the presence of normal circulating T4 and T3 concentrations [40]. Furthermore, an increased risk of fracture was found in healthy post-menopausal women with normal circulating TSH and thyroid hormone levels at the upper end of the normal range [41]. Taken together, these studies demonstrate the adult skeleton is sensitive to even minor alterations in thyroid status.

Studies in adult TR knockout and mutant mice are consistent with these clinical data. Thus, adult TR α knockout and mutant mice, despite systemic euthyroidism, have greatly increased trabecular bone volume and mineralization due to a net reduction in osteoclastic bone resorption [30,31]. These findings demonstrate that deletion or mutation of TR α results in impaired T3 action in skeletal cells. By contrast, adult TR β mutant mice display increased osteoclastic bone resorption and severe osteoporosis due to the effects of thyroid hormone excess in TR α -expressing skeletal cells [30,31,33].

T3 action in skeletal cells

TRA1 and TRB1 mRNAs are expressed in chondrocytes, osteoblasts and osteoclasts, but data regarding TR protein expression are limited by the lack of availability of suitably sensitive and specific antibodies [42]. In growth plate and

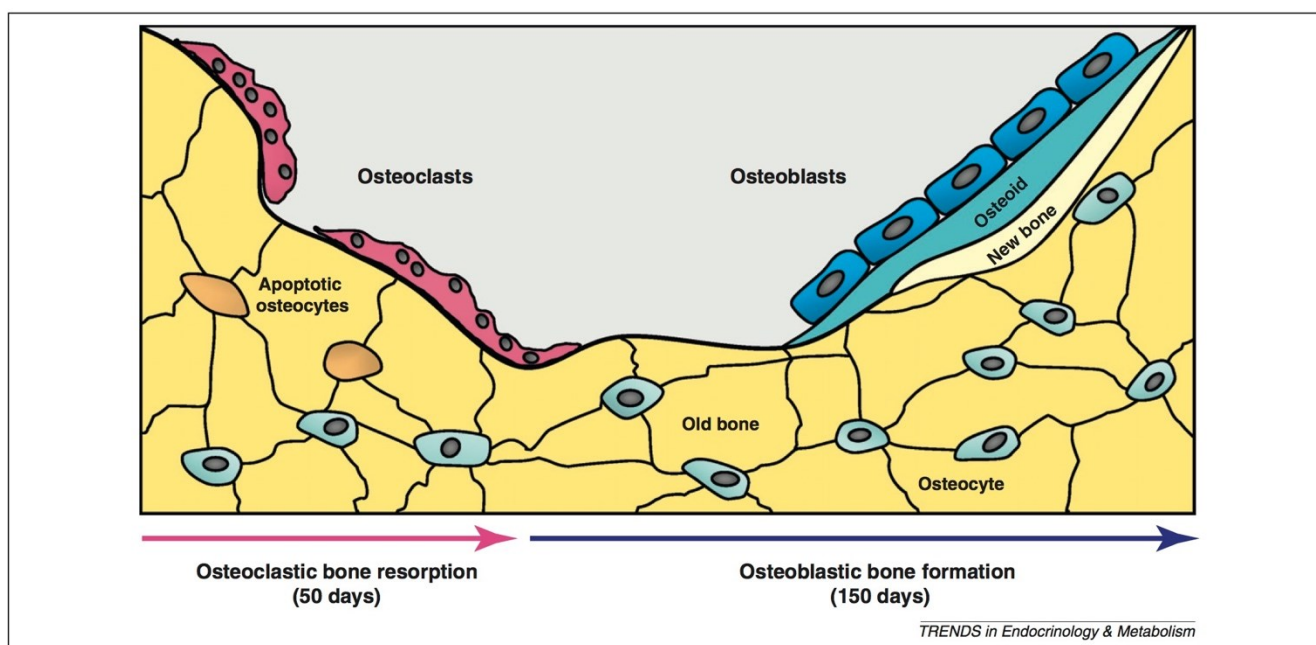


Figure 4. The bone remodeling cycle. Adult bone structure and strength are maintained by the coupled activities of bone-resorbing osteoclasts and bone-forming osteoblasts. Changes in biomechanical loading or areas of microdamage are sensed by a network of osteocyte dendritic processes that ramify throughout the bone. Apoptotic osteocytes at these sites recruit osteoclasts and initiate the remodeling cycle. Osteoclasts resorb areas of damaged bone and communicate with osteoblasts to initiate the subsequent phase of new bone formation and mineralization. Completion of the cycle results in repair of defective bone. The continuous nature of this process at discrete sites throughout the skeleton ensures that bone mass and microarchitecture are maintained in response to loading and injury.

Review

cultured chondrocytes, thyroid hormones inhibit cell proliferation and stimulate hypertrophic differentiation [43–45]. Accordingly, T3 induces markers of hypertrophic chondrocyte differentiation, including alkaline phosphatase and collagen X expression in primary growth-plate chondrocyte cultures, and enhances cartilage matrix mineralization [45]. During endochondral ossification, a paracrine negative feedback loop involving Ihh and PTHrP inhibits hypertrophic chondrocyte differentiation and T3 regulates its set point (Figure 3) [46]. Accordingly, hypothyroidism results in increased PTHrP expression, decreased collagen X synthesis and a failure of hypertrophic differentiation [24]. By contrast, thyrotoxicosis causes reduced expression of the PTHrP receptor with increased expression of fibroblast growth factor receptor-3 (FGFR3) and bone morphogenetic protein-4 (BMP4), all of which promote hypertrophic chondrocyte differentiation [24,26,47]. In addition to inducing chondrocyte hypertrophy, T3 stimulates the expression of proteoglycan and collagen-degrading enzymes including aggrecanase-2 (a disintegrin and metalloproteinase with thrombospondin motifs1, ADAMTS5) and matrix metalloproteinase 13 (MMP13) [48–50].

In osteoblasts, T3 regulates cell differentiation and bone matrix synthesis and degradation. *In vitro* studies demonstrate that T3 stimulates cell proliferation, differentiation and apoptosis by direct and indirect mechanisms. T3 enhances expression and synthesis of the osteoblast differentiation markers collagen I, osteocalcin, alkaline phosphatase, MMP9 and MMP13 in osteoblasts [50–52]. T3 also modulates local paracrine signaling by stimulating osteoblast responses to insulin-like growth factor-1 (IGF1), parathyroid hormone (PTH) and fibroblast growth factors (FGFs) both in cell cultures and *in vivo* [27,53,54]. Although the effects of thyrotoxicosis in adult bone are characterized by increased bone resorption, it is not known whether T3 acts directly in osteoclasts or whether effects on osteoclasts are secondary to the direct actions of T3 in osteoblasts.

Thyroid hormone transporters in bone

Thyroid hormone transporter mRNA expression has been investigated in primary skeletal cells, immortalized bone cell lines and skeletal tissue. *MCT8* mRNA is expressed at all stages of differentiation in primary chondrocytes, osteoblasts and osteoclasts and in chondrogenic ATDC5 cells, osteoblastic MC3T3-E1 cells and osteoclastic RAW 264.7 cells [55] and its expression is regulated by thyroid status [56]. *MCT8*, *LAT1* and *LAT2* expression was also identified in mRNA samples prepared from pre- and postnatal mouse bones, although *NTCP* and *OATP1C1* mRNA expression was undetectable [56]. No data are currently available regarding the expression of *MCT10* mRNA in skeletal cells and the functional role of thyroid hormone transporters in bone has also not been studied.

Individuals with Allan–Herndon–Dudley syndrome display considerable phenotype variability and suffer from hypotonia of the axial muscles, quadriplegia and severe cognitive impairment [57]. Thus, direct effects of *MCT8* deficiency in skeletal cells cannot be determined. *Mct8* knockout mice, however, lack a neurological phenotype [58,59] and thus represent a useful model to study the role

of *MCT8* in skeletal cells, although this has not yet been investigated.

Deiodinases and thyroid hormone metabolism in the skeleton

The roles of the individual deiodinases in bone and skeletal cells have been investigated both *in vivo* and *in vitro*, in various animal models and in well-characterized cell lines and primary cultures.

Type 1 iodothyronine deiodinase

In studies of C3H/HeJ inbred mice, which have a 90% reduction in hepatic D1 activity [60,61], and in D1 knockout mice [11] no skeletal or growth abnormalities were identified. Consistent with these findings, *D1* mRNA expression and enzyme activity were absent from primary chondrocyte, osteoblast and osteoclast cultures [55], as well as immortalized cell lines [62–64]. Thus, D1 does not have a role in the skeleton during either development or adulthood.

Type 2 iodothyronine deiodinase

Studies of chicken embryonic skeletal development identified restricted D2 expression and activity in the perichondrium adjacent to the growth plate. This activity of D2 was inhibited by hedgehog morphogens via stimulation of ubiquitin-mediated degradation of the enzyme (Figure 3) [46]. This finding suggests that D2-mediated control of T3 action in the perichondrium forms an integral part of the Ihh–PTHrP feedback loop. In this model, local hypothyroidism mediated by degradation of D2 results in increased synthesis of PTHrP and consequent inhibition of growth plate chondrocyte differentiation (Figure 3) [46]. This mechanism is consistent with the delayed endochondral ossification and short stature observed in hypothyroidism.

Studies during early mouse development indicate that *D2* mRNA is expressed in the developing limb from embryonic day E14.5 and increases until E18.5, when activity of the enzyme was also found to be increased in thyroid hormone deficiency [65]. These findings further highlight the principle that levels of *D2* mRNA do not necessarily correlate with enzyme activity because D2 is subject to substrate-dependent (i.e. T4-dependent) ubiquitination and proteasome-mediated degradation [9,10]. Thus, in T3-target tissues, D2 activity is upregulated in hypothyroidism but is inhibited in thyrotoxicosis. Accordingly, studies of D2 expression and activity in bone tissue and skeletal cells have provided conflicting results [63,65–67]. Recently, however, a highly specific and sensitive HPLC-based D2 assay demonstrated that D2 activity is only detectable in mature primary osteoblasts but is absent from cultures of primary chondrocytes and osteoclasts [55].

D2 knockout mice (D2KO) display pituitary resistance to thyroid hormones with a 1.4-fold elevated T4 level, a 2-fold increase in TSH, but a normal circulating T3 concentration [68]. D2KO mice have a minor and transient reduction in growth plate height, but normal linear growth, bone formation and cortical bone thickness [12]. Thus, although D2 has been shown to have a functional role in the perichondrium during early skeletal development [46], these

Review

studies indicate its activity is not essential for normal postnatal growth.

By contrast, bones from adult D2KO mice are brittle and have increased mineralization despite normal bone microarchitecture [12]. Dynamic histomorphometry demonstrated normal osteoclastic bone resorption but impaired osteoblast function. The rate of bone formation in D2KO mice was reduced by 50%, resulting in prolongation of the bone remodeling cycle and an extended period of secondary mineralization, which account for the increased bone mineral density and abnormal biomechanical phenotype [12]. These findings are consistent with histomorphometry studies of individuals with hypothyroidism [36,37] and the restricted expression of D2 activity in mature osteoblasts [55].

In summary, thyrotoxicosis causes high bone turnover, osteoporosis and an increased risk of fragility fracture, whereas thyroid hormone deficiency results in low bone turnover and an increased risk of fracture [38,39,55]. Together with understanding obtained from studies of D2KO mice, these observations suggest an essential homeostatic role for D2 in regulating local T3 concentrations in osteoblasts and thus in maintaining optimal bone mineralization and strength (Figure 5) [12].

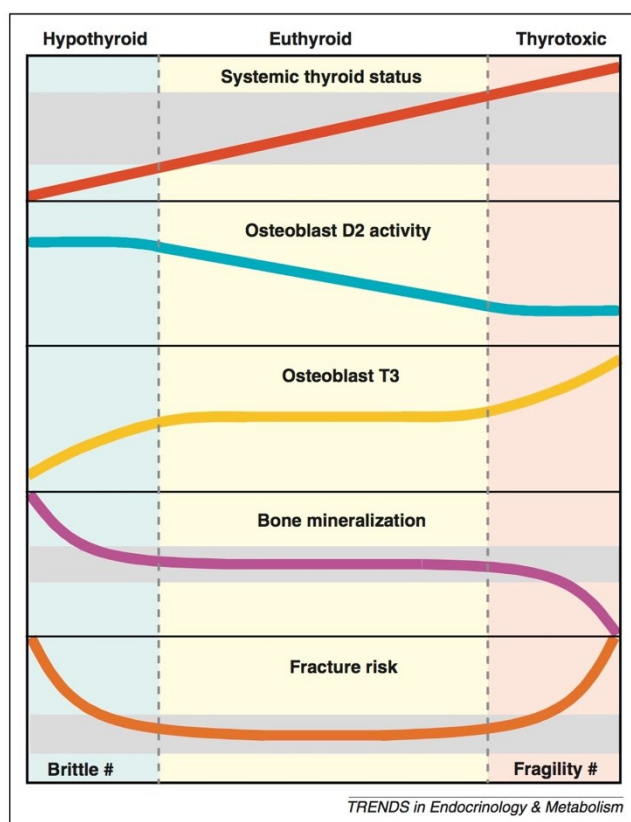


Figure 5. Regulated activity of type 2 deiodinase (D2) in osteoblasts maintains optimal bone mineralization and strength. D2 converts T4 to active T3. D2 activity is inhibited by its substrate T4 and thus enzyme activity is maximal in hypothyroidism and suppressed in thyrotoxicosis. The regulation of D2 activity in osteoblasts is able to maintain a constant intracellular T3 concentration when systemic thyroid hormone concentrations are within the euthyroid range, which thus preserves optimal bone mineralization and strength. The capacity of this local feedback mechanism, however, cannot compensate in overt hypothyroidism or thyrotoxicosis, which lead to increased or reduced bone mineralization and an increased risk of brittle or fragility fractures (#), respectively.

Type 3 iodothyronine deiodinase

Studies in primary growth plate chondrocytes, osteoblasts and osteoclasts demonstrated the presence of D3 expression and activity [55]. However, enzyme activity was only detected in chondrocytes obtained from 3-week-old weaning mice, but not from older animals, which suggests that D3 may reduce T3 availability in chondrocytes to limit linear growth before weaning. This hypothesis is consistent with additional studies demonstrating that D3 is expressed at high levels in the embryonic skeleton, and thus maintains low tissue concentrations of T3 and limits thyroid hormone action. Accordingly, embryonic and early postnatal skeletal development was unaffected by systemic hypothyroidism [65]. Deletion of D3 in knockout mice results in marked abnormalities of systemic thyroid status, including neonatal thyrotoxicosis followed by central hypothyroidism [69,70]. Persistent growth retardation is a consequence of multiple systemic abnormalities that confound interpretation of the skeletal phenotype [69,70]. The physiological role of D3 in the skeleton is therefore currently unknown.

