Differentiation of osteoblasts to osteocytes in 3D type I collagen gels - a novel tool to study osteocyte responses to mechanical loading

A thesis submitted for the Postgraduate Degree of
Doctor of Philosophy

Nicole Scully
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Institute of Molecular and Experimental Medicine,
Cardiff University School of Medicine,
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This work has not been submitted in substance for any other degree or award at this or any other university or place of learning, nor is being submitted concurrently in candidature for any degree or other award.

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# Table of contents

Declaration

Table of contents ................................................................. 1
List of figure .............................................................................. VIII

Declaration ................................................................................. XI

Acknowledgements ...................................................................... XII

Summary ..................................................................................... XIII

Abbreviations ............................................................................ XIV

Chapter 1: Introduction................................................................ 1

1.1 Bone structure ....................................................................... 2
  1.1.1 Macroscopic structure ......................................................... 2
  1.1.1.1 Cortical and cancellous bone ........................................ 2
  1.1.2 Microscopic structure ........................................................ 2

1.2 Bone modelling and remodelling ........................................... 4
  1.2.1 Bone modelling ................................................................. 4
  1.2.2 Bone remodelling ............................................................. 4

1.3 Bone constituents .................................................................. 6
  1.3.1 The composite ................................................................. 6
  1.3.2 Non-collagenous proteins .................................................. 7
  1.3.3 Other components ........................................................... 7

1.4 Bone cells ............................................................................. 8
  1.4.1 Osteoclasts ...................................................................... 8
  1.4.1.1 Morphology ............................................................... 8
  1.4.1.2 Differentiation ............................................................ 9
  1.4.2 Osteoblasts ..................................................................... 9
  1.4.2.1 Morphology ............................................................... 9
  1.4.2.2 Gene expression ........................................................ 10
  1.4.3 Osteocytes ...................................................................... 11
  1.4.3.1 Morphology ............................................................... 11
  1.4.3.2 Gene expression ........................................................ 12
  1.4.3.3 Osteoblast to osteocyte differentiation ......................... 13

1.5 In vivo and in vitro models for the study of osteocytes .............. 15
  1.5.1 In vivo ........................................................................... 15
  1.5.2 In vitro ........................................................................... 16
  1.5.2.1 Primary osteocytes ...................................................... 16
  1.5.2.2 Osteocyte cell lines .................................................... 19
1.5.2.3 MLO-A5 cell line .................................................................................... 19
1.5.2.4 MLO-Y4 cell line .................................................................................... 19
1.5.2.5 HOB-O1-C1 cell line .............................................................................. 20
1.5.3 Differentiation of osteoblasts to osteocytes in vitro .................................... 21
1.5.3.1 IDG-SW3 cell line ................................................................................. 21
1.6 Models of osteoblast to osteocyte differentiation ........................................... 25
1.6.1 Strontium Ranelate ................................................................................... 25
1.6.1.1 Background ............................................................................................ 25
1.6.1.2 Evidence of use of strontium ranelate in cell differentiation .................. 26
1.6.2 IGF-1 ........................................................................................................ 27
1.6.2.1 Background ............................................................................................ 27
1.6.2.2 Evidence of use of IGF-1 in cell differentiation ....................................... 28
1.6.3 FGF-2 ....................................................................................................... 28
1.6.3.1 Background ............................................................................................ 28
1.6.3.2 Evidence of use of FGF-2 in bone cell differentiation ......................... 29
1.6.4 RA ............................................................................................................. 30
1.6.4.1 Background ............................................................................................ 30
1.6.4.2 Evidence of use of RA in cell differentiation .......................................... 31
1.6.5 Vit K .......................................................................................................... 32
1.6.5.1 Background ............................................................................................ 32
1.6.5.2 Evidence of use of Vit K in bone cell differentiation ......................... 33
1.7 Studies of osteoblasts and osteocytes in 3D in vitro cultures ......................... 33
1.8 Mechanical loading of osteocytes ................................................................... 36
1.8.1 Mechanical loading of osteocytes in vivo ..................................................... 36
1.8.1.1 Vertebra loading ....................................................................................... 36
1.8.1.2 Ulna loading ............................................................................................. 37
1.8.1.3 Four point bending .................................................................................. 38
1.8.1.4 Tooth movement models ......................................................................... 39
1.8.1.5 Tail suspension ........................................................................................ 39
1.8.2 Mechanical loading of osteocytes in vitro ................................................... 40
1.8.2.1 Cyclic loading .......................................................................................... 41
1.8.2.2 Fluid flow in vitro .................................................................................... 41
1.9 Bone disease ................................................................................................ 43
1.9.1 Common diseases affecting bone ................................................................. 43
1.9.2 OP .............................................................................................................. 43
1.9.2.1 Background .............................................................................................. 43
1.9.2.2 Incidence and prevalence ....................................................................... 44
1.9.2.3 Diagnosis ................................................................................................ 44

IV
1.9.3 Treatment ........................................................................................................... 45
1.9.4 OA ....................................................................................................................... 45
1.9.4.1 Background ...................................................................................................... 45
1.9.4.2 Incidence and prevalence ............................................................................... 46
1.9.4.3 Diagnosis ......................................................................................................... 46
1.9.4.4 Treatment ......................................................................................................... 46
1.9.5 Less common diseases affecting bone ............................................................... 47
1.9.6 Rheumatoid Arthritis ....................................................................................... 47
1.9.7 PDB ................................................................................................................... 48
1.9.8 OI ........................................................................................................................ 48
1.10 Aims of this study ............................................................................................. 50

2 Materials and Methods .......................................................................................... 51

2.1 Materials .............................................................................................................. 52
2.1.1 General reagents and consumables ................................................................. 52

2.2 Methods ................................................................................................................ 52
2.2.1 Monolayer cell culture .................................................................................... 52
2.2.1.1 MC-3T3 cell line ........................................................................................... 52
2.2.1.2 IDG-SW3 cell line ......................................................................................... 53
2.2.1.3 hOBs cells ..................................................................................................... 54
2.2.1.4 Cell culture maintenance ............................................................................. 56
2.2.1.5 Cell counting ................................................................................................ 57
2.2.1.6 Cryopreservation of cells ............................................................................ 57
2.2.2 Cell culture in 3D ............................................................................................ 58
2.2.2.1 MC-3T3s in 3D collagen type I gels ............................................................... 58
2.2.2.2 IDG-SW3s in 3D collagen type I gels ............................................................. 58
2.2.2.3 hOBs in 3D collagen type I gels ................................................................... 59
2.2.2.4 Mineralising medium for MC-3T3s and hOBs in 3D collagen type I gels ...... 60
2.2.2.5 Cell number and viability in 3D collagen gels ............................................ 60
2.2.3 Analysis of mRNA expression ......................................................................... 61
2.2.3.1 RNA extraction ............................................................................................ 61
2.2.3.2 Sodium acetate precipitation of RNA ......................................................... 61
2.2.3.3 DNase I digest of RNA .................................................................................. 62
2.2.3.4 Determining quantity, purity and quality of RNA in samples ..................... 63
2.2.3.5 Reverse transcription (RT) .......................................................................... 65
2.2.3.6 Isolation of bone cells from mouse femur ................................................. 66
2.2.3.7 Primer design ............................................................................................... 66
2.2.3.8 RT Quantitative polymerase chain reaction (RT-qPCR) ............................. 68
2.2.3.9 Agarose gel electrophoresis ....................................................................... 71
3 Differentiation of osteoblasts (MC-3T3 cells or hOBs) to osteocytes in 3D collagen type I gels

