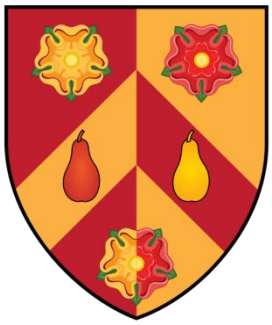
COMPARISON OF THE EFFECTS OF VITAMIN D METABOLITES ON OSTEOBLAST AND OSTEOCYTE BONE CELLS

A DISSERTATION SUBMITTED TO THE UNIVERSITY OF OXFORD





IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF

DOCTOR OF PHILOSPHY

BY

ALLAHDAD ZAREI

WOLFSON COLLEGE & NUFFIELD DEPARTMENT OF ORTHOPAEDICS, RHEUMATOLOGY AND MUSCULOSKELETAL SCIENCES, OXFORD

SUPERVISORS: DR AFSIE SABOKBAR, DR PHILIPPA HULLEY & DR KASSIM JAVAID

Hilary 2015

To Mum

ABSTRACT

While the major source of vitamin D is D_3 from ultraviolet exposure, some supplements supply D_2. The relative potency of vitamin D_2 versus vitamin D_3 remains controversial.

The aims of the current study were, 1. To optimize the *in vitro* model, including use of cell lines, vitamin D concentrations, and outcome biomarkers. 2. To compare the potency of vitamin D_2 and D_3 metabolites on mouse and human bone cellular activity. 3. To explore the expression of VDR in osteoarthritic (OA) bone tissues as well as cellular responses to vitamin D_2 and D_3 metabolites *ex-vivo*.

In mouse 2T3 osteoblasts, at physiological doses, both vitamin D_2 and D_3 metabolites increased ALP activity and mineralisation and up-regulated osteoblastic signature genes and proteins. At supra-physiological doses D_3 metabolites were more potent inhibitors of 2T3 function than D_2 metabolites. Although hBMS cell proliferation was inhibited by both 25(OH)D_2 and D_3, ALP activity was enhanced by both metabolites. However, 25(OH)D_3 was a more potent stimulator of ALP and mineralisation of hBMSCs. D_2 and D_3 equally stimulated expression of *CX43* and *PHEX* markers in osteocytic cell lines. Immunohistochemistry of femoral heads showed much reduced VDR expression in OA osteocytes and osteoclasts, yet both 25(OH)D_2 and D_3 increased OA-hBMSCs mineralisation more than non-OA-hBMSCs *ex-vivo*.

While vitamin D_2 or D_3 increased mouse 2T3 osteoblastic activity at physiological doses, OA and non-OA hBMSCs differentiation was more responsive to 25(OH)D_3. Key bone cells such as osteocyte and osteoclasts expressed less VDR in OA. For the first time vitamin D_2 metabolites have been thoroughly examined and emerged as a potent stimulator of bone cell differentiation, at least *in vitro*. Vitamin D_3 in contrast is confirmed as highly potent in bone cells, but with toxicity at much lower doses than D_2.

ACKNOWLEDGMENTS

First and foremost, my sincere gratitude goes to my supervisors, Afsie Sabokbar, Philippa Hulley, Kassim Javaid, and my co-supervisor Dr Reza Moravat for supporting and guiding me throughout my D.Phil. It has been a privilege to learn from their biological and clinical knowledge and experience. They ensured that I had all the support that I needed throughout this unique journey over the recent years. No words could describe my appreciation to them.

I have been fortunate over the past few years to share this time with many colleagues and friends whom I would like to thank. In particular, Aaron Kwaasi with whom I shared my working place, provided a great environment that made my research fun. Aaron and I had many spirited discussions about life, family and work. There are several others that I spent my time with that I would like to thank for their friendship: Mona A, Nasim Z, Cameron B, Sara A and Srinivasa R. All work presented in this thesis was performed by the candidate alone except for endogenous vitamin D measurements which have been conducted at King's Hospital, courtesy of Dr Caje Moniz and second harmonic generation image of collagen fibres on supplied cultured cells, courtesy of Dr Clarence Yapp and Sirinivasa Rao.

I am extremely grateful to Orthopaedic Research UK for funding my research time, true appreciation to Dr Kassim Javaid for funding my research and sincere gratitude to Professor Raashid Luqmani who provided funding towards the last three months of my time at NDORMS.

I thank my brothers and my sister for encouraging and inspiring me, as well as my nephew Mr Ghasem Zarei who provided endless support throughout my scientific work. This thesis is dedicated to the memory of my dear mother who was very proud of my achievements. She sadly passed away during my training and unfortunately is not able to see the completion of the work. She has been at the forefront of my thoughts as I am writing in her memory, for giving me endless love and every opportunity a son could have wanted.

GLOSSARY

ALP	Alkaline phosphatase
ATP	Adenosine triphosphate
$\alpha_v\beta_3$	Alpha v beta three (VNR)
BAX	BCL2-associated X protein
β-cell	Beta cell
BMP	Bone morphogenetic protein
BMSC	Bone marrow stromal cell
BMD	Bone mineral density
BSP-1	Bone sialoprotein-1
CTR	Calcitonin receptor
CVS	Cardiovascular
CD	Cathelicidin
CATK	Cathepsin K
CX-43	Connexin 43
COL1	Collagen type 1
Cbfa1	core-binding factor subunit alpha-1(Osterix)
CKD	Chronic kidney disease
CDKIs	Cyclin dependent kinase inhibitors
CYP2R1	Cytochrome P450, family 2, subfamily R, polypeptide 1
CYP24A1	Cytochrome P450, family 24, subfamily A, polypeptide 1
CYP27B1	Cytochrome P450, family 27, subfamily B, polypeptide 1
DBP	Vitamin D binding protein
DPBS	Dulbecco’s phosphate-buffered saline
DKK1	Dickkopf-related protein 1
DMEM	Dulbecco`s modified Eagle medium
DMP1	Dentin matrix acidic phosphoprotein1
ENPP	Ectonucleotide pyrophosphatase
ELISA	Enzyme-linked immunosorbant assay
FBS	Foetal bovine serum
FGF23	Fibroblast growth factor 23
HVDRR	Hereditary vitamin D resistant rickets
IHH	Indian Hedgehog
ICAM-1	Inter-cellular adhesion molecule 1
IHC	Immunohistochemistry
IL	Interleukin
IU	International unit
KDa	Kilo Dalton
LC-MS	Liquid chromatography-mass Spectrometry
LRP5	Low density lipoprotein receptor-related protein 5
M-CSF	Macrophage colony-stimulatory factor
MSC	Mesenchymal stem Cell
MEM	Minimum essential medium

MEPE	Matrix extracellular phosphoglycoprotein
MMP	matrix metalloprotease
MS	Multiple sclerosis
NFATc	Nuclear factor of activated T cell cytoplasmic
OA	Osteoarthritis
OB	Osteoblast
OCN	Osteocalcin
OC	Osteoclast
ONC	Osteonectin
OPG	Osteoprotegerin
OPN	Osteopontin
ORP150	Oxygen-Regulated Protein 150
PBMC	Peripheral blood mononuclear Cell
PHEX	Phosphate-regulating neutral endopeptidase on chromosome X
PTH	Parathyroid hormone
PDDR	Pseudo-vitamin D deficiency rickets
PPi	Pyrophosphate
QRT-PCR	Quantitative reverse transcriptase polymerase
RANKL	Receptor activator of nuclear factor Kappa-B ligand
RFU	Relative fluorescence unit
RXR	Retinoid X receptor
Runx-2	Runt-related transcription factor 2
SOST	Sclerostin
SV40	Simian vacuolating virus 40
TGF-β	Transforming growth factor beta
TRAP	Tartrate-resistant acid phosphatase
TLR	Toll like receptor
TCF	Transcription factor 4
TGFβ	Transforming growth factor beta
TB	Tuberculosis
UVB	Ultraviolet B
VDR	Vitamin D receptor
VDRE	Vitamin D response element
VNR	Vitronectin receptor
WNT	Wingless
$1,25(OH)_2D_2$	1-25-dihydroxyvitamin D_2
$1,25(OH)_2D_3$	1-25-dihydroxyvitamin D_3
4MU	4-methylumberlliferone
4MUP	4-methylumberlliferyl phosphate
7DHC	7-dehydrocholesterol
25-OHase	25-hydroxylase
$25(OH)D_3$	25-hydroxy vitamin D_3
$25(OH)D_2$	25-hydroxy vitamin D_2

Table of Contents

ABSTRACT.....**ii**
ACKNOWLEGMENTS.....**iii**
GLOSSARY.....**1**
LIST OF FIGURES.....**9**
LIST OF TABLES.....**12**

1. LITERATURE REVIEW **14**
1.1. History of vitamin D including evolutionary aspects 14
1.2. Vitamin D synthesis: D_3 vs D_2 16
1.3. Sources of vitamin D.....18
1.4. Vitamin D metabolism.....19
1.5. Vitamin D transport 21
1.6. Vitamin D catabolism 22
1.7. Mechanisms of vitamin D action 24
1.8. Regulation of $1,25(OH)_2D$ synthesis and catabolism 27
1.9. Vitamin D actions.....28
1.9.1. Classical actions of vitamin D 28
1.9.2. Non-Classical actions of vitamin D 33
1.10. Vitamin D and diseases 36
1.11. Rational for using ketoconazole.....38
1.12. Vitamin D and musculoskeletal health 40
1.13. Bone and bone cells 41
1.13.1. Osteoblasts.....42
1.13.2. Osteoclasts.....45
1.13.3. Osteocytes.....46
1.14. Vitamin D and bone cells 49
1.14.1. Vitamin D and osteoblasts 49
1.14.2. Vitamin D and osteoclasts 54
1.14.3. Vitamin D and osteocytes 55
1.15. Conflicting Views 56
1.16. Aims of this thesis.....59
2. OPTIMISATION OF OSTEOBLAST-LIKE CELLS FOR *IN VITRO* STUDIES.....**63**

2.1. Aims and objectives 64
2.2. MATERIAL AND METHODS 66
2.2.1. Chemicals and reagents 66
2.2.2. Osteoblast-like cell lines 67
2.2.3. Mycoplasma screening 68
2.2.4. Endogenous levels of vitamin D in *in vitro* studies 69
2.2.5. Cell number measurements 70
2.2.6. VDR, CYP27B1, OPN and OCN protein expression 71
2.2.7. Differentiation markers 73
2.2.7.1. ALP activity 73
2.2.7.2. ALP activity in osteoblast-like cells 74
2.2.8. Quantitative reverse polymerase chain reaction (QRT-PCR) 75
2.2.8.1. RNA extraction 75
2.2.8.2. Complementary DNA synthesis 75
2.2.8.3. Signature gene expression 76
2.2.9. Collagen production 76
2.2.10. Mineralisation 78
2.2.11. Statistical analysis 79
2.3. Results 80
2.3.1. Endogenous level of vitamin D 80
2.3.2. Summary of results 80
2.3.3. 2T3 Cell number 83
2.3.4. ALP activity 86
2.3.5. Signature gene expression 87
2.3.6. Total collagen measurements 89
2.3.7. Protein expression 90
2.4. Mineralisation assay 91
2.5. Discussion 94
3. EFFECTS OF VITAMIN D_2 AND D_3 ON 2T3 OSTEOBLAST ACTIVITIES .. 99
3.1. Introduction 99
3.2. Aims and objectives 105
3.3. Materials and Methods 106
3.3.1. 2T3 cell number in response to vitamin D metabolites 106

3.3.2. 2T3 ALP activity in response to vitamin D metabolites 106
3.3.3. 2T3 osteoblastic signature gene expression in response to vitamin D metabolites 107
3.3.4. 2T3 protein expression in response to vitamin D metabolites 107
3.3.5. 2T3 Mineralisation in response to vitamin D metabolites 108
3.3.6. Influence of ketoconazole on the effects of vitamin D metabolites on 2T3 function 109
3.3.6.1. Effect of ketoconazole on 2T3 cell activity 109
3.3.6.2. Influence of ketoconazole on the effects of vitamin D metabolites on ALP activity in 2T3 cells 109
3.3.6.3. Influence of ketoconazole on the effects of vitamin D metabolites on signature genes in 2T3 cells 110
3.3.6.4. Influence of ketoconazole on the effects of vitamin D metabolites on 2T3 mineralisation..... 110
3.4. Results…… 111
3.4.1. Effect of vitamin D on 2T3 cell number 111
3.4.2. Murine 2T3 differentiation in response to vitamin D 112
3.4.3. 2T3 ALP activity 112
3.4.4. 2T3 osteoblastic signature gene expression 113
3.4.5. 2T3 osteoblastic protein expression 114
3.4.6. Collagen production in 2T3 osteoblasts 115
3.4.7. Effect of vitamin D on 2T3 mineralisation 116
3.4.8. Effects of ketoconazole on 2T3 cellular activity 118
3.4.8.1. 2T3 Cell number 118
3.4.8.2. 2T3 mineralisation 119
3.4.8.3. 2T3 signature genes 120
3.4.9. Influence of ketoconazole on the effects of vitamin D metabolites 121
3.4.9.1. 2T3 ALP activity 121
3.4.9.2. 2T3 Osteoblastic signature gene expression 123
3.4.9.3. 2T3 Mineralisation 124
3.4.10. Effects of BMP-2 on 2T3 differentiation by vitamin D 126
3.5. Discussion…… 132
3.5.1. Comparison of D_2 vs D_3 effects on 2T3 function 133
3.5.2. Influence of ketoconazole on the effects of D_2 and D_3 on 2T3 function 136

3.5.3. Comparison of D_2 vs D_3 effects on 2T3 function ±BMP-2 137
4. D_2 vs D_3 EFFECTS ON PRIMARY HUMAN OSTEOBLAST ACTIVITIES .142
4.1. Introduction…… 142
4.2. Aims and objectives 145
4.3. Materials and Methods 146
4.3.1. Osteoblast cell number 146
4.3.2. Alkaline phosphatase activity 146
4.3.3. Mineralisation 147
4.4. Results…… 148
4.4.1. Effect of vitamin D metabolites on cell number 148
4.4.2. Effect of vitamin D metabolites on ALP activity 149
4.4.2.1. Effect of vitamin D metabolites on Mineralisation 150
4.4.3. Influence of ketoconazole on stimulation of human osteoblast differentiation by vitamin D metabolites 151
4.4.3.1. Effects of ketoconazole on hBMSCs 151
4.4.3.2. ALP activity in response to D_2 and D_3 metabolites ± ketoconazole……..……152
4.4.3.3. Mineralisation..... 155
4.5. Discussion…….. 156
4.5.1. D_2 vs D_3 effects on human primary osteoblasts 156
4.5.2. D_2 vs D_3 effects on human primary cells in the presence of ketoconazole 159
5. MLOY-4 AND MLO-A5 RESPONSES TO VARIOUS METABOLITES OF VITAMIN D_2 AND D_3 164
5.1. Introduction…. 164
5.2. Aims and objectives 167
5.3. Material and methods 168
5.3.1. VDR and CYP27B1 protein expression 169
5.3.2. Osteocytic signature gene expression 169
5.3.3. Osteocyte cell number in response to vitamin D metabolites.....170
5.3.4. Osteocytic signature genes expression in response to vitamin D metabolites 170
5.4. Results…… 172
5.4.1. VDR and CYP27B1 expression 172

5.4.2. Osteocytic signature genes.....172
5.4.3. Osteocyte cell number in response to vitamin D metabolites.....173
5.4.4. Osteocytic markers in response to vitamin D metabolite 175
5.5. Discussion…….. 180
6. VITAMIN D IN OSTEOARTHRITIC BONE **186**
6.1. Introduction…. 186
6.1.1. Osteoarthritis (OA).....186
6.1.2. OA and vitamin D.....190
6.2. Aims and objectives.....192
6.3. Materials and methods 193
6.3.1. Specimens.....193
6.3.2. Immunohistochemistry.....195
6.3.3. Ex-vivo cell culture.....197
6.4. Results…… 198
6.4.1. Changes in OA 198
6.4.2. Ex-vivo cell culture.....202
6.4.2.1. Mineralisation..... 202
6.5. Discussion…….. 206
7. CONCLUSION AND FUTURE WORK **214**
7.1. Summary of the results.....214
7.2. Directions for future work.....227
8. POSTER PRESENTATIONS.....**231**
9. APPENDICES.....**232**
9.1. Appendix 1: Mycoplasma screening of cell lines used in this thesis revealing no infection. 232
9.2. Appendix 2: MG-63 osteoblast-like cell growth over time. 233
9.3. Appendix 3: SaOS-2 osteoblast-like cell growth over time. 234
9.4. Appendix 4: MC3T3 osteoblast-like cell growth over time. 235
9.5. Appendix 5: SaOS-2 osteoblast-like cells did not respond to D_2 and D_3 metabolites at physiological concentrations. 236
9.6. Appendix 6: Osteoblast-like SaOS-2, MG-63 and MC3T3 cell lines did not mineralise in the presence of osteogenic medium over 12 days of culture.....237

9.7. Appendix 7: Vitamin D_3 metabolites are more potent than D_2 at supra-physiological doses in reducing 2T3 osteoblast number. 238

9.8. Appendix 8: Both $1,25(OH)_2D_2$ and $1,25(OH)_2D_3$ active metabolites suppress prostatic cancer cell number in the presence of ketoconazole. 239

9.9. Appendix 9: OPN protein is up-regulated by vitamin D_2 and D_3 metabolites in MBA-15.4 osteoblast-like cells 240

9.10. Appendix 10: Metabolism of $25(OH)D_2$ or D_3 metabolites did not drive 2T3 osteoblast-like cells into adipocytes. 241

9.11. Appendix 11: Vitamin D_2 or D_3 active metabolites did not drive 2T3 osteoblast-like cells into adipocytes. 242

9.12. Appendix 12: Human bone marrow stromal cell activity. 243

10. REFERENCES **244**

LIST OF FIGURES

Figure 1-1: Synthesis of vitamin D_2 (from plant ergosterol) and vitamin D_3 from 7-DHC in the skin. **17**
Figure 1-2: An overview of vitamin D_3 metabolism/production *in vivo*. **20**
Figure 1-3: Pathways for active vitamin D synthesis. **21**
Figure 1-4: Mechanism of renal uptake of bound 25(OH)D. **22**
Figure 1-5: Catabolism of vitamin D_3 and D_2 metabolites **23**
Figure 1-6: Vitamin D receptor.. ..... **26**
Figure 1-7: Regulation of $1,25(OH)_2D$ production.. ..... **28**
Figure 1-8: Renal function of $1,25(OH)_2D_3$. **31**
Figure 1-9: Renal function of $1,25(OH)_2D_3$ **32**
Figure 1-10: Renal function of $1,25(OH)_2D_3$ **33**
Figure 1-11: Extra-renal role of vitamin D. **34**
Figure 1-12: Schematic model of BMSC differentiation into osteoblast **43**
Figure 1-13: Process of osteoblast to osteocyte differentiation **47**
Figure 2-1: SaOS-2 and MG-63 and MC3T3 osteoblast-like cells express VDR and CYP27B1 proteins. **81**
Figure 2-2: SaOS-2 and MG-63 and MC3T3 osteoblast-like cells express OPN and low levels of OCN proteins.. ..... **81**
Figure 2-3: Osteoblast-like cell lines SaOS-2, MG-63 and MC3T3 up-regulate ALP in response to BMP-2.. ..... **82**
Figure 2-4: 2T3 osteoblast-like cell growth over time with different initial cell densities.. ..... **84**
Figure 2-5: 2T3 osteoblast-like cells are viable during 12 days of differentiation. **85**
Figure 2-6: ALP is stimulated by osteogenic differentiation medium or BMP-2 in 2T3 osteoblast-like cells. **86**
Figure 2-7: Osteoblastic signature genes are up-regulated in 2T3 osteoblast-like cell when cultured in osteogenic differentiation medium.. ..... **88**
Figure 2-8: 2T3 osteoblast-like cells produce collagen. **89**
Figure 2-9: 2T3 osteoblast-like cells express OPN, OCN, VDR and CYP27B1 during differentiation. **90**
Figure 2-10: 2T3 osteoblast-like cells mineralised in the presence of osteogenic differentiation medium. **93**
Figure 2-11: Figure illustrates the timeline at which specific osteoblastic signature genes and protein markers are expressed as identified in this study. **97**
Figure 3-1: Vitamin D_3 metabolites are more potent than D_2 in inhibiting 2T3 cell number.. ..... **111**
Figure 3-2: Both vitamin D_2 and D_3 metabolites stimulated 2T3 ALP activity dose and time dependently.. ..... **112**
Figure 3-3: Both $25(OH)D_2$ and D_3 metabolites stimulated 2T3 signature genes. **114**
Figure 3-4: 2T3 osteoblastic proteins stimulated by both vitamin D_2 and D_3 metabolites. **115**
Figure 3-5: Total collagen expression was not affected by physiological doses of vitamin D_2 or D_3 metabolites in 2T3 osteoblastic cell line. **116**

Figure 3-6: Low doses of D_2 and D_3 metabolites increased 2T3 mineralisation, but high doses inhibited mineralisation. **118**
Figure 3-7: Only 80µM ketoconazole reduced 2T3 cell number over time. ... **119**
Figure 3-8: High doses of ketoconazole suppress 2T3 mineralisation…….**118**
Figure 1-9: 10µM Ketoconazole depleted *CYP27B1* and abolished *CYP24A1* in 2T3 osteoblast-like cells……..**119**
Figure 1-10: ALP expression reduced in response to D_2 and D_3 metabolites in the presence of ketoconazole…….**120**
Figure 1-11: 2T3 osteoblastic and vitamin D pathway genes were up-regulated in response to 25(OH)D_2 or 25(OH)D_3 in the presence of ketoconazole……**122**
Figure 1-12: 2T3 mineralisation was abolished in response to co-treatment with D_2 or D_3 metabolites and ketoconazole……**123**
Figure 3-13: 2T3 mineralisation was accelerated by BMP-2……**124**
Figure 1-14: 2T3 mineralisation was accelerated in response to 50nM of 25(OH)D_2 and D_3 in the presence of BMP-2……**126**
Figure 3-15: Quantified Alizarin red staining of 2T3 cells in response to 25(OH)D_2 and D_3 in the presence of BMP-2 on days 5 and 6……**127**
Figure 1-16: 2T3 mineralisation accelerated in response to 0.05nM of 1,25$(OH)_2D_2$ and 1,25$(OH)_2D_3$ active metabolites in the presence of BMP-2……**128**
Figure 3-17: Quantified Alizarin red staining of 2T3 cells in response to the active metabolites 1,25$(OH)_2D_2$ or D_3 in the presence of BMP-2 on days 5 and 6……..**129**
Figure 4-1: Vitamin D_3 metabolites are more potent than D_2 in reducing hBMSC number. **148**
Figure 4-2: Vitamin D_3 metabolites are more potent than D_2 in up-regulation of ALP in hBMSCs. **149**
Figure 4-3: Mineralisation of hBMSCs within 28 days only occurred in the presence of 25(OH)D_3. ..... **151**
Figure 4-4: 10µM ketoconazole had no effects on hBMSC number or ALP over 7 days.. ..... **152**
Figure 4-5: Addition of ketoconazole resulted in higher ALP up-regulation in response to 1,25$(OH)_2D_3$ than 1,25$(OH)_2D_2$, but had no effect when co-treated with 25(OH)D_2 or D_3. ..... **141**
Figure 4-6: Addition of ketoconazole resulted in higher ALP up-regulation in response to 1,25$(OH)_2D_3$ than 1,25$(OH)_2D_2$..... **154**
Figure 4-7: Addition of ketoconazole resulted in higher mineralisation in response to 1,25$(OH)_2D_2$ than 1,25$(OH)_2D_3$, but supressed mineralisation when treated with 25(OH)D_2 or D_3. ..... **156**
Figure 5-1: Osteocyte-like cells express CYP27B1 and VDR at protein levels.. ..... **172**
Figure 5-2: Osteocyte-like cells express low levels of osteocyte genes at transcript levels. **173**
Figure 5-3: Osteocyte-like cell number was reduced by high dose of vitamin D_3 metabolites..…..... **174**
Figure 5-4: Both 1,25$(OH)_2D_2$ and D_3 active metabolites up-regulate osteocytic transcripts in MLOY-4. ..... **176**
Figure 5-5: 25(OH)D_2 and D_3 up-regulate osteocytic transcripts in MLO-A5 cells while active metabolites had little effect. **178**

Figure 6-1: Comparison of normal joint versus late stage OA joint.. ..... **189**
Figure 6-2: Schematic representation of OA femoral head and its various regions depending on macroscopic observations. **195**
Figure 6-3: Subchondral bone thickness varies in OA femoral head..... **198**
Figure 6-4: VDR is expressed by BMSCs, osteoblast, osteocyte and osteoclasts in healthy bone samples.. ..... **199**
Figure 6-5: VDR is expressed by BMSCs, chondrocytes and osteoblasts, in OA bone samples..... **200**
Figure 6-6: 25(OH)D_3 was more potent than D_2 in induction of mineralisation in both healthy and OA BMSCs. **204**
Figure 6-7: 1,25(OH)$_2D_2$ was more potent than D_3 when co-treated with ketoconazole to induce mineralisation in both healthy and OA BMSCs. **205**

LIST OF TABLES

Table 2-1: List of human and murine osteoblast-like cells **67**
Table 2-2: Lists of primary antibodies **72**
Table 2-3: Basal media or sera contain no detectable 25(OH)D_2 or D_3. ..... **80**
Table 2-4: Summary of human and murine osteoblast activities **83**
Table 3-1: The effects of 1,25$(OH)_2D_3$ on rodent osteoblast function **101**
Table 4-1: Summary of the effects of vitamin D_3 metabolites on established human osteoblast-like cells and primary osteoblasts **144**
Table 5-1: Summary of the results of the effects of 100-500nM of 25(OH)D_2 or D_3 and 0.1-0.5nM 1,25$(OH)_2D_2$ and D_3 on MLO-A5 osteocytic signature genes. **179**
Table 6-1: List of the VDR positive cells in non-OA and OA human tissue… **201**

CHAPTER 1: LITERATURE REVEIW

1. LITERATURE REVIEW

1.1. History of vitamin D including evolutionary aspects

The naming of vitamins was initially derived from the concept of “unknown micronutrients” that was essential in growth, in supporting life and in development, from the work of MacCollum [1], as well as that of Osborne and Mendel in the early nineteenth century [2].

During the industrial revolution, when people began to migrate to big cities, bone deformity was reported in children as a consequence of lack of sun exposure [3]. It was also observed that children who lived indoors in Northern European town centres with no sun exposure, developed rickets compared to those living in countryside with higher sun exposure [4]. Hence, it was initially concluded that lack of sunlight exposure was the likely cause of rickets in children [5].

Evidence that sunlight and cod liver oil could cure severe rickets was initially noted in dogs maintained with oat-meal and kept indoors in 1918 by Mellanby [6]. The discovery in 1924 by Steenbock and Black that rickets could be eliminated in rats that were either exposed to sunlight or fed irradiated fat is known as one of the great achievements in medicine [7]. Similar observation on the use of ultraviolet rays with regard to prevention and cure of rickets was simultaneously observed by Hess *et al* [8]. Thereafter experimental approaches using exposure to artificial light or sunlight as well as cod liver oil treatment was found to prevent or cure rickets in children [9-11].

This was followed by the discovery of the pharmacological aspect of irradiated ergosterol (pre-vitamin D_2) with antirachitic potency in children, [8, 12, 13]. The

concept that radiolabeled vitamin D_3 in rats, was converted into a detectable active form in bone, liver and blood had appeared in 1966 [14]. Circulatory $25(OH)D_3$ was isolated, chemically identified, and synthesised by 1969 [15]. The active hormone derived from vitamin D_3 was discovered as $1,25(OH)_2D_3$ and its synthesis *in vitro* was confirmed by 1971 [16, 17]. These findings later led to the discovery of physiological functions of vitamin D that are associated with utilization of calcium and phosphate from the diet [11, 18].

Vitamin D is well known for its effect on calcium and bone homeostasis, but its evolutionary path in different species is not well understood. Nearly 400 million years ago vertebrates moved from the ocean (where they used to obtain their calcium from the sea) onto the land. Thereafter a sophisticated mechanism had to be evolved, to tightly control the calcium and phosphate absorption from the intestine for skeletal mineralisation. This came to involve sunlight [19].

Calcium absorption for animals in the calcium rich sea involves calcium transport by gill's cells, or by direct absorption by skin. Since vertebrates moved from the sea to the land they evolved to obtain calcium from the intestine [19]. This mechanism involved the evolution of vitamin D precursor (7DHC) synthesis by sunlight in the skin and its effect on regulation of calcium uptake in the intestine for skeletal health [19].

Vitamin D existence was first demonstrated in rats, and later on in other species such as dogs, humans, birds, rodents and monkeys [20]. Vitamin D requirement for calcium homeostasis in different species varies based on their evolutionary path [21]. For example flying species cannot produce vitamin D in their skin due to low levels of vitamin D precursor in their skin. Horse, sheep and goats have very high levels of intestinal calcium absorption and their requirement for vitamin

D is lower than other species [21]. Vitamin D production has been suggested to have a major role in the evolution of human skin colour. Early humans (in Africa) are thought to have had a darker skin to avoid excess vitamin D production in their skin as well as protecting against sun exposure, and as they migrated to other continents where sun exposure (Ultraviolet B: UVB) declines, skin de-pigmentation evolved allowing greater efficacy for vitamin D production [21].

1.2. Vitamin D synthesis: D_3 vs D_2

Based on the importance of skin exposure to sunlight and food containing sterols, scientists first discovered the vitamin D_2 precursor ergosterol (the major sterol in fungus) from yeast [22]. Hence the irradiated form of ergosterol as a precursor of ergocalciferol (vitamin D_2) was the first to be discovered and thought to be the same as vitamin D produced in the skin [22]. However, soon after, this assumption was rejected by the same group as they identified a similar compound with a different side chain from pig skin, with the same anti-rachitic effect but different potency compared with vitamin D_2. They identified this cholesterol compound as 7-dehydroxycholestrol (7-DHC), a precursor of vitamin D_3 named as cholecalciferol [23].

We now know that vitamin D_3 synthesis in human is mediated by the skin's exposure to sunshine [3, 22, 24] whereby 7-DHC within the epidermal layers absorbs UVB radiation (wavelength; 290-315 nm) and forms pre-vitamin D_3 which in turn photo-isomerises into vitamin D_3 [25]. On the other hand, vitamin D_2 is purely a plant vitamin D that is not synthesised in humans and that is synthesised by artificial UVB irradiation of ergosterol derived from green plants

and fungi [8].The main difference between the two forms of vitamin D is that vitamin D_2 has an extra double bond on carbon 22-23, as well as an extra methyl group on carbon number 24 (shown in red in **Figure 1-1**).

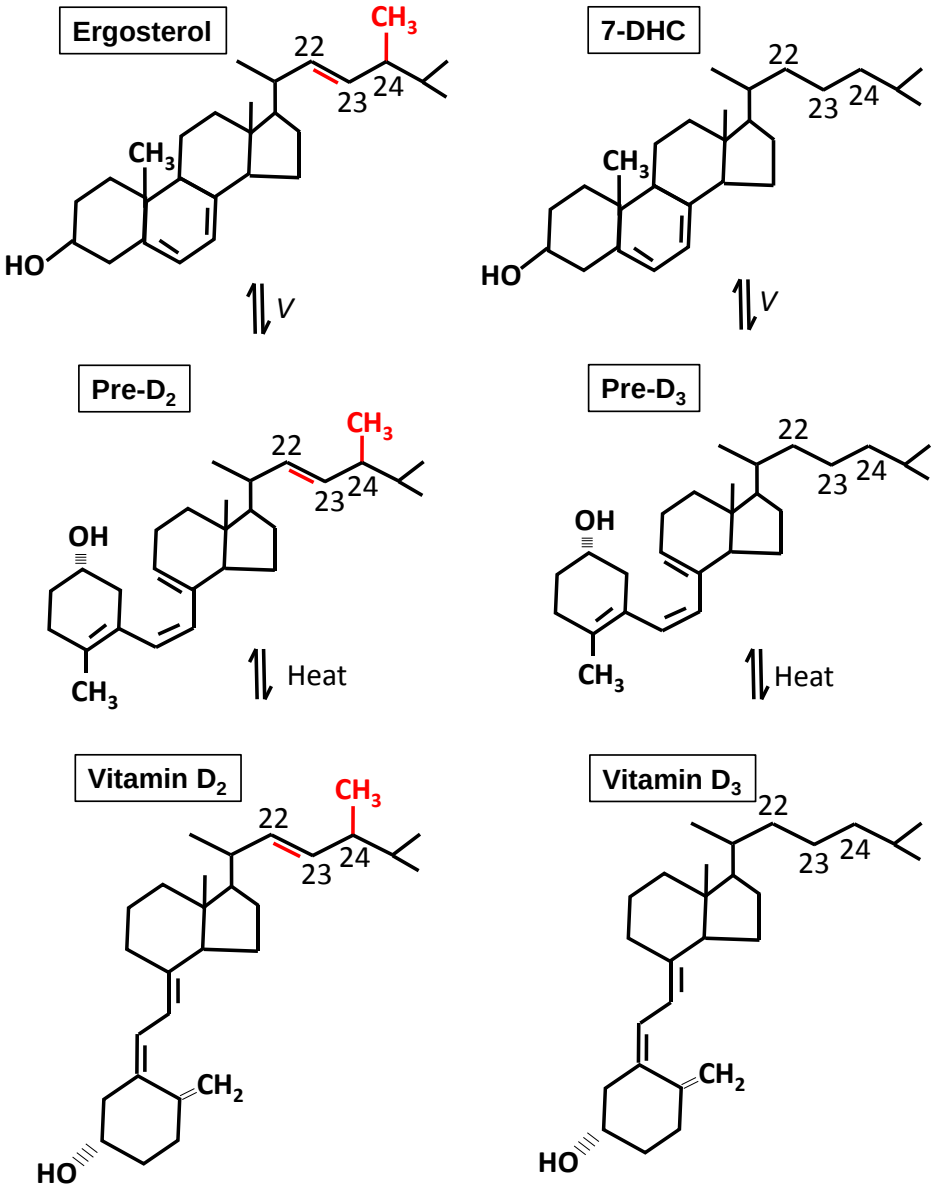


Figure 1-1: Synthesis of vitamin D_2 (from plant ergosterol) and vitamin D_3 from 7-DHC in the skin. For description please see text. Differences between D_2 and D_3; D_2 has an extra double bound on carbon 22-23 and an extra methyl group on carbon 24 shown in red , compared to D_3. Picture re-drawn from Bikle 2009 [26].

1.3. Sources of vitamin D

The major source of vitamin D is by skin exposure to sunlight as vitamin D_3 (>95%), and a variety of factors such as application of sunscreen [27], skin pigmentation [28] and ageing [29] limit the skin's vitamin D production. Very few natural foods, such as oily or fatty fish, contain significant amounts of vitamin D_3 (~100-1000IU[1]), but it is not found in other natural sources such as vegetables, fruits or nuts [30].

A very small amount of vitamin D_2 can be obtained from the diet in fresh mushrooms or in eggs. In addition to natural sources, some countries fortify milk, bread and breakfast cereals with either vitamin D_2 or D_3 [30]. Vitamin D can also be supplemented in a form of multivitamin (400-5000IU of either D_2 or D_3) or as prescribed medicine (800-50,000 IU as in capsule , D_3) [31]. There are vitamin D products for oral use, either as cholecalciferol (vitamin D_3) or ergocalciferol (vitamin D_2) that can be purchased in recommended dose from pharmacies or supermarkets without prescription [30]. Vitamin D is also prepared in higher doses for the prevention or treatment of vitamin D deficiency. These include vitamin D supplements in combination with calcium in treatment of osteoporosis [32].

[1] International Unit (IU): 40IU=1µg [35].

1.4. Vitamin D metabolism

Once vitamin D_3 is formed in the skin, it is believed that it is bound to vitamin D binding protein (DBP) in the circulation and this promotes vitamin D diffusion into the blood capillaries [18]. Exposure of vitamin D_3 to ultra-violet radiation (UVR) in the skin, prior to its transport into the blood is known to result in breakdown and photo-degradation of vitamin D_3 [33, 34]. It is now recognised that sunlight itself is the major factor of regulation of vitamin D_3 production in the skin, hence any excess vitamin D_3 produced by exposure to UVB can also be destroyed by solar UVR [33]. This sophisticated regulatory mechanism in the skin is the reason that over-exposure to sunlight never causes over-production of vitamin D_3 and toxicity [35].

After heat-induced isomerization of pre-vitamin D_3 into vitamin D_3 in the epidermal layer of the skin, vitamin D_3 enters the blood capillaries by an unknown mechanism. After whole body UVB irradiation of healthy volunteers, vitamin D_3 levels in the circulation start to rise at 12 hours, reached its peak levels at 24 hours and maintained this high for a week [33]. The same group showed that isolation of human DBP from the circulation also removed vitamin D_3 from the blood samples of UVB exposed subjects, which led to the conclusion that vitamin D_3 transport is mainly carried out by DBP [33]. When vitamin D_2 was orally administered in healthy individuals and its level was measured over the same time as irradiated subjects, it was shown that blood vitamin D_2 levels stay constant over the whole 24 hours and after ingestion [33]. The pathway of vitamin D_3 in humans is summarised in **Figure 1-2.** There are two major forms of active metabolite of vitamin D, known as 1,25-dihydroxyvitamin D ; $1,25(OH)_2D_2$ and

$1,25(OH)_2D_3$, which are formed from serial hydroxylation in the liver and kidneys [36].

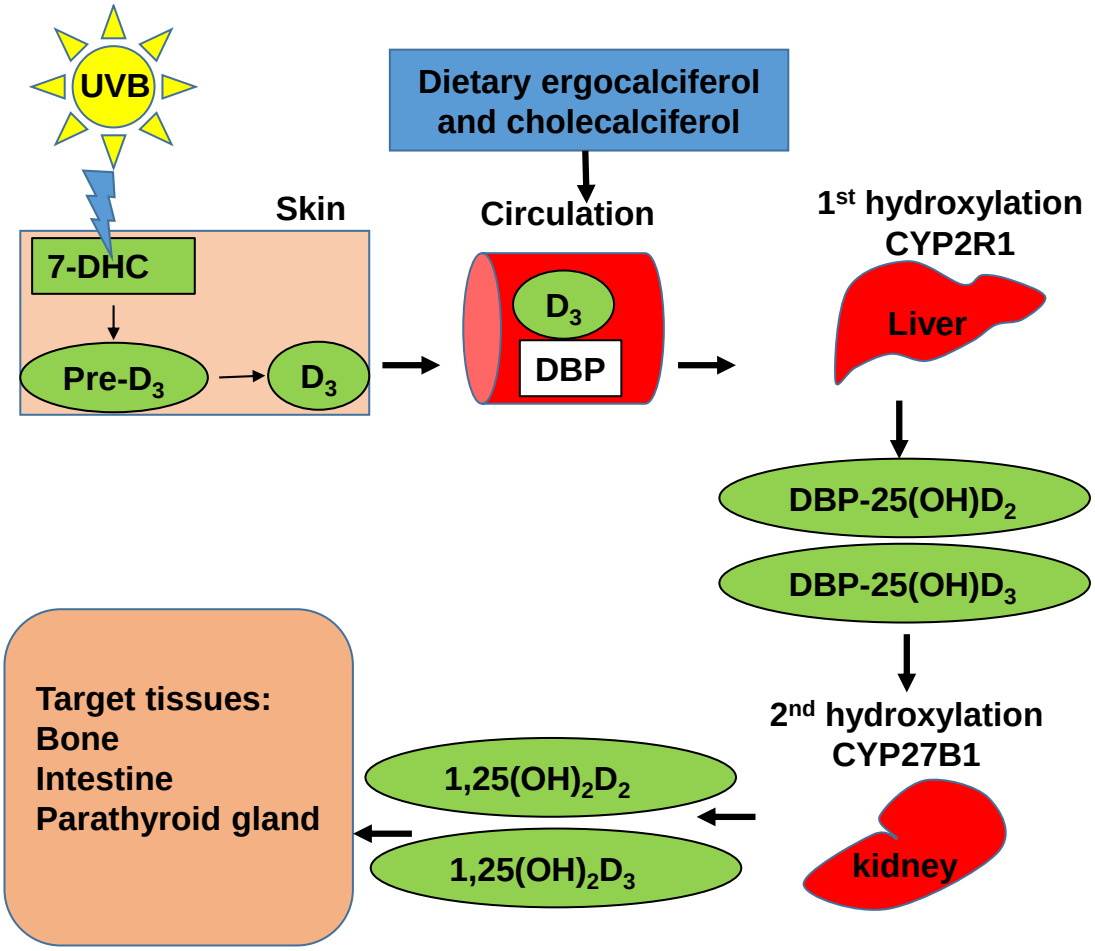


Figure 1-2: An overview of vitamin D_3 metabolism/production *in vivo*. Vitamin D_3 is obtained by either UVB radiation of 7-DHC in the skin or absorbed in the intestine as cholecalciferol, and D_2 absorbed as ergocalciferol (vitamin D_2) from diet. When entering the circulation, vitamin D_3/D_2 bind to DBP and are transported to liver for 25-hydroxylation by CYP2R1 into $25(OH)D_3$ or $25(OH)D_2$. The resulting $25(OH)D_3/D_2$ are further hydroxylated by CYP27B1 in the kidney into active secosteroid hormone as $1,25(OH)_2D_3/D_2$ and exert different effects on the target tissues.

Both forms of vitamin D_3 and D_2 after entering the circulation follow the same metabolic pathways as shown in **Figure 1-3**. Firstly, both forms can be hydroxylated by 25-hydroxylase enzymes (including CYP2R1) of hepatocytes in the liver into $25(OH)D_3$ and $25(OH)D_2$, (on carbon position 25 as shown on

Figure 1-3). Secondly, a further hydroxylation on carbon position 1 by 1α-hydroxylase enzyme (CYP27B1) in the kidney proximal renal tubule, leads to the formation of the main biologically endocrine active forms of vitamin D; $1,25(OH)_2D_3$ and $1,25(OH)_2D_2$, respectively [37]. These hydroxylation sites are shown as red in **Figure 1-3**.

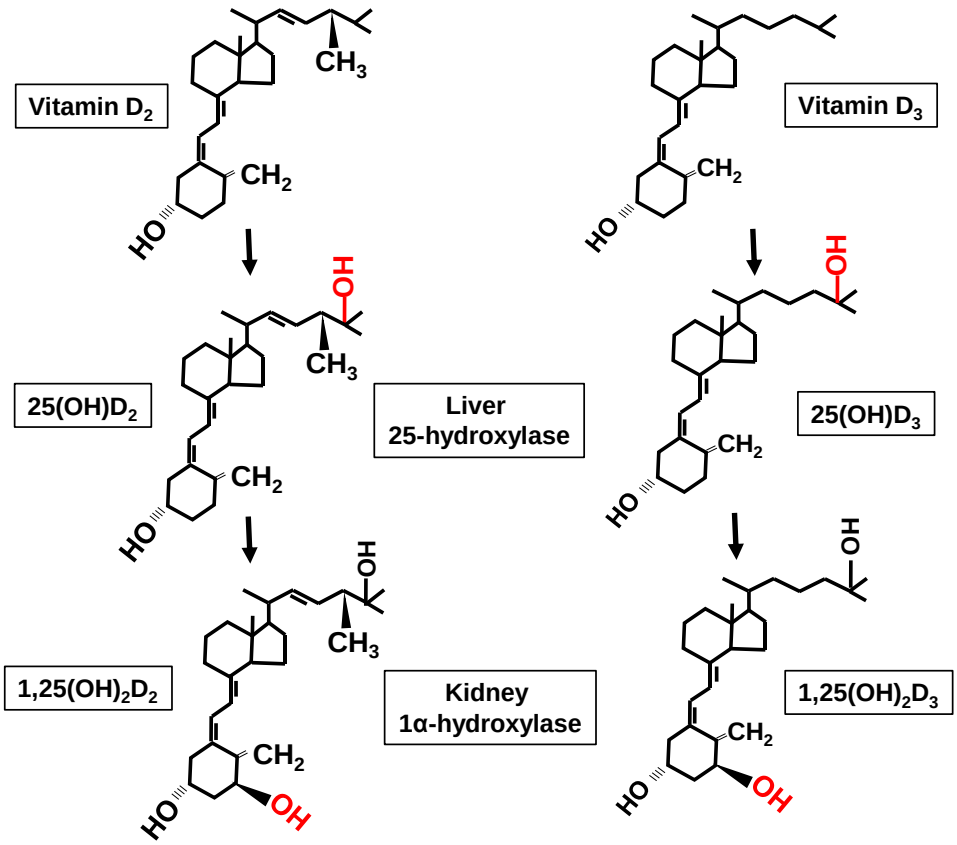


Figure 1-3: Pathways for active vitamin D synthesis. Vitamin D_3 and D_2 in human undergo similar serial hydroxylation on carbon number 25 in the liver by 25 hydroxylase enzyme and carbon number one by 1α-hydroxylase in the kidney into biologically active hormones $1,25(OH)_2D_3$ and D_2. Hydroxylation sites are shown in red.

1.5. Vitamin D transport

More than 99% of both circulatory 25(OH)D and the active forms of $1,25(OH)_2D$ are bound to DBP and albumin (both produced in the liver), with higher affinity

binding to the former than the latter [38]. DBP is degraded by lysosomes in the kidney's proximal tubular cells via its uptake by megalin after which $1,25(OH)_2D$ is synthesised by a mitochondrial 1-αhydroxylase enzyme, CYP27B1 [39]. Although the major site of circulating $1,25(OH)_2D$ production is in the proximal renal tubules (**Figure 1-4**), the CYP27B1 enzyme is found in many extra-renal sites including parathyroid gland, immune cells, epithelia cells and in bone [40].

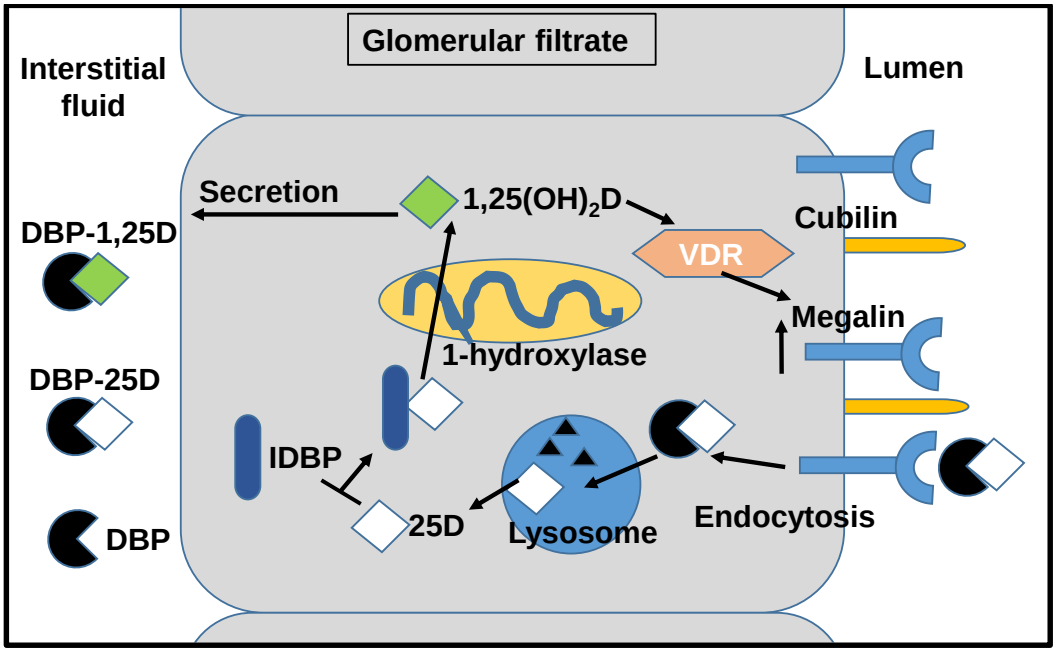


Figure 1-4: Mechanism of renal uptake of bound 25(OH)D. Circulatory 25(OH)D (25D) bound to DBP is filtered by the kidney and internalized via megalin-mediated endocytosis into the proximal tubular cells. Upon release from DBP, 25(OH)D either re-enters the circulation or is delivered by intracellular vitamin D-binding proteins (IDBP) for 1-hydroxylation by mitochondrial CYP27B1 enzyme into $1,25(OH)_2D$. $1,25(OH)_2D$ induces renal megalin expression via VDR, generating a cycle of normal systemic $1,25(OH)_2D$ and 25(OH)D. Picture re-drawn from Nykjaer *et al* [39].

1.6. Vitamin D catabolism

Both $25(OH)D_3$ and $1,25(OH)_2D_3$ are inactivated by 24-hydroxylation by 24-hydroxylase enzyme (CYP24A1) and are converted into $24,25(OH)_2D_3$ and

$1,24,25(OH)_3D_3$ [41, 42]. Vitamin D_2 undergoes similar catabolic processing, giving rise to $24,25(OH)_2D_2$ and $1,24,25(OH)_3D_2$ metabolites, suggesting that the mitochondrial enzymes responsible for these conversions do not discriminate between vitamin D_2 or D_3 side chains [36, 43-45] as shown on **Figure 1-5**.

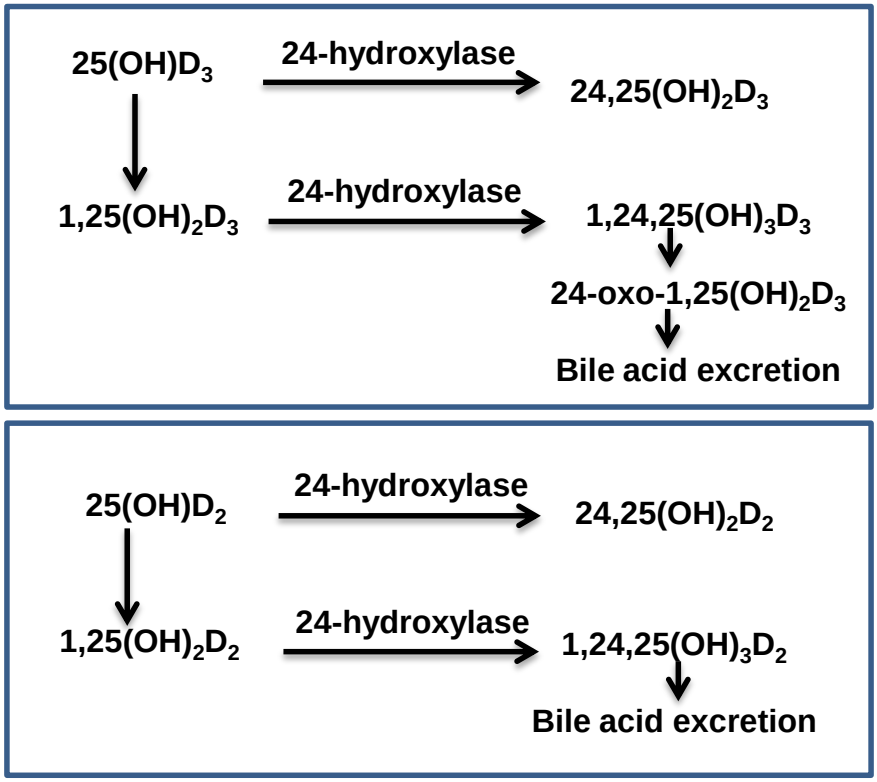


Figure 1-5: Catabolism of vitamin D_3 and D_2 metabolites by 24-hydroxylase enzyme. $25(OH)D_2$ or $25(OH)D_3$ and their active metabolites $1,25(OH)_2D_3$ or D_3 are the most active metabolites of vitamin D with highest biological activities. Other catabolites of D_2 or D_3 have been shown to have minor biological activities [46].

Deactivation of $1,25(OH)_2D_3$ is a two-step process, first hydroxylation into $1,24,25(OH)_3D_3$, then $24\text{-oxo-}1,25(OH)_2D_3$, whereas, $1,25(OH)_2D_2$ deactivation involves one step hydroxylation into $1,24,25(OH)_3D_2$ to be fully deactivated [47]. The circulatory levels of 25(OH)D are known to be 1000-fold higher than $1,25(OH)_2D$, and the 24-hydroxylase has been shown to have higher affinity for

$1,25(OH)_2D$ substrate than 25(OH)D (~10fold) [48, 49]. 24-hydroxylase enzyme is expressed at high levels in the kidney and known to be mainly responsible for inactivation of excess 25(OH)D from the circulation [50, 51]. The inactivation of $1,25(OH)_2D$ by 24-hydroxylase is thought to be involved mainly in all the target cells [52]. The final products of the catabolism of vitamin D metabolites are water soluble and biologically inactive calcitroic acid which is secreted in the bile [53].

1.7. Mechanisms of vitamin D action

Vitamin D, either in the form of vitamin D_2 or D_3, is capable of stimulating intestinal calcium absorption from the diet [54, 55]. This could only be achieved by either vitamin D_2 or D_3 first being activated by the liver (25-hydroxylation) as $25(OH)D_2$ or $25(OH)D_3$ and consequently by kidneys (1α hydroxylation) to $1,25(OH)_2D_2$ or $1,25(OH)_2D_3$ active metabolites [36, 43]. Once the active metabolites are formed they are transported to the intestine or bone targets as primary calcium regulating tissues [31]. When calcium from the diet is sufficient, the active metabolite stimulates calcium absorption from the intestine and stimulates deposition by osteoblasts into the bone [31]. On the other hand if calcium is not sufficient, the active metabolite, $1,25(OH)_2D_3$, can stimulate osteoblasts/osteocytes in bone to activate osteoclasts by producing RANKL, thus inducing bone resorption to raise the circulatory calcium level [31, 56].

$1,25(OH)_2D_3$ as a potent hormone, exerts many of its biological effects by binding to its soluble nuclear receptor, VDR, with high affinity and selectivity [57]. High affinity and selectivity of VDR to bind $1,25(OH)_2D_3$ are the most important characteristic of the physical and functional properties of VDR [58]. Accordingly,

an equilibrium dissociation constant of 10^{-10} M for VDR and 1,25$(OH)_2$D is achieved [59]. Both hydroxyl groups on carbon numbers 1 and 25 in 1,25$(OH)_2$D and 25(OH)D play an important part into high affinity and specificity of VDR binding to its ligand, hence the non-hydroxylated vitamin D precursor does not bind to VDR [59]. The three dimensional structure of VDR ligand binding domain in complex with 1,25$(OH)_2D_3$ has been documented [60]. Human VDR structure has been shown to contain 13 α-helices and three β-sheets to form the core binding pocket for hydrogen binding interactions with 1,25$(OH)_2D_3$ [60].

VDR is a member of a large family of nuclear receptors that include steroid and thyroid hormones, as well as retinoid X receptors (RXR) containing DNA binding domain acting as transcription factors [61]. VDR is expressed in all human tissues and cells in the body for the purpose of classical actions of 1,25$(OH)_2D_3$ on classical target tissues; bone, gut and kidneys or non-classical target tissues; parathyroid gland and pancreas [62]. Hence,1,25$(OH)_2D_3$ has a diverse role in many tissues; by stimulating calcium and phosphate absorption in intestine for bone mineralisation, metabolism of bone calcium and phosphate, as well as differential roles in various tissues and cells such as skin keratinocytes, B and T lymphocytes, renal and intestinal epithelia, cancer cells, chondrocytes and bone cells (osteoblasts, osteocytes, osteoclasts) [63].

As a result of formation of the 1,25$(OH)_2D_3$-VDR-RXR complex (**Figure 1-6**), a cascade of molecular events take place that results in transcription of many genes in the target tissues. In this mechanism, VDR hetero-dimerises with RXR, forming a complex that binds to specific regions of DNA in vitamin D responsive genes known as vitamin D response elements (VDREs) [64, 65]. Formation of this machinery complex results in vitamin D mediated gene transcription. The

specific binding sequence consists of two GGGTCA hexameric repeats separated by 3 nucleotides. VDREs are located in the promoter regions of genes that are important in calcium and phosphate regulation or other vitamin D target tissues for vitamin D metabolism or functions [66].

VDR is homologous to steroid hormone receptors, possessing both a DNA binding domain on the C terminal as well as a ligand binding domain [67]. Many genetic mutations in the VDR gene have been discovered that result in a non-functional or truncated VDR protein, which causes vitamin D deficiency rickets type 2 [68].

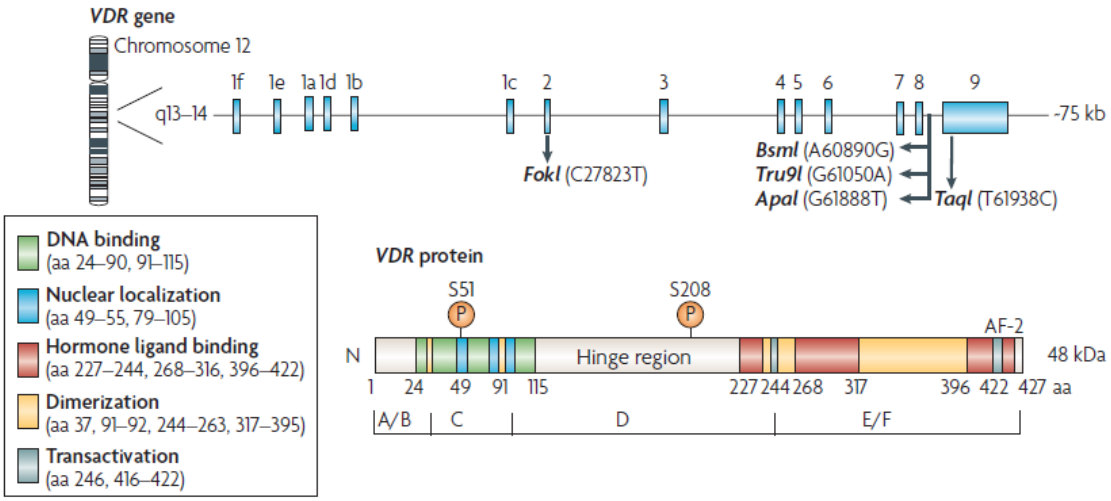


Figure 1-6: Vitamin D receptor. Human *VDR* gene, located on chromosome number 12 (top panel); composing of 1a-1f as promoter and regulatory regions, exons 2-9 encoding A-F (6 domains) of the VDR protein (below panel). Nuclear localization region in blue is responsible for entry of the receptor into the nucleus via nuclear pores. Hormone ligand binding domain in red is involved in binding to active hormone $1,25(OH)_2D$. Upon this binding, VDR is stabilised by phosphorylation (serine 51 and 208; s51, s208) in the DNA binding domain (in green). Hetero-dimerization of VDR and RXR takes place at the dimerization domain (in yellow). DNA binding domain (in green) is the site of binding of vitamin D-VDR-RXR complex into VDREs. Transcription region (in light grey) is the site of interaction of VDR and regulatory proteins for transcription activity. Image adapted from Deeb *et al* with permission [69].

1.8. Regulation of $1,25(OH)_2D$ synthesis and catabolism

PTH stimulates *CYP27B1* in the proximal tubule for $1,25(OH)_2D$ synthesis in the kidneys (**Figure 1-7, arrow 1**) [70]. FGF23, mainly produced by mature osteoblasts and osteocytes in the bone [71], is thought to suppress the production of renal $1,25(OH)_2D$ by inhibition of *CYP27B1* and stimulation of *CYP24A1* in renal proximal tubule (**Figure1-7, arrow 2**). $1,25(OH)_2D$ in turn stimulates FGF23 production by osteocytes (**Figure1-7, arrow 3**) [71]. In addition, in target tissues, $1,25(OH)_2D$ can negatively regulate its own production through VDR mediated expression of CYP24A1 catabolic enzyme [72]. $1,25(OH)_2D$ can also participate in a negative feedback loop by decreasing the PTH gene expression and PTH synthesis by parathyroid glands (**Figure1-7 arrow 4**) [70].

In order to prevent hypercalcemia and hyper-phosphatemia, the catabolism of $1,25(OH)_2D_3$ must be tightly regulated. Therefore the CYP24A1 is regulated by serum calcium and phosphate, $1,25(OH)_2D_3$ itself, as well as parathyroid hormone (PTH) [73, 74]. As long as serum calcium remains low, PTH decreases CYP24A1 levels [75]. When calcium levels normalise to adequate levels, $1,25(OH)_2D_3$ itself stimulates CYP24A1 up-regulation, so mediating its own breakdown and catabolism [76]. During hypophosphatemic conditions, low serum phosphorus suppresses CYP24A1 transcription preventing $1,25(OH)_2D_3$ catabolism [77]. Most of this CYP24A1 regulation of $1,25(OH)_2D_3$ is known to be at a transcriptional level, but the actual mechanism still remains unclear [75].

Although, the regulation and control of non-renal $1,25(OH)_2D$ is not fully understood, it is thought that its action may be mediated by CYP27B1 synthesis

and local CYP24A1 degradation. This regulation in part could be due to the availability of the substrate for $1,25(OH)_2D$ and also the absence of PTH and FGF23 regulation of CYP27B1 in extra renal tissues [78, 79].

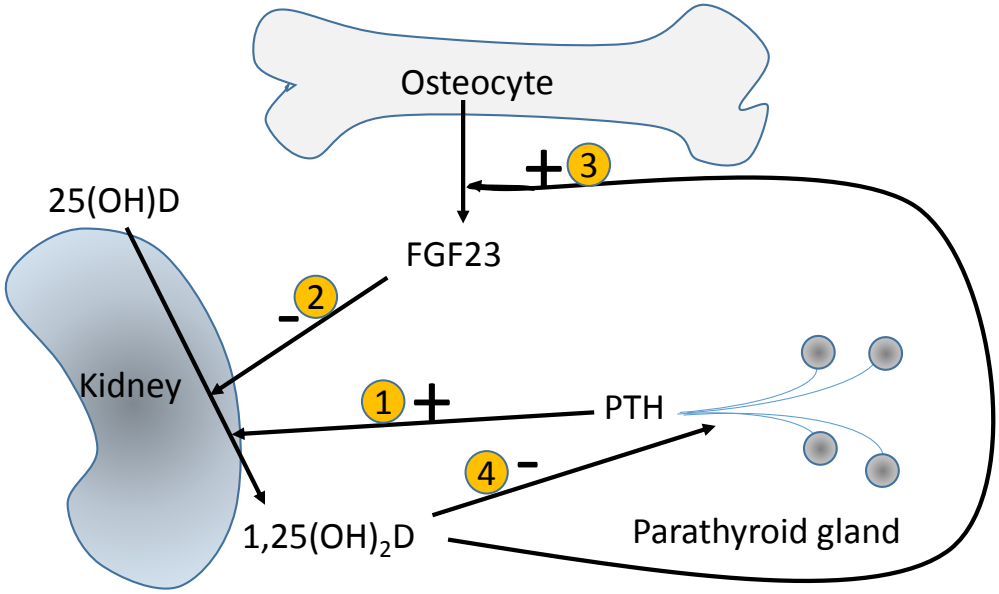


Figure 1-7: Regulation of $1,25(OH)_2D$ production. Interactions of $1,25(OH)_2D$, PTH and FGF23. PTH stimulates (arrow 1) and FGF23 (arrow 2) inhibits $1,25(OH)_2D$ production in kidney. $1,25(OH)_2D$ in turn stimulates FGF23 (arrow 3) and inhibits PTH (arrow 4). Picture re-drawn from Bikle 2009 [26].

1.9. Vitamin D actions

1.9.1. Classical actions of vitamin D

The classical action of $1,25(OH)_2D$ is involved in the regulation of phosphate and calcium across three main target tissues, bone, gut and kidney. The regulated calcium and phosphate are used in skeletal mineralisation, and are therefore important for prevention of rickets or osteomalacia [80]. However, VDR is expressed in all tissues and the role of $1,25(OH)_2D$ is not limited to the classical

target tissues. The endocrine action of $1,25(OH)_2D$ on calcium and phosphate is achieved in concert with PTH and FGF23 [81].

In this mechanism feed forward and feedback regulatory loops are operating between these three hormones. PTH is known to be the major stimulator of $1,25(OH)_2D$ production in the kidneys, and $1,25(OH)_2D$ in turn either directly suppresses transcriptional production of PTH or indirectly by raising circulatory calcium levels [81]. In the intestine $1,25(OH)_2D_3$ directly stimulates calbindin receptor expression for calcium binding and transport from the intestine into the blood circulation [82]. Calcium regulation by $1,25(OH)_2D_3$ provides additional minerals required for osteoblastic bone formation and calcium deposition into the bone [80] as well as for other functions in the body such as neuromuscular activity [83].

The parathyroid gland possess VDR and CYP27B1, hence the regulation of PTH secretion could involve endogenous metabolism of 25(OH)D into $1,25(OH)_2D$ or as well as exogenously by circulatory $1,25(OH)_2D$ [84]. Parathyroid gland also expresses the calcium sensing receptor (CaSR), whereby serum calcium can act on this receptor to suppress PTH secretion [85]. CaSR expression in the parathyroid gland is increased by $1,25(OH)_2D$ and VDR expression in the parathyroid gland is increased by calcium, enhancing a negative feedback on the effects of calcium and $1,25(OH)_2D$ on secretion of PTH [86].

The major role of FG23 is phosphate excretion by inhibition of sodium-dependent phosphate co-transporters in the renal proximal tubules. FGF23 role in regulation of phosphate involves suppression of $1,25(OH)_2D$ production in the kidneys, and $1,25(OH)_2D$ in turn increases FGF23 production in bone [71, 87]. High intake of

phosphate stimulates FGF23 production, an effect which is independent of $1,25(OH)_2D$ on FGF23 regulation of phosphate [70].

Interestingly, high dietary intake of phosphate also increase PTH secretion, an effect which is independent of phosphate inhibitory action of $1,25(OH)_2D$ production [70]. It is not known whether phosphate, similar to calcium, has its own receptor. It is important to know that when the mechanism of $1,25(OH)_2D$ *in vivo* is considered, the effects of PTH and FGF23 also should be considered along with the effects of the calcium and phosphate that they regulate [88]. Role of $1,25(OH)_2D_3$ in regulating calcium and phosphate via the three classical target tissues, bone, kidney and intestine under different conditions are summarized in **Figures 1-8** to **1-10**.

When circulatory phosphate level is normal, but calcium is low in the circulation (**Figure1-8**), CaSR in the parathyroid glands increase PTH (**Figure 1-8.1**), which acts on the kidneys to increase 1α hydroxylase (**Figure 1-8.2**) leading to release of $1,25(OH)_2D_3$ (**Figure 1-8.3**) [89]. $1,25(OH)_2D_3$ acts on the intestine (**Figure 1-8.4**) to absorb calcium. In this situation PTH acts on the kidneys to reduce phosphate absorption [90]. The net effect of this mechanism is an increase in serum calcium while phosphate remains unchanged.

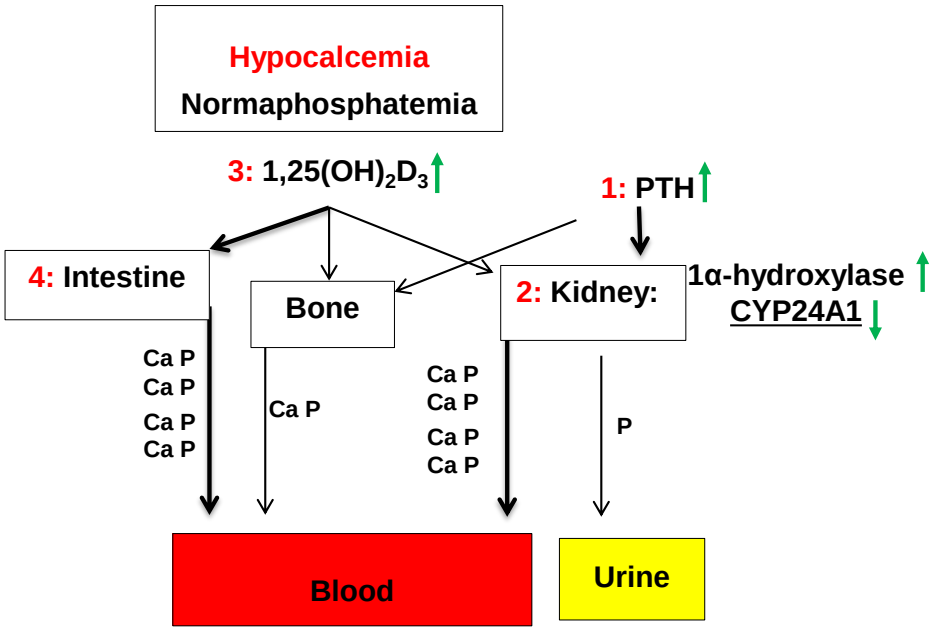


Figure 1-8: Renal function of $1,25(OH)_2D_3$ in hypocalcemia and normophosphatemia. Ca: calcium, P: phosphate

In situations where serum calcium is normal (**Figure 1.9**), but phosphate levels are low, 1α hydroxylase activity in kidneys (**Figure 1-9.1**) increases the circulatory levels of $1,25(OH)_2D_3$ (**Figure 1-9.2**), which in turn stimulates interstitial phosphate re-absorption (**Figure 1-9.3**) [91]. In addition the active metabolite $1,25(OH)_2D_3$ has been shown to increase FGF23 secretion by osteocytes [92].

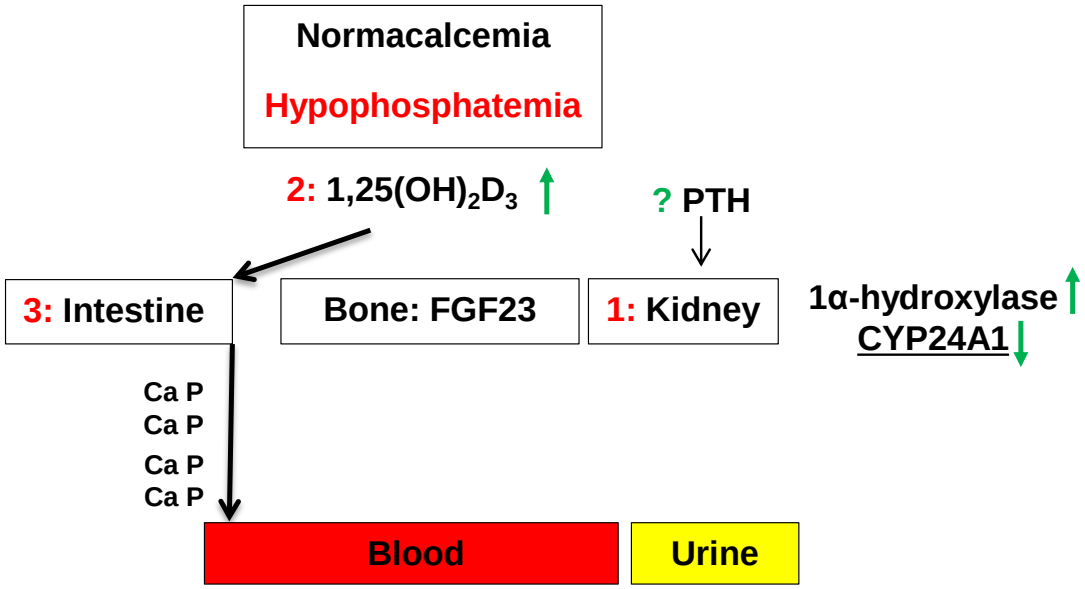


Figure 1-9: Renal function of $1,25(OH)_2D_3$ in normocalcemia and hypo phosphatemia. Ca: calcium, P: phosphate

When both circulatory levels of calcium and phosphate are low (**Figure 1-10**), 1α hydroxylase activity is super-stimulated by low phosphate as well as an increase in PTH (**Figure 1-10.1**). The net result is a high level of circulatory $1,25(OH)_2D_3$ and high PTH (**Figure 1-10.2&3**). High levels of $1,25(OH)_2D_3$ increase interstitial calcium and phosphate re-absorption. High levels of PTH in combination with high $1,25(OH)_2D_3$ lead to a net increase in intestinal calcium and phosphate re-absorption into serum and calcium and phosphate mobilization in the bone [91].

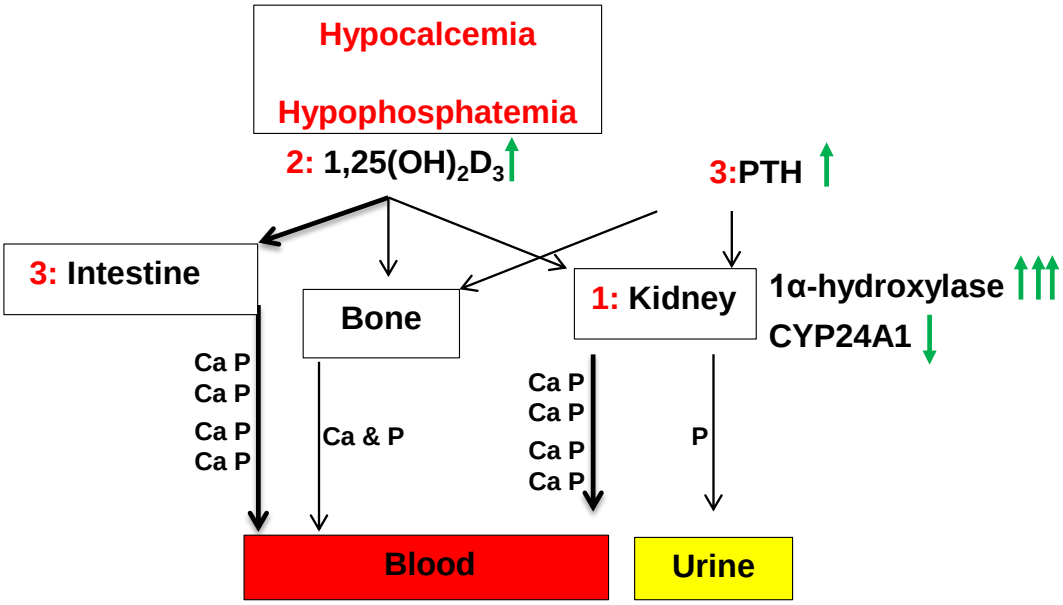


Figure 1-10: Renal function of $1,25(OH)_2D_3$ in hypocalcemia and hypophosphatemia. Ca: calcium, P: phosphate

1.9.2. Non-Classical actions of vitamin D

Although the kidneys are known to be the main site of $1,25(OH)_2D$ production [93], it is now known that other tissues express CYP27B1 for local production of this hormone [94]. It has been shown that placenta [94], bone cells [95], macrophages [96], melanocytes [97] and skin keratinocytes [98] are capable of local $1,25(OH)_2D$ synthesis. The same cells in these tissues also express VDR for the action of circulatory $1,25(OH)_2D$, however the potential physiological role, or auto/paracrine function of extra-renal expression of CYP27B1 and $1,25(OH)_2D$ production compared to kidneys is not fully understood. The metabolism of $1,25(OH)_2D$ from 25(OH)D for non-skeletal functions in multiple targets have been summarised in **Figure1-11**.