Clinical implications of altered thyroid hormone metabolism in bone

To date no mutations in the *DIO1*, *DIO2* or *DIO3* genes have been identified in humans. Nevertheless, the association between skeletal disorders and a common, non-synonymous single nucleotide polymorphism (SNP, rs225014, Thr92Ala) in *DIO2* [71] has been investigated [72]. *In vitro* studies demonstrated that the Thr92Ala substitution in D2 does not affect enzyme activity, half-life or substrate-mediated inhibition [71]. Despite these findings, D2 activity in skeletal muscle and thyroid tissue was reduced by 50% in individuals with the Ala/Ala genotype. Other studies have investigated a polymorphism of unknown functional significance in the 3' untranslated region of the *DIO3* gene (SNP, rs945006) [73].

A genome-wide linkage analysis identified the D2 Thr92Ala polymorphism as a susceptibility locus for osteoarthritis [74]. This association between *DIO2* and osteoarthritis, however, was not replicated in a subsequent association study in the Rotterdam Study population [75] and was not identified in a recent large meta-analysis [76]. Despite this, a meta-analysis of factors that regulate thyroid hormone metabolism identified a possible role for *DIO3* as a disease-modifying locus in osteoarthritis [73]. Taken together, these new findings implicate deiodinase-regulated local availability of T3 in chondrocytes as a possible factor in the pathophysiology of osteoarthritis, although further studies are necessary to investigate this hypothesis and its underlying mechanisms.

In addition to effects in cartilage, a possible role of *DIO2* in osteoporosis has been studied in a mixed population of thyroid cancer patients. Individuals with Thr/Ala and Ala/Ala genotypes had increased markers of bone turnover and a 6% reduction in bone mineral density compared to Thr/Thr individuals, which is suggestive of increased T3 action in bone [71]. However, underlying mechanisms to account for this finding remain obscure because the polymorphism does not affect enzyme activity *in vitro* or results in reduced tissue activity *in vivo*.

Review

Trends in Endocrinology and Metabolism xxx xxxx, Vol. xxx, No. x

Concluding remarks

Thyroid hormones enter target cells via membrane transporter proteins. The MCT8 specific transporter is expressed widely in all bone cell lineages but its functional role in the skeleton is unknown. Expression and function of the other thyroid hormone transporters in bone have not been explored. Thyroid hormone metabolism by the deiodinases is an important determinant of thyroid status in the circulation and in target tissues. D1 contributes to the circulating pool of T3 but has no physiological role in the skeleton. D2 and D3 control the intracellular T3 concentration in some thyroid-hormone-responsive cells and regulate T3 action in target tissues. In the skeleton, D2 activity is restricted to bone-forming osteoblasts and plays a crucial role in maintaining optimal bone mineralization and strength. D3 is expressed in cartilage before weaning and is likely to have an important regulatory role during skeletal development and growth. Recent population studies identified the *DIO2* and *DIO3* genes as susceptibility loci for osteoarthritis. This suggests that control of the T3 supply in cartilage may play a role in articular cartilage maintenance and repair. Investigation of the molecular mechanisms underlying these novel aspects of the local control of T3 action in bone cells and skeletal disease is a major focus for current thyroid research, but remains a significant challenge. The application of Cre-Lox gene-targeting strategies to generate new mouse models will enable the cell-specific mechanisms of T3 action to be determined *in vivo*.

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References

- Bianco, A.C. *et al.* (2002) Biochemistry, cellular and molecular biology, and physiological roles of the iodothyronine selenodeiodinases. *Endocr. Rev.* 23, 38–89
- Abel, E.D. *et al.* (2001) Critical role for thyroid hormone receptor beta2 in the regulation of paraventricular thyrotropin-releasing hormone neurons. *J. Clin. Invest.* 107, 1017–1023
- Forrest, D. *et al.* (1996) Recessive resistance to thyroid hormone in mice lacking thyroid hormone receptor beta: evidence for tissue-specific modulation of receptor function. *EMBO J.* 15, 3006–3015
- Visser, W.E. *et al.* (2011) Thyroid hormone transporters: the knowns and the unknowns. *Mol. Endocrinol.* 25, 1–14
- Dumitrescu, A.M. *et al.* (2004) A novel syndrome combining thyroid and neurological abnormalities is associated with mutations in a monocarboxylate transporter gene. *Am. J. Hum. Genet.* 74, 168–175
- Friesema, E.C. *et al.* (2004) Association between mutations in a thyroid hormone transporter and severe X-linked psychomotor retardation. *Lancet* 364, 1435–1437
- Friesema, E.C. *et al.* (2006) Mechanisms of disease: psychomotor retardation and high T3 levels caused by mutations in monocarboxylate transporter 8. *Nat. Clin. Pract. Endocrinol. Metab.* 2, 512–523
- Cheng, S.Y. *et al.* (2010) Molecular aspects of thyroid hormone actions. *Endocr. Rev.* 31, 139–170
- Gereben, B. *et al.* (2008) Cellular and molecular basis of deiodinase-regulated thyroid hormone signaling. *Endocr. Rev.* 29, 898–938
- St Germain, D.L. *et al.* (2009) Defining the roles of the iodothyronine deiodinases: current concepts and challenges. *Endocrinology* 150, 1097–1107
- Schneider, M.J. *et al.* (2006) Targeted disruption of the type 1 selenodeiodinase gene (*Dio1*) results in marked changes in thyroid hormone economy in mice. *Endocrinology* 147, 580–589
- Bassett, J.H. *et al.* (2010) Optimal bone strength and mineralization requires the type 2 iodothyronine deiodinase in osteoblasts. *Proc. Natl. Acad. Sci. U.S.A.* 107, 7604–7609
- Bianco, A.C. and Kim, B.W. (2006) Deiodinases: implications of the local control of thyroid hormone action. *J. Clin. Invest.* 116, 2571–2579
- Harvey, C.B. and Williams, G.R. (2002) Mechanism of thyroid hormone action. *Thyroid* 12, 441–446
- Abel, E.D. *et al.* (1999) Divergent roles for thyroid hormone receptor beta isoforms in the endocrine axis and auditory system. *J. Clin. Invest.* 104, 291–300
- Forrest, D. *et al.* (1990) Contrasting developmental and tissue-specific expression of alpha and beta thyroid hormone receptor genes. *EMBO J.* 9, 1519–1528
- Kronenberg, H.M. (2003) Developmental regulation of the growth plate. *Nature* 423, 332–336
- Lai, L.P. and Mitchell, J. (2005) Indian hedgehog: its roles and regulation in endochondral bone development. *J. Cell. Biochem.* 96, 1163–1173
- Boersma, B. *et al.* (1996) Catch-up growth after prolonged hypothyroidism. *Eur. J. Pediatr.* 155, 362–367
- Rivkees, S.A. *et al.* (1988) Long-term growth in juvenile acquired hypothyroidism: the failure to achieve normal adult stature. *N. Engl. J. Med.* 318, 599–602
- Segni, M. *et al.* (1999) Special features of Graves' disease in early childhood. *Thyroid* 9, 871–877
- Bookout, A.L. *et al.* (2006) Anatomical profiling of nuclear receptor expression reveals a hierarchical transcriptional network. *Cell* 126, 789–799
- O'Shea, P.J. *et al.* (2003) A thyrotoxic skeletal phenotype of advanced bone formation in mice with resistance to thyroid hormone. *Mol. Endocrinol.* 17, 1410–1424
- Stevens, D.A. *et al.* (2000) Thyroid hormones regulate hypertrophic chondrocyte differentiation and expression of parathyroid hormone-related peptide and its receptor during endochondral bone formation. *J. Bone Miner. Res.* 15, 2431–2442
- O'Shea, P.J. *et al.* (2005) Contrasting skeletal phenotypes in mice with an identical mutation targeted to thyroid hormone receptor alpha1 or beta. *Mol. Endocrinol.* 19, 3045–3059
- Barnard, J.C. *et al.* (2005) Thyroid hormones regulate fibroblast growth factor receptor signaling during chondrogenesis. *Endocrinology* 146, 5568–5580
- Stevens, D.A. *et al.* (2003) Thyroid hormone activates fibroblast growth factor receptor-1 in bone. *Mol. Endocrinol.* 17, 1751–1766
- Wang, L. *et al.* (2007) Thyroid hormone interacts with the Wnt/beta-catenin signaling pathway in the terminal differentiation of growth plate chondrocytes. *J. Bone Miner. Res.* 22, 1988–1995
- Gauthier, K. *et al.* (1999) Different functions for the thyroid hormone receptors TRalpha and TRbeta in the control of thyroid hormone production and post-natal development. *EMBO J.* 18, 623–631
- Bassett, J.H. *et al.* (2007) Thyroid status during skeletal development determines adult bone structure and mineralization. *Mol. Endocrinol.* 21, 1893–1904
- Bassett, J.H. *et al.* (2007) Thyroid hormone excess rather than thyrotropin deficiency induces osteoporosis in hyperthyroidism. *Mol. Endocrinol.* 21, 1095–1107
- Gauthier, K. *et al.* (2001) Genetic analysis reveals different functions for the products of the thyroid hormone receptor alpha locus. *Mol. Cell. Biol.* 21, 4748–4760
- O'Shea, P.J. *et al.* (2006) Characterization of skeletal phenotypes of TRalpha1 and TRbeta mutant mice: implications for tissue thyroid status and T3 target gene expression. *Nucl. Receptor Signal.* 4, e011
- Boyle, W.J. *et al.* (2003) Osteoclast differentiation and activation. *Nature* 423, 337–342
- Harada, S. and Rodan, G.A. (2003) Control of osteoblast function and regulation of bone mass. *Nature* 423, 349–355
- Eriksen, E.F. *et al.* (1986) Kinetics of trabecular bone resorption and formation in hypothyroidism: evidence for a positive balance per remodeling cycle. *Bone* 7, 101–108
- Mosekilde, L. *et al.* (1990) Effects of thyroid hormones on bone and mineral metabolism. *Endocrinol. Metab. Clin. North Am.* 19, 35–63
- Vestergaard, P. and Mosekilde, L. (2002) Fractures in patients with hyperthyroidism and hypothyroidism: a nationwide follow-up study in 16,249 patients. *Thyroid* 12, 411–419

Review

Trends in Endocrinology and Metabolism xxx xxxx, Vol. xxx, No. x

- 39 Vestergaard, P. *et al.* (2005) Influence of hyper- and hypothyroidism, and the effects of treatment with antithyroid drugs and levothyroxine on fracture risk. *Calcif. Tissue Int.* 77, 139–144
- 40 Bauer, D.C. *et al.* (2001) Risk for fracture in women with low serum levels of thyroid-stimulating hormone. *Ann. Intern. Med.* 134, 561–568
- 41 Murphy, E. *et al.* (2010) Thyroid function within the upper normal range is associated with reduced bone mineral density and an increased risk of nonvertebral fractures in healthy euthyroid postmenopausal women. *J. Clin. Endocrinol. Metab.* 95, 3173–3181
- 42 Bassett, J.H. and Williams, G.R. (2009) The skeletal phenotypes of TRalpha and TRbeta mutant mice. *J. Mol. Endocrinol.* 42, 269–282
- 43 Ballock, R.T. and Reddi, A.H. (1994) Thyroxine is the serum factor that regulates morphogenesis of columnar cartilage from isolated chondrocytes in chemically defined medium. *J. Cell Biol.* 126, 1311–1318
- 44 Ballock, R.T. *et al.* (2000) Expression of cyclin-dependent kinase inhibitors in epiphyseal chondrocytes induced to terminally differentiate with thyroid hormone. *Endocrinology* 141, 4552–4557
- 45 Robson, H. *et al.* (2000) Thyroid hormone acts directly on growth plate chondrocytes to promote hypertrophic differentiation and inhibit clonal expansion and cell proliferation. *Endocrinology* 141, 3887–3897
- 46 Dentice, M. *et al.* (2005) The hedgehog-inducible ubiquitin ligase subunit WSB-1 modulates thyroid hormone activation and PTHrP secretion in the developing growth plate. *Nat. Cell Biol.* 7, 698–705
- 47 Lasso, L. *et al.* (2009) Thyroid hormone treatment of cultured chondrocytes mimics in vivo stimulation of collagen X mRNA by increasing BMP 4 expression. *J. Cell. Physiol.* 219, 595–605
- 48 Himeno, M. *et al.* (2002) Impaired vascular invasion of Cbfa1-deficient cartilage engrafted in the spleen. *J. Bone Miner. Res.* 17, 1297–1305
- 49 Makihiro, S. *et al.* (2003) Thyroid hormone enhances aggrecanase-2/ADAM-TS5 expression and proteoglycan degradation in growth plate cartilage. *Endocrinology* 144, 2480–2488
- 50 Pereira, R.C. *et al.* (1999) Triiodothyronine induces collagenase-3 and gelatinase B expression in murine osteoblasts. *Am. J. Physiol.* 277, E496–E504
- 51 Gouveia, C.H. *et al.* (2001) Thyroid hormone stimulation of osteocalcin gene expression in ROS 17/2.8 cells is mediated by transcriptional and post-transcriptional mechanisms. *J. Endocrinol.* 170, 667–675
- 52 Varga, F. *et al.* (2010) T3 affects expression of collagen I and collagen cross-linking in bone cell cultures. *Biochem. Biophys. Res. Commun.* 402, 180–185
- 53 Gu, W.X. *et al.* (2001) Mutual up-regulation of thyroid hormone and parathyroid hormone receptors in rat osteoblastic osteosarcoma 17/2.8 cells. *Endocrinology* 142, 157–164
- 54 Huang, B.K. *et al.* (2000) Insulin-like growth factor I production is essential for anabolic effects of thyroid hormone in osteoblasts. *J. Bone Miner. Res.* 15, 188–197
- 55 Williams, A.J. *et al.* (2008) Iodothyronine deiodinase enzyme activities in bone. *Bone* 43, 126–134
- 56 Capelo, L.P. *et al.* (2009) The monocarboxylate transporter 8 and L-type amino acid transporters 1 and 2 are expressed in mouse skeletons and in osteoblastic MC3T3-E1 cells. *Thyroid* 19, 171–180
- 57 Schwartz, C.E. and Stevenson, R.E. (2007) The MCT8 thyroid hormone transporter and Allan-Herndon-Dudley syndrome. *Best Pract. Res. Clin. Endocrinol. Metab.* 21, 307–321
- 58 Dumitrescu, A.M. *et al.* (2006) Tissue-specific thyroid hormone deprivation and excess in monocarboxylate transporter (mct) 8-deficient mice. *Endocrinology* 147, 4036–4043
- 59 Trajkovic, M. *et al.* (2007) Abnormal thyroid hormone metabolism in mice lacking the monocarboxylate transporter 8. *J. Clin. Invest.* 117, 627–635
- 60 Berry, M.J. *et al.* (1993) Physiological and genetic analyses of inbred mouse strains with a type I iodothyronine 5' deiodinase deficiency. *J. Clin. Invest.* 92, 1517–1528
- 61 Schoenmakers, C.H. *et al.* (1993) Impairment of the selenoenzyme type I iodothyronine deiodinase in C3H/He mice. *Endocrinology* 132, 357–361
- 62 Dreher, I. *et al.* (1998) Selenoproteins are expressed in fetal human osteoblast-like cells. *Biochem. Biophys. Res. Commun.* 245, 101–107
- 63 Gouveia, C.H. *et al.* (2005) Type 2 iodothyronine selenodeiodinase is expressed throughout the mouse skeleton and in the MC3T3-E1 mouse osteoblastic cell line during differentiation. *Endocrinology* 146, 195–200
- 64 LeBron, B.A. *et al.* (1989) Thyroid hormone 5'-deiodinase activity, nuclear binding, and effects on mitogenesis in UMR-106 osteoblastic osteosarcoma cells. *J. Bone Miner. Res.* 4, 173–178
- 65 Capelo, L.P. *et al.* (2008) Deiodinase-mediated thyroid hormone inactivation minimizes thyroid hormone signaling in the early development of fetal skeleton. *Bone* 43, 921–930
- 66 Miura, M. *et al.* (2002) Thyroid hormones promote chondrocyte differentiation in mouse ATDC5 cells and stimulate endochondral ossification in fetal mouse tibias through iodothyronine deiodinases in the growth plate. *J. Bone Miner. Res.* 17, 443–454
- 67 Morimura, T. *et al.* (2005) Expression of type 2 iodothyronine deiodinase in human osteoblast is stimulated by thyrotropin. *Endocrinology* 146, 2077–2084
- 68 Schneider, M.J. *et al.* (2001) Targeted disruption of the type 2 selenodeiodinase gene (*DIO2*) results in a phenotype of pituitary resistance to T4. *Mol. Endocrinol.* 15, 2137–2148
- 69 Hernandez, A. *et al.* (2006) Type 3 deiodinase is critical for the maturation and function of the thyroid axis. *J. Clin. Invest.* 116, 476–484
- 70 Hernandez, A. *et al.* (2007) Type 3 deiodinase deficiency results in functional abnormalities at multiple levels of the thyroid axis. *Endocrinology* 148, 5680–5687
- 71 Canani, L.H. *et al.* (2005) The type 2 deiodinase A/G (Thr92Ala) polymorphism is associated with decreased enzyme velocity and increased insulin resistance in patients with type 2 diabetes mellitus. *J. Clin. Endocrinol. Metab.* 90, 3472–3478
- 72 Heemstra, K.A. *et al.* (2010) The type 2 deiodinase Thr92Ala polymorphism is associated with increased bone turnover and decreased femoral neck bone mineral density. *J. Bone Miner. Res.* 25, 1385–1391
- 73 Meulenbelt, I. *et al.* (2011) Meta-analyses of genes modulating intracellular T3 bio-availability reveal a possible role for the *DIO3* gene in osteoarthritis susceptibility. *Ann. Rheum. Dis.* 70, 164–167
- 74 Meulenbelt, I. *et al.* (2008) Identification of *DIO2* as a new susceptibility locus for symptomatic osteoarthritis. *Hum. Mol. Genet.* 17, 1867–1875
- 75 Kerkhof, H.J. *et al.* (2010) A genome-wide association study identifies an osteoarthritis susceptibility locus on chromosome 7q22. *Arthritis Rheum.* 62, 499–510
- 76 Evangelou, E. *et al.* (2010) Meta-analysis of genome-wide association studies confirms a susceptibility locus for knee osteoarthritis on chromosome 7q22. *Ann. Rheum. Dis.* 70, 349–355