3.1 Introduction ................................................................................................................. 93
3.1.1 3D culture methods for bone cells ........................................................................... 93
3.2 Hypothesis ..................................................................................................................... 95
3.3 Aims: .............................................................................................................................. 95
3.4 Methods ......................................................................................................................... 96
3.4.1 Experimental design ........................................................................................................... 96
3.4.2 Protein secretion, cell number and viability and gene expression ................................. 96
3.4.3 Morphology and IHC analysis ............................................................................................ 97
3.4.4 Statistical analysis of data .................................................................................................. 98
3.5 Results ...................................................................................................................................... 99
3.5.1 Cell number and viability ................................................................................................. 99
3.5.2 Morphology .......................................................................................................................... 103
  3.5.2.1 H&E staining ................................................................................................................... 103
  3.5.2.2 Calcein, phalloidin and DAPI labelling ........................................................................ 104
3.5.3 Protein expression ............................................................................................................... 105
  3.5.3.1 ALP expression .............................................................................................................. 105
  3.5.3.2 E11 expression ............................................................................................................... 105
  3.5.3.3 DMP-1 expression ........................................................................................................ 106
  3.5.3.4 Cx43 expression ........................................................................................................... 106
3.5.4 E11, DMP-1, CX43, sclerostin, MEPE, PHEX and FGF-23 gene expression .............. 114
  3.5.4.1 MC-3T3 cells ................................................................................................................ 114
  3.5.4.2 hOBs cells .................................................................................................................... 115
3.5.5 IL-6, VEGF and RANKL protein secretion ..................................................................... 119
  3.5.5.1 MC-3T3 cells ................................................................................................................ 119
  3.5.5.2 hOBs ............................................................................................................................ 119
3.5.6 Gel stiffness ....................................................................................................................... 122
  3.5.6.1 MC-3T3 cells ................................................................................................................ 122
  3.5.6.2 hOBs ............................................................................................................................ 122
3.6 Discussion ................................................................................................................................. 124

4 The effect of Insulin like Growth Factor-1, Fibroblast Growth Factor-2, Retinoic Acid and
Vitamin K on the differentiation of osteoblast to osteocytes in 3D collagen type I gels ........... 133
4.1 Introduction .............................................................................................................................. 134
  4.1.1 IGF-1 and osteocytes ....................................................................................................... 134
  4.1.2 FGF-2 and osteocytes ..................................................................................................... 135
  4.1.3 RA and osteocytes ........................................................................................................... 137
  4.1.4 Vit K and osteocytes ....................................................................................................... 138
4.2 Hypothesis .............................................................................................................................. 141
4.3 Aims ....................................................................................................................................... 141
4.4 Methods .................................................................................................................................. 142
  4.4.1 Experimental design ....................................................................................................... 142
  4.4.2 Protein secretion, cell number and viability and gene expression ............................. 143
  4.4.3 Morphological and immunohistochemical analysis ..................................................... 145
  4.4.4 Statistical analysis of data ............................................................................................. 145

VII
4.5 Results ................................................................................................................................. 146
Effect of IGF-1, FGF-2, RA and Vit K treatment on MC-3T3 cells................................. 146
4.5.1 Cell number and viability ............................................................................................. 146
  4.5.1.1 IGF-1 or FGF-2 treatment ..................................................................................... 146
  4.5.1.2 RA or Vit K treatment .......................................................................................... 147
4.5.2 Morphology .................................................................................................................. 148
  4.5.2.1 IGF-1 or FGF-2 ................................................................................................... 148
  4.5.2.2 RA or Vit K .......................................................................................................... 149
4.5.3 Protein expression following IGF-1 treatment ............................................................ 150
  4.5.3.1 E11 expression ...................................................................................................... 150
  4.5.3.2 DMP-1 expression ............................................................................................... 151
4.5.4 E11, DMP-1, Cx43, LRP-5, RANKL and sclerostin mRNA expression ............... 158
  4.5.4.1 IGF-1 or FGF-2 treatment .................................................................................. 158
  4.5.4.2 RA or Vit K treatment ......................................................................................... 161
4.5.5 VEGF, IL-6 and FGF-23 protein secretion ................................................................. 168
  4.5.5.1 IGF-1 or FGF-2 treatment .................................................................................. 169
  4.5.5.2 RA or Vit K treatment ........................................................................................ 172
4.5.6 Effect of IGF-1 treatment on MC-3T3 cells in mineralisation medium .............. 174
  4.5.6.1 E11, DMP-1, RANKL, PHEX, MEPE and FGF-23 mRNA expression .......... 174
  4.5.6.2 VEGF and IL-6 protein secretion ...................................................................... 176
4.5.7 Effect of IGF-1 treatment on hOBs ............................................................................... 178
  4.5.7.1 Cell number and viability ..................................................................................... 178
  4.5.7.2 Morphology ......................................................................................................... 179
  4.5.7.3 E11, Cx43, DMP-1 and sclerostin mRNA gene expression ......................... 179
  4.5.7.4 VEGF, IL-6 and RANKL protein secretion ....................................................... 182
4.6 Discussion ......................................................................................................................... 185
  4.6.1 IGF-1 treatment ....................................................................................................... 185
  4.6.2 FGF-2 treatment ..................................................................................................... 190
  4.6.3 RA treatment .......................................................................................................... 194
  4.6.4 Vit K treatment ....................................................................................................... 197
5 Differentiation of IDG-SW3 osteoblasts to osteocytes in 3D collagen type I gels .... 200
  5.1 Introduction .................................................................................................................... 201
  5.1.1 Osteocyte cell line .................................................................................................... 201
  5.1.2 IDG-SW3 cell line ................................................................................................... 203
  5.2 Hypothesis .................................................................................................................... 205
  5.3 Aims ............................................................................................................................... 205
  5.4 Methods ....................................................................................................................... 205
  5.4.1 Experimental design ............................................................................................... 205

VIII
6 The effects of mechanical loading on the IDG-SW3 osteocyte-like cells

6.1 Introduction .............................................................................................................. 237

6.1.1 Osteocytes: the mechanosensory cells .............................................................. 237

6.1.2 Osteocyte responses to mechanical loading ...................................................... 238

6.2 Hypothesis ............................................................................................................... 241

6.3 Aims ....................................................................................................................... 241

6.4 Methods .................................................................................................................. 242

6.4.1 Experimental design .......................................................................................... 242

6.4.2 Assessment of cell number and viability, gene expression, protein secretion and PGE₂ release following mechanical loading ................................................................................. 242

6.4.3 Assessment of morphological and immunohistochemical analysis following mechanical loading .............................................................................................................................. 244

6.4.4 Statistical analysis of data ................................................................................... 244

6.5 Results ..................................................................................................................... 246

6.5.1 Cell number and viability .................................................................................... 246

6.5.2 Morphology ......................................................................................................... 247

6.5.3 RANKL expression ............................................................................................ 247

6.5.4 DMP-1, LRP-5, IL-6, VEGF, sclerostin, RANKL and OPG gene expression ...... 252

6.5.5 IL-6, VEGF and PGE₂ secretion ......................................................................... 256

6.5.5.1 IL-6 protein secretion .................................................................................... 256

6.5.5.2 VEGF protein secretion ................................................................................ 256

6.5.5.3 Re-analysis of IL-6 and VEGF protein secretion data .................................... 257

6.5.5.4 PGE₂ secretion ............................................................................................ 258

6.6 Discussion .............................................................................................................. 262

7 General Discussion .................................................................................................. 271
List of figures

Figure 1.1 The structural organisation of bone ................................................................. 3
Figure 1.2 The bone remodelling process ........................................................................ 6
Figure 1.3 The three different types of bone cells. ......................................................... 8
Figure 1.4 The expression of markers during the transition from a pre-osteoblast to an osteocyte .... 15
Figure 1.5 The regulation of osteoblastic gene expression by FGF signally during osteogenesis. ..... 30
Figure 2.1 An example of the typical spectral pattern for nucleic acid from the Nanodrop .......... 64
Figure 2.2 Results generated from the Aligent 2100 Bioanalyzer ....................................... 64
Figure 2.3 PCR reaction ..................................................................................................... 70
Figure 2.4 The BOSE Electroforce 3200 machine ......................................................... 88
Figure 2.5 The mechanical loading rig .............................................................................. 89
Figure 2.6 The silicone plate attached to the mechanical loading rig .................................. 90
Figure 3.1 Assessment of MC-3T3 and hOB cell number in basal medium in 3D collagen type I gels in a range of seeding densities ................................................................. 101
Figure 3.2 MC-3T3 cells and hOBs cell number and viability in basal and mineralising medium in collagen type I gels ........................................................................................................ 102
Figure 3.3 H&E staining of MC-3T3 cells in 3D collagen type I gels in basal and mineralising medium ............................................................................................................................ 107
Figure 3.4 H&E staining of hOBs cells in 3D collagen type I gels in basal and mineralising medium ................................................................. 108
Figure 3.5 Actin and calcein labelling of MC-3T3 cells and hOBs in basal and mineralising medium in 3D collagen type I gels. ................................................................. 109