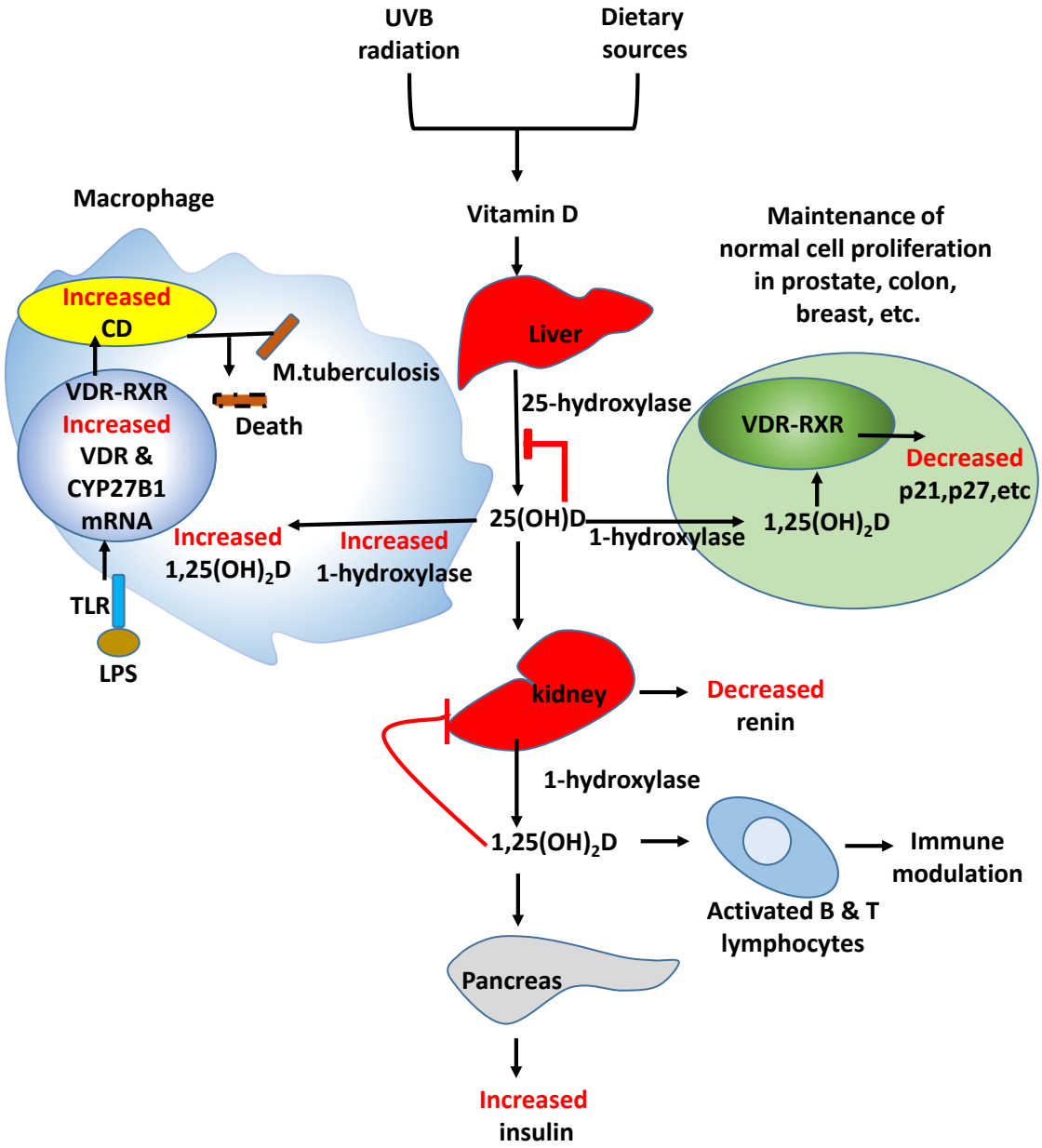


Figure 1-11: Extra-renal role of vitamin D. Synthesised $1,25(OH)_2D$ not only regulates calcium and phosphate metabolism, but also stimulates insulin production and down regulate renin production. As CYP27B1 is expressed in a variety of cells, 25(OH)D can be metabolised locally into $1,25(OH)_2D$ for autocrine action. It is believed that the autocrine metabolism of $1,25(OH)_2D$ play an important role for regulation of cellular proliferation and differentiation (decrease p21/p27), this may decrease the risk of malignancy. Macrophages also express 1-OHase and can produce $1,25(OH)_2D$. VDR and 1-OHase are up-regulated when Toll like receptors 2/1 is stimulated by lipopolysaccharides. The increased production of $1,25(OH)_2D$ by macrophages results in an increase in cathelicidine (CD) expression. CD is a peptide that destroys infectious agents such as *Mycobacterium tuberculosis* (TB). Picture re-drawn from Holick 2006 [99].

Parathyroid gland and pancreas were identified as the two first non-classical targets for the action of $1,25(OH)_2D_3$ [85, 100]. $1,25(OH)_2D_3$ has been shown to inhibit PTH synthesis and secretion as well as preventing the proliferation of PTH producing cells [101]. In addition to VDR expression, parathyroid glands also expresses CYP27B1 for local metabolism of $1,25(OH)_2D_3$ from circulatory $25(OH)D_3$. Although vitamin D has been shown to play an important role in insulin secretion in animal studies, its exact underlying mechanism remains unclear [102].

Human skin keratinocytes in epidermis also express CYP27B1 and are capable of synthesising $1,25(OH)_2D_3$. However at normal physiological levels skin metabolism of $1,25(OH)_2D_3$ does not appear to have substantial impact on the circulatory levels of $1,25(OH)_2D$. Local synthesis of $1,25(OH)_2D$ has a role in the proliferation and differentiation of the target cells, as in keratinocyte, the level of $1,25(OH)_2D$ synthesis and VDR receptor expression declines as cells differentiate [103].

Most of the epithelial cells, in tissues such as prostate [104], cervix [105], mammary gland [106] and endometrium have been shown to express CYP27B1. Expression of this enzyme has been associated with malignancy, suggesting a role for CYP27B1 in cancerous tissues such as colon [107], breast [106], cervical cancers [105] as well as basal or squamous cell carcinoma [108].

Recent studies on the immuno-biology of vitamin D have shown that vitamin D regulates both innate and adaptive immunity (80). Activated T and B cells also have been shown to express CYP27B1 and local production of $1,25(OH)_2D$ in these cells suppresses the action of the adaptive immune system by inhibiting B cell proliferation and differentiation into plasma cells [109] or inhibiting T cell

function [110]. In this case, local production of 1,25$(OH)_2$D and its inhibitory role on the adaptive immune system could favour inflammatory auto-immune diseases such as arthritis, diabetes, multiple sclerosis and Crohn's disease [111]. Local production of 1,25$(OH)_2$D from the circulatory 25(OH)D has been shown to play a role in the innate immune system in killing of mycobacteria by monocytes [112]. Hence vitamin D deficiency has been known to be associated with higher risk of mycobacterial diseases such as tuberculosis [113].

Understanding of the role of UVB-acquired vitamin D in the innate immune system to protect against viral or bacterial infections is rapidly growing [94]. We now know that vitamin D induces the production of antibacterial cathelicidine-related peptides to fight mycobacterium tuberculosis, and protect against septicaemia, pneumonia and seasonal influenza [114-117].

1.10. Vitamin D and diseases

Although vitamin D was originally recognised for its role in reducing the risk of rickets, its role has extended to other organs and has been associated with a number of disorders. Early studies in the 1960s revealed that super-physiological daily doses of vitamin D (400,000IU = 10mg) had adverse effects in a range of animals, in calcification of heart, arteries, and other organs such as kidneys [118]. Multiple sclerosis (MS) is a chronic inflammatory disease of demyelination or lesions of nerves in the cerebrum and throughout the spinal cords [119]. An increase in latitude (lack of sunshine) has been shown to increase the risk of MS in USA [120], Australia [121] and in Europe [122].

Evidence that vitamin D reduces the risk of cancer has been growing recently and several reviews have examined these data and concluded that there is strong evidence for the role of vitamin D in reducing the risk of ovarian, colorectal, breast and prostate cancer in developing countries [123]. The anti-proliferative effect of vitamin D has been shown to be by direct or indirect interference with cell cycle in tumour cells [124, 125].

One of the best understood anti-tumour effects of vitamin D is in prostate cancer. VDR is expressed by prostate epithelial cells as well as prostate cancer cells, hence prostate is a target for vitamin D action [126]. The 1α hydroxylase mRNA and protein also have been shown to be reduced in prostate cancer cells compared to primary prostate epithelial cells [104, 127]. Prostate cancer cell lines PC3, DU145 and LNCaP have been shown to express the catabolising enzyme CYP24A1 differentially, and this expression has also been shown to be up-regulated in these cells [128]. Therefore, inhibition of CYP24A1 in prostate cancer has been an interesting target for future treatment and the use of antifungal imidazole derivatives such as ketoconazole have been under investigation. Ketoconazole has been shown to be an inhibitor of cytochrome p450 enzymes and widely been used as a CYP24A1 hydroxylase inhibitor in *in vitro* studies [129]. Combination treatments with $1,25(OH)_2D_3$ and ketoconazole has been shown to enhance or increase the half-life of the anti-proliferative effect of the vitamin D in prostate cancer cell lines [130].

1.11. Rational for using ketoconazole

Because of its ability to regulate both vitamin D activating and inhibiting enzymes, it is also a useful tool for experimental manipulation of vitamin D metabolism and has been used for this purpose in my study. Ketoconazole is a synthetic derivative of imidazole antifungal medication that interferes with ergosterol biosynthesis in fungus and cholesterol synthesis in human by blocking 14α-landosterol demethylase [131, 132]. Ketoconazole as an antifungal agent has been clinically employed as early as 1980s, due to low toxicity, good patients' tolerance, low degree of inactivation and easy mode of administration [132, 133]. Initial *in vivo* and *in vitro* studies revealed that ketoconazole is a potent inhibitor of adrenal, gonadal and steroidogenesis [134, 135]. Ketoconazole is well known for its action on inhibition of CYP27B1 and CYP24A1 [136]. It has been demonstrated that administration of 900mg/d of ketoconazole resulted in 45% decrease in circulatory levels of $1,25(OH)_2D_3$ in healthy individuals as well as patients with sarcoidosis, tuberculosis and hyperparathyroidism [137-139].

The effect of ketoconazole depends on the abundance of native [25(OH)D] or active [1,25(OH)D] vitamin D metabolites. In the presence of abundant $25(OH)D_2$ or D_3 a mixed effect is obtained from dual inhibition of both CYP27B1 activating and CYP24A1 inhibiting enzymes. Based on selective activity of this inhibitor (ketoconazole), CYP27B1 would be less inhibited than CYP24A1 catabolising enzyme, resulting in less metabolism of active metabolite but equally there will be little catabolism by CYP24A1 and therefore a prolonged response to the active metabolite. However in the presence of active metabolites [$1,25(OH)D_2$ or $–D_3$] the CYP27B1 is redundant and the effect on CYP24A1 dominates, i.e.

1,25(OH)D_2 or –D_3 are prolonged and enhanced. *In vivo* the end effect will depend on the total native vitamin D status as well as the tissue specific level of active metabolites. For example the circulating levels of native vitamin D could produce opposite effects in a tissue with high CYP27B1 levels compared with a tissue low in local activation capability and display a different local response to ketoconazole. An individual dosed with high levels of active metabolite together with ketoconazole will show a systemic increased in response to active vitamin D metabolites. Because of the ability of ketoconazole to rapidly reduce adrenal production and block 24-hydroxylase activity potentiating the activity of high levels of 1,25$(OH)_2D_3$, it has been introduced for treatment of prostate cancer as well as *in vitro* studies on prostate cancer cell lines [140].

Selective inhibition of cytochromes P-450s , *CYP27B1* and *CYP24A1* by ketoconazole has made it a good candidate to study the vitamin D mechanism of activation and catabolism in *in vitro* studies [141] [142]. In *in vitro* studies, inhibition of CYP27B1 in the conversion of 25$(OH)D_3$ into 1,25$(OH)_2D_3$ and on inhibition of CYP24A1 on the catabolism of vitamin D metabolites has been shown to be by micro-molar concentrations (10-30μM) of ketoconazole on kidney cells, skin keratinocytes, bone osteoblast cell lines, bone marrow stromal cells as well as other cell lines [136, 143-147]. Although cytotoxicity with ketoconazole has been shown at concentrations of ≥30μM [148], some studies have used doses of as high as 100 μM [149].

Ketoconazole has been shown to inhibits CYP27B1 expression in human bone marrow stromal cells when treated with 25$(OH)D_3$ and block 1,25$(OH)_2D_3$ metabolism, hence reducing osteoblast markers such as ALP and OCN [150, 151]. Ketoconazole has also been used to enhance the effects of 1,25$(OH)_2D_3$ in

in vitro cultures when cells are treated with the active metabolites $1,25(OH)_2D_3$ by blocking its catabolism enzyme CYP24A1 [142, 144].

1.12. Vitamin D and musculoskeletal health

The major physiological role of vitamin D is optimisation of intestinal calcium absorption for bone and mineral homeostasis, crucial for skeletal growth during childhood [152]. Low circulatory concentrations of 25(OH)D are known to be associated with higher serum PTH (secondary hyperparathyroidism), reduced intestinal calcium absorption, resulting in accelerated bone loss, a decrease in BMD, increase risk of osteoporosis as well as fracture risks [153, 154]. Low circulatory 25(OH)D has been shown to increase the risk of sarcopenia in the elderly, whereas, higher serum levels of vitamin D could influence muscle strength, balance, and reduce the risk of fall and fracture [155-159]. Calcium, vitamin D or bisphosphonates are currently administered for prevention or treatment of osteoporosis [160]. Pre-clinical and clinical efficacy of combination therapy of bisphosphonate with vitamin D_3 in post-menopausal osteoporosis suggests significant clinical improvement [161, 162].

Low dietary vitamin D intake, lack of vitamin D activation or deficiency in its synthesis will lead impaired calcium absorption from the intestine. Impaired calcium absorption induces neuro-muscular symptoms such as seizures or tetany [163]. Long term vitamin D deficiency or resistance to vitamin D results in low calcium and phosphate levels and impaired bone mineralisation in the growing skeleton and growth plate as seen in rickets. The major consequence of vitamin D deficiency on skeletal health during childhood is severe growth

retardation [164]. The absence of calcium in bone results in bone deformities characterized by widening of long bone and bowed legs caused by growth plate abnormalities [164].

1.13. Bone and bone cells

Bone is a metabolically active and highly specialized form of connective tissue responsible for support in vertebrates [165]. It is a dynamic living tissue undergoing constant extracellular matrix mineralisation providing strength and rigidity to the skeleton [165]. Besides being mechanically supportive, it is a major source of inorganic ions responsible for calcium and phosphate homeostasis in the body [166]. The organic matrix of the bone whereby calcium and phosphate in the form of hydroxyapatite is deposited is composed of mainly collagen type 1 [167]. The remaining components of the organic matrix of the bone are proteoglycans and non-collagenous proteins such as OCN, osteonectin (ONC), bone sialoprotein (BSP) and OPN [168]. Bone is a sophisticated organ containing bone marrow, nerves and blood vessels and to fulfil its role, it is continuously shaped, remodelled and repaired by three bone cell types known as osteoblasts, osteoclasts and osteocytes [169]. Bone remodelling is a continuous process in response to on-going micro-structural damage, hormonal triggers and mechanical loading that requires a coordinated action of resorption by osteoclasts and repair/formation by osteoblasts [169].

1.13.1. Osteoblasts

Osteoblast cells are the bone forming cells which originate from bone marrow stromal cells (BMSCs) [170]. BMSCs are pluripotent cells that are located in the bone marrow, muscle and fat and can differentiate into a variety of tissues, including muscle, fat, cartilage and bone [171]. Osteoblast gene expression during proliferation and differentiation and the molecular mechanism that control these have been well studied and extensively documented in human, mouse, rat and other species [172-174]. During differentiation, osteoblasts express distinct markers such as collagen type I (Col I) and alkaline phosphatase (ALP), followed by bone sialoprotein-1 (BSP-1), osteopontin (OPN), osteonecin (ONC) and finally osteocalcin (OCN) [175]. ALP is used as early differentiation marker and OCN is considered as late marker for osteoblast differentiation [175].

Osteoblast differentiation involves several stages including osteoblast proliferation, differentiation, mineralisation and extracellular matrix formation [176]. During this process osteoblast final fate can be either forming bone lining cells, being embedded in matrix as osteocytes, or undergoing apoptosis (**Figure 1-12**) [175].

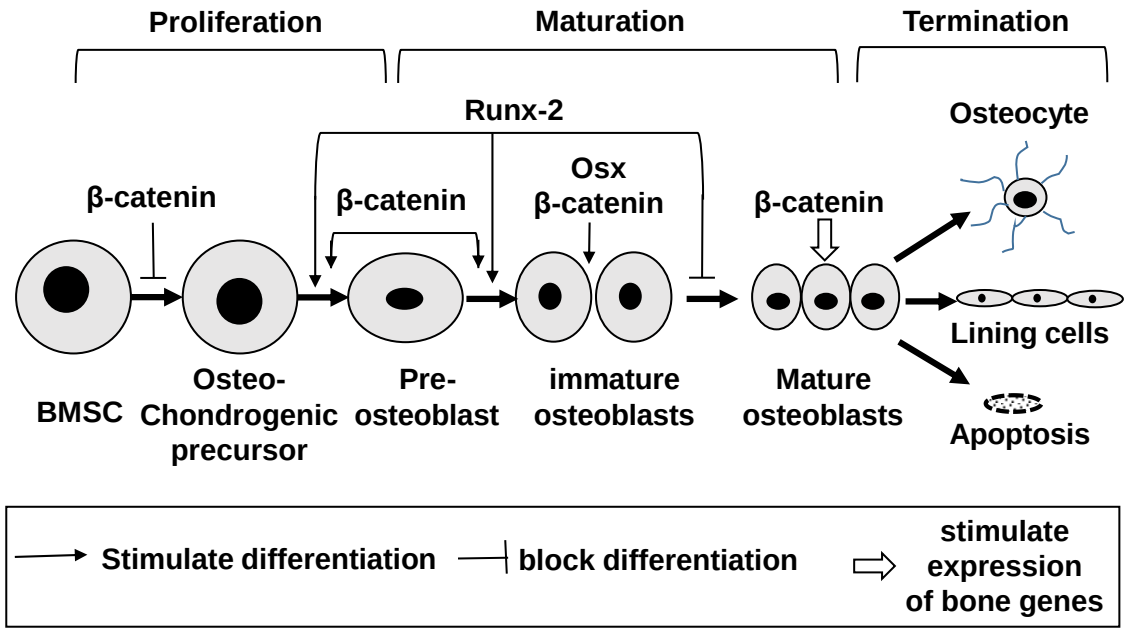


Figure 1-12: Schematic model of BMSC differentiation into osteoblast and the impact of β-catenin, Osterix (Osx) and Runx-2 transcriptional regulators. Picture re-drawn from Krause et al 2009 [175].

BMSC differentiation towards the osteoblast lineage is regulated by mediators such as: bone morphogenetic proteins (BMPs), Indian Hedgehog (IHH), transforming growth factor beta (TGF-β), wnt/βcatenin, PTH and PTH-related peptides (PTH-rP) [175]. Activation of the key transcription factor; Runt-related transcription factor 2 (Runx-2, also known as core-binding factor subunit alpha-1:Cbfa1) and Osterix are the key events and the point of convergence of signal transduction towards osteoblast differentiation and bone formation [177, 178]. Runx-2 is known to be the master switch for osteoblast differentiation as mice with deletion of Runx-2 completely lack osteoblasts and mineralised matrix [179]. It has been shown that Runx-2 can either positively or negatively regulate osteoblastic signature genes such as Col1, ALP, OPN and OCN [177]. Bone morphogenic proteins (BMPs) are expressed by skeletal tissue and are important during skeletal development and bone formation as well as fracture healing [180].

BMP-2 and TGF-β signalling in osteoblasts involves heteromeric complex formation of type I and II transmembrane kinase receptors and Smad as intracellular effector proteins [181]. Phosphorylated Smads can act as transcription factors and stimulate ALP and bone formation [181].

Wnts are secreted glycoproteins that induce their effects via frizzled receptor and co receptors low-density lipoprotein receptor related protein (LRP-5/6) to β-catenin [182]. In the absence of Wnt, β-catenin is targeted for proteasomal degradation, whereas in the presence of Wnt, β-catenin can stimulate ALP and bone formation [182]. Wnt signalling has been shown to drive osteo-chondral precursor cell differentiation into osteoblasts (**Figure 1-12**). Ectopic Wnt signalling stimulates osteoblast differentiation, whereas conditional deletion of β-catenin results in osteo-chondral precursor cell differentiation into chondrocytes [183]. Loss or gain of function mutation in LRP-5 receptor results in decrease or increase osteoblast activity associated with diseases by low or high bone mass respectively [184].

Hedgehog proteins have been shown to be critically important for developments in mammals, as mice deficient in Indian Hedgehog lack osteoblasts in bone [185].

PTH and its related peptide PTHrP can have catabolic as well as anabolic effects on bone as continuous treatment with PTH can result in bone loss, whereas intermittent PTH administration results in bone formation [186]. Transgenic mice lacking PTHrP die perinatally, due to respiratory failure and abnormal bone development [187]. Mice lacking receptor for PTH and PTHrP demonstrate premature chondrocyte maturation [188].

Osteoblasts play a critical role in bone formation as well as regulating osteoclast bone resorption. Osteoblast cells synthesize collagen type I as the most

abundant protein in the bone as well as non-collagenous ECM proteins during osteoid formation and bone mineralisation. Mineral deposition requires a continuous supply of calcium (Ca^{2+}) and phosphate (Pi) in the extracellular fluid at the site of mineralisation, pH of extracellular fluid (pH 7.4), as well as proteins synthesized by osteoblasts [189, 190]. ALP also known as tissue non-specific ALP, is concentrated in the bone and is important for bone mineralisation [189, 191]. ALP hydrolyses phosphate containing substrates such as adenosine triphosphate (ATP) and pyrophosphate (PPi) for bone mineral deposition [191]. PPi in excess is known to block bone mineralisation as it blocks Pi and Ca^{2+} crystal formation [192]. Hydrolysis of pyrimidine nucleoside triphosphates such as ATP produces PPi, is a reaction that is catalysed by ectonucleotide pyrophosphatase enzyme family (ENPP) [193]. Osteoblasts are known to express some family members of these enzymes such as ENPP1 [193, 194]. During bone mineralisation, ALP and ENPP1 play an important role on regulation of PPi and Pi levels [195].

1.13.2. Osteoclasts

Osteoclasts (OC) are the bone resorptive cells originating from hematopoietic lineage [196]. The process of osteoclastogenesis involves pre-osteoclast proliferation, fusion, differentiation and finally maturation into active osteoclasts [197]. These steps are regulated by a number of mediators such as; M-CSF (macrophage colony-stimulatory factor), RANKL (receptor activated of nuclear factor kappa-B ligand), NFATc1 (nuclear factor of activated T cell cytoplasmic) and OPG [198-200]. OCs anchor to the bone surface through alpha-v beta-3

($\alpha_v\beta_3$) integrin, produce hydrochloric acid and proteases such cathepsin-K (CATK) and matrix metalloproteases (MMPs) such as MMP-9 and MMP-13 for bone resorption, a process involved in physiological bone remodelling as well as in pathological conditions such as osteoporosis [201] .

Osteoclasts, being the exclusive bone resorptive cells, are a member of macrophage or monocyte family that can be generated *in vitro* from mononuclear cell precursors residing in various tissues [198, 200]. Pre-osteoclasts for *in vitro* studies can be obtained from spleen cells, bone marrow macrophages as well as peripheral blood mononuclear cells (PBMC) [198]. M-CSF and RANKL, either as membrane-bound or as soluble form produced by osteoblasts as well as other cell types, are the major driver of osteoclast recruitment [198]. RANKL, as a member of the tumor necrosis factor superfamily is the key mediator for osteoclast formation, and M-SCF is known to be involved in osteoclast precursor proliferation, differentiation and survival towards an efficient mature bone resorbing osteoclast [198].

1.13.3. Osteocytes

As osteoblasts become embedded in their mineralised matrix they differentiate into osteocytes. Osteocytes account for 90% of the permanent bone cells in the skeleton and express specific proteins such as podoplanin (E11/gp38), DMP1, sclerostin, matrix extracellular phosphoglycoprotein (MEPE), connexin 43 (CX-43) and PHEX (phosphate-regulating neutral endopeptidase on chromosome X) [202]. The role of many of these proteins is still unknown. Sclerostin protein is encoded by the *SOST* gene and is an antagonist of Wingless signalling pathway

(Wnt) [203]. The loss of function and mutation of the *SOST* gene is associated with sclerosteosis and Van Buchem disease with progressive bone growth and high bone mineral density [204, 205].

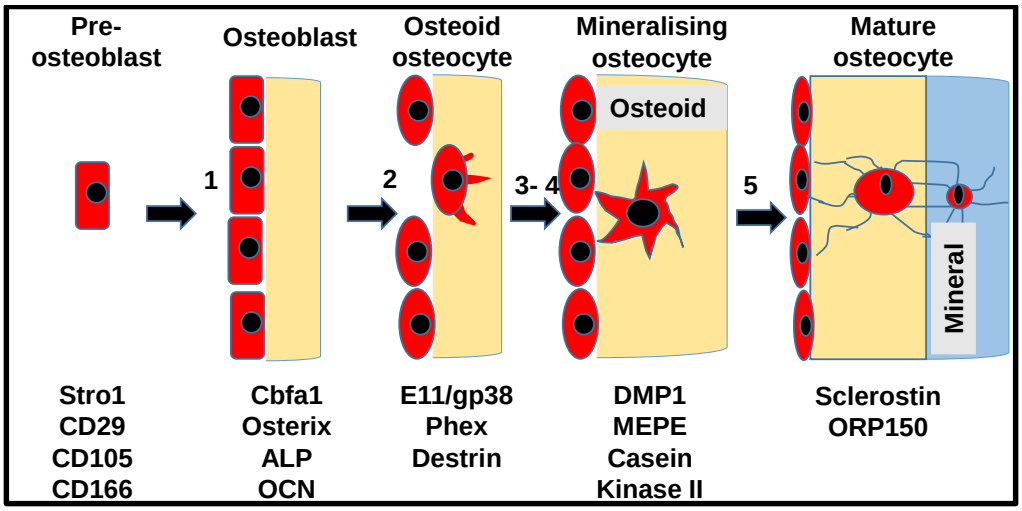


Figure 1-13: Process of osteoblast to osteocyte differentiation and expression of related markers. Pre-osteoblast originated from MSCs expressing surface markers such as: Stro1, CD29, CD105, and CD166. (1) Osteoblasts producing matrix expressing markers: Cbf1, osterix, ALP, OCN. (2) Osteoid embedded osteocyte expressing E11/gp38 (podoplanin) as early osteocyte markers that known to be involved in dendrite formation, whereas Destrin is involved in cytoskeleton regulation. (3-4) Mineralising osteocyte expressing PHEX, DMP1 and MEPE that are known to regulate mineral metabolism [206]. (5) Mature osteocyte expressing sclerostin (known as negative regulator of bone formation). Oxygen-Regulated Protein 150 (ORP150) is thought to be involved in cell viability in hypoxic environment [207]. Picture re-drawn from Lynda Bonewald 2011 [208]

While becoming embedded in osteoid (**Figure 1-13)**, osteoblasts undergo transitional changes accompanied with a reduction in expression of osteoblastic markers such as ALP and acquiring new osteocytic markers (**Figure1-13**) [209]. This transitional stage also involves morphological changes from a cuboidal shape into stellate cells with a much reduced cell body and elongated processes forming an interconnecting network between osteocytes themselves as well as with the bone lining osteoblasts and osteoclasts for molecular signalling [208].

E11, also known as podoplanin or gp38, as an early marker of osteocytes, is known to be involved in regulation of osteocyte branching morphology during the transitional phase of osteoblast embedding into its matrix [210]. It has been widely accepted that mechanical loading has a role in bone mass and unloading (space flight or bed rest) is associated with bone loss [211]. Mechanical stimuli in the form of fluid-flow shear stress has been shown to regulate osteocyte cell viability [212], activity [213] and cellular communication [214] via wnt/β-catenin pathway [215]. It has been postulated that micro-cracks in the extensive canalicular system are another important mechanism for osteocytes to sense and send messages to the neighbouring cells to recruit osteoclasts and initiate bone remodelling [216].

Our knowledge of osteocyte biology has improved with identification of osteocyte specific markers such as PHEX, DMP1, MEPE, FGF23 and sclerostin [207]. Establishment of osteocytic cell lines such as murine MLOY-4 or MLOA-5 expressing some of these proteins has led to a direct molecular or cellular analysis of osteocyte cell biology. The role of osteocytes in mineral metabolism (phosphate homeostasis) has been identified from discovery of a mutation in the DMP1 gene in autosomal recessive hypophosphatemic rickets, with elevated FGF23 [217].

Both healthy and dying osteocytes have been shown to express RANKL and recruit osteoclasts for bone remodelling [218]. Conditioned media from the established osteocyte cell line, MLOY-4, has been shown to promote osteoclastogenesis, supporting the key role of osteocytes in bone homeostasis [213, 219].

1.14. Vitamin D and bone cells

1.14.1. Vitamin D and osteoblasts

In addition to the classical action of $1,25(OH)_2D_3$ on calcium absorption for bone biology, $1,25(OH)_2D_3$ inhibits proliferation and promotes differentiation of many cultured cells via crosstalk with other signal transduction pathways [220]. Recent studies have revealed that $1,25(OH)_2D_3$ or VDR actions in certain tissues is related to its mechanism of interaction with the Wnt/β catenin pathway [221, 222]. For example, in colon cancer the Wnt pathway is known to be the main driving force on proliferation of cancer cells, and $1,25(OH)_2D_3$ has been shown to inhibit the Wnt pathway in colon cancer [223]. VDR has been shown to bind to β catenin reducing its downstream signalling [224]. Furthermore $1,25(OH)_2D_3$ has been shown to induce β catenin nuclear export into the plasma membrane [225]. $1,25(OH)_2D_3$ also has been shown to regulate the level of Wnt inhibitor DKK1 by increasing its levels both at RNA and protein levels in colon cancer cells [226].

Although $1,25(OH)_2D_3$ has been shown to inhibit Wnt signalling in cancer, Wnt or β catenin pathway up-regulation has been described in other cell types such as keratinocytes and osteoblasts [223]. The interplay between $1,25(OH)_2D_3$ or VDR and Wnt/β catenin pathway has been shown to be cell or tissue type specific [222]. $1,25(OH)_2D_3$ has been shown to enhance *SOST* gene (Wnt inhibitor) in human embedded osteoblasts (pre-osteocyte) [227], whereas it suppressed SOST gene in keratinocytes [228]. Similarly, DKK1 expression is induced by $1,25(OH)_2D_3$ in colon cancer, whereas $1,25(OH)_2D_3$ inhibits DKK1 in mouse BMSCs [229, 230].

Wnt signalling has been shown to promote BMSCs differentiation into bone cells while suppressing their differentiation into adipocytes [231, 232]. $1,25(OH)_2D_3$ has been shown to induce the expression of Wnt co-receptor Lrp5 in both mouse primary and osteoblastic cell lines as well as inhibiting DKK1 expression in mouse BMSCs [230, 233]. These effects support the stimulatory role of $1,25(OH)_2D_3$ on Wnt signalling in bone. On the other hand the interplay between these two pathways (vitamin D and Wnt) changes in human osteosarcoma SaOS-2 osteoblast-like cells as $1,25(OH)_2D_3$ inhibits Wnt/β catenin signalling in this cell line [234]. $1,25(OH)_2D_3$ has been shown to have a direct effects on the regulation of genes that mediate signalling pathways such as the Wnt pathway for induction of bone formation. $1,25(OH)_2D_3$ has been shown to antagonise Wnt signalling in MSC differentiation into adipogenic lineage, favouring osteoblast differentiation [235].

The role of vitamin D in bone health is principally the regulation of gene expression during bone formation. In general, vitamin D has been shown to directly or indirectly affect the expression of nearly 2800 genes [236]. This is achieved by vitamin D-VDR-RXR interaction with the vitamin D response elements of the regulatory regions of target genes in the cell [237].

It has been shown that all osteoblasts express VDR and respond to paracrine activity of vitamin D [148, 238, 239]. Osteoblasts can also locally synthesise the active metabolite 1,25(OH)D as they express CYP27B1, as well as having the ability to locally catabolize vitamin D by expressing CYP24A1 enzyme [240].

It has been demonstrated that osteoblasts are capable of metabolising $25(OH)D_3$ into its active metabolite $1,25(OH)_2D_3$, inducing osteoblast signature genes; *COL1*, *OCN*, *OPN*, *OPG*, *RANKL* as well as vitamin D related genes, *VDR*,

CYP27B1 and *CYP24A1* [148]. The extra-renal metabolism and autocrine or paracrine activity of vitamin D_3 metabolites on human and mouse osteoblast function is well documented [241, 242].

It has been shown that well-characterized human primary osteoblasts and human osteosarcoma cell lines (MG63, SaOS-2) express CYP27B1 and CYP24A1. When these cells are incubated with $25(OH)D_3$, they secrete detectable levels of $1,25(OH)_2D_3$ [148, 150, 151, 243]. However, whether these effects on osteoblasts differ with D_2 remains unclear. In both human and mouse osteoblast cell lines, $1,25(OH)_2D_3$ has been shown to have an inhibitory effect on proliferation [176, 244, 245]. $1,25(OH)_2D_3$ has been documented to exert its regulatory effects on early stages of differentiation where a stimulatory effect on mineralisation of osteoblasts derived from mouse, rat and in human bone marrow and human primary osteoblasts derived from bone has been reported [246-250]. In *in vitro* studies $1,25(OH)_2D_3$ has been shown to increase differentiation of human osteoblast-like cells (MG-63), however bone marrow stromal cells from mouse and rat show more varied responses. In studies using a range of hormones and cytokines, differentiation status of the cells and culture conditions, vitamin D_3 has been shown to have both stimulatory as well as inhibitory effects on ALP, OCN as well as mineralisation in mouse and rat cell cultures [251-254]. $1,25(OH)_2D_3$ has been shown to stimulate ALP mRNA and protein in human bone marrow derived osteoblasts *in vitro* [255]. In mouse MC3T3, ALP is also stimulated in early phases of differentiation but not at later stages [250]. $1,25(OH)_2D_3$ has been shown to stimulate the synthesis of collagen type I (both Iα1 & Iα2) in human MG-63 both at mRNA and protein levels [256], [254, 257]. However, $1,25(OH)_2D_3$ has been shown to both stimulate and inhibit collagen

synthesis by mouse primary as well as MC3T3 osteoblast-like cells [250, 258]. *OPN* mRNA and protein expression in human MG-63 and mouse MC3T3 osteoblast-like cells has been shown to be stimulated by $1,25(OH)_2D_3$ [242]. OCN as the late osteoblast differentiation marker has been shown to be strongly induced in human primary osteoblasts as well as in osteoblast-cells such as MG-63 at mRNA and protein levels [242]. In mice primary osteoblasts, the effect of $1,25(OH)_2D_3$ on OCN has been shown to be inhibitory [259, 260]. There is evidence that $1,25(OH)_2D_3$ inhibits OCN gene expression in murine osteoblasts, however $1,25(OH)_2D_3$ has also been shown to increase OCN secretion in neonatal murine calvarial osteoblasts [261].

The anti-proliferative effect of $1,25(OH)_2D_3$ is mediated by up-regulation of p21 and p27 cell cycle inhibitors, suppressing cell cycle progression [262]. The anti-apoptotic effect of $1,25(OH)_2D_3$ in human primary osteoblasts has been shown to be by a decrease in pro-apoptotic BAX protein, as well as an increase in anti-apoptotic protein Bcl [263].

Our current understanding of the role of $1,25(OH)_2D_3$ in mouse, rat and human established osteoblast cell lines as well as BMSCs, as described in this section and later in Chapters 3 and 4, is stimulation of osteoblastogenesis. Having said that, the effects of $1,25(OH)_2D_3$ on osteoblast-like cells derived from different species or bones from different sites (calvarial versus long bone or BMSCs) may vary. The molecular mechanisms underlying species differences are complex and in studies of $1,25(OH)_2D_3$ effects, species differences should be considered [251]. Differential effects of $1,25(OH)_2D_3$ on BMSCs derived from rat, mouse and human have been reported, as $1,25(OH)_2D_3$ increased BMSC number obtained from rat, but inhibited cell number in human and mouse [251]. $1,25(OH)_2D_3$ was

also reported to stimulate ALP in BMSCs from human and rat, but inhibited ALP in mouse BMSCs [251]. However the dose ranges used in these studies are not always comparable and this may explain some of the differences.

In *in vitro* studies, depending on the culture conditions used or on the cell system from different species, i.e. primary cultures or organ cultures, sequential osteoblast marker expression has been shown to vary and is sometimes contradictory [264]. For example, $1,25(OH)_2D_3$ has been shown to stimulate osteoblastic markers and mineralisation in MC3T3 pre-osteoblast cell line as well as mouse primary calvarial cells cultured in osteogenic differentiation medium [250, 265]. On the other hand a few studies have shown that $1,25(OH)_2D_3$ inhibited osteoblastogenesis in primary cells derived from rat calvaria and that $1,25(OH)_2D_3$ rather stimulated adipogenesis in those cells maintained in osteogenic differentiation medium [266, 267]. Rat primary calvarial osteoblasts cultured in osteogenic differentiation medium alone have also been shown to differentiate and mineralise [268, 269].

In the case of primary cultures of mouse and rat osteoblasts, limitations have been noted as primary cells may contain a mixture of heterogeneous osteoblast cells or other lineages such as fibroblasts, all at different stages of differentiation [264]. Nevertheless, osteoblast differentiation from various primary cell culture models when plated accordingly, can produce consistent stages of differentiation events and mineralisation [264]. The effects of $1,25(OH)_2D_3$ on human bone marrow stromal cells, primary cells derived from human bone, has been shown to be stimulatory on *ALP*, Col I, *OPN* ,OCN and mineralisation [242].

It is worth noting that the vast majority of *in vitro* studies make use of vitamin D_3 and that only one cited study reported the use of vitamin D_2 in differentiation of osteoblasts from human umbilical cord blood [270] (see **Table 4.1** in **Chapter 4**).

1.14.2. Vitamin D and osteoclasts

PBMC-derived OCs have been shown to express VDR as well as CYP27B1 capable of metabolising $25(OH)D_3$ into $1,25(OH)_2D_3$, suggesting that metabolism of vitamin D plays a role in osteoclast differentiation [271, 272]. The level of *CYP27B1* transcript expression by PBMCs has been shown to increase during RANKL induced osteoclast differentiation in PBMCs as well as RAW 264.7 murine cell line [151, 271]. $1,25(OH)_2D_3$ treatment of PBMCs has been shown to up-regulate CYP24A1 transcript [271]. Metabolism of $25(OH)D_3$ by mouse RAW 264.7 cell line has been shown to increase the number of multi-nucleated osteoclasts assessed by tartrate-resistant acid phosphatase staining (TRAP), as well as to increase osteoclast multi-nuclearity [271].

Our current understanding of the effect of $1,25(OH)_2D_3$ on osteoclast behaviour is that $1,25(OH)_2D$ promotes osteoclast formation, but inhibits the resorptive activity of mature osteoclasts derived from human PBMCs and murine cell line RAW264.7 [271, 273]. The action of vitamin D on the reduction of osteoclast activity is not very well defined, but it has been suggested that the metabolism of $25(OH)D_3$ into $1,25(OH)_2D_3$ reduces osteoclast resorptive capacity probably by modification of cellular adhesion and a reduction in the migration of resorptive osteoclasts [274]. It has been proposed that the action of $1,25(OH)_2D_3$ on bone resorption might be by an increase in the proliferation of the precursors of

osteoclasts, the macrophages [275] . It has been reported that VDR is not expressed by mature osteoclasts, rather it is expressed in osteoclast precursors [70, 276]. Stimulation of osteoclast formation by $1,25(OH)_2D_3$ has been shown to be by up-regulation of RANKL in osteoblasts and a direct cell to cell contact between osteoclast precursors and osteoblasts [277, 278]. Osteoclast activity can be blocked by OPG, a decoy receptor for RANKL, and OPG has been shown to be down regulated in osteoblasts by $1,25(OH)_2D_3$ [277].

1.14.3. Vitamin D and osteocytes

As previously mentioned the role of many proteins produced by osteocytes are not fully understood; however, the literature suggests that DMP1 is up-regulated by its responsiveness to $1,25(OH)_2D_3$ in osteocytes [279]. Osteocytes are known to be the major source of FGF23 production which suppresses renal $1,25(OH)_2D_3$ synthesis and itself can also be up-regulated by $1,25(OH)_2D_3$ stimulation as was discussed earlier (**Figure 1-7 arrows 2 &3**) [280].

Also as previously mentioned, $1,25(OH)_2D_3$ active hormone has a primary target in regulation of calcium and phosphate and lack of this action leads to bone abnormalities. This process is achieved only by the action of the hormone via its receptor VDR on intestine and bone cells such as osteoblasts and osteocytes. Recent developments in generation of tissue specific (intestine, osteoblast/osteocyte) VDR null mice has provided insights into the role of the $1,25(OH)_2D_3$ in the control of bone and its contribution to calcium and phosphate balance in the circulation [281]. From these animal models we have learnt that with a normal calcium diet, $1,25(OH)_2D_3$ primarily acts on intestinal calcium

reabsorption and indirectly on bone by ensuring that the absorbed calcium and phosphate are stored in bone [281].

When dietary calcium is insufficient, $1,25(OH)_2D_3$ acts directly on osteoblasts and osteocytes to increase RANKL-induced osteoclastic bone resorption and suppress bone mineralisation by osteoblasts to compensate for the low circulatory level of calcium [281]. Target deletion of VDR in osteocytes in mice has shown that osteocyte (and mature osteoblasts) VDR signalling is important to suppress bone mineralisation, and also in RANKL mediated bone resorption to maintain a normal calcium level in the circulation [282].

$1,25(OH)_2D_3$ has been shown to have a direct effect on FGF23 production by osteocytes, which in turn suppresses $1,25(OH)_2D_3$ production by suppressing PTH and CYP27B1 transcription in parathyroid gland as well as inhibition of renal CYP27B1 transcription [283]. Similarly $1,25(OH)_2D_3$ supplementation in *in vivo* studies has confirmed this by an increased level of circulatory FGF23, whereas VDR null mice have no detectable FGF23 [284]. It also must be noted that the local rather than renal production of $1,25(OH)_2D_3$ by osteocytes themselves may play an important role on auto/paracrine activity of osteocyte VDR signalling [285].

1.15. Conflicting Views

While both vitamin D_2 and D_3 were the major players in the eradication of rickets in children in the early 19^{th} century [99]. Shortly after the discovery of vitamin D_2 (*Viosterol*) [20] the equipotency of vitamin D_2 to vitamin D_3 was questioned. The initial comparison of vitamin D_2 and D_3 were conducted by Hess et al in early

1920s, based on their ability to cure rachitic rats [286]. They concluded that plant derived *Viosterol* and vitamin D_3 derived from cod liver oil may have different biological mechanisms in preventing rickets [287]. But when the first set of experiments were conducted to treat rickets in children, it was noted by Hess and colleagues that the same doses of vitamin D_2 applied in rats was not sufficient to cure rickets in children, and the doses of vitamin D_3 used in rats would be toxic in children [286]. It was highly emphasized at the time that the dosage and standardisation of both forms of vitamin D must be discussed and a uniform method of preparation must be adapted [286].

The other main learning curve from such studies was that high levels of vitamin D_3 supplementation can lead to hypercalcemia, whereas this incidence was less and milder with D_2 [287]. Similar experiments were conducted in horses and monkeys receiving mega doses of vitamin D_2 and D_3 metabolites. The outcomes of these studies were that all the animals on high doses of vitamin D_3 developed hypercalcemia, with extensive soft tissue mineralisation and died. In contrast, animals with the same doses of D_2 developed hypercalcemia to a lesser degree, but survived as a consequence of less soft tissue mineralisation [288, 289]. Furthermore, the doses of cod liver oil or vitamin D_3 that cure rickets may not elevate the circulatory phosphate level [286]. Since then, the preparations of vitamin D have advanced and the crystalline forms of vitamin D and their derivatives have been shown to be chemically and biologically active [290]. After the formulation of vitamin D_3 in 1950s, it was determined that vitamin D_3 is 4 times more potent in raising circulatory 25(OH)D than vitamin D_2 per unit mass, therefore vitamin D_2 supplementation in Europe was replaced with vitamin D_3 [291].

The ability of vitamin D_2 and D_3 supplementation to raise serum 25(OH)D has been studied in humans as well as other species. In rats, vitamin D_2 is more effective than D_3 [292], whilst in monkeys [293] vitamin D_3 is 2-3 fold more effective than vitamin D_2 in raising serum 25(OH). In birds, vitamin D_3 is ten-fold more potent than vitamin D_2 [292-294]. In man although both forms of vitamin D_2 and D_3 raised the circulatory 25(OH)D levels, the increase with vitamin D_3 was 70% greater than that with vitamin D_2 and 2.5 fold more vitamin D_2 was needed to raise the same 25(OH)D level as that for vitamin D_3 [295].

Armas et al showed that in subjects receiving a large dose of 50,000IU of D_2 or D_3, both forms of vitamins absorbed equally, but the D_2 levels started to drop after 3 days, whereas subjects on D_3 maintained high vitamin D levels for two weeks [296].

These changes could be explained by the affinities of vitamin D and its metabolites to DBP, the weaker the binding the shorter the half-life in the blood circulation, as well as more rapid clearance [297]. The low affinity binding of D_2 to DBP than D_3 has been shown to be as a result of presence of an extra methyl group on D_2 carbon number 24 [297].

It appears that in studies that found no differences between either forms of D_2 or D_3 in raising circulatory levels of 25(OH)D, 400-2000IU were supplemented on a daily basis [298].

Although the species differences in vitamin D_2 or D_3 treatments have been highlighted since the 1920s, the majority of studies on comparisons have been carried out on rats *in vitro*. The data from these studies indicate that differences between rat and human in vitamin D responsiveness need to be further investigated [297].

The deactivation process (catabolism) of the active metabolites $1,25(OH)_2D_2$ has also been shown to differ from its $1,25(OH)_2D_3$ counterpart. 24-hydroxylation of $1,25(OH)_2D_2$ results in an inactive $1,24,25(OH)_3D_2$, whereas, $1,24,25(OH)_3D_3$ has to undergo additional side chain oxidation to be fully de-activated [47]. In fact $1,24,25(OH)_3D_3$ has been shown to have ~40% higher VDR binding affinities than $1,25(OH)_2D_3$ and could potentially generate biological activities [299].

Despite this evidence, the major form of vitamin D supplementation to date in the United States is still vitamin D_2, although D_3 is widely used in many European countries and elsewhere [300]. Although, there is strong evidence to suggest high doses of vitamin D_3 is capable of preventing rickets and curing osteomalacia there is little evidence to suggest that vitamin D_2 can prevent hip fracture [301]. As such it is debatable to suggest whether vitamin D_2 instead of vitamin D_3 should be the main substitute for supplementation and or fortification [47, 290, 295, 296, 299, 302-304].

1.16. Aims of this thesis

This thesis aims to compare the *in vitro* biological responses of vitamin D_2 and D_3 metabolites in various mouse and human bone cells. The aim is to conduct the first *in vitro* biological comparison of vitamin D_2 and D_3 metabolites directly on bone cells across a wide range of physiological and pharmacological concentrations. It is hoped that the findings of this study would provide a better understanding of the bone cellular responses to vitamin D_2 and D_3 metabolites. This would provide a better discrimination between each metabolite specifically at bone cellular levels and add to the data that informs the clinical applications of

D_2 or D_3 supplementation in vitamin D deficient individuals or in disease specific cases.

A series of cellular and molecular techniques will be used to examine the biological responses of the cells to address the following questions:

1. Are vitamin D_2 metabolites as potent as D_3 to induce mouse or human osteoblast activity?
2. Would osteocyte like cell lines respond similarly or differently to vitamin D_2 and D_3 metabolites
3. Is VDR up-regulated or down-regulated in OA bone specimens compared to controls? Would hBMSCs isolated from OA respond similarly to D_2 and D_3 metabolites compared to healthy controls?

To address these objectives, the chapters are structured as follows:

Chapter 2 describes the general methods used in the current study as well as identification of suitable osteoblast-like cells for the comparison of D_2 and D_3 metabolites. The timeline of osteoblastic signature genes or protein expression will be optimised. An *in vitro* mineralisation assay described as a surrogate of bone formation will be optimised for assessment of vitamin D treatments.

Chapter 3 explores the responses of the candidate osteoblast-like cell to vitamin D_2 and D_3 metabolites. Changes in osteoblast specific signature genes and proteins, as well as mineralisation, will be compared in response to vitamin D_2 and D_3 metabolites.

Chapter 4 compares the biological responses of vitamin D_2 and D_3 metabolites on human primary osteoblasts derived from BMSCs. ALP activities and

mineralisation will be compared in response to addition of different concentrations of vitamin D_2 and D_3 metabolites.

Chapter 5 explores the effects of vitamin D_2 and D_3 metabolites on relevant marker transcript levels of established osteocyte like cells MLO-Y4 and MLO-A5.

Chapter 6 examines the expression of VDR in bone specimens obtained from human OA femoral head biopsies in patients undergoing total hip replacement or healthy bone specimens. In this chapter I will also examine the biological responses of vitamin D_2 and D_3 metabolites in hBMSCs obtained from OA patients versus commercially available hBMSCs obtained from healthy volunteers.

Chapter 7 summarises the data and suggests potential future investigations.

CHAPTER 2: OPTIMISATION OF OSTEOBLAST-LIKE CELLS FOR *IN VITRO* STUDIES

2. OPTIMISATION OF OSTEOBLAST-LIKE CELLS FOR *IN VITRO* STUDIES

The use of established osteoblast-like cells in various *in vitro* culture models has enabled a better understanding of the biology of these cells [305]. Osteoblastic-like cells have been developed for *in vitro* assays as a model to study various aspects of osteoblast activities such as: regulation of osteoblast proliferation and differentiation, the synthesis and secretion of matrix proteins, hormonal regulation and molecular mechanism of bone diseases for drug discovery [305]. Osteoblast-like cells are frequently used in bone research studies due to the limited availability of human primary osteoblast cells [305]. Isolation of human primary osteoblast cells from bone biopsies have been recently advanced and our knowledge of their biology has considerably improved [306, 307].

To study osteoblast cell biology, various cell culture models from different species including mouse, rat, and human have been designed for primary or established osteoblasts, immortalised or osteosarcoma malignant cell lines [305]. There are advantages and disadvantages of using these cell models for *in vitro* studies. The most obvious advantage of using immortalised mouse osteoblast-like cell lines (MC3T3, 2T3), or human osteosarcoma SaOS-2 and MG-63, are unlimited access to cells as well as reliable data reproducibility [308-311]. The greatest disadvantage of using rodent osteoblast-like cell lines is species differences that could complicate extrapolation of data to human clinical outcomes [312, 313]. Human primary cells are obviously the ideal choice as their behaviour is more reflective of humans *in vivo*, and therefore, the data are more clinically applicable [305]. However, the most noted disadvantage of human primary cells use is their

limited access, heterogeneity and long term culture time [305]. Furthermore, human primary cells are also known to have limited proliferation capacity and senesce with aging, a phenomenon known as the “Hayflick limit” [314]. This is thought to explain the observation that culturing of human primary cells from old donors shows slow proliferation rate and reduced osteoblast number but not function due to low population doubling [315].

As previous reports had revealed, established mouse osteoblast-like cells MC3T3 [316], 2T3 [309] and human SaOS-2 [317] and MG-63 [318] as well as human primary osteoblast cells [319] have been shown to be vitamin D responsive. The main focus of this chapter was to ascertain their suitability for *in vitro* studies in this thesis.

2.1. Aims and objectives

The main objectives of this Chapter are:

- To select the most suitable cell line for the vitamin D *in vitro* studies.
- Optimise the cell culture conditions for the selected cell lines.
- Ascertain the expression of specific VDR and CYP27B1 machinery for vitamin D metabolism

Initially culture media and sera will be assessed for endogenous levels of vitamin D_2 and D_3 by liquid chromatography-mass spectrometry (LC-MS). The viability of the cell or cells in culture medium or over prolonged culture in osteogenic differentiation medium will be examined by Alamar blue assay. Mineralisation as an indication of osteoblastic *in vitro* bone formation will be assessed in the

presence of osteogenic differentiation media by Alizarin red staining. By the end of this Chapter I am aiming to answer the following questions:

- Do the culture media or sera necessary for *in vitro* studies contain any endogenous 25(OH)D_2 or D_3?
- How long are the osteoblast cell lines viable in the presence of culture growth media or differentiation media?
- Which osteoblast cell line mineralizes *in vitro* in the presence of differentiation culture condition?
- Do the osteoblast cell lines express VDR and CYP27B1 machinery for the metabolism of addition of exogenous vitamin D?
- Do they express osteoblast specific genes and proteins?

2.2. MATERIAL AND METHODS

2.2.1. Chemicals and reagents

Unless otherwise noted all the chemicals and reagents were purchased from Sigma Aldrich (Poole, UK). Cell culture consumables such as petri dishes, plates and flasks were purchased from Greiner Bio-One, UK. Culture sera (heat inactivated foetal bovine serum: HI-FBS), L-glutamine, penicillin/streptomycin and reagents for Western blotting were purchased from Lonza, UK. The avidin-biotin peroxidase kit and DAB (3,3 diaminobenzidine) substrate were purchased from Vector labs, UK.

Vitamin D_2 [$25(OH)D_2$ and $1,25(OH)_2D_2$] and vitamin D_3 [$25(OH)D_3$ and $1,25(OH)_2D_3$] metabolites (Isosciences, CertiMass™ , USA) were dissolved in absolute ethanol as vehicle at 10^{-3} M concentration as a stock solution and stored in light protected-glass containers at -80^{o}C. Ketoconazole was dissolved in Dimethyl Sulfoxide (DMSO) and used at 10µM concentration. Mouse and human recombinant bone morphogenetic protein 2 (BMP2) were purchased from Peprotech (London, UK), dissolved in DMEM-10%HI-FBS (100µM/µl) and used at 100ng/ml final concentration. Autoradiography films, ECL (Enhanced chemi-luminescence) reagents were purchased from Amersham Pharmacia biotech, UK. Polyvinylidene difluoride (PVDF) membrane was purchased from Millipore Corporation, USA.

2.2.2. Osteoblast-like cell lines

Table 2-1: List of human and murine osteoblast-like cells

Cell line	Origin	Source	Stages
SaOS-2	Bone osteosarcoma	Gifted by Dr Philippa Hulley	Mature osteoblast
MG-63	Bone osteosarcoma	Gifted by Dr Philippa Hulley	Pre-osteoblast
MC3T3	Bone clonal	Gifted by Dr Philippa Hulley	Mature osteoblast
2T3	Bone clonal	Gifted by Dr Steven Harris, San Antonio , USA	Pre-osteoblast
MBA15.4	Bone Marrow stromal	Gifted by Dr Philippa Hulley	Mature osteoblast

Culture growth media for maintenance of osteoblast cells:

- Mouse 2T3, MC3T3 and MBA15.4 culture media:

  Media [Dulbecco's Modified Eagle Medium (DMEM)
- Human SaOS-2: McCoy's 5a medium modified , MG-63: Alpha minimum essential medium (αMEM)
- Both human and mouse media were supplemented with 10 % (v/v) HI-FBS, as well as 10mM glutamine, antibiotics (100 IU/mL penicillin and 10 µg/mL streptomycin)

Osteogenic differentiation medium:

Osteogenic differentiation is often conducted by culturing osteoblast-like cells or HBMSCs in the presence of ascorbic acid and β-glycerophosphate [320]. Addition of osteogenic medium to the cell monolayers at confluency has been shown to result into osteogenesis; expression of collagen, ALP, deposition of calcium and phosphate, formation of ECM and mineralisation. Expression of ALP as previously described in Chapter one (section 1.12.1) results in formation of inorganic phosphate as building blocks for hydroxyapatite formation [321]. Ascorbic acid is a cofactor for prolyl hydroxylase, an enzyme that adds hydroxyl groups onto the proline residues of procollagen units, hence allowing collagen fibre assembly [321]. Expression of ALP during *in vitro* osteoblast differentiation and mineralisation has been shown to be at its highest levels at pH 7.4 and significantly declines at pH 6.9 [190].

For osteoblast differentiation DMEM basal medium will be supplemented with 10% HI-FBS, 100 IU/mL penicillin and 10 µg/mL streptomycin, 5-10mM β-glycerophosphate disodium salt, and 50µg/ml L-ascorbic acid 2-phosphate.

2.2.3. Mycoplasma screening

All the human and murine cell lines were routinely and regularly screened for mycoplasma infection by polymerase chain reaction (PCR), to ensure the cell lines were mycoplasma free. Human and murine osteoblast-like cells were cultured in their culture growth medium in T75 flasks for 3 days. 1ml of medium of each growing cell was collected and spun down at 1500rpm for 5 minutes.

Supernatants were transferred into fresh tubes, spun down at 12,500rpm. Supernatants were discarded and the "pellets" were heated in 50µl 1xTE buffer[2] at 95°C for 3 minutes. A total volume of 10 µl of sample and master mix[3] (Wong-Lee and Lovett primers [322]) was used to perform regular PCR programme[4].

PCR products from each cell culture were checked by 0.5% agarose gel electrophoresis and ethidium bromide staining. Photographs were taken using GelDoc-It ™ TS imaging system (Upland, USA).

2.2.4. Endogenous levels of vitamin D in *in vitro* studies

In order to assess "endogenous" levels of vitamin D used, all the cell culture media and sera were assessed at the start of the study.

All the media were sent to St Bartholomew's London Hospital (Barts) and the London NHS trust and King's College London for assessment of vitamin D levels by liquid chromatography-mass spectrometry (LC-MS). LC-MS is a powerful technique which has a high sensitivity and selectivity, and is based on combined

[2] 10ml 1M Tris-HCL pH 7.5, plus 2ml 500mM EDTA (Ethylene Diamine Tetraacetic Acid)
[3] 7.5µl master mix [5.5 µl water, 1 µl Jumpstart buffer, 0.2 µl dNTP (deoxyribonucleatide triphosphate), 0.3 µl forward primers, 0.3 µl reverse primer, 0.2 µl Tag DNA polymerase] and 2.5 µl of test samples.
[4] PCR programme: 94°C for 90s (denaturation and polymerase activation): 40 cycles of denaturation at 94°C for 30s, annealing at 60°C for 30s, elongation at 72°C for 45s, final elongation 4 minutes at 72°C.

physical separation ability (HPLC) as well as mass analysis capabilities [323, 324]. Samples were analysed by 2 different centres to ensure accuracy.

2.2.5. Cell number measurements

Cell number analysis was performed for all osteoblast cell lines using Alamar blue™ assay (AbDSerotec, Oxford, UK) according to the manufacturer's instruction. Alamar blue™ dye (Resazurin) on its own is non-fluorescent, but it is effectively reduced by cytochrome-b_5 reductase to a red fluorescence colour (Resorufin) in the mitochondria of metabolically active cells and the dye is released into the cell medium [325]. The gold standard cell proliferation assay is ^{3}H-thymidine DNA incorporation during cell division [326]. However, due to cost and issues associated with radiolabeled ^{3}H handling, Alamar blue™ was chosen for the purpose of cell number and viability assays [327].

Human osteosarcoma osteoblast-like cells SaOS-2, MG63, and murine osteoblast-like cells MBA 15.4, MC3T3 and 2T3 were maintained in their culture growth medium in T75 flasks in a humidified incubator at 37°C and 5% CO_2. Osteoblasts like cells were seeded in 24 well plates at different cell densities of 0.25-10 x10^4 over time. After adherence of cells, Alamar blue™ was aseptically added in an amount equal to 10% of total volume in each well.

Plates were returned to the incubator (37°C and 5% CO_2) until the desired colour change (red, 3-6 hours) was observed. Media were collected from each well, and fluorescence was measured (absorbance 540nm, emission 590nm) in 96 well plates using a FluorStar Optima plate reader. Cells were washed carefully with Dulbecco's phosphate buffered saline (DPBS), and returned into the incubator in

fresh culture medium. This process was repeated every day, and the percentage of Alamar blue™ substrate reduction for each cell density was calculated. For all cell densities, the relative fluorescence unit of Alamar blue™ reduction was plotted against duration of the cell cultures.

2.2.6. VDR, CYP27B1, OPN and OCN protein expression

To determine the expression of osteoblast marker OPN, OCN and vitamin D related proteins VDR and CYP27B1, the amount of these proteins was assessed by Western blotting. Human osteoblast-like cells; SaOS-2 (1.24 x 10^4), MG-63 (1x10^4) and mouse MC3T3 cells (1.5x 10^4) were culture in 24 well plates in their culture growth medium until full confluency. Media at this time point (day zero) was replaced with osteogenic differentiation medium and replaced every other day up to 6 days.

2T3 cells (1X10^4) were culture in 24 well plates until full confluency, when medium was replaced with osteogenic differentiation media. Osteogenic differentiation medium was changed every other day up to 10 days. Cell lysates on days 2, 4, 6, 8 and 10 (2T3 cells) or day 6 for other cells were extracted on ice in radioimmunoprecipitation lysis buffer (RIPPA-PI). Total protein content of collected lysates was determined by BCA protein assay kit and assessed by Western blots. Briefly, proteins were fractionated by 10-15% Sodium Dodecyl Sulfate Polyacrylmaide Gel Electrophoresis (SDS-PAGE) in 1xTris-Glycine SDS

buffer[5], transferred[6] (in 1x Transfer buffer) onto PVDF membrane. Membranes ware blocked in 10% BSA-TBST[7] and protein bands detected using anti-mouse OPN, anti-mouse OCN, anti-mouse VDR and CYP27B1 primary antibodies (**Table 2-2**). Membranes were incubated with horseradish peroxidase-conjugated rabbit anti goat and goat anti-mouse (1:4000) secondary antibodies and immunoblots were detected by ECL.

Table 2-2: Lists of primary antibodies, host reactivates, source and dilutions used for Western blots

Antibody name	Host	Source	Dilutions
Anti-mouse OPN	Goat IgG	R&D Systems	1:1000
Anti-human OPN	Mouse IgG	R&D Systems	1:1000
Anti-human VDR	Mouse IgG_{2a}	SANTA CRUZ	1:1000
Anti-human/mouse CYP27B1	Mouse IgG	SANTA CRUZ	1:1000
Anti-mouse OCN	Goat IgG	SANTA CRUZ	1:1000
Anti-human OCN	Goat IgG	SANTA CRUZ	1:1000

[5] **10x Tris-Glycine SDS buffer:** 30.3g Tris-HCL, 144.4g glycine, 10g SDS, pH 8.3 in 1 litre Milli-Q water

[6] **10xtransfer buffer:** 30.3g Tris-HCL, 144.4 glycine, pH 8.3 in 1 litre Milli-Q water

[7] **Blocking buffer:** 10% bovine serum albumin (BSA) in 1xTBST (Tris Buffered Saline with Tween 20, Cell signalling Technology:10x TBST**:** 0.5M Tris, 1.5M NaCl, 0.5% Tween 20, pH 7.4)

2.2.7. Differentiation markers

2.2.7.1. ALP activity

Osteoblastic phenotype was examined by the response to BMP-2. Osteoblast-like cells typically up-regulate ALP and other phenotypic markers in response to BMP2 when cultured and assessed *in vitro*. Osteoblast-like cells SaOS-2 (1.24 x 10^4), MG-63 (1x10^4), 2T3 (0.75 x 10^4), and MC3T3 (1.5x 10^4) cells were seeded in triplicates in 24 well plates and treated with human or murine recombinant BMP-2 (100ng/ml) over 6 days. Plates were removed from incubators, media were aspirated and cells were washed with DPBS and lysed in RIPPA in 2% ready to use mammalian protease inhibitor cocktail (PI) on ice. Cell lysates were collected in labelled microfuge tubes, sonicated for 5 seconds, and centrifuged at 12000 RPM for 20 minutes. Supernatant was collected and total protein content was measured by PIERCE BCA™ protein assay kit (Fisher Scientific-UK Ltd). ALP was measure by substrate 4-methylumbelliferyl phosphate (4MUP) substrate kit (Sigma- Aldrich). Briefly, 10µl of cell lysates were incubated with 100µl of freshly made 4MUP (0.16mM in 50mM Tris pH 6) in white 96 well plates (Fisher Scientific-UK Ltd) for 45 minutes in the dark at 37^{o}C, following an addition of 100µl of 0.6M Na_2CO_3 to stop the reaction. Fluorescence was measured at 360nm excitation, 450nm emission and 435nm cut off using a BMG Optima FluorSTAR plate reader. ALP activities (rate of 4MU production) were calculated by preparing a standard curve of 0-20000 pmol 4-methylumbelliferone (4MU) on the same plate and normalised to the total protein (µg) content of each relevant well or treatment.

2.2.7.2. ALP activity in osteoblast-like cells

Human SaOS-2 (1.24 x 10^4), MG-63 (1x10^4), and mouse MC3T3 osteoblast-like cells (1.5x 10^4) were cultured in 24 well plates and treated every other day with 100ng/ml of human or mouse BMP-2 up to 6 days. Murine 2T3 cells (1x10^4) were plated into 24 well plates in replicates in their culture growth medium to reach confluence. Differentiation was then induced (day zero) by osteogenic differentiation medium. 2T3 osteoblasts were maintained in osteogenic differentiation medium for 3 days ± mouse BMP-2, with media being changed twice on day zero and day two. Cultures were stopped at 12 hour intervals for the assessment of ALP activity in 2T3 and day 6 for other cells.

2T3 cell cultures were stopped at 12 hours intervals, washed with DPBS, fixed in 10% formalin, re-washed with deionised water and incubated with 500µl ALP staining solution for 30 minutes[8]. Cells were washed with deionised water, air dried and scanned with Epson perfection V500 scanner or images captured with Zeiss AX10 microscope.

[8] 200µl Naphthol AS-MX Phosphate Alkaline solution; Sigma 85-5 into 4.8ml FAST BLUE RR SALT; Sigma FBS-25.

2.2.8. Quantitative reverse polymerase chain reaction (QRT-PCR)

2.2.8.1. RNA extraction

2T3 cells were cultured in culture growth medium in triplicates in 6-well plates at $1x10^5$ cell densities until full confluence (overnight). At this time point, the medium was replaced with osteogenic differentiation medium and total RNA was extracted at 6, 12, 24, 48 and 72 hours by RNeasy Mini Kit (QIAGEN® , USA) according to the manufacturer's instructions.

Nucleic acid concentrations were measured by NanoDrop ND-1000 spectrophotometer (Thermo Scientific) at 260nm. The absorbance ratios of 260/280 and 260/230 were used to detect any protein or DNA carryover. Samples with both ratios (260/280 and 260/230) of ≥2 with good yield of RNA were used for complementary DNA synthesis and QRT-PCR. The integrity of total RNA purified with Qiagen RNeasy kit was also checked using 1.5% agarose gel electrophoresis and ethidium bromide staining.

2.2.8.2. Complementary DNA synthesis

A total of 2µg from each extract was treated with RNase-free DNase I (Fisher Scientific-UK Ltd) for 30 min at 37°C. Removal of genomic DNA was terminated by further heat inactivation with 100mM EDTA at 65°C for 10 minutes. First strand cDNA synthesis of template RNA extracts was performed using a Veriti 96 well thermal cycler (Applied Biosystems, Life Technologies, Paisley, UK) using BIO-RAD iScript Reverse Transcription Supermix (BIO-RAD, Life Science,

Hertfordshire, UK) in a final reaction volume of 40µl according to the manufacturer's instruction.

2.2.8.3. Signature gene expression

QRT-PCR was performed with commercially available lyophilized Quanititect® Qiagen primers for osteoblast signature genes; *COL I* (Cat.no: QT00162204), *OPN* (Cat.no: QT00157724), *OCN* (Cat.no: QT00259406), *OPG* (Cat.no: QT00106757), *RANKL* (Cat.no:QT00147385).

Briefly, one µl of one twentieth dilutions of templates were used in a total volume of 10µl reaction in 384 well plates (Applied Biosystems) by 2 step cycling (Polymerase activation 95^{o}C for 2 minutes, 40 cycles of template denaturation at 95^{o}C for 5 seconds, primer annealing and extension at 65^{o}C for 30 seconds) using SYBR green Master Mix (BIOLINE; SensiFAST™ SYBR Lo-Rox Kit, London, UK). The Ct values for treated samples were normalised to housekeeping genes *GAPDH* (Cat.no: QT01658692) and *18S* (Cat.no: QT02448075) and the relative expressions were calculated by (ΔΔCt) [328].

2.2.9. Collagen production

Differentiation of 2T3 cells into matrix-forming osteoblasts was assessed and quantified by Picrosirius Red staining [329]. Sirius Red dye is an anionic dye, that reacts through its sulphonic acid with the basic groups in collagen fibres (type I to V), and does not discriminate between collagen types [329]. The technique is commonly used to stain and determine total collagen content [329]. 2T3 cells were seeded in 24 well plates in replicates at 1x10^{4} and grown until confluency

in their culture medium. Medium at this time point was replaced with osteogenic differentiation medium, and medium was replaced every other day up to 12 days. Collagen content was determined in differentiating 2T3 osteoblasts at days 4, 8 and 12 in osteogenic differentiation culture condition. To assess the extracellular matrix collagen, 2T3 cell layers were washed with DPBS, fixed in Bouin's solution[9] for one hour. Cell mono-layers were washed thoroughly with deionised water, and air dried prior to staining with Picrosirius Red dye[10] for one hour [329]. Excess dye was removed and the cell layers were extensively washed with running water followed by 10 minutes wash with 0.5% HCl-acidified water. Plates were scanned with Epson perfection V500 scanner and high resolution images were captured with Zeiss AX10 microscope or collagen fibres were examined by second-harmonic imaging microscopy. Extraction of the dye was carried out using 0.1M NaOH on an orbital shaker for 30 minutes. The digests were transferred into 96 well plates (100µl) and the absorbance was measured at 540nm.

The production of soluble collagen from the differentiating 2T3 osteoblasts was quantified in collected media from day 4, 8 and 12. Picrosirius Red dye was incubated with collected media (30% media: 70% dye: ratio) mixed gently by pipetting up and down, incubated for one hour and spun at max rpm (15,000) for

[9] **Bouin`s solution:** 15ml picric acid, 5ml 35% formaldehyde and 1ml glacial acid
[10] **Picrosirius red :** 0.5g Sirius Red dye F3BA in 500ml saturated picric acid, pH 2

30 minutes at 10^{o}C (IEC Micromax Centrifuge, Thermo Scientific). The collected pellets from each condition were washed once with PBS and 0.1M acetic acid. The pellet was dissolved in 0.1M NaOH (same volume as the Picrosirius Red dye) and the optical density was measured at 540nm. Rat tail collagen type I (GIBCO, Life Technologies, London, UK) was used to set up a standard curve and for quantification of matrix or soluble collagen for Picrosirius Red staining.

2.2.10. Mineralisation

Osteoblast cell lines can be stimulated to differentiate *in vitro* under the influence of osteoblast differentiation media, also called osteogenic differentiation medium (OSM) containing phosphate and ascorbic acid [330]. Under these conditions osteoblasts can deposit calcium and phosphate into a nodule like structure, which is widely accepted as a surrogate of osteoblast mineralising activity. The most common *in vitro* technique that is currently used to assess and evaluate calcium deposition into the mineralised ECM is Alizarin Red staining [331]. Alizarin red dye stains the deposited calcium in the matrix and produces a red colour. The dye can be extracted by dissolving the ECM monolayer in 10% acetic acid, neutralized in 10% ammonium hydroxide followed by semi-quantification by colorimetric detection at 550nm [332]. Human and murine osteoblast-like cells [SaOS-2 (1.24 x 10^4), MG-63(1x10^4), 2T3 (1 x 10^4), and MC3T3 (1.5x 10^4)] were seeded in triplicates in 24 well plates in their culture medium until full confluency (day zero). Media were replaced with osteogenic differentiation media and were replaced every other day up to 12 days. Cultures were stopped on days 4, 8 or

12 and stained with Alizarin red dye[11]. Viability of the only mineralising cell type, 2T3 cells was assessed on day 12 by live (Calcein-AM) or dead (ethidium bromide) double staining kit according to the manufacturer's instructions (Sigma 04511).

2.2.11. Statistical analysis

All experiments in this thesis were carried out at least in triplicates and data are expressed as ± SEM unless otherwise indicated in the figure legends. Analysis were done using SPSS for windows version 11.0 (SPSS Inco., Chicago, IL, USA) and graphs were plotted using Prism software version 5.1. Quantitative data for multiple comparisons were performed with non-parametric tools using ANOVA to examine the statistical significance for vitamin D_2 and D_3 dose-response treatments. When data allowed parametric tools, t test for two groups or one-way ANOVA for multiple comparisons were performed. P values of less than 0.05 were considered as significant.

[11] **Alizarin red dye:** 1.5g Alizarin red dye in 100ml Milli-Q water, pH 4.1-4.2

2.3. Results

2.3.1. Endogenous level of vitamin D

To assess endogenous levels of vitamin D metabolites in culture, media and sera samples were assessed for 25(OH)D_2 and D_3 content. Serum and media measurements by LC-MS at both Barts (commercial) and the London NHS Trust and King`s Hospital (courtesy of Dr Caje Moniz) showed no detectable levels (nmol/L) of endogenous vitamin D_3/D_2 in the media or sera (**Table 2-3**).

	LC-MS at Barts (nmol/L)		**LC-MS at Kings (nmol/L)**	
	25(OH)D_3	25(OH)D_2	25(OH)D_3	25(OH)D_2
Media	Sample 1	Sample 2	Sample 3	Sample 4
αMEM	< 5 (2)	< 5 (2)	< 5 (2)	< 5 (2)
DMEM	< 5 (2)	< 5 (2)	< 5 (2)	< 5 (2)
MEM	< 5 (2)	< 5 (2)	< 5 (2)	< 5 (2)
Serum	Sample 1	Sample 2	Sample 3	Sample 4
HI-FBS	< 5 (2)	< 5 (2)	< 5 (2)	< 5 (2)
Culture medium	< 5 (2)	< 5 (2)	< 5 (2)	< 5 (2)

Table 2-3: Basal media or sera contain no detectable 25(OH)D_2 or D_3. Endogenous vitamin D in culture media and sera samples as measured by LC-MS (nmol/L). To ensure the accuracy of measurements and sensitivity of the assays (~ 5nmol/L detection limit) samples were assayed at two centres. (n): number of assays in each centre.

2.3.2. Summary of results

Human osteosarcoma osteoblast-like cells SaOS-2 and MG-63 as well as murine MC3T3 expressed the vitamin D related proteins, VDR and CYP27B1 (**Figure 2-1**).

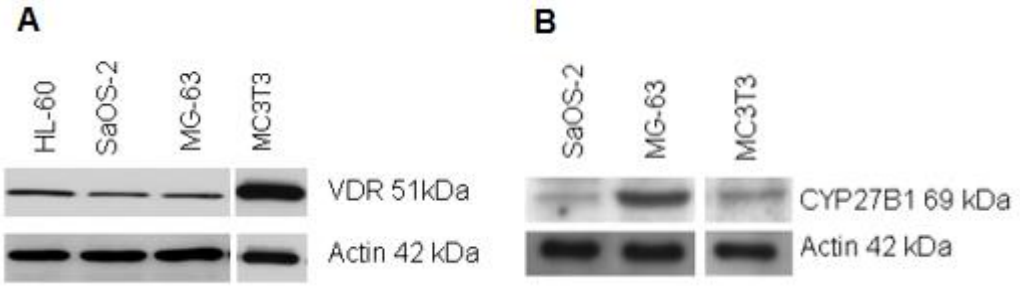


Figure 2-1: SaOS-2 and MG-63 and MC3T3 osteoblast-like cells express VDR and CYP27B1 proteins. Western blots of 20µg extracts of human SaOS-2, MG-63 and murine MC3T3 cell lysates for expression of (A) VDR and (B) CYP27B1. Cells were cultured in 24 well plates [SaOS-2 (1.24 x 10^4), MG-63 (1x10^4 and MC3T3 (1.5x 10^4)] until full confluency then in osteogenic differentiation medium over 6 days. Total protein contents were measured by BCA protein assay kit. Proteins (20µg) were fractionated by 10-15% SDS-PAGE, transferred onto PVDF membrane and detected using anti-VDR/CYP27B1 primary antibody. HL-60 cell lysate was used as positive control for VDR expression in blot (A). Note that blot A and B has been cropped and reassembled as indicated by the gap.

Both human osteoblast-like cells SaOS-2 and MG-63 as well as mouse MC3T3 expressed OPN and low levels of OCN after 6 days of culture in osteogenic differentiation medium (**Figure2-2 A&B**).