Osteoarthritis and Cartilage



Brief Report

Quantitative X-ray microradiography for high-throughput phenotyping of osteoarthritis in mice

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SUMMARY

Objective: To investigate and validate digital X-ray microradiography as a novel, high-throughput and cost-effective screening approach to identify abnormal joint phenotypes in mice.

Method: Digital X-ray microradiography was used to quantify the subchondral bone mineral content (BMC) in the medial tibial plateau. Accuracy and reproducibility of the method were determined in 22 samples from C57BL/6(B6Brd;B6Dnk;B6N-Tyr^{C-Brd}) wild-type mice. The method was then validated in wild-type mice that had undergone surgical destabilisation of medial meniscus (DMM) and in a genetically modified mouse strain with an established increase in trabecular bone mass.

Results: The measurement of subchondral BMC by digital X-ray microradiography had a coefficient of variation of 3.6%. Digital X-ray microradiography was able to demonstrate significantly increased subchondral BMC in the medial tibial plateau of male mice 4 and 8 weeks after DMM surgery and in female mice 8 weeks after surgery. Furthermore, digital X-ray microradiography also detected the increase in subchondral BMC in a genetically modified mouse strain with high trabecular bone mass.

Conclusion: Quantitation of subchondral BMC by digital X-ray microradiography is a rapid, sensitive and cost-effective method to identify abnormal joint phenotypes in mice of both genders at several ages.

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Introduction

Osteoarthritis is the commonest joint disorder; it causes inexorable joint dysfunction, pain and disability as no drugs are available to prevent or delay disease progression. Patients are asymptomatic in the early stages of osteoarthritis and develop problems only after significant cartilage erosion has occurred. The aetiology of osteoarthritis is complex and multi-factorial with major genetic and environmental contributions¹. Its heritability is estimated to be between 40 and 65% but currently identified disease susceptibility loci account for only a fraction of this heritability². Thus, there is an urgent need to advance understanding of the pathogenesis of osteoarthritis and define new molecular pathways that facilitate the development of novel treatments.

Animal models are essential for *in vivo* study of disease mechanisms, drug targets and treatment responses³. A limited number of mouse models of spontaneous cartilage disorders have been described, including the STR/ort and STR-1N mouse. These models reinforce the importance of genetics in disease susceptibility but their use is limited by variable disease penetrance⁴. Surgically induced models of osteoarthritis are also used to investigate disease pathogenesis and response to treatment. However, this approach requires significant expertise and experience, and requires large numbers of mice at considerable expense. The best characterised and most reliable surgical model requires destabilisation of the medial meniscus (DMM), resulting in a high incidence of osteoarthritis in male mice 8 weeks following surgery⁵. Nevertheless, there is now a clear need to develop robust and rapid screening approaches to identify joint abnormalities in mouse models of spontaneous osteoarthritis.

The International Knockout Mouse Consortium and Medical Research Council *N*-ethyl-*N*-nitrosourea mutagenesis programmes aim to disrupt each of the >20,000 protein-coding genes⁶. These initiatives provide a unique opportunity to identify novel genetic determinants and *in vivo* models of osteoarthritis and define the cellular and molecular basis of disease. Nevertheless, to capitalise on these resources and identify mice with outlier joint phenotypes,

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new high-throughput, sensitive, specific and cost-effective joint phenotyping methods are needed.

Established osteoarthritis is characterised by articular cartilage destruction and subchondral bone sclerosis. Articular cartilage degradation progresses from surface fibrillation to formation of fissures and erosions, and finally cartilage loss with exposure of underlying bone. These changes are associated with increased chondrocyte hypertrophy, cartilage calcification and osteophyte formation¹. Subchondral bone distributes load-bearing compressive forces and comprises the cortical plate underlying the articular cartilage and the adjacent trabecular bone. Early osteoarthritis is characterised by thinning of the cortical plate due to accelerated remodelling, whereas subchondral sclerosis is the hallmark of established disease⁷.

The gold standard histological assessment of articular cartilage type, architecture and integrity in accordance with Osteoarthritis Research Society International (OARSI) recommendations⁴ is laborious, expensive and unsuitable for high-throughput screening. Micro-computed tomography (CT) analysis of osteoarthritis in mice is also time consuming; suitable high-resolution imaging equipment is expensive and not widely available and the vast quantity of data generated requires an extensive archive⁸.

We previously established that quantitative digital X-ray microradiography is a sensitive and specific method for determining bone mineral content (BMC)⁹ and also demonstrated its suitability for high-throughput phenotyping¹⁰. Thus, we generated reference data for BMC in C57BL/6(B6Brd;B6Dnk;B6N-Tyr^{c-Brd}) wild-type mice and subsequently screened 100 knockout strains from the IKMC to identify nine outlier strains with either high or low BMC¹⁰. Since subchondral bone sclerosis is a consistent and established feature of both human osteoarthritis and surgically induced mouse models of osteoarthritis^{11,12}, analysis of subchondral BMC represents a suitable target for high-throughput joint phenotype screening approaches.

We hypothesised, therefore, that digital X-ray microradiography can be used to quantify subchondral BMC and identify osteoarthritis phenotypes in genetically modified mice. We, therefore, studied knee joints from mice that had undergone DMM surgery^{5,13} and genetically modified mice with increased trabecular bone mass^{14,15}.

Methods

Animals

Studies were conducted in accordance with the Animals (Scientific Procedures) Act 1986 and recommendations of the Weatherall report, and were approved by the Imperial College Ethical Review Committee. All wild-type mice used for DMM surgery were of the same C57BL/6(B6Brd;B6Dnk;B6N-Tyr^{c-Brd}) genetic background¹⁰. Additional studies were performed using TR α ^{0/0} and control mice originally derived in a mixed C57BL/6 and 129Sv genetic background^{14,15}, and subsequently backcrossed onto C57BL/6N for more than 20 generations.

DMM surgery

Transection of the right medial meniscotibial ligament was performed at 10 weeks of age and sham surgery was performed on the left knee⁵. Males were sacrificed 4 weeks ($n = 6$ per group) or 8 weeks ($n = 12$ per group) post-surgery. Females were sacrificed 8 weeks post-surgery ($n = 6$ per group).

Sample preparation

Lower limb samples were fixed in 10% neutral buffered formalin overnight and stored in 70% ethanol. Soft tissue was removed but integrity of the knee capsule and patella was preserved [Fig. 1(A)].

Histological analysis

Knees were decalcified in 10% Ethylenediaminetetraacetic acid (EDTA) for 10 days and embedded in paraffin. 5 μ m coronal sections were cut at 80 μ m intervals at 16 levels (10 sections per level) through the entire thickness of the joint, stained with safranin O and fast green, according to OARSI recommendations⁴. Qualitative analysis was performed to confirm the expected presence of cartilage degradation that co-localised with subchondral bone thickening in the medial tibial plateau following DMM surgery¹¹.

X-ray microradiography

Knee joints were held in a flexed position of 105° using synthetic rubber adhesive within a 37 mm diameter plastic ring. This resulted in consistent location of the apex of the knee joint 12 mm above the mount with the tibial growth plate in a vertical plane. The patella was used as a landmark to ensure correct orientation of the joint [Fig. 1(B)].

Anterior–posterior projection X-ray images were recorded at 10 μ m pixel resolution using a Faxitron MX-20 variable kV point projection X-ray source and digital image system (Qados, Cross Technologies plc, Berkshire, UK) operating at 26 kV for 15 s and 5 $\times$ magnification. Images were calibrated by X-raying a digital micrometer. The relative medial tibial plateau subchondral BMC was determined by comparison with standards included in each image (1 mm diameter steel, aluminium and polyester wires) [Fig. 1(B)]. The 2368 $\times$ 2340 16 bit DICOM images were converted to 8 bit TIFF using ImageJ (<http://rsb.info.nih.gov/ij/>) and the grey scale histogram was stretched from the polyester (grey level 0) to steel (grey level 255) standards. Increasing gradations of mineralization density were represented in 16 equal intervals by a pseudo-colour scheme^{9,10}.

Image analysis

A 1000 μ m $\times$ 250 μ m area of the medial tibial plateau was selected as representative of subchondral bone and defined as the region of interest (ROI) [Fig. 1(C)]. A single montage was generated from the individual ROIs from all mice within one experimental group. The relative and cumulative frequency distributions of grey levels were determined and compared between groups as indicated in the figures^{9,10} [Fig. 1(D)].

Statistics

As grey level frequency distributions were not normally distributed [Fig. 1(D)], the median value was determined for each sample. To define the variation in median BMC within the population of 22 female, 16-week-old wild-type mice [Fig. 1(D)] the mean and standard deviation of the median BMC values were determined. The Kolmogorov–Smirnov test was used to compare grey level cumulative frequency distributions between DMM and sham-operated mice, and between TR α ^{0/0} and control mice. P values for the D statistic were $D \geq 6.01$, $P < 0.05$; $D \geq 7.20$, $P < 0.01$; and $D \geq 8.62$, $P < 0.001$ ⁹.