Figure 3.6 ALP protein expression in MC-3T3 cells in 3D collagen type I gels in basal and mineralising medium ................................................................. 110

Figure 3.7 E11 protein expression in MC-3T3 cells in 3D collagen type I gels in basal and mineralising medium ................................................................. 111

Figure 3.8 DMP-1 protein expression in MC-3T3 cells in 3D collagen type I gels in basal and mineralising medium ................................................................. 112

Figure 3.9 CX43, Actin and DAPI labelling of MC-3T3s in basal medium in 3D collagen type I gels. .................................................................................................. 113

Figure 3.10 E11, DMP-1, Cx43, sclerostin, MEPE, PHEX and FGF-23 gene expression in MC-3T3 cells in basal and mineralising medium in 3D collagen type I gels. .................................................. 117

Figure 3.11 E11, DMP-1 and sclerostin gene expression in hOBs in basal and mineralising medium in 3D collagen type I gels. .................................................. 118

Figure 3.12 IL-6 secretion in MC-3T3 cells in basal and mineralising medium in 3D collagen type I gels. .................................................................................... 120

Figure 3.13 IL-6, VEGF and RANKL secretion in hOBs in basal and mineralising medium in 3D collagen type I gels ................................................................. 121

Figure 3.14 Gel stiffness in MC-3T3 cells and hOBs in basal and mineralising medium in 3D collagen type I gels ................................................................. 123

Figure 4.1 MC-3T3 cell number and viability in 3D collagen type I gels following treatment with (1) IGF-1 or (2) FGF-2 ......................................................... 152

Figure 4.2 MC-3T3 cell number and viability in 3D collagen type I gels following treatment with (1) RA or (2) Vit K ......................................................... 153

Figure 4.3 H&E staining of MC-3T3 cells in 3D collagen type I gels following treatment with IGF-1 or FGF-2 ................................................................. 154

Figure 4.4 H&E staining of MC-3T3 cells in 3D collagen type I gels following treatment with RA or Vit K ................................................................. 155

Figure 4.5 E11 Protein expression in MC-3T3s in 3D collagen type I gels following treatment with IGF-1 ................................................................. 156

Figure 4.6 DMP-1 protein expression in MC-3T3s in 3D collagen type I gels following treatment with IGF-1 ................................................................. 157

Figure 4.7 E11, DMP-1, Cx43, LRP-5, RANKL and FGF-23 mRNA expression in MC-3T3 cells in 3D collagen type I gels following treatment with IGF-1 ................................................................. 164

Figure 4.8 E11, DMP-1, Cx43, LRP-5, RANKL and FGF-23 mRNA expression in MC-3T3 cells in 3D collagen type I gels following treatment with FGF-2 ................................................................. 165

Figure 4.9 E11, DMP-1, Cx43, LRP-5, RANKL and FGF-23 mRNA expression in MC-3T3 cells in 3D collagen type I gels following treatment with RA ................................................................. 166

Figure 4.10 E11, DMP-1, Cx43, LRP-5, RANKL and FGF-23 mRNA expression in MC-3T3 cells in 3D collagen type I gels following treatment with Vit K ................................................................. 167

Figure 4.11 Sclerostin mRNA expression in MC-3T3 cells in 3D collagen type I gels following treatment with IGF-1 ................................................................. 168

Figure 4.12 VEGF, IL-6 and FGF-23 protein secretion in MC-3T3 cells in 3D collagen type I gels following treatment with IGF-1 ................................................................. 171
Figure 6.3 H&E staining of IDG-SW3 cells in 3D collagen type I gels following mechanical loading. ........................................................................... 250

Figure 6.4 IHC of RANKL in IDG-SW3 cells in 3D collagen type I gels following mechanical loading. ........................................................................... 251

Figure 6.5 Gene expression of known mechano-regulated factors in IDG-SW3 cells in 3D collagen type I gels following mechanical loading ........................................................................... 255

Figure 6.6 Gene expression of RANKL and OPG in IDG-SW3 cells in 3D collagen type I gels following mechanical loading ........................................................................... 256

Figure 6.7 IL-6 and VEGF secretion in IDG-SW3 cells maintained in 3D collagen type I gels following mechanical loading. ........................................................................... 258

Figure 6.8 Measurement of PGE2 secretion by IDG-SW3 cells maintained in 3D collagen type I gels and following mechanical loading. ........................................................................... 261

Figure 6.9 Factors that are up-regulated and down-regulated post mechanical loading in IDG-SW3 osteocyte cells in 3D collagen type I gels. .......................................................... 269

List of tables

Table 1.1: Characteristics of different primary osteoblasts options available ........................................... 23
Table 1.2: Characteristics of most commonly used osteoblast cell lines available ..................................... 24
Table 2.1: Human patient samples used throughout my thesis ..................................................................... 56
Table 2.2: Concentration and volume of reagents used for a RT reactions ...................................................... 65
Table 2.3: Mouse (Mus musculus) primer sequences used for RT-qPCR reactions ........................................ 67
Table 2.4: Human (Homo sapiens) primer sequences used for RT-qPCR reactions ........................................ 68
Table 2.5: Concentrations and volumes of reagents used for RT-qPCR reactions ........................................ 69
Table 2.6: Reagents required for PCR reactions .......................................................................................... 72
Table 2.7: Reagents required for ligation reactions ....................................................................................... 74
Table 2.8: Antibodies used for IHC staining ............................................................................................... 79
Table 2.9: Specifications for individual DuoSet ELISAs ............................................................................. 83
Table 4.1: Concentrations and source of factors used for treating MC-3T3 cells or hOBS .............................. 143
Table 4.2: Results following a two-way ANOVA investigating the effect of time, treatment and the interaction between time and treatment on cell number ........................................... 146
Table 4.3: Results following a two-way ANOVA investigating the effect of time, treatment and the interaction between time and treatment on gene expression ........................................... 158
Table 4.4: Results following a two-way ANOVA investigating the effect of time, treatment and the interaction between time and treatment on VEGF and IL-6 ................................................................ 168
Table 4.5: Results following a two-way ANOVA investigating the effect of time, treatment and the interaction between time and treatment on E11, DMP-1, RANKL, PHEX, MEPE and FGF-23 .... 174
Table 5.1: Characteristics of some of the currently available osteocyte-like cell lines .............................. 202
Table 6.1: Summary of published osteocytic cell responses following mechanical loading in vivo or in vitro ..................................................................................................................... 240
Table 6.2: PGE2 secretion in experiments 1 and 3 ......................................................................................... 260
Table 7.1: Summary of cell number and viability, gene expression and protein secretion results ................... 274
Table 7.2: Summary of cell number and viability, gene expression and protein secretion results for MC-3T3 cells following treatment with IGF-1, FGF-2, RA or Vit K .................................................................... 276
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XIV
Summary

Osteocytes are currently regarded as being pivotal in maintaining bone homeostasis. They differentiate from osteoblasts, are embedded in mineralised matrix and difficult to isolate. Current models for *in vitro* osteocyte studies are limited. Others have suggested that osteoblasts in 3-dimensional (3D) cultures differentiate to osteocytes. This study aimed to develop 3D cultures enabling differentiation of osteoblasts to osteocytes, which could be used for studies of osteocyte differentiation and responses to mechanical loading. Furthermore, the effects of external compounds on osteoblast differentiation in 3D were assessed.

Mouse (MC-3T3, IDG-SW3) and human primary osteoblasts (hOBs) were maintained in type I collagen gels in either non-osteogenic or osteogenic media, and +/- compounds such as insulin-like growth factor-1 (IGF-1). Furthermore, mechanical loading (5 mins, 10 Hz, 2.5 N) was applied to 3D cultures and responses characterised.

Cells were viable in collagen gels for 25 days, and expressed mRNA for mature osteocyte markers *e.g.* sclerostin in osteogenic medium. Furthermore, IGF-1 up-regulated mRNA expression of osteocyte markers and other molecules (*e.g.* receptor activator of the nuclear factor kappa-β ligand - RANKL - 43-fold) in MC-3T3 cells, indicating modulation of cell differentiation and function. Osteocyte markers were expressed earlier in IDG-SW3 cells in 3D compared to published marker expression profiles in 2D monolayer cultures. Following mechanical loading, known mechanosensitive markers were modulated in IDG-SW3 cells in 3D, for example, RANKL and vascular endothelial growth factor (VEGF) up-regulated and sclerostin down-regulated post-loading.