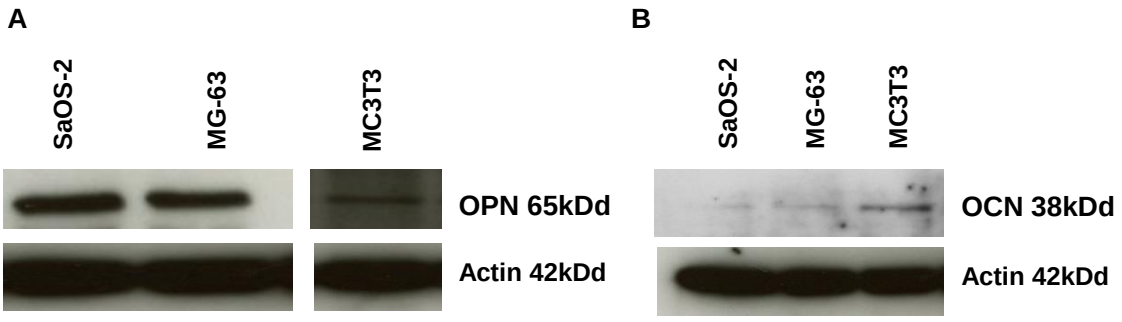


Figure 2-2: SaOS-2 and MG-63 and MC3T3 osteoblast-like cells express OPN and low levels of OCN proteins. Western blots of 20µg extracts of human SaOS-2, MG-63 and murine MC3T3 cell lysates for expression of (A) OPN and (B) OCN (50 µg). Cells were seeded in 24 well plates [SaOS-2 (1.24 x 10^4), MG-63 (1x10^4), and MC3T3 (1.5x 10^4)] until full confluency, then in osteogenic differentiation medium over 6 days. Total protein contents were measured by BCA protein assay kit. Proteins (OPN: 20µg and OCN: 50 µg) were fractionated by 10-15% SDS-PAGE, transferred onto PVDF membrane and detected using anti-OPN and anti-OCN primary antibodies.

Addition of recombinant BMP-2 significantly up-regulated ALP in SaOS-2 ($P<0.05$), MG-63 and MC3T3 ($P<0.001$) compared to untreated cells (**Figure 2-3 A-C**).

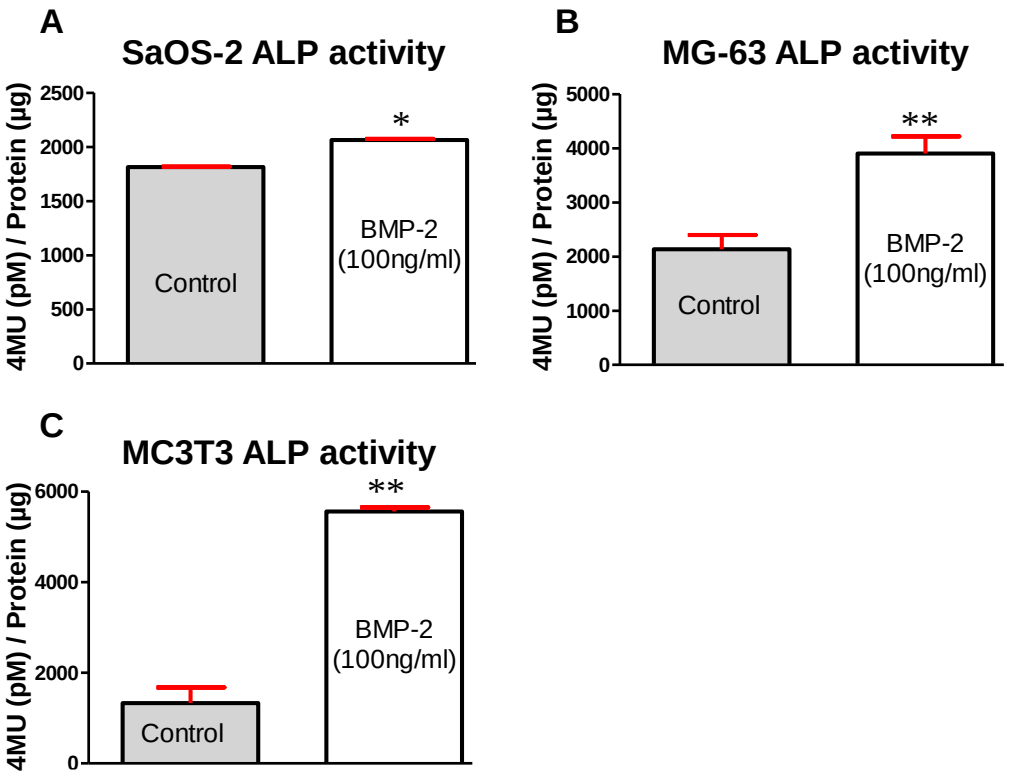


Figure 2-3: Osteoblast-like cell lines SaOS-2, MG-63 and MC3T3 up-regulate ALP in response to BMP-2. ALP response of (A) human SaOS-2, (B) MG-63 and murine (C) MC3T3 osteoblast in response to BMP-2. SaOS-2 (1.24×10^4), MG-63(1×10^4), and MC3T3 (1.5×10^4) were treated every other day with 100ng/ml of human or mouse BMP-2 for 6 days. ALP enzymatic activities were measured as described in M&M and normalised to the total protein content of the cells. (Data are presented as mean ±SEM of n=6 wells, (Student t-test), * $P<0.05$ and ** $P<0.001$ for comparisons of BMP-2 treated conditions in (white bars) to untreated controls (grey bars).

Summary of optimisation of all osteoblast-like cells are shown in **Table 2-4**.

Of all the cell lines, murine MBA-15.4 was the only cell line which repeatedly gave mycoplasma positivity (data not shown) and hence it was decided not to use this cell line for subsequent investigations.

Table 2-4: Summary of human and murine osteoblast activities. Vit: vitamin, Min: mineralisation, ND: not determined

Cell line	VDR	CYP27B1	ALP	OCN	OPN	Min	VitD ALP response
SaOS-2	√	√	√	√	√	X	≥1000nM
MG-63	√	√	√	√	√	X	≥1000nM
MC3T3	√	√	√	√	√	X	≥1000nM
MBA15.4	ND	ND	√	ND	√	ND	≤500nM
2T3	√	√	√	√	√	√	≤500nM

Since only murine 2T3 osteoblast-like cells mineralised over 12 days in the presence of osteogenic differentiation medium (DMEM supplemented with 10%HI-FBS, 10mM βGP, 50µg/ml ascorbate-2-phosphate) this cell line was selected as the preferred model with which to study other osteoblastic activities. All subsequent studies and detailed optimisations presented in the following sections are on murine 2T3 osteoblast-like cells.

2.3.3. 2T3 Cell number

Growth curves from Alamar blue assays indicated that murine 2T3 osteoblast cells maintained their proliferative activity at different cell densities until they reached full confluency in their culture growth medium containing 10% HI-FBS. Full confluency occurred for 12,500 seeding density at 30hrs, for 10,000 cells at day 2, and for 7,500 at day 4 (**Figure 2-4**). After full confluency (10,000 and 12,500 cell densities), cells continued to grow in multilayers up to 6 days, then rolled up and folded at the edges of the wells hence 2T3 proliferation assays were discontinued after day 6 in this culture medium of 10%HI-FBS. For MG-63, SaOS-2 and MC3T3 cell measurements please see **Appendices 2-4**.

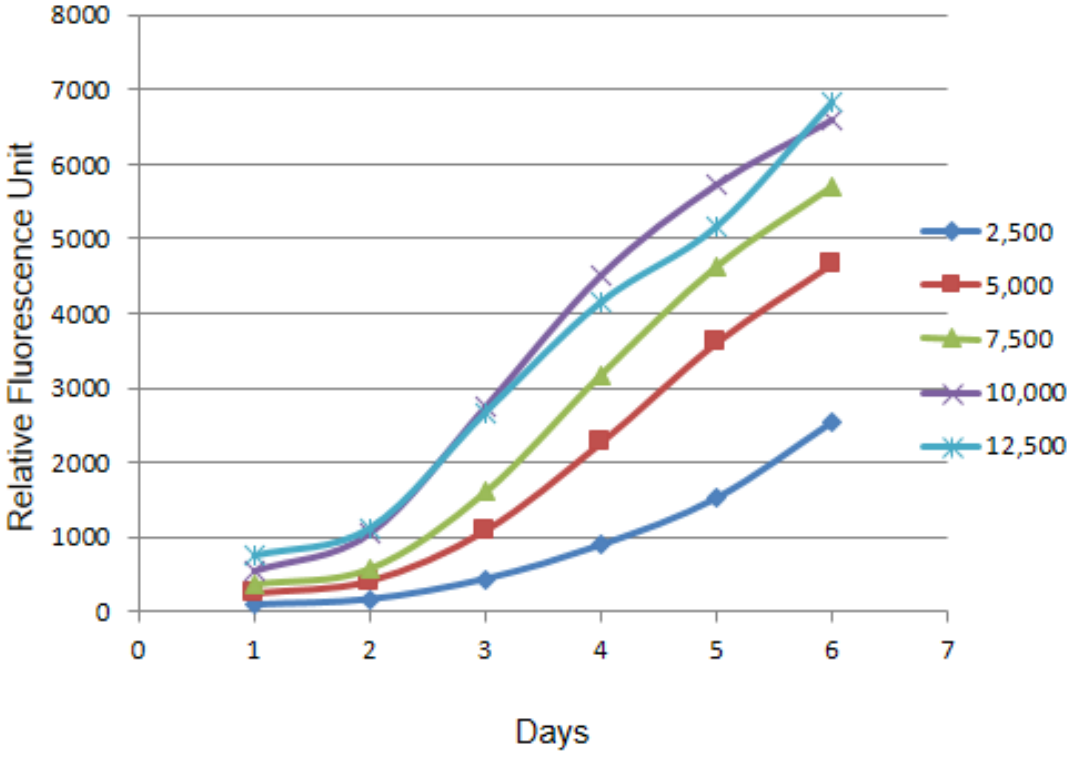


Figure 2-4: 2T3 osteoblast-like cell growth over time with different initial cell densities. 2T3 cells cultured with DMEM-10% HI-FBS and assessed by Alamar blue™ assay. Data presented as mean of relative fluorescence units (n= 6 wells) from different cells densities grown over 6 days in culture growth medium.

Growth of mouse 2T3 cells in their culture growth media supplemented with osteogenic differentiation medium during differentiation was assessed using Alamar blue assay. Cellular activity, as measured by fluorescence increased in the first 6 days of post confluency, indicative of an increase in the number of cells. The number of cells remained constant from day 6 up to day12 (**Figure 2-5A**). Live and dead staining of cells within the mineralised matrix at full confluency on day 12 showed very few dead cells as shown in **Figure 2-5E**.

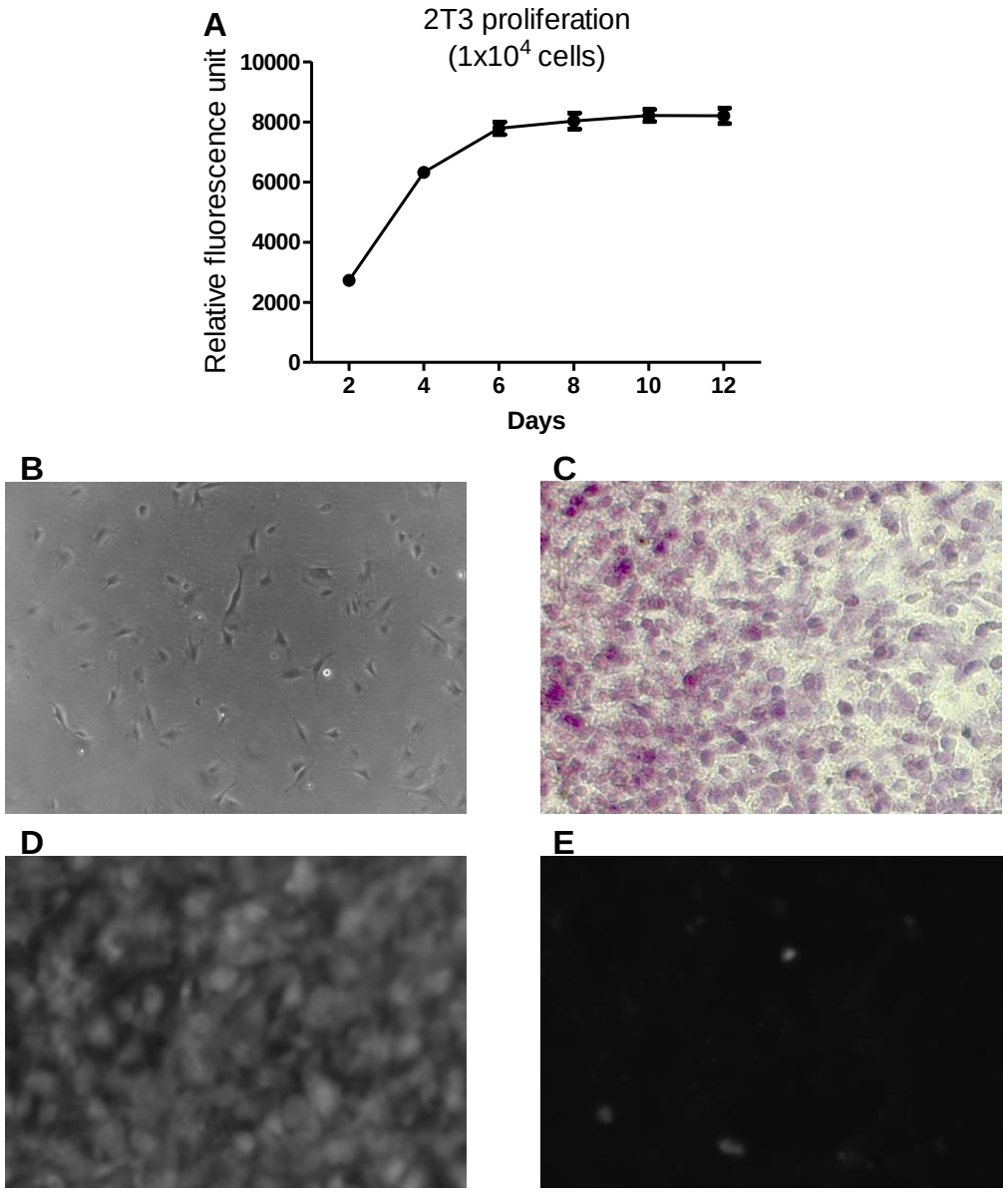


Figure 2-5: 2T3 osteoblast-like cells are viable during 12 days of differentiation. (A) Growth of 2T3 cells in the presence of osteogenic differentiation medium over 12 days of culture. $1x10^4$ cells were grown from day zero to full confluency when osteogenic differentiation medium was introduced and cell numbers were assessed fluorimetrically by Alamar blue™. Data are presented as mean ±SEM, n= 6 wells. (B) Phase contrast image of 2T3 cells on day zero, (C) cells at full confluency on day 2, (cells fixed in 10% formalin and counterstained with haematoxylin), (D) phase contrast image of Live and (E) Dead staining with calcein-AM and ethidium homodimer respectively. Images were taken by ZEISS AX10 microscope, scale bar in B-E X10 magnification.

2.3.4. ALP activity

ALP activity in 2T3 cells was accelerated in osteogenic differentiation medium after 48 hours in culture and reached a maximum at 72 hours (**Figure 2-6A)**. ALP activity was significantly up regulated by up to nearly 3-fold in response to the addition of BMP2 (**Figure 2-6C**).

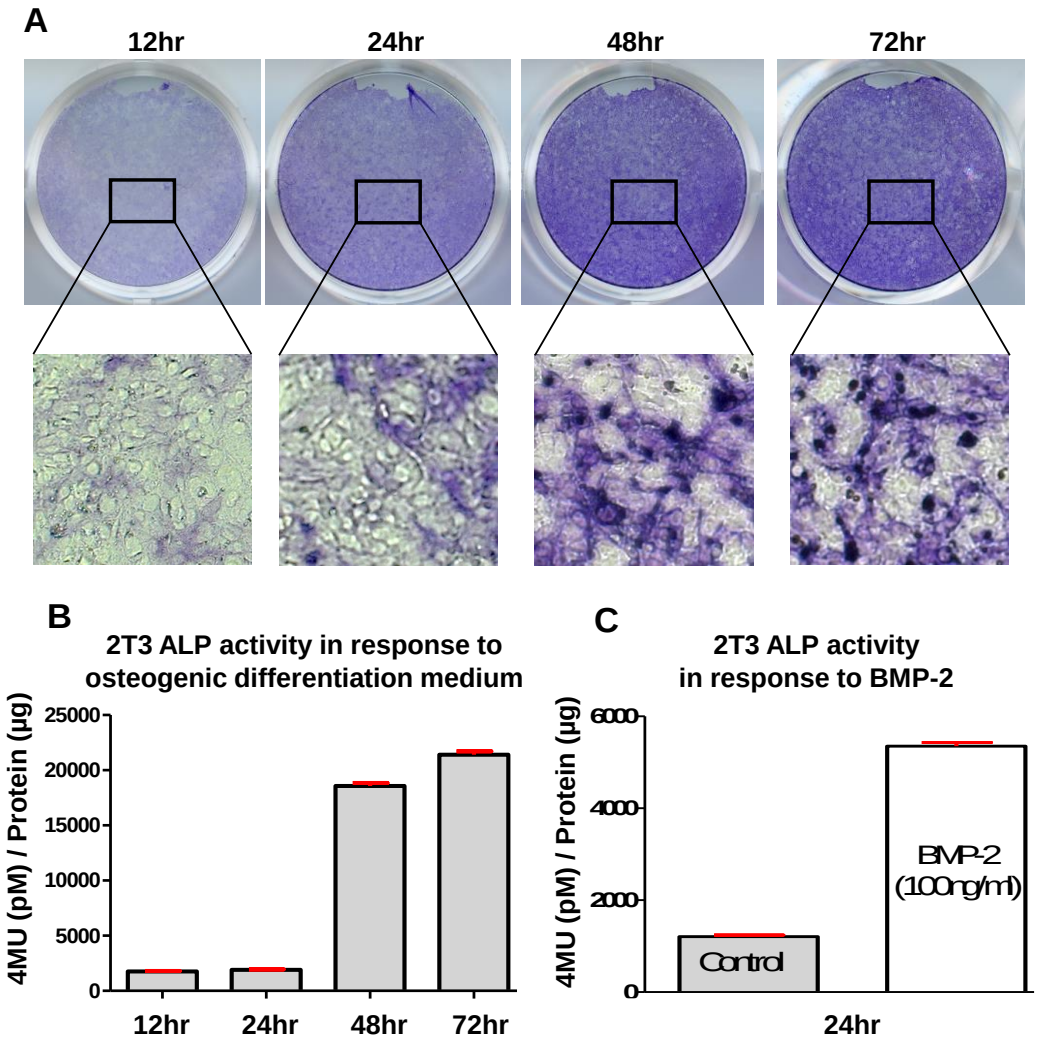


Figure 2-6: ALP is stimulated by osteogenic differentiation medium or BMP-2 in 2T3 osteoblast-like cells. (A) Representative scans and high resolution images (Scale bar 10X) of the effects of osteogenic differentiation media on ALP enzyme activity [rate of 4MU production (pM) per µg protein] in mouse 2T3 cells over 72 hours culture. (B) Enzymatic activities of ALP assessed over 3 days. (C) Effect of 100ng/ml BMP-2 on mouse 2T3 ALP activity. Values are mean ± SEM from triplicates experiments (Student t-test) as percentage of increase in ALP compared to 12 hours in (B), (C) an increase in ALP (white bar) in response to BMP-2 over 24 hours compared to untreated cells (grey bar) * P<0.001.

2.3.5. Signature gene expression

To determine the baseline expression of 2T3 osteoblastic signature genes, COL1, OCN, OPN as well as RANKL and OPG marker QRT-PCR were performed (**Figure 2-7**). When 2T3 cells were maintained in osteogenic differentiation medium over 72 hours, there was up-regulation of early osteoblastic genes COL1 (9-fold) and OPN (2-fold) as well as OPG (4-fold) and RANKL (20-fold) genes after 24 hours compared to baseline (6 hours) (**Figure 2-7**A-D). The late osteoblast signature gene OCN was up-regulated by osteogenic differentiation medium after 72 hours culture by 4-fold compared to baseline (6 hours cultures) (**Figure 2-7**E).

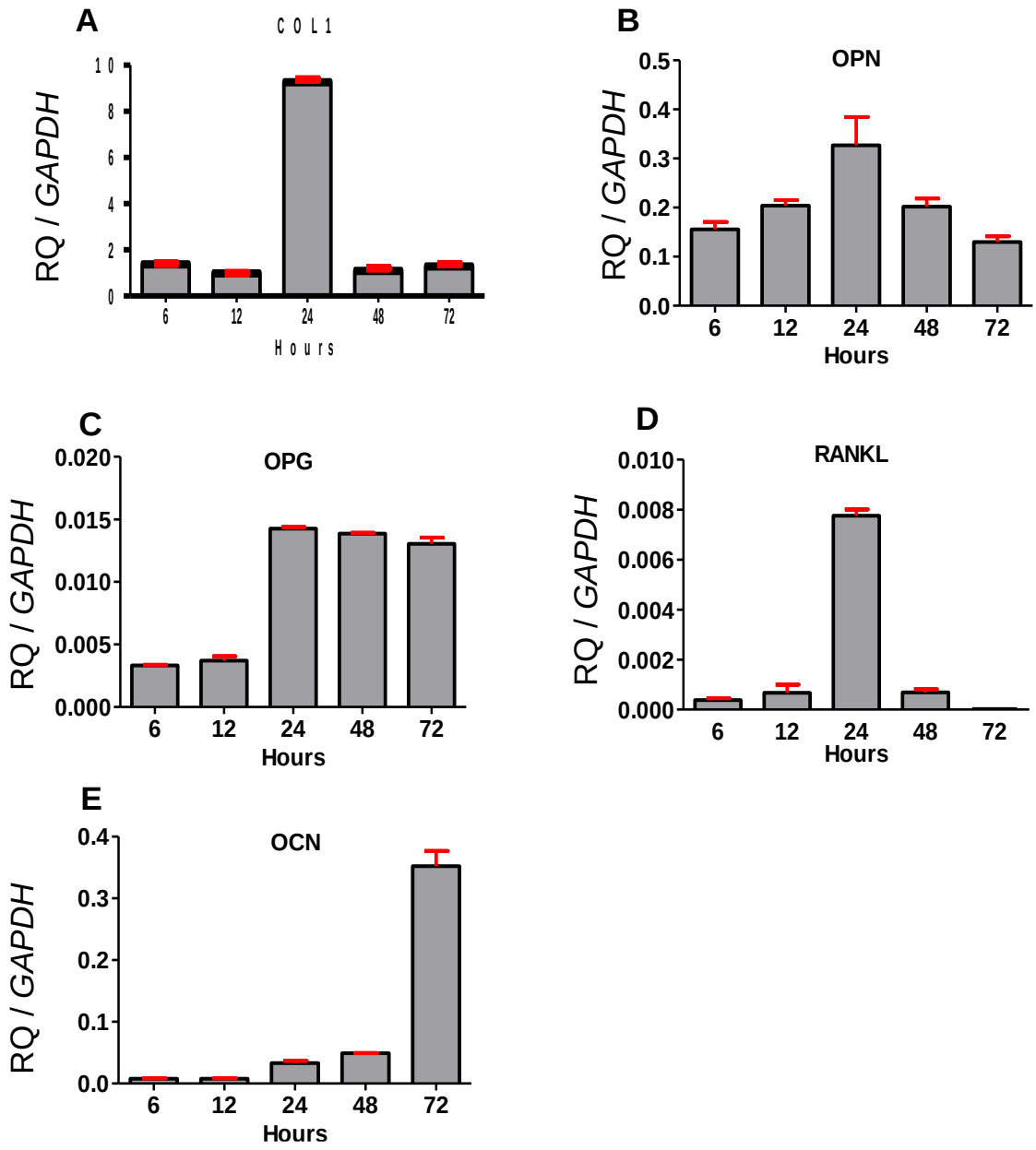


Figure 2-7: Osteoblastic signature genes are up-regulated in 2T3 osteoblast-like cell when cultured in osteogenic differentiation medium. QRT-PCR analysis of (A): *COL1*, (B): *OPN*, (C): *OPG*, (D): *RANKL* and (E): *OCN* expression in 2T3 cells cultured in osteogenic differentiation media. The magnitude of increase in expression of each gene (relative quantity: RQ) is normalised to the house-keeping gene *GAPDH*. Values are mean ± S.D from triplicate reactions.

2.3.6. Total collagen measurements

2T3 cells were cultured in replicates and maintained in osteogenic differentiation medium over 12 days, with the medium replaced every other day. Cell cultures were stopped on days 4, 8 and 12 for collagen quantification. Sirius red staining revealed that 2T3 osteoblast cells deposited collagen into the extracellular matrix over time (**Figure 2-8 B**). Cells also secreted collagen into the media, with a 12-fold increase from day 8 compared to day 4 (**Figure 2-8 C**).

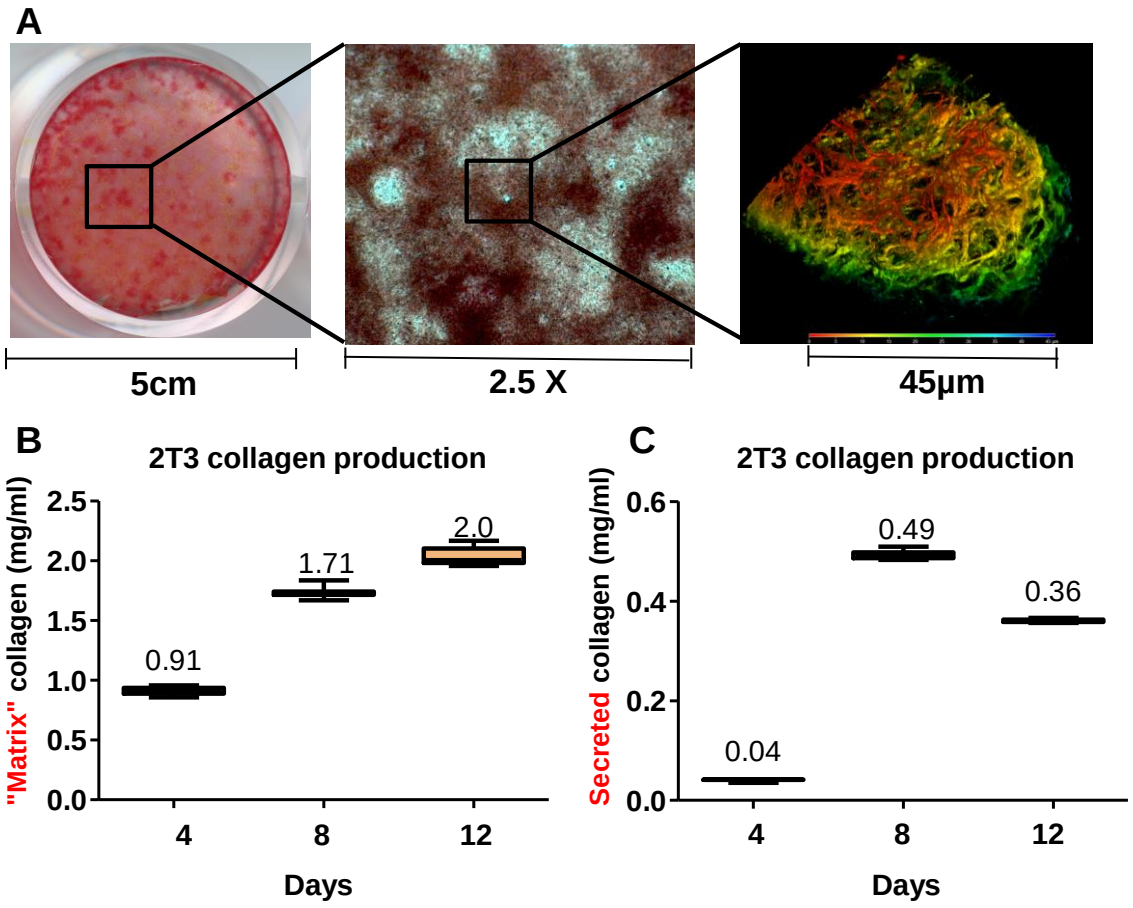


Figure 2-8: 2T3 osteoblast-like cells produce collagen. Panel (A) representative scan (left) of collagen staining on day 12, Sirius red staining at 2.5X magnification by ZEISS AX10 microscope (middle) and 3D representation of collagen fibres as observed by second harmonic generation (right), red top, yellow middle and green bottom layer (courtesy of Dr Clarence Yapp, Botnar Research Centre) . (B) Quantified matrix and (C) secreted collagen produced by 2T3 cells. Amount of collagen (mg) is normalized to the total protein concentration of each relevant time point. Data are representative of 4 independent wells from 3 separate experiments.

2.3.7. Protein expression

Western blot analysis of differentiating 2T3 osteoblasts revealed that these cells up-regulate OPN and OCN over time while differentiating in the presence of osteogenic medium (**Figure 2-9 A & B**). Cells also expressed VDR, as well as the activating 25(OH)D-1α hydroxylase enzyme, CYP27B1, at protein level during the differentiation process (**Figure 2-9 C & D,** respectively).

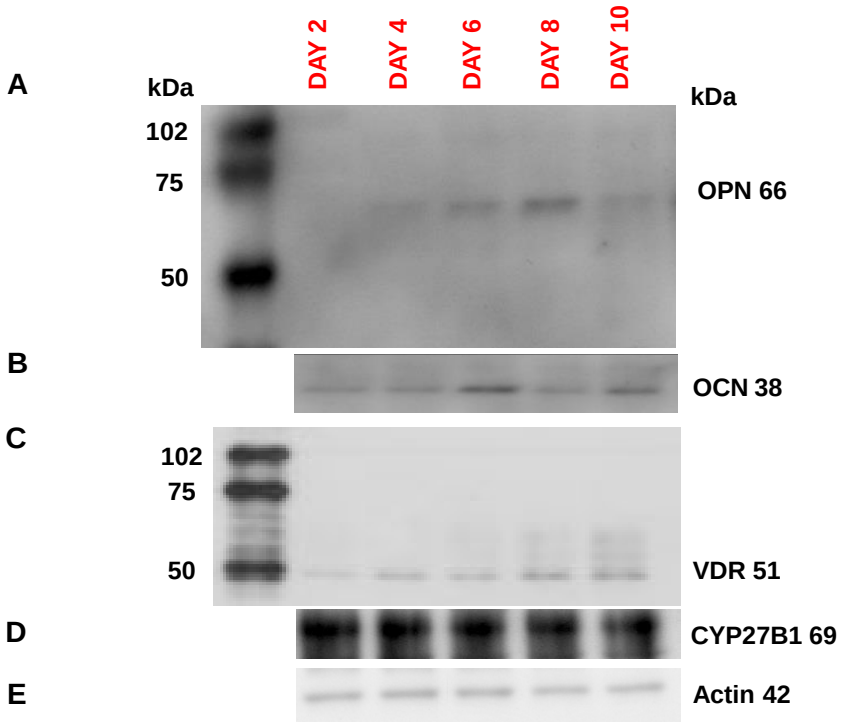


Figure 2-9: 2T3 osteoblast-like cells express OPN, OCN, VDR and CYP27B1 during differentiation. Western blots of 20µg extracts of 2T3 cell lysates from days 2-10 of OSM treatment for expression of (A) OPN, (B) OCN, (C) VDR and (D) CYP27B1. Membrane was stripped[12] and re-blotted for beta-actin as loading control (E).

[12] **Stripping buffer:** 1.87 glycine, 10g SDS, pH2.2

2.4. Mineralisation assay

Mouse and human osteoblast-like cells were grown to confluency in their culture growth medium and then maintained in the absence or presence of osteogenic differentiation medium containing 5-10mM β-glycerophosphate disodium salt, and 50µg/ml L-ascorbic acid 2-phosphate up to 12 days. No mineralisation occurred in SaOS-2, MG-63 or MC3T3 osteoblast-like cells over 12 days in the presence or absence of osteogenic differentiation medium (**Appendix 6**).

No mineralisation occurred in 2T3 cells maintained in the absence of osteogenic differentiation medium on days 4, 8 or 12 (**Figure 2-10**, **top row**). Osteogenic medium containing 5mM β-glycerophosphate disodium salt showed weak mineralisation as shown in **Figure 2-10** middle row. Mineralisation in 2T3 cells only formed in the presence of 10mM β-glycerophosphate disodium salt within 8-12 days (**Figure 2-10, bottom row**).

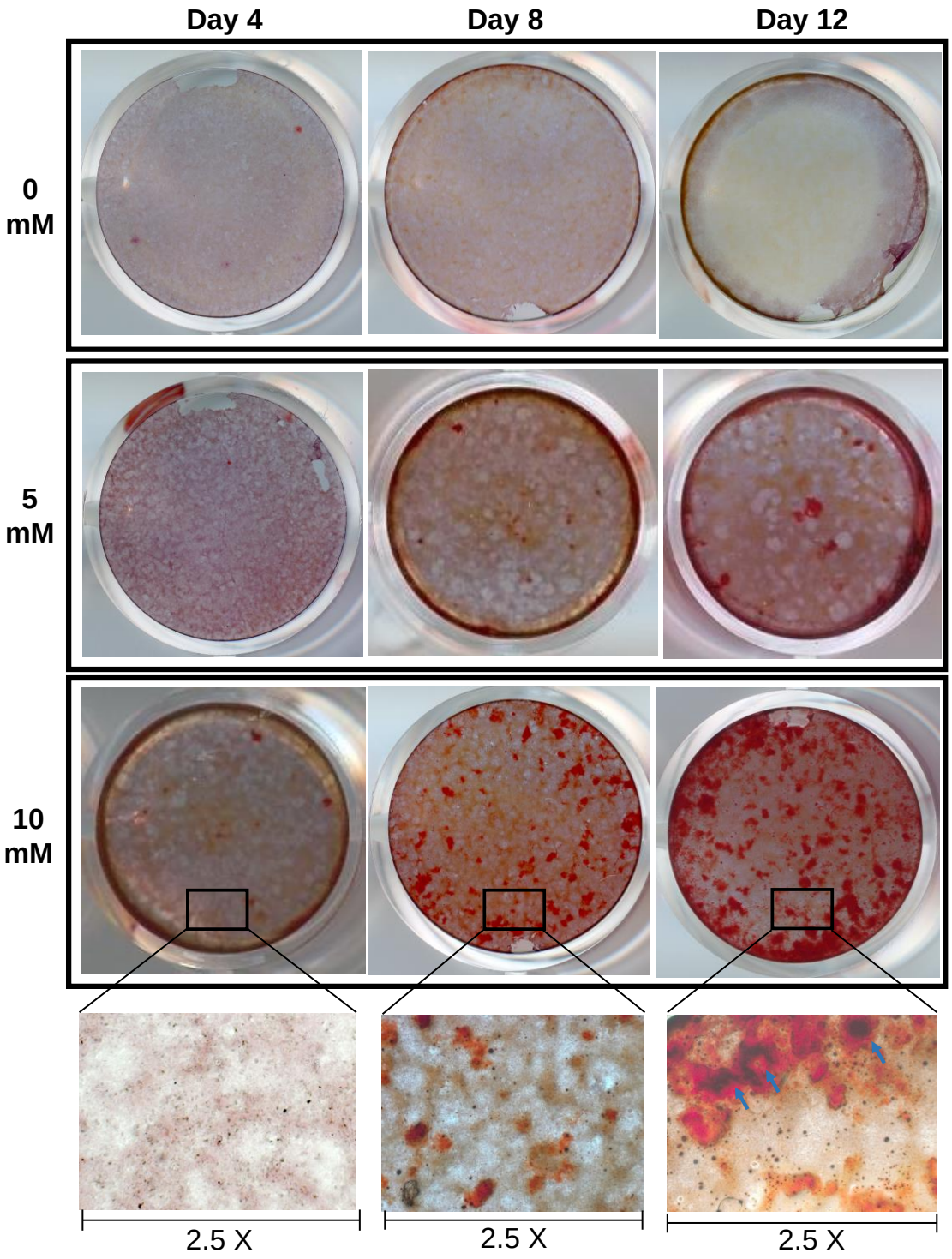

Day 4
Day 8
Day 12
0 mM
5 mM
10 mM
2.5 X
2.5 X
2.5 X

Figure 2-10: 2T3 osteoblast-like cells mineralised in the presence of osteogenic differentiation medium. Mineralisation by mouse 2T3 osteoblast cells over 12 days culture in the absence (1st row) or presence of osteogenic differentiation media containing 5mM (2nd row) or 10mM (3rd row) β-glycerophosphate. 2T3 cells were seeded at $1x10^4$ in 24 well plates and maintained in their culture growth medium to confluency (day 0) when culture media was replaced by osteogenic differentiation media. Media was replaced every other day and cultures were stopped at days 4, 8 and 12 and stained with Alizarin red. Alizarin red staining indicates calcium deposition. Plates were scanned with Epson perfection V500 PHOTO scanner or Zeiss microscope (scale bar X2.5). Blue arrows in high resolution image of day 12 indicate "mineralisation deposits".

2.5. Discussion

Initially all the cell lines that were examined for the presence of mycoplasma infections were positive. All the cryo-preserved murine MBA15.4 cells were mycoplasma contaminated, hence, this cell line was disregarded for future experiments. Except for MBA15.4, new batches of human and murine osteoblast-like cells were obtained and screened for mycoplasma, passaged and cryo-preserved for future vitamin D treatment.

Culture media such as DMEM and αMEM are defined basal solutions with known ingredients of inorganic salts, and essential amino acids for maintenance of cells in *in vitro* cell culture [333]. The measurements obtained from LC-MS from two different centres confirmed that there were no detectable levels of 25(OH)D in either media or sera, ensuring that the *in vitro* studies of vitamin D will be solely due to the exogenous addition of vitamin D metabolites.

Osteoblast viability, cytotoxicity or proliferation are usually assessed in culture conditions of DMEM 10% HI-FBS at 0.7-1x10^4 cell densities. Osteoblast viability by Alamar blue assay showed that 2T3 cells are viable at the various cell densities as assessed over 6 days in 10% HI-FBS and up to 12 days post-confluence in osteogenic differentiation media.

The gold standard osteoblast activity assay involves calcium deposition or mineralisation, which is indicative of *in vitro* osteoblast “bone formation” and terminally differentiated function [331]. All osteoblast-like cells (MG63, SaOS2, MC3T3, 2T3) were examined for their abilities to mineralise in the presence of different concentrations of βGP containing osteogenic differentiation media. In the current study addition of high concentration of 10mM of βGP and ascorbate

over 12 days failed to induce mineralisation in SaOS-2, MG-63 or MC3T3 osteoblast-like cells. Only the murine pre-osteoblast-like 2T3 cells were capable of mineralising within 12 days in the presence of 10mM of βGP and ascorbate. Assessments of osteoblast mineralisation after day 12 was stopped as cell sheets rolled over and assembled in the centre of wells.

Previous studies have shown that addition of dexamethasone and BMP-2 in osteogenic differentiation media results in enhanced osteoblast differentiation by up-regulation of ALP, collagen and OCN [319, 334]. Mineralisation of MC3T3 [250] and human osteosarcoma osteoblast-like cells SaOS-2 [335] and MG-63 have been shown to take longer than up to 8 weeks, with the addition of dexamethasone and BMP-2 to βGP and ascorbate [305]. In the current study the cellular activities of MG-63, SaOS-2 and MC3T3 declined over a week of culture, hence long term culture of these established cell lines post-confluency are not recommended.

Murine MC3T3 osteoblast-like cell are the most commonly used cell line *for in vitro* studies [264], and have been shown to up-regulate osteoblastic signature gene expression of *ALP*, *COLI* and *OCN* after 12 days in culture [336]. However, mineralisation in MC3T3 has been shown to take up 2-6 weeks [305, 336].

SaOS-2 expressed the osteoblastic markers ALP, OCN, OPN and vitamin D related proteins VDR and CYP27B1, but preliminary vitamin D treatments had no effects on ALP activity (**Appendix 5**). SaOS-2 cells have been previously shown to exhibit limited response to $1,25(OH)_2D_3$ metabolite [310].

The responsiveness of MG-63 cell line to $1,25(OH)_2D_3$ has been shown to be similar to human osteoblasts [337]. Although human MG-63 osteoblast-like cells have been shown to be vitamin D responsive [318], inconsistent data exists

regarding their mineralisation abilities [338]. MG-63 cells have been shown to up-regulate ALP and OCN (secreted into culture medium) with 10nM $1,25(OH)_2D_3$ over 72 h [337]. In a different study, *COL1* mRNA was shown to be stimulated from 3-48h in response to 100nM of $1,25(OH)_2D_3$ in MG-63 [256]. MG-63 cells were also shown to peak ALP expression by 12 days, up-regulate collagen I by 24 days, OCN by 28 days and to mineralise by 29 days [338, 339]. Other reports have shown that MG-63 expressed low levels of ALP and do not mineralise [340, 341].

In the present study, 2T3 cells did not require the addition of dexamethasone or BMP-2, which was advantageous as any additional effects in 2T3 osteoblast activity for future studies will be directly due to addition of vitamin D metabolites. Osteoblast differentiation *in vitro* has been shown to involve up regulation of osteoblastic signature markers, in a sequence with early markers such as ALP, COL 1 and OPN followed by OCN as a late marker of differentiation [175]. Assessment of 2T3 osteoblast-like cells in the presence of osteogenic differentiation medium showed that this cell line behaves like osteoblasts; expressing the osteoblastic genes (*COL1*, *OPN* and *OCN*) and up-regulate ALP, total collagen, OPN and OCN proteins when the mineralisation initiates after day 8 onward.

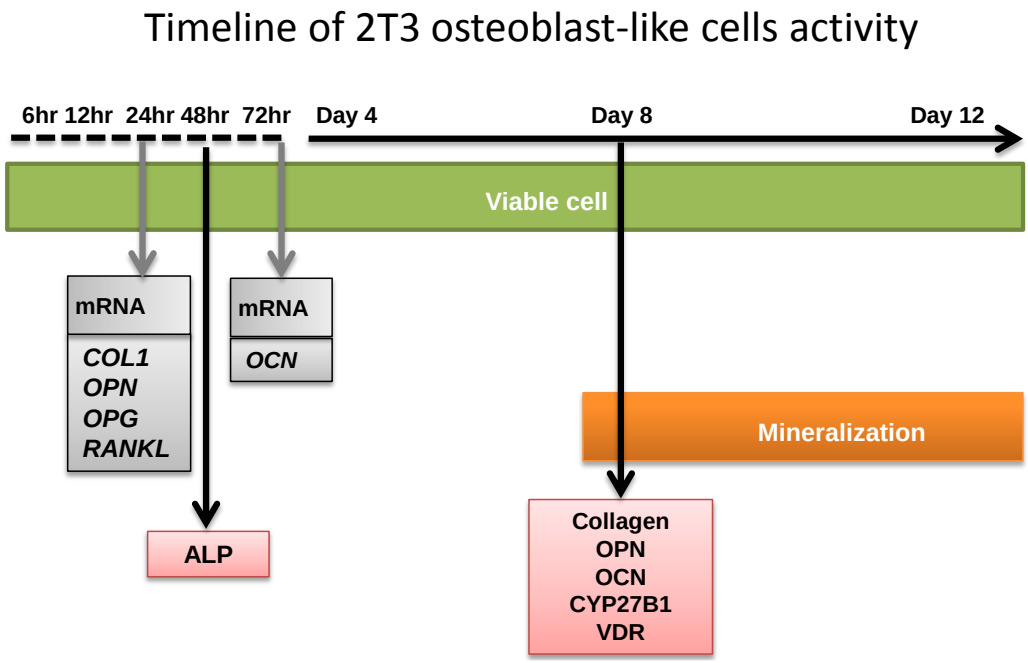


Figure 2-11: Figure illustrates the timeline at which specific osteoblastic signature genes and protein markers are expressed as identified in this study. It is worth noting that cells maintained in the presence of osteogenic differentiation medium and in the absence of BMP-2 or DEX. 2T3 osteoblastic signature genes, *COL1*, *OPN*, *OPG* and *RANKL* as early transcripts were detected at 24 hours, *OCN* as late marker at 72 hours, up-regulation of ALP protein at 48 hours. Collagen, OPN and OCN, CYP27B1 and VDR protein expression were up-regulated from day 8 onward. *In vitro* bone mineralisation initiates at day 4 and increases from day 8 onwards.

Time course of the 2T3 transcript levels in this Chapter correlates with the finding of Ghosh-Choudhury *et al*, who were the first to characterise the cells [309].

CHAPTER 3: EFFECTS OF VITAMIN D_2 AND D_3 ON 2T3 OSTEOBLAST ACTIVITIES

3. EFFECTS OF VITAMIN D_2 AND D_3 ON 2T3 OSTEOBLAST ACTIVITIES

3.1. Introduction

Increasing evidence suggests that bone cells are directly regulated by vitamin D [342]. The role of vitamin D is principally the regulation of gene expression during bone formation. This is achieved by vitamin D-VDR interaction with the vitamin D response elements in the regulatory regions of the target genes [237]. Screening of the mouse genome has identified nearly 3000 conserved VDREs (also Chapter1 section 1.7) [237].

$1,25(OH)_2D_3$ has been documented to exert its stimulatory effects on early stages of differentiation and on mineralisation as noted *in vitro* in osteoblast-like cells derived from mouse, rat, as well as human bone marrow and primary osteoblasts [241, 246-250]. Osteoblasts can synthesise the active metabolite $1,25(OH)D_3$ as they express CYP27B1, and also have the ability to catabolise $1,25(OH)_2D_3$ by expressing CYP24A1 enzyme [240]. $1,25(OH)_2D_3$ hormone induces osteoblast signature genes; *COL1*, *OCN*, *OPN*, *OPG*, *RANKL* as well as vitamin D related genes, *VDR*, *CYP27B1* and *CYP24* [148].

Table 3-1 summarises the *in vitro* effects of $1,25(OH)_2D_3$ on some of the established rodent osteoblast cell lines. Generally, $1,25(OH)_2D_3$ is known to have an inhibitory effect on proliferation of mouse osteoblast-like cell MC3T3, as well as rat ROS-17/2.8 osteoblast-like cells [343, 344]. A number of studies have shown that osteoblast differentiation in mouse and rat shows varied responses when treated *in vitro* with $1,25(OH)_2D_3$ [251]. For instance differential effects of

vitamin D_3 have been reported in a study by Li *et al*. They reported that rat primary osteoblast cell number as well as ALP expression were increased by $1,25(OH)_2D_3$ (0.1-10nM), but the same concentrations of $1,25(OH)_2D_3$ resulted in apoptosis and ALP inhibition in mouse primary osteoblasts [251]. In studies using different stages of the cells (pre- or mature osteoblast-like cells as well as primary osteoblasts) and using different culture conditions (such as regular culture condition 10%HI-FBS or osteogenic differentiation medium), $1,25(OH)_2D_3$ has been shown to have both stimulatory as well as inhibitory effects on ALP and OCN and mineralisation in rodent cells [251-254].

In mouse MC3T3 osteoblast-like cells ALP is stimulated by $1,25(OH)_2D_3$ in the early phase of differentiation but not late stages [250]. Furthermore, $1,25(OH)_2D_3$ has also been shown to stimulate collagen synthesis in mouse MC3T3 osteoblasts-like cells, but inhibit collagen promoter activity both *in vivo* and in *vitro* on primary mouse osteoblasts from calvaria [250, 258].

OPN mRNA expression and protein up-regulation is stimulated by $1,25(OH)_2D_3$ in MC3T3 as well as several osteoblast cells of rat origin [242]. In mice the induction of OCN by $1,25(OH)_2D_3$ has been shown to be stage dependent, as stimulatory in early differentiation and inhibitory in the maturation stage [259, 260].

Table 3-1:
The effects of 1,25$(OH)_2D_3$ on rodent osteoblast function. *Italics: transcript level*, non-Italics: protein level. K: ketoconazole. Arrows indicate up or down regulation.

Osteoblast	Dose (nM)	Response	Ref
Mouse			
MC3T3	100nM	↑*VDR*,↑*CYP24*,↓cell number	[345]
MC3T3	10nM	↑*OPN*,↑*CYP24*,↑VDR	[346]
MC3T3	0.3nM	↑OCN,↑ALP,↑Col,↑ mineralisation	[250]
MC3T3	10nM	↓Cell number	[343]
MC3T3	10nM	↑ OCN	[347]
2T3	10nM	↓ *ALP*, ↑ *OCN*	[309]
Primary	10nM	↑*OPN*, ↓*OCN*, no change *Col1*	[348]
Primary	10nM	↑OPN, ↑CYP24, ↑VDR	[346]
Primary	1-10nM	↓ ALP	[251]
Rat			
ROS 17/2.8	10nM	↑OPN &↑OCN	[349]
ROS 17/2.8	10-100nM	↑ALP	[350]
ROS 17/2.8	10-100nM	↑ALP,↓cell number	[344]
ROS 17/2.8	10-100nM	↓Col	[351]
ROS 17/2.8	10nM	↓BSP	[352]
ROS 17/2.8	10nM	↑OCN	[353]
ROS 17/2.8	1nM +K	↑VDR	[144]
Primary	1-10nM	↑ALP	[251]

BMSCs differentiation towards osteoblast lineage is a well-defined process resulting in osteoblast mineralisation of extracellular matrix and termination whereby osteoblasts either die, become bone lining cells or become embedded

in their matrix as osteocytes [175]. In *in vitro* studies using mouse osteoblast-like MC3T3 cells, treatment with $1,25(OH)_2D_3$ (0.1nM) has been shown to stimulate mineralisation [250]. But treatment with higher doses of $1,25(OH)_2D_3$ (100nM) has been shown to inhibit mineralisation in mouse primary osteoblast cultures [354]. As summarised in **Table 3-1**, all findings to date have been focused exclusively on vitamin D_3 effects on mouse or rat osteoblasts and there is still considerable controversy.

Since the discovery of vitamin D_2 and development of Viosterol for clinical applications, to our knowledge no comparison has been carried out on the effects of vitamin D_2 and D_3 metabolites directly on osteoblast cell cultures. Soon after discovery of vitamin D_2, its bioassay activity was assessed based on its ability to calcify rat tibiae *in vivo* [299]. As discussed in Chapter One there have been conflicting views on the potencies of D_2 and D_3 in humans and other species.

Only a small number of studies have compared the potencies of $1,25(OH)_2D_2$ and $1,25(OH)_2D_3$ metabolites on non-skeletal cells. As an example, anti-proliferative activities of $1,25(OH)_2D_2$ and $1,25(OH)_2D_3$ in chronic myelogenous leukaemia cell line RWLEU-4 have shown that higher concentrations of $1,25(OH)_2D_2$ (IC_{50}: 11nM) are needed to achieve 50% inhibition of cell number compared to $1,25(OH)_2D_3$ (IC_{50}: 6nM) [355]. Furthermore, analysis of $1,25(OH)_2D_2$ and $1,25(OH)_2D_3$ VDR binding affinities in bovine thymus, chick and rat intestine, in pig kidney cells as well as human cancer cells demonstrated that both $1,25(OH)_2D_2$ and $1,25(OH)_2D_3$ active metabolites have similar VDR binding affinities [356].

Osteoblast cells as exclusive bone forming cells are also the main targets for extra-renal metabolism of $25(OH)D_3$ to $1,25(OH)_2D_3$, but the metabolism of

25(OH)D_2 or its autocrine effects on osteoblasts have not been investigated [151, 241]. It has been shown that in bone osteoblasts, the activating enzyme 25(OH)D-1α hydroxylase (*CYP27B1*), is responsible for metabolism of 1,25(OH)$_2D_3$ and the catabolising enzyme 24-hydroxylase (*CYP24A1*) for deactivation of 1,25(OH)$_2D_3$ itself [148]. It has been shown that when human and mouse osteoblasts are cultured in the presence of 25(OH)D_3, cells metabolised significant amounts of 1,25(OH)$_2D_3$ [148, 241]. An increase in metabolism of 25(OH)D_3 into 1,25(OH)$_2D_3$ by osteoblast cells in culture also has been shown to coincide with *CYP27B1* up-regulation [151]. Specific knockdown of *CYP27B1* using small interfering RNA (siRNA) in osteoblast cells has shown that 1,25(OH)$_2D_3$ production and transcription of osteoblastic genes is reduced [148]. Depletion of *CYP27B1* by ketoconazole drug also has been shown to significantly reduce 1,25(OH)$_2D_3$ production in osteoblast cells [151].

Ketoconazole has been shown to be a strong but differential inhibitor of *CYP27B1* and *CYP24A1* [129, 142]. Co-treatment with ketoconazole and 25(OH)D_3 metabolite has been shown to block 1,25(OH)$_2D_3$ metabolism by human osteoblasts, hence resulting in an inhibition of osteoblast markers such as *OCN* and *ALP* [150, 151].

Ketoconazole also has been used in a variety of *in vitro* vitamin D studies to study the mechanism of *CYP24A1* activities. By co-administration of ketoconazole and 1,25(OH)$_2D_3$, the lifetime of 1,25(OH)$_2D_3$ hormone in culture conditions has been shown to increase. The increase in the 1,25(OH)$_2D_3$ cellular responses in the presence of ketoconazole has been shown to be due to VDR mediated inhibition of *CYP24A1* [142, 144]. As highlighted in section 1.11 of the introduction the effect of ketoconazole depends on the abundance of native [25(OH)D] or active

[1,25(OH)D] vitamin D metabolites. In the presence of abundant 25(OH)D_2 or D_3 a mixed effect is obtained from dual inhibition of both CYP27B1 activating and CYP24A1 inhibiting enzymes. Based on selective activity of this inhibitor (ketoconazole), CYP27B1 would be less inhibited than CYP24A1 catabolising enzyme, resulting in less metabolism of the active metabolite but equally there will be little catabolism by CYP24A1 and therefore a net increase in active metabolite. However in the presence of active metabolites [1,25(OH)D_2 or –D_3] the CYP27B1 is redundant and the effect on CYP24A1 dominates, i.e. 1,25(OH)D_2 or –D_3 are prolonged and enhanced. *In vivo* the end effect will depend on the total native vitamin D status as well as the tissue specific level of active metabolites. For example the circulating levels of native vitamin D could produce opposite effects in a tissue with high CYP27B1 levels compared with a tissue low in local activation capability and display a different local response to ketoconazole. An individual dosed with high levels of active metabolite together with ketoconazole will show a systemic increased in response to active vitamin D metabolites.

Beside VDR mediated genomic effects of 1,25$(OH)_2D_3$, protein-disulfide isomerase-associated 3 (Pdia3) has been shown to be a membrane receptor for 1,25$(OH)_2D_3$ in MC3T3 mouse osteoblast-like cells [357]. It has been suggested that Pdia3 may have an important function in initiation of the 1,25$(OH)_2D_3$ stimulated membrane signalling pathway. 1,25$(OH)_2D_3$ co-treated with BMP2 showed a synergistic increase in osteoblastic markers in mouse MC3T3 cells [357]. In *in vitro* osteoblast studies, BMP-2 treatment alone has been shown to increase osteoblast differentiation and mineralisation [358]. Co-treatment of BMP-2 with vitamin D_3 has been shown to result in an accelerated, synergistic

effect on osteoblast differentiation and mineralisation, involving VDR and Pdia3 [357]. As these collective data indicate, the function of vitamin D in rodent osteoblasts is still far from clear. Even more evident is the lack of data on vitamin D2 and these questions will be the subject of this Chapter.

3.2. Aims and objectives

In the previous chapter it was demonstrated that 2T3 cells were the most suitable cells for the current *in vitro* study.

The aims of this Chapter are to compare the effects of D_2 and D_3 metabolites on osteoblastic activities. 2T3 osteoblastic activities will be assessed in response to various physiological and supra-physiological concentrations of 25(OH)D_2 and D_3 and their active metabolites.

By the end of this chapter I aim to answer the following questions;

- Do vitamin D_2 and D_3 metabolites exert similar effects on 2T3 osteoblast cell number?
- What are the vitamin D metabolite concentrations that induce osteoblastic differentiation and activity over time?
- What are the effects of prolonged treatments with vitamin D_2 and D_3 metabolites on mineralisation
- How does ketoconazole influence the effects of vitamin D metabolites on ALP, osteoblastic signature genes and osteoblast mineralisation activities

in mouse osteoblast, and is there a difference in this influence between vitamin D_2 or D_3 metabolites?

- How does co-treatment of osteoblasts with BMP-2 affect their response to vitamin D_2 and D_3 metabolites?

3.3. Materials and Methods

Unless otherwise noted the same reagents and methods have been used as previously described in Chapter 2.

3.3.1. 2T3 cell number in response to vitamin D metabolites

2T3 osteoblast cells were seeded in their culture medium (DMEM-10%HI-FBS), in multiple 96 well plates at $5x10^3$ cells per well in eight replicates. Cells were then treated with 25(OH)D_2 and 25(OH)D_3 (50-1000nM) and their active metabolites 1,25$(OH)_2D_2/D_3$ (0.05-1nM) for 24 hours. After 21 hours 10% Alamar blue™ was added to the wells and incubated for a further 4 hours under standard tissue culture conditions. Fluorescence activities of treated or control wells were measured using a BMG FluorStar Optima plate reader.

3.3.2. 2T3 ALP activity in response to vitamin D metabolites

Murine 2T3 cells were re-plated into 24 well plates at $1x10^4$ in four replicates in their culture medium (DMEM-10%HI-FBS) to reach confluence (48h). Differentiation was then induced by addition of osteogenic differentiation medium in the presence or absence of 25(OH)D_2 and 25(OH)D_3 (100-500nM) and 1,25$(OH)_2D_2/D_3$ (0.1-0.5nM).

Cell lysates of vitamin D treatments were collected from 12-48 hour time points and ALP activity was assessed as previously described in Chapter 2.

3.3.3. 2T3 osteoblastic signature gene expression in response to vitamin D metabolites

Preliminary studies indicated that a single dose of 200nM of 25(OH)D_2/D_3 was the most suitable concentration for comparisons of vitamin D metabolites. 2T3 cells over their 19th ,20th and 21st passages were cultured in their culture medium (DMEM-10%HI-FBS) in triplicates in 6-well plates at 1x10^5 cell density until full confluence (24h), followed by the addition of osteogenic differentiation medium in the presence or absence of 200nM of 25(OH)D_2/D_3, or ethanol as control.

Total RNA from each treatment condition was extracted after 24h by RNeasy Mini Kit (Qiagen) as described earlier in Chapter 2 according to the manufacturer's instructions. The effects of vitamin D metabolite on osteoblast signature genes *COL1*, *OPN*, *RANKL, OPG*, as well as vitamin D related genes *VDR*, *CYP27B1* and *CYP24* were assessed and compared with untreated cells containing ethanol as vehicle control.

The Ct values for treated samples were normalised to housekeeping genes *GAPDH* and the relative expressions were calculated using ΔΔCt with amplification and accuracy of 98-100%.

3.3.4. 2T3 protein expression in response to vitamin D metabolites

To determine the effects of different metabolites of vitamin D on endogenous levels of VDR, CYP27B1, CYP24A1, as well as on the expression of osteoblast

differentiation markers OPN and OCN, murine 2T3 osteoblasts were treated with 100-500nM concentrations of 25(OH)D_2/D_3 or 0.01-0.5nM of 1,25(OH)$_2D_2$/D_3 in osteogenic differentiation medium for 8 days. Protein expression of the above markers was determined by Western blotting under reducing conditions. To ensure equal loading for the Western blot analysis anti beta- actin antibody was used to detect endogenous actin.

To determine the effects of vitamin D metabolites on total collagen production 2T3 cells were treated with 200 and 500nM of 25(OH)D_2 or D_3 and 0.2 and 0.5 nM of 1,25(OH)$_2D_2$ or D_3 active metabolites in osteogenic differentiation medium. Total collagen in the extracellular matrix and secreted collagen in the medium was assessed by Picrosirius Red as described in Chapter 2.

3.3.5. 2T3 Mineralisation in response to vitamin D metabolites

2T3 osteoblast cells were cultured in 96, 48 or 24 well plates at 0.1-1x10^4 in replicates in their culture medium (DMEM-10%HI-FBS) to reach confluence (24-48h). Medium at this time point was replaced with osteogenic differentiation medium and presence of 50-1000nM of 25(OH)D_2 or D_3 or 0.05-1nM of 1,25(OH)$_2D_2$ or D_3 ± recombinant mouse BMP-2.

Media and treatments were replaced every other day for up to 8 days. Cultures were stopped on days 5 and 6 for vitamin D treatments in the presence of BMP-2 and on day 8 in the absence of BMP-2 and stained with Alizarin red dye and plates were scanned with EPSON EXPRESSION 1640XL scanner. Mineralisation was quantified by addition of 800 µl of 10% acetic acid and 200µl

of the extractions were transferred into a 96 well plate prior to colorimetric reading at 550nm as previously described in Chapter 2.

3.3.6. Influence of ketoconazole on the effects of vitamin D metabolites on 2T3 function

3.3.6.1. Effect of ketoconazole on 2T3 cell activity

2T3 osteoblast cells were seeded in their culture medium (DMEM-10%HI-FBS) in multiple 96 well plates at $5x10^3$ cells or in 6 well plates at $1x10^5$ per well in replicates until confluency. Medium at this time point was replaced with osteogenic differentiation medium ± 0.25-80µM ketoconazole for 8 days for cell number and mineralisation, or ±10µM ketoconazole for 24 hours for gene expression studies. After 24 hours total RNA was extracted and assessed for CYP27B1 or CYP24A1 QRT-PCR as explained in section 3.3.3. After 8 days treatments 10% Alamar blue™ was added and fluorescence activities were measured as described previously. Parallel 96 well plates were stopped on day 8 for mineralisation by Alizarin red staining as explained earlier.

3.3.6.2. Influence of ketoconazole on the effects of vitamin D metabolites on ALP activity in 2T3 cells

Murine 2T3 cells were re-plated into 24 well plates at $1x10^4$ in replicates in their culture medium (DMEM-10%HI-FBS) to reach confluence (48h). Differentiation was then induced by addition of osteogenic differentiation medium in the presence or absence of $25(OH)D_2$ and $25(OH)D_3$ (200nM) or $1,25(OH)_2D_2/D_3$

(0.2nM) and ± 10µM ketoconazole. Cell lysates of all conditions were collected and ALP activity was assessed as previously described.

3.3.6.3. Influence of ketoconazole on the effects of vitamin D metabolites on signature genes in 2T3 cells

2T3 cells were cultured as in section 3.3.3 and the influence of ketoconazole (10µM) on the effects of 200nM of 25(OH)D_2/D_3 on osteoblast signature genes *COL1*, *OPN*, *RANKL, OPG*, as well as vitamin D related genes *VDR*, *CYP27B1* and *CYP24* were determined by QRT-PCR as described above.

3.3.6.4. Influence of ketoconazole on the effects of vitamin D metabolites on 2T3 mineralisation

2T3 osteoblast cells were cultured in 48 well plate at $1x10^4$ in replicates in their culture medium (DMEM-10%HI-FBS) to reach confluence (24h). Medium at this time point was replaced with osteogenic differentiation medium and presence of 200nM of 25(OH)D_2/D_3 or 0.2nM of 1,25$(OH)_2D_2$/D_3 ± 10µM ketoconazole. Cultures were stopped on day 6 and stained with Alizarin red dye, and mineralisation was quantified by 10% acetic acid extraction.

3.4. Results

3.4.1. Effect of vitamin D on 2T3 cell number

When murine 2T3 osteoblasts were exposed to 50-1000nM 25(OH)D_2 and D_3 or 0.05-1nM of 1,25(OH)$_2D_2$ and D_3, both 25(OH)D_2 and D_3 reduced cell number at doses above 500nM (**Figure 3-1A**). However, the magnitude of the decrease was greater with 25(OH)D_3 in 2T3 cells at ≥500nM concentrations (P<0.001). While 1,25(OH)$_2D_3$ at ≥0.5nM concentrations reduced cell numbers significantly (P<0.001), 1,25(OH)$_2D_2$ did not have such an effect, and, if anything, showed a tendency to increase osteoblast numbers at 0.2nM (**Figure 3-1B**: P<0.001). 1,25(OH)$_2D_3$ reduced 2T3 cell number at concentrations of ≥0.5nM (P<0.001).

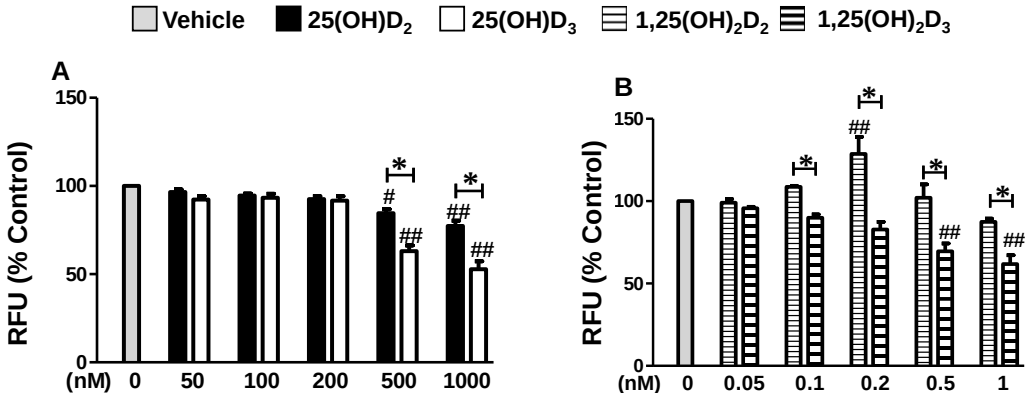


Figure 3-1: Vitamin D_3 metabolites are more potent than D_2 in inhibiting 2T3 cell number. Comparative effects of (A) 25(OH)D_2, 25(OH)D_3, (B) 1,25(OH)$_2D_2$ and 1,25(OH)$_2D_3$ on mouse 2T3 cell number. 2T3 osteoblasts were treated over 24 hours with 50-1000nM of 25(OH)D_2/D_3 and 0.05-1nM of 1,25(OH)$_2D_2$ /D_3. Cell number was measure by Alamar blue™ assay. Results of triplicate experiments are presented as percentage of relative fluorescence unit (RFU) of treated cells against vehicle (control) as mean ±SEM (non-parametric Kruskal Wallis test). * P<0.001 for comparisons between D_2 and D_3 metabolites, and # P<0.05, ## P<0.001 comparisons between treatments and control (grey).

3.4.2. Murine 2T3 differentiation in response to vitamin D

3.4.3. 2T3 ALP activity

At 100-200nM concentrations and in combination with osteogenic differentiation medium, both 25(OH)D_2 and D_3 increased ALP activity in 2T3 cells within 24h of treatment, 12h ahead of control cells treated with osteogenic differentiation medium alone (**Figure 3-2A-B**:P<0.001). In the same cells, 0.1-0.5 nM 1,25$(OH)_2D_2$ and D_3 also induced ALP 36 h earlier than controls (**Figure 3-2D-F**; P<0.001). There were no significant differences between the effects of D_2 and D_3 metabolites on ALP in mouse cells.

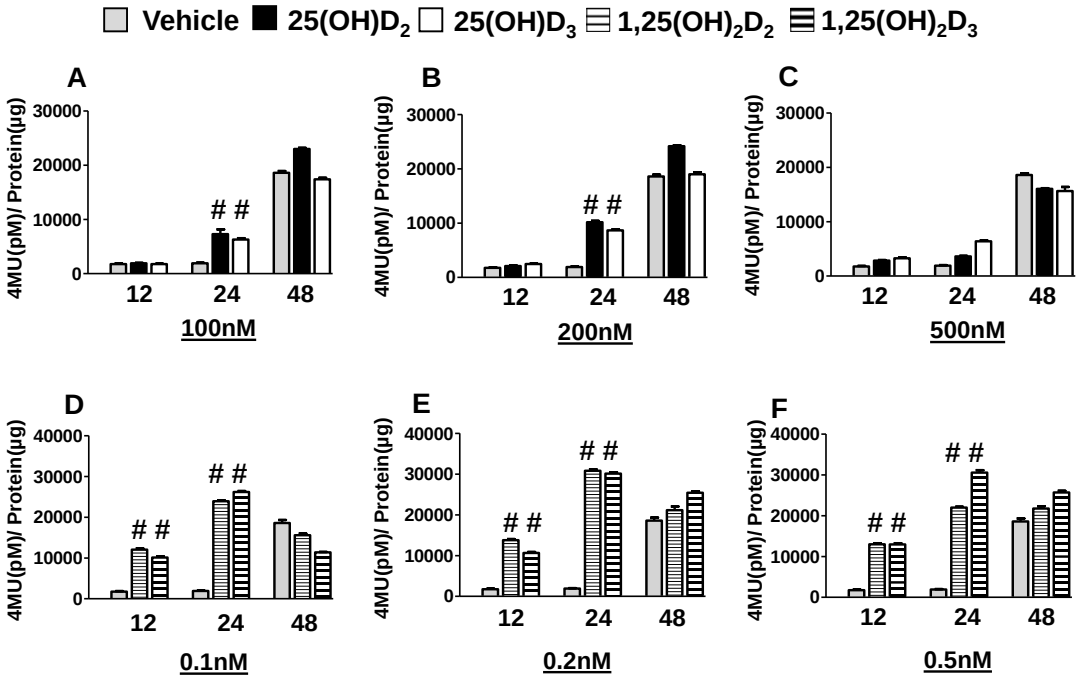


Figure 3-2: Both vitamin D_2 and D_3 metabolites stimulated 2T3 ALP activity dose and time dependently. Effects of vitamin D_2 and D_3 metabolites on ALP enzymatic activity in mouse 2T3 cells (A-F). 2T3 cells were grown in their culture medium (DMEM-10%HI-FBS) for 48h until confluency (0h), and were then treated once with either 100-500 nM 25$(OH)D_2/D_3$ (A-C) or 0.1-0.5 nM 1,25$(OH)_2D_2/D_3$ (D-F). ALP enzymatic activities were measure at 12, 24 and 48 h in osteogenic differentiation medium (see Methods). Values are mean ± SEM (non-parametric Kruskal Wallis test) from triplicate experiments. # P<0.001 for comparisons between treatments and control (grey).

3.4.4. 2T3 osteoblastic signature gene expression

When murine 2T3 osteoblasts were treated with 200nM 25(OH)D_2 or D_3 for 24h in osteogenic differentiation medium, there was up-regulation of *OPG* (**Figure 3-3A**, D3: P<0.01) in response to 25(OH)D_3 only, *RANKL* (**Figure 3-3B**, $D_2 = D_3$: P<0.001) *COL1* (**Figure 3-3C**, $D_2 = D_3$: P<0.001), *OPN* (**Figure 3-3D**, $D_2 = D_3$: P<0.001), *VDR* (**Figure 3-3E** , D_2 :P<0.01, and D_3; P<0.001, respectively,$D_3>D_2$) and *CYP27B1* (**Figure 3-3G**, D_2: P<0.001 and D_3 ;P<0.01, $D_2>D_3$) compared with the controls. There was no detectable change in *CYP24A1* in response to 25(OH)D_2 over 24h, whereas D_3 significantly up-regulated *CYP24A1* (**Figure3-3F,** P<0.001). There was no significant differences between 25(OH)D_2 and D_3 in upregulation of *RANKL* and *COL1* and *OPN* (**Figure 3-3B,C&D**), whereas only 25(OH)D_3 significantly increased *OPG* (P<0.01). 25(OH)D_3 was also more potent in upregulation of *VDR* compared to 25(OH)D_2 (**Figure 3-3E**, D_2 vs D_3 :P<0.001). 25(OH)D_2 was more potent than 25(OH)D_3 in up-regulation of *CYP27B1* (**Figure 3-3G**, P<0.05).

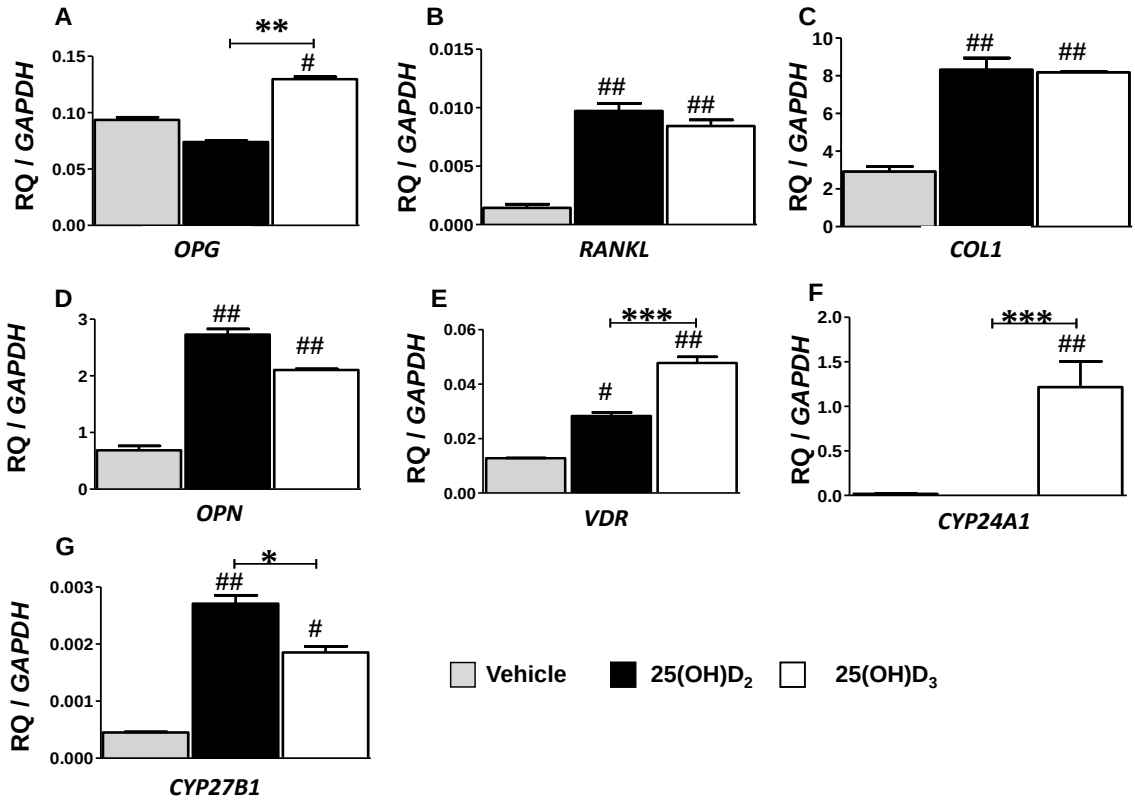


Figure 3-3: Both 25(OH)D_2 and D_3 metabolites stimulated 2T3 signature genes. Effects of vitamin D metabolites on the relative quantity (RQ) of murine 2T3 osteoblast signature gene expression; (A): *OPG,* (B): *RANKL,* (C): *COL1*, (D): *OPN* and vitamin D-related gene expression *VDR* (E), *CYP24A1* (F) and *CYP27B1* (G) during differentiation. 2T3 cells were cultured for 24h in the presence of 200nM 25(OH)D_2 (black bars), D_3 (white bars) or vehicle (grey bars) in osteogenic differentiation medium. The magnitude of increase in expression of each gene is normalised to the house keeping gene *GAPDH*. Values are mean ± S.D. from triplicate reactions (one way Anova). * P<0.05, ** P<0.01, and *** P<0.001 for comparisons between D_2 and D_3 metabolites, and # P<0.01, ## P<0.001 for comparison of vitamin D metabolite treatments against control (grey).

3.4.5. 2T3 osteoblastic protein expression

The protein expression of VDR, CYP27B1, OPN and OCN were examined in response to 100-500nM 25(OH)D_2 and D_3 and 0.1-0.5nM 1,25(OH)$_2D_2$ or D_3 in 2T3 osteoblasts cultured in osteogenic differentiation medium for 8 days. Western blots shows that there was a modest increase in VDR, CYP27B1, OPN

and OCN at 200nM 25(OH)D_2 or D_3, whereas a reduction of these proteins was observed with 500nM of either, with D_3 being more potent (**Figure 3-4**, left panel). However, there was a dose-dependent increase in VDR, OPN and OCN protein expression in response to the active metabolites 1,25(OH)$_2D_2$ / D_3, where 0.2-0.5nM 1,25(OH)$_2D_2$ exerted the highest response (**Figure 3-4**, right panel).