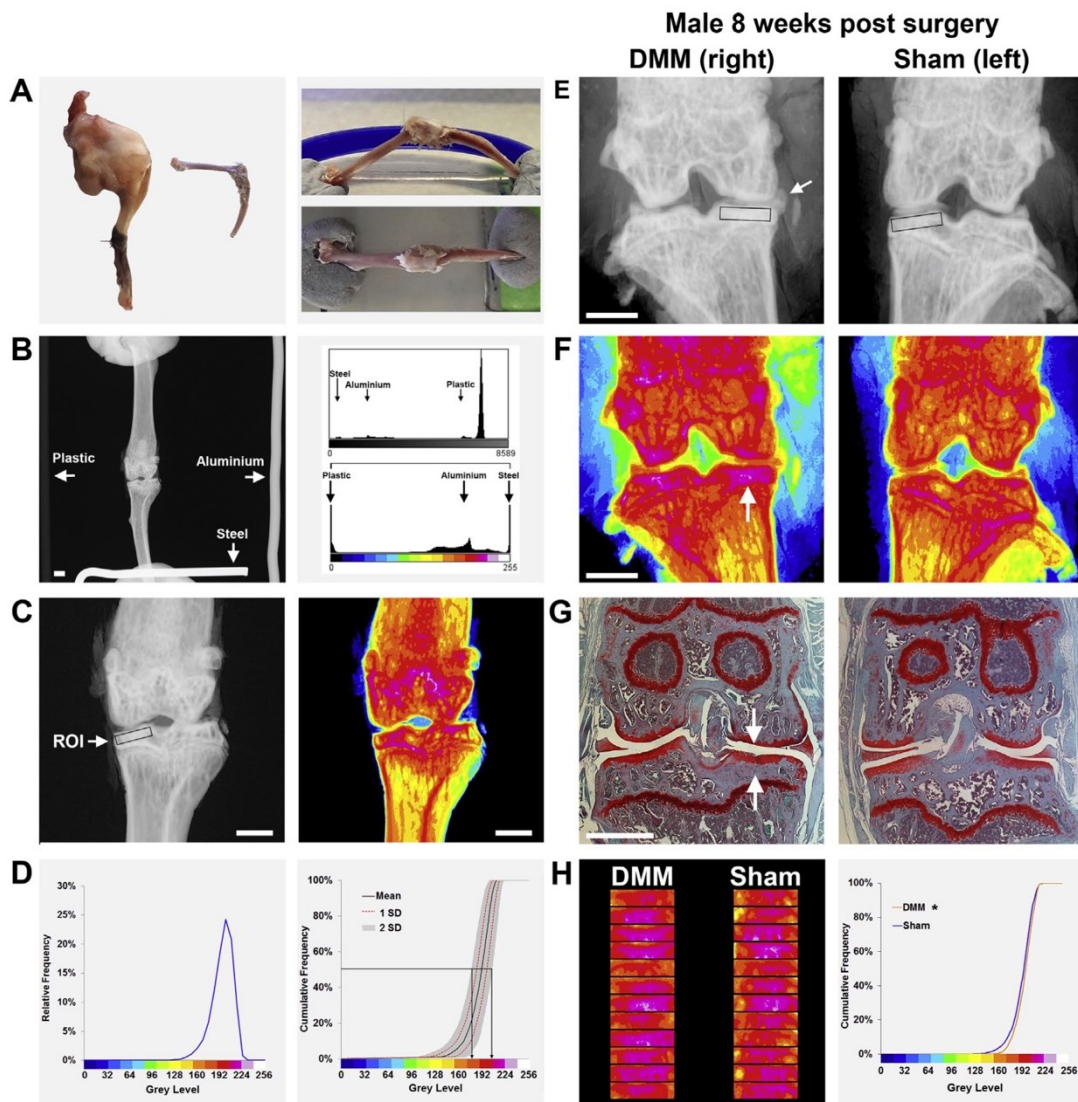


Fig. 1. Quantitative digital X-ray microradiography of subchondral bone. (A) Left panel shows mouse lower limb before and after removal of muscle to expose the articulated femur and tibia with preservation of the knee joint capsule and the patella. Right panels show lateral and anterior–posterior views of the knee fixed such that the tibial growth plate lies in a vertical plane. (B) Left panel shows a Faxitron MX-20 X-ray microradiograph illustrating the essential requirement to orientate the knee joint with the patella in the mid-sagittal plane. The recommended organisation of plastic, aluminium and steel calibration standards is also shown. White bar = 1000 μ m. The upper right panel shows a histogram of grey levels derived from the original 16 bit DICOM X-ray microradiograph with the location of the calibration standards indicated. The large peak on the right represents the background. The lower right panel shows the histogram after stretching the grey scale distribution to the plastic and steel standards, converting to an 8 bit TIFF format and pseudo-colouring the resultant image using a 16 colour look-up table. (C) Left panel shows a grey scale image of the left knee joint with the medial tibial plateau subchondral bone ROI (1000 μ m $\times$ 250 μ m box, ROI) indicated. Right panel shows a pseudo-coloured TIFF image of the same grey scale image in which lower BMC is green and yellow and higher BMC is red and purple. White bar = 1000 μ m. (D) Left panel shows a representative example of a relative frequency histogram of grey levels within the medial tibial plateau subchondral bone ROI. Right panel shows the cumulative frequency histogram of relative tibial plateau subchondral BMC in $n = 22$ female, 16-week-old wild-type mice (mean $\pm$ 2.0SD reference interval in grey, $\pm$ 1.0SD red dotted lines). Median grey levels at the limits of the 2.0SD reference interval are shown. (E) Grey scale images of the right (8 weeks after surgical DMM) left knee (following sham surgery) joints. The white arrow indicates medial displacement of the medial meniscus following DMM surgery. Boxes indicate the medial tibial plateaux subchondral bone ROIs. White bar = 1000 μ m. (F) Corresponding pseudo-coloured images in which lower BMC is green and yellow and higher BMC red and purple. The white arrow indicates increased subchondral BMC in the medial tibial plateau following DMM surgery. White bar = 1000 μ m. (G) Coronal sections through the knee joint at the level of the tibial indication of the anterior cruciate ligament stained with safranin O and fast green. Upper arrow indicates a defect in the articular cartilage surface of the medial tibial plateau, and the lower arrow indicates increased subchondral bone following DMM surgery. White bar = 1000 μ m. (H) Left panel shows a montage of the medial tibial plateaux ROIs from male mice 8 weeks following DMM ($n = 12$) or sham ($n = 12$) surgery. Right panel shows the cumulative frequency histograms of tibial plateau subchondral BMC following DMM or sham surgery. Kolmogorov–Smirnov test, DMM vs sham, $*P < 0.05$.

Results

To determine the accuracy and reproducibility of digital X-ray microradiography, we analysed knee joints from 22 female wild-type C57BL/6(B6Brd;B6Dnk;B6N-Tyr^{c-Brd}) mice. The 2.0SD reference interval for relative subchondral BMC was 178–206 with a median grey level of 192 and coefficient of variation (CV) of 3.6% [Fig. 1(D)].

To validate this novel imaging approach, DMM was selected as an established surgical model of osteoarthritis^{5,13} and histology was performed according to OARS recommendations⁴ and analysed qualitatively. X-ray microradiography of joints from male mice 8 weeks following DMM surgery confirmed displacement of the medial meniscus and demonstrated increased medial tibial plateau subchondral BMC [Fig. 1(E), (F)]. Importantly these changes co-localised with articular cartilage lesions and histological evidence

of increased subchondral bone [Fig. 1(G)]. Quantitative ROI analysis demonstrated a significant increase in medial tibial plateau subchondral BMC 8 weeks following DMM surgery compared to the contralateral sham-operated knee [Fig. 1(H)].

To investigate sensitivity of the method, knees were analysed from males 4 weeks following DMM surgery and from females 8 weeks post-surgery [Fig. 2(A), (B)]. Medial tibial plateau subchondral BMC was increased 4 weeks post-surgery in males ($P < 0.001$) and also in females 8 weeks post-surgery ($P < 0.001$) compared to the sham-operated knee.

To determine whether the method could be applied for rapid detection of altered subchondral BMC in genetically modified mice, we analysed knees from control and $TR\alpha^{0/0}$ mice¹⁵, which we previously demonstrated exhibit increased trabecular bone mass¹⁴ [Fig. 2(C)]. Quantitative X-ray microradiography identified the marked increase in medial tibial plateau subchondral BMC in $TR\alpha^{0/0}$ mice compared to control mice (median grey level 210 vs 192; $TR\alpha^{0/0}$ vs control; $P < 0.001$).

Discussion

Osteoarthritis is a major cause of pain and disability, its pathogenesis is poorly defined, and no drugs are available that prevent the onset or progression of disease. Advancement of the understanding of disease pathogenesis has been limited by a lack of suitable mouse models, but the generation of knockout mice for all

protein-coding genes⁶ now provides a timely opportunity to identify new *in vivo* models of osteoarthritis. These models will be essential to investigate the cellular and molecular basis of osteoarthritis and identify novel signalling pathways for future therapeutic targeting.

Nevertheless, identification of knockout strains with outlier joint phenotypes requires development of novel, robust, high-throughput and cost-effective screening approaches since current methods are too labour intensive, time consuming and costly.

We have previously shown that X-ray microradiography is an ideal high-throughput bone phenotyping method for determination of BMC in knockout mice^{9,10}. We, thus, investigated this approach for the quantitation of subchondral BMC, which is characteristically increased in established osteoarthritis⁷. Induction of osteoarthritis by transection of the right medial meniscotibial ligament^{5,13} was used to validate the use of X-ray microradiography for identification of osteoarthritis phenotypes in mice. Establishment of a normal reference range for medial tibial plateau subchondral BMC revealed a CV of only 3.6%, which is similar to the CV of 1.7% previously determined for whole femur BMC¹⁰, thus demonstrating excellent sensitivity of the method.

Digital X-ray microradiography clearly demonstrated increased subchondral BMC following DMM surgery, and these changes colocalised with the characteristic histological abnormalities in mice following surgically induced osteoarthritis^{5,13}. Group sizes of only six animals were sufficient to identify increased subchondral

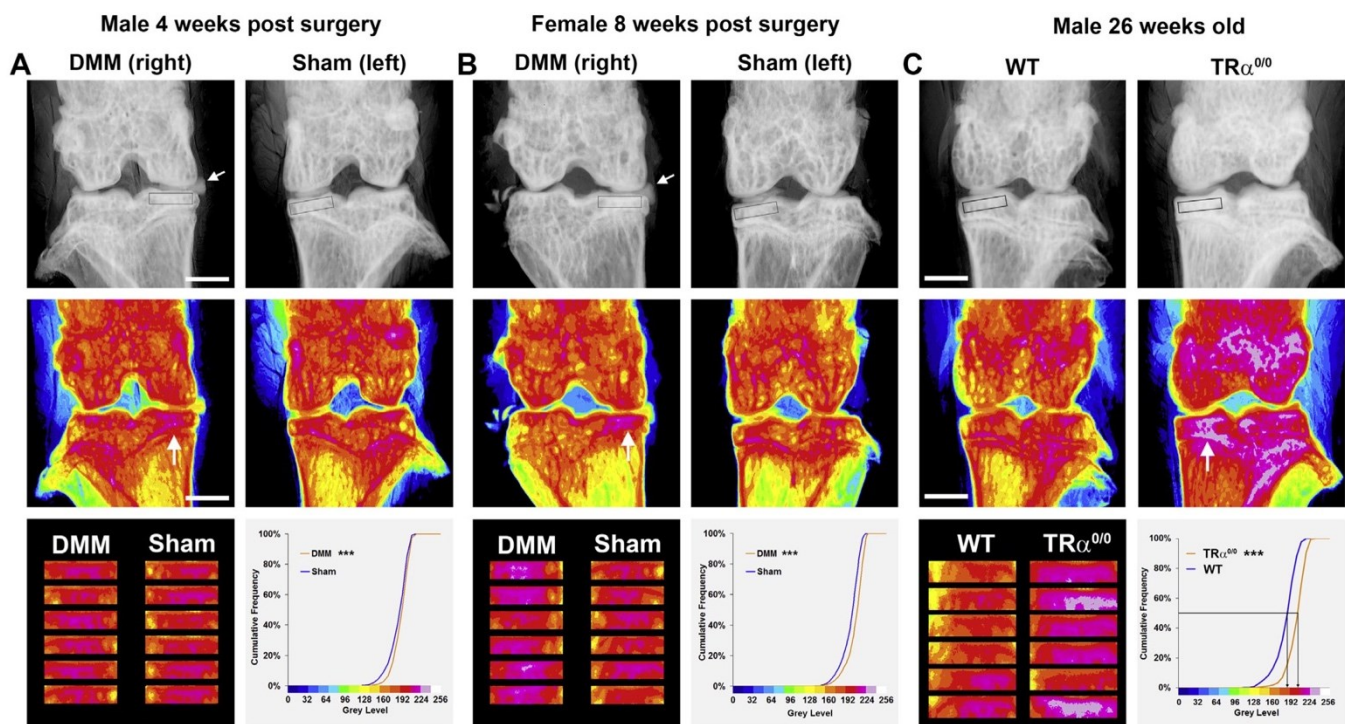


Fig. 2. Increased subchondral BMC in male and female mice following DMM surgery and in $TR\alpha^{0/0}$ mice. (A) Upper panels show grey scale images of the right and left knees 4 weeks after DMM or sham surgery in male mice. The white arrow indicates displacement of the medial meniscus following DMM surgery. Boxes indicate subchondral bone ROIs. Middle panels show corresponding pseudo-coloured images. The white arrow indicates increased subchondral BMC following DMM surgery. Lower left panel shows the ROI montage from male mice 4 weeks following DMM or sham surgery ($n = 6$ per group). Lower right panel shows the cumulative frequency histograms of tibial plateau subchondral BMC following DMM or sham surgery. Kolmogorov–Smirnov test, DMM vs sham, $***P < 0.001$. White bar = 1000 μm . (B) Upper panels show grey scale images of the right and left knees 8 weeks after DMM or sham surgery in female mice. The white arrow indicates displacement of the medial meniscus following DMM surgery. Boxes indicate subchondral bone ROIs. Middle panels show corresponding pseudo-coloured images. The white arrow indicates increased subchondral BMC following DMM surgery. Lower left panel shows the ROI montage from female mice 8 weeks following DMM or sham surgery ($n = 6$ per group). Lower right panel shows the cumulative frequency histograms of tibial plateau subchondral BMC following DMM or sham surgery. Kolmogorov–Smirnov test, DMM vs sham, $***P < 0.001$. White bar = 1000 μm . (C) Upper panels show grey scale images of the left knee from 26-week-old male wild-type and $TR\alpha^{0/0}$ mice. Boxes indicate subchondral bone ROIs. Middle panels show corresponding pseudo-coloured images. The white arrow indicates increased subchondral BMC in $TR\alpha^{0/0}$ mice. Lower left panel shows the ROI montage from wild-type and $TR\alpha^{0/0}$ mice ($n = 6$ per group). Lower right panel shows the cumulative frequency histograms of tibial plateau subchondral BMC with the ROI median grey level in wild-type and $TR\alpha^{0/0}$ mice indicated. Kolmogorov–Smirnov test, $TR\alpha^{0/0}$ vs wild type, $***P < 0.001$. White bar = 1000 μm .

BMC 4 weeks after DMM surgery in males and 8 weeks post-surgery in females (in which provocation of osteoarthritis is less penetrant⁵). Comparing groups of six samples also allowed the difference in subchondral BMC in TR $\alpha^{0/0}$ mice compared to genetically identical controls to be identified. Importantly, these group sizes are commensurate with the number of samples available from knockout lines generated for the International Mouse Phenotyping Consortium⁶.

These data indicate that X-ray microradiography is a sensitive and robust technique for quantitation of subchondral BMC, but a number of technical points need to be considered. Firstly, all soft tissue should be removed whilst carefully maintaining the knee joint capsule intact. Secondly, joints should be mounted at a constant angle of flexion such that the tibial growth plate is in a vertical plane with the patella central. Thirdly, X-ray intensity and exposure should be optimised for each imaging system to ensure the full dynamic range of the detector is used. Finally, standards should be included within every image for internal calibration^{9,10}.