This 3D model enables differentiation of osteoblasts to osteocytes in an environment akin to osteocytes *in vivo*. External compounds accelerated cell differentiation, and this was also accelerated in 3D compared to monolayer. Furthermore, the 3D model enabled osteocyte mechanical loading. This model can be used with human cells, will further our understanding of osteocyte differentiation, and inform on osteocyte function including their responses to mechanical loading.
## Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACPAs</td>
<td>anti-citrullinated protein antibodies</td>
</tr>
<tr>
<td>ALP</td>
<td>alkaline phosphate</td>
</tr>
<tr>
<td>Ank</td>
<td>progressive ankylosis</td>
</tr>
<tr>
<td>ANOVA</td>
<td>analysis of variance</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>ATRA</td>
<td>all trans retinoic acid</td>
</tr>
<tr>
<td>BCP</td>
<td>biphasic calcium phosphate</td>
</tr>
<tr>
<td>bFGF</td>
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<td>RNasin RI</td>
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</tr>
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<td>total activity</td>
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<td>transforming growth factors</td>
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<td>total knee replacement</td>
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<td>weight/weight</td>
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<td>two dimensional</td>
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<td>three dimensional</td>
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<td>$C_t$ gene of interest - $C_t$ of calibrator (housekeeping gene)</td>
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<td>$2^{\Delta(\Delta C_t \text{ gene of interest} - \Delta C_t \text{ normaliser of assay})}$</td>
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Chapter 1: Introduction
1.1 Bone structure

1.1.1 Macroscopic structure

1.1.1.1 Cortical and cancellous bone

Bone can be sub-divided into cortical (compact) and cancellous (trabecular) bone, which differ due to the porosity or density [1, 2]. In a cross-section, long bones (e.g. femur) have a tightly packed cortical shell and a porous cancellous interior, whereas flat bones (e.g. calvaria) have dense cortical layers on the outer surfaces and a thin cancellous structure within [3]. Cancellous bone is made up of “irregular, sinuous convolutions of lamellae” which are thin plate of bone matrix which is the basic structural unit of mature bone whereas cortical bone is made up of “regular, cylindrically shaped lamellae” [3].

1.1.2 Microscopic structure

Cortical bone is composed of osteons (200-250 µm in diameter), which have numerous concentric layers or lamellae in the bone matrix [4]. Osteons run parallel to the long axis on the bone [5], and reside inside a central canal called the Haversian canal and are composed of collagen fibres which contain assemblies of collagen fibrils, bone mineral crystals, collagen molecules and non-collagenous proteins (Figure 1.1). In between the lamellae, small cavities called lacunae are located, where osteocytes and canaliculi reside. Although the arrangement and the orientation of the substance of the lamellae is still not understood, it is known that they are 3-7 µm thick [6]. Canaliculi are tiny fluid-filled passageways that extend from one lacunae to the other and from the Haversian canal to other lacunae [7]. The canaliculi enable osteocyte cells within the mineralised matrix to communicate with other cells [4]. Haversian canals, located within osteons are hollow channels which enable
blood, lymph vessels and nerve fibres to pass through [7]. Smaller Volkmann canals connect osteons and supply nutrients to all cells [7].

Other types of bone include:

1. **Woven bone**: which is another form of cortical bone where mineralised collagen fibres display no distinct organisational patterns [3]

2. **Lamellar bone**: where the lamellae can be located lateral to the outer surface of the bone (do not form osteons) and also contains woven bone tissue around the complete circumference of the bone [3]. and

3. **Cancellous bone**: which is composed of an interconnecting framework of trabeculae [3].

![Figure 1.1:](image) The structural organisation of bone highlighting the macrostructure (cortical and cancellous bone), microstructure (osteons, Haversian systems and lamellae) and the nanostructure (collagen fibre assemblies of collagen fibrils, bone mineral crystals, collagen molecules and non-collagenous proteins). Adapted from Rho et al. 1998 [3].
1.2 Bone modelling and remodelling

1.2.1 Bone modelling

Bone modelling is the process where bones change their overall shape in response to physiological influences or mechanical forces which results in a steady adjustment of the skeleton. Furthermore, bone modelling also takes into account bone size, shape and spatial distribution of its mineralised mass to the prevailing loads [8, 9].

1.2.2 Bone remodelling

Bone remodelling is a continuous, co-ordinated process that results in the formation and the degradation of bone, to maintain bone homeostasis [10]. Remodelling can be described as a balance between bone formation which is co-ordinated by the osteoblasts and bone resorption which is controlled by the osteoclasts (Figure 1.2) [11]. Bone remodelling is a highly-organised process where osteoclasts, osteoblasts and osteocytes activities are co-ordinated in basic multi-cellular units (BMUs). It is believed that the whole adult skeleton undergoes remodelling every decade [10]. In healthy bone, the number of BMUs, the resorption rate and the bone formation rate remain constant [12].

There are 4 distinct phases in the bone remodelling process - (1) activation phase, (2) resorption phase, (3) reverse phase and (4) formation phases:

**Activation phase:** This phase involves the detection of signal that initiates remodelling [12]. The signal can be a micro-fracture, a change in mechanical loading (sensed by the osteocyte) or factors released into the bone micro-environment (e.g. parathyroid hormone - PTH, oestrogen, interleukin-6 - IL-6) that activate the bone
lining cells [12, 13]. This results in the increased surface expression of RANKL by the cells, and this interacts with receptor activator of the nuclear factor kappa (RANK) expressed by the pre-osteoclasts, resulting in the differentiation of pre-osteoclasts to mature, multinucleated osteoclasts [13].

**Resorption phase:** This is when the differentiated osteoclasts polarize and stick to the bone surface and dissolve the bone surface. This process requires the (i) acidification of the bone matrix to destroy the inorganic component and (ii) release of lysosomal enzymes (cathepsin K, matrix metalloproteinase-9 - MMP-9). When the osteoclasts have finished dissolving the bone they undergo apoptosis [13]. In healthy humans the resorption phase takes 20 days.

**Reverse phase:** After osteoclast-mediated resorption, the lacunae are covered with undigested demineralised collagen matrix [14]. The reverse cells which are macrophage-like cells remove the debris produced during matrix degradation [13], and prepares the bone surface for osteoblast mediated bone formation [12]. Although the role of the reverse cells has not yet being fully determined, it is believed that their role may be to receive or produce coupling signals that enable the transition from bone resorption to bone formation within the BMUs [14].

**Formation phase:** The formation phase is the final step in this process and involves osteoblasts being recruited into the resorbed areas of the bone. These osteoblasts are recruited by several growth factors (e.g. bone morphogenic proteins - BMPs, fibroblast growth factors - FGFs), as they are released following the resorption of the bone matrix [13]. The osteoblasts promote the mineralisation of this matrix.
completing the bone remodelling process [13]. The formation phase takes 100 days in healthy humans.

**Figure 1.2: The bone remodelling process.** Bone remodelling is initiated when different inputs lead to the activation of the lining cells which increased surface expression of RANKL. RANKL then interacts with RANK and this triggers osteoclast differentiation (Activation phase). Osteoclasts then resorb the bone using the resorption markers TRACP-5b, calcium, D-Pyr, Ntx, and ICTP (Resorption phase) which enables the release of factors usually stored in the bone matrix and recruit the osteoblasts to the resorbed bone. When recruited these osteoblasts produce the new bone matrix, and promote its mineralisation using the formation markers (osteocalcin, PINP/PICP and BSAP-Formation phase) which completes the bone remodelling process. Abbreviations: TRACP-5b-tartrate-resistant acid phosphatase 5b, D-pyr-Deoxypyridinoline, Ntx-N-terminal telopeptide, ICTP- Carboxy-terminal crosslinked telopeptide of type I collagen, PINP-procollagen type I N-terminal, BSP-bone sialoprotein.