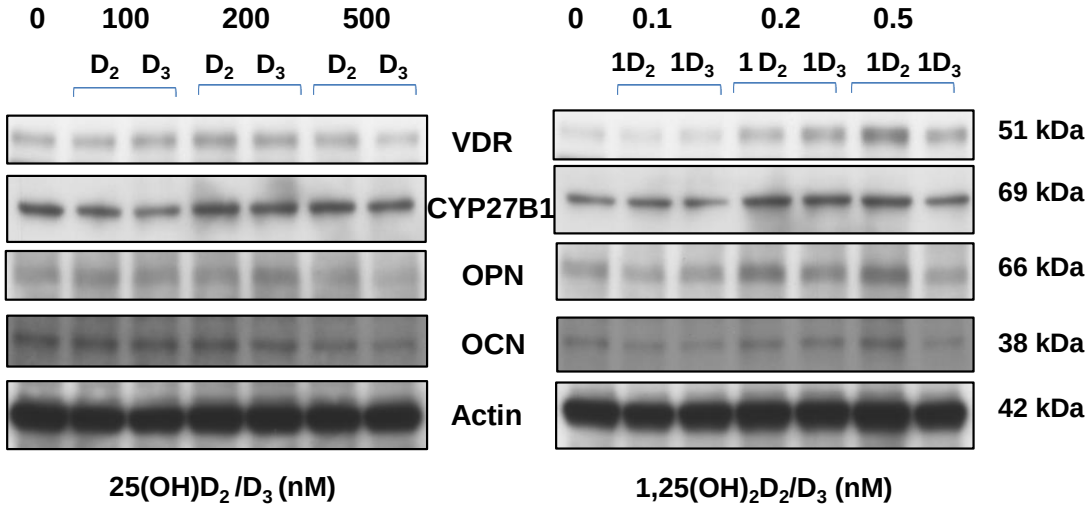


Figure 3-4: 2T3 osteoblastic proteins stimulated by both vitamin D_2 and D_3 metabolites. Western blots of 20 µg extract of 2T3 cell lysates for VDR, CYP27B1, OPN or OCN on day 8 have been shown in response to 100-500nM 25(OH)D_2 (D_2) or 25(OH)D_3 (D_3) (left panel) and 0.1- 0.5 nM 1,25(OH)$_2D_2$ ($1D_2$), 1,25(OH)$_2D_3$ ($1D_3$) (right panel). Actin was used as loading control.

3.4.6. Collagen production in 2T3 osteoblasts

The total amount of matrix and collagen secreted into the medium by 2T3 cells in response to vitamin D metabolites in osteogenic differentiation medium was quantified on day 4 by Picrosirius Red binding (**Figure 3-5A&B** respectively). Addition of 200nM or 0.2nM of either vitamin D metabolites had no effects on matrix collagen production (**Figure 3-5A&B**). Addition of both 500nM 25(OH)D_2 and D_3 significantly (**Figure 3-5A**, D_2: $P<0.05$ and D_3: $P<0.01$ respectively,

D_3>D_2: P<0.05) reduced matrix collagen. Similarly 0.5nM of $1,25(OH)_2D_3$ was more potent than D_2 in inhibition of matrix collagen (**Figure 3-5A**: D3: P<0.01, D_2: P<0.05, D_3>D_2: P<0.05). Secreted collagen production was unaffected by vitamin D metabolites on day 4 (**Figure 3-5B**).

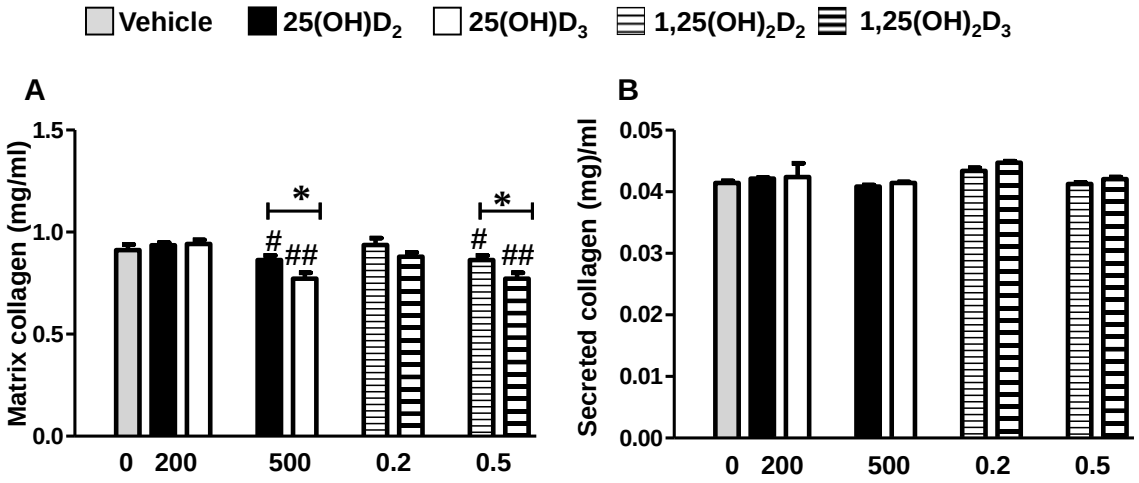
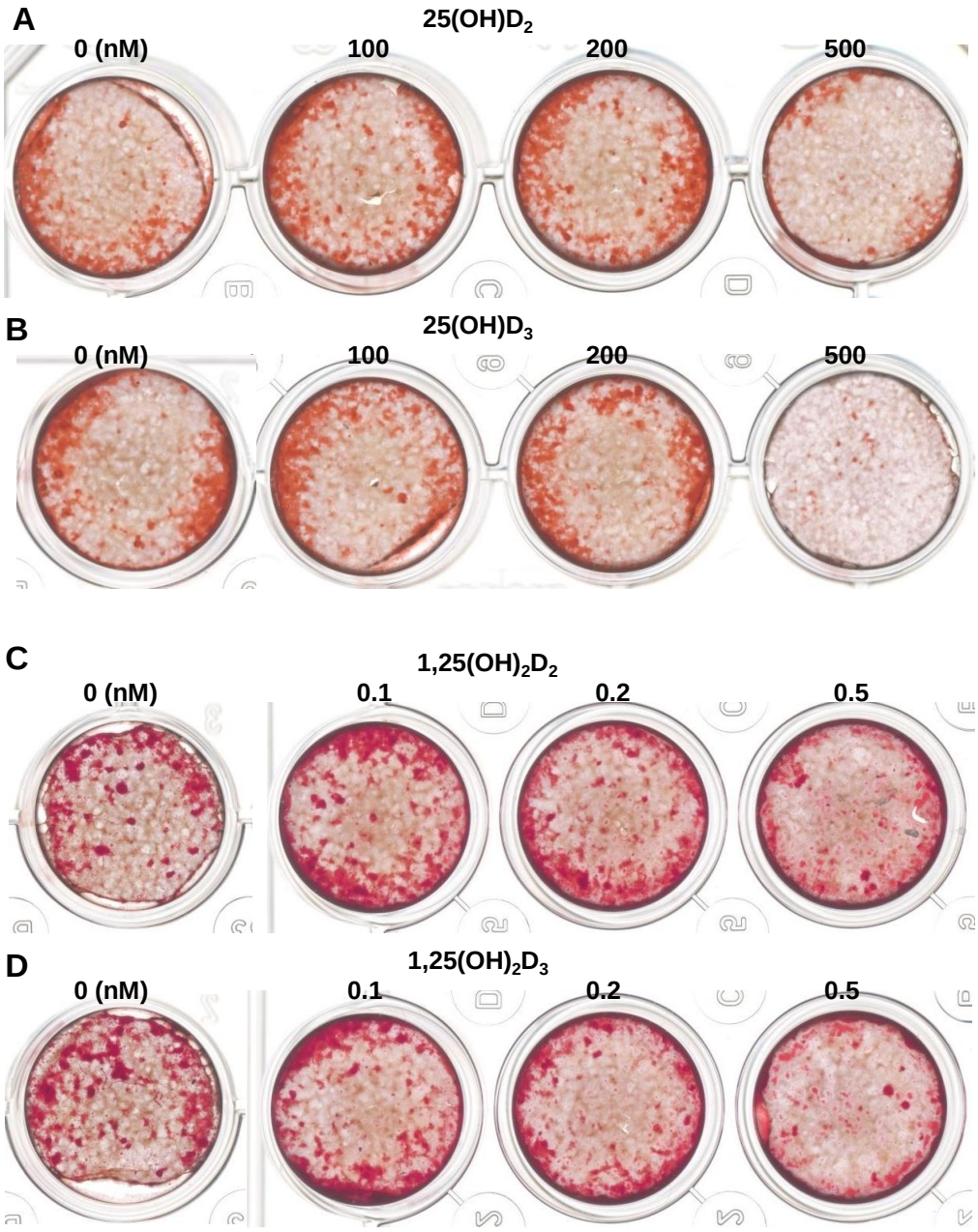


Figure 3-5: Total collagen expression was not affected by physiological doses of vitamin D_2 or D_3 metabolites in 2T3 osteoblastic cell line. Comparative effects of $25(OH)D_2$, $25(OH)D_3$ (200-500nM), $1,25(OH)_2D_2$ and $1,25(OH)_2D_3$ (0.2-0.5nM) on mouse collagen production (A&B). 2T3 cells were cultured in differentiation media in 24 well plates for 4 days in the presence of (A) 200/500nM $25(OH)D_2$ / D_3 or (B) 0.2-0.5nM of their active metabolites $1,25(OH)_2D/D_3$. Amount of collagen deposited into the extracellular matrix or secretory collagen into the medium were quantified by Picrosirius red. Data presented as mean ± SEM (non-parametric Kruskal Wallis test) treated versus untreated controls (grey) from triplicate experiments. * P<0.01 for comparison between treatments and # P<0.05 and ## P<0.01 for comparisons between treatments and control (grey).

3.4.7. Effect of vitamin D on 2T3 mineralisation

Pre-osteoblast murine 2T3 cells were cultured to full confluence in their culture medium (DMEM-10%HI-FBS) and treated over 8 days with 100-500nM of either $25(OH)D_2$ or D_3 (**Figure 3-6A**-**B**) or 0.1-0.5nM of either $1,25(OH)_2D_2$ or D_3 (**Figure 3-6C-D**) in osteogenic differentiation medium. Quantification of Alizarin red staining revealed that compared to vehicle, 200nM $25(OH)D_2$ and D_3 equally induced a modest increase in 2T3 mineralisation (**Figure 3-6E**: P<0.05). There

was an increase in 2T3 mineralisation in response to $1,25(OH)_2D_2$ and D_3 at 0.1-0.2nM compared to vehicle, with 0.1nM $1,25(OH)_2D_2$ being slightly more stimulatory than D_3 (**Figure 3-6F**: P<0.05). High doses of either $25(OH)D_2$ or D_3 or 0.5nM of $1,25(OH)_2D_2$ or D_3 significantly inhibited mineralisation in 2T3 osteoblasts (**Figure 3-6A-D**: P<0.001). This inhibitory effect was more evident with $25(OH)D_3$ or its active metabolite $1,25(OH)_2D_3$ compared to $25(OH)D_2$ or $1,25(OH)_2D_2$ (**Figure 3-6E-F**: P<0.001).



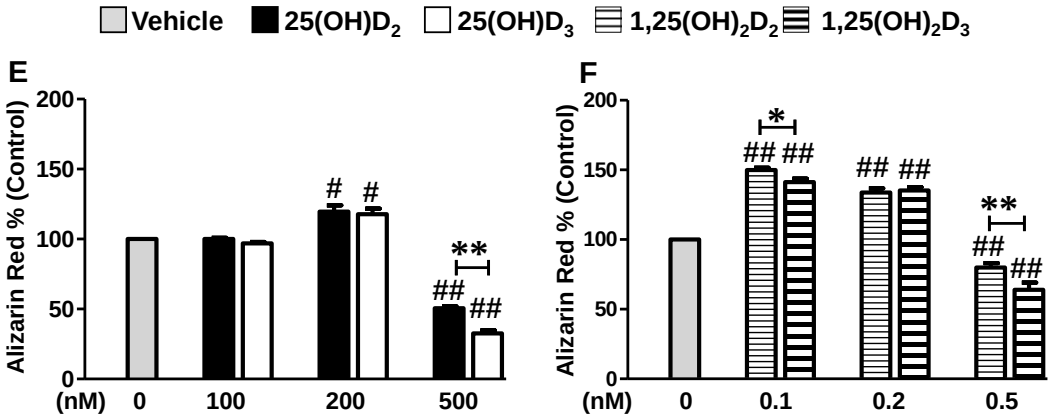


Figure 3-6: Low doses of D_2 and D_3 metabolites increased 2T3 mineralisation, but high doses inhibited mineralisation. Effects of vitamin D_2 and D_3 on mineralisation by mouse 2T3 cells. Cells were cultured in osteogenic differentiation media for 8 days in the presence of (A) 100-500nM of either 25(OH)D_2 or (B) 25(OH)D_3 , (C) 0.1- 0.5nM or either 1,25(OH)$_2D_2$ and (D) 1,25(OH)$_2D_3$. After washing and staining with Alizarin red, the cells were photographed. Corresponding data from the quantification of staining by acetic acid extractions are presented in panels (E) for 25(OH)D_2 and D_3 and (F) for 1,25(OH)$_2D_2$ and D_3. Values are mean ± SEM (non-parametric Kruskal Wallis test) as percentage of untreated control (grey) from triplicate experiments. * P<0.05, and ** P<0.001 for comparisons between D_2 and D_3 metabolites, and # P<0.05, ## P<0.001 for comparison of vitamin D metabolite treatments against control (grey).

3.4.8. Effects of ketoconazole on 2T3 cellular activity

3.4.8.1. 2T3 Cell number

Addition of prolonged treatments of 0.25 to 40µM of ketoconazole over 8 days had no effect on 2T3 cell number (**Figure 3-7**). Only a high concentration of 80 µM of ketoconazole reduced 2T3 cell number compared to untreated cells (**Figure 3-7**, P<0.001).

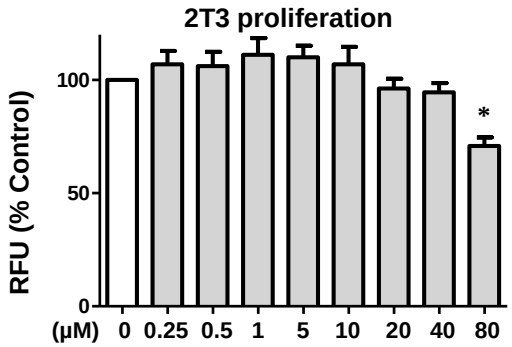


Figure 3-7: Only 80µM ketoconazole reduced 2T3 cell number over time. Effect of ketoconazole on mouse 2T3 cell number over 8 days of culture in osteogenic differentiation medium assessed by Alamar blue™ assay. $5x10^3$ cells were grown in their culture medium until confluence, when medium was replaced with osteogenic differentiation medium ± 0.25-80µM ketoconazole. Cell numbers were assessed fluorimetrically as percentage of relative fluorescence units (RFU) of ketoconazole treated cells against vehicle as control. Data are presented as mean ±SEM, (Unpaired Student t-test, n= 6 wells). * P<0.001 for the comparison of presence of ketoconazole compared to untreated cells (white bar).

3.4.8.2. 2T3 mineralisation

To determine the effects of ketoconazole on 2T3 osteoblast mineralisation, 2T3 cells were cultured in osteogenic media containing various concentrations (0.25-80µM) of ketoconazole over 8 days (**Figure 3-8**). Quantification of Alizarin red staining of mineralisation revealed that prolonged treatments with doses of 0.25-5µM of ketoconazole had no effects on mineralisation in 2T3 cells (**Figure 3-8B**). Doses of above 10µM dose dependently suppressed mineralisation, whereby 80µM of ketoconazole abolished 2T3 mineralisation (**Figure 3-8A-B**, 10µM; P<0.05, 20-80µM; P<0.001 respectively).

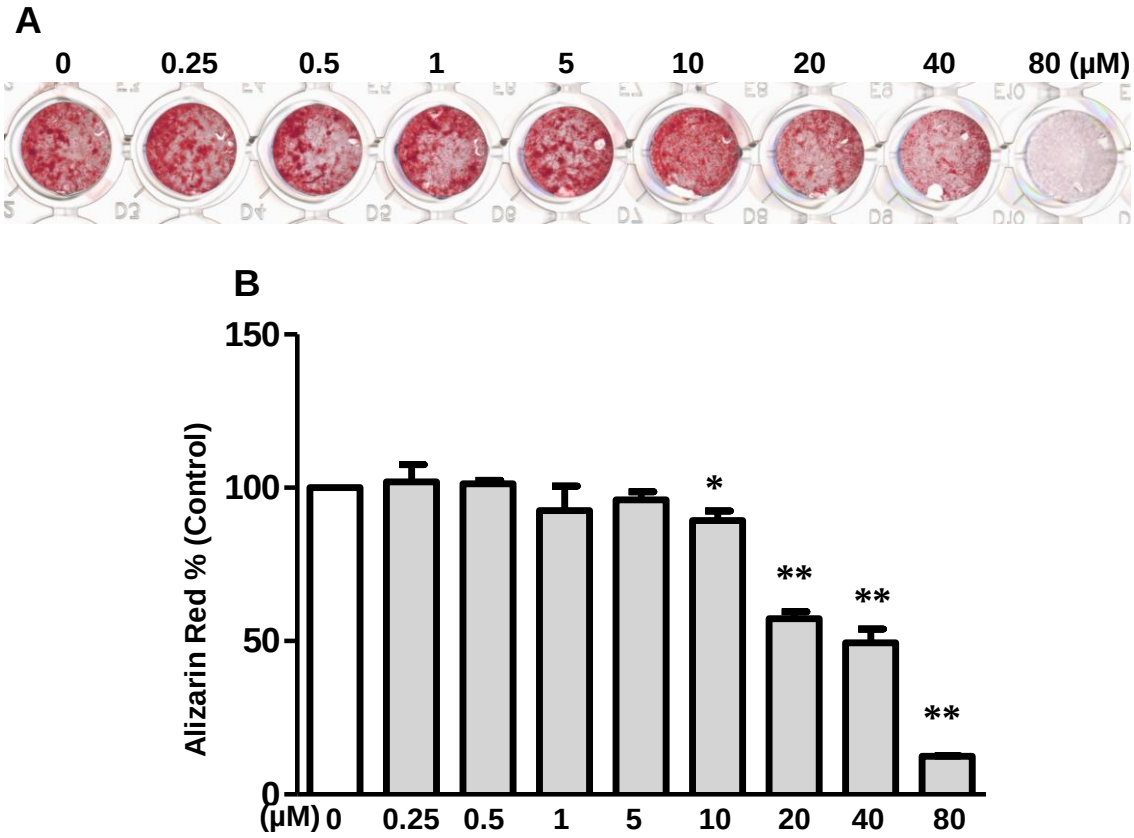


Figure 3-8: High doses of ketoconazole suppress 2T3 mineralisation. (A) Representative images of 2T3 mineralisation in response to 0-80µM ketoconazole over 8 days of culture in osteogenic differentiation medium. (B) Quantification of Alizarin red dye staining on day 8 by 10% acetic acid extraction. 2T3 osteoblast-like cells were cultured at $5x10^3$ in 96 well-plates overnight in DMEM-10%FBS and 8 days in oestrogenic differentiation medium. Data presented as mean ± SEM of quadruple wells, unpaired Student t-test. * P<0.05, and ** P<0.001 for the comparison of ketoconazole (grey) compared to untreated cells in white bar.

3.4.8.3. 2T3 signature genes

When 2T3 cells were cultured in osteogenic media and ketoconazole over 24h, addition of 10µM ketoconazole significantly decreased *CYP27B1* (average 2.1-fold, **Figure 3-9A**, P<0.01), and abolished *CYP24A1* (**Figure 3-9B**, P<0.001) expression compared to untreated cells.

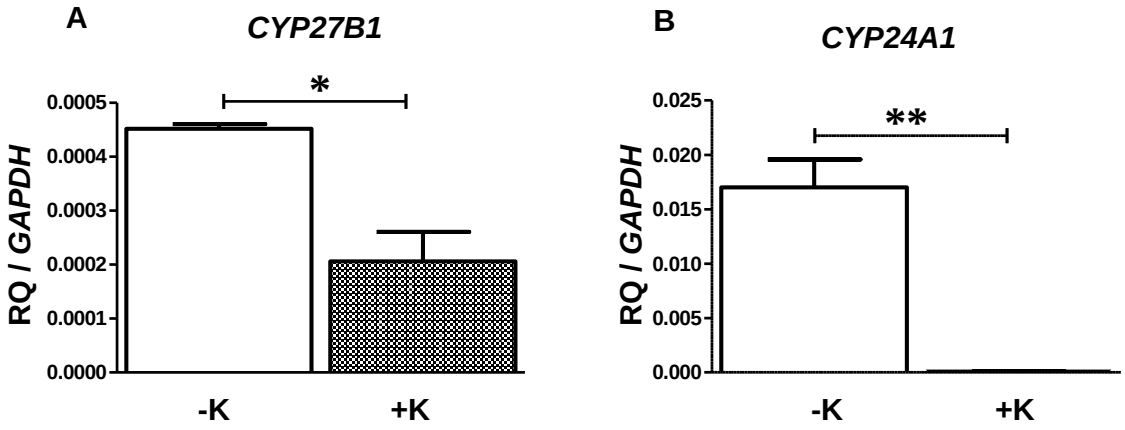


Figure 3-9: 10μM Ketoconazole depleted *CYP27B1* and abolished *CYP24A1* in 2T3 osteoblast-like cells. Effect of ketoconazole on the relative quantity (RQ) of murine 2T3 osteoblast vitamin D pathway genes CYP27B1 (A) and CYP24A1 (B) assessed by QRT-PCR. 2T3 cells were cultured in osteogenic differentiation medium ±10μM ketoconazole. The magnitude of change in expression of each gene is normalised to the house keeping gene *GAPDH*. Values are mean ± S.D. from triplicate reactions, unpaired Student t-test. * P<0.01, and ** P<0.001 for the comparison of presence or absence of ketoconazole (K).

3.4.9. Influence of ketoconazole on the effects of vitamin D metabolites

3.4.9.1. 2T3 ALP activity

To assess the effects of ketoconazole on osteogenic activity stimulated by vitamin D metabolites, 2T3 cells were treated for 6-24 hour with 200nM 25(OH)D_2/D_3 or 0.2nM 1,25(OH)$_2D_2$/D_3, either in the presence or in absence of 10μM ketoconazole. Compared to untreated controls at 24 hour, both 25(OH)D_2 and D_3 significantly (**Figure 3-10A**, P<0.001) up-regulated ALP in the absence of ketoconazole. Similarly, in the absence of ketoconazole, both 1,25(OH)$_2D_2$ and D_3 significantly up-regulated ALP compared to untreated controls at 24 hours (**Figure 3-10B**, P<0.001). In the absence of ketoconazole there was no significant difference between 200nM of 25(OH)D or 0.2nM 1,25(OH)$_2$D

metabolites. Ketoconazole treatment equally diminished the effects of both 25(OH)D_2 and D_3 on ALP activity at 24hour (**Figure 3-10A**: P<0.001 for D_2 and D_3). There was no significant difference between the degrees of inhibition of ALP for 25(OH)D_2 versus 25(OH)D_3 at 24 hour. A similar ALP inhibition by ketoconazole was observed at 24 hour for 1,25$(OH)_2$D metabolites (**Figure 3-10B**). However, the inhibition by ketoconazole was less on the 1,25$(OH)_2D_2$ metabolite compared with the 1,25$(OH)_2D_3$ (P<0.01).

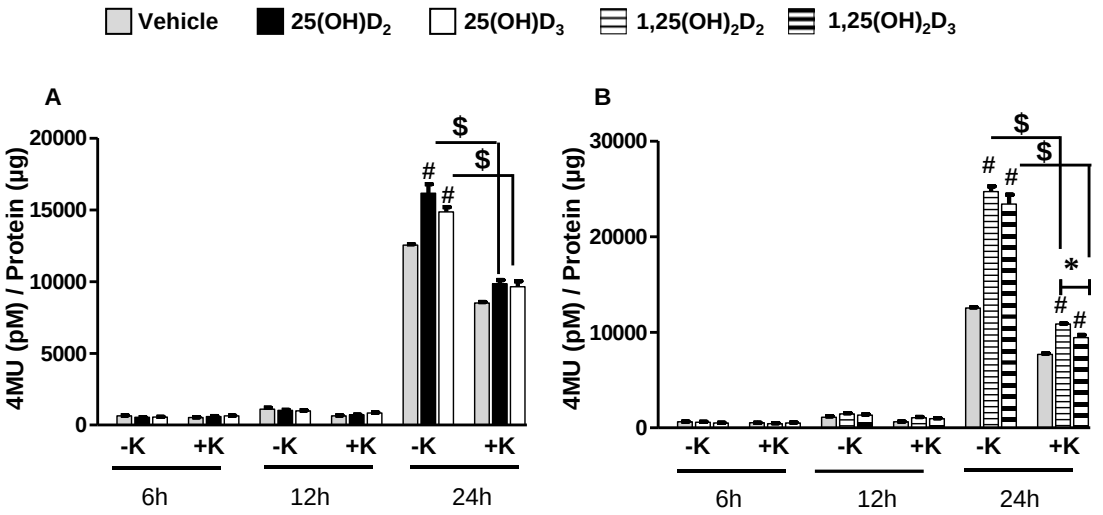


Figure 3-10: ALP expression reduced in response to D_2 and D_3 metabolites in the presence of ketoconazole. Influence of 10µM ketoconazole on the effects of (A) 200nM 25(OH)D_2 or 25(OH)D_3, and (B) 0.2nM 1,25$(OH)_2D_2$ or D3 on 2T3 ALP activities. Values are mean ±SEM (Two-way ANOVA: Bonferroni Post Hoc Test) of 4MU activity (normalised to protein content) of treated cells against untreated control (grey, 6h) from triplicate experiments. * P<0.01 for comparisons between D_2 and D_3 metabolites at 24 h in the presence of ketoconazole, $ P<0.001 for comparisons between D_2 and D_3 metabolites in the presence and absence of ketoconazole at 24 hour, and # P<0.001 comparison of vitamin D treatments to control (grey) at 24 hour in the presence or absence of ketoconazole.

3.4.9.2. 2T3 Osteoblastic signature gene expression

Ketoconazole co-treatment with 25(OH)D_2 had no effect on *OPG* compared to cells treated with ketoconazole alone, whereas D_3 treatments increased *OPG* (**Figure 3-11A**, P<0.001). In the presence of ketoconazole, both 25(OH)D_2 and D_3 equally up-regulated *RANKL* (**Figure 3-11 B**, P<0.001) and *OPN* (**Figure 3-11C**, P<0.01) compared to ketoconazole treated cells. Both 25(OH)D_2 and D_3 in the presence of ketoconazole significantly up-regulated *COL1* (**Figure 3-11D**; P<0.001, D_3>D_2: P<0.001), *CYP24A1* (**Figure 3-11E**, D_2: P<0.01, D_3: P<0.001, D_3>D_2: P<0.01), *CYP27B1* (**Figure 3-11 F**, P<0.01, D_3>D_2: P<0.05) and VDR (**Figure 3-11 G**; P<0.001, D_3>D_2: P<0.01) transcripts compared to ketoconazole treated cells.

Ketoconazole co-treatment increased *OPN* mRNA with 200nM of both 25(OH)D_2 and D_3 (**Figure 3-11C**). Co-treatment with ketoconazole did not enhance the effects of D_2 on *COL1* or *VDR* (**Figure 3-11D&G**) mRNA, but differential responses were observed on mRNA expression of *CYP24A1* (**Figure 3-11E,** enhanced) and *CYP27B1* (**Figure 3-11F,** reduced) in 2T3 cells. The effects of 200nM of 25(OH)D_3 on *COL1* and *CYP24A1* (**Figure 3-11D-E**) were enhanced with addition of ketoconazole at mRNA level, but not *CYP27B1* or *VDR* (**Figure 3-11F-G**). Addition of ketoconazole alone non-specifically reduced *OPG* (**Figure 3-11A**), increased *RANKL* (**Figure 3-11B**) and *OPN* (**Figure 3-11C**), but had no effects on *COL1* (**Figure 3-11D**) or *VDR* (**Figure 3-11G**) transcripts in 2T3 osteoblast-like cells.

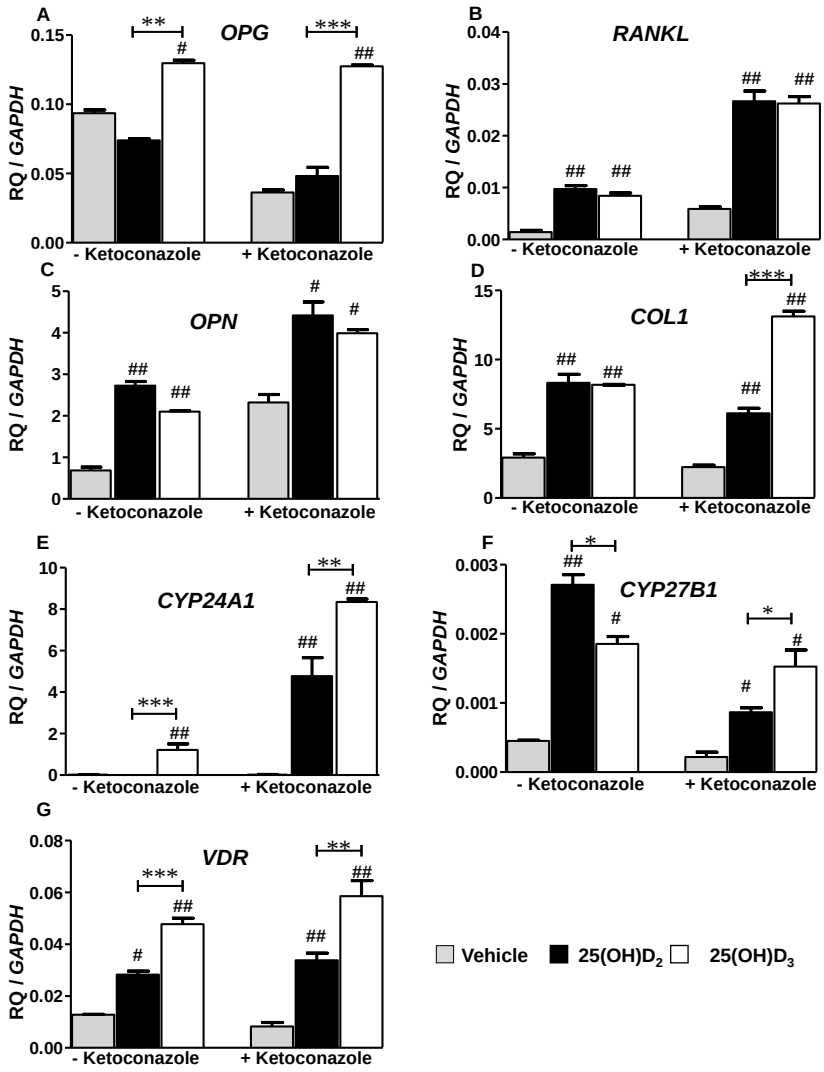


Figure 3-11: 2T3 osteoblastic and vitamin D pathway genes were up-regulated in response to 25(OH)D_2 or 25(OH)D_3 in the presence of ketoconazole. Influence of addition of 10μM ketoconazole on the effects of 200 nM 25(OH)D_2 or 25(OH)D_3, on 2T3 signature genes *OPG* (A), *RANKL* (B), *OPN* (C), *COL1* (D) as well as vitamin D-related genes *CYP24A1* (E), (F) *CYP27B1* and *VDR* (G) were assessed by QRT-PCR, Values are mean ± S.D. from triplicate reactions, (one way Anova, Welch correction). * P<0.05, ** P<0.01, and *** P<0.001 for comparisons between D_2 and D_3 metabolites, and # P<0.01, ## P<0.001 comparison of treatments to control (grey).

3.4.9.3. 2T3 Mineralisation

Addition of 200nM of 25(OH)D_2/D_3 (**Figure3-12A**) or 0.2nM 1,25(OH)$_2D_2$/D_3 (**Figure3-12B**) equally increased mineralisation in the absence of ketoconazole. Co-treatment with ketoconazole and vitamin D metabolites abolished 2T3

mineralisation. Inhibition of mineralisation was greater for D_3 metabolites than D_2 (**Figure3-12A-C**: *P*<0.001).

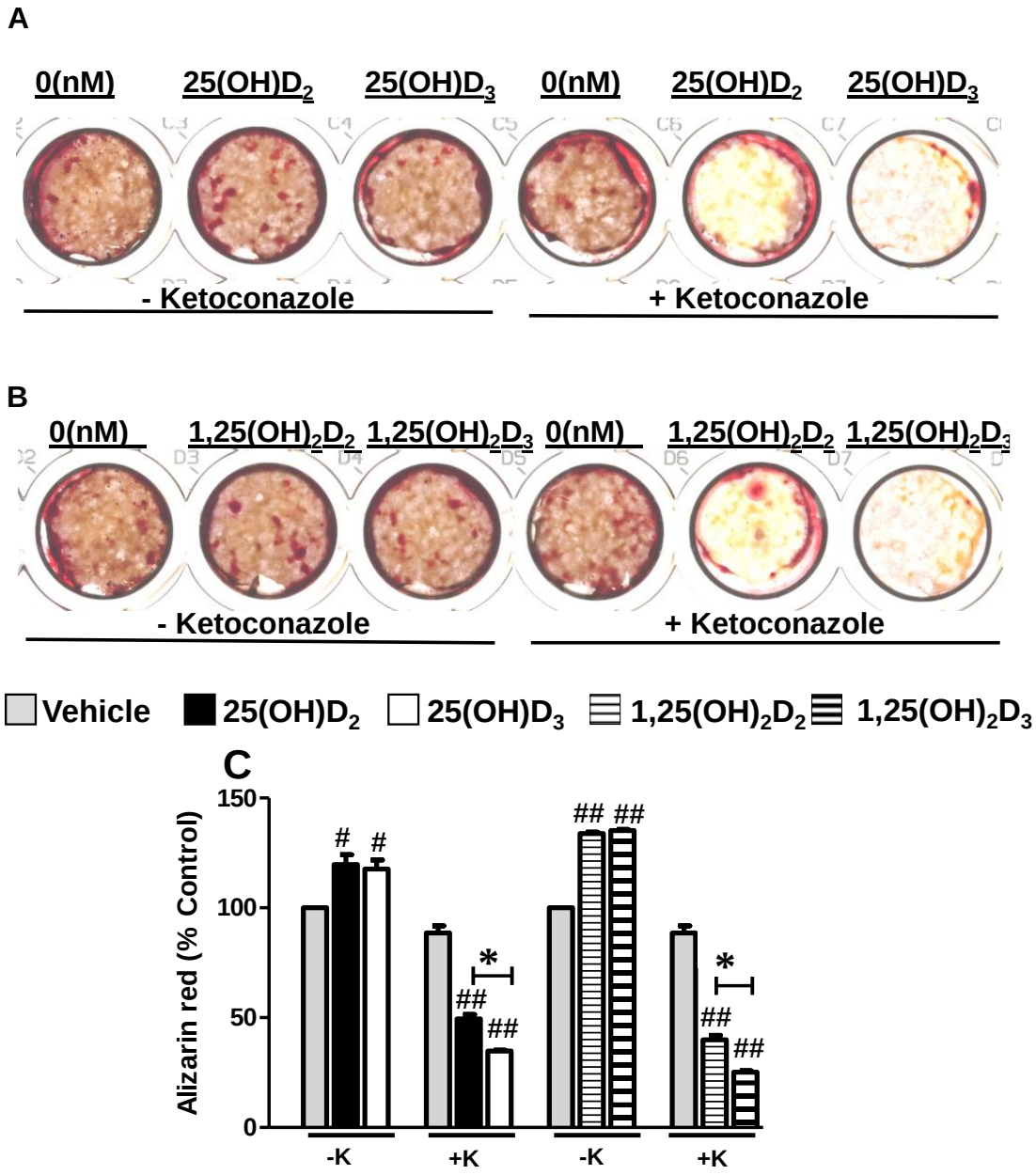


Figure 3-12: 2T3 mineralisation was abolished in response to co-treatment with D_2 or D_3 metabolites and ketoconazole. Representative images of Alizarin red of 2T3 cells treated with 200 nM 25(OH)D_2 / 25(OH)D_3 (A) or (B) 0.2 nM 1,25(OH)$_2D_2$ or 1,25(OH)$_2D_3$ in the presence or absence of ketoconazole over a 6 days mineralisation period, (C) quantified staining of Alizarin red by 10% acetic acid. Values are mean ±SEM (two-way ANOVA, Bonferroni Post Hoc Test) as percentage of untreated controls (grey) from triplicate experiments. * P<0.001 for comparisons between D_2 and D_3 metabolites, and # P<0.05, ## P<0.01 comparison of treatments to control (grey).

3.4.10. Effects of BMP-2 on 2T3 differentiation by vitamin D

In a series of separate experiments, 2T3 cells were further cultured in osteogenic differentiation medium, plus vitamin D, as well as 100ng/ml recombinant mouse BMP-2. Addition of BMP-2 alone accelerated mineralisation in 2T3 osteoblast-like cells over 6 days compared to the absence of BMP-2 (**Figure 3-13**).

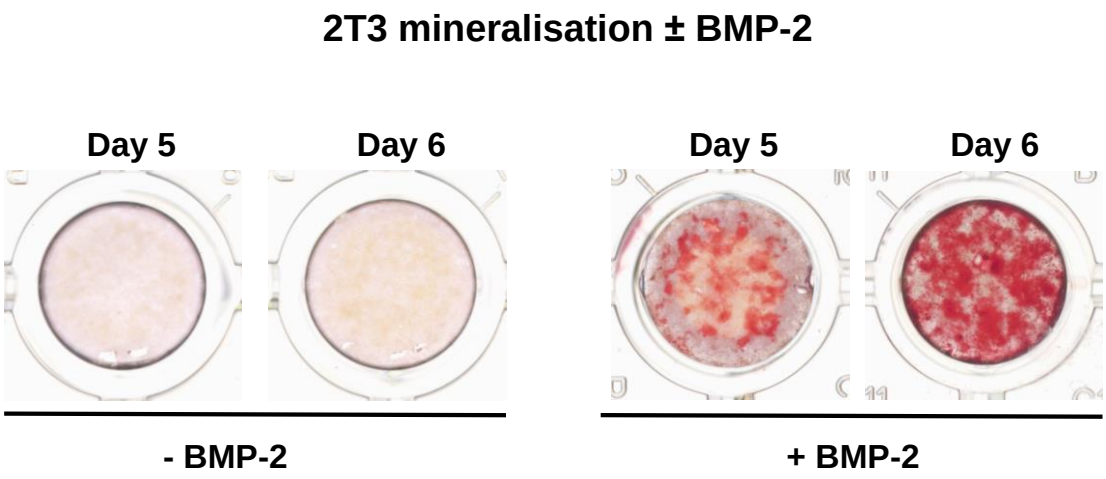


Figure 3-13: 2T3 mineralisation was accelerated by BMP-2. Representative images of 2T3 osteoblast mineralisation in the presence (right) or absence of BMP-2 (left). 2T3 osteoblast-like cells were seeded at $5x10^3$ in 96 well-plates in DMEM-10%FBS until confluency and grown for 5-6 days in osteogenic differentiation medium. Cultures were stopped on days 5 and 6 and stained with Alizarin red dye.

Addition of BMP-2 resulted in an earlier mineralisation on **day 5**, where the increase in mineralized matrix was more evident in response to lower concentrations of 25(OH)D_2 and D_3 (**Figure 3-14A&B**). On day 5; both 50nM of 25(OH)D_2 and 25(OH)D_3 (**Figure 3-15**; $P<0.01$ and $P<0.001$ respectively) increased mineralisation, where 25(OH)D_3 (**Figure 3-14E**: $P<0.01$) were more potent than 25(OH)D_2 counterpart. On day 5 doses of 100nM of either 25(OH)D_2 or D_3 had no stimulatory or inhibitory effects on mineralisation compared to vehicle and doses of greater than 200-1000nM of both 25(OH)D_2 and 25(OH)D_3 significantly suppressed mineralisation to the same extent (**Figure 3-15:** $P<0.001$).

On **day 6** and in the presence of BMP-2, 50nM of either 25(OH)D2 or D_3 equally increased 2T3 mineralisation (**Figure 3-5**: $P>0.001$). Doses of 100nM of either 25(OH)D_2, or D_3 had no effect, whereas doses of ≥ 200nM of 25(OH)D_2 and D_3 equally abolished mineralisation (**Figure 3-5**: $P<0.001$) compared to untreated control.

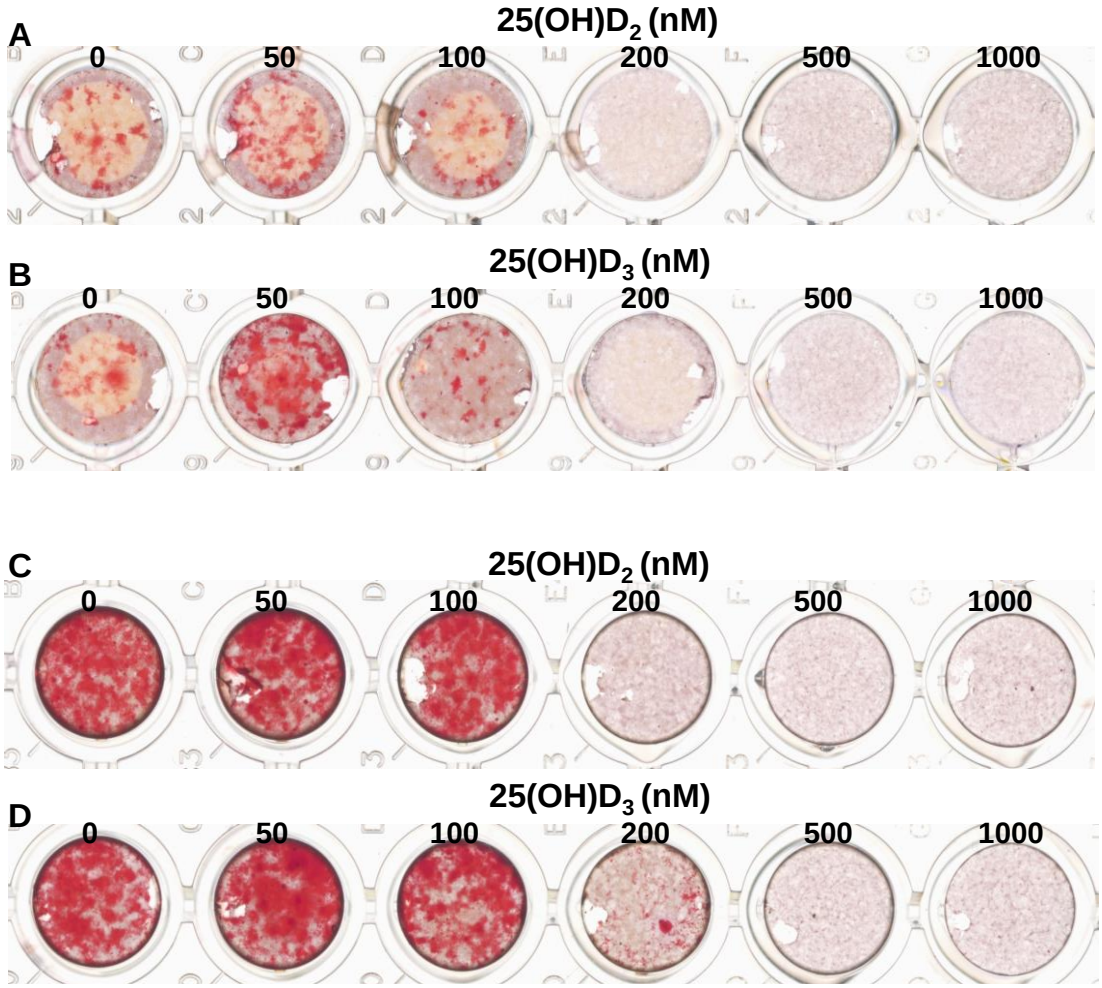


Figure 3-14: 2T3 mineralisation was accelerated in response to 50nM of 25(OH)D_2 and D_3 in the presence of BMP-2. 2T3 cells were cultured in osteogenic differentiation medium (96 well plate format) in the presence of 50-1000nM of 25(OH)D_2 (A) day 5 & (C) day 6, or 25(OH)D_3 (B) day 5 & (D) day 6 as well as BMP-2. Cultures were stopped on days 5 or 6, washed with DPBS, stained with Alizarin red dye, washed with 70% ethanol and deionised water, air dried and scanned with an Epson AX10 scanner. For quantification see **Figure 15**.

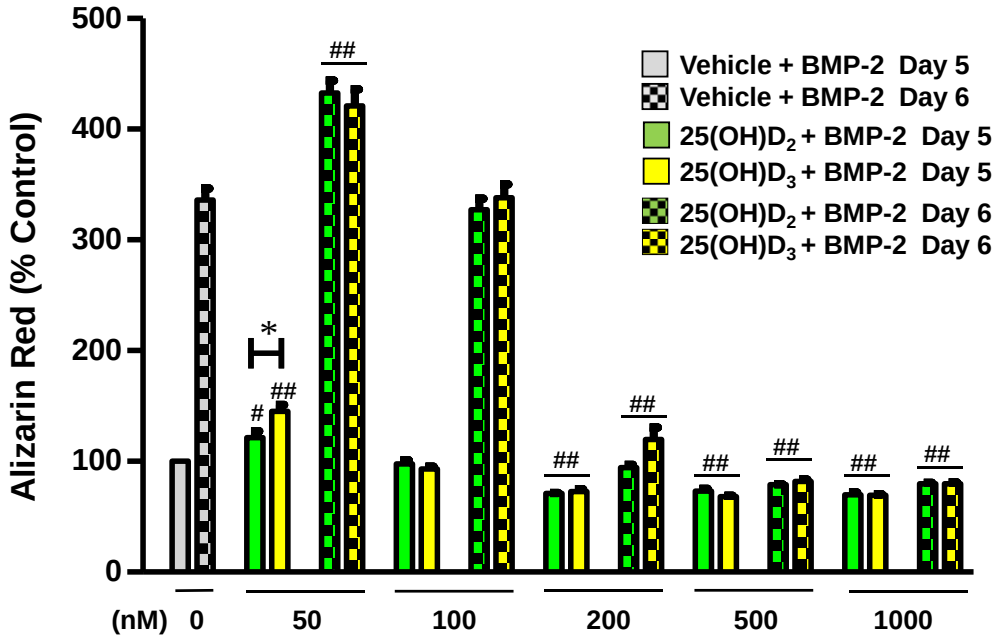


Figure 3-15: Quantified Alizarin red staining of 2T3 cells in response to 25(OH)D_2 and D_3 in the presence of BMP-2 on days 5 and 6. Results of triplicate experiments are presented as percentage of untreated control (grey on day 5) as mean ±SEM assessed by One-way-ANOVA, Tukey Post Hoc Test. * P<0.01 for comparisons between D_2 and D_3 metabolites, and # P<0.01, ## P<0.001 comparison of treatments to control on day 5 and 6. Vitamin D_2 and D_3 treatments on day 5 have been compared to control on day 5 and vitamin D treated cells on day 6 to untreated cells on day 6.Solid line on bars (50nM on day 6 and 200-1000nM of 25(OH)D_2, D_3 on days 5 or 6 represents significant difference of both metabolites to their controls.

On day 5, 0.05nM of the active metabolites 1,25$(OH)_2D_2$ and D_3 (**Figure 3-16A-B**) increased mineralisation (**Figure 17**; P<0.01 and P<0.001 respectively), where 1,25$(OH)_2D_3$ was more potent than its 1,25$(OH)_2D_2$ counterpart (**Figure 3-17**; P<0.001). On day 5, doses of above 0.1nM of 1,25$(OH)_2D_3$ dose-dependently suppressed mineralisation (**Figure 3-17:** 0.1-1nM; P<0.001), whereas doses of ≥0.2nM of 1,25$(OH)_2D_2$ were inhibitory (**Figure 3-17:** 100nM no effects, 200nM P<0.05 and 500-1000nM: P<0.001 respectively). On day 6; doses of 0.05-0.2nM of 1,25$(OH)_2D_2$ had no effects on mineralisation compared to untreated cells, whereas, 0.1-0.2nM of 1,25$(OH)_2D_3$ significantly suppressed

mineralisation (**Figure 3-7:** P<0.001, D_3>D_2: P<0.001). Doses of 0.5-1nM of both 1,25$(OH)_2D_2$ and 1,25$(OH)_2D_3$ equally abolished mineralisation compared to untreated cells (**Figure 3-7:** P<0.001).

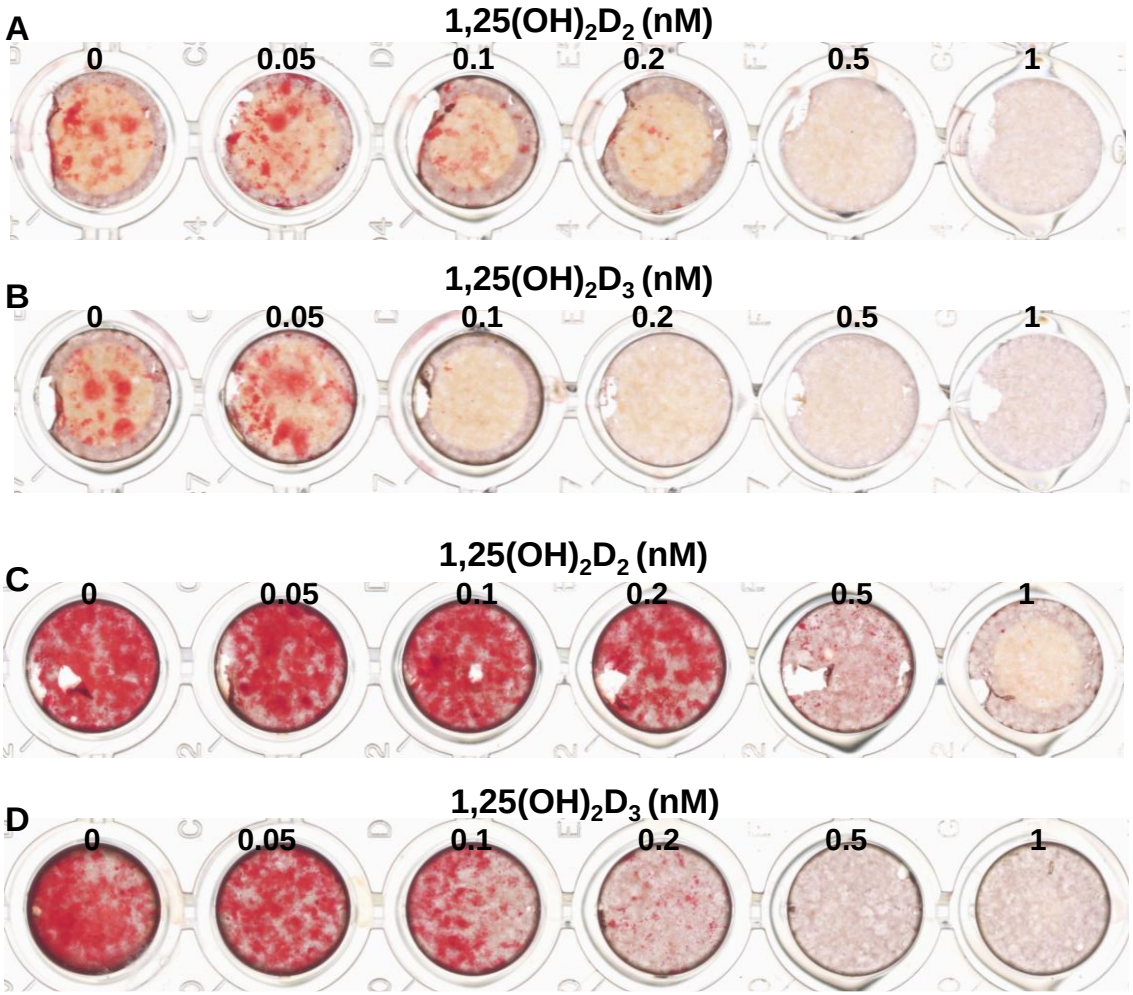


Figure 3-16: 2T3 mineralisation accelerated in response to 0.05nM of 1,25$(OH)_2D_2$ and 1,25$(OH)_2D_3$ active metabolites in the presence of BMP-2. Effects of co-treatment with BMP-2 and vitamin D metabolites on 2T3 osteoblast mineralisation. 2T3 cells were cultured in osteogenic differentiation medium (96 well plate format) in the presence of 0.05-1nM of active metabolites 1,25$(OH)_2D_2$ (A) day 5 & (C) day 6 or 1,25$(OH)_2D_3$ (B) day 5 & (D) day 6 as well as BMP-2. For quantification see **Figure 17**.

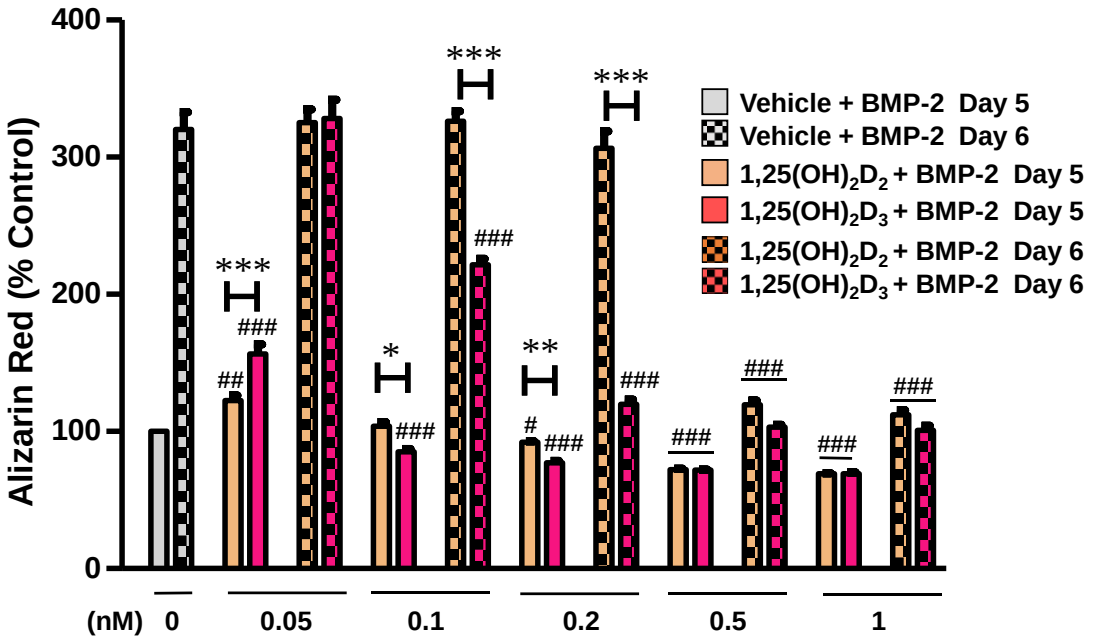


Figure 3-17: Quantified Alizarin red staining of 2T3 cells in response to the active metabolites $1,25(OH)_2D_2$ or D_3 in the presence of BMP-2 on days 5 and 6. Results of triplicate experiments are presented as percentage of untreated controls (grey on day 5) as mean ±SEM assessed by One-way-ANOVA, Tukey Post Hoc Test. * P<0.05, ** P<0.01 and *** P<0.001 for comparisons between D_2 and D_3 metabolites, and # P<0.05, ## P<0.01 and ### P<0.001 comparison of treatments to controls on days 5 or 6. Vitamin D_2 and D_3 active metabolite treatments on day 5 have been compared to control on day 5 and vitamin D treated cells on day 6 to untreated cells on day 6.Solid line on bars 0.5-1nM of $1,25(OH)_2D_2$, D_3 on days 5 and 6 represents significant difference of both metabolites to their controls.

3.5. Discussion

Both vitamin D_2 and D_3 have been widely used in the clinical treatment of rickets [99] and there are numerous studies detailing their comparative effects in raising circulatory levels of 25(OH)D, their DBP affinities, or rate of clearance from the circulation [296, 299, 303]. However, it is less clear whether they have equivalent potencies directly on bone cells. Despite this lack of evidence, vitamin D_3 is widely favoured over D_2 since it is regarded as more clinically potent [299]. Recently, concerns have been raised that vitamin D_3 is associated with a higher risk of vascular calcification than D_2 [359]. It would therefore be useful to know if vitamin D_2 and D_3 are equipotent in their direct effects on bone since vitamin D_2 may be therapeutically less hypercalcemic than D_3 [360].

The first challenge to overcome when designing this study was the choice of cell model. There are numerous mouse and human cell lines that *in vitro* respond to osteogenic medium with increased ALP and other differentiation markers of the osteoblastic lineage [305]. However, most of the cell lines do not proceed to mineralisation. An initial assessment of human MG63, SaOS2 and murine MC3T3 and 2T3 osteoblastic cell lines confirmed ALP up-regulation under osteogenic conditions but only the 2T3 cells mineralised avidly within 8-12 days. The murine 2T3 cell line was selected because this rapid program of differentiation permits high throughput screening of multiple doses of vitamin D metabolites and time points with several vitamin D isoforms. QRT-PCR analysis in the 2T3 cells revealed that, transcripts for *COL1*, *OPN*, *OPG* and *RANKL* were detectable by 24h, and *OCN* by 72h. ALP was typically induced within 48h by

osteogenic medium. The onset of mineralisation in 2T3 cells in the presence of osteogenic differentiation medium was within 8 days.

Comparisons between D_2 and D_3 metabolites on mouse 2T3 cellular differentiation in this chapter were investigated under three different conditions:

- Comparison of D_2 and D_3 effects on 2T3 function
- Influence of ketoconazole on the effects of D_2 and D_3 on 2T3 function
- Comparison of D_2 vs D_3 effects on 2T3 function ±BMP-2

3.5.1. Comparison of D_2 vs D_3 effects on 2T3 function

Vitamin D_2 and D_3 were compared both as native 25(OH)D_2 and 25(OH)D_3 and active metabolites 1,25$(OH)_2D_2$ and 1,25$(OH)_2D_3$ across a physiological to pharmacological dose range of 0-1000nM native vitamin D or 0.05-1nM active metabolite. Consistent with previous literature vitamin D became anti-proliferative in 2T3 at higher doses. However D_2 was consistently less inhibitory than D_3, with inhibition of 2T3 cells from 500-1000nM 25(OH)D_3 versus 500-1000nM 25(OH)D_2 and 0.5-1nM 1,25$(OH)_2D_3$ metabolite versus 0.5-1nM 1,25$(OH)_2D_2$.

ALP activity was strongly induced by both vitamin D_2 and D_3 metabolites in 2T3 cells. ALP activity levels increased a full 24h earlier in 2T3 cells treated with vitamin D metabolites versus osteogenic medium alone for doses of 100-200nM 25(OH)D_2,D_3 and 36 hours for doses of 0.1-0.5nM active metabolite 1,25$(OH)_2D_2$ and D_3. Correlating with effects on cell number, higher doses of native 25(OH)D_2, 25(OH)D_3 and 1,25$(OH)_2D_2$ and 1,25$(OH)_2D_3$ active metabolites inhibited ALP activity in 2T3 cells, with >500nM native D [25(OH)D] and >0.5nM of active

metabolite [1,25$(OH)_2D_3$], where D_3 was significantly more potent than D_2 metabolites.

Inhibitory effects of vitamin D metabolites at high concentrations were however assay specific, so that doses of 500nM of vitamin D_2 that impaired ALP activity successfully induced protein expression of a range of osteoblast differentiation markers. Thus OPN, OCN and VDR were up-regulated in 2T3 cells by 500nM or 0.5nM doses of 25$(OH)D_2$ or 1,25$(OH)_2D_2$. At the protein level, there was a closer correlation of ALP activity with impaired OPN, OCN and VDR protein production within the high dose of 500nM of 25$(OH)D_3$, and 0.5nM of 1,25$(OH)_2D_3$. The inhibitory effects of vitamin D_3 metabolites at high doses are probably as a result of a decrease in cell number as assessed by Alamar blue assay. Alamar blue assay of 2T3 osteoblast cells differentiating in response to 500nM of 25$(OH)D_2$, D_3 or 0.5nM 1,25$(OH)_2D_2$ and D_3 confirmed D_3 metabolites to reduce 2T3 cell number more than D_2 metabolites (**Appendix 7**).

The desired end point of osteoblast activity *in vitro* is mineralisation. This was induced by 200nM and 0.1-0.2nM doses for native 25$(OH)D_2$, 25$(OH)D_3$ and 1,25$(OH)_2D_2$ and 1,25$(OH)_2D_3$ active metabolite in 2T3 cells, but unlike ALP activity there was no acceleration of the timescale for mineralisation. Inhibition of mineralisation was apparent with doses of 500nM of 25$(OH)D_2$, 25$(OH)D_3$ and 0.5nM of 1,25$(OH)_2D_2$ and 1,25$(OH)_2D_3$ active metabolite, but notably D_2 was less toxic than D_3. An equal increase in mineralisation in response to both 200nM of 25$(OH)D_2$ and 25$(OH)D_3$ metabolites was associated with an increase in *COL1* and *OPG* transcript, as well as OPN and OCN at protein levels. Similarly an increase in mineralisation in response to 1,25$(OH)_2D_2$ and 1,25$(OH)_2D_3$ active metabolite was also associated with an increase in OPN and OCN proteins

assessed by Western blotting. It appears that in 2T3 pre-osteoblast cell line total collagen production is not affected in response to physiological doses of 25(OH)D or 1,25$(OH)_2$D active metabolites. Although total collagen content has been shown to be stimulated by 1,25$(OH)_2D_3$ (1nM over 4 hours) in MC3T3 osteoblast cell line [250], data in the current study revealed that high concentrations of 25(OH)D or 1,25$(OH)_2$D metabolites (D_3>D_2) inhibited collagen production over 4 days of culture. Others have shown that *COLI* is decreased in primary mouse osteoblast by 10nM 1,25$(OH)_2D_3$ [348].

Overall, in osteogenic differentiation medium no differences were observed between D_2 and D_3 at physiological doses on mouse osteoblast activity evidenced by mineralisation assays. The limitation of using mouse 2T3 osteoblast-like cells was that they were very sensitive to addition of phosphate, so the majority of osteoblastic signature genes and ALP were up-regulated within hours of culture in osteogenic differentiation medium. Hence the comparisons of vitamin D metabolites were assessed by single dose treatments of vitamin D metabolites in this restricted time frame. In this culture condition, vitamin D_2 and D_3 were equi-potent at physiological concentrations, but had different potencies at pharmacological doses [≥500nM of 25(OH)D and ≥0.5nM of active metabolites: D_3>D_2).

Compared to other studies as summarised in Table 3-1, the concentrations that I used were much lower than other investigators have reported. Mouse 2T3 osteoblastic activities were suppressed by treatments with doses of above 500nM of 25(OH)D or 0.5nM of 1,25$(OH)_2$D. Finally, unlike an increase on the onset of matrix formation and accelerated mineralisation in response to 1,25$(OH)_2D_3$ that has been previously observed in human foetal osteoblast-like

cell (SV-40), vitamin D metabolites did not show such an effect in mouse 2T3 osteoblast-like cells [361]. Compared to previous reports that osteogenic medium alone or in combination with1,25$(OH)_2D_3$ drives primary rat calvaria cells into adipocytes, in the current study osteogenic medium alone or in combination with vitamin D_2 or D_3 metabolites had no effects on adipogenesis in mouse 2T3 osteoblast-like cells [267] (**Appendix 10-11**).

3.5.2. Influence of ketoconazole on the effects of D_2 and D_3 on 2T3 function

In order to further probe the effect of vitamin D metabolites, the vitamin D sensitising drug ketoconazole was used. This drug inhibits CYP27B1 and CYP24A1 enzymes and is used clinically in 2 ways [142, 144]. In the absence of vitamin D supplementation it produces androgen depletion by inhibiting the activating enzymes [362]. However it is also used to enhance the effective dose and duration of vitamin D treatment for prostate cancer because when given together with an excess of 1,25$(OH)_2D_3$ its inhibitory effect on CYP24A1 dominates, resulting in stabilisation of active metabolite levels and cell death of vitamin D resistant prostate cancer cells [140].

In this study I tested relevant doses of ketoconazole on 2 prostate cancer cell lines and demonstrated markedly decreased cell number when ketoconazole was combined with vitamin D (**Appendix 8**). Use of ketoconazole in bone cell culture is complex since when combined with native 25(OH)D it will inhibit both activating (CYP27B1) and inhibitory (CYP24A1) enzymes. However when used

with 1,25$(OH)_2$D active metabolite only the effect on inhibitory CYP24A1 dominates, because activating enzyme (CYP27B1) is not needed.

Initially I tested to be sure that the dose of ketoconazole (10µM) used in the current cell culture experiment did not caused toxicity as assessed by Alamar blue assay over 8 days of culture. Secondly I further confirmed the previous finding that 10µM ketoconazole selectively inhibits *CYP27B1* and *CYP24A1* up-regulation using QRT-PCR.

Overall, single dose treatments with ketoconazole displayed marked enhancement of the effect of 200nM of 25$(OH)D_2$ and 25$(OH)D_3$ metabolites, increasing the induction of *OPN* and *CYP24A1* transcripts, but at protein levels ALP was suppressed. Prolonged treatments with ketoconazole and 200nM of 25$(OH)D_2$, 25$(OH)D_3$ or 0.2nM of their active metabolites abolished mineralisation of 2T3, with D_3 being more potent than D_2 metabolites.

3.5.3. Comparison of D_2 vs D_3 effects on 2T3 function ±BMP-2

In the current studies, both 25$(OH)D_2$, 25$(OH)D_3$ and 1,25$(OH)_2D_2$ and 1,25$(OH)_2D_3$ active metabolites treatments caused an up-regulation of VDR protein as assessed by Western blotting on day 8. Binding of vitamin D to its receptor VDR represents an important molecular event that results in biological action of vitamin D in target cells. A few studies have shown higher binding affinities of D_3 metabolites to VDR than D_2 counterparts [299].

In addition to the systemic effects of 1,25$(OH)_2D_3$ on calcium and phosphate homeostasis, 1,25$(OH)_2D_3$ has been shown to directly affect mouse bone tissue development [363]. Murine osteoblasts possess two receptors for rapid

responses to $1,25(OH)_2D_3$; the traditional VDR , as well as protein disulphide isomerase family A member 3 (Pdia3) [364]. Both of these receptors have been shown to contribute to a rapid response to $1,25(OH)_2D_3$, resulting in an accelerated osteoblastic gene up-regulation. Previous studies *in vivo* or on the established mouse osteoblast cell line MC3T3 demonstrated that vitamin D_3, co-treated with BMP-2, resulted in an accelerated synergistic effect on mineralisation due to a cross talk between vitamin D_3 and BMP-2 on mouse osteoblastogenesis involving both VDR and Pdia3 [357] [358].

In order to further investigate whether the combination of vitamin D and BMP-2 in the current experimental design on mineralisation in mouse 2T3 cells would facilitate the comparison of vitamin D metabolites mineralisation assays were conducted using co-treatment with BMP-2.

In the presence of BMP-2, both D_2 and D_3 at lowest physiological doses (D_3>D_2) resulted in an earlier onset of mineralisation on day 5. Co-treatments with vitamin D_2 and D_3 metabolites on 2T3 mineralisation assay revealed that vitamin D_3 metabolites were more potent both at physiological doses of 50nM of $25(OH)D_3$ and 0.05nM of $1,25(OH)_2D_3$ on induction of mineralisation as well as more potent inhibitor of mineralisation with pharmacological doses of 0.1-0.2nM of $1,25(OH)_2D_3$ active metabolites. Compared to initial experiments (section 3.5.1), where in the absence BMP-2 doses of 200nM of either $25(OH)D_2$, D_3 equally increased mineralisation, addition of BMP-2 and doses of >200nM of either $25(OH)D_2$, D_3 metabolite equally abolished 2T3 mineralisation. Interestingly, the doses of 0.1nM of $1,25(OH)_2D_2$ that previously increased mineralisation in the absence of BMP-2 had no effect on mineralisation in the presence of BMP-2, whereas similar does of $1,25(OH)_2D_3$ (0.1nM) abolished mineralisation.

Based on current findings in this Chapter, it could be postulated that the higher binding affinities of D_3 metabolites to VDR in 2T3 osteoblast cells is probably one of the main causes of such an accelerated response to vitamin D_3 metabolites compared with D_2 counterparts. However although this is much discussed in the literature, there is only one publication on comparisons of D_2 and D_3 metabolites to VDR from rat thymus [356] which in fact shows similar binding affinity. The fact that there are structural differences between D_2 and D_3 metabolites (presence of an extra methyl group on carbon 24 and a double bound on carbon 22-23 in D_2) [297] suggests more work should be done to clarify this.

The effects of vitamin D_3 on mouse osteoblast activity in previous studies were shown to be both stimulatory as well as inhibitory. Literature review (**Table 3-1**) revealed that supra-physiological doses of 1, 10 and 100nM of $1,25(OH)_2D_3$ have been used to study osteoblast cellular activities, the doses that I found to be potent inhibitors of ALP or mineralisation in mouse cells.

As mentioned earlier, assessment of osteoblastic markers or activity was conducted over hours of stimulation with vitamin D_3, using MC3T3 mouse cell line [250, 348]. However, in my assessments I not only demonstrated the comparisons at transcripts and protein levels, I also showed effects of vitamin D metabolites on mouse 2T3 mineralisation.

Overall, vitamin D_2 and D_3 metabolites in the presence of BMP-2 affected mineralisation differentially suggesting that different potencies exist between the vitamin D metabolites. Although it would have been very interesting to further explore the synergy between BMP-2 and D_2 vs. D_3, time and budget precluded further studies. So in the next Chapter I will compare D_2 and D_3 metabolites on

human primary cells, where the timing will be more representative to the human *in vivo* situation.

In conclusion, I have demonstrated that vitamin D_2 and its active metabolite largely match vitamin D_3 in terms of osteogenic activity. While a slightly higher dose of D_2 is needed to induce the same level of osteoblastic function, this may be counterbalanced by the reduced toxicity. This was consistently observed across multiple different assays and also in combination with ketoconazole and may prove clinically useful in the combined treatment setting or in patients at risk of vascular calcification.

CHAPTER4: D_2 vs D_3 EFFECTS ON HUMAN PRIMARY OSTEOBLAST ACTIVITIES

4. D_2 vs D_3 EFFECTS ON PRIMARY HUMAN OSTEOBLAST ACTIVITIES

4.1. Introduction

Human MSCs have the capabilities to differentiate into lineages such as osteoblasts, adipocytes, chondrocytes, myoblasts and fibroblasts [175, 365]. Currently there are few human established osteoblast-like cells lines such as MG-63, SaOS-2 and SV-40 immortalised primary cells for *in vitro* studies [305]. However in recent years the use of human primary osteoblasts derived from BMSCs or cancellous bone has increased, as they are more reflective of the human in *vivo* response [305]. Although concerns have been raised about heterogeneity of primary human cells, these cells are more relevant to human clinical studies [366]. Furthermore, as mentioned earlier in Chapter 2 human primary cells are known to exhibit slow proliferation , reduced osteoblast number but not function when differentiated in *in vitro* due to the “Hayflick limit” on replicative capacity [315].

The effects of $1,25(OH)_2D_3$ on human osteoblast differentiation in *in vitro* studies is reported to be stimulatory, both at transcript and protein levels as well as mineralisation. Similar to rodent osteoblasts, it has been shown that well-characterized human primary osteoblasts and established human osteoblast-like cells express *VDR*, *CYP27B1* and *CYP24*A1 [148]. When these cells are incubated with $25(OH)D_3$, they secrete detectable levels of $1,25(OH)_2D_3$ [148, 150, 151, 243]. Most studies involving the effects of vitamin D_3 on human osteoblasts have involved treatment with $1,25(OH)_2D_3$ active hormone. **Table 4-1** summarizes the effect of $1,25(OH)_2D_3$ on established osteoblast-like cell lines

MG-63, SV-40-immortalised and human primary osteoblasts derived from bone biopsies or bone marrow. Similar to other non-skeletal cell lines, $1,25(OH)_2D_3$ has been shown to inhibit cellular proliferation of human primary osteoblasts as well as established osteoblast-like cell lines (**Table 4-1**).

$1,25(OH)_2D_3$ has been shown to stimulate *ALP* mRNA and protein in hBMSCs [255]. $1,25(OH)_2D_3$ also stimulates the synthesis of collagen type I (both Iα1 & Iα2) in human primary osteoblasts both at mRNA and protein levels [254, 257]. *OPN* mRNA expression and protein up-regulation by $1,25(OH)_2D_3$ in hBMSCs and MG-63 osteoblasts have been shown to be stimulated [242]. OCN as the late osteoblast differentiation marker has been shown to be strongly induced in human primary osteoblasts at mRNA and protein levels [242].

Table 4-1
Summary of the effects of vitamin D_3 metabolites on established human osteoblast-like cells and primary osteoblasts at transcript level (in italics), protein level (non-italics) and mineralisation in *in vitro* studies. +K stands for addition of ketoconazole, min stands for mineralisation

Human	Metabolite	Dose	Effect	Ref
MG63	$1,25(OH)_2D_3$	100nM	↑*VDR*, ↓cell number	[367]
MG63	$1,25(OH)_2D_3$	10nM	↑CYP24, ↑OCN	[368]
MG63	$1,25(OH)_2D_3$	100nM	↑VDR,↑ALP,↑OCN,↓cell number	[369]
MG63	$1,25(OH)_2D_3$	1-100nM	↓ALP, ↑OCN	[370]
MG63	$1,25(OH)_2D_3$	>1nM	↑ALP	[251]
MG-63	$25(OH)D_3$	900nM	↑CYP24	[148]
SV-40	$1,25(OH)_2D_3$	10nM	↑ALP, ↑min, ↑*OCN*	[151]
SV-40	25(OH)D3	1000nM	↑min, ↑*OCN*, ↑*CYP24*	[151]
SV-40	$25(OH)D_3$	200nM+k	↓CYP24, ↓ALP, ↓OCN, ↓ CYP27	[151]
SV-40	$25(OH)D_3$	500nM+k	No 1,25(OH)2D3 (HPLC),	[151]
SV-40	$1,25(OH)_2D_3$	10nM	↑*ALP*,↑*OCN*,↑*OPN*,↑ min	[371]
SV-40	$24,25(OH)_2D_3$	100nM	↑ALP, ↑min	[372]
SV-40	$1,25(OH)_2D_3$	10nM	↑ALP, ↑min	[361, 372]
Primary	$25(OH)D_3$	100nM	↑OCN, ↑OPN, ↑RANKL	[148]
Primary	$1,25(OH)_2D_3$	100nM	↑ALP, ↑col, ↓cell number	[373]
Primary	$1,25(OH)_2D_3$	10-100nM	↓cell number, ↑ALP	[374]
Primary	$1,25(OH)_2D_3$	10nM	↑min	[265]
Primary	$1,25(OH)_2D_3$	10nM	↑OCN, ↑RANKL, ↑OPG	[375]
Primary	$1,25(OH)_2D_3$	100nM	↑min, ↑ Col, ↑ALP	[376]
Primary	$1,25(OH)_2D_3$	1nM	↓CYP27, ↓VDR, ↑CYP24, ↑OPN	[148]
Primary	$1,25(OH)_2D_3$	100nM	↓CYP27	[148]
Primary	25(OH)D3	1000nM	↓CYP27	[148]
Primary	$1,25(OH)_2D_3$	10nM	↑ALP	[251]
Primary	$1,25(OH)_2D_3$	0.01-10nM	↑ALP, ↑CYP24	[377]
Primary	$25(OH)D_3$	0.01-10nM	↑ALP, ↑ CYP27(10nM)	[377]
Primary	$25(OH)D_3$	1000nM+k	↓CYP27	[377]
Primary	$1,25(OH)_2D_3$	10-100nM	↓cell number, ↑*CYP24*, ↑*ALP*,	[150]
Primary	$25(OH)D_3$	100nM	no change cell number & *CYP24*	[150]
Primary	$25(OH)D_3$	1000nM	↑CYP24, ↑ALP, ↑BSP	[150]
Primary	$25(OH)D_3$	1000nM+k	↓ALP, ↓BSP	[150]
Primary	$25(OH)D_3$	10nM+k	↓ CYP27	[145]
Primary	$1,25(OH)_2D_2$	100nM	↑*OCN*, ↑ALP, ↑min	[270]

Silencing CYP27B1 by siRNA, or CYP27B1 depletion by ketoconazole in human BMSCs and human SV-40 cell line, inhibited the production of $1,25(OH)_2D_3$ in $25(OH)D_3$ treated cells [145, 151]. The inhibition of the metabolism of $25(OH)D_3$ into $1,25(OH)_2D_3$ coincided with reduction of human osteoblast markers ALP protein and *OCN* mRNA [151]. Ketoconazole also has been used to inhibit CYP24A1 activities in vitamin D assays as previously described [48]. $1,25(OH)_2D_3$ co-treatment with ketoconazole has been shown to increase the cellular responsiveness to $1,25(OH)_2D_3$ [144]. Ketoconazole–mediated inhibition of CYP24A1 has also been shown to be associated with 40% increase in up-regulation of VDR [144] .