In conclusion, these studies demonstrate that quantitation of subchondral BMC by digital X-ray microradiography is a rapid new method to identify abnormal joint phenotypes in mice. It should be used as an initial high-throughput screening tool to identify mutant mice for further investigation. Such detailed studies, however, are costly and labour intensive, requiring high-resolution micro-CT, surgical provocation and gold standard OARSI histological scoring. Thus, the role of this novel method is in the cost-effective and robust identification of mouse strains with joint abnormalities that merit detailed analysis^{4,11}.

Overall, we report the novel application of digital X-ray microradiography that, in combination with complementary methods of joint analysis, will help to identify new genetic determinants and animal models for osteoarthritis.

Author contributions

GRW (graham.williams@imperial.ac.uk) and JHDB (d.bassett@imperial.ac.uk) take responsibility for the integrity of the work as a whole from its inception to the finished article. JAW acquired, assembled, analysed and interpreted data, and drafted the article. SAM acquired and analysed data, and helped draft the article. SG acquired and analysed data, and helped draft the article. AG analysed and interpreted data, and helped draft the article. JGL interpreted data and helped draft the article. GRW conceived and designed the studies, provided statistical advice, interpreted data, and drafted and critically revised the article for important intellectual content. JHDB conceived and designed the studies, provided statistical advice, analysed and interpreted data, prepared final figures and critically revised the article. All authors approved the final article. JAW, GRW and JHDB obtained funding from Arthritis Research UK, the Wellcome Trust and Imperial College London.

Conflict of interest

JAW receives grant support from Arthritis Research UK, GRW and JHDB receive grant support from Arthritis Research UK and the Wellcome Trust. There are no other competing interests for any of the authors.

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References

1. Lories RJ, Luyten FP. The bone–cartilage unit in osteoarthritis. *Nat Rev Rheumatol* 2011;7:43–9.
2. Reynard LN, Loughlin J. Insights from human genetic studies into the pathways involved in osteoarthritis. *Nat Rev Rheumatol* 2013;9:573–83.
3. Vincent TL, Williams RO, Maciewicz R, Silman A, Garside P. Mapping pathogenesis of arthritis through small animal models. *Rheumatology (Oxford)* 2012;51:1931–41.
4. Glasson SS, Chambers MG, Van Den Berg WB, Little CB. The OARSI histopathology initiative – recommendations for histological assessments of osteoarthritis in the mouse. *Osteoarthritis Cartil* 2010;18(Suppl 3):S17–23.
5. Glasson SS, Blanchet TJ, Morris EA. The surgical destabilization of the medial meniscus (DMM) model of osteoarthritis in the 129/SvEv mouse. *Osteoarthritis Cartil* 2007;15:1061–9.
6. White JK, Gerdin AK, Karp NA, Ryder E, Buljan M, Bussell JN, et al. Genome-wide generation and systematic phenotyping of knockout mice reveals new roles for many genes. *Cell* 2013;154:452–64.
7. Burr DB, Gallant MA. Bone remodelling in osteoarthritis. *Nat Rev Rheumatol* 2012;8:665–73.
8. Botter SM, van Osch GJ, Waarsing JH, Day JS, Verhaar JA, Pols HA, et al. Quantification of subchondral bone changes in a murine osteoarthritis model using micro-CT. *Biorheology* 2006;43:379–88.
9. Bassett JH, van der Spek A, Gogakos A, Williams GR. Quantitative X-ray imaging of rodent bone by Faxitron. *Methods Mol Biol* 2012;816:499–506.
10. Bassett JH, Gogakos A, White JK, Evans H, Jacques RM, van der Spek AH, et al. Rapid-throughput skeletal phenotyping of 100 knockout mice identifies 9 new genes that determine bone strength. *PLoS Genet* 2012;8:e1002858.
11. Botter SM, Glasson SS, Hopkins B, Clockaerts S, Weinans H, van Leeuwen JP, et al. ADAMTS5^{-/-} mice have less subchondral bone changes after induction of osteoarthritis through surgical instability: implications for a link between cartilage and subchondral bone changes. *Osteoarthritis Cartil* 2009;17:636–45.
12. Saito T, Fukui A, Mabuchi A, Ikeda T, Yano F, Ohba S, et al. Transcriptional regulation of endochondral ossification by HIF-2 α during skeletal growth and osteoarthritis development. *Nat Med* 2010;16:678–86.
13. Glasson SS, Askew R, Sheppard B, Carito B, Blanchet T, Ma HL, et al. Deletion of active ADAMTS5 prevents cartilage degradation in a murine model of osteoarthritis. *Nature* 2005;434:644–8.
14. Bassett JH, O'Shea PJ, Sriskantharajah S, Rabier B, Boyde A, Howell PG, et al. Thyroid hormone excess rather than thyrotropin deficiency induces osteoporosis in hyperthyroidism. *Mol Endocrinol* 2007;21:1095–107.
15. Gauthier K, Plateroti M, Harvey CB, Williams GR, Weiss RE, Refetoff S, et al. Genetic analysis reveals different functions for the products of the thyroid hormone receptor alpha locus. *Mol Cell Biol* 2001;21:4748–60.

Adult Mice Lacking the Type 2 Iodothyronine Deiodinase Have Increased Subchondral Bone but Normal Articular Cartilage

Julian A. Waung, J. H. Duncan Bassett, and Graham R. Williams

Background: Although osteoarthritis (OA) is the commonest joint disorder and has a rising prevalence as the population ages, no drugs are available that prevent or delay the onset and progression of disease. Recent studies identified the *DIO2* gene encoding type 2 deiodinase (D2) as a susceptibility locus for OA, and further data suggest deiodinase-regulated local availability of triiodothyronine (T3) in the joint plays an important role in cartilage maintenance and repair. To investigate the hypothesis that reduced tissue T3 availability protects joints from development of OA, the joint phenotypes of adult mice lacking D2 (D2KO) or lacking both D1 and D2 (D1D2KO), the only enzymes that catalyze conversion of the prohormone thyroxine to active T3, were determined.

Methods: Knee joints were prepared from male 16-week-old adult wild type (WT; $n=9$), D2KO ($n=5$), and D1D2KO ($n=3$) mice. Articular cartilage pathology was scored using the Osteoarthritis Research Society International (OARSI) histopathology scale for murine OA to determine the severity and extent of disease. Digital X-ray microradiography was used to determine the area and mineral content of subchondral bone immediately beneath the articular cartilage surface.

Results: There were no differences in maximum and standardized OA scores, cartilage erosion indices, or articular cartilage cellularity among WT, D2KO, and D1D2KO mice. Subchondral bone area did not differ among genotypes, but mineral content was markedly increased in both D2KO and D1D2KO mice compared to WT.

Conclusions: Although adult D2KO mice have normal articular cartilage and no other features of spontaneous joint damage, they exhibit increased subchondral bone mineral content.

Introduction

OSTEOARTHRITIS (OA) IS THE COMMONEST joint disorder worldwide, and its prevalence is increasing in the ageing population (1). The disease affects all tissues of the joint (2), leading to pain, progressive dysfunction, and disability. OA is a major cause of time off work, second only to ischemic heart disease in the United States (1). Despite this increasing health burden, no drugs are currently available that prevent or delay the onset and progression of joint damage. Characteristic pathological changes in OA include damage to cartilage lining the joint surface, sclerosis of underlying subchondral bone (3), osteophyte formation, and synovial thickening. Articular chondrocytes undergo abnormal terminal differentiation, and the balance between synthesis and destruction of cartilage matrix favors loss of matrix due to increased production of degrading enzymes (2). Although understanding of the pathogenesis of OA has progressed in recent years, its complex etiology is multifactorial with important

genetic and environmental contributions (2). The heritability of OA is between 40% and 65%, although known disease susceptibility loci account for only a small fraction (4). Currently, these loci have mostly identified regulatory rather than structural proteins as modulators of OA risk.

Recent data suggest that altered thyroid hormone availability in articular cartilage or joint tissues may play a key role in predisposition to OA (5–9). Normal euthyroid status is maintained by the hypothalamic-pituitary-thyroid axis via a negative feedback loop in which thyroid hormones produced by the thyroid gland inhibit synthesis and secretion of thyrotropin (TSH) from the anterior pituitary. The main hormone secreted by the thyroid gland is the prohormone thyroxine (T4), which is converted to the physiologically active metabolite 3,5,3'-L-triiodothyronine (T3) by removal of an outer ring iodine atom. Thyroid hormones enter peripheral tissues, including bone and cartilage (10), via specific membrane transporters. In target cells, the type 2 iodothyronine deiodinase (D2) converts T4 to T3, whereas the type 3 enzyme (D3)

irreversibly inactivates T3 and prevents activation of T4 by removal of an inner ring iodine. Thus, the balance of D2 and D3 activities determines local T3 availability and tissue thyroid hormone responsiveness (11–13).

The D2 enzyme is labile due to substrate-induced ubiquitination and proteasome-mediated degradation (12,13), and its specific activity is difficult to measure, especially at low levels. Nevertheless, in-depth studies of iodothyronine metabolism in mouse skeletal tissues, cell lysates, and intact primary cell cultures have shown that specific D2 enzyme activity is restricted to mature bone-forming osteoblasts and not detectable in bone-resorbing osteoclasts or growth plate chondrocytes (10). A recent study using quantitative reverse transcription polymerase chain reaction (RT-qPCR) identified *Dio2* mRNA expression in rat articular cartilage at levels comparable to brown adipose tissue, although D2 enzyme activity was not measured (14). By contrast, functional D3 enzyme, a much more stable protein with readily detectable activity, was identified in osteoblasts, osteoclasts, and growth plate chondrocytes, with activity highest in cells derived from developing and younger animals (10). It is unknown whether D3 is expressed in articular cartilage or whether D2 expression in articular cartilage is regulated during joint development. The type 1 deiodinase (D1), which converts T4 to T3 mainly in the liver and kidney and generates a large proportion of the circulating T3, is not expressed in bone or cartilage and appears to have no physiological role in the skeleton (10).

T3 regulates cartilage maturation and is essential for endochondral ossification, bone modeling, and longitudinal growth (15). A role for thyroid hormones in the pathogenesis of OA has been proposed. Although chondrocytes resist terminal differentiation in healthy articular cartilage, a process resembling endochondral ossification occurs during the progression of OA in which articular chondrocytes undergo hypertrophic differentiation with acceleration of cartilage mineralization (16,17). Increased activities of ADAMTS5 (a disintegrin and metalloproteinase with thrombospondin motifs-5) (18,19) and MMP13 (matrix metalloproteinase-13) (20) in OA cause degradation of cartilage matrix, and these enzymes are regulated in the growth plate by T3 (21–24), which also induces hypertrophic chondrocyte differentiation at this location (14,21,25).

A genome-wide linkage analysis of siblings with generalized OA found a nonsynonymous coding variant (rs225014; Thr92Ala) that identified *DIO2* as a disease-susceptibility locus (6). Replication studies in Asian and European cohorts confirmed this by identifying a *DIO2* haplotype containing the minor allele of rs225014 and major allele of rs12885300 that was associated with symptomatic OA (6). The relationship between this haplotype and OA, however, was not replicated in the Rotterdam Study population (26), and *DIO2* was not identified as a susceptibility locus in a more recent meta-analysis (27). Nevertheless, further data suggest the risk allele of rs225014 may be expressed at higher levels than the protective allele in OA cartilage from heterozygous patients (28), possibly because of epigenetic modifications (29). Increased *DIO2* mRNA and protein expression has also been documented by RT-qPCR and immunohistochemical staining in cartilage obtained from joints affected by end-stage OA (14,28). Additionally, the rs12885300 *DIO2* polymorphism has been suggested to influence the association between hip shape and

OA susceptibility by possibly increasing vulnerability of articular cartilage to abnormal hip morphology (30). Furthermore, studies in transgenic rats that overexpress human D2 in chondrocytes indicate transgenic animals have an increased susceptibility to OA following surgical destabilization of the knee (14). Finally, a recent meta-analysis studying factors that regulate thyroid hormone metabolism and action identified a possible role for *DIO3* as a disease-modifying locus in OA (5).

Together, these data suggest that deiodinase-regulated local availability of T3 in joint tissues may play an important role in articular cartilage renewal and repair, particularly as the polymorphisms in *DIO2* and *DIO3* that are associated with OA do not influence systemic thyroid status (31). Overall, current findings suggest that increased T3 availability in OA may be detrimental to joint maintenance and articular cartilage homeostasis. Thus, it was hypothesized that reduced tissue T3 availability protects joints from damage and the development of OA. To investigate, the joint phenotypes of adult mice lacking D2 (D2KO) or lacking both D1 and D2 (D1D2KO) were determined.

Materials and Methods

Mice

D1KO or D2KO mice were generated after targeted disruption of *Dio1* or *Dio2* in 129/SvJ embryonic stem cells. Mice were bred and maintained as homozygous colonies in a C57BL/6 background at Dartmouth Medical School animal facility using protocols approved by the Institutional Review Board (32,33). D1KO and D2KO mice were crossed to obtain double heterozygous F1 progeny, which were used to generate a colony of D1D2KO homozygous double mutants (12,34). Mice were housed under conditions of controlled lighting and temperature and sacrificed at 16 weeks of age. Bone samples were shipped to Imperial College London for phenotyping studies in which nine wild type (WT), five D2KO, and three D1D2KO male mice were used for detailed histological analysis of the joints. Histological sections from four wild type, four D2KO, and three D1D2KO mice were prepared at directly comparable levels for subchondral bone analysis.

Preparation of histological sections

Lower limbs were fixed in 10% neutral buffered formalin for 24 h and decalcified in 10% EDTA, pH7.4, for 7–10 days. Knee joints were embedded in paraffin and 5 μ m thick midline sections were cut at 80 μ m intervals. Slides were stained for cartilage proteoglycans using Safranin-O Fast-Green (35) and imaged using a Leica MZ75 binocular microscope (Leica AG, Heerbrugg, Switzerland), KL1500 light source, and DFC320 digital camera.

Analysis of articular cartilage

Articular cartilage pathology was scored by a blinded observer using the Osteoarthritis Research Society International (OARSI) histopathology scale for murine OA (35). A score of 0 represents normal cartilage; 0.5 represents loss of Safranin-O staining without structural changes; 1 represents small fibrillations without loss of cartilage; and 2 represents vertical clefts down to the layer immediately below the superficial layer and some loss of surface lamina. Vertical clefts

or erosions to the calcified cartilage extending to <25%, 25–50%, 50–75%, or >75% of the articular surface are assigned scores of 3, 4, 5, or 6 respectively. Using this system, the severity and extent of osteoarthritis is expressed as a maximum score for each joint and as a cumulative score over the whole joint (35). Because the numbers of histological sections obtained differed between individual mice, a standardized OA score was determined. Thus, a reference histological atlas of a 16-week-old male C57BL/6 WT mouse knee was constructed using 5 μ m thick coronal sections cut at 80 μ m intervals through the intact joint to yield 16 levels of sectioning, of which nine passed directly through the joint cavity (Supplementary Fig. S1; Supplementary Data are available online at [www.liebertpub.com.thy](http://www.liebertpub.com/thy)). The total articular surface was determined for the reference atlas and each experimental animal (Fig. 1A and B). A standardized OA score for each experimental animal was defined as: cumulative OA score from all histological sections $\times$ (reference articular surface/experimental animal articular surface). A validated erosion index was also determined to assess the extent of cartilage lesions as a measure of OA progression (36,37): the length of eroded articular cartilage was measured and divided by the total length of articular cartilage surface and expressed as a percentage (Fig. 1C). The articular cartilage cellularity was also determined and defined as the mean number of chondrocytes per mm^2 .