### 1.3 Bone constituents

#### 1.3.1 The composite

Bone is a composite material whose extracellular matrix (ECM) is composed of mineral, collagen, water, non-collagenous proteins and lipids in decreasing proportions which is dependent on age, species and site. The mineral phase of bone contains a highly substituted analogue of the naturally occurring mineral
hydroxyapatite, where the substitutions can be carbonate, magnesium, and acid phosphate along with other trace elements. The mineral content varies with diet and environment. The minerals strengthen the collagen composite, increasing the mechanical resistance of the tissue, and provide a source of calcium, phosphate and magnesium for systemic mineral homeostasis. Collagen type I is the main component of the bone matrix fibre network. Collagen type I is a triple helical molecule that has two identical α1(I) and a genetically different α2(I) chain [15]. Although the bone matrix is predominately composed of collagen type I it also contains trace amounts of Collagen type III, V and fibril-associated collagens.

1.3.2 Non-collagenous proteins
The non-collagenous proteins in bone main functions are to organise the ECM, regulate cell-matrix and mineral matrix interactions, and co-ordinate the mineralisation process. There are many different types of non-collagenous proteins in bone including serum-derived proteins (e.g. albumin, α2HS glycoprotein), proteoglycans (e.g. chondroitin sulphate, hyaluronan, decorin, fibromodulin), glycosylated proteins (e.g. ALP, osteonectin, tetraneectin) and gla containing proteins (e.g. matrix gla protein, osteocalcin, periostin, protein S). However, it should be noted that some are far more abundant than others (e.g. osteocalcin).

1.3.3 Other components
These account for minor components of the bone ECM and include enzymes, growth factors, water and lipids. Growth factors found in the bone matrix include IGF-1 and IGF-2, transforming growth factor-beta (TGF-β), acidic FGF and basic FGF (bFGF), platelet-derived growth factor (PDGF) and BMPs [16].
1.4 Bone cells

There are three different types of bone cells - the osteoclasts, osteoblasts and osteocytes (Figure 1.3). The osteocyte is the most abundant cell in bone comprising > 90-95 % of total bone cells, whereas 4–6 % of the bone cell population are osteoblasts and and ~1-2 % osteoclasts.

![Figure 1.3: The three different types of bone cells.](image)

The osteoclasts and osteoblasts are located on the surface of the bone, and the osteocytes are embedded in the mineralised matrix on bone.

1.4.1 Osteoclasts

1.4.1.1 Morphology

Osteoclasts are multinucleated giant cells whose function is to resorb bone. They are derived by the fusion of hematopoietic cells of the monocyte and macrophage lineage during the initial stage of the differentiation process. The formation of osteoclasts requires a number of steps, which include the commitment of the hematopoietic precursors to the osteoclastic lineage, proliferation of these committed cells, as well as fusion of the mononuclear cells to multinucleated pre-osteoclasts. [17].

[8]
1.4.1.2 Differentiation

Osteoclast differentiation is dependent on a number of signalling molecules which include macrophage colony stimulating factor (M-CSF) and RANKL [18]. The role of RANKL is to activate the signal that enables precursor cells to differentiate into osteoclasts and is expressed by osteoblasts [19], but more recently has been shown to also be expressed by osteocytes [20, 21]. M-CSF is secreted by osteoblasts and its function is to provide the survival signal for the osteoclasts [22], support osteoclast proliferation [17] and promote their differentiation [22, 23] Osteoclasts express CD45 (leucocyte common antigen), CD68 (pan-macrophage marker) and CD51 (vitronectin receptor) [24]. Immature osteoclasts differentiate in the presence of RANKL and CSF-1 (polypeptide growth factor) which induce expression of genes including tartrate-resistant acid phosphatase (TRAP), cathepsin K, calcitonin receptor, MMP-9 and β3-integrin [25, 26]. Following differentiation to a mature osteoclast, RANKL, hormones and cytokines control the bone resorbing activity and mature osteoclast survival [27].

1.4.2 Osteoblasts

1.4.2.1 Morphology

Osteoblasts are multi-potent cells that are derived from mesenchymal stem cells (MSCs) [28]. These MSCs also can differentiate into bone marrow stromal cells, chondrocytes, muscle cells and adipocytes [28]. Following the termination of the bone forming phase osteoblasts can (1) secrete osteoid (non-mineralised bone matrix) and then can differentiate to osteocytes, (2) change into inactive osteoblasts and be known as bone lining cells or (3) initiate programmed cell death (apoptosis) [29]. It is also thought that osteoblasts may differentiate into cells that deposit
chondroid or chondroid bone [29]. Osteoblasts play an essential role in creating and maintaining the skeletal architecture as they deposit the bone matrix and regulate osteoclasts [30].

### 1.4.2.2 Gene expression

Pre-osteoblasts express CD29, CD105, CD166 and Stro-1 (Figure 1.4) [31]. Osteoblasts differentiate in the presence of growth factors which include BMPs [32], TGFs [33], IGF-1 [34], VEGF [35] as well as glucocorticoids [36]. BMPs, particularly BMP 2 and 4, are the only factors that have the ability to initiate osteoblastogenesis (the formation of osteoblasts) from the uncommitted progenitors, or from MSCs of the adult bone marrow [28].

BMPs induce transcription of the osteoblast specific factor 2 (Osf2) gene which is also known as Cbfa1 or Runx2 [28]. Runx2 is a transcription factor that drives activation of a number of osteoblast-specific genes, including osteopontin, BSP, type 1 collagen, and osteocalcin, ALP (Figure 1.4) [37, 38]. Knocking out Osf2 in mice prevents osteoblast differentiation and bone development [39]. Osf2 and osterix are expressed by the matrix producing osteoblast, and these genes are required for osteoblast differentiation. Osteoblasts become embedded in their ECM and mineral deposits extend along and within collagen fibrils [40]. The late osteoblasts generate osteocalcin which is also expressed in osteocytes [31]. The control of the expression of osteoblastic specific genes such as osteocalcin and ALP is controlled by the distal-less 5 gene (Dlx5), which is also know to play a role in mineralization of these cells [41]. Furthermore, BMP-4 is responsible for the transcription of Dlx5 [41].
1.4.3 Osteocytes

1.4.3.1 Morphology

Osteocytes are terminally differentiated [42], mature bone cells [43] which are spindle-shaped and located within the three dimensional (3D) mineralized bone matrix [44]. Osteocytes reside in a fluid-filled network which is composed of “widely spaced lacunae” [45, 46]. These cells react to mechanical strain and as a result send signals to control resorption or formation [47]. Osteocytic cells are connected to each other as well as other cells that are located on the bone surface through numerous spindle shaped, dendritic processes, which can be found in tiny canals that are called canaliculi [46]. Both the canaliculi and the lacunae are fluid filled and are composed of a proteoglycan rich ECM which has a role in the diffusion of soluble factors released by osteocytes [45].

It is believed that osteocytes control the recruitment of the BMUs in response to mechanical stimulation. It is also thought that they play a role in the “modulation of osteoblast activity” and the “recruitment of osteoblasts which differentiate into osteocytes” [48, 49]. Osteocytes have a half-life of approximately 25 years, significantly higher than an active osteoblast with a life span of approximately 3 months [29]. It is well established that osteocyte numbers decrease with age [50]. Furthermore, defective osteocyte function may be responsible for numerous bone diseases, especially glucocorticoid induced bone fragility and osteoporosis in the adult ageing skeleton [31].
1.4.3.2 Gene expression

Osteocytes express E11 (gp38/podoplanin), CD44, fimbrin, phosphate-regulating endopeptidase homolog x-linked (PHEX), matrix extracellular phosphoglycoprotein (MEPE, also known as OF45), dentin matrix protein-1 (DMP-1), sclerostin, fibroblast growth factor 23 (FGF-23) and ORP150 at various stages during their differentiation. However the gene expression in early and mature osteocytes is very different, and some of these markers overlap in expression with osteoblasts, while others are observed in specific stages of osteocyte differentiation (Figure 1.4).