It is interesting to note that in both **Table 3-1** (in Chapter 3) and **Table 4-1** almost all our knowledge on the effects of vitamin D on osteoblast function is based on treatments with $1,25(OH)_2D_3$ in rodent and human established or primary osteoblast cells and the majority of the studies focus on the doses of 1, 10 and 100nM of $1,25(OH)_2D_3$. Literature review revealed that no-one has looked into the effects of vitamin D_2 metabolites on human osteoblasts.

4.2. Aims and objectives

The aim of this Chapter is to compare the dose-response potencies of various concentrations of vitamin D_2 and D_3 metabolites on human primary osteoblast activities. For this purpose human bone marrow stromal cells will be maintained in osteoblast differentiation medium and the effects of physiological and pharmacological doses of vitamin D metabolites will be compared with respect to ALP and mineralisation activities.

The main objective of this Chapter is to determine whether vitamin D_2 and D_3 metabolites exert similar effects on human osteoblasts to those observed in murine 2T3 osteoblast-like cells.

4.3. Materials and Methods

Unless otherwise noted the same reagents and methods have been used as previously described in Chapter 2.

4.3.1. Osteoblast cell number

For routine cell culture commercially available hBMSCs (Lonza Group Ltd: passage 1-3) were cultured in αMEM-10%HI-FBS in T75 flasks. Cells were seeded in multiple 96-well plates at $5x10^3$ cell density per well in replicates in the presence or absence of different concentrations of $25(OH)D_2$ and $25(OH)D_3$ (50-1000nM) and their active metabolites $1,25(OH)_2D_2$ or $1,25(OH)_2D_3$ (0.05-10nM) for 24 h or ± 0.25-80μM ketoconazole in osteogenic differentiation medium every other day for 7 days. Upon completion of the assays 10% Alamar blue™ solution reagent was added to the wells and incubated for 3 hours under standard tissue culture conditions. Plates were removed and assayed at 490nm using a BMG FluorStar Optima plate reader.

4.3.2. Alkaline phosphatase activity

HBMSCs were re-plated at $2x10^4$ into 24/48 well plates in replicates in their culture medium of αMEM-10%HI-FBS to reach confluence, when differentiation

was induced by osteogenic differentiation medium[13] in the presence or absence of 25(OH)D_2 and 25(OH)D_3 (100-1000nM) and their active metabolites 1,25(OH)$_2D_2$ or 1,25(OH)$_2D_3$ (0.01-10nM) and ± 10µM ketoconazole. HBMSCs were cultured for 7 days, during which cells were treated every other day for up to 7 days. Upon completion of treatment duration, ALP enzymatic activities of each treated condition was determined fluorometrically as previously described in Chapter 2.

4.3.3. Mineralisation

For mineralisation assays, cells were plated 2x10^4 in 96 well plates in replicates and cultured in their culture medium (αMEM-10%HI-FBS) to confluence. Cells on this day (day zero) were introduced to osteogenic differentiation medium and presence of 100-1000nM concentrations of 25(OH)D_2, 25(OH)D_3 or 0.01-10nM of 1,25(OH)$_2D_2$ and 1,25(OH)$_2D_3$ or ±10µM ketoconazole and media was replaced every other day up to 28 days. On completion of the assays cultures were stopped and stained with Alizarin red dye and treatment groups were quantified by acetic acid extraction.

[13] **Osteogenic differentiation medium:** culture medium (αMEM-10% HI-FBS) supplemented with 10mM β-glycerophosphate disodium salt, 50µg/ml L-ascorbic acid 2-phosphate

4.4. Results

4.4.1. Effect of vitamin D metabolites on cell number

HBMSC numbers decreased in response to both 25(OH)D_2 and D_3 at ≥500nM for D_2 and ≥200nM for D_3 (**Figure 4-1A**). At 200nM concentration, 25(OH)D_3 was more potent in arresting proliferation than 25(OH)D_2 ($P<0.01$). HBMSC numbers showed only a small trend of a decrease as a result of treatment with 1,25$(OH)_2D_3$ metabolites ($P<0.05$ only for 1,25$(OH)_2D_3$ at 1nM; **Figure 4-1B**).

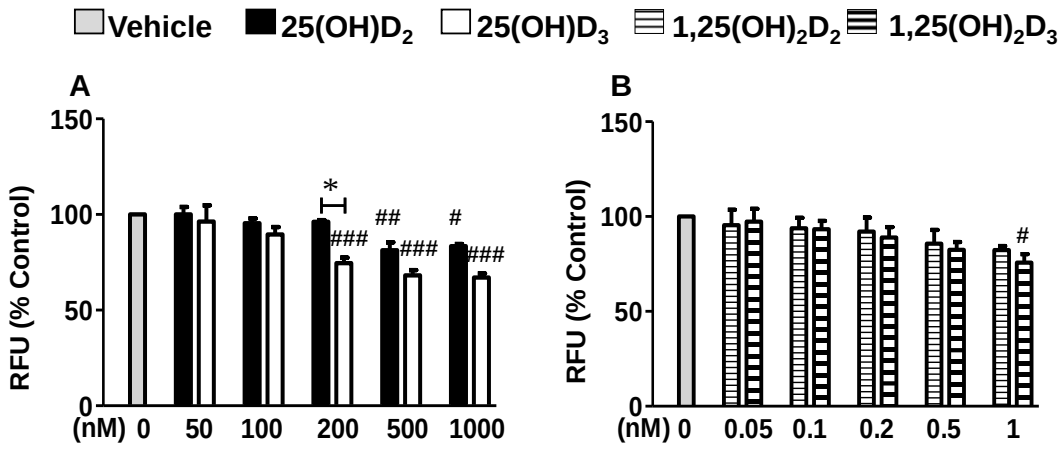


Figure 4-1: Vitamin D_3 metabolites are more potent than D_2 in reducing hBMSC number. Comparative effects of (A): 25(OH)D_2, 25(OH)D_3, (B): 1,25$(OH)_2D_2$ and 1,25$(OH)_2D_3$ on hBMSC cell number. HBMSCs were treated over 24 h with 50-1000nM of 25(OH)D_2/D_3 and 0.05-1nM of their active metabolites 1,25$(OH)_2D_2$ /D_3. Cell number was measure by Alamar blue™ assay. Results of triplicate experiments are presented as percentage of relative fluorescence unit (RFU) of treated cells against untreated controls (grey) as mean ±SEM (non-parametric Kruskal Wallis test). * $P<0.01$ for comparisons between D_2 and D_3 metabolites, and # $P<0.05$, ## $P<0.01$, ### $P<0.001$ comparison between treatments to controls (grey).

4.4.2. Effect of vitamin D metabolites on ALP activity

When hBMSCs were cultured in the presence of osteogenic differentiation medium there was an induction of ALP activity after 7 days of treatment with 500-1000nM 25(OH)D_2 and 25(OH)D_3 ($P<0.001$ for both D_2 and D_3 versus controls; **Figure 4-2A**). After 7 days of treatment, 1,25(OH)$_2D_2$ and 1,25(OH)$_2D_3$ increased ALP activity, but only at a dose of 10nM ($P<0.001$ for both 1,25(OH)$_2D_2$ and D_3 versus control (**Figure 4-2B**). Both 25(OH)D_3 (at 500-1000nM) and 1,25(OH)$_2D_3$ metabolites (at 10nM) were more potent than their D_2 counterparts in ALP up-regulation in hBMSCs ($P<0.001$).

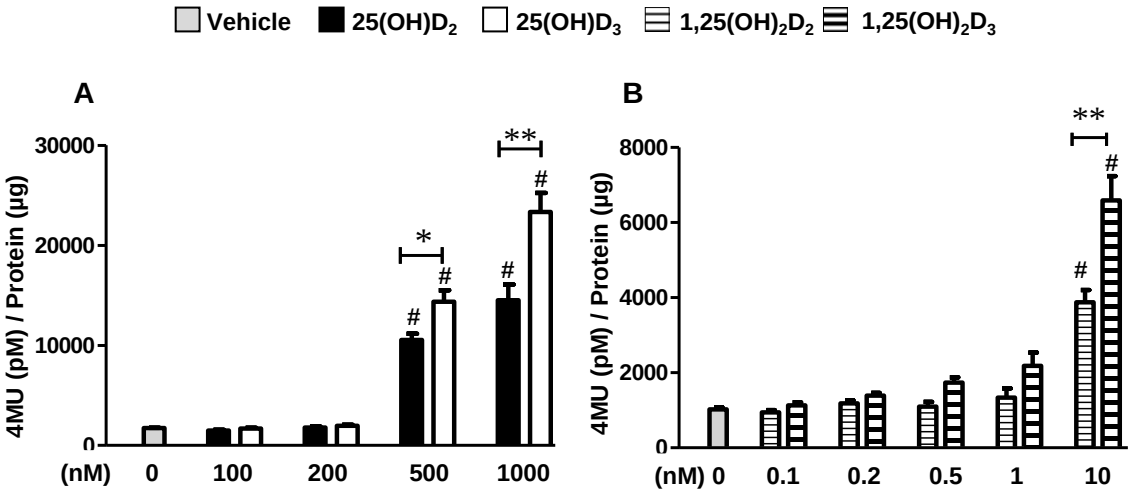
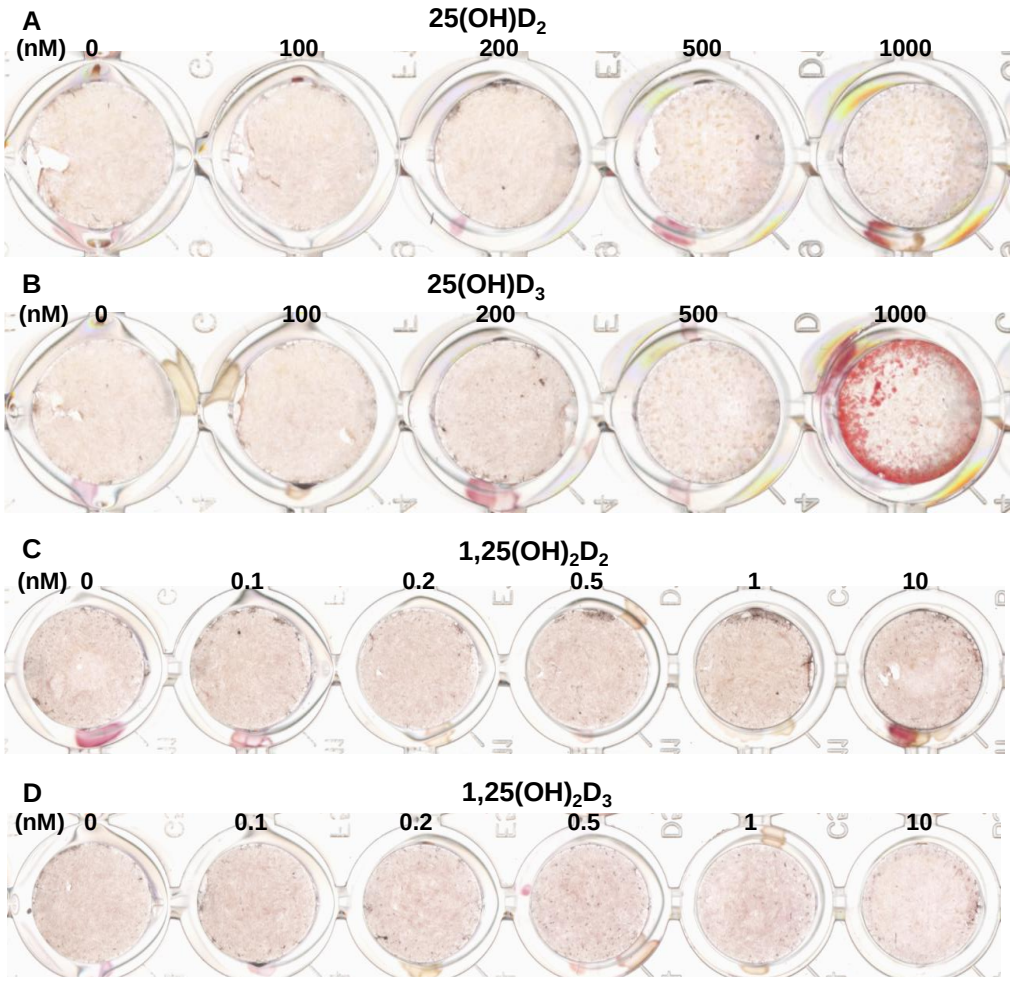


Figure 4-2: Vitamin D_3 metabolites are more potent than D_2 in up-regulation of ALP in hBMSCs. Comparative effects of vitamin D_2 and D_3 metabolites on ALP enzymatic activity in human osteoblasts. HBMSCs were grown in 10% HI-FBS for 4 days until confluency (day zero) and treated with (A): 100-1000nM concentrations of 25(OH)D_2 / D_3 metabolites or (B): 0.1-10nM of 1,25(OH)$_2D_2$/D_3 every other day (days; 0, 2, 4 and 6). Culture was stopped on day 7 and ALP activity was measured. ALP activities were normalised to the total protein content of each treatment. Results of triplicate experiments are presented as 4MU (pM) activity of treated versus untreated controls as mean ±SEM (non-parametric Kruskal Wallis test). * $P<0.01$ and ** $P<0.001$ for comparisons between D_2 and D_3 metabolites, and # $P<0.001$ for comparisons between treatments and controls (grey).

4.4.2.1. Effect of vitamin D metabolites on Mineralisation

Concentrations of 100 to 500nM of either 25(OH)D_2 or D_3 (**Figure 4-3A-B**) had no effect on the induction of mineralisation in hBMSCs. Mineralisation was only significantly increased in hBMSCs in the presence of 1000nM 25(OH)D_3 (**Figure 4-3 B&E**, P<0.001) versus 25(OH)D_2 and control. None of the active metabolites 1,25(OH)$_2D_2$/D_3 were capable of inducing mineralisation in hBMSCs as indicated in **Figure 4-3C-D &F**.



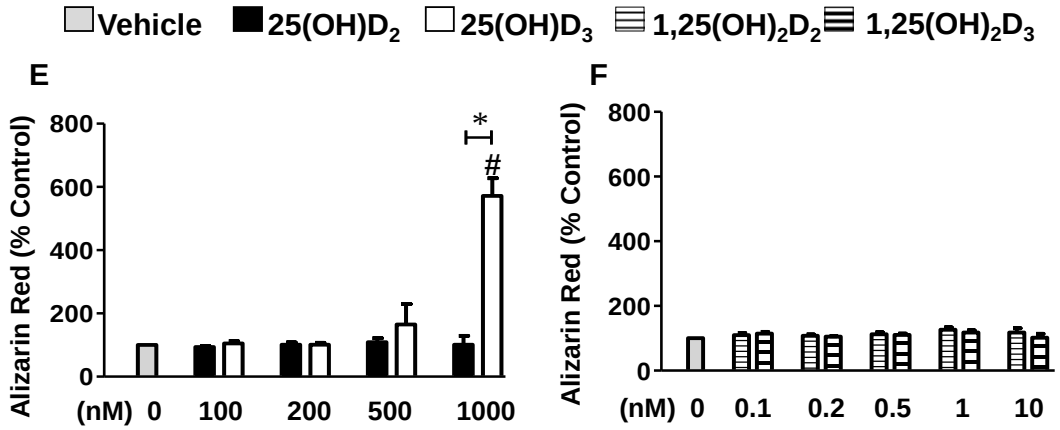


Figure 4-3: Mineralisation of hBMSCs within 28 days only occurred in the presence of 25(OH)D_3. Comparison of the effects of vitamin D metabolites on mineralisation in hBMSCs. HBMSCs cells were cultured in the presence of osteogenic differentiation medium in multiple 96 well plates for 28 days in the presence of vitamin D_2 / D_3 metabolites. On day 28, cells were washed with DPBS, fixed with ethanol, stained with Alizarin red and photographed. Representative images of Alizarin red staining of; hBMSCs in the presence of 100-1000nM of (A) 25(OH)D_2 , (B) 25(OH)D_3, or 0.1-10nM of (C) 1,25$(OH)_2D_2$ and (D) 1,25$(OH)_2D_3$. Lower panels show quantified Alizarin red staining by acetic acid extraction of treatments with 10-1000nM of (E) 25(OH)D_2 and 25(OH)D_3 or (F) 0.1-10nM of 1,25$(OH)_2D_2$ or 1,25$(OH)_2D_3$. Data are presented as percentage of untreated controls as mean ±SEM, (non-parametric Kruskal Wallis test) n=8. * P<0.001 for comparisons between D_2 and D_3 metabolites, and # P<0.001 for comparisons between treatments and controls (grey).

4.4.3. Influence of ketoconazole on stimulation of human osteoblast differentiation by vitamin D metabolites

4.4.3.1. Effects of ketoconazole on hBMSCs

Addition of 0.25-20µM ketoconazole compared to vehicle had no effect on hBMSC number over 7 days as assessed by Alamar Blue (**Figure 4-4A**). However doses of 40-80µM of ketoconazole significantly reduced cell number (**Figure 4-4A**: P<0.01). Treatments of hBMSCs with 10µM ketoconazole over 7 days had no effects on ALP up-regulation (**Figure 4-4B**).

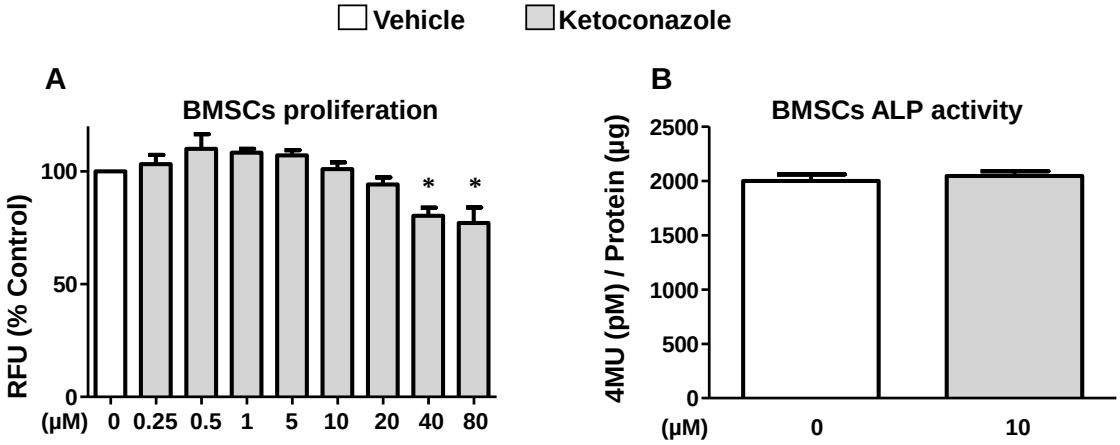


Figure 4-4: 10μM ketoconazole had no effects on hBMSC number or ALP over 7 days. (A) HBMSCs were treated with 0.25-80μM ketoconazole or vehicle (DMSO) over 7days and cell number was assessed by Alamar Blue™ assay as described earlier. (B) HBMSCs were cultured in αMEM-10%HI-FBS until confluency, then medium was replaced with osteogenic differentiation medium and presence or absence 10μM ketoconazole or DMSO as vehicle every other day up to 7days. ALP activities were assessed enzymatically as described earlier. Data presented as mean ±SEM (Student t-test) of percentage of RFU of ketoconazole treated cells versus untreated cells in (A) from 6 wells and 4MU (pM) enzymatic activity of cells treated with 10μM ketoconazole versus untreated cells (white bar) in (B) from quadruple wells. * P<0.01 for the comparison of presence of ketoconazole compared to untreated cells (white bar).

4.4.3.2. ALP activity in response to D_2 and D_3 metabolites ± ketoconazole

Ketoconazole did not alter the enhanced ALP activities seen in hBMSCs with 100-1000nM 25(OH)D_2 25(OH)D_3 (**Figure 4-5 A&B**). However, when these cells were treated with 1,25$(OH)_2D_2$ or 1,25$(OH)_2D_3$ and ketoconazole all doses (0.1-10nM) of 1,25$(OH)_2D_2$ and D_3 increased ALP activity significantly compared to vehicle (P<0.001), with 1,25$(OH)_2D_3$ being more potent than 1,25$(OH)_2D_2$ at 0.5-10nM (**Figure 4-5 C&D**).

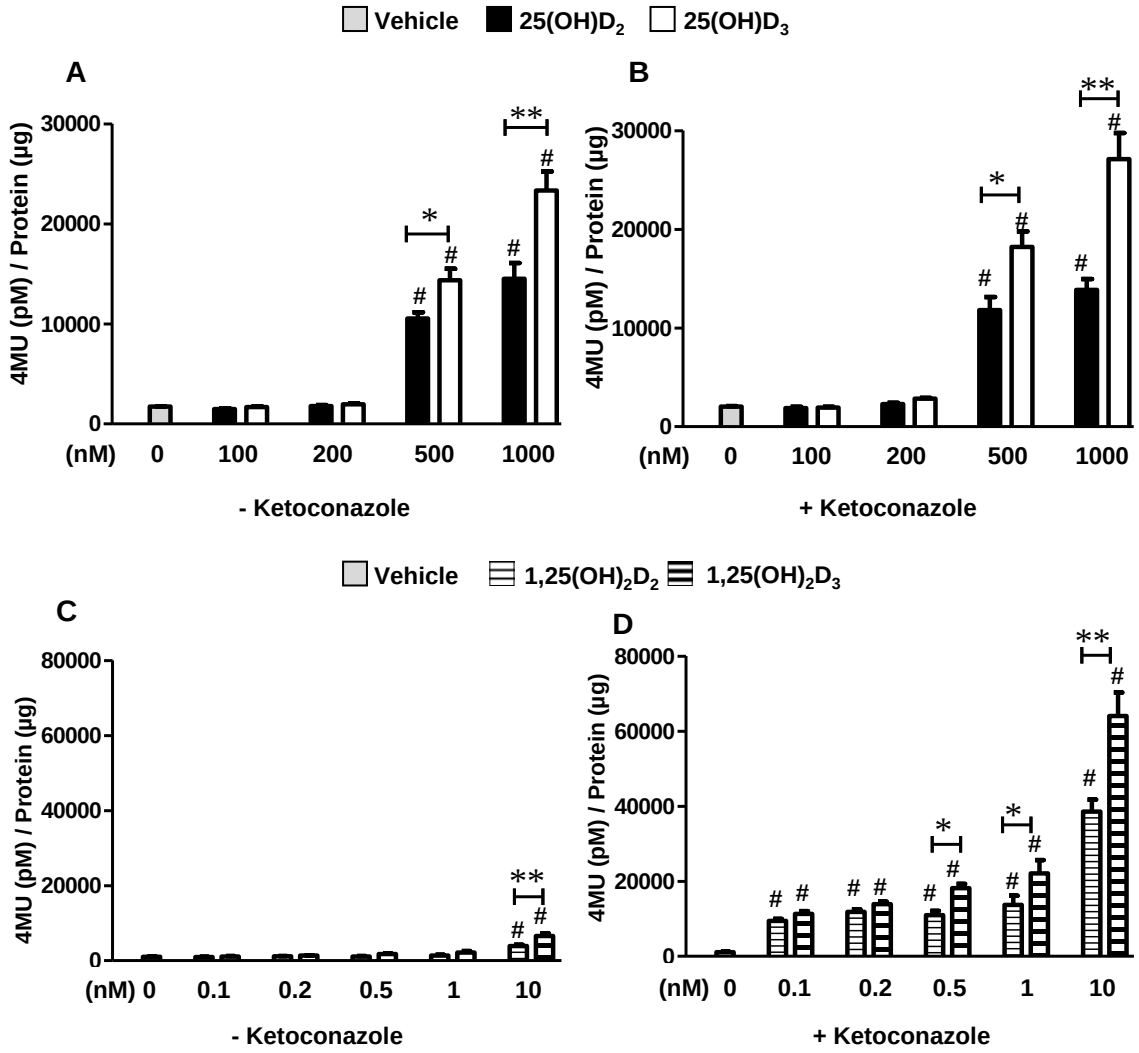


Figure 4-5: Addition of ketoconazole resulted in higher ALP up-regulation in response to $1,25(OH)_2D_3$ than $1,25(OH)_2D_2$, but had no effect when co-treated with $25(OH)D_2$ or D_3. HBMSCs were cultured for 7 days in differentiation medium for ALP enzymatic activities in the presence of 100-1000nM of $25(OH)D_2$ or $25(OH)D_3$ ± 10 ketoconazole (A&B) or 0.1-10nM of $1,25(OH)_2D_2$ or $1,25(OH)_2D_3$ ± 10µM ketoconazole (C&D). ALP activities were normalised to total protein content. Results of triplicate experiments are presented as pM 4MU activity of treatments versus untreated controls (grey) as mean ±SEM (non-parametric Kruskal Wallis test). * P<0.01 and ** P<0.001 for comparisons between D_2 and D_3 metabolites, and # P<0.001 comparisons between treatments and controls (grey).

There was a 10-fold increase in ALP up-regulation in hBMSCs in response to 10nM of $1,25(OH)_2D_2$ and D_3 when cells were co-treated with ketoconazole (**Figure 4-6**: P<0.001). At 10nM concentration, $1,25(OH)_2D_3$ was more potent that its counterpart $1,25(OH)_2D_2$ in up-regulation of ALP in hBMSCs, both in the absence or presence of ketoconazole (**Figure 4-6**: P<0.001).

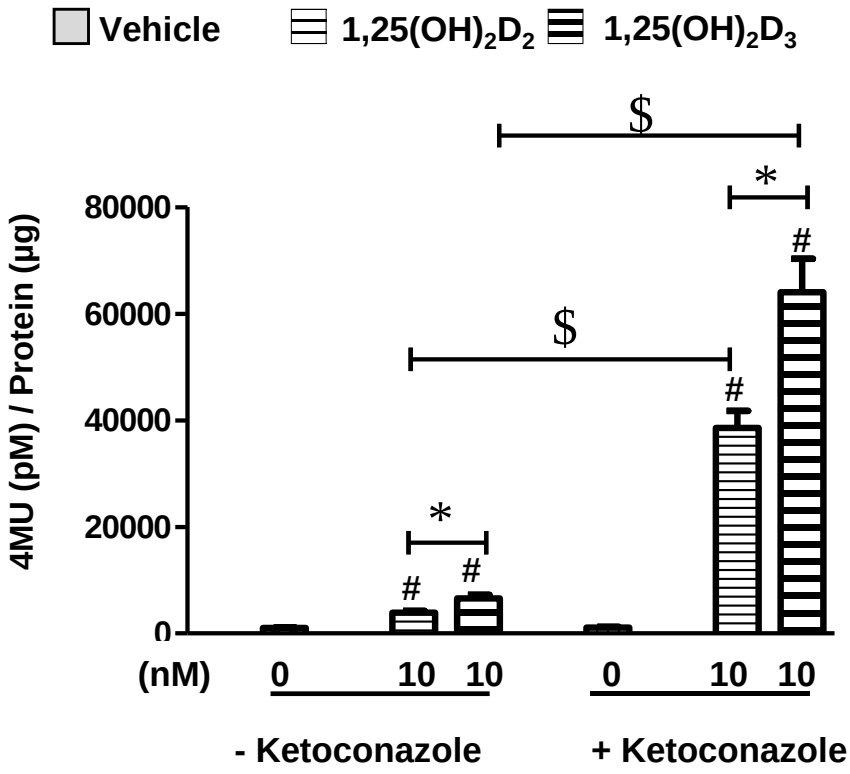
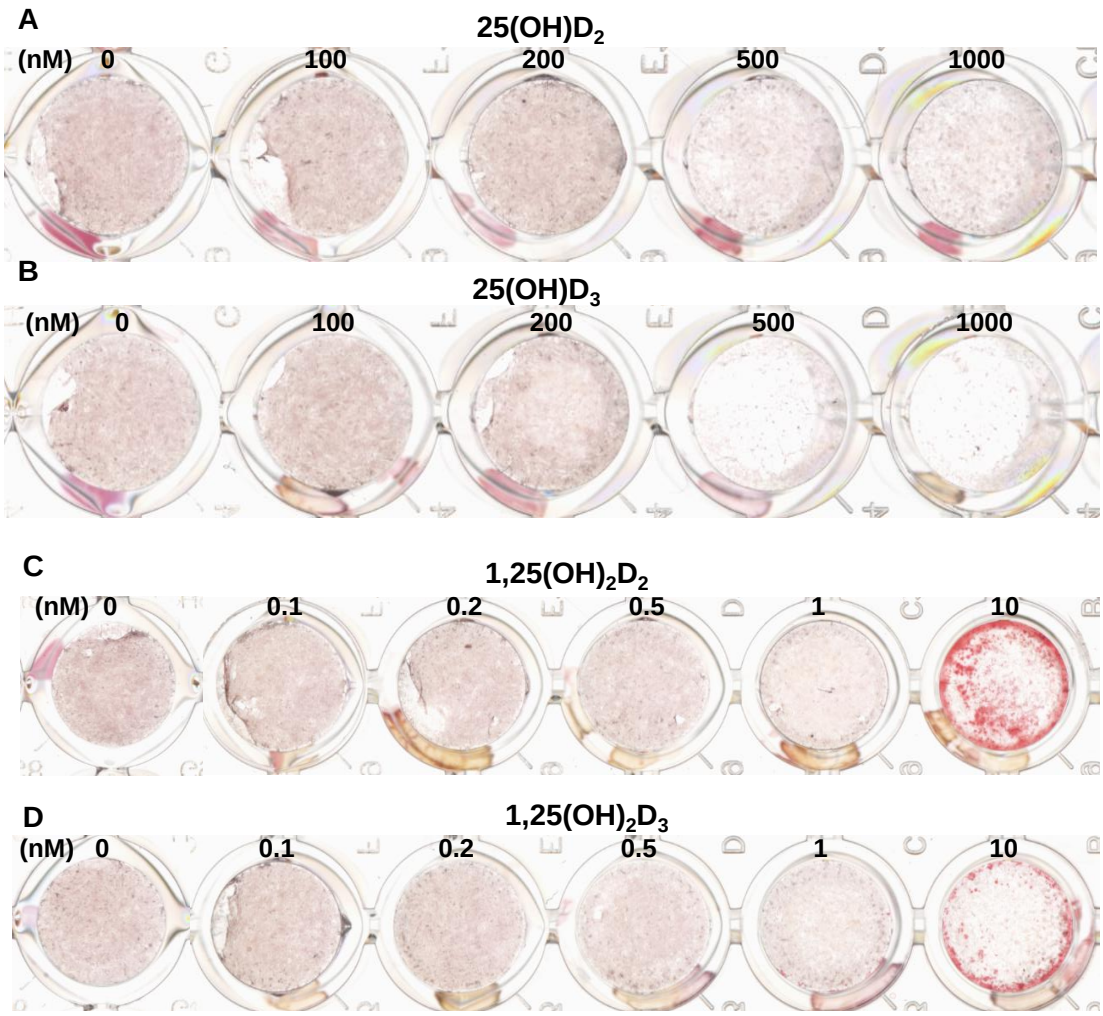


Figure 4-6: Addition of ketoconazole resulted in higher ALP up-regulation in response to $1,25(OH)_2D_3$ than $1,25(OH)_2D_2$. Influence of 10µM ketoconazole on the effects of vitamin D_2 and D_3 active metabolites on ALP up-regulation in hBMSCs. Cells were cultured for 7 days in differentiation medium for ALP enzymatic activities in the presence of 10nM of $1,25(OH)_2D_2$ or $1,25(OH)_2D_3$ ± 10µM ketoconazole. ALP activities were normalised to total protein content. Results are presented as pM of 4MU activity of treatments versus untreated controls (grey) as mean ±SEM of triplicate experiments (non-parametric Kruskal Wallis test). * P<0.001 and for comparisons between D_2 and D_3 metabolites, # P<0.001 comparisons between treatments and controls (0nM: grey) and $ P<0.001 for comparison of D_2 or D_3 metabolites with or without ketoconazole.

4.4.3.3. Mineralisation

Addition of 10µM ketoconazole with 100-1000nM 25(OH)D_2 or 25(OH)D_3 abolished mineralisation in hBMSCs dose-dependently with 25(OH)D_3 being a more potent inhibitor at 500-1000nM compared to 25(OH)D_2 ($P<0.01$) (**Figure 4-7 A**, **B&E**). Co-treatment with ketoconazole enhanced mineralisation at 10nM concentration of either active metabolite (**Figure 4-7C&D**), where 10nM 1,25$(OH)_2D_2$ was more potent than its counterpart 1,25$(OH)_2D_3$ ($P<0.001$) (**Figure 4-7F**).



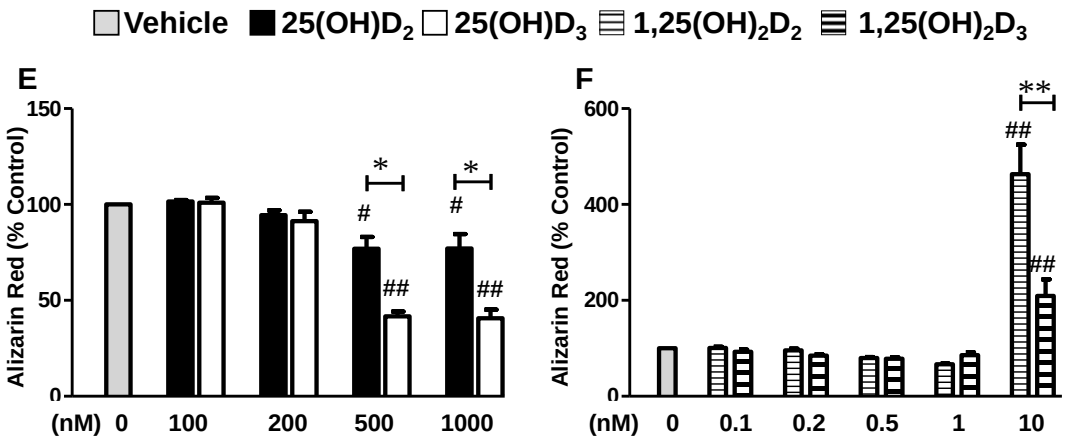


Figure 4-7: Addition of ketoconazole resulted in higher mineralisation in response to $1,25(OH)_2D_2$ than $1,25(OH)_2D_3$, but supressed mineralisation when treated with $25(OH)D_2$ or D_3. HBMSCs were cultured for 28 d in the presence of 10μM ketoconazole as well as 100-1000nM of (A) $25(OH)D_2$, (B) $25(OH)D_3$ and 0.1-10nM (C) $1,25(OH)_2D_2$ or (D) $1,25(OH)_2D_3$. Alizarin red staining of cells in the presence of 100-1000nM of $25(OH)D_2$ /D_3 (E) and 0.1-10nM of $1,25(OH)_2D_2$ and D_3 (F) were quantified by acetic acid extraction and plotted against untreated cells (grey) ±SEM (non-parametric Kruskal Wallis test) n=8. * P<0.01 and ** P<0.001 for comparisons between D_2 and D_3 metabolites, and # P<0.05, ## P<0.001 comparisons between treatments and controls (grey).

4.5. Discussion

4.5.1. D_2 vs D_3 effects on human primary osteoblasts

The current study presents the first detailed description of the effects of vitamin D_2 metabolites on hBMSCs differentiation into primary osteoblasts. It has been previously shown that hBMSCs express VDR and CYP27B1, the latter capable of metabolizing $25(OH)D_3$ into $1,25(OH)_2D_3$ and can differentiate into osteoblasts as a results of $1,25(OH)_2D_3$ stimulation. However, little is known about the direct role of vitamin D_2 metabolites and their potencies compared to D_3 metabolites on hBMSC differentiation. The outcome of the current study will provide a better understanding of the potencies of D_2 metabolites on autocrine regulation of human osteoblast differentiation and skeletal health.

$25(OH)D_3$ has previously been shown to have multiple effects on hBMSCs function, including inhibition of cellular proliferation and promotion of osteoblast differentiation, similar to data for its active metabolites $1,25(OH)_2D_3$. In the present study I have also shown that both $25(OH)D_2$ and $25(OH)D_3$ equally decreased cell number at high concentrations of ≥500nM. Interestingly, $1,25(OH)_2D_2$ had no inhibitory effects on hBMSC number, unlike $1,25(OH)_2D_3$, which reduced cell number at high concentration (1nM). It is also important to note that $1,25(OH)_2D_2$ did slightly increase mouse 2T3 cell number (Chapter 3), whereas it had no effect on hBMSC number.

It has been previously shown that unlike established osteoblast-like cells, high concentration $25(OH)D_3$ treatment (1000nM) is required to induce human primary osteoblast differentiation from hBMSCs [150]. My study also suggest that prolonged treatment with physiological doses of $25(OH)D_3$ (50-200nM) or 0.05-0.5nM of $1,25(OH)_2D_3$ had no effect on hBMSC induction of ALP and mineralisation. Similarly, 50-200nM of $25(OH)D_2$ or 0.05-0.5nM of its active metabolite $1,25(OH)_2D_2$ had no effects on stimulation of hBMSCs.

Both $25(OH)D_2$ and $25(OH)D_3$ (≥500nM) or their active metabolites at 10nM concentration up-regulated ALP in human primary osteoblasts over 7 days of culture, the effect being more evident with D_3 metabolites.

Prolonged (28 days) treatments of human primary osteoblasts with 1000nM of $25(OH)D_3$ only resulted in mineralisation. The absence of mineralisation by $25(OH)D_2$ correlated with lower potency of this metabolite in reducing cell number and lower ALP up-regulation compared to its counterpart $25(OH)D_3$.

This study suggests that the autocrine pathway of $25(OH)D_3$ metabolism locally also regulates osteoblast differentiation from hBMSCs perhaps independently of

kidney derived $1,25(OH)_2D_3$. Although I did not measure how much $1,25(OH)_2D_3$ or $1,25(OH)_2D_2$ was metabolised during this process, I would assume that hBMSCs were more capable of producing $1,25(OH)_2D_3$ which led to higher mineralisation compared to D_2 and this could be by possibly higher up-regulation of CYP27B1 when treated with $25(OH)D_3$.

Neither of the active metabolites $1,25(OH)_2D_2$ or $1,25(OH)_2D_3$ had an effect on hBMSCs mineralisation over 28 days of culture in osteogenic differentiation medium. The best explanation for this is that I used lower concentrations (10 fold) of the active metabolites and no other co-factors such as BMP compared to other publications [376].

In summary $25(OH)D_3$ was more potent than D_2 on reducing cellular proliferation and inducing osteoblast differentiation from hBMSCs by an increase in ALP. Although I only showed the differential responses of human primary osteoblast to D_2 and D_3 metabolites, by cell number, ALP and mineralisation, it would be interesting to find out whether osteoblast signature genes or protein expression are also affected. The limitation of working with human primary osteoblast was lack of high cell number as well as lengthy mineralisation assays. For future experiments the use of mouse or rat bone marrow stromal cells would be a better choice.

Finally, in the previous Chapter, and in the absence of ketoconazole, I showed that, the differences between D_2 and D_3 metabolites on mouse 2T3 osteoblast activity were mainly evident at supra-physiological doses. Similarly, there were no differences between D_2 or D_3 metabolites at physiological concentrations (50-100nM or 0.05-0.2 of the active metabolites) on human primary cell number, ALP and mineralisation. However, vitamin D_3 metabolites remained more potent than

D_2 at high concentrations, the only difference was that high concentrations of vitamin D in mouse tended to suppress cellular activities, but stimulated human primary osteoblast function.

4.5.2. D_2 vs D_3 effects on human primary cells in the presence of ketoconazole

In an attempt to identify the possible explanation for such differences between D_2 and D_3 metabolites on human primary cells, I took advantage of the influence of ketoconazole on the effects of vitamin D_2 and D_3. As previously described ketoconazole inhibits both CYP27B1 and CYP24A1 enzymatic machinery for conversion of 25(OH)D into $1,25(OH)_2D$, as well as inhibiting the catabolism of $1,25(OH)_2D$ into $1,24,25(OH)_3D$.

Initially we showed that addition of ketoconazole had no effects on human osteoblast cell number over 24 hours or ALP activity over 7 days.

When human primary cells were co-treated with ketoconazole and $25(OH)D_2$ and $25(OH)D_3$, ALP up-regulation remained more responsive to D_3 than D_2. Overall, addition of ketoconazole did not alter ALP responsiveness of human primary cells, when cells were co-treated with 25(OH)D metabolites. This is probably due to CYP27B1 depletion by ketoconazole and inhibition of $1,25(OH)_2D$ metabolism. However, partial depletion of CYP27B1 over 7 days resulted in higher ALP up-regulation in response to $25(OH)D_3$ suggesting that primary cells probably metabolise more $1,25(OH)_2D_3$ than D_2. Measurements of $1,25(OH)_2D_2$ or D_3 metabolites from the collected media at this stage of this project was beyond the scope of current study, but would have been interesting to confirm.

Co-administration of ketoconazole and $1,25(OH)_2D_3$, as mentioned earlier has been shown to increases the half-life of $1,25(OH)_2D_3$ hormone in osteoblasts as well as keratinocyte culture conditions. Subsequently, the increase in the $1,25(OH)_2D_3$ cellular responsiveness of osteoblast cells in the presence of ketoconazole was confirmed to be due to inhibition of *CYP24A1* [142, 144].

In the current study, when primary cells were treated with $1,25(OH)_2D_2$ or D_3 metabolites, although I did not examine the inhibition of CYP24A1 at transcript or protein levels, I indirectly showed this removal of catabolism by an enhanced ALP up-regulation. Consistent with equal ALP up-regulation in mouse 2T3 osteoblasts by either $1,25(OH)_2D_2$ or D_3 (0.1-0.2nM), human primary cells also equally up-regulated ALP in response to physiological doses of the active metabolites (0.1-0.2nM). Similarly, in human primary osteoblasts, the differences between D_2 and D_3 (D_3>D_2) were only observed at higher concentrations of the active metabolites (0.5-10nM) by ALP responsiveness.

Addition of ketoconazole with $25(OH)D_2$ and $25(OH)D_3$ in hBMSCs abolished mineralisation over 28 days of culture with both metabolites. Compared to ALP assays, where primary cells were treated on days 0, 2, 4 and 6, in mineralisation assays primary cells were treated from day 0-28 (14 times).

It is interesting to note that the same doses of $25(OH)D_2$ and $25(OH)D_3$ (500-1000nM) that stimulated ALP over a short course of treatment inhibited mineralisation with the same potencies (D_3>D_2). An inhibition of mineralisation as seen macroscopically from the impaired extra-cellular matrix (**Figure 4-5**: D_3>D_2) could be to an overdose or overexposure of cells to vitamin D metabolites. Due to lack of enough primary cells, I was not able to confirm this with Live and Dead staining or total DNA content.

Mineralisation was only increased with ketoconazole plus either $1,25(OH)_2D$ metabolites (D_2>D_3). The use of ketoconazole could be interpreted in two possible ways, bearing in mind that initially I used lower doses (10nM) than others previously used (100nM). One explanation is that using vitamin D-sensitizing ketoconazole enhanced the presence of $1,25(OH)_2D$ metabolites in the cultures to induce more cellular activity, as was observed by an increase in mineralisation. In this way, the doses of the active metabolites (10nM of $1,25(OH)_2D_2$ and $1,25(OH)_2D_3$) that previously did not increase mineralisation (absence of ketoconazole), by an increase in the half-life of $1,25(OH)_2D$ with ketoconazole became stimulatory [$1,25(OH)_2D_2$ >$1,25(OH)_2D_3$]. The probable reason why $1,25(OH)_2D_2$ resulted in higher mineralisation than D_3, could be that prolonged exposure (28 days) of the cells to $1,25(OH)_2D_3$ eventually could lead to toxicity. In contrast, prolonged exposure of the cells to the $1,25(OH)_2D_2$ concentrations that had no effects in the absence of ketoconazole, could favour the osteoblast differentiation and mineralisation. The future experiment to explore this could be Western blot analysis of osteoblast markers such as OCN or OPN in response to the active metabolites and ketoconazole as well as cell viability by Alamar blue assay over 28 days.

One of the other main findings of this study was that the doses of D_2 metabolites assessed here were not potent inhibitors of human cells at high concentrations compared to D_3 metabolites. As established in the literature, accidental vitamin D intoxication could occur with supplementation of high doses of either D_2 or D_3. For ethical reasons, there has not been any study examining vitamin D toxicity in humans, however, daily mega dose supplementation of vitamin D_2 and D_3 in horses and monkeys have been performed. The outcomes of these studies were

that all the animals on high doses of vitamin D_3 developed hypercalcemia, with extensive soft tissue mineralisation and died. In contrast, animals with the same doses of D_2 developed hypercalcemia to a lesser degree, but survived as a consequence of less soft tissue mineralisation [288, 289].

In conclusion, although further experiments on human BMSCs from more donors are required to confirm this, however, this preliminary data revealed that $25(OH)D_3$ is a better metabolite for human primary osteoblast mineralisation than $25(OH)D_2$. This may strengthen the use of vitamin D_3 as a better supplement for vitamin D deficient patients. Although both $1,25(OH)_2D_2$ and D_3 were capable of increasing human primary osteoblast mineralisation, it appears that the $1,25(OH)_2D_2$ active metabolite may indeed be a better choice of treatments for severe diseases such as CKD where $1,25(OH)_2D$ treatment is the only choice.

CHAPTER 5: MLOY-4 AND MLO-A5 RESPONSES TO VARIOUS METABOLITES OF VITAMIN D_2 AND D_3

5. MLOY-4 AND MLO-A5 RESPONSES TO VARIOUS METABOLITES OF VITAMIN D_2 AND D_3

5.1. Introduction

With the discovery of osteocytic sclerostin as a regulator of bone density and the site of osteosclerotic bone diseases, the establishment of osteocyte cell lines has helped research to focus on the role of osteocytes in bone homeostasis. There have been exciting discoveries of new proteins, hormones, factors and pathways in osteocytes that regulate calcium and phosphate [207]. These finding could open new complex processes and mechanism, but will create opportunities for new therapeutic approaches. There is currently little literature on the role of vitamin D metabolites in osteocytes and the current study may begin to fill the gaps by making use of osteocyte-like cell lines.

Calcium and phosphate homeostasis in the body is a complex mechanism mediated by many cells in different organs, and achieved by interplay of hormones, such as $1,25(OH)_2D_3$, and PTH [70]. The interaction between these hormones and osteocyte proteins such as Phex and Dmp1 are not well understood.

$1,25(OH)_2D_3$ active metabolite, PTH and FGF23 are responsible for regulation of circulatory levels of calcium and phosphate and consequently bone mineralisation [70]. Osteocytes are known to be the major source of FGF23 production, and FGF23 is known to directly antagonise $1,25(OH)_2D_3$, by reducing its activating hydroxylation steps (reduce CYP27B1) in the kidney [378]. This results in an increase in the catabolism of $1,25(OH)_2D_3$ (increase in CYP24A1)

and subsequently restricts calcium and phosphate absorption [378, 379]. FGF23 itself can also be up-regulated by $1,25(OH)_2D_3$ stimulation providing a feedback loop between these factors (**Figure1-7**) [280]. In *in vivo* rat studies, injections of $1,25(OH)_2D_3$ led to a dose response increase in the circulatory levels of FGF23 [379].

PTH stimulation of CYP27B1 in kidneys and $1,25(OH)_2D_3$ stimulation of FGF23 expression by osteocytes closes the loop of the parathyroid-kidney-bone axis of calcium and phosphate regulation [70]. Although osteocytes are known to express Phex and Dmp1, effects of vitamin D on these proteins remain unclear. It is currently known that mutations in Phex causes X-linked dominant hypophosphatemic rickets [380] and Dmp1 mutation can cause autosomal recessive hypophosphatemic rickets, and as a result both cases are associated with high levels of FGF23 [279].

Phex and Dmp-1 could be the potential regulatory links in between $1,25(OH)_2D_3$ and FGF23, but currently the exact underlying mechanisms are unknown. Phex expression has been shown to be down regulated by $1,25(OH)_2D_3$ (100nM) in terminally differentiated MC3T3 osteoblasts, suggesting a possible role in bone mineralisation [354]. The effect of vitamin D_3 metabolite on the endogenous levels of Dmp1 has been examined in rat UMR-106 osteosarcoma cell line. Treatments of UMR-106 cells with 100nM $1,25(OH)_2D_3$ metabolite has been shown to up-regulate DMP1 both at mRNA (8h) and protein level (24h) [279]. In contrast, in another study, $1,25(OH)_2D_3$ treatment of established murine osteocyte cell lines, MLO-Y4 and MLO-A5, resulted in VDR mediated dose-dependent (0.01,10 and 100nM) suppression of *Dmp-1* mRNA [381]. This further

suggests an effect of vitamin D on Dmp-1 in osteocytes that may contribute to local mineral homeostasis.

Recent development of tissue specific, intestinal, osteoblast or osteocyte VDR null mice (VDR-/-) have provided great insights into the role of the $1,25(OH)_2D_3$ on the control of bone and its contribution to calcium and phosphate balance in the circulation [382]. When on a normal calcium diet, $1,25(OH)_2D_3$ primarily acts on intestinal calcium reabsorption and indirectly on bone by ensuring that the absorbed calcium and phosphate are stored in bone [382, 383]. However, if the calcium is not sufficient from the diet, $1,25(OH)_2D_3$ acts directly on osteoblasts and osteocytes to increase osteoclastic bone resorption and suppress bone mineralisation by osteoblasts to compensate for the low circulatory level of calcium [382].

Primary osteocyte cells have been shown to express high levels of *RANKL* and induce osteoclastogenesis when co-cultured with PBMCs, hence osteocytes could play an important role in bone homeostasis via RANKL mediated osteoclast bone resorption [199]. Expression of RANKL by osteocytes and the underlying mechanisms of osteocyte regulation of local bone remodelling is a new and interesting topic, since osteoblasts have long been thought to be the main source of RANKL [384].

It has been postulated that micro cracks caused by mechanical loading on bone would disrupt the cross talk between osteocytes [385]. This could lead to recruitment of osteoclasts to the site of damage, possibly by direct cell-cell contact of osteocytes to osteoclasts as well as expression of RANKL by osteocytes for local bone remodelling [385].

Currently there are only two osteocyte-like cell lines in research known as MLO-Y4 and MLO-A5 cells [386]. The MLO-A5 line is known as a late osteoblast or pre-osteocyte lineage that can differentiate in *in vitro* studies [387].

When MLO-A5 cells are maintained in a low concentration of osteogenic differentiation medium supplemented with ascorbic acid and phosphate, cells differentiate which is associated with an increase in up-regulation of osteocyte markers such as *Dmp1*, *Phex*, *SOST* and *E11* [387]. Removal of osteogenic media from these cells led to down-regulation of osteocytic markers *E11*, *Dmp1* and *SOST*, while osteoblast markers such as *OCN* and *COL I* increased [387].

Compared to MLO-A5, MLOY-4 are a more mature osteocyte-like cell line expressing some osteocyte markers such as *connexin-43*, *Dmp1*, *Phex*, *E11*, but not *SOST* or *FGF23.* There is very limited information in the literature on the role of vitamin D on the differentiation of these cells.

When MLO-A5 were exposed to 25(OH)D_3 (100nM), they up-regulate *CYP27B1* as well as *CYP24A1* and metabolise 1,25(OH)$_2D_3$ [388]. Addition of 25(OH)D_3 (100nM, 48h) also resulted in an increase in *OPG* mRNA, but reduced *RANKL* at transcript levels in MLO-A5 [285]. The same group showed that MLOY-4 also express *CYP27B1* at mRNA level, and when MLOY-4 were exposed to exogenous 25(OH)D_3 at 200nM, *CYP24A1* was up-regulated, which is indicative that 1,25(OH)$_2D_3$ is being metabolized and could cause down-regulation of *Phex* [388].

5.2. Aims and objectives

The main aim of this Chapter is to ascertain the effect of vitamin D_2 and D_3 metabolites on osteocyte biology. To address this established murine osteocyte-

like MLOY-4 and MLO-A5 cell lines will be used and the following questions will be addressed:

A. Do these cells possess VDR and CYP27B1 for processing exogenous vitamin D metabolites?
B. Do these cells express osteocytic markers *CX-43*, *Phex*, *Dmp1*, *FGF23*, *SOST*, *OPG* and *RANKL*?
C. What effect do various concentrations of 25(OH)D_2, 25(OH)D_3 and their respective active metabolites have on MLOY-4 or MLO-A5 cell number?
D. Are there any transcriptional changes in osteocyte-like cells in response to D_2 or D_3 metabolites?

5.3. Material and methods

Osteocyte-like cells were kindly provided by Professor Lynda Bonewald, University of Missouri-Kansas City, USA. These cells were isolated from transgenic mice carrying the OCN promoter driven by SV40 large T antigen and are well established and characterised [386]. For regular cell maintenance both cell lines were grown on collagen-coated plates (BD BioCoat™ UK) in αMEM supplemented with 5% HI-FBS and 5% foetal calf serum (HI-FCS) and essential ribonucleotides, as instructed by Professor Bonewald's lab and previous publications.

5.3.1. VDR and CYP27B1 protein expression

MLOY-4 and MLO-A5 were plated at $1x10^4$ per 4 and 24 well plates over 24hr and 6 days respectively. Human pro-myelocytic leukaemia cell line, HL-60 has been shown to express VDR, hence this cell line was used as a positive control for VDR expression by Western blots [389]. HL-60 cells were cultured in 24 well plates at $1x10^4$ in RPMI-1640 medium supplemented with 10%HI-FBS for 48 hours. Whole cell lysates for all cell lines were prepared in 100µl lysate buffer containing 2% protease inhibitor (RIPPA-2%PI) and the total protein amounts were measured using the Pierce BCA protein detection kit. Equal amounts of protein (20µg) were separated under reducing conditions by 12-15 % SDS-PAGE and were transferred onto PVDF membrane. Membranes were blocked in 5% milk in TBS-Tween-20 overnight. Anti-actin 1:8000 (Sigma Aldrich), anti VDR, anti CYP27B1 primary antibodies (1:1000) (Santa Cruz Biotechnology) were used for overnight incubation of the membranes in 5% milk-TBST to detect the related proteins respectively. Goat anti-mouse secondary antibody (R&D Systems, UK, 1:4000) conjugated with horseradish peroxidase was used for chemiluminescence detection with ECL substrate kit.

5.3.2. Osteocytic signature gene expression

MLOY-4 cells were cultured in αMEM supplemented with 5%HI-FBS and 5%HI-FCS at $1x10^5$ in 60mm collagen-coated petri dishes in replicates for 24 hours. MLO-A5 were seeded at $1x10^4$ in replicates in 6 well plates in regular culture conditions supplemented with 5mM βGP and 50µg/ml ascorbate (osteogenic differentiation medium). Total RNA from each treatment group was extracted by

RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions as previously described in Chapter 2. Osteocytic signature genes *CX-43* (Cat.no:QT00173635), *Phex* (Cat.no:QT00102067), *Dmp1* (Cat.no:QT01078210), *FGF23* (Cat.no:QT01166557), *SOST* (Cat.no:QT00294322), *OPG* (Cat.no:QT00106757) and *RANKL* (Cat.no:QT00147385) were assessed and normalised to the housekeeping genes *GAPDH* (Cat.no: QT01658692).

5.3.3. Osteocyte cell number in response to vitamin D metabolites

MLOY-4 and MLO-A5 cells were seeded in αMEM supplemented with 5%HI-FBS and 5%HI-FCS at $1x10^3$ in collagen-coated 96 well plates in replicates in the presence or absence of 100-500nM of $25(OH)D_2$, or $25(OH)D_3$ and 0.1-0.5nM of $1,25(OH)_2D_2$ and $1,25(OH)_2D_3$, or ethanol (as vehicle) over 24 hours. After 21 hours 10% Alamar blue™ was added to the wells and plates were incubated under standard tissue culture conditions at 37°C, 5% CO_2. Plates were removed and cell number was assayed using a BMG FluorStar Optima plate reader.

5.3.4. Osteocytic signature genes expression in response to vitamin D metabolites

MLOY-4 was cultured in αMEM supplemented with 5%HI-FBS and 5%HI-FCS at $1x10^5$ in 60mm collagen-coated petri dishes in replicates in the presence of

200nM[14] of 25(OH)D_2, 25(OH)D_3 and 0.2nM of 1,25$(OH)_2D_2$ and 1,25$(OH)_2D_3$ or ethanol (vehicle) as control for 24 hours. MLO-A5 cells were seeded at $1x10^4$ in replicates in 6 well plates in osteogenic differentiation medium[15] in the presence of 100-500nM of 25(OH)D_2 or 25(OH)D_3, 0.1-0.5nM of 1,25$(OH)_2D_2$ and 1,25$(OH)_2D_3$ or ethanol over 6 days, with complete medium and treatments changed every other day. Upon completion of the treatments total RNA from each condition was extracted by RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions as previously described in Chapter 2. Osteocytic signature genes *CX-43*, *Phex*, *Dmp1*, *FGF23*, *SOST*, *OPG* and *RANKL* were quantified in response to treatment with vitamin D_2 and D_3 and normalised to the housekeeping genes, *GAPDH.*

[14] The concentration of 200nM of 25(OH)D_3 has previously shown to stimulate MLOY-4 osteocytic markers [282].
[15] It has been established that osteocytic markers are up-regulated in MLO-A5 cells when they are cultured in osteogenic differentiation medium [386].

5.4. Results

5.4.1. VDR and CYP27B1 expression

Western blot analyses revealed that VDR and CYP27B1 were expressed in both MLOY-4 and MLO-A5 indicating their ability to metabolise vitamin D metabolites **Figure 5-1**.

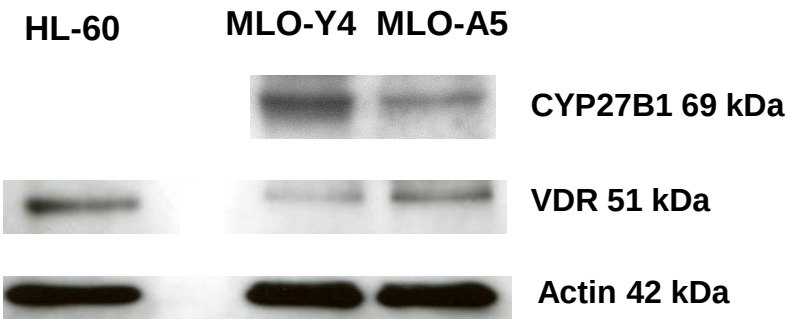


Figure 5-1: Osteocyte-like cells express CYP27B1 and VDR at protein levels. Western blot analyses of VDR and CYP27B1 of 20 µg of cell lysate extracts from MLOY-4 and MLO-A5 cells on 24h and day 6 cultures in αMEM supplemented with 5%HI-FBS and 5%HI-FCS. HL-60 cell lysate was loaded as a positive control as these cells are known to express VDR [389].

5.4.2. Osteocytic signature genes

Osteocyte-like cells were shown to express osteocytic markers as indicated in **Figure 5-2A.** As expected MLOY-4 expressed high levels of osteocytic transcripts of *Dmp1*, *Phex*, *CX-43*, *FGF23* and low levels of *SOST*, *OPG* and *RANKL*. MLO-A5 also expressed *CX-43*, *Phex*, *Dmp1*, as well as low levels of *FGF23*, *RANKL* and *SOST* (**Figure 5-2B**).

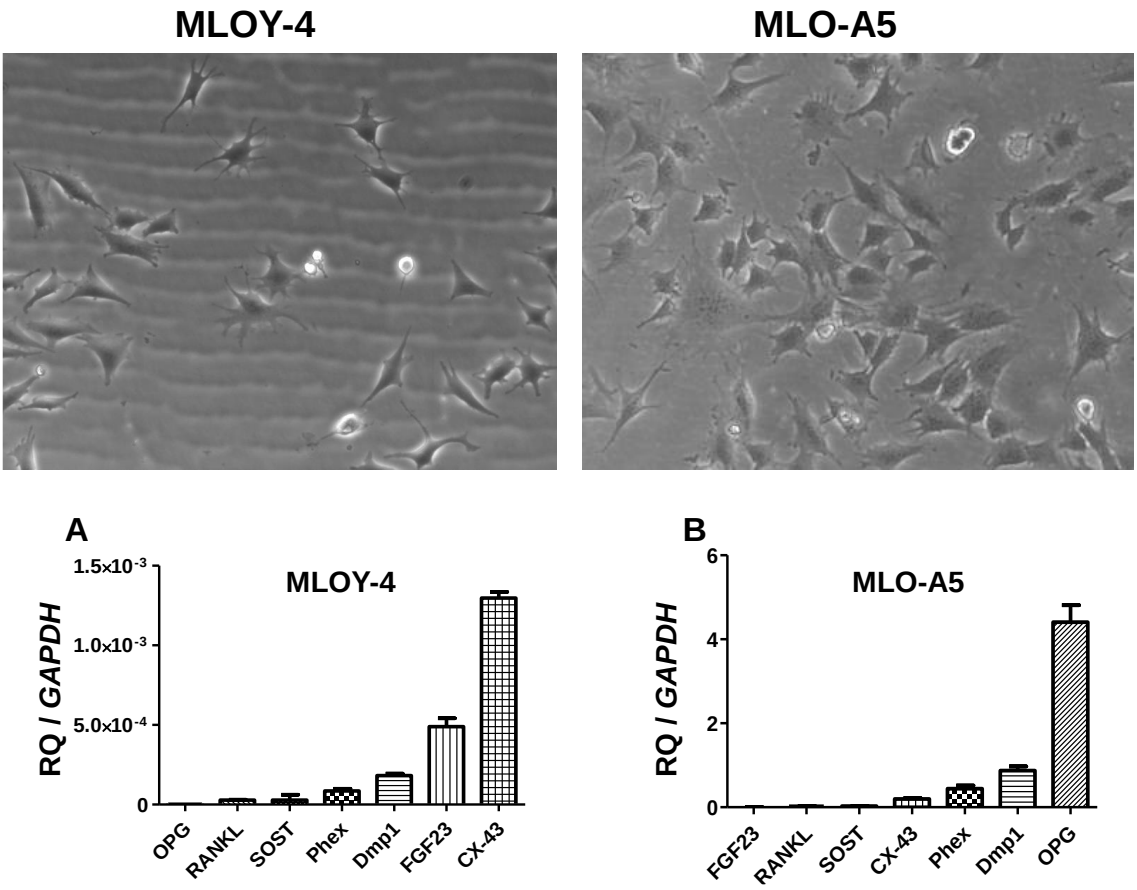


Figure 5-2: Osteocyte-like cells express low levels of osteocyte genes at transcript levels. Top panel phase contrast image of MLOY-4 (left) and MLO-A5 (right) osteocyte-like cells grown on collagen type one coated plates. Images taken by ZEISS AX10 microscope, 10X magnifications**.** Below panel, baseline expression of osteocytic markers: *SOST*, *Phex*, *Dmp1*, *FGF23*, and *CX-43* as well as *OPG* and *RANKL* in (A): MLOY-4 maintained in their recommended growth conditions over 24 hours and (B): MLO-A5 osteocyte like cells maintained in osteogenic media over 6 days. Values are means ±S.D of triplicate reactions.

5.4.3. Osteocyte cell number in response to vitamin D metabolites

In order to compare the effects of 25(OH)D_2 or 25(OH)D_3, 1,25(OH)$_2D_2$ and 1,25(OH)$_2D_3$ metabolites on osteocyte cell number, I performed Alamar blue assays. A reduction in viable cells was observed in both osteocytic cells in response to 500nM concentration of 25(OH)D_3 compared to untreated cells (**Figure 5-3 A**: MLOY-4: P<0.01, & **Figure 5-3C**: MLO-A5: P<0.05). 500nM

concentration of 25(OH)D_3 was more potent than 25(OH)D_2 in reducing MLOY-4 (**Figure 5A**: D_2 vs D_3: P<0.01) and MLO-A5 (**Figure 5C** D_2 vs D_3: P<0.05). Treatment of these cells with 0.5 nM of 1,25(OH)$_2D_3$ also reduced both osteocyte cell numbers (**Figure 5-3B**: MLOY4: P<0.05 & **Figure 5-3D**: MLO-A5: P<0.01). In contrast, 1,25(OH)$_2D_2$ metabolites had no effect on osteocyte cell number at similar concentrations compared to control.

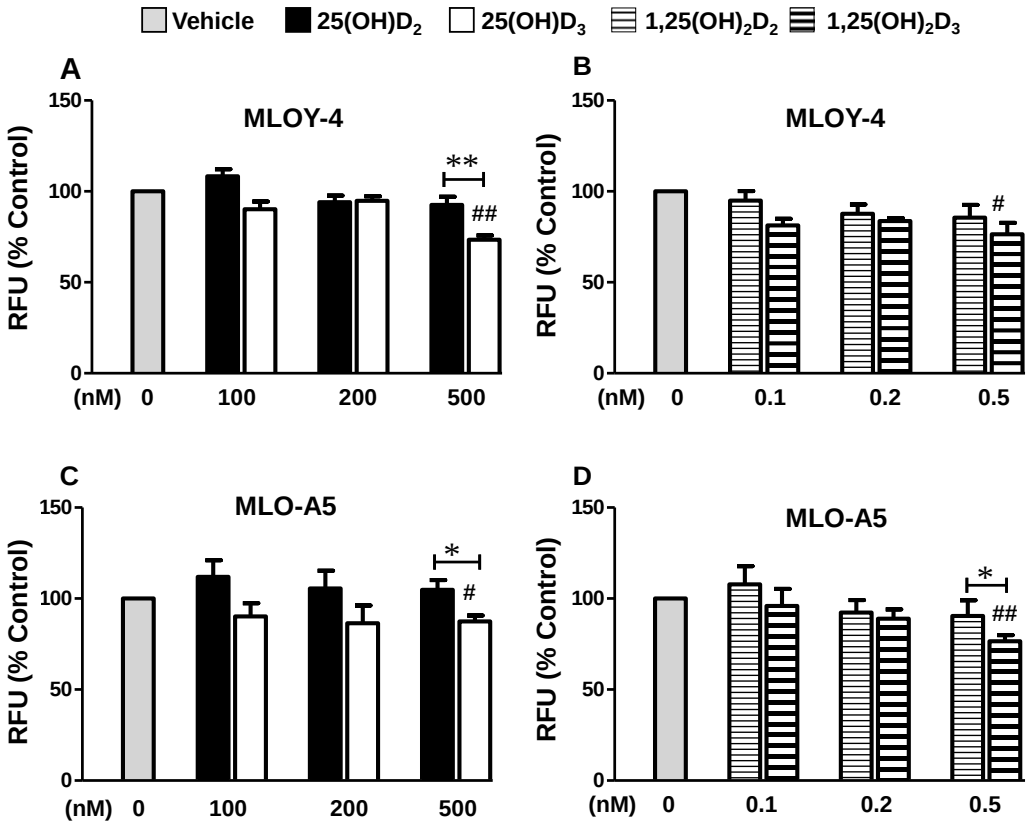


Figure 5-3: Osteocyte-like cell number was reduced by high dose of vitamin D_3 metabolites. Comparative effects of 25(OH)D_2, D_3 (100-500nM), 1,25(OH)$_2D_2$ and D_3 (0.1-0.5nM) on MLOY-4 (A-B) and MLO-A5 (C-D) osteocyte cell number. Results are presented as percentage of relative fluorescence unit of treated cells versus untreated control in grey as mean ±SEM (non-parametric Kruskal Wallis test). * P<0.05 and ** P<0.01 for comparisons between D_2 and D_3 metabolites, and # P<0.05 and ## P<0.01 comparison of treatments to vehicle.

5.4.4. Osteocytic markers in response to vitamin D metabolite

To determine the effects of $25(OH)D_2$ and $25(OH)D_3$ or their active metabolites $1,25(OH)_2D_2$ and $1,25(OH)_2D_3$ on relative expression of osteocyte signature genes *CX-43, Phex, Dmp1*, *SOST* and *FGF23* as well as *OPG* and *RANKL* I performed QRT-PCR. When MLOY-4 cells were treated with the 200nM of $25(OH)D_2$ and $25(OH)D_3$ only *FGF-23* was up-regulated ($P<0.01$) by either metabolite (**Figure 5-4G**, $D_2 > D_3$: $P<0.01$). Neither $25(OH)D_2$ or D_3 metabolites had an effect on *CX-43*, *Phex*, *SOST*, *OPG* and *RANKL* (**Figure 5-4 A, B, D, E&F**). Only $25(OH)D_3$ metabolite decreased *Dmp1* ($P<0.01$) in MLOY-4 compared to D_2 and untreated control (**Figure 5-4C**). When MLOY-4 were treated with 0.2nM $1,25(OH)_2D_2$ or $1,25(OH)_2D_3$, there was an up-regulation of *CX-43* ($P<0.01$), *Phex* ($P<0.01$), *RANKL* ($P<0.01$) and *FGF23* ($P<0.01$) by both metabolites compared with the controls (**Figure 5-4A, B, F&G**, respectively). Only 0.2nM $1,25(OH)_2D_3$ suppressed *Dmp1* ($P<0.05$) and 0.2nM of $1,25(OH)_2D_2$ showed an increase in *OPG* ($P<0.05$) compared to untreated control (**Figure 5-4C&E**).

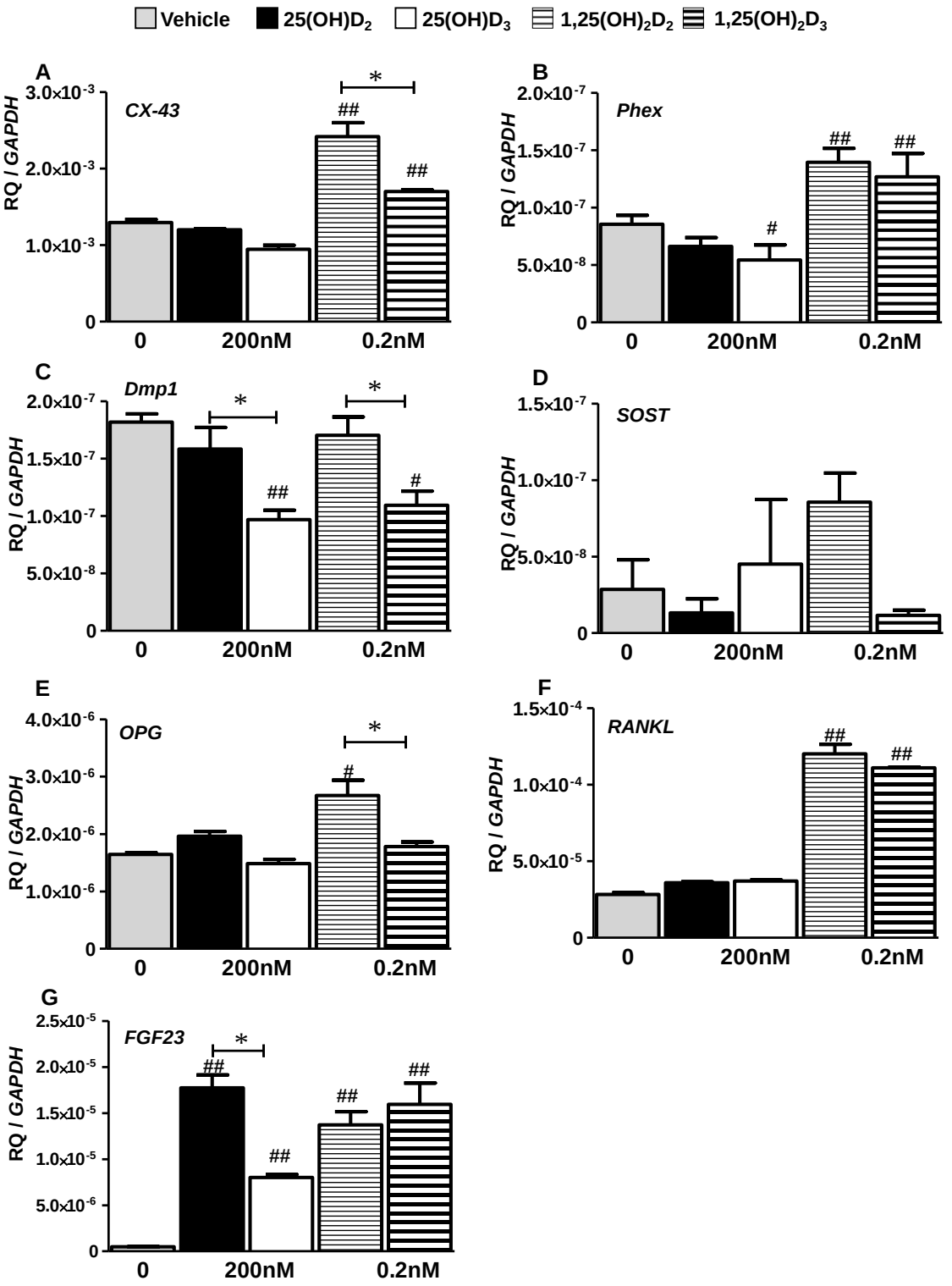


Figure 5-4: Both $1,25(OH)_2D_2$ and D_3 active metabolites up-regulate osteocytic transcripts in MLOY-4. Effects of 200nM of $25(OH)D_2$, D_3 and 0.2nM of $1,25(OH)_2D_2$ and D_3 on the expression of (A) *CX-43*, (B) *Phex*, (C) *DMP1*, (D) *SOST*, (E) *OPG*, (F) *RANKL* and (G) *FGF23* in MLOY-4 over 24 hours culture. Values are mean ± S.D. from triplicate reactions, (one way Anova, Welch correction). * P<0.01, for comparisons between D_2 and D_3 metabolites, and # P<0.05, ## P<0.01 for comparison of vitamin D metabolite against control (grey).

Table 5-1 summarises the dose-response effects of 100-500nM 25(OH)D_2, 25(OH)D_3 and 0.1-0.5nM 1,25$(OH)_2D_2$ or 1,25$(OH)_2D_3$ on MLO-A5 cells. Both 25(OH)D_2 and 25(OH)D_3 up-regulated *Phex* and *Dmp1* at 100nM with D_2 being more potent than D_3 (Figure **5-5 B&C**: P<0.01), whereas only 100nM of 25(OH)D_2 up-regulated *RANKL* and *FGF23* (**Figure 5-5 F&G**: P<0.05 & P<0.01 respectively) compared to untreated cells. Doses of 200-500nM of both 25(OH)D_2 and D_3 up-regulated *OPG* with 25(OH)D_2 being more potent than 25(OH)D_3 (**Figure 5-5E**: P<0.01). Doses of 200nM of either 25(OH)D_2 or 25(OH)D_3 had no effects on *CX-43*, *SOST*, or *FGF23* (**Figure 5-5AD&G**), whereas compared to untreated cells, 200nM of 25(OH)D_2 and D_3 equally up-regulated *Phex* (**Figure 5-5B**).