Histological analysis of subchondral bone area

Histological sections were imaged in 8-bit Tiff format. The subchondral bone region of interest (ROI) between the articular cartilage and epiphyseal growth plate of the tibia was contoured using ImageJ (<http://rsb.info.nih.gov/ij/>), and the subchondral bone area relative to tissue area (BA/TA) determined (Fig. 1D and E).

Subchondral bone mineral content

Quantitative digital X-ray microradiography was used to determine relative bone mineral content in tibial subchondral bone as described (38,39). Increasing gradations of mineralization density were represented in 16 equal intervals by a pseudocolor scheme (Fig. 2). The bone compartment proximal to the tibial growth plate was selected as the subchondral bone ROI. Relative and cumulative frequency distributions of gray levels in ROIs were determined and compared among genotypes.

Statistics

Individual comparisons between WT and mutant mice for the maximum OA score and range were analyzed using the Mann–Whitney *U*-test. Comparisons between WT and mutant mice for mean standardized OA score, erosion index, and

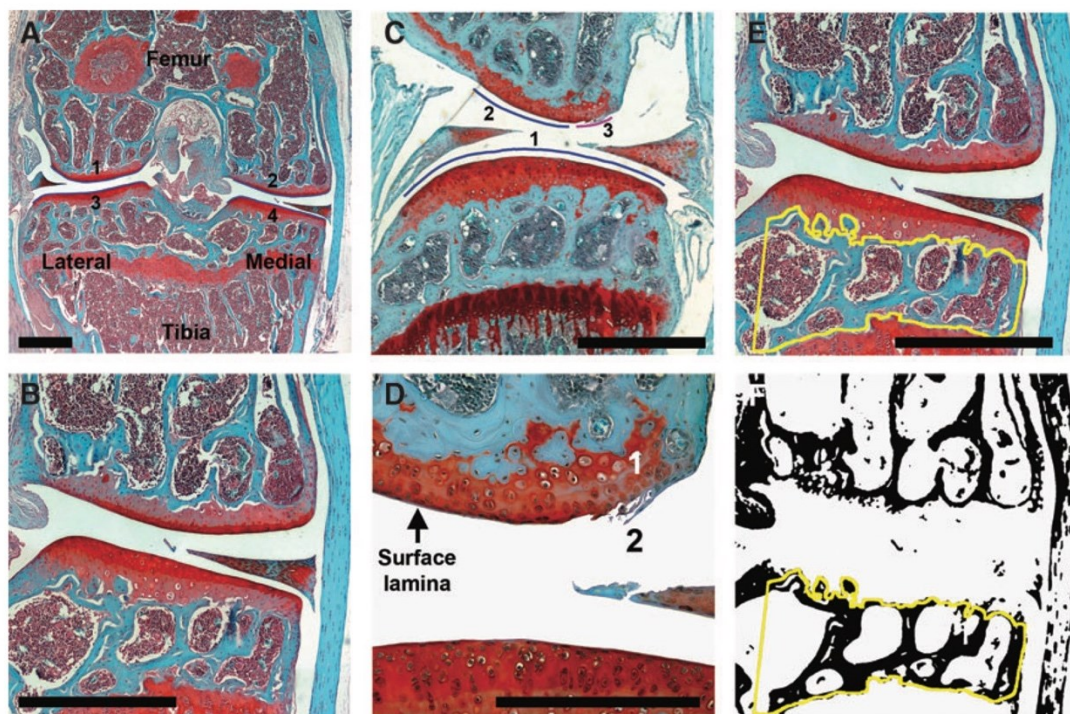


FIG. 1. Articular cartilage and subchondral bone analysis in wild type (WT) C57BL/6 mice. (A) Right knee mid-joint coronal section from a 16-week-old male C57BL/6 mouse stained with Safranin-O Fast-Green. Numbered blue lines (1–4) delineate the articular surfaces. Scale bar=0.5 mm. (B) Magnified view of medial joint compartment showing normal articular cartilage (red) and subchondral bone (green). Scale bar=0.5 mm. (C) Erosion index is calculated by measuring the eroded surface (3) divided by total articular surface (1+2+3). Scale bar=0.5 mm. (D) High power view of the eroded surface shown in panel (C). The intact surface lamina in undamaged articular cartilage is indicated; area number 1 shows loss of safranin O staining, and area number 2 shows fibrillations without loss of cartilage. Scale bar=0.25 mm. (E) Subchondral bone region of interest enclosed by yellow line. Scale bar=0.5 mm. (F) Articular cartilage and marrow space after thresholding in Image J to allow the subchondral bone area in the region of interest to be determined automatically.

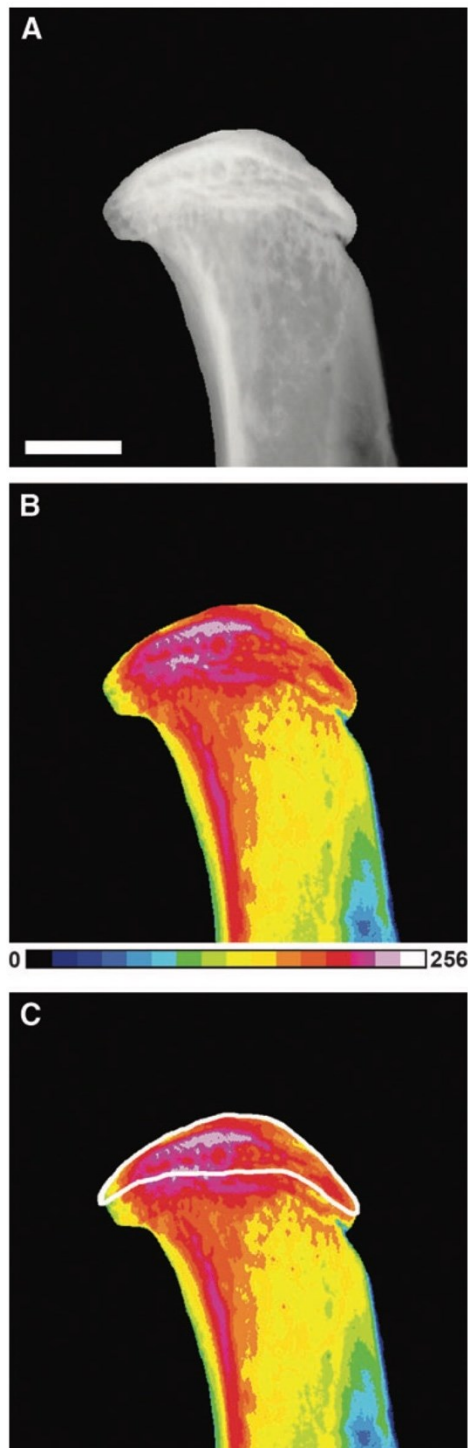


FIG. 2. Analysis of subchondral bone mineral content in WT C57BL/6 mice. (A) Gray-scale digital X-ray micro-radiograph of proximal tibia. (B) Pseudocolored images representing gradations of relative bone mineral content (BMC) in which blue-green is low BMC and pink-white is high BMC. (C) Subchondral bone region of interest is indicated within the white boundary.

subchondral bone area were analyzed by unpaired Student's *t*-test. *p*-Values <0.05 were considered significant. The Kolmogorov–Smirnov test was used to compare gray-level cumulative frequency distributions in digital X-ray micrographs between WT and mutant mice. *p*-Values for the *D* statistic were $D > 6.01$, $p < 0.05$; $D \geq 7.20$, $p < 0.01$; and $D \geq 8.62$, $p < 0.001$ (39).

Results

Length of articular cartilage

The articular cartilage surface in the reference atlas was determined in sections from the nine levels that passed directly through the joint cavity (Supplementary Fig. S1 and Fig. 1). The measured complete articular cartilage surface in the reference joint was 27.4 mm, and this length was used to calculate standardized OA scores. In sections from experimental samples, the measured articular cartilage surface was: WT 5.0 ± 1.2 , $n = 9$; D2KO 6.0 ± 1.1 , $n = 5$; D1D2KO 10.99 ± 2.99 , $n = 3$ (mm $\pm$ SEM; ANOVA; not significant).

Articular cartilage is not altered in D2KO mice

There were no differences in maximum and standardized OA scores or erosion indices between WT and D2KO mice (Fig. 3). The maximum OA scores were: WT 3 (0–3), $n = 9$; D2KO 4 (0.5–4), $n = 5$ (maximum score (range); Mann–Whitney *U*-test; not significant). The standardized OA scores were: WT 7.8 ± 2.1 , $n = 9$; D2KO 10.9 ± 3.6 , $n = 5$ (mean standardized score $\pm$ SEM; Student's *t*-test; not significant). The erosion indices were WT 2.7 ± 1.4 , $n = 9$; D2KO 6.1 ± 2.7 , $n = 5$ (mean % $\pm$ SEM; Student's *t*-test; not significant). There were also no differences in these parameters in D1D2KO mice: maximum OA score (0–2), $n = 3$; standardized OA score 9.6 ± 5.4 , $n = 3$; erosion index 5.5 ± 3.3 , $n = 3$ (Mann–Whitney *U*-test and Student's *t*-tests vs. WT or D2KO mice; not significant).

Articular cartilage cellularity also did not differ between WT and D2KO mice: WT 1412 ± 82 , $n = 9$; D2KO 1705 ± 161 , $n = 5$ (mean number of chondrocytes per mm² $\pm$ SEM; Student's *t*-test; not significant). Articular cartilage cellularity also did not differ in D1D2KO mice: 1493 ± 251 , $n = 3$ (Student's *t*-test vs. WT or D2KO mice; not significant).

Subchondral bone area is unchanged but bone mineral content is increased in D2KO mice

The subchondral bone area did not differ between WT and D2KO mice: WT $64 \pm 7.6\%$, $n = 4$; D2KO $56 \pm 10.3\%$, $n = 4$ (mean BA/TA; Student's *t*-test; not significant; Fig. 3) and was also not different in D1D2KO mice: $52 \pm 3.8\%$, $n = 3$ (Student's *t*-test vs. WT or D2KO mice; not significant). Despite this, subchondral bone mineral content was markedly increased in D2KO mice ($n = 4$) compared to WT ($n = 5$), Kolmogorov–Smirnov test ($p < 0.001$; Fig. 4), and a similar increase was evident in D1D2KO mice ($n = 2$; $p < 0.001$).

Discussion

It is demonstrated that 16-week-old adult male D2KO mice, which have a normal circulating T3 concentration (33) but lack the D2 enzyme that regulates intracellular T3 availability, have increased subchondral bone mineral

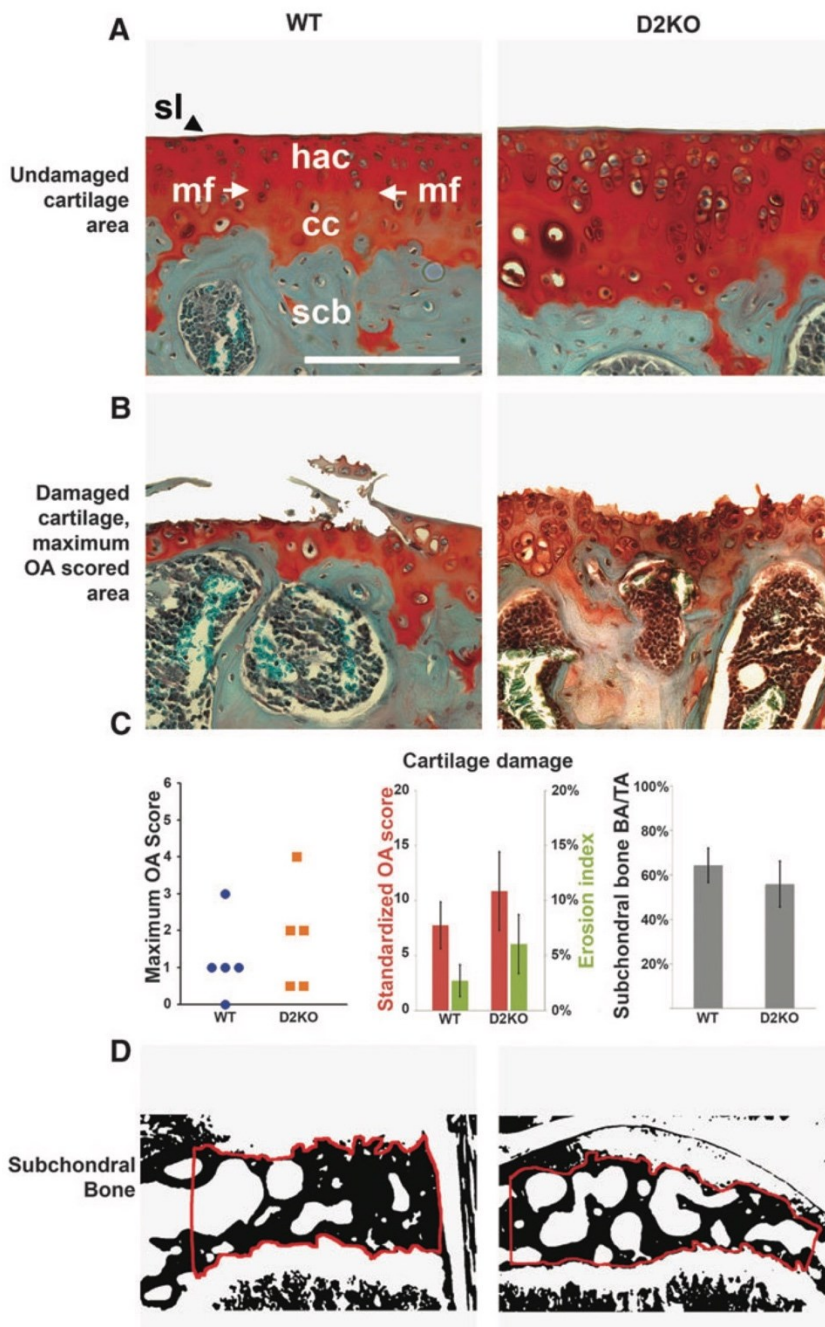


FIG. 3. Osteoarthritis (OA) pathology and scores in WT and D2KO mice. **(A)** High-powered views showing normal articular cartilage with an OARSI Score of zero in WT and D2KO mice. Scale bar = 50 μ m. sl, surface lamina; hac, hyaline articular cartilage; mf, mineralizing front; cc, calcified cartilage; scb, subchondral bone. **(B)** Maximum OA scores for WT (3 = vertical clefts or erosions to the calcified cartilage extending to <25% of articular surface) and D2KO (4 = vertical clefts or erosions to the calcified cartilage extending to 25–50% of articular surface) mice. **(C)** Graphs showing maximum OA scores in WT and D2KO mice; measures of cartilage damage (mean standardized OA score in red bars, mean erosion index in green bars); and mean subchondral bone area (BA/TA). Error bars represent standard error of the mean, with no significant differences among genotypes for all parameters. **(D)** Subchondral bone regions of interest in WT and D2KO mice.

content but normal articular cartilage and no other features of OA. Additional deletion of D1 in D1D2KO mice, which also have a normal circulating T3 concentration (12), did not affect the phenotype observed in D2KO mice, supporting the notion that D1 has no physiological role in the skeleton (10).