E11 expression is highly expressed in both early embedding osteocytes and young osteocytes [51]. E11 functions by generating dendrites [31, 52]. CD44 is up-regulated in osteocytes when compared to osteocytes [53], and is a hyaluronic acid receptor associated with E11 as well as being linked to the cytoskeleton [54]. Fimbrin is expressed in all osteocytes and although its role is not known it is thought to be involved in dendritic branching [51]. PHEX plays an important role in phosphate metabolism and is expressed in early and mature osteocytes, but is greatly up-regulated in mature osteocytes [51, 55, 56]. MEPE inhibits bone formation and regulates phosphate metabolism and is expressed in late osteoblasts and all stages of osteocytes, being up-regulated in mature osteocytes [57, 58]. DMP-1 plays a role in phosphate metabolism and mineralisation and is expressed in both early and mature osteocytes [59-61]. Sclerostin is an inhibitor of bone formation expressed in mature osteocytes [62, 63]. FGF-23 is expressed in early and mature osteocytes and is known to induce hypophosphatemia. It is also believed that FGF-23 acts as a “bone messenger” whose key role is informing the kidneys when bone is not in need of phosphate and calcium [61]. ORP150 is expressed by the mature osteocyte and
protects the cells from hypoxia [64]. In the ECM, DMP-1 is believed to be necessary for the down regulation of both osteoblast markers and osteocyte differentiation [61]. Furthermore, biomineralisation and mineral metabolism are controlled by PHEX, MEPE and DMP-1 [31].

1.4.3.3 Osteoblast to osteocyte differentiation

Osteoblast to osteocyte differentiation is influenced by (1) the type of ossification \textit{i.e.} intramembranous, perichondral or endochondral, (2) the bone type that is being formed (woven or lamellar bone), (3) where the bone is located, (4) the species and (5) the age or gender of the individual [29].

Osteocytogenesis has been proposed to be a passive process in which a sub-population of osteoblasts can become encased in the osteoid that mineralises or an invasive process reliant upon the cleavage of collagen and other matrix molecules [31]. Osteoblasts transform themselves from a polygonal cell into a cell that has dendrites located on its mineralizing front, which then leads to the dendrites broadening to either the vascular space or the bone surface [31]. Mineralisation is controlled by the osteoid osteocyte. During osteocytogenesis there is also the formation of cell processes called lamellipodia and pseudopodia which extend out from the osteocyte cell body [51].

During osteocytogenesis, the mesenchymal cell shortens and the osteoblasts differentiate, along with the initiation of deposition of collagen type I, II and III fibres. When the initial matrix (osteoid) is laid down, bone size is increased as osteoblasts line up along the edge of the bone spicule to deposit more bone matrix.
When the osteoid has reached its thickness (approximately 9 microns), the surplus osteoblasts become trapped [29]. Morphological studies have shown that any osteoblast from the osteogenic layer (endosteum or periosteum) are capable of entering the bone matrix and transform into an osteocyte [65].

Both electron and light microscopy have revealed that the dendritic shape of a mature osteocyte is a derivative of the original rounded osteoblast that has been subjected to a number of morphological and ultra-structural changes [65]. There is a reduction in the size of the cell body which is proportional to the forming of the cytoplasmic processes. This is approximately 30% at the mineralising osteocyte stage and by approximately 70% when the osteocyte has reached maturity. During this process there is also a reduction in the number of cytoplasmic organelles, and the nucleus-to-cytoplasm ratio increases [66].
Figure 1.4: The expression of markers during the transition from a pre-osteoblast to an osteocyte, where the genes in red highlight the ones investigated in this thesis, adapted from Bonewald et al. 2010 [31].

1.5 *In vivo* and *in vitro* models for the study of osteocytes

1.5.1 *In vivo*

It is difficult to study osteoblast to osteocyte differentiation *in vivo* [67]. The generation of numerous visual transgenes driven by cell type-specific promoters has aided the dissection of osteoblast and osteocyte functions. These visual transgenes use green fluorescent protein (GFP) expression linked to specific promoters of
osteoblast and osteocyte genes which include collagen type I promoters (Col3.6kb and Col2.3kb) in preosteoblasts, osteoblasts and osteocytes [68], osteocalcin-GFP promoter for mature osteoblasts [69, 70], or DMP-1 promoters in mineralising and mature osteocytes [42] [60, 71]. Since DMP-1 expression is localised to preosteocytes and osteocytes in chicken, rat and murine models [60], GFP or lacZ expression were linked to different lengths of the DMP-1 promoter to encourage activation of DMP-1 in osteoblast-osteocyte lineages [42]. A DMP-1 transgene has also been generated utilizing a topaz variant of eGFP controlled by the 8kb DMP-1 promoter, localised to preosteocytes and osteocytes within the bone [71].

1.5.2 In vitro

In vitro there are several options to study osteocytes which include primary osteocytes and osteocyte-like cell lines (MLO-A5, MLO-Y4, HOB-O1-C1) but they each have limitations (discussed below). Primary osteocytes are difficult to study as they are terminally differentiated cells embedded in 3D mineralised bone matrix. No single cell-line fully represents the in vivo mature osteocyte. As osteocytes in vivo are in 3D, there is a need to develop a new in vitro model where mature osteocytes in a 3D mineralised can be studied in order to understand their biology and function.

1.5.2.1 Primary osteocytes

In 2006, osteocytes were described as being poorly characterised not only because of their location, but because of the lack of primary osteocyte isolation methods [72]. In recent years this has changed with the development of new primary osteocyte isolation methods [73]. However, there are still limitations to these isolation methods, in that the osteocyte-like cultures can be contaminated with proliferating
cells [74]. To date primary osteocytes have been isolated from animal (chicken, rat or mouse) or human bones.

The first published study to report isolation of primary osteocytes was by Van der Plas and Nijweide in 1992 [75] and described the isolation and culture of osteocytes from 18 day old chicken calvariae. Cells were isolated by serial digestions of collagenase with ethylenediaminetetraacetic acid (EDTA), followed by purification of a 95% osteocyte specific population using the osteocyte specific monoclonal antibody OB 7.3 (which targets PHEX [55]) bound to protein G-conjugated magnetic beads [75]. The isolated osteocytes adhered to plastic in monolayer culture, and had numerous processes which branched out to make contact with other osteocytes. Other studies on similar cells isolated using these methods characterised this cell population by comparing them to isolated osteoblast-like cells, and reported that they were differentiated post-mitotic cells that had lower ALP activity that measured in osteoblast-like cell populations [76]. Immunocytochemical studies on cells isolated in the same way, showed that the osteocyte population rarely expressed collagen type I and fibronectin, but had high expression of osteocalcin, osteopontin and osteonectin [77]. Following a few days in culture, these isolated cells, formed what appeared to be an interconnected cell network similar to the osteocyte network in bone [75]. These osteocyte cells also expressed MEPE [76], DMP-1 [77] and PHEX [74] which are known to be more highly expressed in osteocytes compared to osteoblasts. Furthermore these cells expressed sclerostin [62] and E11 [52], and were also CD44 positive [78].
Primary osteocytes have also been isolated using different cell fractions digested from newborn rat calvariae or rat cortical bone. Methods of isolation included either (1) repeated enzymatic digestions or calcification or (2) using outgrowths of cells from pre-digested tissue to isolate the osteocytes based on their morphology when these cells were maintained on matrigel or type I collagen-coated surfaces [72, 74, 79]. Similarly to the chicken osteocytes, these isolated cells were negative or faintly positive for ALP, but highly expressed osteocalcin, PHEX and DMP-1. These cells also had irregular membrane staining for connexin-43 (Cx43) [72].

There has been a recent publication that has highlighted a method of isolating and culturing long bone osteocytes from skeletally mature young (4 month old) and aged (22 month old) mice [73]. This methodology involved serial collagenase digestions combined with EDTA based decalcification. The remaining bone fragments were then broken up using a tissue homogenizer and the homogenised material was then placed into culture to yield an outgrowth of osteocyte-like cells [73]. These cells had osteocyte-like morphology and expressed osteocyte markers (E11, sclerostin and MEPE). This methodology has the potential to increase our understanding of age-related changes in osteocyte function [73].

To date there is only one published study where human osteocyte-like cells were isolated [80]. Osteocytes-like cells were isolated from human mandibular bone by placing bone strips in collagen type I coated dishes, following washing in phosphate buffered saline (PBS) and EDTA. Following 1 week of culture, the outgrowth of fibroblast cells were recovered and transferred into another dish. The remaining
outgrowths of flat cells became dendritic, and were used as osteocytes when they reached confluence [80].

1.5.2.2 Osteocyte cell lines

Three cell lines are currently available for the study of osteocyte biology. These are the MLO-A5, MLO-Y4 and HOB-O1-C1 cell lines. The mouse-derived MLO-A5 [81] and MLO-Y4 [82] cells were developed by the Bonewald group whereas the HOB-O1-C1 [83] cells were developed by the Bodine group and are a human derived cell line.