Active metabolites, 1,25$(OH)_2D_2$ or 1,25$(OH)_2D_3$ at 0.1-0.5nM, had no significant effect on *RANKL* or *FGF23* expression (**Figure 5-5 F&G**). *Cx-43* was equally suppressed by: 0.1nM of 1,25$(OH)_2D_2$ or 1,25$(OH)_2D_3$ **(Figure 5-5A**), and 02.nM of 1,25$(OH)_2D_2$ or 1,25$(OH)_2D_3$ (**Figure 5-5 A**: P<0.05 and P<0.01, respectively). 1,25$(OH)_2D_2$ and 1,25$(OH)_2D_3$ equally up-regulated *Phex* at 0.5nM (**Figure 5-5B**). *OPG* was only up-regulated in response to 0.1nM of 1,25$(OH)_2D_3$ (**Figure 5-5E**, P<0.05), whereas 0.2-0.5nM of either active metabolites equally down-regulated *OPG* (**Figure 5-5E**: P<0.05). 0.1nM of either active metabolites had no effects on *Dmp1* (**Figure 5-5C**), whereas only 1,25$(OH)_2D_3$ at 0.2-0.5nM suppressed *Dmp1* (**Figure 5-5C**: P<0.01).

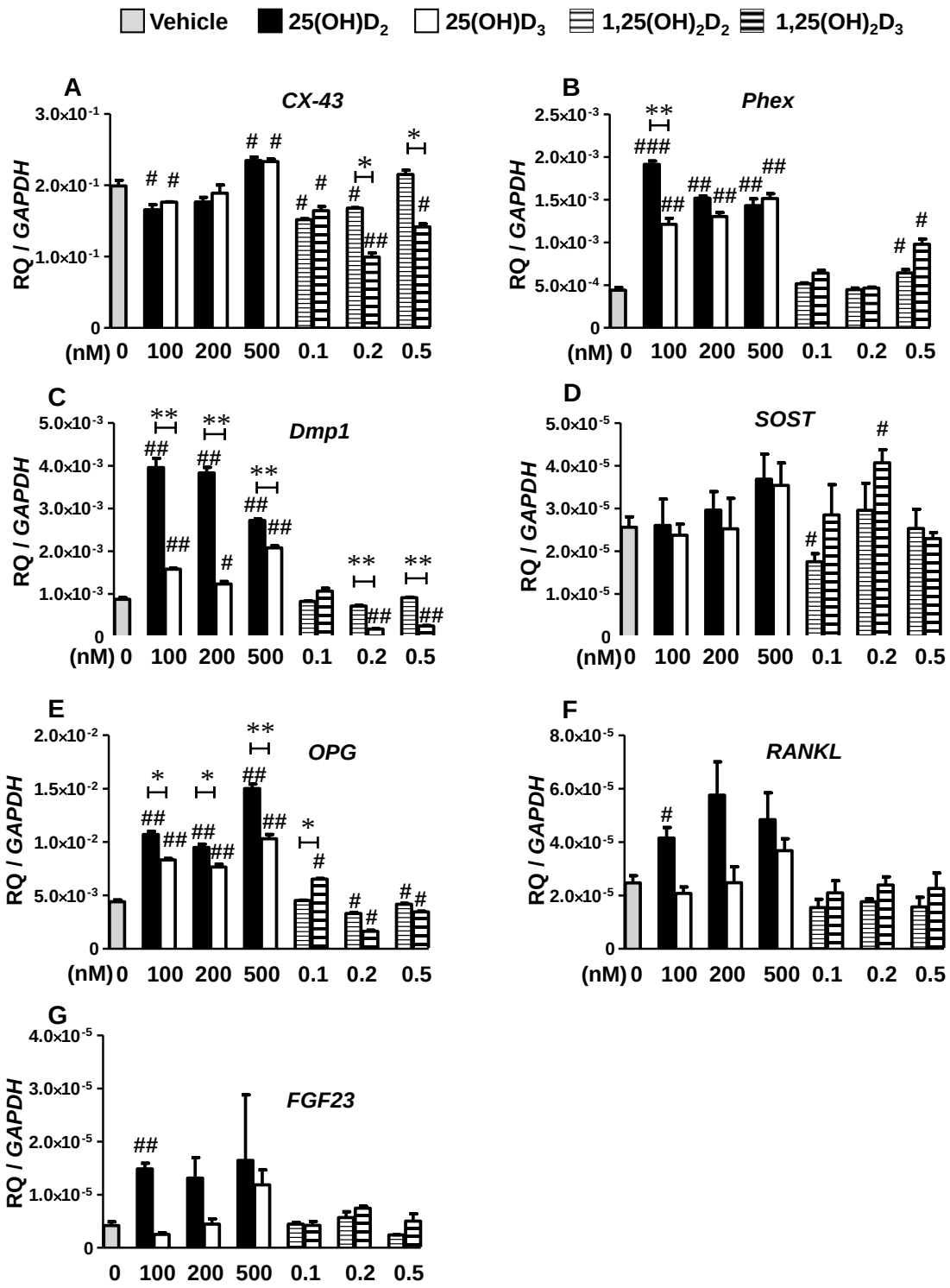


Figure 5-5. 25(OH)D_2 and D_3 up-regulate osteocytic transcripts in MLO-A5 cells while active metabolites had little effect. Effects of 100-500nM of 25(OH)D_2, D_3 and 0.1-0.5nM of 1,25(OH)$_2D_2$ and D_3 on the expression of (A) *CX-43*, (B) *DMP1*, (C) *SOST*, (D) *Phex*, (E) *RANKL*, (F) *OPG* and (G) *FGF23* in MLO-A5 osteocytic cells. Values are mean ± S.D. from triplicate reactions (one way Anova, Welch correction). * P<0.05, ** P<0.01 for comparisons between D_2 and D_3 metabolites, and # P<0.05, ## P<0.01 AND ### P<0.001 for comparison of vitamin D metabolites against control (grey).

Table 5-1

Summary of the results of the effects of 100-500nM of 25(OH)D_2 or D_3 and 0.1-0.5nM 1,25(OH)$_2D_2$ and D_3 on MLO-A5 osteocytic signature genes. ↓: decrease in gene expression: =: equi-potent, ↑: increase in gene expression, − no effect.

	25(OH)D_2/D_3 (nM)			**1,25(OH)$_2D_2$/D_3 (nM)**		
Gene	100	200	500	0.1	0.2	0.5
CX-43	↓ D_2=D_3	− D_2= D_3	↑ D_2=D_3	↓ D_2=D_3	↓ D_2<D_3	↓ D_3
Phex	↑ D_2>D_3	↑ D_2=D_3	↑ D_2=D_3	− D_2= D_3	− D_2= D_3	↑ D_2=D_3
Dmp1	↑ D_2>D_3	↑ D_2>D_3	↑ D_2>D_3	− D_2= D_3	↓ D_3	↓ D_3
SOST	− D_2= D_3	− D_2= D_3	− D_2= D_3	↓ D_2	↑ D_3	− D_2= D_3
OPG	↑ D_2>D_3	↑ D_2>D_3	↑ D_2>D_3	↑ D_3	↓ D_2=D_3	↓ D_2=D_3
RANKL	↑ D_2	− D_2= D_3	− D_2= D_3	− D_2= D_3	− D_2= D_3	− D_2= D_3
FGF23	↑ D_2	− D_2= D_3	− D_2= D_3	− D_2= D_3	− D_2= D_3	− D_2= D_3

5.5. Discussion

To date the direct effect and comparison of vitamin D_2 and D_3 metabolites on osteocyte cells has not been investigated. There is currently little literature on the effects of vitamin D_2 metabolites on established osteocytes *in vitro*. Better information on the effects of vitamin D_2 and D_3 metabolites may help to understand the relevance of vitamin D to osteocyte function, as well as the cross talk of osteocyte cells to other bone cells such as osteoclasts that may lead to identification of new therapeutic targets.

In the current study I initially showed that osteocyte-like cells, MLO-Y4 and MLO-A5, do possess the vitamin D metabolising machinery, VDR and CYP27B1 proteins for extra-renal production of 1,25(OH)D that is in line with previous publications [388]. Furthermore, both cell lines were responsive to 25(OH)D_2 and D_3, as up-regulation of osteocyte transcripts were observed. This suggested that osteocytes were capable of metabolising both circulatory 25(OH)D_2 and D_3 for an autocrine function. Assessment of osteocyte cell number revealed that only the highest concentrations of vitamin D_3 metabolites had an inhibitory effect on numbers for both osteocyte cell lines.

MLO-A5 is known to be a pre-osteocyte, capable of mineralisation within the embedded osteoid matrix and differentiation into mature osteocyte by up-regulating osteocyte markers such as *Phex* and *Dmp1* [388]. Here I also confirmed that when MLO-A5 cells were cultured in osteogenic differentiation medium, expression of *CX-43*, *Phex* and *Dmp1* were up-regulated, but cells expressed very low levels of *SOST* and *FGF23*.

When MLO-A5 cells were treated with vitamin D_2 and D_3 metabolites, both 25(OH)D_2 and 25(OH)D_3 were capable of increasing early stages of pre-

osteocyte differentiation by an increase in osteocytic markers such as *Dmp1* and *Phex*. This was mostly notable with the lowest concentration (100nM) of either 25(OH)D_2 or D_3 (D_2>D_3). Similarly, *OPG* was also up-regulated by 100-500nM of both 25(OH)D_2 and D_3 (D_2>D_3). It has been previously shown that 100nM treatment of 25(OH)D_3 in MLOA-5 inhibited *RANKL* expression on day 2, but had no effects on day 9 [285]. I also showed here that doses of 100-500 nM of 25(OH)D_3 had no effects on RANKL expression on day 6, whereas 25(OH)D_2 significantly up-regulated RANKL at 100nM compared to 25(OH)D_3.

However, the findings on local metabolism of 1,25$(OH)_2D_2$ or 1,25$(OH)_2D_3$ in pre-osteocyte MLO-A5 suggests that vitamin D_2 and D_3 may not only have local effects on osteocyte maturation (*CX-43*), but also may be involved in the endocrine effects (*Phex* & *Dmp1*) on renal phosphate absorption.

There is currently little known about the effects of vitamin D metabolites on MLOY-4 cells. Only 200nM dose of 25(OH)D_3 inhibited *Dmp1* but not its counterpart 25(OH)D_2. MLOY-4 cells were more sensitive in up-regulation of *FGF23* in response to both 25(OH)D_2 & 25(OH)D_3 (D_2>D_3) than MLO-A5. It is interesting to note that local metabolism of 25(OH)D_2 and 25(OH)D_3 showed a pattern of inhibition of *Dmp1* and *Phex* that co-incided with significant up-regulation of *FGF23*. Hence this may suggest that local metabolism of vitamin D by osteocytes may have an autocrine effect on regulation of *FGF23*.

It has been shown that single subcutaneous injection of 1,25$(OH)_2D_3$ (6µg/kg body weight) over 24 hours can significantly increase *FGF23* mRNA in cells subsequently isolated from tibial and calvarial bone (flash frozen in liquid nitrogen) [71]. Similarly, the same investigators showed that doses of 100nM of

$1,25(OH)_2D_3$ also significantly up-regulated *FGF23* in UMR-106 rat osteosarcoma cell line [71].

In this study I showed that single dose treatment with $1,25(OH)_2D_2$ or $1,25(OH)_2D_3$ metabolites equally up-regulated *FGF23*, as well as *RANKL* and *Phex* transcripts in MLOY-4 cells.

The effect of the vitamin D metabolites on mature osteocytes (MLO-Y4) seems different from pre-osteocyte MLO-A5. Addition of 200nM of either $25(OH)D_2$ or $25(OH)D_3$ had no effect on the maturation of the MLOY-4 cells, as no effects on *CX-43* were observed. This may be in line with the fact that mature osteocytes are fully differentiated cells, although MLOY-4 do not express SOST and could not be considered fully mature. However, findings in this chapter suggest that the local metabolism of $25(OH)D_2$ or $25(OH)D_3$ in mature osteocytes (modelled by MLOY-4) could indeed have an effect on suppression of renal phosphate absorption as a consequence of FGF23 up-regulation.

Current data on the effects of active metabolites on MLOY-4 suggests that circulatory levels of $1,25(OH)_2D_2$ or $1,25(OH)_2D_3$ could be sensed by mature osteocytes. As *FGF23* and *Phex* were highly up-regulated in MLOY-4 in response to both active metabolites (D_2=D_3), this further confirms that osteocytic cells are a major source of FGF23. Whether up-regulation of *Phex* in MLOY-4 in response to both active metabolites (D_2=D_3) could have an autocrine effects on osteocyte cells for *FGF23* is something that needs to be further studied. A dose and time course assessment of $1,25(OH)_2D_2$ or $1,25(OH)_2D_3$ on MLOY-4 would better explain this phenomenon.

The co-culture of osteocyte-like cell MLOY-4 and mouse spleen marrow cells in the presence of 10nM of $1,25(OH)_2D_3$ has been shown to support osteoclast

formation, activation and resorption [390]. This data is further supported by MLOY-4 significantly up-regulating RANKL and OPG in response to $1,25(OH)_2D_3$ [390]. In this study I showed that local metabolism of both $25(OH)D_2$ and $25(OH)D_3$ (D_2>D_3) significantly up-regulated *OPG*, but not *RANKL* during osteocyte maturation in MLO-A5. However local metabolism of either $25(OH)D_2$ or $25(OH)D_3$ had no effect on *OPG* and *RANKL* expression in MLOY-4. A co-culture of osteocyte-like cells with mouse spleen marrow cells or PBMCs would provide further insight onto the role of local metabolism of various concentrations of $25(OH)D_2$ or $25(OH)D_3$ on osteoclast activities.

When MLOY-4 cells were treated with 0.2nM of either active metabolites $1,25(OH)_2D_2$ or $1,25(OH)_2D_3$, I showed that both equally up-regulate *RANKL*, but only D_2 was capable of up-regulating *OPG*. Similarly, a dose and time-dependent treatment of MLOY-4 with $1,25(OH)_2D_3$ would confirm *OPG* expression in this cell type.

The outcome of the present study on MLOY-4 response to active metabolites further confirmed and improved our understanding that osteocytes could be in cross-talk to neighbouring osteoclasts for local bone remodelling. The effect of vitamin D metabolites on *SOST* expression in my study was inconsistent, due to the fact that MLO-Y4 expressed only a single copy of transcript in recommended culture conditions. There was no significant alteration in *SOST* gene expression in MLO-A5 in response to $25(OH)D_2$ or D_3, possibly due to duration of the cultures that probably need to exceed 6 days.

The preliminary finding of my study suggest that osteocytes could locally respond to endocrine action of vitamin D and may have a paracrine action away from skeleton on phosphate regulation for bone health. The significance of local

metabolism of $1,25(OH)_2D$ by osteocytes is high as they account for > 90% of bone cells. The fact that both *RANKL* and *OPG* expression were affected in osteocytes suggest that local metabolism of vitamin D metabolites (either D_2 or D_3) could regulate osteoclast function and activity. Furthermore the responsiveness of mature MLOY-4 to $1,25(OH)_2D_2$ or $1,25(OH)_2D_3$ and up-regulation of *RANKL* adds another pathway for bone remodelling that is influenced by osteocytes.

The current data on the effect of vitamin D metabolites in osteocytes are consistent with up-regulation of osteocyte marker gene expression. Furthermore an up-regulation of *Phex* and *Dmp1* genes in osteocytes in response to vitamin D metabolites adds to the known involvement of osteocytes in endocrine regulation of renal phosphate.

CHAPTER 6: VITAMIN D IN human OSTEOARTHRITIC BONE

6. VITAMIN D IN OSTEOARTHRITIC BONE

6.1. Introduction

6.1.1. Osteoarthritis (OA)

Osteoarthritis (OA) is considered to be one of the most common causes of disability in adults [391]. The risk of OA increases between age 45 to 75 (49% women and 42% men) with women more likely than men to seek treatment for OA in UK [391]. The most common reason for people undergoing hip (93%) or knee (98%) replacement is OA and the average cost of a replacement in UK is about £7400 [392].

Many factors, such as age, gender, obesity, joint injury or abnormalities and some rare genetic factors are known to be involved in or cause OA [391]. The underlying factors explaining why OA starts from age 40 onwards among older people and why OA is more common and severe in hand and knee in women are not fully known [393]. The alterations in the joint structure seen in OA are known to have some underlying genetic factors, involving COL1A1, VDR genes as well as genes in the WNT signalling pathway [394, 395].

OA was initially considered as a disorder of the articular cartilage, however it is now commonly accepted that all the joint is affected, including synovium, subchondral and trabecular bone and cartilage [396]. Destruction of articular cartilage is known to be the primary feature of OA [397]. The major characteristics of bone changes associated with OA are the presence of

subchondral bone cysts, osteophyte formation and thickening of subchondral bone plate underlying the cartilage defect [396].

Diagnosis of OA is made primarily on the basis of joint space narrowing due to loss of articular cartilage and a variety of grading systems such as Mankin grading system and Osteoarthritis Society International histopathological system are used (MANKIN, OARSI) [398, 399]. The relationship between the changes in cartilage and bone in OA has not been fully understood and established. This in part could be due to the fact that the disease is usually diagnosed at late stages of the progression and also the majority of the studies have focused on the late or end stages of the disease [397].

The articular cartilage is composed of a network of different collagen types (type II, VI, IX and XI) supporting and constraining aggregating proteoglycans and aggrecans [397, 400]. The articular cartilage extends from superficial to deep zone regions containing chondrocytes with different morphology and functions [400]. Superficial chondrocytes are responsible for providing a low friction, lubricating boundary, whereas chondrocytes in the deep zone exist in a hypoxic environment [400]. Alteration of the composition of the collagen matrix leads to deterioration of properties and structure of cartilage during the progression of OA [397]. In OA, cartilage gradually loses proteoglycans, followed by degradation of collagen by up-regulation of degradative proteinases [397]. In addition, a pattern of clustering of chondrocytes with increased proliferation and catabolic activity has been observed [401].

Bone is separated from the cartilage by a calcified cartilage zone [401]. During progression of OA, while cartilage is being degraded, the underlying bone architecture is re-modelled into a thick subchondral plate to adapt the skeleton to

changes in mechanical loading (**Figure 6-1**) [402]. The features of early stages of OA include an increase in vascularity and a reduced bone mass, whereas, the characteristics of late stage include an increase in bone remodelling and thickening of subchondral bone plate [397]. Another alteration of bone in OA is formation of cysts and subchondral lesions [403], associated with up-regulation and over expression of matrix metallo-proteinases [404]. Osteophytes, as a skeletal outgrowth, are localized on the joint margins and are another feature of OA, with not fully defined underlying pathogenic mechanisms [405].

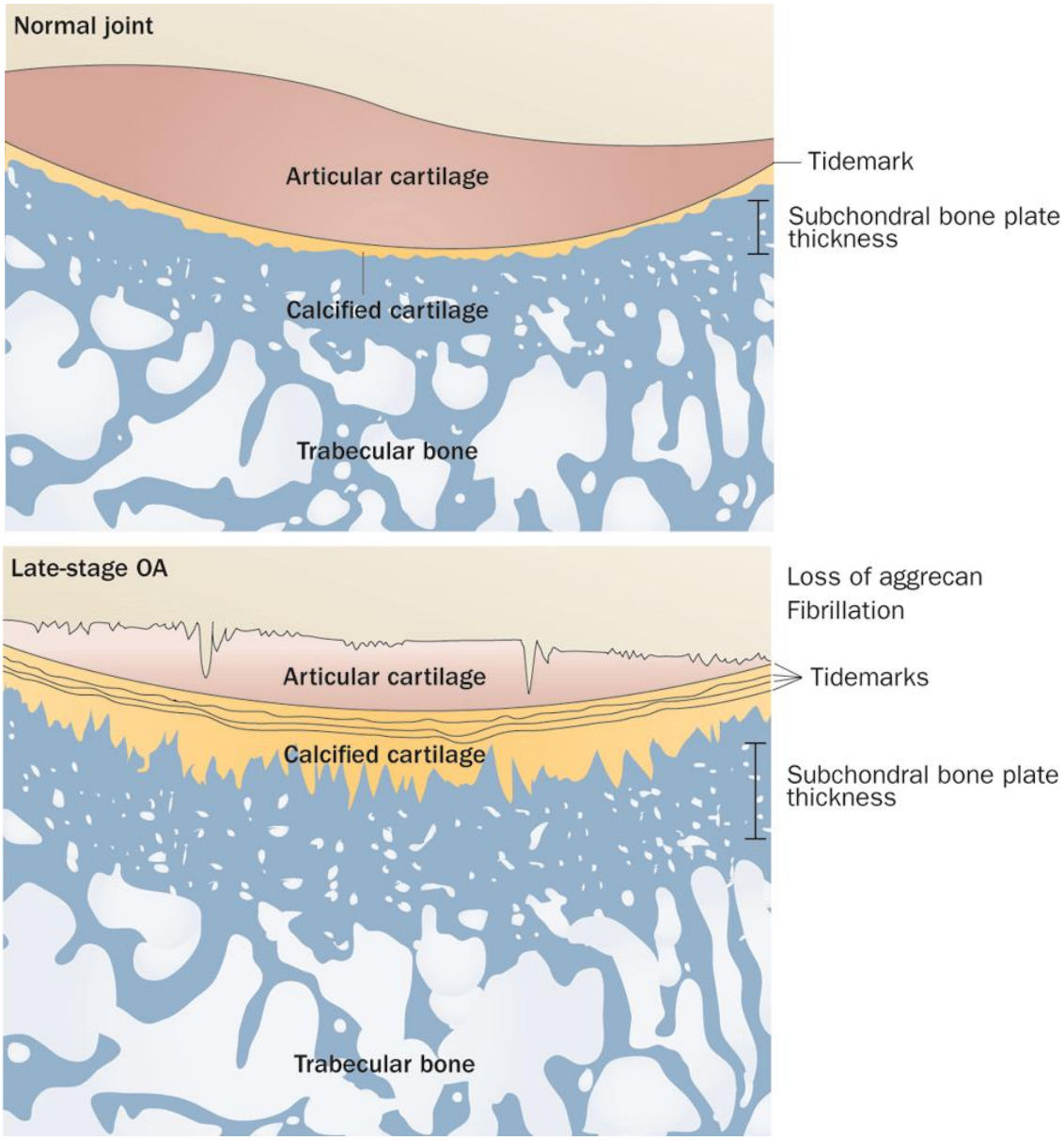


Figure 6-1: Comparison of normal joint versus late stage OA joint. Normal joint (top panel) comprises of subchondral bone plate positioned beneath the articular cartilage separated by a thin tidemark line from the calcified cartilage and trabecular bone extending into bone marrow beneath it. Late stage OA joint (below panel) architecturally differs with normal joint, with thicker subchondral bone plate, and thinner trabecular bone. Image adapted from Burr *et al* 2012 with permission [397].

6.1.2. OA and vitamin D

Vitamin D deficiency frequently co-exists with OA among the older population [30]. As VDR expression is present in cartilage, bone and muscle, vitamin D can exert multiple biological functions among these targets and could play a beneficial role in the OA joint [406, 407]. Furthermore low vitamin D levels could result in endothelial cell dysfunction, increase in proliferation of vascular smooth muscle cells, and an increase in inflammation, all of which could play a role in the aetiology of OA [408, 409]. A recent systematic review on the current evidence of serum vitamin D and its effects on OA concluded that vitamin D may be implicated in structural changes in human OA rather than only in the symptoms [410].

Current literature review revealed that:

- There is moderate evidence demonstrating that vitamin D is not associated with cartilage volume loss or changes in cartilage defects assessed by MRI [411, 412]
- There is a strong link between low circulatory vitamin D levels and loss of cartilage and the incidence or progression of joint narrow spacing (Framingham and Rotterdam studies) [413, 414]
- There is moderate evidence demonstrating that low levels of vitamin D are positively associated with progression of knee OA assessed by standard radiographic atlas [413, 414]

- There is limited evidence to show a positive association between vitamin D and join narrow spacing in knee OA, or there was conflicting evidence between vitamin D and knee OA progression or osteophytes [412, 413]
- There is limited evidence between association of vitamin D and prevalence of symptomatic knee OA (diagnosis based on clinical examination for pain, stiffness and dysfunction) [415, 416]
- There is no association between circulatory levels of vitamin D and incidence of symptomatic knee OA [417]. There was insufficient evidence for the association of circulatory vitamin D and radiographic OA in hand [418] and hip [419]
- There is limited evidence between association of vitamin D with the incidence or progression of hip OA [420]

It has been shown that there is a positive association of optimal levels of vitamin D and bone mass density (BMD) of femoral neck, suggesting that vitamin D supplementation may enhance BMD [421]. Since bone and cartilage are responsive to vitamin D, the expression of VDR in human OA specimens has been investigated by immunohistochemistry (IHC) [406]. It has been demonstrated that VDR is up-regulated in human OA primary chondrocytes compared to normal chondrocytes from early autopsy cases [406]. This up-regulation of VDR may suggest a possible regulatory role of vitamin D in modulation of chondrocyte behaviour or pathogenesis in OA [406].

Bone tissue, as a well-known target for vitamin D, has been one of the first to be assessed for expression of VDR. VDR expression has been shown in foetal and adult rat bone by $1,25(OH)_2D_3$ binding assay as well as ELISA [62]. VDR

expression has been demonstrated in osteoprogenitor cells, bone lining osteoblasts, osteocytes as well as chondrocytes in healthy tissues [62].

Human bone marrow derived osteoblasts from OA patients have been shown to express VDR and CYP27B1, the molecular machinery to metabolize and respond to vitamin D_3, up-regulating osteoblast differentiation markers [145].

Previous studies have also revealed abnormal cellular activities in OA subchondral bone plate [422]. An increase in bone remodelling in OA has been shown to be correlated with the severity of this disease [423].

Cantatore *et al* examined the vitamin D_3 metabolism of human primary osteoblasts from cancellous bone of healthy subjects and compared to OA subchondral bone osteoblasts. They showed high OCN expression in osteoblasts isolated from OA bone [424]. Similar study by Hilal et al, showed high ALP and OCN expression of OA subchondral bone osteoblasts compared to osteoblasts isolated from non-OA subjects in response to $1,25(OH)_2D_3$ [422]. It is interesting to note that in both these studies, where primary osteoblasts have been isolated from OA subchondral bone plate, the greater responses to vitamin D_3 in OCN and ALP expressions was related to the extent of the overlying articular cartilage damage [424].

6.2. Aims and objectives

The aim of this Chapter is to assess the VDR expression in bone biopsies obtained from OA patients undergoing total hip replacement and compare with samples from healthy individuals. The direct role of vitamin D_2 and D_3 metabolites on differentiation of human bone marrow stromal cells (OA-hBMSCs) into

osteoblasts will be assessed and compared to commercially available human bone marrow stromal cells (non-OA-hBMSCs) obtained from healthy individuals. By the end of this Chapter I aim to answer the following questions;

- Are the structural parameters of the subchondral bone plate different in different regions of the OA bone joint?
- Which cell types in what regions in the OA femoral head express VDR and is this pattern different to healthy control specimens?
- Do OA-hBMSCs compared to non-OA-hBMSCs respond differently to vitamin D_2 or D_3 metabolites?

6.3. Materials and methods

6.3.1. Specimens

Femoral heads of 4 patients undergoing total hip replacement (50-55 years old male) were collected under ethical approval and in compliance with the University of Oxford Musculoskeletal Bio-bank and HTA ethical procedures. Specimens were clinically graded as late stage OA with full cartilage damage, and stored in 4% paraformaldehyde[16] for fixation at 4°C (overnight). From each femoral head,

[16] **Paraformaldehyde solution:** 4 g paraformaldehyde in 100 ml TBST, pH to 7.4

cylindrical cores of 6x15 mm were taken longitudinally from different regions of femoral heads as illustrated in **Figure 6-2**.

Core samples were decalcified with 0.5M EDTA (pH 7.4) for three weeks at 4°C. Control paraffin embedded sample of human femoral heads [non-osteoarthritic (non-OA)] were kindly provided by the Pathology Department, Nuffield Orthopaedic Centre under Professor Nick Athanasou's guidance. Samples were obtained from patients with informed consent and in full compliance with UK Human Tissue Act and Multicentre Research Ethics Committee requirements and with Helsinki Declaration of 1975, as revised in 1983. The OA cores were then embedded in paraffin and 5µm sections were cut longitudinally and mounted on adhesive glass slides (Lecia Biosystems, UK). Slides of each core and non-OA samples were deparaffinised in xylene and rehydrated through a graded series of alcohols to water. Decalcified sections were stained for 15 minutes with Mayer's Haematoxylin and 8 minutes with Eosin (Lecia Biosystems, UK). Slides were washed under running water for 30 seconds, dehydrated in ethanol, cleared in xylene, and cover slipped with DEPEX mounting medium (Fisher Scientific). Photographs of subchondral bone plates of representative slides from each core were captured at 10 X magnifications using an Olympus BX40 light microscope. Cross sectional areas of subchondral bone plate surfaces from each slide was measured using ImageJ software (version 1.47) in μm^2.

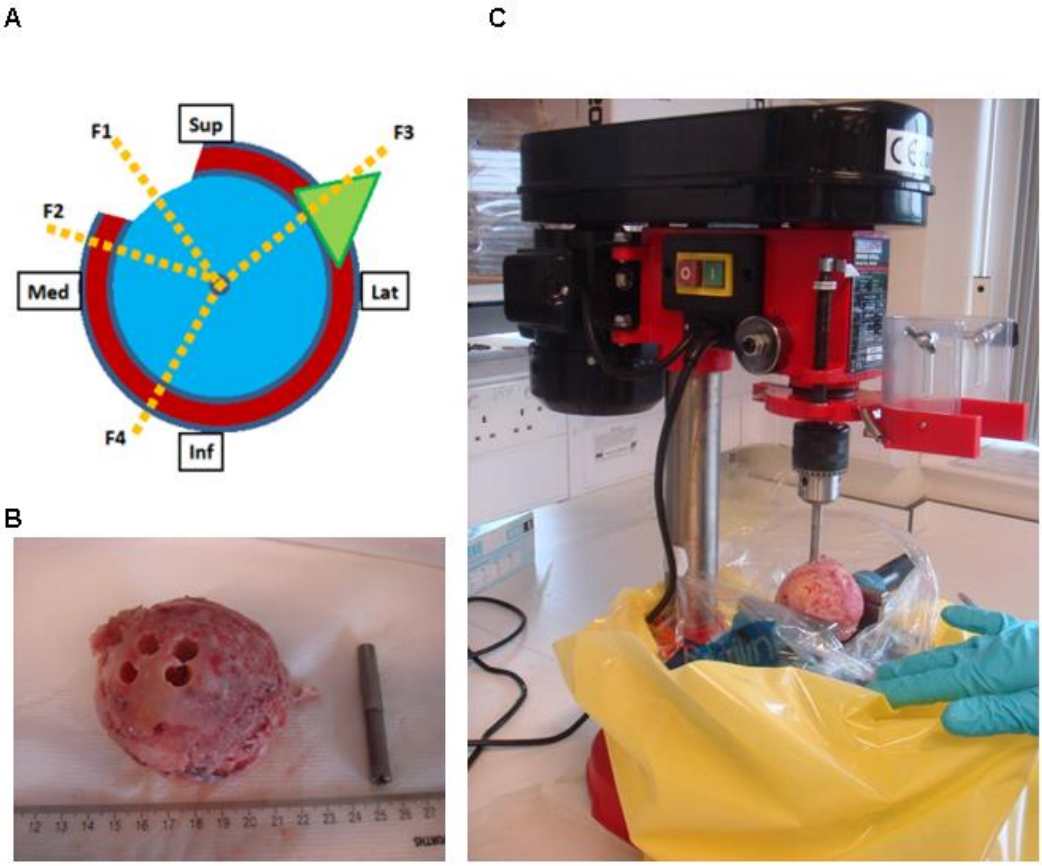


Figure 6-2: Schematic representation of OA femoral head and its various regions depending on macroscopic observations. F1; complete absence of cartilage, F2; partial cartilage defect, F3; base of osteophytes (green triangle), F4; normal and undamaged cartilage (red). (Sup); Superior, (Med); Medial, (Lat); Lateral and (Inf); inferior. (B) Representative image of a human femoral head where the cores taken from with a holesaw, (C) table-drill (Toolmaster, Oxford, UK).

6.3.2. Immunohistochemistry

Slides of each osteoarthritic core (**Figure 6-2**) or non-OA (healthy bone with undamaged cartilage, equivalent to F4) were deparaffinised in xylene and rehydrated through a graded series of alcohols to water. Slides were washed with TBS buffer and endogenous peroxidase activity was quenched by 3% hydrogen peroxide for 30 minutes. Antigen retrieval was performed on an 85°C hot plate

using citrate buffer[17] for 10 minutes. Non-specific reactivity was blocked in TBST-5%BSA for 30 minutes at room temperature. Representative slides of each core from four OA patients, and control slides were incubated with mouse anti-human VDR monoclonal antibody (Santa Cruz Biotechnology: SC-13133) at a dilution of 1:200 overnight at 4°C.

After primary antibody incubation reaction, secondary biotinylated rabbit anti mouse antibody (Vector Laboratories) was applied for 30 minutes at room temperature. Sections then were rinsed with TBS buffer, and visualised using the avidin-biotin peroxidise diluted at 1:200 for 30 minutes. Sections were rinsed with TBS buffer and treated with DAB (Vector labs, UK: 3, 3 diaminobenzidine). Sections were counterstained with haematoxylin for 10 seconds, washed under running water for 30 seconds, dehydrated in ethanol, cleared in xylene, and cover slipped with mounting medium. Sections were examined using an Olympus BX40 light microscope; photographs were captured at 40-200 X magnifications. For general morphological analysis, decalcified sections were stained with Mayer's H&E, and negative control sections were incubated with the non-immunized mouse IgG2a (R&D Systems, UK).

[17] **Citric buffer:** 10mM anhydrous citric acid, 0.05% Tween 20, pH 6.0

6.3.3. Ex-vivo cell culture

For human osteoblast studies; human bone marrow stromal cells as control (non-OA- hBMSC) were purchased from Lonza (19 years old, healthy male volunteer), and human primary bone marrow stromal cells (OA-hBMSCs: 55 years old male) were obtained in compliance with the University of Oxford Musculoskeletal Bio-bank and HTA ethical procedures. Human OA primary cells were obtained from bone chips of trabecular bone using a modification of the method described by James Gallagher [425]. Briefly, bone chips were thoroughly washed with DPBS, digested with collagenase with rotation at 37^{o}C for 3 hours, re-washed with DPBS and placed in petri dishes in αMEM 10%HI-FBS. Medium was replaced every other day, cells were allowed to grow until full confluency and cryo-preserved for future experiments (Courtesy of Dr Philippa Hulley).

For differentiation assays, cells from both sources were plated in 96 well plate ($2x10^4$) formats in replicates and cultured in αMEM-10% HI-FBS to confluence. Cells on this day (day zero) were introduced to osteogenic differentiation medium (10mM βGP, 50µg/ml ascorbic acid and 100nM dexamethasone) and presence of 1000nM concentrations of 25(OH)D_2, D_3 or 10nM of 1,25$(OH)_2D_2$ or D_3 ± ketoconazole (10µM). Media were replaced every other day up to 4 weeks. On completion of the assays, cells were washed with DPBS, fixed in 70% ethanol, washed with deionised water and stained with Alizarin red dye for assessment of mineralisation and staining from each condition was quantified by acetic acid extraction as described earlier.

6.4. Results

6.4.1. Changes in OA

In an attempt to identify whether the presence or absence of cartilage, partial cartilage defect or presence of osteophytes would affect the thickness of the subchondral plate, the structural parameters of subchondral plate were evaluated (**Figure 6-3**). Subchondral plate thickness was significantly thicker at F1 compared to F2 and F4 (P=0.005) as well as F3 (P=0.001). There were no significant differences between subchondral plate between F2 and F4, whereas, both F2 and F4 subchondral plate thicknesses were significantly higher than F3 (**Figure 6-3;** P=0.004).

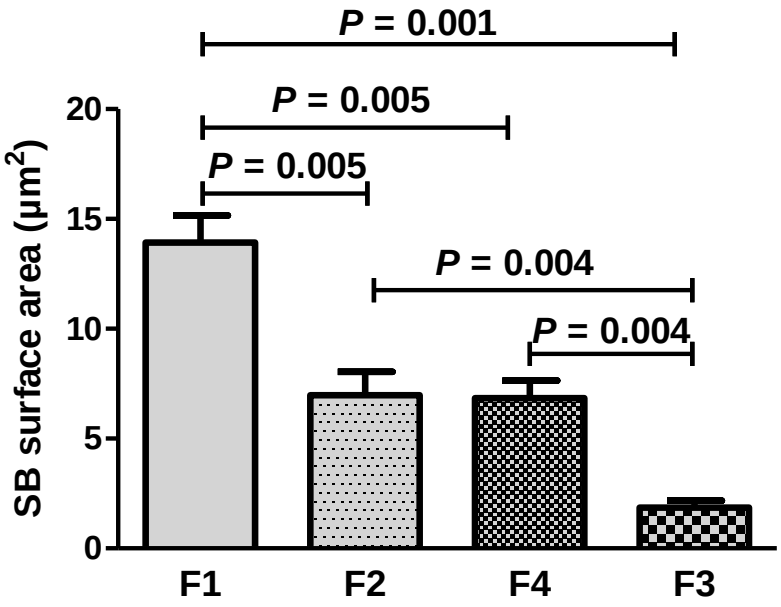


Figure 6-3: Subchondral bone thickness varies in OA femoral head. Cylindrical cores were taken from 4 OA femoral head biopsies ; F1; full cartilage defect, F2; partial cartilage defect, F3; osteophyte, F4; normal or undamaged cartilage (see schematic in Figure 6.2). Cores were fixed and decalcified in EDTA, sectioned longitudinally at 5µm, stained with H&E. Images of subchondral bone surfaces were taken at 10X magnifications from each slides and cross sectional areas of subchondral bone plate (SB) thickness were measured by ImageJ software (μm^2). Data are presented as mean ±SEM from 3 slides per each core from 4 femoral head biopsies (One-way Anova: Tukey Post Hoc test).

Immunohistochemical (IHC) staining of control bone sections (**Figure 6-4**) revealed VDR expression in bone lining osteoblasts in subchondral bone (**Figure 6-4F**, red arrows), as well as trabecular bone multi-nucleated bone resorbing osteoclasts (**Figure 6-4G,** black arrow), as well as newly embedded osteocytes (**Figure 6-4F,** green arrows). Note that BMSCs in the bone marrow region on slide 2F (black arrow) are also VDR positive. Mature osteocytes did not show any VDR staining in trabecular bone specimens (**Figure 6-4G,** blue arrows).

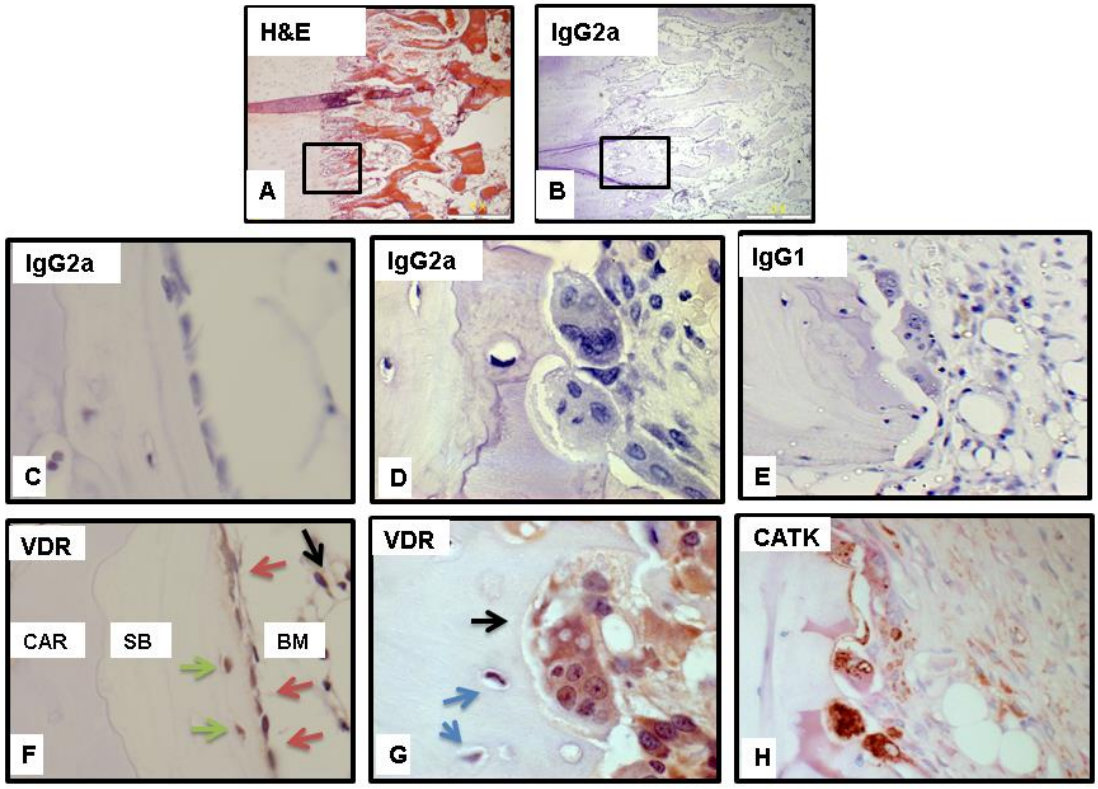


Figure 6-4: VDR is expressed by BMSCs, osteoblast, osteocyte and osteoclasts in healthy bone samples. Serial 5µm sections of healthy human bone femoral head. (A): H&E staining, (B): negative staining, (C&D): IGg2a and (E): IGg1 negative staining, (F&G): IHC of VDR, and (H): Cathepsin K (CATK). Positive cross-reactivity of VDR was observed in bone lining osteoblasts (red arrows in F), newly embedded osteocytes (green arrows in F), but not mature osteocytes (blue arrows in G), bone resorbing multi-nucleated osteoclast (black arrow in G). (CAR): cartilage, (SB): subchondral bone, (TB): trabecular bone, (BM): bone marrow. A&B 4X, C, D, F, &G 60X, E&H 20X magnifications.

IHC staining for VDR in OA bone revealed staining in only bone lining osteoblasts in the subchondral plate and trabecular bone (**Figure 6-5F,** red arrows) (**Figure 6-2A: F1**) and in chondrocytes in the articular cartilage region (**Figure 6-5H,** black arrows) (**Figure 6-2A: F4**). There was no VDR immuno-reactivity in newly embedded osteocytes (**Figure 6-5F,** green arrows), and osteoclasts in OA samples. VDR staining was also observed in BMSCs in the OA bone samples (**Figure 6-5G,** blue arrows).

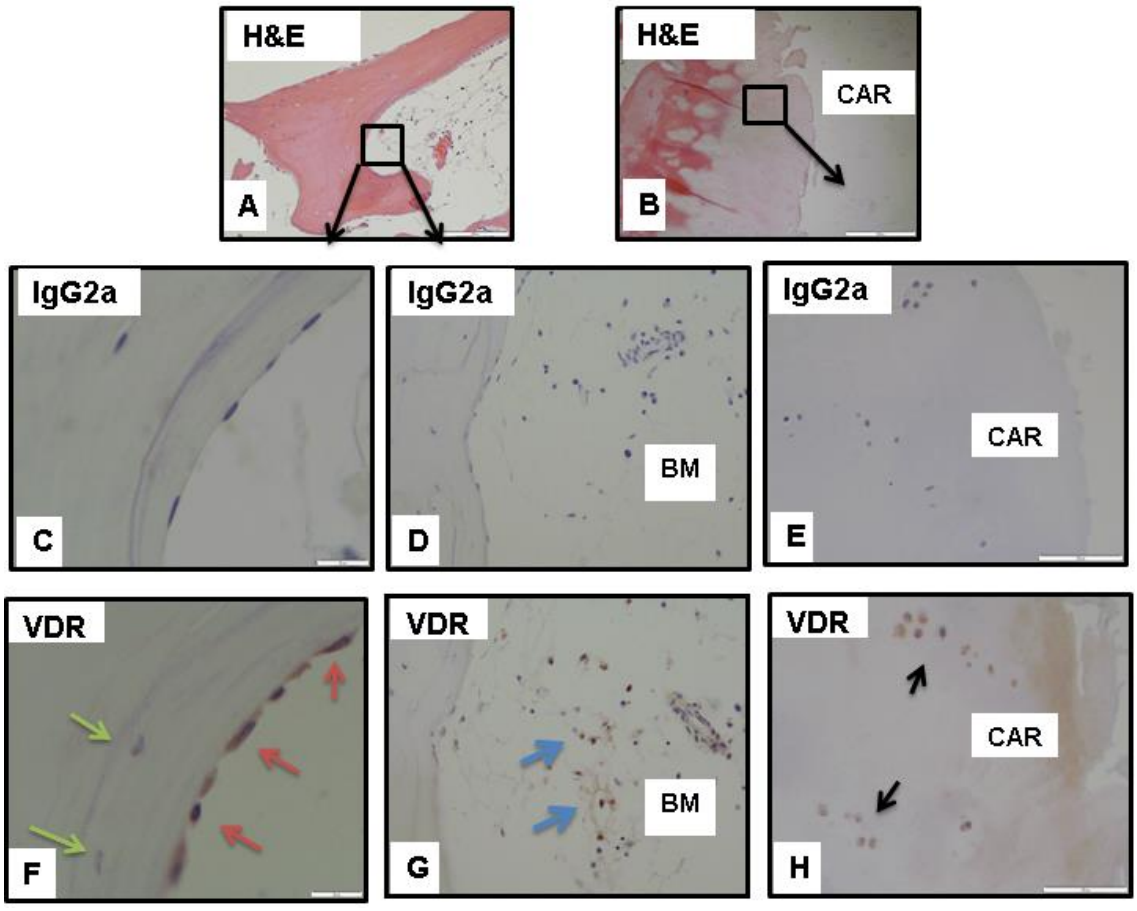


Figure 6-5: VDR is expressed by BMSCs, chondrocytes and osteoblasts, in OA bone samples. Repeated serial 5µm sections of OA femoral head: H&E staining of trabecular bone (A), cartilage (CAR) (B), VDR immuno-reactivity of bone lining osteoblasts is shown by red arrows in (F), BMSCs, blue arrows in (G) and clustered chondrocytes, black arrows in (H). C, D&E negative staining of adjacent section of F, G&H with IgG2a. Newly embedded osteocytes in (F) shown by green arrows did not show any VDR cross reactivity. BM: bone marrow, CAR: cartilage, A &B 4X, D&G 20X, and C, E, F, and H 60X magnifications.

The summary of VDR immuno-reactive cells in OA and non-OA tissues is listed in **Table 6-1.** Non-OA control bone specimen had no pathological abnormalities such as cartilage defect or osteophytes and VDR expression was present in bone lining osteoblasts, newly embedded osteocytes, bone resorbing osteoclasts, calcifying chondrocytes as well as BMSCs. However, VDR expression in OA tissue was restricted to bone lining osteoblasts in F1 with full cartilage defect and thickest subchondral plate, in clustered chondrocytes in F4 as well as expression by BMSCs.

Table 6-1
List of the VDR positive cells in non-OA and OA human tissue

Cell Type	Non-OA (F4)	OA (F1)	OA (F4)
Osteoblast	**+**	**+**	**+**
Osteoclast	**+**	**-**	**-**
Osteocytes (osteoid)	**+**	**-**	**-**
Mature Osteocytes	**-**	**-**	**-**
Superficial chondrocytes	**-**	**-**	**+**
Calcifying chondrocytes	**+**	**-**	**-**
BMSCs	**+**	**+**	**+**

(+): Presence, (-) absence of staining

6.4.2. Ex-vivo cell culture

6.4.2.1. Mineralisation

I also examined the effects of 1000nM of 25(OH)D_2 and 25(OH)D_3 ±10μM ketoconazole on human primary osteoblast mineralisation (**Figure 6-6A-B**). When non-OA hBMSCs were treated with vitamin D, compared to controls, 1000nM of 25(OH)D_2 had no effects on mineralisation, whereas 1000nM of 25(OH)D_3 increased mineralisation significantly (**Figure 6-6C** :P<0.001). 1000nM of either 25(OH)D_2 (**Figure 6-6C**: P<0.01) or 25(OH)D_3 (**Figure 6-6C**, P<0.001) increased mineralisation in OA-hBMSCs, with 25(OH)D_3 being more potent than 25(OH)D_2 (**Figure 6-6C**: P<0.001). Compared to controls, 1000nM of 25(OH)D_2 increased mineralisation in OA-hBMSCs (**Figure 6-6C**: P<0.01) but not in non-OA hBMSCs. Similarly, 25(OH)D_3 also was more potent in mineralisation in OA-hBMSCs than non-OA hBMSCs (**Figure 6-6C**: P<0.05).

Addition of ketoconazole with 1000nM 25(OH)D_2 or 25(OH)D_3 abolished the mineralisation in OA-hBMSCs as well as non-OA hBMSCs, with 25(OH)D_3 being a more potent inhibitor compared to 25(OH)D_2 (P<0.01) in cells from both sources (**Figure 6-6B&D**).

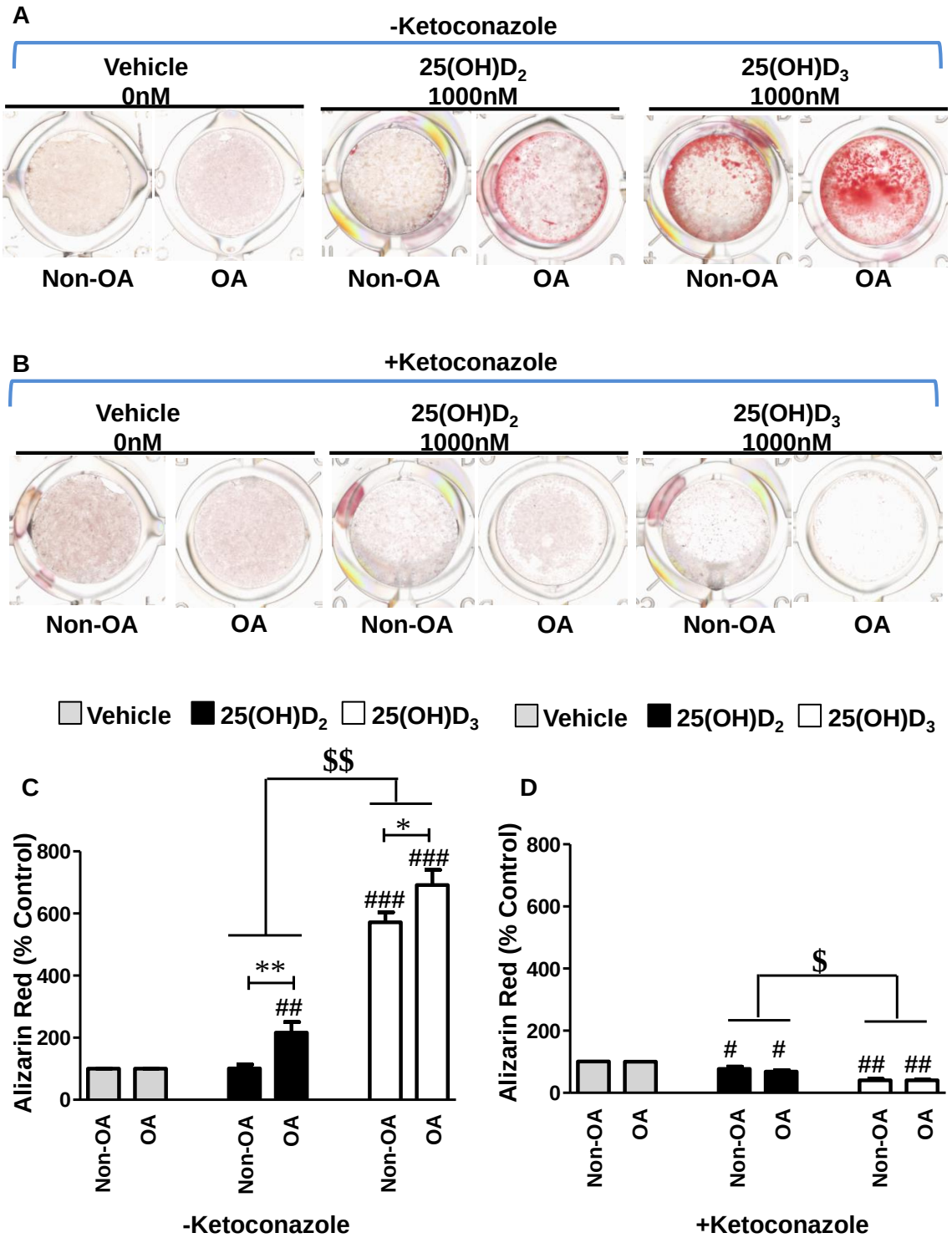

A
-Ketoconazole
Vehicle 0nM
25(OH)D2 1000nM
25(OH)D3 1000nM
Non-OA
OA
B
+Ketoconazole
Vehicle 0nM
25(OH)D2 1000nM
25(OH)D3 1000nM
Non-OA
OA
Vehicle
25(OH)D2
25(OH)D3
C
Alizarin Red (% Control)
$$
*
###
###
**
##
Non-OA
OA
-Ketoconazole
D
$
#
#
##
##
+Ketoconazole

Figure 6-6: 25(OH)D_3 was more potent than D_2 in induction of mineralisation in both healthy and OA BMSCs. Comparison of effects of vitamin D metabolites on mineralisation in Non-OA hBMSCs (19 years old) and OA–hBMSCs (55 years old). Cells were cultured in differentiation media in multiple 96 well plates for 4 weeks in (A) the absence of ketoconazole and presence of 1000nM of 25(OH)D_2 , 25(OH)D_3 metabolites or (B) presence of 10µM ketoconazole as well as 1000nM of 25(OH)D_2 or 25(OH)D_3. On completion of the treatments, cells were washed with DPBS, fixed with ethanol, stained with Alizarin red and photographed. Lower panels are quantified Alizarin red staining by acetic acid extraction of (C) 1000nM of 25(OH)D_2 and 25(OH)D_3 and absence of ketoconazole or (D) presence of 1000nM of 25(OH)D_2, D_3 as well as presence of ketoconazole. Quantified Alizarin data (8 well) are presented as percentage of untreated controls (grey) as mean ±SEM (Two-way ANOVA: Bonferroni Post Hoc Test). * P<0.05, ** P<0.01 for comparisons between OA and Non-OA, $ P<0.05 and $$ P<0.001 for comparisons between vitamin D metabolites, and # P<0.05, ## P<0.01, ### P<0.001 comparison of treatments to controls (grey).

OA-hBMSCs and non-OA hBMSCs were also cultured in the absence of ketoconazole and 10nM of active metabolites 1,25(OH)$_2$$D_2$ or 1,25(OH)$_2$$D_3$ (**Figure 6-7A**) or absence of ketoconazole and vitamin D active metabolites **(Figure 6-7B**) in osteogenic differentiation medium over 4 weeks. Treatments with 10nM of the active metabolites 1,25(OH)$_2$$D_2$ or 1,25(OH)$_2$$D_3$ without ketoconazole had no effect on induction of mineralisation in non-OA hBMSCs or OA-hBMSCs (**Figure 6-7A&C**).

Co-treatment with ketoconazole enhanced mineralisation at 10nM concentration of either active metabolite in non-OA BMSCs (**Figure 6-7B&D**, 1,25(OH)$_2$$D_2$: P<0.001, and 1,25(OH)$_2$$D_3$: P<0.01) where 10nM 1,25(OH)$_2$$D_2$ was more effective than 1,25(OH)$_2$$D_3$ (P<0.001). 10nM concentration of 1,25(OH)$_2$$D_2$ only increased OA-hBMSCs mineralisation (**Figure 6-7B&D**, P<0.01) but not 1,25(OH)$_2$$D_3$ compared to controls. 10nM 1,25(OH)$_2$$D_2$ was more potent in induction of mineralisation in Non-OA hBMSCs compared to OA-hBMSCs (**Figure 6-7D**, P<0.001).

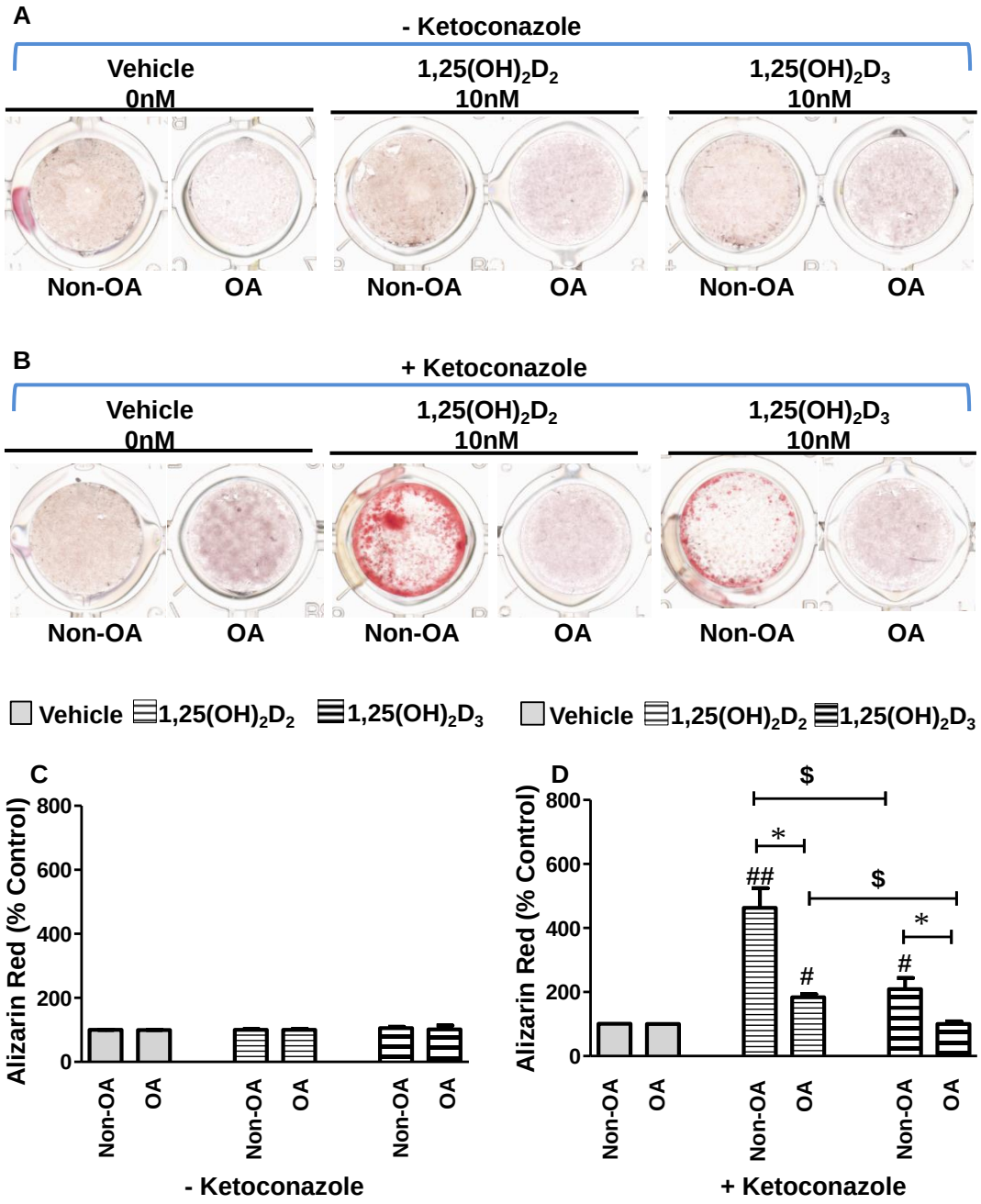


Figure 6-7: $1,25(OH)_2D_2$ was more potent than D_3 when co-treated with ketoconazole to induce mineralisation in both healthy and OA BMSCs. Comparative effects of $1,25(OH)_2D_2$ or D_3 active metabolites ± ketoconazole on non-OA hBMSCs and OA-hBMSCs. (A) Cells were cultured in the presence 10nM of active metabolites $1,25(OH)_2D_2$ or $1,25(OH)_2D_3$ and absence of ketoconazole or (B) 10nM of $1,25(OH)_2D_2$ or $1,25(OH)_2D_3$ and presence of ketoconazole. (C) Alizarin red staining of 10nM of $1,25(OH)_2D_2$, D_3 and absence of ketoconazole or (D) presence of ketoconazole, 8 wells were quantified by acetic acid and plotted against untreated controls (grey) ±SEM (Two-way ANOVA: Bonferroni Post Hoc Test). * P<0.001 for comparisons between OA and Non-OA, $ P<0.001 for comparisons between vitamin D metabolites vitamin, and # P<0.01, ## P<0.001, comparison of treatments to untreated cells (grey).

6.5. Discussion

In OA, an increase in bone mass density has been documented with a possible positive association with circulatory levels of vitamin D (Framingham Study), as well as an association between radiographic OA and VDR genotype. However, the expression of VDR in OA bone cells, or the direct effects of vitamin D metabolites on OA bone osteoblast cell differentiation has not been fully investigated. Specifically, changes in VDR expression in OA imply parallel changes in sensitivity to circulating or supplemented vitamin D which may have consequences for the higher bone mass density evident in OA.

Histological assessments in OA bone biopsies from 4 individuals showed that bone lining osteoblasts consistently express VDR, but newly embedded or mature osteocytes and osteoclasts did not. Osteoblasts are known to be the only bone forming cells and whether higher BMD in OA is due to expression of VDR by osteoblasts, but not osteoclasts needs to be further studied.

Immunohistochemical analysis of VDR in non-OA bone suggests that VDR expression is probably involved in the normal metabolic activities of the main bone cell types. This includes the observation that chondrocytes in the deep zone of the cartilage require vitamin D metabolism during osteochondral ossification and calcification to bone. This is in line with previous publications in literature that vitamin D metabolism by chondrocytes in the deep zone facilitates cartilage calcification [426].

It is well known that osteocyte formation from BMSC precursor involves a process of differentiation and maturation and embedding into mineralized matrix osteoid as newly embedded osteocytes. Expression of VDR in BMSCs, bone lining osteoblasts and newly embedded osteocytes in non-OA bone suggests that

vitamin D metabolism may be involved throughout these differentiation processes.

In OA the normal metabolic activities of the cells such as osteoblasts [422], chondrocytes [406] and osteocytes [427, 428] and the cross talk between these cells may be hampered [429]. It has been shown that osteoblasts isolated from OA bone are more active in expressing OCN, ALP and when co-cultured with non-OA chondrocytes increase factors such as glycosaminoglycan that influence cartilage metabolism [422, 430].

In OA bone it has also been described that osteocytes macroscopically do not look healthy with rather abnormal lacunae compared to osteocytes from normal bone [428]. In this study I did not see any VDR expression in mature osteocytes in OA or non-OA bone. The fact that only newly embedded osteocytes in non-OA bone expressed VDR suggests that there may be a problem with vitamin D response in OA osteocytes.

My data in Chapter 5 showing that vitamin D up-regulates osteocytic differentiation markers in the MLO-A5 cell line may indicate an important role for vitamin D in normal osteocyte formation and maturation. Since no VDR was expressed in mature osteocytes in either OA or non-OA tissue, it is not clear to us under what specific physiological conditions mature osteocytes would express VDR for local metabolism of vitamin D or if VDR up regulation is only needed for differentiation.

Bone mineral density is known to increase in OA and it seems logical that bone has to compensate for the absence of cartilage to cope with the mechanical loading changes [421]. Although findings in this Chapter suggests a decrease in VDR expression across the joint tissues with loss in both osteoclasts and

osteocytes, both osteoblast and BMSCs retain VDR expression in OA bone, indicating their ongoing vitamin D sensitivity.

In OA bone the VDR expression in osteoblasts was seen in cells lining the bone surfaces located under a full thickness cartilage defect (core **F1, Figure 6-2A**). A further cross sectional area analysis of bone of the cores obtained from different regions of OA femoral head biopsies revealed that subchondral bone plate was thicker in core **F1** (full thickness cartilage defect) than other regions. The absence of VDR expression in osteoblast cells in cores **F2**, **F3** or **F4** was also correlated with lower subchondral plate thicknesses. Findings in this study suggests that an increase in bone density in OA is possibly site specific (core **F1**) and there is probably an increase in the activities of VDR in positive osteoblasts and BMSCs. One could argue the fact that the bone has to become thicker under the absence of overlying cartilage (**F1**) to compensate for the body load on the joint.

In contrast to this, the subchondral bone plate was at its lowest thickness in **F3**, where not only cartilage is intact, but also a thick osteophyte was present. The bone/ subchondral bone plate in **F3** probably receives the lowest mechanical loading or lower shock absorption throughout the cartilage as well as osteophyte. Interestingly, the VDR expression was absent in subchondral plate bone lining osteoblasts in **F3**.

Whether elevated VDR is also a response to this mechanical loading in subchondral bone underlying the cartilage is unclear. It was, however, evident that OA-hBMSCs were more vitamin D sensitive *in vitro* as shown by higher Alizarin red staining in OA-hBMSCs in response to $25(OH)D_2$ and $25(OH)D_3$ than non-OA-hBMSCs. Similar observation has been made previously as OA primary osteoblasts are more vitamin D responsive than non-OA osteoblasts [422].

Comparison of Alizarin red staining in cells from both sources revealed that 25(OH)D_3 was more potent than 25(OH)D_2. The OA-hBMSCs mineralized more in response to 25(OH)D_2 than non-OA-hBMSCs. The trend was seen also in response to 25(OH)D_3, which was stronger in OA-hBMSCs than non-OA-hBMSCs. The possible explanation for this could be a higher expression of VDR or CYP27B1, or lower CYP24A1 in OA-hBMSCs than the non-OA-hBMSCs. Although I did not look for VDR and CYP27B1 or CYP24A1 expression at protein levels in these cells due to lack of enough cells, it would be interesting to confirm these markers in future studies.

Present results in this chapter suggesting that hBMSCs may have higher vitamin D metabolism in OA might link to higher bone mass density in OA and provision of higher shock absorbing capacity that might lower the cartilage degradation in the joint [431, 432]. Due to constraining ethical agreements I had no information regarding circulatory vitamin D levels for the 4 patients. However macroscopic analysis of all femurs revealed presence of osteophytes which are features of possibly low vitamin D levels in OA patients, as has been previously reported [414]. However, histological analysis in this study showed no VDR expression in the osteophytes or the cysts in the bone marrow specimens that were stained.

An aim of this study was to compare the effects of 1,25$(OH)_2D_2$ and 1,25$(OH)_2D_3$ metabolites on bone marrow derived primary osteoblast cells. Interestingly, OA-hBMSCs showed lower mineralisation in response to 1,25$(OH)_2$D metabolites than non-OA-hBMSCs in contrast to the results with 25(OH)D. Results in this chapter revealed that 1,25$(OH)_2D_2$ is more potent than 1,25$(OH)_2D_3$ in increasing *in vitro* bone formation both in OA-hBMSCs and non-OA-hBMSCs. But I was

surprised that hBMSCs in OA were less responsive to both active metabolites of $1,25(OH)_2D_2$ and $1,25(OH)_2D_3$ compared to non-OA-hBMSCs.

In the absence of ketoconazole the active metabolites $1,25(OH)_2D_2$ and $1,25(OH)_2D_3$ at 10nM had no effects on mineralisation in BMSCs from OA or non-OA. The data is in line with previous finding on the effects of the active metabolites on non-OA cells in Chapter 4 where no mineralisation was observed. The differential responsiveness of the OA-BMSCs to the active metabolites in the presence of ketoconazole could be due to higher baseline expression of VDR in OA cells compared to non-OA cells. Higher VDR expression by OA cells could up-regulate CYP24 hydroxylase catabolising enzyme when cells were exposed to the active metabolites. Having said that, present findings on mineralisation assays on BMSCs from either OA or non-OA cells revealed that prolonged (4 weeks) treatments with $1,25(OH)_2D_2$ increases mineralisation more than its $1,25(OH)_2D_3$ counterpart. Prolonged treatments of 10nM of $1,25(OH)_2D_3$ and CYP24A1 inhibition in the presence of ketoconazole in OA-BMSCs could possibly cause an overdose and toxicity. The effects were consistently observed throughout the previous experiments when mouse or human primary cells were exposed to high concentrations of D_2 and D_3, where D_3 metabolites tended to be more inhibitory than D_2.

At this stage of present study it cannot be explained why local metabolism of $25(OH)D_2$ or $25(OH)D_3$ had an effect on OA-hBMSCs, but addition of $1,25(OH)_2D_2$ or $1,25(OH)_2D_3$ did not affect the OA-hBMSCs to the same degree as they did in non-OA-hBMSCs. However, it could be that the differential responses of OA and non-OA cells to the active metabolites due to baseline expression of vitamin D receptor and metabolising or catabolising enzymes

CYP27B1 or CYP24 hydroxylase. It would be interesting to investigate if isolated primary OA cells differentially express VDR, CYP27B1 or CYP24A1 compared to non-OA primary cells. The commercial BMSCs (non-OA hBMSCs) are derived from a 19 years old male donor, whereas OA hBMSCs are from an older male donor (55) undergoing total hip replacement. Generally the access to cells or tissues from healthy donors are very limited and age differences in the current study might influence the differential vitamin D responsiveness of the cells. However in both OA and non-OA hBMSCs the responsiveness of the cells to $25(OH)D_3$ and $1,25(OH)_2D_2$ are the same and these metabolites are more potent than their counterparts, $25(OH)D_2$ and $1,25(OH)_2D_3$.

OA-BMSCs were more responsive to both $25(OH)D_2$ and $25(OH)D_3$ compared to non-OA cells, hence it would be interesting to measure the circulatory levels 25(OH)D in OA patients prior to hip or knee replacement and assess whether this responsiveness is as a result of higher VDR or CYP27B1 expression at cellular levels in cells isolated from these patients compared to healthy individuals. The non-responsiveness of OA-hBMSCs to the active metabolites in present study however correlates with previous finding that there is no association between radiographic OA changes in the hip with serum $1,25(OH)_2D_3$ among the populations studied [420].

Similar to other publications in literature VDR expression was observed in clustered chondrocytes in my IHC studies [406]. It has been shown that the synthesis of the proteins that make up the articular cartilage matrix or metalloproteinase enzymes that break it down are tightly coupled and regulated by chondrocytes in normal cartilage, the balance which is lost in OA cartilage [404, 406].

In conclusion, findings in this chapter suggest that VDR expression is altered among the bone cells in OA and restricted to clusters of chondrocytes, bone lining osteoblasts and BMSCs, suggesting the balance of bone cellular activities may be shifted to more bone formation by differentiation of BMSCs into osteoblast rather than osteoclast differentiation with bone resorption. In contrast, VDR expression in non-OA healthy bone suggests that vitamin D metabolism seems to be involved in cellular activities and differentiation of deep zone chondrocytes, BMSCs differentiation into bone lining osteoblasts and newly embedded osteocytes as well as osteoclast activities.

$25(OH)D_3$ supplementation of osteoarthritic or non-OA individuals seems to have a better outcome on *in vitro* bone formation and differentiation of BMSCs, possibly higher metabolism of $25(OH)D_3$ into $1,25(OH)_2D_3$ is the underlying mechanism. Since $1,25(OH)_2D_3$ half-life in circulation is ~15 hours the exact concentrations of this active metabolite to induce *in vivo* BMSCs differentiation into osteoblasts remains unclear. However, high concentrations of prolonged (4 weeks) $1,25(OH)_2D_3$ treatment seems to lead to inhibition of *in vitro* hBMSC activities, whereas prolonged $1,25(OH)_2D_2$ is beneficial to hBMSC differentiation into osteoblasts. Therefore, it would seem more sensible to administer $1,25(OH)_2D_2$ active metabolites to patients such as renal failure patients if active metabolite was the last line of treatment.

CHAPTER 7: CONCLUSION AND FUTURE WORK

7. CONCLUSION AND FUTURE WORK

7.1. Summary of the results

A review of the literature revealed that many controversies exist on potencies of D_2 and D_3 metabolites. Our current understanding of the effects of vitamin D on various tissues or cells from different species is based largely on *in vitro* studies using vitamin D_3 metabolites. As vitamin D directly affects bone cells, surprisingly there have not been any *in vitro* studies on the effect of vitamin D_2 metabolites on bone cells. It also appears that we are now moving away from D_2 supplementation in humans based on lower potencies of D_2 metabolites. These findings presented the case for my study on vitamin D_2 metabolites on bone cell functions aiming to answer the main question of the current project, "Are D_2 metabolites as potent as D_3 metabolites on bone cell biological activity"?

In order to investigate the potencies of vitamin D_2 and D_3 metabolites it was aimed to identify candidate cell lines to conduct my experiments. The D_3 literature in bone is confusing, perhaps due to the wide range of cell lines used. Data on effective doses and rationale for choosing these are unsatisfactory and may have been complicated by failure to use primary cells, frequent use of non-mineralising cell lines, and possible species differences between mouse, rat and human.

In **Chapter 2** I screened available human and mouse osteoblast cell lines for use, in addition to human primary osteoblasts, for studies on the comparison of vitamin D metabolites. Established osteoblast-like cell lines are known to mineralise in the presence of higher concentrations of phosphate than mouse or rat primary cells. Currently bone formation of osteoblast-like cells under influence of osteogenic medium is debatable as these cells are not expected to form bone

nodules as primary rat and mouse cells do. Over the duration of this project there was no access to mouse or rat primary bone tissue or cells to conduct the vitamin D treatments and comparisons. However the mouse 2T3 osteoblast-like cell line that I optimised expressed all the candidate markers, mineralised rapidly and seemed the best candidate cell line for extensive dose and time studies. All the osteoblast differentiation markers that were assessed at transcript or protein levels as well as mineralisation activity or vitamin D related markers, VDR, CYP27B1 and CYP24A1 were responsive to both D_2 and D_3. An increase in mouse 2T3 differentiation as an end point activity of mouse osteoblasts was observed with either D_2 or D_3 metabolites which were assessed in **Chapter 3**. This increase in osteoblastic activity was observed at physiological doses of $25(OH)D_2$, $25(OH)D_3$ (200nM) and of the active metabolites $1,25(OH)_2D_2$ and $1,25(OH)_2D_3$ (0.1-0.2nM).

I noted that when mouse 2T3 cells were treated with the lower dose range of 50-100nM, no effects on osteoblastic activities were observed (D_2=D_3). With 200nM of $25(OH)D_2$, $25(OH)D_3$, or 0.1-0.2nM of their active metabolites, both D_2 and D_3 metabolites were equipotent in increasing 2T3 activities (D_2=D_3). However, the differences between D_2 and D_3 were evident at supra-physiological doses of ≥ 500nM of $25(OH)D_2$, $25(OH)D_3$, or ≥0.5nM of their active metabolites. At these high doses an inhibition of cellular activities were observed, where D_3 consistently was more potent than D_2.

It was also noted that, currently in the literature, high doses of $1,25(OH)_2D_3$ (1, 10 and 100nM) have been used to assess the effects of vitamin D_3 on established osteoblast-like cell activities over short stimulation assays. However in my hands prolonged (4-12 days) exposure of cells to these high doses resulted in complete

2T3 cellular inhibition (mainly with D_3). Therefore this raises questions about choice of doses in several published studies which would have encountered toxicity if they had prolonged the experiments. I assessed three established mouse osteoblastic cell lines and the doses of >500nM of 25(OH)D_2, 25(OH)D_3 and 1, 10 and 100nM of either 1,25(OH)$_2D_2$ or 1,25(OH)$_2D_3$ were potent inhibitors of ALP activities in these cells (2T3, MBA15.4 and MC3T3). As an example, doses of greater than 500nM of 25(OH)D_2, D_3 and >0.5nM of either active metabolite abolished 2T3 mineralisation. My study overall covered various concentrations for these comparisons on mouse 2T3, identifying the doses that increased osteoblastic activity which was more relevant to *in vivo* studies.