D2KO mice have a generalized increase in bone mass and mineralization resulting in bones with reduced toughness and increased susceptibility to fracture (34). The abnormality results from disruption of a critical function for D2 in osteoblasts, in which a higher T3 concentration relative to other skeletal cells is maintained in order to optimize bone mineralization (34). In

this study, it is demonstrated that adult D2KO mice also have increased mineral content in subchondral bone but no abnormalities of articular cartilage or other pathological features of spontaneous OA.

It is important to consider whether the subchondral bone abnormality in D2KO mice reflects a potential role for D2 in the pathogenesis of OA. This is a complex question because OA may result from primary abnormalities in articular chondrocytes, changes in adjacent subchondral bone that lead to secondary cartilage damage and degeneration, or from defects in both tissues. Although it is well established that both subchondral bone remodeling and articular cartilage

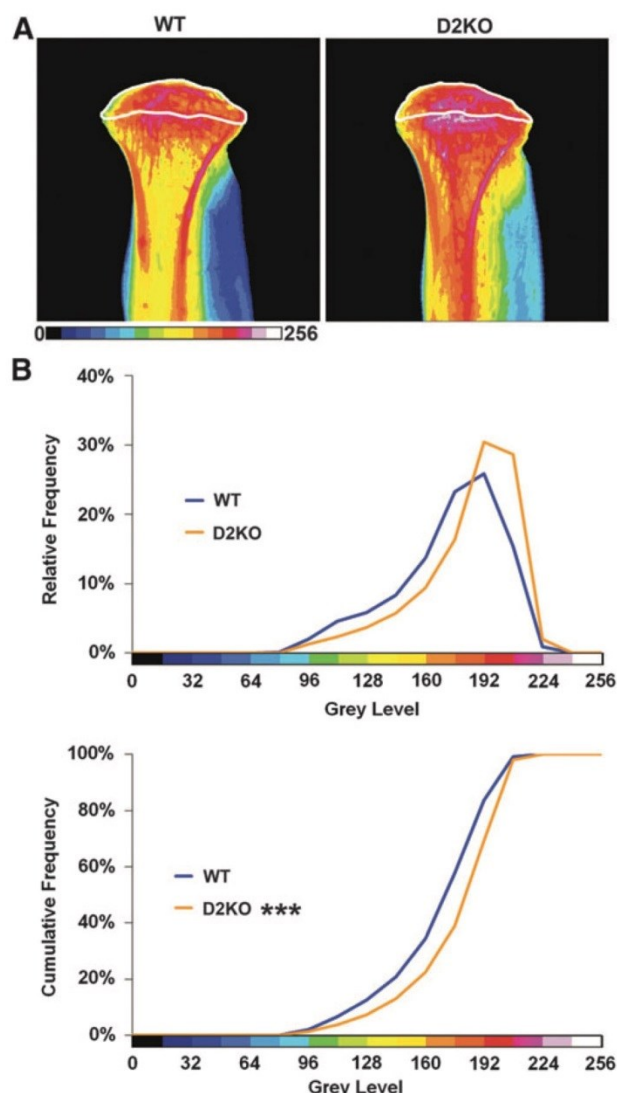


FIG. 4. Subchondral bone mineral content in WT and D2KO mice. (A) Pseudocolored images of proximal tibias from WT and D2KO mice in which blue-green is low BMC, and pink-white is high BMC. Subchondral bone region of interest is indicated within the white boundary. (B) Relative and cumulative frequency histograms demonstrating increased subchondral bone mineral content in D2KO mice. Kolmogorow–Smirnov test; *** $p < 0.001$ (WT $n = 5$, D2KO $n = 4$).

damage occur in OA, it is not clear whether these alterations occur simultaneously or whether subchondral bone changes result in cartilage damage or *vice versa* (3,9,40,41). For example, cathepsin K knockout mice have increased subchondral bone mineralization and are protected from OA (42), whereas rabbits treated with parathyroid hormone have increased subchondral bone mineral density but worsened articular cartilage integrity (43). The current findings demonstrate abnormal subchondral bone in the absence of articular cartilage damage, suggesting that any effect of D2 deletion on OA pathogenesis would likely be mediated via primary effects in subchondral bone osteoblasts.

In this respect, it is essential to consider the cells in which D2 enzyme activity is actually present; chondrocytes, osteoblasts, or both cell types. In detailed studies demonstrating that functional D2 enzyme is restricted to bone-forming osteoblasts, it was shown that measurable D2 mRNA expression does not necessarily correlate with detectable enzyme activity (10). An important reason for this discrepancy is that D2 protein is labile, undergoing rapid degradation following exposure to increasing concentrations of T4 in a local negative feedback loop (13,44). A further difficulty is that available antibodies for detection of D2 by immunohistochemistry or Western blotting are insufficiently sensitive or unreliable. It has not been established, therefore, whether functional D2 is genuinely expressed in cartilage. Thus, *Dio2* mRNA expression was demonstrated in growth plate cartilage but measurable enzyme activity using a high sensitivity assay could not be detected (10), whereas others have shown *Dio2* mRNA expression in rat articular cartilage (14) and increased levels of *Dio2* mRNA (14) and protein (28) in human articular cartilage from joints resected for end-stage OA, although in each case enzyme activity was not determined. The significance of apparently increased D2 expression in end-stage OA cartilage is therefore uncertain; it is also not known whether increased D2 expression would represent a secondary response to joint destruction or whether it might precede cartilage damage and be a causative factor in disease progression.

In this context, transgenic rats overexpressing *Dio2* in chondrocytes had increased susceptibility to OA following provocation by surgical destabilization of the knee (14). Nevertheless, a causal relationship between increased D2 expression and OA susceptibility was not established because D2 activity was not determined in transgenic rats overexpressing the enzyme (14). This is an important point because in a previous study, transgenic mice overexpressing D2 in the heart surprisingly displayed only mild thyrotoxic changes in cardiomyocytes (45), presumably because the phenotype was mitigated by T4-induced degradation of the overexpressed enzyme (44). Furthermore, in a recent study, siRNA-mediated inhibition of *DIO2* mRNA expression in primary human chondrocytes resulted in decreased expression of liver X receptor α (LXR α) and increased expression of interleukin-1 β (IL1 β) and IL1 β -induced cyclooxygenase-2 (COX2) expression (46). At odds with the notion that deletion or inhibition of D2 might protect against OA, these findings indicate that suppression of D2 results in a pro-inflammatory response in cartilage. The authors suggest that D2 has an anti-inflammatory role in chondrocytes resulting in inactivation of LXR α and suppression of IL-1 β and COX2 (46). Thus, the apparent increase in D2 expression observed in end-stage human OA (14,28) could represent a consequence of disease progression resulting from activation of pro-inflammatory pathways such as the nuclear factor κ B (NF κ B) pathway, which is known to stimulate D2 expression (47,48).

In light of these conflicting findings, it is prudent to reconsider the original hypothesis that reduced tissue T3 availability protects joints from damage and the development of OA. Genetic studies proposed *DIO2* as a susceptibility gene for OA based on identification of the rs225014 (Thr92Ala) polymorphism. The functional effect of this polymorphism on enzyme activity is, however, uncertain. Initial *in vitro* transfection studies demonstrated the Thr92Ala substitution in D2 did not

affect protein half-life, enzyme activity, or its sensitivity to substrate-mediated inhibition (49). Nevertheless, analysis of thyroid and skeletal muscle samples from subjects homozygous for the Ala risk allele revealed D2 enzyme velocity was reduced by 50% compared to heterozygous individuals or subjects homozygous for the protective Thr allele (49), although it was subsequently suggested this finding may have resulted from a methodological artifact (50,51). The initial genetic studies suggesting that reduced D2 activity is associated with increased susceptibility to OA (6) were based on the assumption that the Thr92Ala polymorphism results in reduced enzyme activity (49). However, studies showing that transgenic rats overexpressing D2 in articular cartilage had increased susceptibility to OA (14) were not consistent with this conclusion, and recent findings of allelic imbalance and increased expression of D2 protein in OA cartilage (28,29) further suggest that increased D2 activity may in fact be detrimental for articular cartilage maintenance. On the other hand, a large genome-wide association study and a recent meta-analysis both failed to identify *DIO2* as a disease-susceptibility locus for OA (26,27), while a recent study in primary human chondrocytes actually suggests an anti-inflammatory role for D2 in cartilage (46). Currently, it remains unclear, therefore, whether variation in *DIO2* plays a role in osteoarthritis pathogenesis, although the independent identification of *DIO3* as a disease susceptibility locus (5) strongly suggests a role for control of local tissue T3 availability in the regulation of joint homeostasis.

Overall, it is not certain whether the original hypothesis that reduced tissue T3 availability protects joints from damage and the development of OA is likely to be correct or whether the reverse is more probable. Alternatively, it is possible that tissue T3 deficiency or excess are each detrimental for optimal articular cartilage homeostasis, thus accounting for the apparently contradictory literature. Indeed, OA pathophysiology is complex, with examples of increased or decreased signaling in both the Wnt/ β -catenin (52,53) and transforming growth factor- β pathways (54,55) having deleterious effects on joint integrity. The finding of increased subchondral bone mineral content in D2KO mice will thus require careful study to determine its pathophysiological significance; that is, whether loss of D2 protects joints from OA or whether it results in increased disease susceptibility. Future studies will require the use of an OA provocation model, such as surgical destabilization of the medial meniscus (56), or study of the consequences of ageing to determine whether deletion of D2 accelerates or delays the onset and progression of predictable joint destruction. Additional studies to investigate the temporo-spatial specific role of D2 in cartilage could also be performed by crossing mice harboring floxed *Dio2* alleles (57) with *Agc1*-CreERT2 mice (58) that conditionally express Cre-recombinase in cartilage following treatment with tamoxifen.

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References

1. Arden N, Nevitt MC 2006 Osteoarthritis: epidemiology. *Best Pract Res Clin Rheumatol* **20**:3–25.
2. Lories RJ, Luyten FP 2011 The bone-cartilage unit in osteoarthritis. *Nat Rev Rheumatol* **7**:43–49.
3. Burr DB, Gallant MA 2012 Bone remodelling in osteoarthritis. *Nat Rev Rheumatol* **8**:665–673.
4. Reynard LN, Loughlin J 2013 Insights from human genetic studies into the pathways involved in osteoarthritis. *Nat Rev Rheumatol* **9**:573–583.
5. Meulenbelt I, Bos SD, Chapman K, van der Breggen R, Houwing-Duistermaat JJ, Kremer D, Kloppenburg M, Carr A, Tsezou A, Gonzalez A, Loughlin J, Slagboom PE 2011 Meta-analyses of genes modulating intracellular T3 bioavailability reveal a possible role for the *DIO3* gene in osteoarthritis susceptibility. *Ann Rheum Dis* **70**:164–167.
6. Meulenbelt I, Min JL, Bos S, Riyazi N, Houwing-Duistermaat JJ, van der Wijk HJ, Kroon HM, Nakajima M, Ikegawa S, Uitterlinden AG, van Meurs JB, van der Deure WM, Visser TJ, Seymour AB, Lakenberg N, van der Breggen R, Kremer D, van Duijn CM, Kloppenburg M, Loughlin J, Slagboom PE 2008 Identification of *DIO2* as a new susceptibility locus for symptomatic osteoarthritis. *Hum Mol Genet* **17**:1867–1875.
7. Loughlin J 2011 Osteoarthritis year 2010 in review: genetics. *Osteoarthritis Cartilage* **19**:342–345.
8. Bos SD, Slagboom PE, Meulenbelt I 2008 New insights into osteoarthritis: early developmental features of an ageing-related disease. *Curr Opin Rheum* **20**:553–559.
9. Goldring MB 2013 Insight into the function of *DIO2*, a susceptibility gene in human osteoarthritis, as an inducer of cartilage damage in a rat model: is there a role for chondrocyte hypertrophy? *Osteoarthritis Cartilage* **21**:643–645.
10. Williams AJ, Robson H, Kester MH, van Leeuwen JP, Shalet SM, Visser TJ, Williams GR 2008 Iodothyronine deiodinase enzyme activities in bone. *Bone* **43**:126–134.
11. Cheng SY, Leonard JL, Davis PJ 2010 Molecular aspects of thyroid hormone actions. *Endocr Rev* **31**:139–170.
12. Galton VA, Schneider MJ, Clark AS, St Germain DL 2009 Life without thyroxine to 3,5,3'-triiodothyronine conversion: studies in mice devoid of the 5'-deiodinases. *Endocrinology* **150**:2957–2963.
13. Gereben B, Zavacki AM, Ribich S, Kim BW, Huang SA, Simonides WS, Zeold A, Bianco AC 2008 Cellular and molecular basis of deiodinase-regulated thyroid hormone signaling. *Endocr Rev* **29**:898–938.
14. Nagase H, Nagasawa Y, Tachida Y, Sakakibara S, Okutsu J, Suematsu N, Arita S, Shimada K 2013 Deiodinase 2 upregulation demonstrated in osteoarthritis patients cartilage causes cartilage destruction in tissue-specific transgenic rats. *Osteoarthritis Cartilage* **21**:514–523.
15. Waung JA, Bassett JH, Williams GR 2012 Thyroid hormone metabolism in skeletal development and adult bone maintenance. *Trends Endocrinol Metab* **23**:155–162.
16. Dreier R 2010 Hypertrophic differentiation of chondrocytes in osteoarthritis: the developmental aspect of degenerative joint disorders. *Arthritis Res Ther* **12**:216.