1.5.2.3 MLO-A5 cell line

The MLO-A5 cell line was developed from cells from the long bones of transgenic mice expressing the SV40 large T-antigen driven by the osteocalcin promoter [81]. They are described as late osteoblastic/pre-osteocytic cells which are responsible for triggering the mineralisation of the osteoid and were selected based on their dendritic or stellate morphology following adherence to collagen type I coated surfaces. The MLO-A5 cells mineralise in sheets in the presence or absence of beta-glycerophosphate and ascorbic acid and express high levels of ALP, collagen type I, PTH/PTH-related peptide, BSP and osteocalcin. This cell line has mainly been used to study (1) osteoblast to osteocyte transition, (2) mineralisation and (3) the effect of mechanical loading on these processes [84-87].

1.5.2.4 MLO-Y4 cell line

The MLO-Y4 cell line is an early osteocyte cell line and was also developed from cells isolated from the long bones of transgenic mice expressing the SV40 large T-
antigen driven by the osteocalcin promoter [82]. They were isolated following serial collagenase digestions with the cells obtained maintained on collagen coated surfaces. Typical of osteocytes, these cells have a stellate morphology and express early osteocyte marker proteins (low ALP, high osteocalcin, E11 and Cx43). Atypical of osteocytes, these cells proliferate, and do not mineralise or produce sclerostin [88]. These cells have mainly been used to study (1) modulation of osteocyte apoptosis and cell death [89-95], (2) function and control of gap junctions and hemi-channels [96-98] and (3) effects of mechanical loading, especially in relation to fluid flow studies [91, 99-101]. Studies using these cells have also shown that they support osteoclastogenesis in the absence of osteotropic factors [45, 102, 103], and that they can regulate the activity of osteoblasts [104], mesenchymal progenitor cells [105] and endothelial cells [106].

1.5.2.5 HOB-O1-C1 cell line

The HOB-O1-C1 cells were the first ever conditionally transformed human pre-osteocytic cell line to be developed [83]. They were developed by extracting primary adult bone cells following protease digestion of cancellous human bone chips. Following this, the cells were infected with adenovirus-ori- SV40 tsA 209, which encodes for a temperature-sensitive large T-antigen so that they contained the temperature-sensitive large T-antigen and were then immortalised to establish the cell line [83]. These cells proliferate at 34°C as they express the mutant T-antigen and stop dividing at 39-40°C. At 39°C, the HOB-O1-C1 cells exhibit pre-osteocytic cellular processes that form gap junctions, mineralise rapidly and respond to PTH, tumour necrosis factor alpha (TNFα) and IL1β. They also have low expression of
ALP, and express CD44 at both temperatures. However, this cell line has not been further used to study osteocytes [107].

1.5.3 Differentiation of osteoblasts to osteocytes in vitro

Investigating the differentiation of osteoblast cells to osteocytes can provide an alternative way to study osteocytes in vitro. Furthermore, a mouse cell line (IDG-SW3) has been developed that differentiates from a pre-osteoblast to a mature osteocyte [108]. Other osteoblast options available are primary cells (human [109], mouse [110], rat [111], ovine [112], bovine [113], rabbit [114]) or cell lines (human – SaOs-2 [115], MG-63 [116] or mouse – MC-3T3 [117]). Table 1.1 and Table 1.2 below describe these cell types in more detail highlighting benefits and limitations.

1.5.3.1 IDG-SW3 cell line

The IDG-SW3 cell line is a mouse osteoblast to mature osteocyte cell line [108]. These cells proliferate in the presence of interferon gamma (INFγ) at 33°C, and stop proliferating and differentiate in the absence of INFγ at 37°C as they contain a temperature sensitive mutant of the SV40 large tumor antigen which is regulated by the INF-γ-inducible H-2K$^b$ promoter ($H-2K^b$-tsA58) enabling continuous proliferation and immortalisation [92]. They also contain the DMP-1 GFP reporter transgene as the mice the cells are isolated from contain a DMP-1 cis-regulatory system which promotes the GFP expression which is specific to the long bones and the calvaria and is related to endogenous DMP-1 expression [92]. This cell line is unique in that it has the ability to differentiate from a late osteoblastic phenotype to a mature osteocyte-like phenotype that also mineralises in culture. Unlike the MLO-Y4 and MLO-A5 cell lines, and following differentiation, these cells express the mature
osteocyte markers sclerostin and FGF-23 following 21 days of culture at 37°C. They also express DMP-1 and MEPE also at 37°C which were present at each time points in osteogenic medium but were elevated following 14 days of culture.

Since their establishment in 2011, there have been 5 publications using this cell line. The initial paper describes their generation and includes details on their morphology and gene expression at various stages of differentiation [108]. One study found that direct cell-cell contact between these cells and calvarial osteoblast [118], up-regulated osteoblast differentiation genes in the osteoblasts, and osteocyte specific genes in the osteocytes when compared to the individual cell populations without cell-cell contact [118]. Another study found that short term treatment of mature IDG-SW3 osteocytes (35 days) with vitamin D3 and extracellular phosphate, increased the expression of FGF-23, N-acetylgalactosaminytransferase, DMP-1, PHEX, MEPE, and ectonucleotide pyrophosphatase/ phosphodiesterase family member 1 [119]. This cell line was also used to investigate the epigenetic changes and responses to the 1, 25-dihydroxyvitamin D3 (vitamin D3) hormone during osteocytogenesis [120]. During osteocytogenesis gene expression changes were controlled by both genetic and epigenetic components. Furthermore, this group hypothesised that it is these changes that are responsible for the osteocyte phenotype as well as the reduced sensitivity to vitamin D3 [120]. Finally, IDG-SW3 cells were used to investigate if FGF-23 was a substrate for secretory phosphorylation and revealed a significant role for the phosphorylation of FGF-23 in bone [121]. These studies highlight the benefits of this cell line to the osteocyte field to further understand what happens at various stages during the transition of an osteoblast to an osteocyte, as well as to investigate osteocytes [107].
### Table 1.1: Characteristics of different primary osteoblasts options available (adapted from Czekanska et al. 2012) [137]

<table>
<thead>
<tr>
<th>Name</th>
<th>Isolation</th>
<th>Characteristics</th>
<th>Benefits</th>
<th>Limitations</th>
<th>Reference</th>
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### Chapter 1

#### Introduction

<table>
<thead>
<tr>
<th>Origin</th>
<th>Characteristics</th>
<th>Benefits</th>
<th>Limitations</th>
<th>References</th>
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<tbody>
<tr>
<td>MC-3T3-E1 clone 14</td>
<td>- Isolated from newborn mouse calvaria clones&lt;br&gt;- Pre-osteoblastic phenotype&lt;br&gt;- High BSP&lt;br&gt;- High osteocalcin&lt;br&gt;- Contain PTH/PTHrP receptor&lt;br&gt;- High collagen type I&lt;br&gt;- High ALP</td>
<td>- Unlimited number of cells&lt;br&gt;- Homogenous&lt;br&gt;- Differentiate from pre-osteoblast to mature osteoblast&lt;br&gt;- Ease of maintenance&lt;br&gt;- Mineralise (under mineralising conditions)&lt;br&gt;- Proliferate</td>
<td>- Interspecies differences&lt;br&gt;- May have cellular replicative senescence</td>
<td>Wang et al., 1999 [117]&lt;br&gt;Sudo et al., 1983 [138]&lt;br&gt;Quarles et al., 1992 [139]&lt;br&gt;Grigoriadis et al., 1985 [140]</td>
</tr>
<tr>
<td>MG-63</td>
<td>- Isolated from a juxtacortical osteosarcoma in the distal diaphysis of the left femur of a 14 yr old male&lt;br&gt;- Immature osteoblast&lt;br&gt;- Rapid cell growth&lt;br&gt;- High ALP&lt;br&gt;- High osteocalcin&lt;br&gt;- High osteonectin</td>
<td>- Unlimited number of cells&lt;br&gt;- Response to hormones similar to hOBs&lt;br&gt;- No interspecies differences&lt;br&gt;- Ease of maintenance&lt;br&gt;- Proliferate</td>
<td>- Stay in pre-osteoblast state&lt;br&gt;- Cell mineralisation inconstitent</td>
<td>Heremans et al., 1978 [141]&lt;br&gt;Clover et al., 1992 [142]&lt;br&gt;Olivares-Navarrete et al., 2008 [116]&lt;br&gt;Kumarasuriyar et al., 2009 [143]&lt;br&gt;Saldana et al., 2011 [144]&lt;br&gt;Pierschbacher et al., 1988 [145]</td>
</tr>
<tr>
<td>SaOS-2</td>
<td>- Isolated from an 11 year old Caucasian female in 1975&lt;br&gt;- Mature osteoblast phenotype&lt;br&gt;- High ALP&lt;br&gt;- Form calcified matrix similar to collagen&lt;br&gt;- Express PTH and 1,25(OH)2D3 receptors</td>
<td>- Unlimited number of cells&lt;br&gt;- Homogenous&lt;br&gt;- Mineralises&lt;br&gt;- Similar cytokine to growth factor expression profile to hOBs cells&lt;br&gt;- No interspecies differences&lt;br&gt;- Ease of maintenance&lt;br&gt;- Proliferate</td>
<td>- Do not demonstrate the range of osteoblastic phenotype changes&lt;br&gt;- Sensitive to Pi substrates</td>
<td>Masuda et al., 1987 [115]&lt;br&gt;Murray et al., 1987 [146]&lt;br&gt;Bilbe et al., 1996 [147]&lt;br&gt;Rodan et al., 1987 [148]&lt;br&gt;Fernandes et al., 2007 [149]&lt;br&gt;Rao et al., 1996 [150]</td>
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**Table 1.2:** Characteristics of most commonly used osteoblast cell lines available for research (adapted from Czekanska et al. 2012) [137]
1.6 Models of osteoblast to osteocyte differentiation