It appears from the literature that, in the *in vivo* studies where they found no differences between a raise in the circulatory levels of 25(OH)D_2 and 25(OH)D_3 (D_2=D_3), those cohorts were supplemented with concentrations of 400-2000IU vitamin D_2 or D_3 over a short period [433, 434]. In contrast, the studies that found differences between a raise in circulatory 25(OH)D_2 and 25(OH)D_3 (D_3>D_2) were based on supplementation with concentration of >4000IU [296, 303].

In the present study on the mouse 2T3 osteoblast activities, both 25(OH)D_2 and 25(OH)D_3 exerted similar effects on osteoblast activities at low concentrations. Although it is premature to compare or correlate *in vitro* osteoblast treatment with *in vivo* vitamin D supplementation, however, if we assumed that supplementation of either vitamin D_2 or D_3 raised equal circulatory levels of 25(OH)D_2 and 25(OH)D_3, then either D_2 or D_3 at physiological levels (200nM) might have similar effects on mouse osteoblast activities.

However, the increase in mouse osteoblast activity as a consequence of the local metabolism of 25(OH)D_2 or 25(OH)D_3 in the presence of other co-factors such

as BMP-2 could be achieved at very low physiological concentrations of 50nM of either D_2 or D_3. In this case vitamin D_3 metabolites were observed to be more potent than D_2.

Although 2T3 mouse osteoblast was the best cell line to choose and conduct this study because of its reliable mineralisation, one major downfall of using this cell line was its rapid responsiveness to exogenous phosphate and calcium, where mineralisation was saturated within hours rather than days in the presence of BMP-2 and vitamin D metabolites. Hence assessments of the differences between D_2 and D_3 could not be fully evaluated over time. For this reason the future experiments for vitamin D comparisons could be evaluated in rat or mouse bone primary cells which also require lower concentrations of phosphate (2.5-5mM) than was used in the current study.

As bone formation is the key function of osteoblasts *in vivo,* one of aims of the current project was to examine the effects of vitamin D metabolites on mineralisation of primary human osteoblasts *in vitro*. As mineralisation occurred over weeks it was decided to use ketoconazole to conduct vitamin D comparisons in hBMSCs with their prolonged 4-5 week mineralisation time course and it was therefore also of interest to test the effect of this drug in 2T3 cells. Ketoconazole is commonly used for CYP27B1 and/or CYP24A1 depletion at µM concentrations and thus acts as a vitamin D sensitizer, particularly when used together with active metabolites where the inhibition of CYP27B1 can have no effect. As expected, ketoconazole enhanced the effect of vitamin D active metabolites in 2T3 cells by inducing toxicity, probably through effectively delivering a higher dose, in line with its reported effects in prostate cancer and other cell lines [130,

140]. In contrast ketoconazole enhanced the vitamin D induced mineralisation of hBMSCs without tipping them into the toxic dose range (see discussion below).

In **Chapter 4** the effects of D_2 and D_3 and their active metabolites were further investigated on hBMSCs differentiation into primary osteoblasts. Human primary osteoblast differentiation from BMSCs is a lengthy process of up to 4 weeks, which required addition of osteogenic differentiation medium and vitamin D treatments every 48 hours. HBMSCs as well as primary osteoblasts isolated from bone samples are currently used in research to study vitamin D_3 effects. The slow proliferation rate of these cells in thought to be due to Hayflick limits where cells are only capable of limited replications, particularly when sourced from older donors. There is also concern about the efficiency of the mineralisation or nodule formation by hBMSCs from old donors. Overall the access to human primary osteoblasts from healthy individuals is extremely limited and currently osteoarthritic biopsies from old patients undergoing total hip or knee replacements are the only source for hBMSCs.

Compared to established osteoblast-like cell lines, it has been shown that higher doses of vitamin D are required to increase human primary osteoblast activities. In this thesis in Chapter 4, I also made similar observations, as human BMSCs did not respond to the physiological doses that mouse 2T3 cells did (Chapter 3). In hBMSC cultures, an increase in ALP activities was observed at doses of ≥500nM of $25(OH)D_2$, $25(OH)D_3$ and >0.5nM of either active metabolites (D_3>D_2), the doses that previously abolished 2T3 activities. Similar to the mouse 2T3, D_2 metabolites also increased hBMSC activities, suggesting that hBMSCs also metabolise $25(OH)D_2$ locally, as well as being responsive to the circulatory

$1,25(OH)_2D_2$ which *in vivo* would come from renal metabolism, an effect that has not been reported previously.

Interestingly an increase in hBMSC mineralisation was observed with $25(OH)D_3$, not $25(OH)D_2$, suggesting higher potency of D_3 over D_2. Prolonged incubation of the hBMSCs with $1,25(OH)_2D_3$ appeared to cause inhibition of cellular activity, but not with $1,25(OH)_2D_2$.

My main aim of this study was to identify whether there was any difference between either metabolite on human primary cells by simple mineralisation assays. I showed that such a difference exists, as non-OA hBMSCs were highly $25(OH)D_3$, and mainly $1,25(OH)_2D_2$ rather than $1,25(OH)_2D_3$ responsive. Similarly hBMSCs derived from OA bone were more responsive to $25(OH)D_3$ but showed less mineralisation with $25(OH)D_2$. Determining the downstream causes of such differences in detail was beyond the scope of this thesis. The use of ketoconazole over long culture assays in both OA and non-OA BMSCs not only showed no inhibition of cellular activity, but also prolonged depletion of CYP24A1 consequently increased mineralisation in the presence of $1,25(OH)_2D_2$. Hence the toxicity as a result of µM use of ketoconazole can be ruled out in hBMSCs.

As well as an increase in mineralisation observed with hBMSCs in response to $25(OH)D_3$, OPN protein was up-regulated more in response to $25(OH)D_3$ than $25(OH)D_2$ in both mouse 2T3 and MBA-15.4 cells, suggesting that native $25(OH)D_3$ is more potent than $25(OH)D_2$ in both human and mouse. But on the other hand $1,25(OH)_2D_2$ both in mouse and human was more capable of stimulating an increase in OPN protein in 2T3 and MBA15.4 and an increase in mineralisation in hBMSCs in response to D_2 was also observed. Although 2T3

cells were used for most studies, preliminary work was also carried out on the mouse MBA 15.4 and is included here for comparison.

The lower mineralisation in hBMSC response to $1,25(OH)_2D_3$ compared to $1,25(OH)_2D_2$ does not necessarily mean $1,25(OH)_2D_3$ was less potent than $1,25(OH)_2D_2$. In fact, prolonged exposure of hBMSCs to $1,25(OH)_2D_3$ could indeed become toxic and inhibitory, whereas exposure to $1,25(OH)_2D_2$ may not. This pattern of inhibition at high doses was consistently observed throughout this study, where high doses of D_3 metabolites were observed to be more potent than D_2. This finding is also in line with previous publications where supplementation with mega doses of D_2 and D_3 in bovine, rat and horse caused vascular calcification and death in response to D_3, whereas lower hypercalcemia and reduced vascular calcification was observed in response to D_2 metabolites [288, 289].

Generally, in order for $1,25(OH)_2D_2$ or $1,25(OH)_2D_3$ to exert any genomic action on their target bone cell:

1. Bound DBP-$25(OH)D_2$ or DBP-$25(OH)D_3$ must gain entry by megalin endocytosis to the target cells that express *CYP27B1* gene
2. 25(OH)D must be released from DBP in the target cells and be transported into the mitochondria by IDBPs
3. $25(OH)D_2$ or $25(OH)D_3$ must be converted by mitochondrial CYP27B1 into $1,25(OH)_2D_2$ or $1,25(OH)_2D_3$
4. Metabolites must bind and form RXR-VDR-$1,25(OH)_2D_2$ or RXR-VDR-$1,25(OH)_2D_3$ complexes for activation or repression of vitamin D responsive genes.

5. RXR-VDR-1,25$(OH)_2D_2$ or RXR-VDR-1,25$(OH)_2D_3$ could up-regulate target genes ALP, COL1, OCN, OPN, RANKL, OPG, VDR and CYP24A1

Following genomic activity 1,25$(OH)_2D_2$ or 1,25$(OH)_2D_3$ may be inactivated [47] by CYP24 hydroxylase where:

A. 1,25$(OH)_2D_2$ catabolised to → 1,24,25$(OH)_3D_2$ (inactive catabolite with no biological activity)
B. 1,25$(OH)_2D_3$ catabolised to →1,24,25$(OH)_3D_3$ [biologically active,~40 times higher VDR binding affinity than 1,25$(OH)_2D_3$] , followed by a further inactivation step→ 24-keto-1,25$(OH)_2D_3$ (inactive) [47]

Currently, the difference between circulatory un-hydroxylated vitamin D_2 and D_3 *in vivo* is thought to be due to lower affinity of vitamin D_2 to DBPs. The differential effects of hydroxylated 25(OH)D_2 and D_3 *in vivo* on DBPs in circulation has been postulated to be due to an extra methyl group on carbon 24 in D_2 [297, 299, 435]. In the current study cells were exposed to the hydroxylated 25(OH)D and 1,25$(OH)_2$D metabolites. Similar to the mouse 2T3 osteoblast assays, in Chapter 4 I aimed to identify whether any differences between D_2 and D_3 existed in hBMSC activities such as ALP and mineralisation. Hence I did not measure the endogenous DBPs in the culture conditions, and at this stage it cannot be concluded if hBMSC higher mineralisation responsiveness to 25(OH)D_3 was due to higher 25(OH)D_3-DBP endocytosis than 25(OH)D_2-DBP. In other words whether hBMSC (or 2T3 for that matter) equally uptake 25(OH)D_2 or D_3 or not? Although I treated the hBMSCs with equi-molar concentrations of vitamin D_2 and D_3 metabolites with exact culture conditions and showed that differences exists between the two metabolites, it could not be determined what concentrations of

the end product of vitamin D, i.e., $1,25(OH)_2D_2$ or $1,25(OH)_2D_3$ eventually reached the target destination in the nucleus for genomic actions. Furthermore the effects of vitamin D_2 and D_3 catabolites were also not examined in the current study.

It has been shown that kidney cells metabolise $25(OH)D_3$ into $1,25(OH)_2D_3$ by CYP27B1 activity, and $1,25(OH)_2D_3$ product within the same cell increases megalin to regulate further $25(OH)D_3$ entry into the cells. At this stage of current study, it is not clear if the hBMSCs metabolise more $1,25(OH)_2D_3$ than $1,25(OH)_2D_2$ when the cells are exposed to $25(OH)D_3$ and $25(OH)D_2$, as an increase in ALP and mineralisation in hBMSCs in response to both $25(OH)D_2$ and $25(OH)D_3$ ($D_3>D_2$) was observed. My assumption here is hBMSC may indeed metabolise more $1,25(OH)_2D_3$ when treated with $25(OH)D_3$, and an increase in mineralisation in response to $25(OH)D_3$ could result in higher hBMSC osteoblastic gene and protein expression. Whether an increase in mineralisation in response to $25(OH)D_3$ was due to higher uptake of DBP-$25(OH)D_3$ or higher binding affinity of IDBPs to $25(OH)D_3$, or more up-regulation of megalin by $1,25(OH)_2D_3$ are questions that need to be further investigated.

Furthermore, I did not determine if up-regulation of megalin differs when cells metabolised $1,25(OH)_2D_3$ or $1,25(OH)_2D_2$ to regulate further $25(OH)D_3$ or D_2 entry into the cells. The intercellular binding affinities of free $25(OH)D_2$ or $25(OH)D_3$ metabolites by IDBPS into the mitochondria for 1-α hydroxylation by CYP27B1 is also unclear.

Although hBMSC have been shown to express VDR, CYP27B1 and CYP24A1 I did not perform expression studies in hBMSCs. However since hBMSCs up-

regulated ALP in response to both D_2 and D_3 metabolites it could be assumed that hBMSCs possess these essential regulators.

One of the effects of $1,25(OH)_2D$ hormone in treated cells is VDR up-regulation and consequently, CYP24A1 up-regulation for inactivation or catabolism of $1,25(OH)_2D$ itself. Since $1,25(OH)_2D_2$ in mouse 2T3 osteoblasts showed more VDR up-regulation at protein levels by Western blotting (dose-response), it could be postulated that in hBMSC $1,25(OH)_2D_2$ and $1,25(OH)_2D_3$ would induce differential levels of VDR or CYP24A1 up-regulation.

Although side chain differences exist between $1,25(OH)_2D_2$ and $1,25(OH)_2D_3$ (extra methyl group on C 24 in D_2), the receptor binding affinity of these two active hormones has been reported in one study to be similar [356]. Upon binding of the active metabolites to VDR, up-regulation of 24-hydroxylase is one of the main targets for catabolism of $1,25(OH)_2D_3$. In the current study observation of lower mineralisation in hBMSCs in response to $1,25(OH)_2D_3$ compared to D_2, suggests that prolonged exposure of hBMSCs to $1,25(OH)_2D_3$ would up-regulate more CYP24A1 and faster catabolism of $1,25(OH)_2D_3$ could occur. In contrast, in the case of $1,25(OH)_2D_2$, it appears that prolonged treatment or exposure of the cells to this hormone, accompanied with possibly slower rate of CYP24A1 up-regulation seems to cause stimulation rather than inhibition. ..

Currently our understanding of the role of osteocytes in bone is very limited due to limited access to the cells and lack of osteocyte cell lines that express all the osteocytic transcripts or proteins. Osteocytes are the most abundant cells in the bone and expression of key proteins such as sclerostin, Phex, DMP1 or FGF23 has been shown to play an important role in regulation of bone formation (via sclerostin) as well as phosphate homeostasis (by FGF23, Phex and Dmp1).

FGF23 also has been shown to be involved in the regulation of renal $1,25(OH)_2D$ metabolism. In the current project (**Chapter 5**) it was noted that the murine osteocyte-like cell lines MLOY-4 and MLOA-5 expressed low levels of some key osteocytic markers such as SOST and FGF23 that I would have liked to investigate. The preliminary data on the effect of vitamin D on osteocytes-like cell lines suggests that MLOY-4 and MLO-A5 cells are involved in local metabolism of both vitamin D_2 and D_3. This suggests that local metabolism of vitamin D by osteocytes is not only involved in differentiation of osteocyte-like cells MLOY-4 and MLOA-5, but also that up-regulation of RANKL and OPG in osteocytes could directly act on the activity of osteoclasts that are essential in bone resorption and release of calcium and phosphate into the circulation.

Furthermore, an elevation of *DMP1* and *Phex* in response to both vitamin D metabolites in osteocyte-like cells suggests that local metabolism of vitamin D metabolites could participate in regulation of the levels of FGF23 in bone. The up-regulation of *FGF23* in osteocyte-like cell lines by vitamin D metabolites in the current study provides further insight into the understanding that FGF23 is produced by osteocytes and could act in an endocrine fashion on distant target organs.

Similar to the mouse osteoblasts and hBMSCs, in osteocyte-like cells, vitamin D_2 and D_3 metabolism and response differed, where high doses of D_3 metabolites led to more inhibition of osteocytes than D_2, as cell number was reduced by high concentrations of D_3 metabolites. In the current study osteocytes were investigated at transcript levels, and further assessment on protein expression still needs to be performed. Currently the role of vitamin D metabolites on human osteocytes remains unknown as no human osteocyte-like cell line exists.

Furthermore osteocytes are embedded in the bone matrix and never divide, hence it would be difficult to translate the effects of any treatments on established osteocyte-like cells that undergo cellular division in *in vitro* assays.

To explore whether osteoblasts and osteocytes from diseased tissue might respond differently to vitamin D metabolites than normal healthy cells, VDR expression was examined in whole femoral heads from OA patients and also in primary cells derived from OA bone (**Chapter 6**). Overall, it appears that VDR expression in OA tissues is less than normal joint and restricted to bone underlying the main site of cartilage injury. VDR expression by chondrocytes has been reported previously and further investigation of chondrocytes was beyond the scope of this study. Immunohistochemical assessment of healthy bone tissue revealed that VDR expression was present in most cell types except mature osteocytes and chondrocytes.

In healthy bone, BMSCs, bone lining osteoblasts, newly embedded osteocytes as well resorbing osteoclasts expressed VDR. This suggests that VDR expression and local metabolism of vitamin D by these cells is involved in the function of BMSCs, bone lining osteoblasts, newly embedded osteocytes and resorbing osteoclasts, as well as possibly a cross talk between these cells. The reduction in expression of VDR in most cell types in OA suggests that the balance is probably interrupted in OA, and apparently is shifted to just differentiation of BMSCs to osteoblasts. This might however be important in compensation for mechanical loading that is no longer provided by the degraded overlying cartilage.

Surprisingly the expression of VDR was evident in multi-nucleated osteoclasts with clear resorption lacunae on the bone surfaces. These cells were further

stained with cathepsin K to confirm that they were osteoclasts (Fig 6.4H). It has been reported that vitamin D_3 metabolism is involved in osteoclast precursors and that VDR expression is absent in mature osteoclasts [81]. My histological analysis of healthy tissues revealed that bone resorbing mature osteoclasts indeed express VDR, however assessments of vitamin D metabolites on osteoclast assays were also beyond the scope of the current study. One main limitation of the histological analysis was that immunohistochemistry is not a quantitative assay, but rather diagnostic and qualitative. Furthermore, histological staining in healthy bone samples were conducted in replicates from a sample from one patient as access to tissues from healthy donors is extremely limited. Other limitation was age differences between OA and non-OA samples, as non-OA sample was from younger donor (19) and OA samples from a much older patient (50-55).

Epidemiological or randomised control trials have revealed that supplementation of vitamin D deficient OA patients increases the circulatory levels of 25(OH)D, but there is no convincing evidence to suggest that OA patients would benefit from this increased vitamin D status [410]. Here in my preliminary data it can be postulated that VDR expression is down regulated in OA bone osteocytes and osteoclasts and that probably the cross talk between these cells is hampered. Although I did not look at the VDR protein expression in the primary cells isolated from OA patients versus healthy individuals, $25(OH)D_2$ and $25(OH)D_3$ treatments of human cells revealed higher mineralisation in OA-BMSCs compared to non-OA cells, which was unexpected if VDR is important and it would be important to assess whether the level of VDR in OA derived hBMSCs in fact increased under culture conditions. Furthermore, the aim of my study in Chapter 6 was to identify

differences between D_2 and D_3 metabolites in primary cells, which I did, finding 25(OH)D_3 >25(OH)D_2. A higher responsiveness of OA-BMSCs than non-OA could be due to an alteration in the vitamin D regulating proteins such as VDR, CYP27B1 or CYP24A1 which needs to be further evaluated. However as mentioned earlier comparison between healthy and diseased cells is currently limited by the lack of tissue or cells from healthy donors.

7.2. Directions for future work

Question 1: Why does BMP2 supplementation permit use of a much lower dose of vitamin D_2 to achieve the same osteoblastic response? Is this a useful strategy in slowly differentiating hBMSC which show inhibition after 4 weeks of high doses of D_3 supplementation, but might respond well to a lower dose in combination with BMP-2?

Because 2T3 responded to much lower doses of D_2 and D_3 in the presence of BMP2, this may be a way to accelerate hBMSC response in the presence of BMP2.

Proposed experiments: Dose response [10, 50 and 100nM of 25(OH)D_3 / 25(OH)D_2 and 0.01, 0.05 and 0.1nM of active metabolites] and time dependent treatments of rat, mouse or hBMSCs with vitamin D_2 and D_3 metabolites under the influence of osteogenic differentiation medium (2.5-5mM phosphate) in the presence or absence of BMP-2.To complete my data set, the possibility to speed up all assays would allow:

a. Measurement of produced 1,25$(OH)_2D_2$ and 1,25$(OH)_2D_3$ metabolites by LC-MS (new data)
b. ALP activity assessment on days 3 and 6 (confirmation)

c. Western blot analysis of vitamin D related proteins: VDR, CYP27B1 and CYP24A1, as well as osteoblast differentiation markers OCN and OPN on days 7 and 14 (new data)
d. Ratio of secretory RANKL:OPG production by ELISA (new data)
e. Mineralization assays by Alizarin red staining (confirmation)

Question 2: How could the differential toxicity of D_3 versus D_2 be explained, because of the amount or type of active metabolite produced? Equally can greater potency of D_3 versus D_2 be explained by differential effect of activating and inactivating enzymes?

Experiments:

To investigate contribution of CYP27B1 to the differential effects of D_2 versus D_3, CYP27B1 silencing of vitamin D_2 and D_3 treated hBMSCs

a. Western blot confirmation of CYP27B1 knock down
b. Measurement of $1,25(OH)_2D_2$ and $1,25(OH)_2D_3$ metabolites by LC-MS
c. Assessment of ALP activity

Repeat for CYP24A1 to see effect of catabolic enzyme

Another explanation for different potencies of D_2 versus D_3 is the production of different catabolites with D_3 making $1,24,25(OH)_3$ D_3 which has 40% higher VDR binding compared to D_3 but its D_2 counterpart has no biological activity [47].

Experiments: Effects of $24,25(OH)_2D_2$, $24,25(OH)_2D_3$ and $1,24,25(OH)_3$ D_2 and D_3 on ALP expression in hBMSCs in osteogenic differentiation medium in response to 1000nM of $25(OH)D_3$ or $25(OH)D_2$ and 10nM of active metabolites

Question 3: A key finding of this thesis was that osteocytic cell lines up-regulate RANKL and OPG in response to D_2 and D_3. Furthermore, osteoclasts in bone sections were shown to express VDR even when they were actively resorbing, which is a novel finding. Since osteocytes are now known to be key regulators of osteoclastic activity I would like to explore further the relevance of this cross-talk. Also I would like to assess whether D_3 is more potent than D_2 and if this is beneficial or harmful

1. measure OPG and RANKL release from D_2 versus D_3 treated osteocytes
2. co-culture osteocytes with PBMCs and quantify osteoclast formation using:
    a) TRAP assay, with and without D_2 or D_3
    b) Assess effect of vitamin D treated osteocytes on osteoclastic bone resorption using measurements of resorption pit on dentine by Toluidine blue staining

Question 4: Histological data in current study suggested down-regulation of VDR in OA joint tissues, except for the osteoblasts underlying the cartilage lesion. However in culture, OA osteoblasts responded with greater sensitivity to D_2 and D_3 than normal osteoblasts. To investigate a possible cause:

a. Based on finding in Chapter 6 OA-BMSCs expected to express higher VDR, CYP27B1 and CYP24A1 compared to Non-OA BMSCs. It can be postulated that a higher vitamin D related machinery (VDR and CYP27B1) would make OA-BMSCs more vitamin D sensitive and responsive than Non-OA BMSCs. This would be quantified by QRT-PCR and Western blot

b. It is also predicted that high levels of VDR or CYP27B1 would make OA-BMSCs more 1,25$(OH)_2D_3$ responsive.

Question 5: Given the literature on increased risk of vascular calcification with D_3 and finding of reduced toxicity with D_2 in current study, are there differential responses of vascular cells to D_2 and D_3 metabolites?

Experiment: Dose response and time dependent vitamin D_2 and D_3 treatment of human vascular smooth muscle cells (VSMCs) in osteogenic media culture conditions and assessment of calcification.

Method: Differentiation of vascular smooth muscle cells in osteogenic differentiation medium in response to 1000nM of 25$(OH)D_3$ or 25$(OH)D_2$ and 10nM of active metabolites as well as BMP-2 and possibly dexamethasone since these are not osteoblast lineage.

8. POSTER PRESENTATIONS

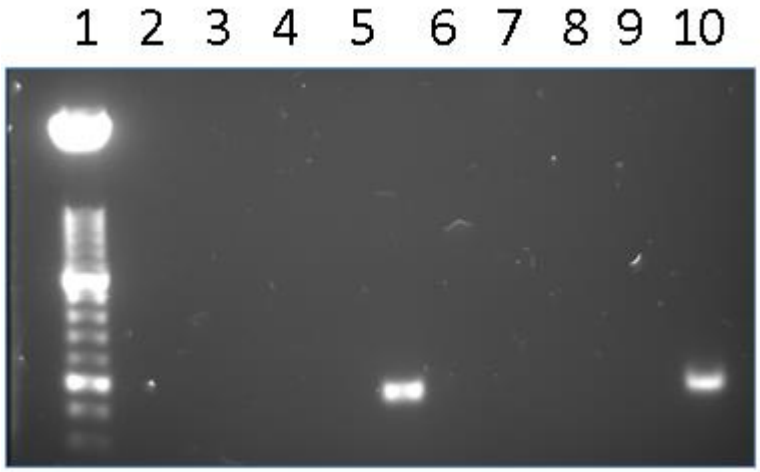
1. **Allahdad Zarei**, Francis Lam, David Mahoney, Guillaume Mabilleau, Afsie Sabokbar, Morovat Alireza. *Comparison of the Biological Activities of Vitamins D_2 and D_3 on Osteoblast Differentiation and Activity*. Journal of Bone and Mineral Research, 32nd Annual Meeting of the American Society for Bone and Mineral Research, Volume 25, Issue Supplement S1, MO0244, pages S363-S502, October 2010.

2. **A. Zarei**, F. Lam, D.J. Mahoney, G. Mabilleau, M.K. Javaid, A. Sabokbar, A. Morovat. *Differential osteoblastic activities of vitamin D2 and D3*. Abstract accepted for oral presentation at ECTS DPhil training course in Ljubljana, Slovenia, September 2011.

3. R.C. Poulsen, **A. Zarei**, A. Sabokbar, P.A. Hulley. *Tendon, a vitamin D-responsive tissue: (Why the British weather may not just be bad for your bones).* International Journal of Experimental, Aug 2013;94(4):A1-A21

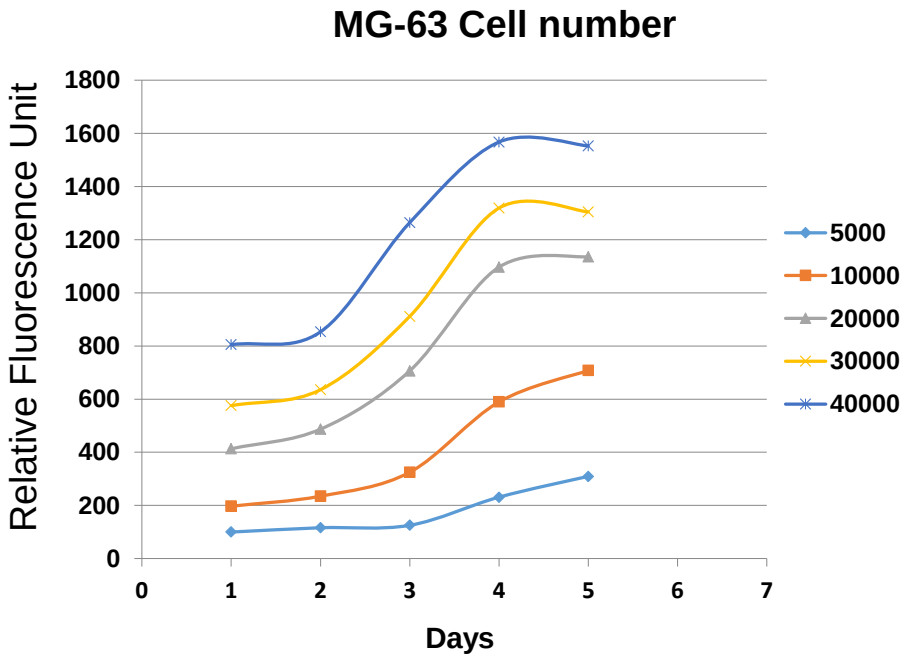
9. APPENDICES

9.1. Appendix 1: Mycoplasma screening of cell lines used in this thesis revealing no infection.



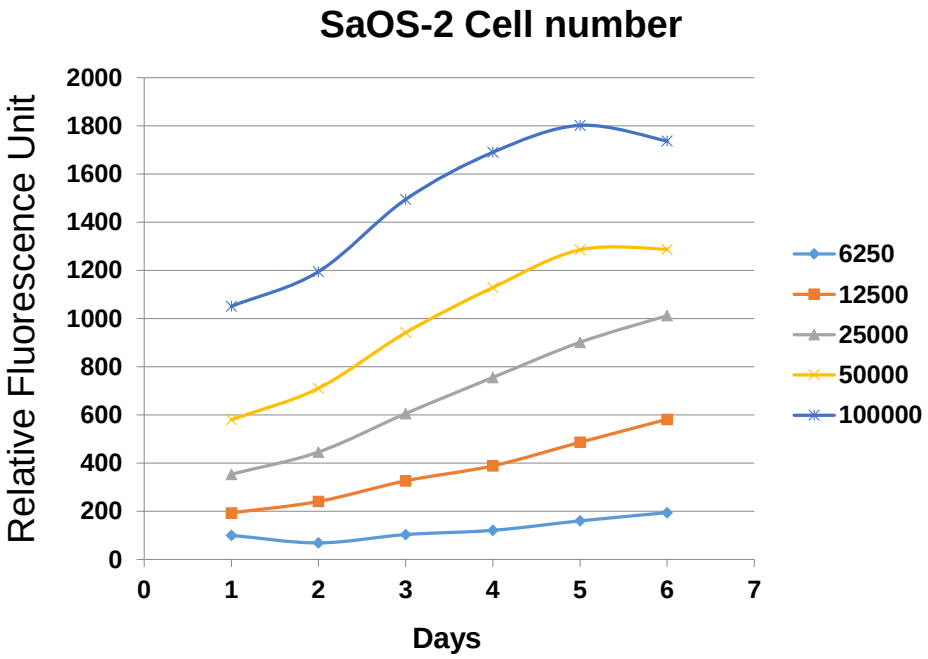
Representative analysis of mycoplasma PCR of murine osteocyte and osteoblast-like cells: lane 1; 1000bp ladder, lane2: MLOA-5, lane 3: MLOY-4, lane 4: negative control, lane 6: SaOS-2 cells, lane 7: MG-63, lane 8: 2T3, lane 9: MC3T3. Lanes 5 and 10 are mycoplasma positive samples with bands at ~500bp.

9.2. Appendix 2: MG-63 osteoblast-like cell growth over time.



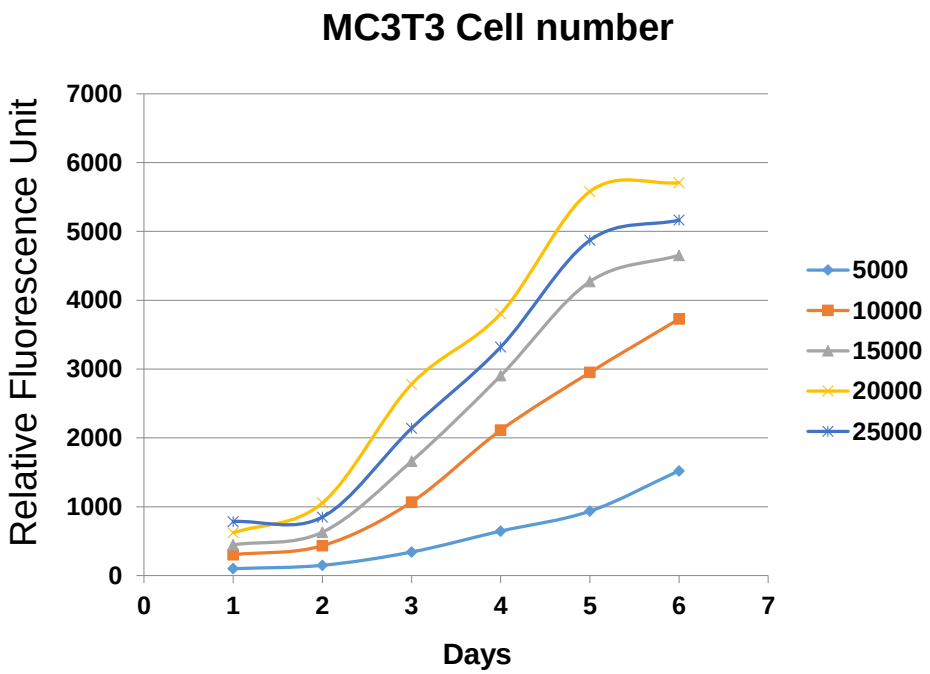
MG-63 cell number when cells cultured with αMEM-10% HI-FBS and assessed by Alamar blue™ assay. Full confluency occurred overnight for 3-4x10^4 seeding densities, for 2x10^4 at 30 hours, 1x10^4 cell density at 48 hours and 5x10^3 over 5 days. Data presented as mean of relative fluorescence units (n= 6 wells) from different cells densities grown over 5 days in culture growth medium.

9.3. Appendix 3: SaOS-2 osteoblast-like cell growth over time.



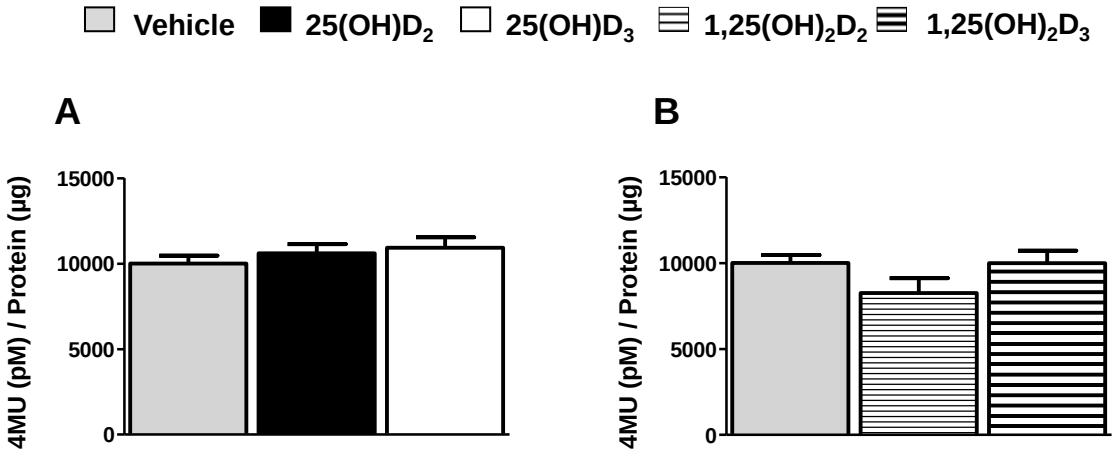
SaOS-2 cell number when cells cultured with McCoy`s 5a medium modified-10% HI-FBS and assessed by Alamar blue™ assay. Full confluency occurred overnight for $1x10^5$ seeding density, for $5x10^4$ at 24 hours, $2.5x10^3$ cell density at 30 hours,12,500 over 48 hours and 6,250 over 6 days. Data presented as mean of relative fluorescence units (n= 6 wells) from different cells densities grown over 6 days in culture growth medium.

9.4. Appendix 4: MC3T3 osteoblast-like cell growth over time.



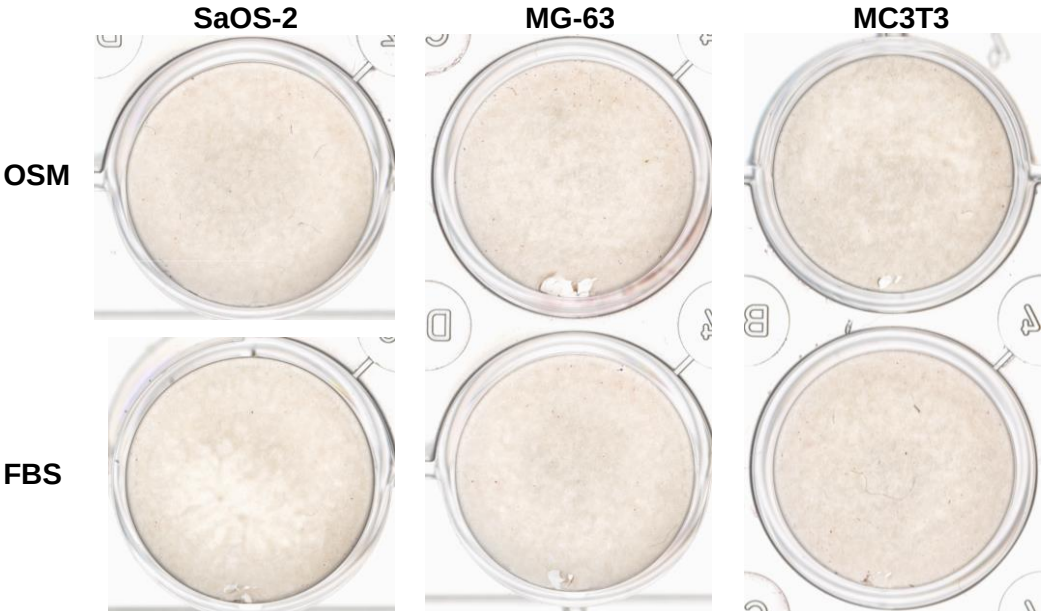
Cells cultured with DMEM-10% HI-FBS and assessed by Alamar blue™ assay. Full confluency occurred for $2.5x10^3$ seeding density at 24 hours, for $2x10^4$ at 30 hours, $1.5x10^4$ cell density at 48 hours, $1x10^4$ over 72 hours and $5x10^3$ over 6 days. Data presented as mean of relative fluorescence units (n= 6 wells) from different cells densities grown over 6 days in culture growth medium.

9.5. Appendix 5: SaOS-2 osteoblast-like cells did not respond to D_2 and D_3 metabolites at physiological concentrations.



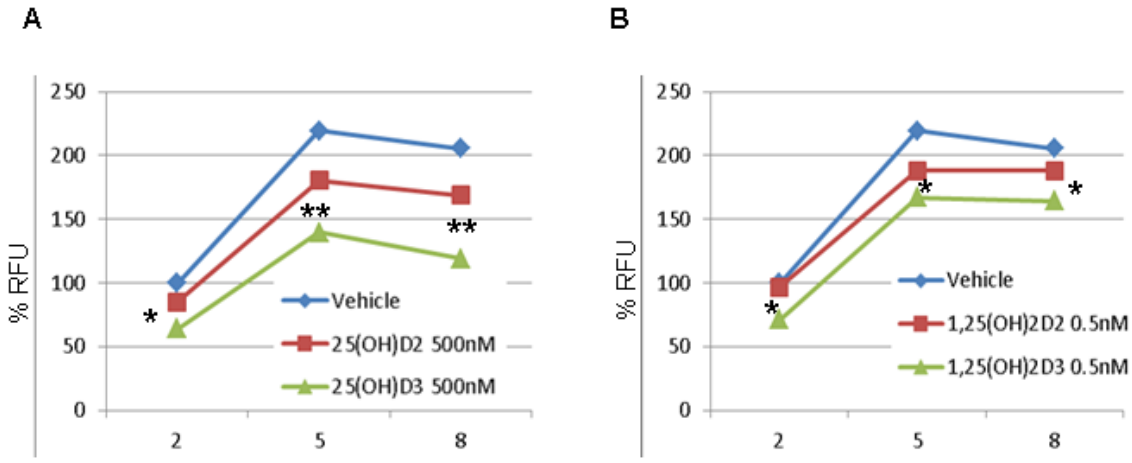
Effects of vitamin D_2 and D_3 metabolites on ALP enzymatic activity in human SaOS-2 osteoblast cell line. SaOS-2 cells were grown in their culture medium until confluency, then introduced to osteogenic differentiation medium and treated every other day with either (A) 200nM $25(OH)D_2/D_3$ or (B) 0.2nM of $1,25(OH)_2D_2$ and D_3 over 6 days. Data are presented as ± SEM (non-parametric Kruskal Wallis test) from triplicate experiments.

9.6. Appendix 6: Osteoblast-like SaOS-2, MG-63 and MC3T3 cell lines did not mineralise in the presence of osteogenic medium over 12 days of culture.



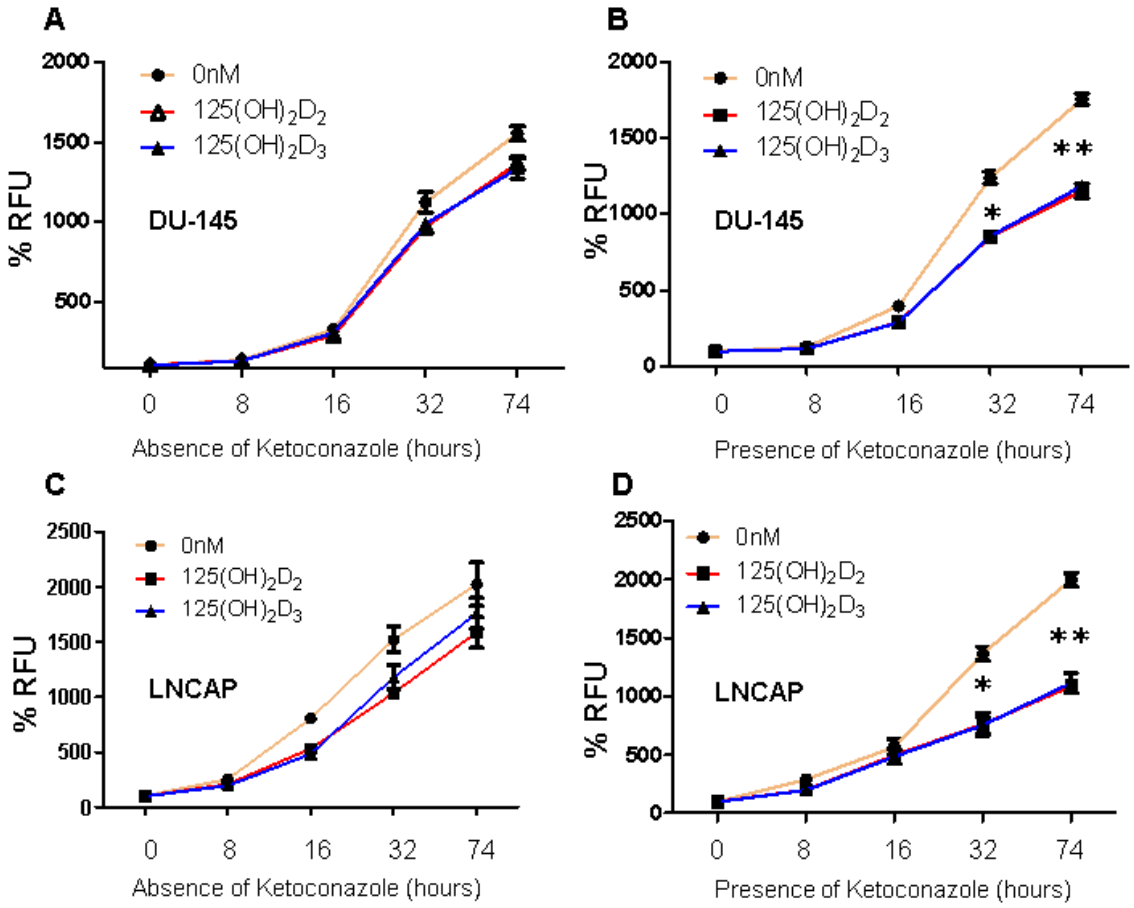
Representative images of osteoblast –like cells in the presence (top row) or absence (below row; FBS) of osteogenic medium (OSM) containing10mM β-glycerophosphate, and 50µg/ml L-ascorbic acid over 12 days. Osteoblast-like cells were grown to full confluency in their growth culture containing10%FBS, when media was replaced with OSM for 12 days. Cultures were stopped on day 12, fixed in 70% ethanol, washed with deionised water and stained with Alizarin red staining solution. Plates were washed with deionised water and scanned with Epson AX10 scanner.

9.7. Appendix 7: Vitamin D_3 metabolites are more potent than D_2 at supra-physiological doses in reducing 2T3 osteoblast number.



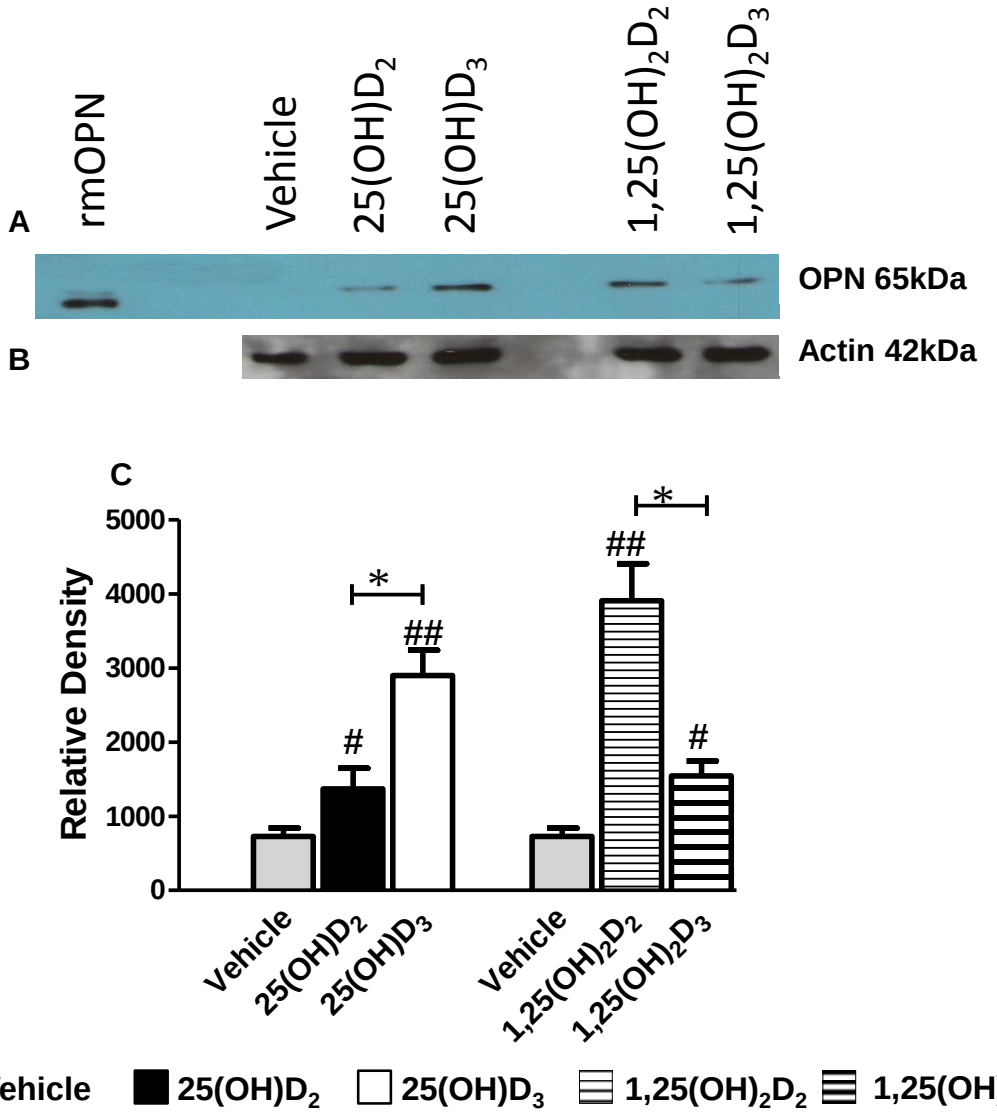
Comparative effects of $25(OH)D_2$, $25(OH)D_3$, $1,25(OH)_2D_2$ and $1,25(OH)_2D_3$ on mouse cell number in osteogenic differentiation medium. 2T3 osteoblasts were treated every other day with (A) 500nM of $25(OH)D_2/D_3$ and (B) 0.5nM of their active metabolites $1,25(OH)_2D_2$ /D_3. Cellular metabolic activities were measure by Alamar blue™ assay on days 2, 5 and 8. Results are presented as percentage of mean of treated cells versus untreated controls (n=6 wells) (non-parametric Kruskal Wallis test). Both D_3 metabolites significantly reduced cell number more than D_2 metabolites on days 2, 5 and 8. * $P<0.05$, ** $P<0.01$ for comparisons between D_2 and D_3 metabolites.

9.8. Appendix 8: Both $1,25(OH)_2D_2$ and $1,25(OH)_2D_3$ active metabolites suppress prostatic cancer cell number in the presence of ketoconazole.



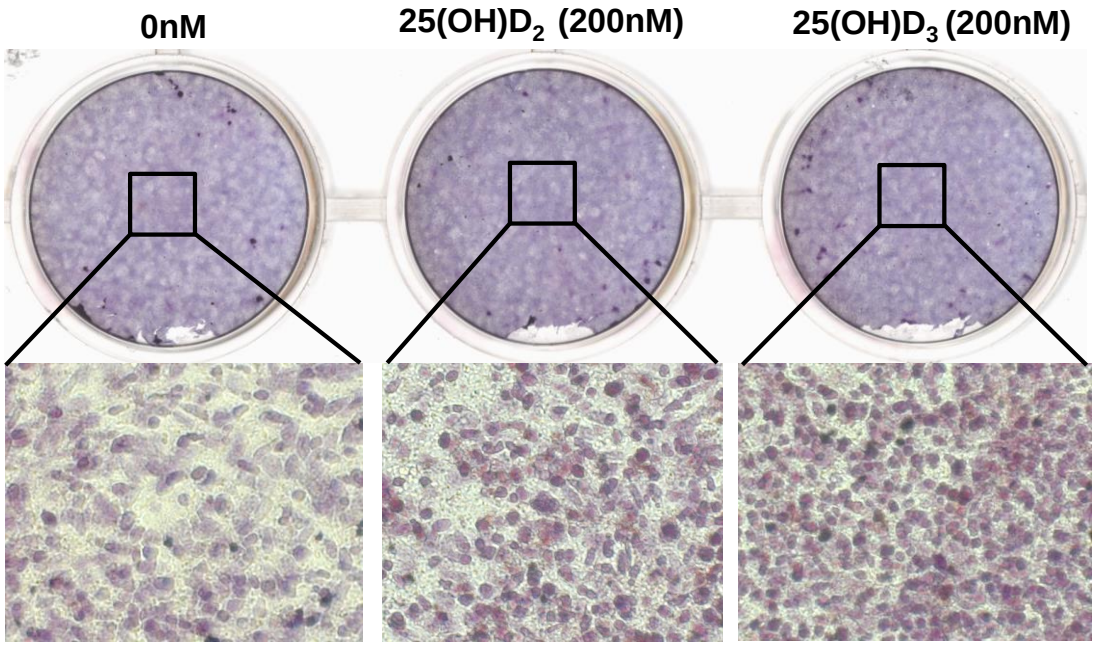
Comparative effects of 0.5nM of the active metabolites $1,25(OH)_2D_2$ and $1,25(OH)_2D_3$ ± ketoconazole on DU-145 and LNCAP prostate cancer cell lines (A-D). DU145 (A&B) and LNCAP (C&D) were seeded in replicates in multiple 96 well plates at $3x10^3$ cells per well in RPMI-1640-10%HI-FBS in the presence of $1,25(OH)_2D_2/D_3$ ± 10µM ketoconazole respectively and cellular activity was measured by Alamar blue™ as described in Materials and Methods. Data presented as mean ±SEM of 6 wells, (non-parametric Kruskal Wallis test) *P<0.01 and **P<0.001 for comparison of treatments to control.

9.9. Appendix 9: OPN protein is up-regulated by vitamin D_2 and D_3 metabolites in MBA-15.4 osteoblast-like cells



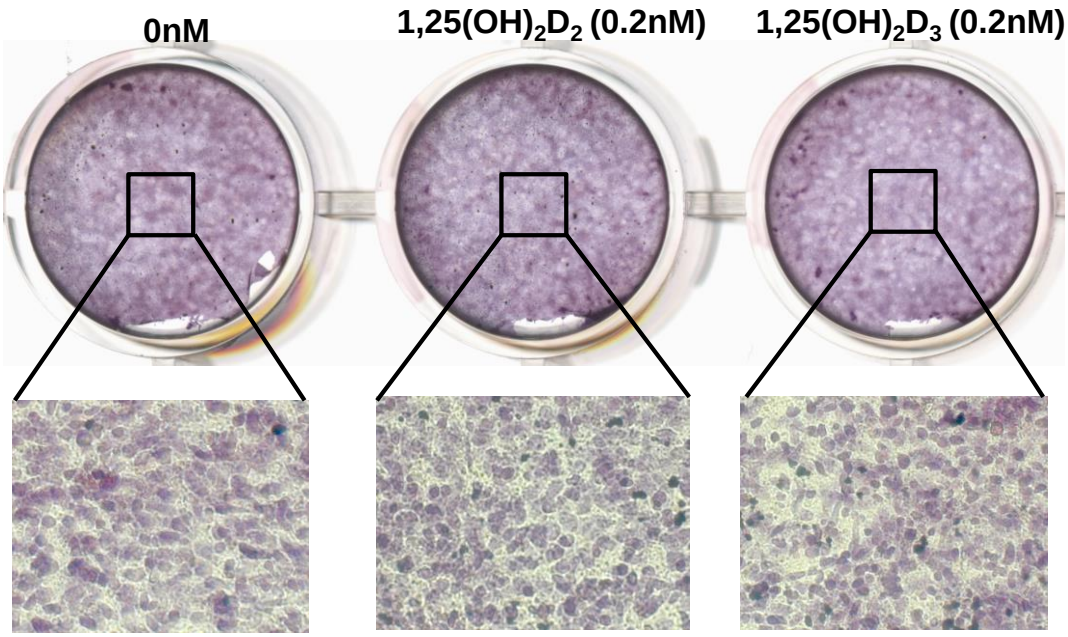
OPN protein is up-regulated by vitamin D_2 and D_3 metabolites. Representative Western blot of the effects of vitamin D metabolites on 20µg of OPN protein in mouse osteoblast-like MBA-15.4. (A) Western blot image of the effects of 10nM of 25(OH)D_2, 25(OH)D_3 or 0.01nM of their active metabolites 1,25(OH)$_2D_2$ and 1,25(OH)$_2D_3$ on OPN protein expression in mouse MBA-15.4. (B) Semi-quantification of the relative expression of mouse OPN. (Analysis was undertaken using imageJ software). Results of triplicates experiments are presented as relative densities of treated versus untreated controls (grey) as mean ±SEM (unpaired Student t-test.* $P<0.01$ for comparisons between D_2 and D_3 metabolites, and # $P<0.05$, ## $P<0.001$comparison of treatments to vehicle (grey).

9.10. Appendix 10: Metabolism of 25(OH)D_2 or D_3 metabolites did not drive 2T3 osteoblast-like cells into adipocytes.



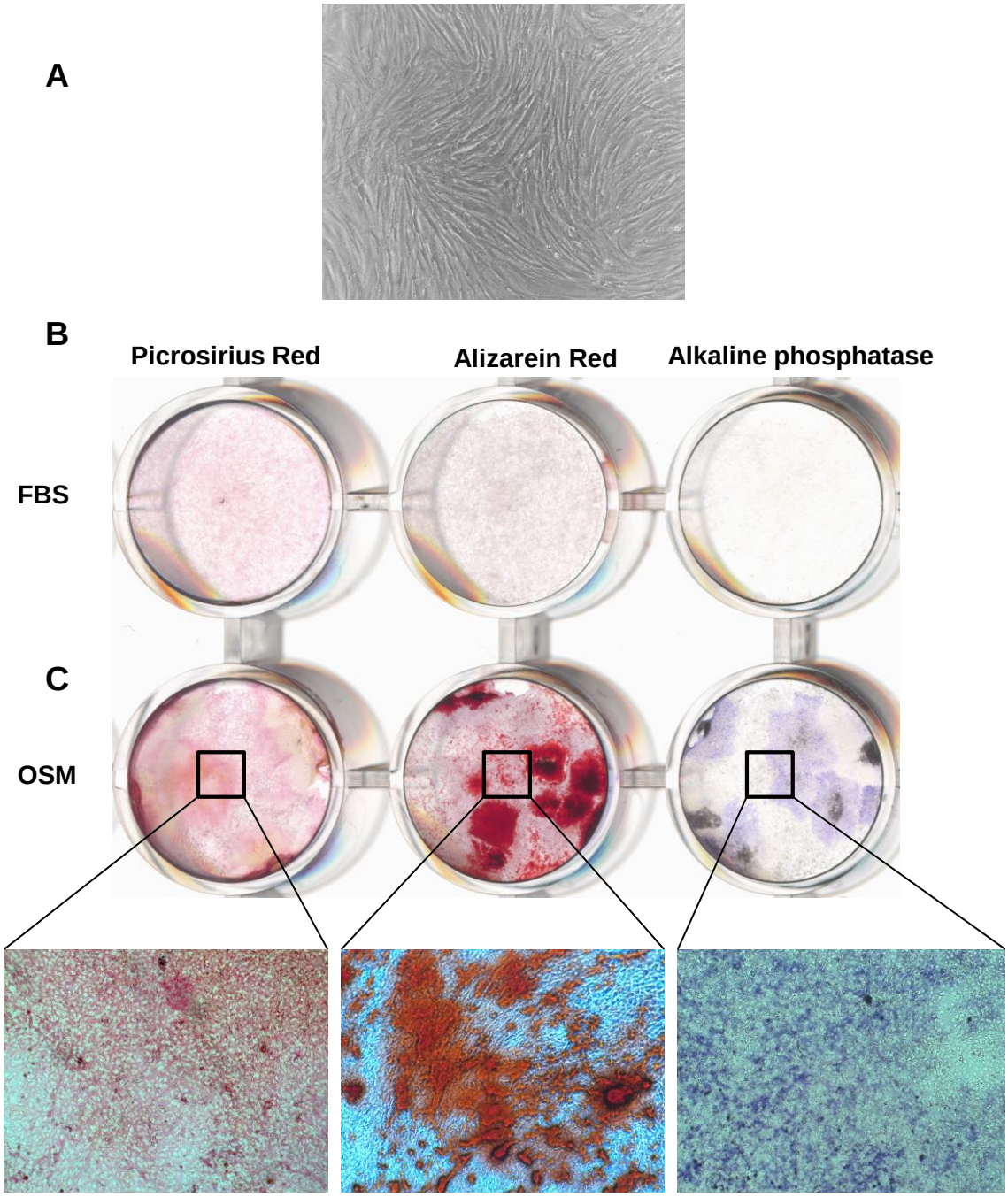
Cells were cultured in osteogenic differentiation media for 12 days in the presence or absence of 200nM of either 25(OH)D_2 or 25(OH)D_3. After washing with DPBS, cells were fixed with 10% formalin for 10 minutes, washed with deionised water and stained with Oil Red O staining solution for 30 minutes. Staining solution was removed and cells were washed with deionised water and counterstained with haematoxylin for 30 seconds. Cells were washed with water, air dried, scanned (representative of three wells) with Epson perfection V500 PHOTO scanner or photographed with Zeiss microscope (field of view 20X).

9.11. Appendix 11: Vitamin D_2 or D_3 active metabolites did not drive 2T3 osteoblast-like cells into adipocytes.



Cells were cultured in osteogenic differentiation media for 12 days in the presence or absence of 0.2nM of either $1,25(OH)_2D_2$ or $1,25(OH)_2D_3$. After washing with DPBS, cells were fixed with 10% formalin for 10 minutes, washed with deionised water and stained with Oil Red O staining solution for 30 minutes as described on previous page. Top panel representative scans of oil red counterstaining with haematoxylin and below high resolution photographs with Zeiss microscope (field of view 20X).

9.12. Appendix 12: Human bone marrow stromal cell activity.



HBMSC (Lonza Group Ltd) were cultured in 24 well plates in replicates at $1x10^4$ in αMEM-10% HI-FBS medium until full confluency (A), when cells were either maintained in the presence of (B) medium containing 10%FBS or (C) osteogenic differentiation medium of 10mM βGP over 5 weeks. Cultures were washed with DPBS, fixed in 10% formalin, washed with deionised water and stained with Picrosirius red, Alizarin red, or ALP staining solutions. (B) Representative scans of high resolution staining of collagen, calcium deposit and ALP. Lower panel in (B) high resolution (2.5X) micrographs by ZEISS light microscope of corresponding staining.

10. REFERENCES

1. McCollum, E., N. Simmonds, and W. Pitz, *The relation of the unidentified dietary factors, the fat-soluble A and watersoluble B of the diet to the growth promoting properties of milk.* Journal of Biological Chemistry, 1916. **27**(1): p. 33-43.
2. T.B. Osborne, L.B.M., The role of vitamines in the diet, The Journal of Biological Chemistry. 31 (1917) 149-163.
3. Sniadecki J, *Cited by W Mozolowski: "Jedrzej Sniadecki (1768–1883) on the cure of rickets."* Nature, 1939: p. 143:121.
4. Holick, M.F., *Vitamin D: a D-Lightful health perspective.* Nutrition Reviews, 2008. **66**(10 Suppl 2): p. S182-94.
5. Mozołowski, W.J.S., *On the Cure of Rickets. Nature. 1939: p.121-143.*
6. Mellanby, T., *The part played by an "accessory factor" in the production of experimental rickets.* Journal of Physiology, 1918. **52**: p. 11-14.
7. Steenbock, H. and A. Black, *Fat-soluble vitamins XVII. The induction of growth-promoting and calcifying properties in a ration by exposure to ultra-violet light.* Journal of Biological Chemistry, 1924. **61**(2): p. 405-422.
8. Hess, A.F., *PRiority as to the activation of foods by ultraviolet rays.* Journal of the American Medical Association, 1930. **94**(15): p. 1163-1164.
9. H. Chick, E.J.P., E.M. Hume, Studies of rickets in Vienna 1919-1922, Medical Research Council, Special Report No. 77 (1923).
10. O'Riordan, J.L. and O.L. Bijvoet, *Rickets before the discovery of vitamin D.* BoneKEy reports, 2014. **3**.
11. Orr, W.J., et al., *THe calcium and phosphorus metabolism in rickets, with special reference to ultraviolet ray therapy.* American Journal of Diseases of Children, 1923. **26**(4): p. 362-372.
12. Hess, A.F. and L.J. Unger, *PRophylactic therapy for rickets in a negro community.* Journal of the American Medical Association, 1917. **LXIX**(19): p. 1583-1586.
13. Wilson, M.G., *The prevention of rickets: The influence of the routine administration of cod liver oil in the prevention of rickets in infants.* American Journal of Diseases of Children, 1926. **31**(5): p. 603-630.
14. Lund, J. and H.F. DeLuca, *Biologically active metabolite of vitamin D3 from bone, liver, and blood serum.* Journal of lipid research, 1966. **7**(6): p. 739-744.
15. Blunt, J. and H.F. DeLuca, *The synthesis of 25-hydroxycholecalciferol. A biologically active metabolite of vitamin D3.* Biochemistry, 1969. **8**(2): p. 671-675.
16. DeLuca, H., et al., *Isolation and identification of 1, 25-dihydroxycholecalciferol. A metabolite of vitamin D active in intestine.* Biochemistry, 1971. **10**(14): p. 2799-2804.
17. Semmler, E., et al., *The synthesis of 1α, 25-dihydroxycholecalciferol. A metabolically active form of vitamin D3.* Tetrahedron Letters, 1972. **13**(40): p. 4147-4150.
18. Holick, M.F., et al., *Photosynthesis of previtamin D3 in human skin and the physiologic consequences.* Science, 1980. **210**(4466): p. 203-5.
19. Bouillon, R. and T. Suda, *Vitamin D: calcium and bone homeostasis during evolution.* BoneKEy reports, 2014. **3**.
20. DeLuca, H.F., *Evolution of our understanding of vitamin D.* Nutrition Reviews, 2008. **66**(10 Suppl 2): p. S73-87.
21. Bouillon, R. and T. Suda, *Vitamin D: calcium and bone homeostasis during evolution.* Bonekey Reports, 2014. **3**: p. 480.
22. Fieser LD, F.M., *Vitamin D. In: Steroids.* Reinhold, New York,, 1959: p. pp 90–168.

23. Wolf, G., *The discovery of vitamin D: the contribution of Adolf Windaus.* Journal of Nutrition, 2004. **134**(6): p. 1299-302.
24. Askew, F.A., et al., *The Distillation of Vitamin D.* Proceedings of the Royal Society of London. Series B, Containing Papers of a Biological Character, 1930. **107**(748): p. 76-90.
25. DeLuca, H.F. and H.K. Schnoes, *Metabolism and mechanism of action of vitamin D.* Annual Review of Biochemistry, 1976. **45**: p. 631-66.
26. Bikle, D., *Nonclassic actions of vitamin D.* The Journal of Clinical and Endocrinology Metabolism, 2009. **94**(1): p. 26-34.
27. Matsuoka, L.Y., et al., *Sunscreens suppress cutaneous vitamin D3 synthesis.* The Journal of Clinical and Endocrinology Metabolism, 1987. **64**(6): p. 1165-8.
28. Clemens, T.L., et al., *Increased skin pigment reduces the capacity of skin to synthesise vitamin D3.* Lancet, 1982. **1**(8263): p. 74-6.
29. Holick, M.F., L.Y. Matsuoka, and J. Wortsman, *Age, vitamin D, and solar ultraviolet.* Lancet, 1989. **2**(8671): p. 1104-5.
30. Holick, M.F., *Vitamin D Deficiency.* The New England Journal of Medicine, 2007. **357**(3): p. 266-281.
31. DeLuca, H.F., *Overview of general physiologic features and functions of vitamin D.* The American Journal of Clinical Nutrition 2004. **80**(6 Suppl): p. 1689S-96S.
32. Hanley, D.A., et al., *Vitamin D in adult health and disease: a review and guideline statement from Osteoporosis Canada.* Canadian Medical Association Journal, 2010. **182**(12): p. E610-8.
33. Haddad, J.G., et al., *Human plasma transport of vitamin D after its endogenous synthesis.* The Journal of Clinical Investigation, 1993. **91**(6): p. 2552-5.
34. Webb, A., B. DeCosta, and M. Holick, *Sunlight Regulates the Cutaneous Production of Vitamin D3 by Causing Its Photodegradation*.* The Journal of Clinical Endocrinology & Metabolism, 1989. **68**(5): p. 882-887.
35. Moan, J., et al., *Addressing the health benefits and risks, involving vitamin D or skin cancer, of increased sun exposure.* Proceeding of the National Academy of Sciences U S A, 2008. **105**(2): p. 668-73.
36. Jones, G., H.K. Schnoes, and H.F. DeLuca, *Isolation and identification of 1,25-dihydroxyvitamin D2.* Biochemistry, 1975. **14**(6): p. 1250-6.
37. Holick, M.F., H.K. Schnoes, and H.F. DeLuca, *Identification of 1,25-dihydroxycholecalciferol, a form of vitamin D3 metabolically active in the intestine.* Proceeding of the National Academy of Sciences U S A, 1971. **68**(4): p. 803-4.
38. Bikle, D.D., et al., *Assessment of the free fraction of 25-hydroxyvitamin D in serum and its regulation by albumin and the vitamin D-binding protein.* The Journal of Clinical and Endocrinology Metabolism, 1986. **63**(4): p. 954-9.
39. Nykjaer, A., et al., *An endocytic pathway essential for renal uptake and activation of the steroid 25-(OH) vitamin D3.* Cell, 1999. **96**(4): p. 507-15.
40. Hewison, M., et al., *Extra-renal 25-hydroxyvitamin D3-1alpha-hydroxylase in human health and disease.* The Journal of Steroid Biochemistry and Molecular Biology, 2007. **103**(3-5): p. 316-21.
41. Holick, M.F., et al., *Isolation and identification of 24,25-dihydroxycholecalciferol, a metabolite of vitamin D made in the kidney.* Biochemistry, 1972. **11**(23): p. 4251-5.
42. Holick, M.F., et al., *1,24,25-Trihydroxyvitamin D3. A metabolite of vitamin D3 effective on intestine.* The Journal of Biological Chemistry, 1973. **248**(19): p. 6691-6.
43. Suda, T., et al., *The isolation and identification of 25-hydroxyergocalciferol.* Biochemistry, 1969. **8**(9): p. 3515-20.
44. Cheng, J.B., et al., *De-orphanization of cytochrome P450 2R1: a microsomal vitamin D 25-hydroxilase.* The Journal of Biological Chemistry, 2003. **278**(39): p. 38084-93.

45. Jones, G., et al., *Hepatic activation and inactivation of clinically-relevant vitamin D analogs and prodrugs.* Anticancer Research, 2006. **26**(4A): p. 2589-95.
46. Bouillon, R., W.H. Okamura, and A.W. Norman, *Structure-function relationships in the vitamin D endocrine system.* Endocrine Reviews, 1995. **16**(2): p. 200-57.
47. Horst, R.L., et al., *24-Hydroxylation of 1,25-dihydroxyergocalciferol. An unambiguous deactivation process.* The Journal of Biological Chemistry, 1986. **261**(20): p. 9250-6.
48. Ohyama, Y. and K. Okuda, *Isolation and characterization of a cytochrome P-450 from rat kidney mitochondria that catalyzes the 24-hydroxylation of 25-hydroxyvitamin D3.* The Journal of Biological Chemistry, 1991. **266**(14): p. 8690-5.
49. Tomon, M., H.S. Tenenhouse, and G. Jones, *Expression of 25-hydroxyvitamin D3-24-hydroxylase activity in Caco-2 cells. An in vitro model of intestinal vitamin D catabolism.* Endocrinology, 1990. **126**(6): p. 2868-75.
50. Pedersen, J.I., et al., *25-Hydroxyvitamin D3-24-hydroxylase in rat kidney mitochondria.* The Journal of Biological Chemistry, 1983. **258**(2): p. 742-6.
51. Makin, G., et al., *Target cell metabolism of 1,25-dihydroxyvitamin D3 to calcitroic acid. Evidence for a pathway in kidney and bone involving 24-oxidation.* Biochemical Journal, 1989. **262**(1): p. 173-80.
52. Lohnes, D. and G. Jones, *Further metabolism of 1 alpha,25-dihydroxyvitamin D3 in target cells.* Journal of Nutritional Science and Vitaminology (Tokyo), 1992. **Spec No**: p. 75-8.
53. Reddy, G.S. and K.Y. Tserng, *Calcitroic acid, end product of renal metabolism of 1,25-dihydroxyvitamin D3 through the C-24 oxidation pathway.* Biochemistry, 1989. **28**(4): p. 1763-1769.
54. Chapuy, M.C., P. Chapuy, and P.J. Meunier, *Calcium and vitamin D supplements: effects on calcium metabolism in elderly people.* The American Journal of Clinical Nutrition 1987. **46**(2): p. 324-8.
55. Heaney, R.P., et al., *Calcium absorptive effects of vitamin D and its major metabolites.* The Journal of Clinical and Endocrinology Metabolism, 1997. **82**(12): p. 4111-6.
56. Suda, T., et al., *Vitamin D and bone.* Journal of Cellular Biochemistry, 2003. **88**(2): p. 259-66.
57. Campbell, F.C., et al., *The yin and yang of vitamin D receptor (VDR) signaling in neoplastic progression: operational networks and tissue-specific growth control.* Biochemical Pharmacology, 2010. **79**(1): p. 1-9.
58. Mellon, W.S. and H.F. DeLuca, *An equilibrium and kinetic study of 1,25-dihydroxyvitamin D3 binding to chicken intestinal cytosol employing high specific activity 1,25-dehydroxy[3H-26, 27] vitamin D3.* Archives of Biochemistry and Biophysics, 1979. **197**(1): p. 90-5.
59. Wecksler, W.R., W.H. Okamura, and A.W. Norman, *Studies on the mode of action of vitamin D--XIV. Quantitative assessment of the structural requirements for the interaction of 1 alpha, 25-dihydroxyvitamin D3 with its chick intestinal mucosa receptor system.* Journal of Steroid Biochemistry, 1978. **9**(10): p. 929-37.
60. Vanhooke, J.L., et al., *Molecular structure of the rat vitamin D receptor ligand binding domain complexed with 2-carbon-substituted vitamin D3 hormone analogues and a LXXLL-containing coactivator peptide.* Biochemistry, 2004. **43**(14): p. 4101-10.
61. Germain, P., et al., *Overview of nomenclature of nuclear receptors.* Pharmacological reviews, 2006. **58**(4): p. 685-704.
62. Wang, Y., J. Zhu, and H.F. DeLuca, *Where is the vitamin D receptor?* Archives of Biochemistry and Biophysics, 2012. **523**(1): p. 123-33.
63. Norman, A.W., *Minireview: vitamin D receptor: new assignments for an already busy receptor.* Endocrinology, 2006. **147**(12): p. 5542-8.

64. Carlberg C, D.T., Frank C(2005) Molecular basis of the diversity of vitamin D target genes. In: Feldman D, Pike JW, Glorieux FH (eds) vitamin D, vol 18, 2nd edn. Elsevier Academic Press, San Diego,CA,pp 313-325.
65. Toell, A., P. Polly, and C. Carlberg, *All natural DR3-type vitamin D response elements show a similar functionality in vitro.* Biochemical Journal, 2000. **352 Pt 2**: p. 301-9.
66. Carlberg, C., et al., *Two nuclear signalling pathways for vitamin D.* Nature, 1993. **361**(6413): p. 657-60.
67. Pike, J.W. and M.B. Meyer, *The vitamin D receptor: new paradigms for the regulation of gene expression by 1,25-dihydroxyvitamin D(3).* Endocrinology Metabolism Clinics North America, 2010. **39**(2): p. 255-69, table of contents.
68. Brooks, M.H., et al., *Vitamin-D-dependent rickets type II. Resistance of target organs to 1,25-dihydroxyvitamin D.* The New England Journal of Medicine, 1978. **298**(18): p. 996-9.
69. Deeb, K.K., D.L. Trump, and C.S. Johnson, *Vitamin D signalling pathways in cancer: potential for anticancer therapeutics.* Nature Review Cancer, 2007. **7**(9): p. 684-700.
70. Bikle, D., et al., *Chapter 28. Vitamin D: Production, Metabolism, Mechanism of Action, and Clinical Requirements*, in *Primer on the Metabolic Bone Diseases and Disorders of Mineral Metabolism*. 2009, John Wiley & Sons, Inc. p. 141-149.
71. Kolek, O.I., et al., *1alpha,25-Dihydroxyvitamin D3 upregulates FGF23 gene expression in bone: the final link in a renal-gastrointestinal-skeletal axis that controls phosphate transport.* American Journal of Physiology Gastrointestinal Liver Physiology, 2005. **289**(6): p. G1036-42.
72. Zierold, C., H.M. Darwish, and H.F. DeLuca, *Two vitamin D response elements function in the rat 1,25-dihydroxyvitamin D 24-hydroxylase promoter.* The Journal of Biological Chemistry, 1995. **270**(4): p. 1675-8.
73. Tanaka, Y. and H.F. DeLuca, *Stimulation of 24,25-dihydroxyvitamin D3 production by 1,25-dihydroxyvitamin D3.* Science, 1974. **183**(130): p. 1198-1200.
74. Tanaka, Y., R.S. Lorenc, and H.F. DeLuca, *The role of 1,25-dihydroxyvitamin D3 and parathyroid hormone in the regulation of chick renal 25-hydroxyvitamin D3-24-hydroxylase.* Archives of Biochemistry and Biophysics, 1975. **171**(2): p. 521-526.
75. Shinki, T., et al., *Parathyroid hormone inhibits 25-hydroxyvitamin D3-24-hydroxylase mRNA expression stimulated by 1 alpha,25-dihydroxyvitamin D3 in rat kidney but not in intestine.* The Journal of Biological Chemistry, 1992. **267**(19): p. 13757-62.
76. Ohyama, Y., et al., *Identification of a vitamin D-responsive element in the 5'-flanking region of the rat 25-hydroxyvitamin D3 24-hydroxylase gene.* The Journal of Biological Chemistry, 1994. **269**(14): p. 10545-50.
77. Wu, S., et al., *Expression of the renal 25-hydroxyvitamin D-24-hydroxylase gene: regulation by dietary phosphate.* American Journal of Physiology, 1996. **271**(1 Pt 2): p. F203-8.
78. Ren, S., et al., *Alternative splicing of vitamin D-24-hydroxylase: a novel mechanism for the regulation of extrarenal 1,25-dihydroxyvitamin D synthesis.* The Journal of Biological Chemistry, 2005. **280**(21): p. 20604-11.
79. Liu, P.T., et al., *Toll-Like Receptor Triggering of a Vitamin D-Mediated Human Antimicrobial Response.* Science, 2006. **311**(5768): p. 1770-1773.
80. Underwood, J.L. and H.F. DeLuca, *Vitamin D is not directly necessary for bone growth and mineralization.* American Journal of Physiology, 1984. **246**(6 Pt 1): p. E493-8.
81. Bikle, D., et al., *Chapter 28. Vitamin D: Production, Metabolism, Mechanism of Action, and Clinical Requirements*, in *Primer on the Metabolic Bone Diseases and Disorders of Mineral Metabolism*. 2013, John Wiley & Sons, Inc. p. 239.
82. Schachter, D. and S.M. Rosen, *Active transport of Ca45 by the small intestine and its dependence on vitamin D.* American Journal of Physiology, 1959. **196**(2): p. 357-62.