17. Lin AC, Seeto BL, Bartoszko JM, Khoury MA, Whetstone H, Ho L, Hsu C, Ali SA, Alman BA 2009 Modulating hedgehog signaling can attenuate the severity of osteoarthritis. *Nat Med* **15**:1421–1425.
18. Glasson SS, Askew R, Sheppard B, Carito B, Blanchet T, Ma HL, Flannery CR, Peluso D, Kanki K, Yang Z, Majumdar MK, Morris EA 2005 Deletion of active ADAMTS5 prevents cartilage degradation in a murine model of osteoarthritis. *Nature* **434**:644–648.
19. Stanton H, Rogerson FM, East CJ, Golub SB, Lawlor KE, Meeker CT, Little CB, Last K, Farmer PJ, Campbell IK, Fourie AM, Fosang AJ 2005 ADAMTS5 is the major aggrecanase in mouse cartilage *in vivo* and *in vitro*. *Nature* **434**:648–652.
20. Billingham RC, Dahlberg L, Ionescu M, Reiner A, Bourne R, Rorabeck C, Mitchell P, Hambor J, Diekmann O, Tschesche H, Chen J, Van Wart H, Poole AR 1997 Enhanced cleavage of type II collagen by collagenases in osteoarthritic articular cartilage. *J Clin Invest* **99**:1534–1545.
21. Chen-An P, Andreassen KV, Henriksen K, Karsdal MA, Bay-Jensen AC 2013 Investigation of chondrocyte hypertrophy and cartilage calcification in a full-depth articular cartilage explants model. *Rheumatol Int* **33**:401–411.
22. Himeno M, Enomoto H, Liu W, Ishizeki K, Nomura S, Kitamura Y, Komori T 2002 Impaired vascular invasion of Cbfa1-deficient cartilage engrafted in the spleen. *J Bone Miner Res* **17**:1297–1305.
23. Makihiro S, Yan W, Murakami H, Furukawa M, Kawai T, Nikawa H, Yoshida E, Hamada T, Okada Y, Kato Y 2003 Thyroid hormone enhances aggrecanase-2/ADAM-TS5 expression and proteoglycan degradation in growth plate cartilage. *Endocrinology* **144**:2480–2488.
24. Pereira RC, Jorgetti V, Canalis E 1999 Triiodothyronine induces collagenase-3 and gelatinase B expression in murine osteoblasts. *Am J Physiol* **277**:E496–504.
25. Robson H, Siebler T, Stevens DA, Shalet SM, Williams GR 2000 Thyroid hormone acts directly on growth plate chondrocytes to promote hypertrophic differentiation and inhibit clonal expansion and cell proliferation. *Endocrinology* **141**:3887–3897.
26. Kerkhof HJ, Lories RJ, Meulenbelt I, Jonsdottir I, Valdes AM, Arp P, Ingvarsson T, Jhamai M, Jonsson H, Stolk L, Thorleifsson G, Zhai G, Zhang F, Zhu Y, van der Breggen R, Carr A, Doherty M, Doherty S, Felson DT, Gonzalez A, Halldorsson BV, Hart DJ, Hauksdottir VB, Hofman A, Ioannidis JP, Kloppenburg M, Lane NE, Loughlin J, Luyten FP, Nevitt MC, Parimi N, Pols HA, Rivadeneira F, Slagboom EP, Styrkarsdottir U, Tsezou A, van de Putte T, Zmuda J, Spector TD, Stefansson K, Uitterlinden AG, van Meurs JB 2010 A genome-wide association study identifies an osteoarthritis susceptibility locus on chromosome 7q22. *Arthritis Rheum* **62**:499–510.
27. Evangelou E, Kerkhof HJ, Styrkarsdottir U, Ntzani EE, Bos SD, Esko T, Evans DS, Metrustry S, Panoutsopoulou K, Ramos YF, Thorleifsson G, Tsilidis KK, Arden N, Aslam N, Bellamy N, Birrell N, Blanco FJ, Carr A, Chapman K, Day-Williams AG, Deloukas P, Doherty M, Engstrom G, Helgadottir HT, Hofman A, Ingvarsson T, Jonsson H, Keis A, Keurentjes JC, Kloppenburg M, Lind PA, McCaskie A, Martin NG, Milani L, Montgomery GW, Nelissen RG, Nevitt MC, Nilsson PM, Ollier WE, Parimi N, Rai A, Ralston SH, Reed MR, Riancho JA, Rivadeneira F, Rodriguez-Fontenla C, Southam L, Thorsteinsdottir U, Tsezou A, Wallis GA, Wilkinson JM, Gonzalez A, Lane NE, Lohmander LS, Loughlin J, Metspalu A, Uitterlinden AG, Jonsdottir I, Stefansson K, Slagboom PE, Zeggini E, Meulenbelt I, Ioannidis JP, Spector TD, van Meurs JB, Valdes AM 2014 A meta-analysis of genome-wide association studies identifies novel variants associated with osteoarthritis of the hip. *Ann Rheum Dis* **73**:2130–2136.
28. Bos SD, Bovee JV, Duijnisveld BJ, Raine EV, van Dalen WJ, Ramos YF, van der Breggen R, Nelissen RG, Slagboom PE, Loughlin J, Meulenbelt I 2012 Increased type II deiodinase protein in OA-affected cartilage and allelic imbalance of OA risk polymorphism rs225014 at DIO2 in human OA joint tissues. *Ann Rheum Dis* **71**:1254–1258.
29. Bomer N, den Hollander W, Ramos YF, Bos SD, van der Breggen R, Lakenberg N, Peppers BA, van Eeden AE, Darvishan A, Tobin EW, Duijnisveld BJ, van den Akker EB, Heijmans BT, van Roon-Mom WM, Verbeek FJ, van Osch GJ, Nelissen RG, Slagboom PE, Meulenbelt I 2014 Underlying molecular mechanisms of DIO2 susceptibility in symptomatic osteoarthritis. *Ann Rheum Dis* [Epub ahead of print]; DOI: 10.1136/annrheumdis-2013-204739.
30. Waarsing JH, Kloppenburg M, Slagboom PE, Kroon HM, Houwing-Duistermaat JJ, Weinans H, Meulenbelt I 2011 Osteoarthritis susceptibility genes influence the association between hip morphology and osteoarthritis. *Arthritis Rheum* **63**:1349–1354.
31. Panicker V, Cluett C, Shields B, Murray A, Parnell KS, Perry JR, Weedon MN, Singleton A, Hernandez D, Evans J, Durant C, Ferrucci L, Melzer D, Saravanan P, Visser TJ, Ceresini G, Hattersley AT, Vaidya B, Dayan CM, Frayling TM 2008 A common variation in deiodinase 1 gene DIO1 is associated with the relative levels of free thyroxine and triiodothyronine. *J Clin Endocrinol Metab* **93**:3075–3081.
32. Schneider MJ, Fiering SN, Thai B, Wu SY, St Germain E, Parlow AF, St Germain DL, Galton VA 2006 Targeted disruption of the type 1 selenodeiodinase gene (Dio1) results in marked changes in thyroid hormone economy in mice. *Endocrinology* **147**:580–589.
33. Schneider MJ, Fiering SN, Pallud SE, Parlow AF, St Germain DL, Galton VA 2001 Targeted disruption of the type 2 selenodeiodinase gene (DIO2) results in a phenotype of pituitary resistance to T4. *Mol Endocrinol* **15**:2137–2148.
34. Bassett JH, Boyde A, Howell PG, Bassett RH, Galliford TM, Archanco M, Evans H, Lawson MA, Croucher P, St Germain DL, Galton VA, Williams GR 2010 Optimal bone strength and mineralization requires the type 2 iodothyronine deiodinase in osteoblasts. *Proc Natl Acad Sci U S A* **107**:7604–7609.
35. Glasson SS, Chambers MG, Van Den Berg WB, Little CB 2010 The OARS histopathology initiative—recommendations for histological assessments of osteoarthritis in the mouse. *Osteoarthritis Cartilage* **18**:S17–23.
36. Sondergaard BC, Catala-Lehnen P, Huebner AK, Bay-Jensen AC, Schinke T, Henriksen K, Schilling S, Haberland M, Nielsen RH, Amling M, Karsdal MA 2012 Mice overexpressing salmon calcitonin have strongly attenuated osteoarthritic histopathological changes after destabilization of the medial meniscus. *Osteoarthritis Cartilage* **20**:136–143.
37. Hoegh-Andersen P, Tanko LB, Andersen TL, Lundberg CV, Mo JA, Heegaard AM, Delaisse JM, Christgau S 2004 Ovariectomized rats as a model of postmenopausal osteoarthritis: validation and application. *Arthritis Res Ther* **6**:R169–180.

38. Waung JA, Maynard SA, Gopal S, Gogakos A, Logan JG, Williams GR, Bassett JH 2014 Quantitative X-ray micro-radiography for high-throughput phenotyping of osteoarthritis in mice. *Osteoarthritis Cartilage* **22**:1396–1400.
39. Bassett JH, van der Spek A, Gogakos A, Williams GR 2012 Quantitative X-ray imaging of rodent bone by Faxitron. *Methods Mol Biol* **816**:499–506.
40. Burr DB, Schaffler MB 1997 The involvement of subchondral mineralized tissues in osteoarthritis: quantitative microscopic evidence. *Microsc Res Tech* **37**:343–357.
41. van der Kraan PM, van den Berg WB 2012 Chondrocyte hypertrophy and osteoarthritis: role in initiation and progression of cartilage degeneration? *Osteoarthritis Cartilage* **20**:223–232.
42. Hayami T, Zhuo Y, Wesolowski GA, Pickarski M, Duong le T 2012 Inhibition of cathepsin K reduces cartilage degeneration in the anterior cruciate ligament transection rabbit and murine models of osteoarthritis. *Bone* **50**:1250–1259.
43. Orth P, Cucchiari M, Wagenpfeil S, Menger MD, Madry H 2014 PTH [1–34]-induced alterations of the subchondral bone provoke early osteoarthritis. *Osteoarthritis Cartilage* **22**:813–821.
44. Dentice M, Bandyopadhyay A, Gereben B, Callebaut I, Christoffolete MA, Kim BW, Nissim S, Mornon JP, Zavadacki AM, Zeold A, Capelo LP, Curcio-Morelli C, Ribeiro R, Harney JW, Tabin CJ, Bianco AC 2005 The Hedgehog-inducible ubiquitin ligase subunit WSB-1 modulates thyroid hormone activation and PTHrP secretion in the developing growth plate. *Nat Cell Biol* **7**:698–705.
45. Carvalho-Bianco SD, Kim BW, Zhang JX, Harney JW, Ribeiro RS, Gereben B, Bianco AC, Mende U, Larsen PR 2004 Chronic cardiac-specific thyrotoxicosis increases myocardial beta-adrenergic responsiveness. *Mol Endocrinol* **18**:1840–1849.
46. Cheng AW, Bolognesi M, Kraus VB 2012 DIO2 modifies inflammatory responses in chondrocytes. *Osteoarthritis Cartilage* **20**:440–445.
47. Roman-Blas JA, Jimenez SA 2006 NF-kappaB as a potential therapeutic target in osteoarthritis and rheumatoid arthritis. *Osteoarthritis Cartilage* **14**:839–848.
48. Zeold A, Doleschall M, Haffner MC, Capelo LP, Menyherth J, Liposits Z, da Silva WS, Bianco AC, Kacsokovics I, Fekete C, Gereben B 2006 Characterization of the nuclear factor-kappa B responsiveness of the human dio2 gene. *Endocrinology* **147**:4419–4429.
49. Canani LH, Capp C, Dora JM, Meyer EL, Wagner MS, Harney JW, Larsen PR, Gross JL, Bianco AC, Maia AL 2005 The type 2 deiodinase A/G (Thr92Ala) polymorphism is associated with decreased enzyme velocity and increased insulin resistance in patients with type 2 diabetes mellitus. *J Clin Endocrinol Metab* **90**:3472–3478.
50. Acosta BM, Bianco AC 2010 New insights into thyroid hormone replacement therapy. *F1000 Med Rep* **2**:34.
51. Larsen PR 2009 Type 2 iodothyronine deiodinase in human skeletal muscle: new insights into its physiological role and regulation. *J Clin Endocrinol Metab* **94**:1893–1895.
52. Zhu M, Tang D, Wu Q, Hao S, Chen M, Xie C, Rosier RN, O'Keefe RJ, Zuscik M, Chen D 2009 Activation of beta-catenin signaling in articular chondrocytes leads to osteoarthritis-like phenotype in adult beta-catenin conditional activation mice. *J Bone Miner Res* **24**:12–21.
53. Zhu M, Chen M, Zuscik M, Wu Q, Wang YJ, Rosier RN, O'Keefe RJ, Chen D 2008 Inhibition of beta-catenin signaling in articular chondrocytes results in articular cartilage destruction. *Arthritis Rheum* **58**:2053–2064.
54. Shen J, Li J, Wang B, Jin H, Wang M, Zhang Y, Yang Y, Im HJ, O'Keefe R, Chen D 2013 Deletion of the transforming growth factor beta receptor type II gene in articular chondrocytes leads to a progressive osteoarthritis-like phenotype in mice. *Arthritis Rheum* **65**:3107–3119.
55. Zhen G, Wen C, Jia X, Li Y, Crane JL, Mears SC, Askin FB, Frassica FJ, Chang W, Yao J, Carrino JA, Cosgarea A, Artemov D, Chen Q, Zhao Z, Zhou X, Riley L, Sponseller P, Wan M, Lu WW, Cao X 2013 Inhibition of TGF-beta signaling in mesenchymal stem cells of subchondral bone attenuates osteoarthritis. *Nat Med* **19**:704–712.
56. Glasson SS, Blanchet TJ, Morris EA 2007 The surgical destabilization of the medial meniscus (DMM) model of osteoarthritis in the 129/SvEv mouse. *Osteoarthritis Cartilage* **15**:1061–1069.
57. Fonseca TL, Correa-Medina M, Campos MP, Wittmann G, Werneck-de-Castro JP, Arrojo e Drigo R, Mora-Garzon M, Ueta CB, Caicedo A, Fekete C, Gereben B, Lechan RM, Bianco AC 2013 Coordination of hypothalamic and pituitary T3 production regulates TSH expression. *J Clin Invest* **123**:1492–1500.
58. Henry SP, Jang CW, Deng JM, Zhang Z, Behringer RR, de Crombrughe B 2009 Generation of aggrecan-CreERT2 knockin mice for inducible Cre activity in adult cartilage. *Genesis* **47**:805–814.

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