83. Amling, M., et al., *Rescue of the skeletal phenotype of vitamin D receptor-ablated mice in the setting of normal mineral ion homeostasis: formal histomorphometric and biomechanical analyses.* Endocrinology, 1999. **140**(11): p. 4982-7.
84. Darwish, H.M. and H.F. DeLuca, *Analysis of binding of the 1,25-dihydroxyvitamin D3 receptor to positive and negative vitamin D response elements.* Archives of Biochemistry and Biophysics, 1996. **334**(2): p. 223-34.
85. Silver, J., et al., *Regulation by vitamin D metabolites of parathyroid hormone gene transcription in vivo in the rat.* The Journal of Clinical Investigation, 1986. **78**(5): p. 1296-301.
86. Canaff, L. and G.N. Hendy, *Human calcium-sensing receptor gene. Vitamin D response elements in promoters P1 and P2 confer transcriptional responsiveness to 1,25-dihydroxyvitamin D.* The Journal of Biological Chemistry, 2002. **277**(33): p. 30337-50.
87. Liu, S., et al., *Fibroblast growth factor 23 is a counter-regulatory phosphaturic hormone for vitamin D.* Journal of the American Society of Nephrology, 2006. **17**(5): p. 1305-15.
88. Chen, T.C., et al., *Role of vitamin D metabolites in phosphate transport of rat intestine.* Journal of Nutrition, 1974. **104**(8): p. 1056-60.
89. Garabedian, M., et al., *Control of 25-hydroxycholecalciferol metabolism by parathyroid glands.* Proceeding of the National Academy of Sciences U S A, 1972. **69**(7): p. 1673-6.
90. Forte, L.R., G.A. Nickols, and C.S. Anast, *Renal adenylate cyclase and the interrelationship between parathyroid hormone and vitamin D in the regulation of urinary phosphate and adenosine cyclic 3',5'-monophosphate excretion.* The Journal of Clinical Investigation, 1976. **57**(3): p. 559-68.
91. Garabedian, M., et al., *Response of intestinal calcium transport and bone calcium mobilization to 1,25-dihydroxyvitamin D3 in thyroparathyroidectomized rats.* Endocrinology, 1974. **94**(4): p. 1022-7.
92. Liu, S., et al., *Pathogenic role of Fgf23 in Hyp mice.* American Journal of Physiology Endocrinology Metabolism, 2006. **291**(1): p. E38-49.
93. Fraser, D.R. and E. Kodicek, *Unique biosynthesis by kidney of a biological active vitamin D metabolite.* Nature, 1970. **228**(5273): p. 764-6.
94. Adams, J.S. and M. Hewison, *Extrarenal expression of the 25-hydroxyvitamin D-1-hydroxylase.* Archives of Biochemistry and Biophysics, 2012. **523**(1): p. 95-102.
95. Turner, R.T., et al., *In vitro synthesis of 1 alpha,25-dihydroxycholecalciferol and 24,25-dihydroxycholecalciferol by isolated calvarial cells.* Proceeding of the National Academy of Sciences U S A, 1980. **77**(10): p. 5720-4.
96. Adams, J.S. and M.A. Gacad, *Characterization of 1 alpha-hydroxylation of vitamin D3 sterols by cultured alveolar macrophages from patients with sarcoidosis.* The Journal of Experimental Medicine, 1985. **161**(4): p. 755-65.
97. Frankel, T.L., et al., *The synthesis of vitamin D metabolites by human melanoma cells.* The Journal of Clinical and Endocrinology Metabolism, 1983. **57**(3): p. 627-31.
98. Bikle, D.D., et al., *Neonatal human foreskin keratinocytes produce 1,25-dihydroxyvitamin D3.* Biochemistry, 1986. **25**(7): p. 1545-8.
99. Holick, M.F., *Resurrection of vitamin D deficiency and rickets.* The Journal of Clinical Investigation, 2006. **116**(8): p. 2062-72.
100. Tanaka, Y., et al., *In vitro production of 1,25-dihydroxyvitamin D3 by rat placental tissue.* Proceeding of the National Academy of Sciences U S A, 1979. **76**(10): p. 5033-5.
101. Demay, M.B., et al., *Sequences in the human parathyroid hormone gene that bind the 1,25-dihydroxyvitamin D3 receptor and mediate transcriptional repression in response to 1,25-dihydroxyvitamin D3.* Proceeding of the National Academy of Sciences U S A, 1992. **89**(17): p. 8097-101.

102. Kadowaki, S. and A.W. Norman, *Demonstration that the vitamin D metabolite 1,25(OH)2-vitamin D3 and not 24R,25(OH)2-vitamin D3 is essential for normal insulin secretion in the perfused rat pancreas.* Diabetes, 1985. **34**(4): p. 315-20.
103. Pillai, S., D.D. Bikle, and P.M. Elias, *1,25-Dihydroxyvitamin D production and receptor binding in human keratinocytes varies with differentiation.* The Journal of Biological Chemistry, 1988. **263**(11): p. 5390-5.
104. Hsu, J.Y., et al., *Reduced 1alpha-hydroxylase activity in human prostate cancer cells correlates with decreased susceptibility to 25-hydroxyvitamin D3-induced growth inhibition.* Cancer Research, 2001. **61**(7): p. 2852-6.
105. Friedrich, M., et al., *Analysis of 25-hydroxyvitamin D3-1alpha-hydroxylase in cervical tissue.* Anticancer Research, 2002. **22**(1A): p. 183-6.
106. Segersten, U., et al., *25-Hydroxyvitamin D3 1alpha-hydroxylase expression in breast cancer and use of non-1alpha-hydroxylated vitamin D analogue.* Breast Cancer Research, 2005. **7**(6): p. R980-6.
107. Bareis, P., et al., *25-hydroxy-vitamin d metabolism in human colon cancer cells during tumor progression.* Biochemical and Biophysical Research Communications, 2001. **285**(4): p. 1012-7.
108. Mitschele, T., et al., *Analysis of the vitamin D system in basal cell carcinomas (BCCs).* Lab Investigation, 2004. **84**(6): p. 693-702.
109. Chen, S., et al., *Modulatory effects of 1,25-dihydroxyvitamin D3 on human B cell differentiation.* The Journal of Immunology, 2007. **179**(3): p. 1634-47.
110. Lemire, J.M., et al., *Immunosuppressive actions of 1,25-dihydroxyvitamin D3: preferential inhibition of Th1 functions.* Journal of Nutrition, 1995. **125**(6 Suppl): p. 1704S-1708S.
111. Ponsonby, A.L., A. McMichael, and I. van der Mei, *Ultraviolet radiation and autoimmune disease: insights from epidemiological research.* Toxicology, 2002. **181-182**: p. 71-8.
112. Rook, G.A., et al., *Vitamin D3, gamma interferon, and control of proliferation of Mycobacterium tuberculosis by human monocytes.* Immunology, 1986. **57**(1): p. 159-63.
113. Ustianowski, A., et al., *Prevalence and associations of vitamin D deficiency in foreign-born persons with tuberculosis in London.* The Journal of Infection, 2005. **50**(5): p. 432-7.
114. Liu, P.T. and R.L. Modlin, *Human macrophage host defense against Mycobacterium tuberculosis.* Current Opinion in Immunology, 2008. **20**(4): p. 371-6.
115. Grant, W.B., *Solar ultraviolet-B irradiance and vitamin D may reduce the risk of septicemia.* Dermatoendocrinology, 2009. **1**(1): p. 37-42.
116. Muhe, L., et al., *Case-control study of the role of nutritional rickets in the risk of developing pneumonia in Ethiopian children.* Lancet, 1997. **349**(9068): p. 1801-4.
117. Aloia, J.F. and M. Li-Ng, *Re: epidemic influenza and vitamin D.* Epidemiology and Infection, 2007. **135**(7): p. 1095-6; author reply 1097-8.
118. Seelig, M.S., *Vitamin D and cardiovascular, renal, and brain damage in infancy and childhood.* Annals of the New York Academy of Science, 1969. **147**(15): p. 539-82.
119. Noseworthy, J.H., et al., *Multiple sclerosis.* The New England Journal of Medicine, 2000. **343**(13): p. 938-52.
120. Wallin, M.T., W.F. Page, and J.F. Kurtzke, *Multiple sclerosis in US veterans of the Vietnam era and later military service: race, sex, and geography.* Annals of Neurology, 2004. **55**(1): p. 65-71.
121. van der Mei, I.A., et al., *Regional variation in multiple sclerosis prevalence in Australia and its association with ambient ultraviolet radiation.* Neuroepidemiology, 2001. **20**(3): p. 168-74.

122. Kurtzke, J.F., *Geographic distribution of multiple sclerosis: An update with special reference to Europe and the Mediterranean region.* Acta Neurologica Scandinavica, 1980. **62**(2): p. 65-80.
123. Garland, C.F., et al., *What is the dose-response relationship between vitamin D and cancer risk?* Nutrition Reviews, 2007. **65**(8 Pt 2): p. S91-5.
124. Verlinden, L., et al., *Action of 1,25(OH)2D3 on the cell cycle genes, cyclin D1, p21 and p27 in MCF-7 cells.* Molecular and Cellular Endocrinology, 1998. **142**(1-2): p. 57-65.
125. Ylikomi, T., et al., *Antiproliferative action of vitamin D.* Vitamins and Hormones, 2002. **64**: p. 357-406.
126. Kivineva, M., et al., *Localization of 1,25-dihydroxyvitamin D3 receptor (VDR) expression in human prostate.* The Journal of Steroid Biochemistry and Molecular Biology, 1998. **66**(3): p. 121-7.
127. Chen, T.C., et al., *Prostatic 25-hydroxyvitamin D-1alpha-hydroxylase and its implication in prostate cancer.* Journal of Cellular Biochemistry, 2003. **88**(2): p. 315-22.
128. Miller, G.J., et al., *Vitamin D receptor expression, 24-hydroxylase activity, and inhibition of growth by 1alpha,25-dihydroxyvitamin D3 in seven human prostatic carcinoma cell lines.* Clinical Cancer Research, 1995. **1**(9): p. 997-1003.
129. Loose, D.S., et al., *Ketoconazole blocks adrenal steroidogenesis by inhibiting cytochrome P450-dependent enzymes.* The Journal of Clinical Investigation, 1983. **71**(5): p. 1495-9.
130. Ly, L.H., et al., *Liarozole acts synergistically with 1alpha,25-dihydroxyvitamin D3 to inhibit growth of DU 145 human prostate cancer cells by blocking 24-hydroxylase activity.* Endocrinology, 1999. **140**(5): p. 2071-6.
131. Sonino, N., *The endocrine effects of ketoconazole.* Journal of Endocrinological Investigation, 1986. **9**(4): p. 341-7.
132. Borgers, M., H. Van den Bossche, and M. De Brabander, *The mechanism of action of the new antimycotic ketoconazole.* American Journal of Medicine, 1983. **74**(1b): p. 2-8.
133. Graybill, J.R., D.J. Drutz, and A.L. Murphy, *Ketoconazole: a major innovation for treatment of fungal disease.* Annals of Internal Medicine, 1980. **93**(6): p. 921-3.
134. Pont, A., et al., *Ketoconazole blocks adrenal steroid synthesis.* Annals of Internal Medicine, 1982. **97**(3): p. 370-2.
135. Pont, A., et al., *Ketoconazole blocks testosterone synthesis.* Archives of Internal Medicine, 1982. **142**(12): p. 2137-40.
136. Henry, H.L., *Effect of ketoconazole and miconazole on 25-hydroxyvitamin D3 metabolism by cultured chick kidney cells.* Journal of Steroid Biochemistry, 1985. **23**(6a): p. 991-4.
137. Adams, J.S., et al., *Ketoconazole decreases the serum 1,25-dihydroxyvitamin D and calcium concentration in sarcoidosis-associated hypercalcemia.* The Journal of Clinical and Endocrinology Metabolism, 1990. **70**(4): p. 1090-5.
138. Saggese, G., et al., *Ketoconazole decreases the serum ionized calcium and 1,25-dihydroxyvitamin D levels in tuberculosis-associated hypercalcemia.* The American Journal of Diseases of Children, 1993. **147**(3): p. 270-3.
139. Glass, A.R. and C. Eil, *Ketoconazole-induced reduction in serum 1,25-dihydroxyvitamin D and total serum calcium in hypercalcemic patients.* The Journal of Clinical and Endocrinology Metabolism, 1988. **66**(5): p. 934-8.
140. Peehl, D.M., E. Seto, and D. Feldman, *Rationale for combination ketoconazole/ vitamin D treatment of prostate cancer.* Urology, 2001. **58**(2 Suppl 1): p. 123-6.
141. Murray, M. and G.F. Reidy, *Selectivity in the inhibition of mammalian cytochromes P-450 by chemical agents.* Pharmacological Reviews, 1990. **42**(2): p. 85-101.
142. Schuster, I., et al., *Selective inhibition of vitamin D hydroxylases in human keratinocytes.* Steroids, 2001. **66**(3-5): p. 409-22.

143. Napoli, J.L., C.A. Martin, and R.L. Horst, *Induction, inhibition, and analysis of vitamin D metabolism in cultured cells.* Methods in Enzymology, 1991. **206**: p. 491-501.
144. Reinhardt, T.A. and R.L. Horst, *Ketoconazole inhibits self-induced metabolism of 1,25-dihydroxyvitamin D3 and amplifies 1,25-dihydroxyvitamin D3 receptor up-regulation in rat osteosarcoma cells.* Archives of Biochemistry and Biophysics, 1989. **272**(2): p. 459-65.
145. Zhou, S., M.S. LeBoff, and J. Glowacki, *Vitamin D metabolism and action in human bone marrow stromal cells.* Endocrinology, 2010. **151**(1): p. 14-22.
146. Kota, B.P., J.D. Allen, and B.D. Roufogalis, *The effect of vitamin D3 and ketoconazole combination on VDR-mediated P-gp expression and function in human colon adenocarcinoma cells: implications in drug disposition and resistance.* Basic and Clinical Pharmacology and Toxicology, 2011. **109**(2): p. 97-102.
147. Loose, D.S., et al., *Ketoconazole blocks adrenal steroidogenesis by inhibiting cytochrome P450-dependent enzymes.* Journal of Clinical Investigation, 1983. **71**(5): p. 1495.
148. Atkins, G.J., et al., *Metabolism of vitamin D3 in human osteoblasts: evidence for autocrine and paracrine activities of 1 alpha,25-dihydroxyvitamin D3.* Bone, 2007. **40**(6): p. 1517-28.
149. van Driel, M., et al., *Evidence for auto/paracrine actions of vitamin D in bone: 1alpha-hydroxylase expression and activity in human bone cells.* Faseb j, 2006. **20**(13): p. 2417-9.
150. Geng, S., S. Zhou, and J. Glowacki, *Effects of 25-hydroxyvitamin D(3) on proliferation and osteoblast differentiation of human marrow stromal cells require CYP27B1/1alpha-hydroxylase.* Journal of Bone and Mineral Research, 2011. **26**(5): p. 1145-53.
151. van Driel, M., et al., *Evidence for auto/paracrine actions of vitamin D in bone: 1alpha-hydroxylase expression and activity in human bone cells.* The Journal of the Federation of American Societies of Experimental Biology, 2006. **20**(13): p. 2417-9.
152. Peacock, M., *Calcium absorption efficiency and calcium requirements in children and adolescents.* The American Journal of Clinical Nutrition, 1991. **54**(1): p. 261S-265S.
153. Chapuy, M.-C., et al., *Prevalence of vitamin D insufficiency in an adult normal population.* Osteoporosis International, 1997. **7**(5): p. 439-443.
154. Lips, P., *Vitamin D deficiency and secondary hyperparathyroidism in the elderly: consequences for bone loss and fractures and therapeutic implications.* Endocrine reviews, 2001. **22**(4): p. 477-501.
155. Visser, M., D.J. Deeg, and P. Lips, *Low vitamin D and high parathyroid hormone levels as determinants of loss of muscle strength and muscle mass (sarcopenia): the Longitudinal Aging Study Amsterdam.* Journal of Clinical Endocrinology & Metabolism, 2003. **88**(12): p. 5766-5772.
156. Bischoff, H.A., et al., *Effects of vitamin D and calcium supplementation on falls: a randomized controlled trial.* Journal of Bone and Mineral Research, 2003. **18**(2): p. 343-351.
157. Bischoff-Ferrari, H.A., E.J. Orav, and B. Dawson-Hughes, *Effect of cholecalciferol plus calcium on falling in ambulatory older men and women: a 3-year randomized controlled trial.* Archives of internal medicine, 2006. **166**(4): p. 424-430.
158. Bischoff-Ferrari, H.A., et al., *Fracture prevention with vitamin D supplementation: a meta-analysis of randomized controlled trials.* Journal of the American Medical Association, 2005. **293**(18): p. 2257-2264.
159. Harvey, N.C., et al., *Maternal Antenatal Vitamin D Status and Offspring Muscle Development: Findings From the Southampton Women's Survey.* The Journal of Clinical Endocrinology & Metabolism, 2013. **99**(1): p. 330-337.

160. Roux, C., et al., *New insights into the role of vitamin D and calcium in osteoporosis management: an expert roundtable discussion.* Current Medical Research and Opinion®, 2008. **24**(5): p. 1363-1370.
161. Schacht, E., L. Dukas, and F. Richy, *Combined therapies in osteoporosis: bisphosphonates and vitamin D-hormone analogs.* Journal of Musculoskeletal and Neuronal Interactions 2007. **7**(2): p. 174.
162. Russell, R. and M. Rogers, *Bisphosphonates: from the laboratory to the clinic and back again.* Bone, 1999. **25**(1): p. 97-106.
163. Mallet, E., et al., *[Symptomatic rickets in adolescents].* Archives of Pediatrics, 2004. **11**(7): p. 871-8.
164. Holick, M.F., *Vitamin D status: measurement, interpretation, and clinical application.* Annals of Epidemiology, 2009. **19**(2): p. 73-8.
165. Scott, T.G., et al., *Advances in bionanomaterials for bone tissue engineering.* Journal of Nanoscience and Nanotechnology, 2013. **13**(1): p. 1-22.
166. Barrere, F., C.A. van Blitterswijk, and K. de Groot, *Bone regeneration: molecular and cellular interactions with calcium phosphate ceramics.* International Journal of Nanomedicine, 2006. **1**(3): p. 317-32.
167. POSNER, A.S., 1973. Bone mineral on the molecular level. Federation proceedings, 32(9), pp. 1933-1937.
168. Landis, W.J., *The strength of a calcified tissue depends in part on the molecular structure and organization of its constituent mineral crystals in their organic matrix.* Bone, 1995. **16**(5): p. 533-44.
169. Kular, J., et al., *An overview of the regulation of bone remodelling at the cellular level.* Clinical Biochemistry, 2012. **45**(12): p. 863-73.
170. Caplan, A.I. and S.P. Bruder, *Mesenchymal stem cells: building blocks for molecular medicine in the 21st century.* Trends in Molecular Medicine, 2001. **7**(6): p. 259-64.
171. Jiang, Y., et al., *Pluripotency of mesenchymal stem cells derived from adult marrow.* Nature, 2002. **418**(6893): p. 41-9.
172. Cheng, S.L., et al., *Differentiation of human bone marrow osteogenic stromal cells in vitro: induction of the osteoblast phenotype by dexamethasone.* Endocrinology, 1994. **134**(1): p. 277-86.
173. Choi, J.Y., et al., *Expression patterns of bone-related proteins during osteoblastic differentiation in MC3T3-E1 cells.* Journal of Cellular Biochemistry, 1996. **61**(4): p. 609-18.
174. Ducy, P., T. Schinke, and G. Karsenty, *The Osteoblast: A Sophisticated Fibroblast under Central Surveillance.* Science, 2000. **289**(5484): p. 1501-1504.
175. Krause, C., et al., *Chapter 2. Signal Transduction Cascades Controlling Osteoblast Differentiation*, in *Primer on the Metabolic Bone Diseases and Disorders of Mineral Metabolism*. 2009, John Wiley & Sons, Inc. p. 10-16.
176. Stein, G.S. and J.B. Lian, *Molecular mechanisms mediating proliferation/differentiation interrelationships during progressive development of the osteoblast phenotype.* Endocrine Reviews, 1993. **14**(4): p. 424-42.
177. Harada, H., et al., *Cbfa1 isoforms exert functional differences in osteoblast differentiation.* The Journal of Biological Chemistry, 1999. **274**(11): p. 6972-8.
178. Kern, B., et al., *Cbfa1 contributes to the osteoblast-specific expression of type I collagen genes.* The Journal of Biological Chemistry, 2001. **276**(10): p. 7101-7.
179. Otto, F., et al., *Cbfa1, a candidate gene for cleidocranial dysplasia syndrome, is essential for osteoblast differentiation and bone development.* Cell, 1997. **89**(5): p. 765-71.
180. Chen, D., M. Zhao, and G.R. Mundy, *Bone morphogenetic proteins.* Growth Factors, 2004. **22**(4): p. 233-41.

181. Massague, J., J. Seoane, and D. Wotton, *Smad transcription factors.* Genes and Development, 2005. **19**(23): p. 2783-810.
182. Clevers, H., *Wnt/beta-catenin signaling in development and disease.* Cell, 2006. **127**(3): p. 469-80.
183. Hill, T.P., et al., *Canonical Wnt/beta-catenin signaling prevents osteoblasts from differentiating into chondrocytes.* Developmental Cell, 2005. **8**(5): p. 727-38.
184. Van Wesenbeeck, L., et al., *Six novel missense mutations in the LDL receptor-related protein 5 (LRP5) gene in different conditions with an increased bone density.* American Journal of Human Genetics, 2003. **72**(3): p. 763-71.
185. Long, F., et al., *Ihh signaling is directly required for the osteoblast lineage in the endochondral skeleton.* Development, 2004. **131**(6): p. 1309-18.
186. Rubin, M. and J. Bilezikian, *New anabolic therapies in osteoporosis.* Endocrinology and metabolism clinics of North America, 2003. **32**(1): p. 285-307.
187. Karaplis, A.C., et al., *Lethal skeletal dysplasia from targeted disruption of the parathyroid hormone-related peptide gene.* Genes and Development, 1994. **8**(3): p. 277-89.
188. Lanske, B., et al., *PTH/PTHrP receptor in early development and Indian hedgehog-regulated bone growth.* Science, 1996. **273**(5275): p. 663-6.
189. Anderson, H.C., R. Garimella, and S.E. Tague, *The role of matrix vesicles in growth plate development and biomineralization.* Frontiers in Bioscience, 2005. **10**: p. 822-37.
190. Brandao-Burch, A., et al., *Acidosis inhibits bone formation by osteoblasts in vitro by preventing mineralization.* Calcified Tissue International, 2005. **77**(3): p. 167-74.
191. Hamade, E., et al., *Chick embryo anchored alkaline phosphatase and mineralization process in vitro.* European Journal of Biochemistry, 2003. **270**(9): p. 2082-90.
192. Fleisch, H., et al., *Effect of condensed phosphates on calcification of chick embryo femurs in tissue culture.* American Journal of Physiology 1966. **211**(3): p. 821-5.
193. Johnson, K.A., et al., *Osteoblast tissue-nonspecific alkaline phosphatase antagonizes and regulates PC-1.* American Journal of Physiology Regulatory Integrative and Comparative Physiology, 2000. **279**(4): p. R1365-77.
194. Hsu, H.H., *Purification and partial characterization of ATP pyrophosphohydrolase from fetal bovine epiphyseal cartilage.* The Journal of Biological Chemistry, 1983. **258**(6): p. 3463-8.
195. Hessle, L., et al., *Tissue-nonspecific alkaline phosphatase and plasma cell membrane glycoprotein-1 are central antagonistic regulators of bone mineralization.* Proceeding of the National Academy of Sciences U S A, 2002. **99**(14): p. 9445-9.
196. Nijweide, P.J., E.H. Burger, and J.H. Feyen, *Cells of bone: proliferation, differentiation, and hormonal regulation.* Physiol Rev, 1986. **66**(4): p. 855-86.
197. Boyle, W.J., W.S. Simonet, and D.L. Lacey, *Osteoclast differentiation and activation.* Nature, 2003. **423**(6937): p. 337-42.
198. Suda, T., et al., *Modulation of osteoclast differentiation and function by the new members of the tumor necrosis factor receptor and ligand families.* Endocrine Reviews, 1999. **20**(3): p. 345-57.
199. Nakashima, T., et al., *Evidence for osteocyte regulation of bone homeostasis through RANKL expression.* Nature Medicine, 2011. **17**(10): p. 1231-4.
200. Ross, F.P. and Asbmr, *Chapter 3. Osteoclast Biology and Bone Resorption*, in *Primer on the Metabolic Bone Diseases and Disorders of Mineral Metabolism*. 2009, John Wiley & Sons, Inc. p. 16-22.
201. Teitelbaum, S.L., Y. Abu-Amer, and F.P. Ross, *Molecular mechanisms of bone resorption.* Journal of Cellular Biochemistry, 1995. **59**(1): p. 1-10.
202. Bonewald, L.F., *Osteocytes as dynamic multifunctional cells.* Annals of the New York Academy of Science, 2007. **1116**: p. 281-90.

203. Baron, R. and M. Kneissel, *WNT signaling in bone homeostasis and disease: from human mutations to treatments.* Nature Medicine, 2013. **19**(2): p. 179-92.
204. Hamersma, H., J. Gardner, and P. Beighton, *The natural history of sclerosteosis.* Clinical Genetics, 2003. **63**(3): p. 192-7.
205. Staehling-Hampton, K., et al., *A 52-kb deletion in the SOST-MEOX1 intergenic region on 17q12-q21 is associated with van Buchem disease in the Dutch population.* American Journal of Medical Genetics, 2002. **110**(2): p. 144-52.
206. Ubaidus, S., et al., *FGF23 is mainly synthesized by osteocytes in the regularly distributed osteocytic lacunar canalicular system established after physiological bone remodeling.* Journal of Electron Microscopy (Tokyo), 2009. **58**(6): p. 381-92.
207. Guo, D., et al., *Identification of osteocyte-selective proteins.* Proteomics, 2010. **10**(20): p. 3688-98.
208. Bonewald, L.F., *The amazing osteocyte.* Journal of Bone and Mineral Research, 2011. **26**(2): p. 229-38.
209. Franz-Odendaal, T.A., B.K. Hall, and P.E. Witten, *Buried alive: how osteoblasts become osteocytes.* Developmental Dynamics, 2006. **235**(1): p. 176-90.
210. Wetterwald, A., et al., *Characterization and cloning of the E11 antigen, a marker expressed by rat osteoblasts and osteocytes.* Bone, 1996. **18**(2): p. 125-32.
211. Robling, A.G., et al., *Shorter, more frequent mechanical loading sessions enhance bone mass.* Medicine and Science in Sports Exercise, 2002. **34**(2): p. 196-202.
212. Kitase, Y., et al., *Mechanical induction of PGE2 in osteocytes blocks glucocorticoid-induced apoptosis through both the beta-catenin and PKA pathways.* Journal of Bone and Mineral Research, 2010. **25**(12): p. 2657-68.
213. Kramer, I., et al., *Osteocyte Wnt/beta-catenin signaling is required for normal bone homeostasis.* Molecular and Cell Biology, 2010. **30**(12): p. 3071-85.
214. Xia, X., et al., *Prostaglandin promotion of osteocyte gap junction function through transcriptional regulation of connexin 43 by glycogen synthase kinase 3/beta-catenin signaling.* Molecular and Cell Biology, 2010. **30**(1): p. 206-19.
215. Bonewald, L.F. and M.L. Johnson, *Osteocytes, mechanosensing and Wnt signaling.* Bone, 2008. **42**(4): p. 606-15.
216. Verborgt, O., G.J. Gibson, and M.B. Schaffler, *Loss of osteocyte integrity in association with microdamage and bone remodeling after fatigue in vivo.* Journal of Bone and Mineral Research, 2000. **15**(1): p. 60-7.
217. Feng, J.Q., et al., *Loss of DMP1 causes rickets and osteomalacia and identifies a role for osteocytes in mineral metabolism.* Nature Genetics, 2006. **38**(11): p. 1310-5.
218. Kogianni, G., V. Mann, and B.S. Noble, *Apoptotic bodies convey activity capable of initiating osteoclastogenesis and localized bone destruction.* Journal of Bone and Mineral Research, 2008. **23**(6): p. 915-27.
219. Heino, T.J., T.A. Hentunen, and H.K. Vaananen, *Osteocytes inhibit osteoclastic bone resorption through transforming growth factor-beta: enhancement by estrogen.* Journal of Cellular Biochemistry, 2002. **85**(1): p. 185-97.
220. James, C.F., et al., *Vitamin D and cancer: a review of molecular mechanisms.* Biochemical Journal, 2012. **441**(1): p. 61-76.
221. Mulholland, D.J., et al., *Interaction of nuclear receptors with the Wnt/β-catenin/Tcf signaling axis: Wnt you like to know?* Endocrine reviews, 2005. **26**(7): p. 898-915.
222. Beildeck, M.E., E.P. Gelmann, and S. Byers, *Cross-regulation of signaling pathways: an example of nuclear hormone receptors and the canonical Wnt pathway.* Experimental cell research, 2010. **316**(11): p. 1763-1772.
223. Larriba, M.J., et al., *Vitamin D Is a Multilevel Repressor of Wnt/b-Catenin Signaling in Cancer Cells.* Cancers, 2013. **5**(4): p. 1242-1260.

224. Pálmer, H.G., et al., *Vitamin D3 promotes the differentiation of colon carcinoma cells by the induction of E-cadherin and the inhibition of β-catenin signaling.* The Journal of cell biology, 2001. **154**(2): p. 369-388.
225. Henderson, B.R., *Nuclear-cytoplasmic shuttling of APC regulates β-catenin subcellular localization and turnover.* Nature cell biology, 2000. **2**(9): p. 653-660.
226. Aguilera, O., et al., *The Wnt antagonist DICKKOPF-1 gene is induced by 1alpha,25-dihydroxyvitamin D3 associated to the differentiation of human colon cancer cells.* Carcinogenesis, 2007. **28**(9): p. 1877-84.
227. Sutherland, M.K., et al., *Unique regulation of< i> SOST</i>, the sclerosteosis gene, by BMPs and steroid hormones in human osteoblasts.* Bone, 2004. **35**(2): p. 448-454.
228. Haussler, M.R., et al., *The nuclear vitamin D receptor controls the expression of genes encoding factors which feed the "Fountain of Youth" to mediate healthful aging.* The Journal of steroid biochemistry and molecular biology, 2010. **121**(1): p. 88-97.
229. Kaler, P., L. Augenlicht, and L. Klampfer, *Macrophage-derived IL-1β stimulates Wnt signaling and growth of colon cancer cells: a crosstalk interrupted by vitamin D3.* Oncogene, 2009. **28**(44): p. 3892-3902.
230. Cianferotti, L. and M.B. Demay, *VDR-mediated inhibition of DKK1 and SFRP2 suppresses adipogenic differentiation of murine bone marrow stromal cells.* Journal of cellular biochemistry, 2007. **101**(1): p. 80-88.
231. Kennell, J.A. and O.A. MacDougald, *Wnt signaling inhibits adipogenesis through β-catenin-dependent and-independent mechanisms.* Journal of Biological Chemistry, 2005. **280**(25): p. 24004-24010.
232. Kang, S., et al., *Wnt signaling stimulates osteoblastogenesis of mesenchymal precursors by suppressing CCAAT/enhancer-binding protein α and peroxisome proliferator-activated receptor γ.* Journal of Biological Chemistry, 2007. **282**(19): p. 14515-14524.
233. Fretz, J.A., et al., *1, 25-Dihydroxyvitamin D3 regulates the expression of low-density lipoprotein receptor-related protein 5 via deoxyribonucleic acid sequence elements located downstream of the start site of transcription.* Molecular Endocrinology, 2006. **20**(9): p. 2215-2230.
234. Yang, H., et al., *Snail-1 regulates VDR signaling and inhibits 1, 25 (OH)-D< sub> 3</sub> action in osteosarcoma.* European journal of pharmacology, 2011. **670**(2): p. 341-346.
235. Bodine, P.V. and B.S. Komm, *Wnt signaling and osteoblastogenesis.* Reviews in Endocrine and Metabolic Disorders, 2006. **7**(1-2): p. 33-9.
236. Ramagopalan, S.V., et al., *A ChIP-seq defined genome-wide map of vitamin D receptor binding: associations with disease and evolution.* Genome Research, 2010. **20**(10): p. 1352-60.
237. Wang, T.T., et al., *Large-scale in silico and microarray-based identification of direct 1,25-dihydroxyvitamin D3 target genes.* Molecular Endocrinology, 2005. **19**(11): p. 2685-95.
238. Sandgren, M.E., M. Bronnegard, and H.F. DeLuca, *Tissue distribution of the 1,25-dihydroxyvitamin D3 receptor in the male rat.* Biochemical and Biophysical Research Communications, 1991. **181**(2): p. 611-6.
239. Langub, M.C., et al., *Characterization of vitamin D receptor immunoreactivity in human bone cells.* Bone, 2000. **27**(3): p. 383-7.
240. St-Arnaud, R., *The direct role of vitamin D on bone homeostasis.* Archives of Biochemistry and Biophysics, 2008. **473**(2): p. 225-30.
241. Letton, R.W., P. Fanti, and H.H. Malluche, *Regulation of 25-hydroxyvitamin D3 metabolism in cultures of osteoblastic cells.* Journal of Bone and Mineral Research, 1990. **5**(8): p. 815-23.
242. van Driel, M., H.A. Pols, and J.P. van Leeuwen, *Osteoblast differentiation and control by vitamin D and vitamin D metabolites.* Curr Pharm Des, 2004. **10**(21): p. 2535-55.

243. Howard, G.A., et al., *Human bone cells in culture metabolize 25-hydroxyvitamin D3 to 1,25-dihydroxyvitamin D3 and 24,25-dihydroxyvitamin D3.* The Journal of Biological Chemistry, 1981. **256**(15): p. 7738-40.
244. Chen, T.L., C.M. Cone, and D. Feldman, *Effects of 1 alpha,25-dihydroxyvitamin D3 and glucocorticoids on the growth of rat and mouse osteoblast-like bone cells.* Calcified Tissue International, 1983. **35**(6): p. 806-11.
245. van den Bemd, G.J., et al., *Differential effects of 1,25-dihydroxyvitamin D3-analogs on osteoblast-like cells and on in vitro bone resorption.* The Journal of Steroid Biochemistry and Molecular Biology, 1995. **55**(3-4): p. 337-46.
246. Shionome, M., et al., *1 alpha,25-dihydroxyvitamin D3 modulation in lipid metabolism in established bone marrow-derived stromal cells, MC3T3-G2/PA6.* Journal of Cellular Biochemistry, 1992. **48**(4): p. 424-30.
247. Rickard, D.J., I. Kazhdan, and P.S. Leboy, *Importance of 1,25-dihydroxyvitamin D3 and the nonadherent cells of marrow for osteoblast differentiation from rat marrow stromal cells.* Bone, 1995. **16**(6): p. 671-8.
248. Beresford, J.N., et al., *The effects of dexamethasone and 1,25-dihydroxyvitamin D3 on osteogenic differentiation of human marrow stromal cells in vitro.* Archives of Oral Biology, 1994. **39**(11): p. 941-7.
249. Koshihara, Y., et al., *Vitamin K2 promotes 1alpha,25(OH)2 vitamin D3-induced mineralization in human periosteal osteoblasts.* Calcified Tissue International, 1996. **59**(6): p. 466-73.
250. Matsumoto, T., et al., *Stimulation by 1,25-dihydroxyvitamin D3 of in vitro mineralization induced by osteoblast-like MC3T3-E1 cells.* Bone, 1991. **12**(1): p. 27-32.
251. Li, Y., et al., *Species difference exists in the effects of 1alpha,25(OH)(2)D(3) and its analogue 2-methylene-19-nor-(20S)-1,25-dihydroxyvitamin D(3) (2MD) on osteoblastic cells.* The Journal of Steroid Biochemistry and Molecular Biology, 2008. **112**(1-3): p. 110-6.
252. Siggelkow, H., et al., *1,25 dihydroxyvitamin-D3 attenuates the confluence-dependent differences in the osteoblast characteristic proteins alkaline phosphatase, procollagen I peptide, and osteocalcin.* Calcified Tissue International, 1999. **64**(5): p. 414-21.
253. Bodine, P.V., S.K. Vernon, and B.S. Komm, *Establishment and hormonal regulation of a conditionally transformed preosteocytic cell line from adult human bone.* Endocrinology, 1996. **137**(11): p. 4592-604.
254. Lomri, A., et al., *Characterization of endosteal osteoblastic cells isolated from mouse caudal vertebrae.* Bone, 1988. **9**(3): p. 165-75.
255. Hicok, K.C., et al., *Development and characterization of conditionally immortalized osteoblast precursor cell lines from human bone marrow stroma.* Journal of Bone and Mineral Research, 1998. **13**(2): p. 205-17.
256. Franceschi, R.T., P.R. Romano, and K.Y. Park, *Regulation of type I collagen synthesis by 1,25-dihydroxyvitamin D3 in human osteosarcoma cells.* The Journal of Biological Chemistry, 1988. **263**(35): p. 18938-45.
257. Tasaki, Y., R. Takamori, and Y. Koshihara, *Prostaglandin D2 metabolite stimulates collagen synthesis by human osteoblasts during calcification.* Prostaglandins, 1991. **41**(4): p. 303-13.
258. Bedalov, A., et al., *1,25-Dihydroxyvitamin D3 inhibition of col1a1 promoter expression in calvariae from neonatal transgenic mice.* Biochimica et Biophysica Acta, 1998. **1398**(3): p. 285-93.
259. Staal, A., et al., *Regulation of osteocalcin production and bone resorption by 1,25-dihydroxyvitamin D3 in mouse long bones: interaction with the bone-derived growth factors TGF-beta and IGF-I.* Journal of Bone and Mineral Research, 1998. **13**(1): p. 36-43.

260. Chen, T.L. and D. Fry, *Hormonal regulation of the osteoblastic phenotype expression in neonatal murine calvarial cells.* Calcified Tissue International, 1999. **64**(4): p. 304-9.
261. van Driel, M., H.A. Pols, and J.P. van Leeuwen, *Osteoblast differentiation and control by vitamin D and vitamin D metabolites.* Current Pharmaceutical Design, 2004. **10**(21): p. 2535-55.
262. Ryhanen, S., et al., *Inhibition of MG-63 cell cycle progression by synthetic vitamin D3 analogs mediated by p27, Cdk2, cyclin E, and the retinoblastoma protein.* Biochemical Pharmacology, 2003. **66**(3): p. 495-504.
263. Duque, G., et al., *Vitamin D inhibits Fas ligand-induced apoptosis in human osteoblasts by regulating components of both the mitochondrial and Fas-related pathways.* Bone, 2004. **35**(1): p. 57-64.
264. Kartsogiannis, V. and K.W. Ng, *Cell lines and primary cell cultures in the study of bone cell biology.* Molecular and Cellular Endocrinology, 2004. **228**(1-2): p. 79-102.
265. Shevde, N.K., et al., *A potent analog of 1alpha,25-dihydroxyvitamin D3 selectively induces bone formation.* Proceeding of the National Academy of Sciences U S A, 2002. **99**(21): p. 13487-91.
266. Ishida, H., et al., *Characterization of the 1,25-(OH)2D3-induced inhibition of bone nodule formation in long-term cultures of fetal rat calvaria cells.* Endocrinology, 1993. **132**(1): p. 61-6.
267. Bellows, C.G., et al., *1,25-dihydroxyvitamin D3 stimulates adipocyte differentiation in cultures of fetal rat calvaria cells: comparison with the effects of dexamethasone.* Endocrinology, 1994. **134**(5): p. 2221-9.
268. Bellows, C.G., J.E. Aubin, and J.N. Heersche, *Physiological concentrations of glucocorticoids stimulate formation of bone nodules from isolated rat calvaria cells in vitro.* Endocrinology, 1987. **121**(6): p. 1985-92.
269. Bellows, C.G. and J.E. Aubin, *Determination of numbers of osteoprogenitors present in isolated fetal rat calvaria cells in vitro.* Developmental Biology, 1989. **133**(1): p. 8-13.
270. Toai, T.C., et al., *In vitro culture and differentiation of osteoblasts from human umbilical cord blood.* Cell Tissue Bank, 2010. **11**(3): p. 269-80.
271. Kogawa, M., et al., *The metabolism of 25-(OH)vitamin D3 by osteoclasts and their precursors regulates the differentiation of osteoclasts.* The Journal of Steroid Biochemistry and Molecular Biology, 2010. **121**(1-2): p. 277-80.
272. Adams, J.S., et al., *Constitutive expression of a vitamin D 1-hydroxylase in a myelomonocytic cell line: a model for studying 1,25-dihydroxyvitamin D production in vitro.* Journal of Bone and Mineral Research, 1990. **5**(12): p. 1265-9.
273. Kogawa, M., et al., *Osteoclastic metabolism of 25(OH)-vitamin D3: a potential mechanism for optimization of bone resorption.* Endocrinology, 2010. **151**(10): p. 4613-25.
274. Kogawa, M., et al., *Modulation of osteoclastic migration by metabolism of 25OH-vitamin D3.* The Journal of Steroid Biochemistry and Molecular Biology, 2013. **136**: p. 59-61.
275. Sahota, O. and D. Hosking, *Update on calcium and vitamin D metabolism.* Current Orthopaedics, 1999. **13**(1): p. 53-63.
276. Menaa, C., et al., *1,25-Dihydroxyvitamin D3 hypersensitivity of osteoclast precursors from patients with Paget's disease.* Journal of Bone and Mineral Research, 2000. **15**(2): p. 228-36.
277. Yasuda, H., et al., *Osteoclast differentiation factor is a ligand for osteoprotegerin/osteoclastogenesis-inhibitory factor and is identical to TRANCE/RANKL.* Proceeding of the National Academy of Sciences U S A, 1998. **95**(7): p. 3597-602.
278. Raisz, L.G., et al., *1,25-dihydroxycholecalciferol: a potent stimulator of bone resorption in tissue culture.* Science, 1972. **175**(4023): p. 768-9.

279. Farrow, E.G., et al., *Molecular analysis of DMP1 mutants causing autosomal recessive hypophosphatemic rickets.* Bone, 2009. **44**(2): p. 287-94.
280. Tang, W.J., et al., *Autocrine/paracrine action of vitamin D on FGF23 expression in cultured rat osteoblasts.* Calcified Tissue International, 2010. **86**(5): p. 404-10.
281. Lieben, L. and G. Carmeliet, *Vitamin D signaling in osteocytes: effects on bone and mineral homeostasis.* Bone, 2013. **54**(2): p. 237-43.
282. Lieben, L., et al., *Normocalcemia is maintained in mice under conditions of calcium malabsorption by vitamin D-induced inhibition of bone mineralization.* The Journal of Clinical Investigation, 2012. **122**(5): p. 1803-15.
283. Ben-Dov, I.Z., et al., *The parathyroid is a target organ for FGF23 in rats.* The Journal of Clinical Investigation, 2007. **117**(12): p. 4003-8.
284. Shimada, T., et al., *Vitamin D receptor-independent FGF23 actions in regulating phosphate and vitamin D metabolism.* American Journal of Physiology Renal Physiology, 2005. **289**(5): p. F1088-95.
285. Turner, A.G., et al., *The Local Production of 1,25(Oh)D Promotes Osteoblast and Osteocyte Maturation.* The Journal of Steroid Biochemistry and Molecular Biology, 2013.
286. Hess Af, L.J.R.H., *NEwer aspects of the therapeutics of viosterol (irradiated ergosterol).* Journal of the American Medical Association, 1930. **94**(24): p. 1885-1889.
287. Hess, A.F., J.M. Lewis, and H. Rivkin, *NEwer aspects of the therapeutics of viosterol (irradiated ergosterol).* Journal of the American Medical Association, 1930. **94**(24): p. 1885-1889.
288. Hunt, R.D., F.G. Garcia, and R.J. Walsh, *A comparison of the toxicity of ergocalciferol and cholecalciferol in rhesus monkeys (Macaca mulatta).* The Journal of Nutrition, 1972. **102**(8): p. 975-86.
289. Harrington, D.D. and E.H. Page, *Acute vitamin D3 toxicosis in horses: case reports and experimental studies of the comparative toxicity of vitamins D2 and D3.* The Journal of the American Veterinary Medical Association, 1983. **182**(12): p. 1358-69.
290. Huber, W. and O.W. Barlow, *Chemical and biological stability of crystalline vitamins D2 and D3 and their derivatives.* Journal of Biological Chemistry, 1943. **149**(1): p. 125-137.
291. Klinke K, B.W., Gleiss J. Zur dosierung des Vigantol D3 bei Fruhgeborenen. [The dosing of premies with Vigantol/vitamin D3. Deutsche Medizinische Wochenschrift. 1954;79:370 –1.
292. Horst, R.L., J.L. Napoli, and E.T. Littledike, *Discrimination in the metabolism of orally dosed ergocalciferol and cholecalciferol by the pig, rat and chick.* Biochemical Journal, 1982. **204**(1): p. 185-9.
293. Marx, S.J., et al., *Differences in mineral metabolism among nonhuman primates receiving diets with only vitamin D3 or only vitamin D2.* The Journal of Clinical Endocrinology and Metabolism, 1989. **69**(6): p. 1282-90.
294. Hoy, D.A., C.F. Ramberg, Jr., and R.L. Horst, *Evidence that discrimination against ergocalciferol by the chick is the result of enhanced metabolic clearance rates for its mono- and dihydroxylated metabolites.* The Journal of Nutrition, 1988. **118**(5): p. 633-8.
295. Mastaglia, S.R., et al., *Vitamin D2 dose required to rapidly increase 25OHD levels in osteoporotic women.* European Journal of Clinical Nutrition, 2006. **60**(5): p. 681-7.
296. Armas, L.A., B.W. Hollis, and R.P. Heaney, *Vitamin D2 is much less effective than vitamin D3 in humans.* The Journal of Clinical Endocrinology and Metabolism, 2004. **89**(11): p. 5387-91.
297. Hollis, B.W., *Comparison of equilibrium and disequilibrium assay conditions for ergocalciferol, cholecalciferol and their major metabolites.* Journal of Steroid Biochemistry, 1984. **21**(1): p. 81-6.

298. Chan, J., *Vitamin D update for nutrition professionals.* Vegetarian Nutrrition, 2009. **18**: p. 1–2.
299. Houghton, L.A. and R. Vieth, *The case against ergocalciferol (vitamin D2) as a vitamin supplement.* The American Journal of Clinical Nutrition 2006. **84**(4): p. 694-7.
300. Holick, M.F., et al., *Evaluation, treatment, and prevention of vitamin D deficiency: an Endocrine Society clinical practice guideline.* The Journal of Clinical Endocrinology and Metabolism, 2011. **96**(7): p. 1911-30.
301. Cooper, L., et al., *Vitamin D supplementation and bone mineral density in early postmenopausal women.* The American Journal of Clinical Nutrition 2003. **77**(5): p. 1324-9.
302. PARK, E.A., *The therapy of rickets* Journal of the American Medical Association., 1940. **115**(5): p. 370-379.
303. Trang, H.M., et al., *Evidence that vitamin D3 increases serum 25-hydroxyvitamin D more efficiently than does vitamin D2.* The American Journal of Clinical Nutrition 1998. **68**(4): p. 854-8.
304. Holmberg, I., et al., *25-Hydroxylase activity in subcellular fractions from human liver. Evidence for different rates of mitochondrial hydroxylation of vitamin D2 and D3.* Scandinavian Journal of Clinical and Laboratory Investigation, 1986. **46**(8): p. 785-90.
305. Czekanska, E.M., et al., *In search of an osteoblast cell model for in vitro research.* European Cells and Materials, 2012. **24**: p. 1-17.
306. Bard, D.R., et al., *Isolation of living cells from mature mammalian bone.* Nature, 1972. **236**(5345): p. 314-5.
307. Gallagher, J.A., *Human osteoblast culture.* Methods in Molecular Medicine, 2003. **80**: p. 3-18.
308. Sudo, H., et al., *In vitro differentiation and calcification in a new clonal osteogenic cell line derived from newborn mouse calvaria.* The Journal of Cell Biology, 1983. **96**(1): p. 191-8.
309. Ghosh-Choudhury, N., et al., *Immortalized murine osteoblasts derived from BMP 2-T-antigen expressing transgenic mice.* Endocrinology, 1996. **137**(1): p. 331-9.
310. Murray, E., et al., *Characterization of a human osteoblastic osteosarcoma cell line (SAOS-2) with high bone alkaline phosphatase activity.* Journal of Bone and Mineral Research, 1987. **2**(3): p. 231-8.
311. Billiau, A., et al., *Human interferon: mass production in a newly established cell line, MG-63.* Antimicrobial Agents and Chemotherapy, 1977. **12**(1): p. 11-5.
312. Pearce, A.I., et al., *Animal models for implant biomaterial research in bone: a review.* European Cells and Materials, 2007. **13**: p. 1-10.
313. Grigoriadis, A.E., et al., *Subclone heterogeneity in a clonally-derived osteoblast-like cell line.* Bone, 1985. **6**(4): p. 249-56.
314. Hayflick, L., *The limited in vitro lifetime of human diploid cell strains.* Experimental cell research, 1965. **37**(3): p. 614-636.
315. Stenderup, K., et al., *Aging is associated with decreased maximal life span and accelerated senescence of bone marrow stromal cells.* Bone, 2003. **33**(6): p. 919-926.
316. Lian, J.B., et al., *Species-specific glucocorticoid and 1,25-dihydroxyvitamin D responsiveness in mouse MC3T3-E1 osteoblasts: dexamethasone inhibits osteoblast differentiation and vitamin D down-regulates osteocalcin gene expression.* Endocrinology, 1997. **138**(5): p. 2117-27.
317. Rao, L.G., et al., *Effects of 1alpha,25-dihydroxy-16ene, 23yne-vitamin D3 on osteoblastic function in human osteosarcoma SaOS-2 cells: differentiation-stage dependence and modulation by 17-beta estradiol.* Bone, 1996. **19**(6): p. 621-7.

318. Franceschi, R.T., W.M. James, and G. Zerlauth, *1 alpha, 25-dihydroxyvitamin D3 specific regulation of growth, morphology, and fibronectin in a human osteosarcoma cell line.* Journal of Cellular Physiology, 1985. **123**(3): p. 401-9.
319. Jorgensen, N.R., et al., *Dexamethasone, BMP-2, and 1,25-dihydroxyvitamin D enhance a more differentiated osteoblast phenotype: validation of an in vitro model for human bone marrow-derived primary osteoblasts.* Steroids, 2004. **69**(4): p. 219-26.
320. Jaiswal, N., et al., *Osteogenic differentiation of purified, culture-expanded human mesenchymal stem cells in vitro.* Journal of Cellular Biochemistry, 1997. **64**(2): p. 295-312.
321. Krause, U., A. Seckinger, and C.A. Gregory, *Assays of osteogenic differentiation by cultured human mesenchymal stem cells.* Methods in Molecular Biology, 2011. **698**: p. 215-30.
322. Eldering, J.A., et al., *Development of a PCR method for mycoplasma testing of Chinese hamster ovary cell cultures used in the manufacture of recombinant therapeutic proteins.* Biologicals, 2004. **32**(4): p. 183-93.
323. Zerwekh, J.E., *Blood biomarkers of vitamin D status.* The American Journal of Clinical Nutrition 2008. **87**(4): p. 1087S-91S.
324. Chen, H., et al., *Measurement of 25-hydroxyvitamin D3 (25OHD3) and 25-hydroxyvitamin D2 (25OHD2) in human serum using liquid chromatography-tandem mass spectrometry and its comparison to a radioimmunoassay method.* Clinica Chimica Acta, 2008. **391**(1-2): p. 6-12.
325. Anoopkumar-Dukie, S., et al., *Resazurin assay of radiation response in cultured cells.* The British Journal of Radiology, 2005. **78**(934): p. 945-7.
326. Sidman, R.L., I.L. Miale, and N. Feder, *Cell proliferation and migration in the primitive ependymal zone; An autoradiographic study of histogenesis in the nervous system.* Experimental Neurology, 1959. **1**(4): p. 322-333.
327. Ansar Ahmed, S., R.M. Gogal Jr, and J.E. Walsh, *A new rapid and simple non-radioactive assay to monitor and determine the proliferation of lymphocytes: an alternative to [3H]thymidine incorporation assay.* Journal of Immunological Methods, 1994. **170**(2): p. 211-224.
328. Livak, K.J. and T.D. Schmittgen, *Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method.* Methods, 2001. **25**(4): p. 402-8.
329. Junqueira, L.C., G. Bignolas, and R.R. Brentani, *Picrosirius staining plus polarization microscopy, a specific method for collagen detection in tissue sections.* The Histochemical Journal, 1979. **11**(4): p. 447-55.
330. Schoeters, G.E., et al., *Mineralization of adult mouse bone marrow in vitro.* Cell Tissue Kinet, 1988. **21**(5): p. 363-74.
331. Puchtler, H., S.N. Meloan, and M.S. Terry, *On the history and mechanism of alizarin and alizarin red S stains for calcium.* Journal of Histochemical Cytochemistry, 1969. **17**(2): p. 110-24.
332. Gregory, C.A., et al., *An Alizarin red-based assay of mineralization by adherent cells in culture: comparison with cetylpyridinium chloride extraction.* Analytical Biochemistry, 2004. **329**(1): p. 77-84.
333. Miller, D.J., *Sydney Ringer; physiological saline, calcium and the contraction of the heart.* The Journal of Physiology, 2004. **555**(Pt 3): p. 585-7.
334. Yamanouchi, K., Y. Gotoh, and M. Nagayama, *Dexamethasone enhances differentiation of human osteoblastic cells in vitro.* Journal of Bone and Mineral Metabolism, 1997. **15**(1): p. 23-29.
335. Rodan, S.B., et al., *Characterization of a human osteosarcoma cell line (Saos-2) with osteoblastic properties.* Cancer Research, 1987. **47**(18): p. 4961-6.

336. Wang, D., et al., *Isolation and characterization of MC3T3-E1 preosteoblast subclones with distinct in vitro and in vivo differentiation/mineralization potential.* Journal of Bone and Mineral Research, 1999. **14**(6): p. 893-903.
337. Clover, J. and M. Gowen, *Are MG-63 and HOS TE85 human osteosarcoma cell lines representative models of the osteoblastic phenotype?* Bone, 1994. **15**(6): p. 585-91.
338. Kumarasuriyar, A., et al., *Glycosaminoglycan composition changes with MG-63 osteosarcoma osteogenesis in vitro and induces human mesenchymal stem cell aggregation.* Journal of Cellular Physiology, 2009. **218**(3): p. 501-11.
339. Lian, J.B. and G.S. Stein, *Concepts of osteoblast growth and differentiation: basis for modulation of bone cell development and tissue formation.* Critical Reviews in Oral Biology and Medicine, 1992. **3**(3): p. 269-305.
340. Pierschbacher, M.D., et al., *An adhesion variant of the MG-63 osteosarcoma cell line displays an osteoblast-like phenotype.* Ciba Foundation Symposium, 1988. **136**: p. 131-41.
341. Saldana, L., et al., *In search of representative models of human bone-forming cells for cytocompatibility studies.* Acta Biomaterialia, 2011. **7**(12): p. 4210-21.
342. Anderson, P.H. and G.J. Atkins, *The skeleton as an intracrine organ for vitamin D metabolism.* Molecular Aspects of Medicine, 2008. **29**(6): p. 397-406.
343. Eelen, G., et al., *Microarray analysis of 1alpha,25-dihydroxyvitamin D3-treated MC3T3-E1 cells.* The Journal of Steroid Biochemistry and Molecular Biology, 2004. **89-90**(1-5): p. 405-7.
344. Majeska, R.J. and G.A. Rodan, *The effect of 1,25(OH)2D3 on alkaline phosphatase in osteoblastic osteosarcoma cells.* The Journal of Biological Chemistry, 1982. **257**(7): p. 3362-5.
345. Verlinden, L., et al., *Characterization of the condensin component Cnap1 and protein kinase Melk as novel E2F target genes down-regulated by 1,25-dihydroxyvitamin D3.* The Journal of Biological Chemistry, 2005. **280**(45): p. 37319-30.
346. Kim, S., N.K. Shevde, and J.W. Pike, *1,25-Dihydroxyvitamin D3 stimulates cyclic vitamin D receptor/retinoid X receptor DNA-binding, co-activator recruitment, and histone acetylation in intact osteoblasts.* Journal of Bone and Mineral Research, 2005. **20**(2): p. 305-17.
347. Varga, F., et al., *Osteocalcin attenuates T3- and increases vitamin D3-induced expression of MMP-13 in mouse osteoblasts.* Endocrine Journal, 2009. **56**(3): p. 441-50.
348. Thomas, G.P., et al., *Changing RANKL/OPG mRNA expression in differentiating murine primary osteoblasts.* Journal of Endocrinology, 2001. **170**(2): p. 451-60.
349. Khoury, R., et al., *Target gene activation by 1,25-dihydroxyvitamin D3 in osteosarcoma cells is independent of calcium influx.* Endocrinology, 1994. **135**(6): p. 2446-53.
350. Manolagas, S.C., D.W. Burton, and L.J. Deftos, *1,25-Dihydroxyvitamin D3 stimulates the alkaline phosphatase activity of osteoblast-like cells.* The Journal of Biological Chemistry, 1981. **256**(14): p. 7115-7.
351. Harrison, J.R., et al., *1,25-Dihydroxyvitamin D3 inhibits transcription of type I collagen genes in the rat osteosarcoma cell line ROS 17/2.8.* Endocrinology, 1989. **125**(1): p. 327-33.
352. Oldberg, A., et al., *Regulation of bone sialoprotein mRNA by steroid hormones.* The Journal of Cell Biology, 1989. **109**(6 Pt 1): p. 3183-6.
353. Markose, E.R., et al., *Vitamin D-mediated modifications in protein-DNA interactions at two promoter elements of the osteocalcin gene.* Proceeding of the National Academy of Sciences U S A, 1990. **87**(5): p. 1701-5.
354. Ecarot, B. and M. Desbarats, *1,25-(OH)2D3 down-regulates expression of Phex, a marker of the mature osteoblast.* Endocrinology, 1999. **140**(3): p. 1192-9.

355. Clark, J.W., et al., *Metabolism and biological activity of 1,25(OH)2D2 and its metabolites in a chronic myelogenous leukemia cell line, rwleu-4.* Bioorganic & Medicinal Chemistry Letters, 1993. **3**(9): p. 1873-1878.
356. Reinhardt, T.A., C.F. Ramberg, and R.L. Horst, *Comparison of receptor binding, biological activity, and in vivo tracer kinetics for 1,25-dihydroxyvitamin D3, 1,25-dihydroxyvitamin D2, and its 24 epimer.* Archives of Biochemistry and Biophysics, 1989. **273**(1): p. 64-71.
357. Chen, J., et al., *Protein-disulfide isomerase-associated 3 (Pdia3) mediates the membrane response to 1,25-dihydroxyvitamin D3 in osteoblasts.* The Journal of Biological Chemistry, 2010. **285**(47): p. 37041-50.
358. Chen, J., et al., *Mineralization of three-dimensional osteoblast cultures is enhanced by the interaction of 1alpha,25-dihydroxyvitamin D3 and BMP2 via two specific vitamin D receptors.* Journal of Tissue Engineering and Regenerative Medicine, 2013.
359. Cardus, A., et al., *Differential effects of vitamin D analogs on vascular calcification.* Journal of Bone and Mineral Research, 2007. **22**(6): p. 860-6.
360. St-Arnaud, R., et al., *1alpha,24(S)(OH)2D2 normalizes bone morphology and serum parathyroid hormone without hypercalcemia in 25-hydroxyvitamin D-1-hydroxylase (CYP27B1)-deficient mice, an animal model of vitamin D deficiency with secondary hyperparathyroidism.* The Journal of Clinical Investigation, 2008. **31**(8): p. 711-7.
361. Woeckel, V.J., et al., *1Alpha,25-(OH)2D3 acts in the early phase of osteoblast differentiation to enhance mineralization via accelerated production of mature matrix vesicles.* Journal of Cellular Physiology, 2010. **225**(2): p. 593-600.
362. Lionakis, M.S., G. Samonis, and D.P. Kontoyiannis, *Endocrine and metabolic manifestations of invasive fungal infections and systemic antifungal treatment.* Mayo Clinic Proceedings, 2008. **83**(9): p. 1046-60.
363. Lee, C.S., et al., *Coordinated tether formation in anatomically distinct mice growth centers is dependent on a functional vitamin D receptor and is tightly linked to three-dimensional tissue morphology.* Bone, 2011. **49**(3): p. 419-27.
364. Huhtakangas, J.A., et al., *The vitamin D receptor is present in caveolae-enriched plasma membranes and binds 1 alpha,25(OH)2-vitamin D3 in vivo and in vitro.* Molecular Endocrinology, 2004. **18**(11): p. 2660-71.
365. Aubin, J.E., et al., *Osteoblast and chondroblast differentiation.* Bone, 1995. **17**(2 Suppl): p. 77S-83S.
366. Kasperk, C., et al., *Human bone cell phenotypes differ depending on their skeletal site of origin.* The Journal of Clinical and Endocrinology Metabolism, 1995. **80**(8): p. 2511-7.
367. Sutton, A.L., et al., *The 1,25(OH)2D3-regulated transcription factor MN1 stimulates vitamin D receptor-mediated transcription and inhibits osteoblastic cell proliferation.* Molecular Endocrinology, 2005. **19**(9): p. 2234-44.
368. van Leeuwen, J.P., et al., *Evidence for coordinated regulation of osteoblast function by 1,25-dihydroxyvitamin D3 and parathyroid hormone.* Biochimica et Biophysica Acta, 1996. **1312**(1): p. 54-62.
369. Finch, J.L., et al., *Relative potencies of 1,25-(OH)(2)D(3) and 19-Nor-1,25-(OH)(2)D(2) on inducing differentiation and markers of bone formation in MG-63 cells.* Journal of the American Society of Nephrology, 2001. **12**(7): p. 1468-74.
370. Gidley, J., et al., *Lysophosphatidic acid cooperates with 1alpha,25(OH)2D3 in stimulating human MG63 osteoblast maturation.* Prostaglandins and Other Lipid Mediators, 2006. **80**(1-2): p. 46-61.
371. Miyahara, T., et al., *A highly potent 26,27-Hexafluoro-1a,25-dihydroxyvitamin D3 on calcification in SV40-transformed human fetal osteoblastic cells.* Calcified Tissue International, 2002. **70**(6): p. 488-95.

372. van Driel, M., et al., *Evidence that both 1alpha,25-dihydroxyvitamin D3 and 24-hydroxylated D3 enhance human osteoblast differentiation and mineralization.* Journal of Cellular Biochemistry, 2006. **99**(3): p. 922-35.
373. Beresford, J.N., J.A. Gallagher, and R.G. Russell, *1,25-Dihydroxyvitamin D3 and human bone-derived cells in vitro: effects on alkaline phosphatase, type I collagen and proliferation.* Endocrinology, 1986. **119**(4): p. 1776-85.
374. Chae, H.J., et al., *ERK MAP Kinase is required in 1,25(OH)2D3-induced differentiation in human osteoblasts.* Immunopharmacology and Immunotoxicology, 2002. **24**(1): p. 31-41.
375. Atkins, G.J., et al., *RANKL expression is related to the differentiation state of human osteoblasts.* Journal of Bone and Mineral Research, 2003. **18**(6): p. 1088-98.
376. Sutherland, M.K., et al., *Unique regulation of SOST, the sclerosteosis gene, by BMPs and steroid hormones in human osteoblasts.* Bone, 2004. **35**(2): p. 448-54.
377. Geng, S., et al., *Vitamin D metabolism in human bone marrow stromal (mesenchymal stem) cells.* Metabolism, 2013. **62**(6): p. 768-77.
378. Shimada, T., et al., *Cloning and characterization of FGF23 as a causative factor of tumor-induced osteomalacia.* Proceeding of the National Academy of Sciences U S A, 2001. **98**(11): p. 6500-5.
379. Shimada, T., et al., *FGF-23 is a potent regulator of vitamin D metabolism and phosphate homeostasis.* Journal of Bone and Mineral Research, 2004. **19**(3): p. 429-35.
380. *A gene (PEX) with homologies to endopeptidases is mutated in patients with X-linked hypophosphatemic rickets. The HYP Consortium.* Nature Genetics, 1995. **11**(2): p. 130-6.
381. Nociti, F.H., Jr., et al., *Vitamin D Represses Dentin Matrix Protein 1 in Cementoblasts and Osteocytes.* Journal of Dental Research, 2013.
382. Bouillon, R., et al., *Vitamin D action: lessons from VDR and Cyp27b1 null mice.* Pediatric Endocrinology Reviews, 2013. **10 Suppl 2**: p. 354-66.
383. *Dietary Reference Intakes for Calcium, Phosphorus, Magnesium, Vitamin D, and Fluoride*. 1997: The National Academies Press.
384. Kim, S., et al., *Activation of receptor activator of NF-kappaB ligand gene expression by 1,25-dihydroxyvitamin D3 is mediated through multiple long-range enhancers.* Molecular and Cell Biology, 2006. **26**(17): p. 6469-86.
385. Rumpler, M., et al., *Microcracks and osteoclast resorption activity in vitro.* Calcified Tissue International, 2012. **90**(3): p. 230-8.
386. Kato, Y., et al., *Establishment of an osteocyte-like cell line, MLO-Y4.* Journal of Bone and Mineral Research, 1997. **12**(12): p. 2014-23.
387. Prideaux, M., et al., *Extracellular matrix mineralization promotes E11/gp38 glycoprotein expression and drives osteocytic differentiation.* PLoS One, 2012. **7**(5): p. e36786.
388. Turner, A.G., et al., *The local production of 1,25(OH)D promotes osteoblast and osteocyte maturation.* The Journal of Steroid Biochemistry and Molecular Biology, 2013.
389. Folgueira, M.A., et al., *Expression of vitamin D receptor (VDR) in HL-60 cells is differentially regulated during the process of differentiation induced by phorbol ester, retinoic acid or interferon-gamma.* The Journal of Steroid Biochemistry and Molecular Biology, 1998. **66**(4): p. 193-201.
390. Zhao, S., et al., *MLO-Y4 osteocyte-like cells support osteoclast formation and activation.* Journal of Bone and Mineral Research, 2002. **17**(11): p. 2068-79.
391. *Arthritis Research UK : Data and statistic on osteoarthritis* 2013
392. de Verteuil, R., et al., *A systematic review of the clinical effectiveness and cost-effectiveness and economic modelling of minimal incision total hip replacement approaches in the management of arthritic disease of the hip.* Health Technology Assessment, 2008. **12**(26): p. iii-iv, ix-223.

393. Felson, D.T., et al., *Osteoarthritis: new insights. Part 1: the disease and its risk factors.* Annals of Internal Medicine, 2000. **133**(8): p. 635-46.
394. Peach, C.A., A.J. Carr, and J. Loughlin, *Recent advances in the genetic investigation of osteoarthritis.* Trends in Molecular Medicine, 2005. **11**(4): p. 186-91.
395. Loughlin, J., et al., *Association analysis of the vitamin D receptor gene, the type I collagen gene COL1A1, and the estrogen receptor gene in idiopathic osteoarthritis.* Journal of Rheumatology, 2000. **27**(3): p. 779-84.
396. Goldring, M.B. and S.R. Goldring, *Articular cartilage and subchondral bone in the pathogenesis of osteoarthritis.* Annals of the New York Academy of Science, 2010. **1192**: p. 230-7.
397. Burr, D.B. and M.A. Gallant, *Bone remodelling in osteoarthritis.* Nature Reviews Rheumatology, 2012. **8**(11): p. 665-73.
398. Mankin, H.J., et al., *Biochemical and metabolic abnormalities in articular cartilage from osteo-arthritic human hips. II. Correlation of morphology with biochemical and metabolic data.* The Journal of Bone and Joint Surgery American volume, 1971. **53**(3): p. 523-37.
399. Pritzker, K.P., et al., *Osteoarthritis cartilage histopathology: grading and staging.* Osteoarthritis Cartilage, 2006. **14**(1): p. 13-29.
400. Ulrich-Vinther, M., et al., *Articular cartilage biology.* Journal of the American Academy of Orthopaedic Surgeons, 2003. **11**(6): p. 421-30.
401. Staines, K.A., et al., *Cartilage to bone transitions in health and disease.* Journal of Endocrinology, 2013. **219**(1): p. R1-r12.
402. Martin, R.B., *Targeted bone remodeling involves BMU steering as well as activation.* Bone, 2007. **40**(6): p. 1574-80.
403. Resnick, D., G. Niwayama, and R.D. Coutts, *Subchondral cysts (geodes) in arthritic disorders: pathologic and radiographic appearance of the hip joint.* American Journal of Roentgenology, 1977. **128**(5): p. 799-806.
404. Tetlow, L.C., D.J. Adlam, and D.E. Woolley, *Matrix metalloproteinase and proinflammatory cytokine production by chondrocytes of human osteoarthritic cartilage: associations with degenerative changes.* Arthritis and Rheumatology, 2001. **44**(3): p. 585-94.
405. van der Kraan, P.M. and W.B. van den Berg, *Osteophytes: relevance and biology.* Osteoarthritis Cartilage, 2007. **15**(3): p. 237-44.
406. Tetlow, L.C. and D.E. Woolley, *Expression of vitamin D receptors and matrix metalloproteinases in osteoarthritic cartilage and human articular chondrocytes in vitro.* Osteoarthritis Cartilage, 2001. **9**(5): p. 423-31.
407. Wolff, A.E., A.N. Jones, and K.E. Hansen, *Vitamin D and musculoskeletal health.* Nature Clinical Practice Rheumatology, 2008. **4**(11): p. 580-8.
408. Miller, R.R., et al., *Association of serum vitamin D levels with inflammatory response following hip fracture: the Baltimore Hip Studies.* Journal of Gerontology Series A Biological Science & Medical Science, 2007. **62**(12): p. 1402-6.
409. Orfanidou, T., et al., *1,25-Dihydroxyvitamin D(3) and extracellular inorganic phosphate activate mitogen-activated protein kinase pathway through fibroblast growth factor 23 contributing to hypertrophy and mineralization in osteoarthritic chondrocytes.* Experimental Biology and Medicine (Maywood), 2012. **237**(3): p. 241-53.
410. Cao, Y., et al., *Association between serum levels of 25-hydroxyvitamin D and osteoarthritis: a systematic review.* Rheumatology (Oxford), 2013. **52**(7): p. 1323-34.
411. Ding, C., et al., *Serum levels of vitamin D, sunlight exposure, and knee cartilage loss in older adults: the Tasmanian older adult cohort study.* Arthritis and Rheumatology, 2009. **60**(5): p. 1381-9.

412. Felson, D.T., et al., *Low levels of vitamin D and worsening of knee osteoarthritis: results of two longitudinal studies.* Arthritis and Rheumatology, 2007. **56**(1): p. 129-36.
413. Bergink, A.P., et al., *Vitamin D status, bone mineral density, and the development of radiographic osteoarthritis of the knee: The Rotterdam Study.* Journal of Clinical Rheumatology, 2009. **15**(5): p. 230-7.
414. McAlindon, T.E., et al., *Relation of dietary intake and serum levels of vitamin D to progression of osteoarthritis of the knee among participants in the Framingham Study.* Annals of Internal Medicine, 1996. **125**(5): p. 353-9.
415. Muraki, S., et al., *Association of vitamin D status with knee pain and radiographic knee osteoarthritis.* Osteoarthritis Cartilage, 2011. **19**(11): p. 1301-6.
416. Heidari, B., P. Heidari, and K. Hajian-Tilaki, *Association between serum vitamin D deficiency and knee osteoarthritis.* International Orthopaedics, 2011. **35**(11): p. 1627-31.
417. Konstari, S., et al., *Association of 25-hydroxyvitamin D with the incidence of knee and hip osteoarthritis: a 22-year follow-up study.* Scandinavian Journal of Rheumatology, 2012. **41**(2): p. 124-31.
418. Kalichman, L. and E. Kobyliansky, *Association between circulatory levels of vitamin D and radiographic hand osteoarthritis.* Rheumatology International, 2012. **32**(1): p. 253-7.
419. Chaganti, R.K., et al., *Association of 25-hydroxyvitamin D with prevalent osteoarthritis of the hip in elderly men: the osteoporotic fractures in men study.* Arthritis and Rheumatology, 2010. **62**(2): p. 511-4.
420. Lane, N.E., et al., *Serum vitamin D levels and incident changes of radiographic hip osteoarthritis: a longitudinal study. Study of Osteoporotic Fractures Research Group.* Arthritis and Rheumatology, 1999. **42**(5): p. 854-60.
421. Bischoff-Ferrari, H.A., et al., *Positive association between serum 25-hydroxyvitamin D level and bone density in osteoarthritis.* Arthritis and Rheumatology, 2005. **53**(6): p. 821-6.
422. Hilal, G., et al., *Osteoblast-like cells from human subchondral osteoarthritic bone demonstrate an altered phenotype in vitro: possible role in subchondral bone sclerosis.* Arthritis and Rheumatology, 1998. **41**(5): p. 891-9.
423. Dieppe, P., et al., *Prediction of the progression of joint space narrowing in osteoarthritis of the knee by bone scintigraphy.* Annals of the Rheumatic Diseases, 1993. **52**(8): p. 557-63.
424. Cantatore, F.P., et al., *Osteocalcin synthesis by human osteoblasts from normal and osteoarthritic bone after vitamin D3 stimulation.* Clinical Rheumatology, 2004. **23**(6): p. 490-5.
425. Gallagher, J.A., *Human osteoblast culture*, in *Bone Research Protocols*. 2003, Springer. p. 3-18.
426. Dean, D.D., et al., *Vitamin D metabolites regulate matrix vesicle metalloproteinase content in a cell maturation-dependent manner.* Calcified Tissue International, 1996. **59**(2): p. 109-16.
427. Power, J., et al., *Sclerostin and the regulation of bone formation: Effects in hip osteoarthritis and femoral neck fracture.* Journal of Bone and Mineral Research, 2010. **25**(8): p. 1867-76.
428. van Hove, R.P., et al., *Osteocyte morphology in human tibiae of different bone pathologies with different bone mineral density--is there a role for mechanosensing?* Bone, 2009. **45**(2): p. 321-9.
429. Lajeunesse, D., et al., *Subchondral bone morphological and biochemical alterations in osteoarthritis.* Osteoarthritis Cartilage, 1999. **7**(3): p. 321-2.
430. Westacott, C.I., et al., *Alteration of cartilage metabolism by cells from osteoarthritic bone.* Arthritis and Rheumatology, 1997. **40**(7): p. 1282-91.

431. Radin, E.L., I.L. Paul, and M.J. Tolkoff, *Subchondral bone changes in patients with early degenerative joint disease.* Arthritis and Rheumatology, 1970. **13**(4): p. 400-5.
432. Dedrick, D.K., et al., *A longitudinal study of subchondral plate and trabecular bone in cruciate-deficient dogs with osteoarthritis followed up for 54 months.* Arthritis and Rheumatology, 1993. **36**(10): p. 1460-7.
433. Alshahrani, F. and N. Aljohani, *Vitamin D: deficiency, sufficiency and toxicity.* Nutrients, 2013. **5**(9): p. 3605-16.
434. Biancuzzo, R.M., et al., *Fortification of orange juice with vitamin D(2) or vitamin D(3) is as effective as an oral supplement in maintaining vitamin D status in adults.* The American Journal of Clinical Nutrition 2010. **91**(6): p. 1621-6.
435. Sommerfeldt, J.L., et al., *Metabolism of orally administered [3H]ergocalciferol and [3H]cholecalciferol by dairy calves.* Journal of Nutrition, 1983. **113**(12): p. 2595-600.