Since i) it is difficult to study primary osteocytes \textit{in vivo} or \textit{in vitro}, and ii) the available osteocyte cell models for \textit{in vitro} studies are limited, it is currently a high priority in the osteocyte research field to develop new models to study these cells. Since osteoblasts \textit{in vivo} differentiate to osteocytes, one avenue which could be explored further is the possibility of harnessing this osteoblast to osteocyte differentiation pathway to provide novel \textit{in vitro} tools to study osteocytes. This section will delineate the current understanding of the effect of external factors to modulate this differentiation process. The literature reports that several compounds (strontium ranelate, FGF-2, Retinoic Acid (RA) and Vitamin K (Vit K) enhance the differentiation of osteoblasts to osteocytes, and these will be discussed in more detail below. There is also some evidence that IGF-1 is necessary for the differentiation of other cell types such as MSCs to osteoblasts, and although its role in osteocyte differentiation is still unknown, the possible use of IGF-1 to enhance differentiation of osteoblasts to osteocytes will also be discussed.

1.6.1 Strontium Ranelate

1.6.1.1 Background

Strontium ranelate was an anti-osteoporosis (OP) treatment [151] that was used for the treatment of postmenopausal OP to reduce the risk of vertebral and hip fractures [152-155], but due to patient complications it has recently been taken off the market. \textit{In vivo} strontium ranelate has been shown to induce a positive bone balance in experimental osteopenic models and it promotes osteoblast replication,
differentiation and survival in vitro [152, 156-159]. However the molecular mechanism of how strontium ranelate acts on the bone forming cells has not yet been characterised. It is hypothesised that Strontium acts on the seven-transmembrane-spanning extracellular calcium-sensing receptor expressed by the osteoblast cells [160], and influences osteoblastic replication and survival via three different pathways which include mitogen-activated protein kinases, ERK1/2 phosphatidylinositol 3-kinase, and phospholipase C [161-163]. Strontium ranelate can also act independently of the calcium-sensing receptor in osteoblasts [163].

1.6.1.2 Evidence of use of strontium ranelate in cell differentiation

Treatment of human primary osteoblasts with strontium ranelate promoted osteoblastic proliferation and in vitro mineralisation, and also increased expression of the osteocyte markers, sclerostin and DMP-1 [164]. Another study confirmed that human primary osteoblasts treated with strontium ranelate increased mineralisation, but in contrast to Atkins et al. 2009, caused reduced sclerostin expression [165]. Similarly, in bone marrow stromal cells strontium ranelate treatment promoted osteoblastic differentiation and mineralisation, increased ALP activity, OCN mRNA expression and PGE$_2$ expression and inhibited cyclooxygenase-2 (COX-2) activity [152]. Following mechanical stimulation strontium ranelate treatment affected the paracrine signalling (Nitric Oxide and PGE$_2$) in MLO-Y4 osteocytes, osteoblasts and osteoclast precursors [166]. Other studies have shown that strontium ranelate treatment increased osteoblastic differentiation in mouse cell lines, bone marrow-derived stromal cells [157, 158], rat MSCs [167-169] and human MSCs [170, 171] or induced mineralisation [172].
1.6.2 IGF-1

1.6.2.1 Background

IGF-1 is a member of the IGF system which is composed of 3 ligands (Insulin, IGF-1 and IGF-2), 3 receptors (insulin receptor, IGF-1 receptor and IGF-2 receptor) and a minimum of 6 high affinity binding proteins that work together to control cellular metabolism, proliferation, differentiation and apoptosis in the majority of cells [173, 174]. The IGF system also contains IGF high affinity binding proteins and high affinity binding proteins proteases [174]. IGF-1 and IGF-2 mediate the activity of growth hormone (GH, somatotropin), and were originally called somatomedians [175], prior to being renamed because of their similarity with insulin [176]. GH is produced and secreted by the pituitary gland and induces body growth [177]. GH binds to the GH receptor in the liver and activates signalling pathways that enables the transcription of numerous genes including IGF-1 [178]. The intracellular activity of IGF-1 is mediated by the tyrosine kinase IGF-1 receptor which controls proliferation and differentiation [179]. IGF-1 can also bind with lower affinity to the insulin receptor [180].

IGF-1 is produced by numerous tissues which include the liver, bone, muscle and brain [181]. Bone is a major target organ for IGF-1 [16, 182, 183] and IGF-1 is necessary for bone formation, developmental bone growth and bone resorption [184-186]. Circulating IGF-1 increases from birth to puberty and reaches maximum levels during puberty before decreasing after this age [187].
1.6.2.2 Evidence of use of IGF-1 in cell differentiation

Osteocytes produce IGF-1 in significant amounts [188]. Bone derived IGF-1 does not have much of an impact on circulating IGF-1 levels but acts as an autocrine/paracrine effector that regulates bone turnover [189]. Furthermore, the circulating liver-derived IGF-1 may also play a role in bone modelling and remodelling [189]. In rodents, where the IGF-1 gene was knocked-out, there was impaired longitudinal and periosteal bone expansion during postnatal growth [184], however, when the hepatic IGF-1 was knocked-out there was no significant difference in bone growth in early life even though circulating IGF-1 levels were reduced significantly [190]. Furthermore, it is believed that IGF-1 derived from different bone cells has different regulatory roles in bone homeostasis [191, 192]. When osteocyte derived IGF-1 was deficient, bone responses to mechanical loading, developmental bone growth, bone turnover, responses to calcium stress and fracture repair were all affected [189]. Similar to osteoblast derived IGF-1 and chondrocyte derived IGF-1, osteocyte derived IGF-1 has been shown to play a necessary role in the regulation of developmental bone growth [191, 192].

1.6.3 FGF-2

1.6.3.1 Background

FGF-2 is a member of the FGF family which are a family of 22 genes encoding structurally related proteins [193]. The FGF family is comprised of 6 subfamilies, which are subdivided by sequence similarities or by biochemical and functional properties [194]. Most members of the FGF family contain 4 distinct FGF receptor tyrosine kinase molecules in the active or bound form [194]. The FGF family also contains FGF receptors which have unique ligand binding properties formed by
alternative mRNA splicing [195, 196]. The FGF family controls the proliferation and differentiation of various cell types [197, 198], but specifically control the expression of differentiation genes in osteoblasts [199, 200].

1.6.3.2 Evidence of use of FGF-2 in bone cell differentiation

FGF-2 regulates the expression of a number of genes involved in osteoprogenitor cell replication, osteoblast differentiation (as shown in Figure 1.5) and apoptosis [194, 201]. FGF signalling regulates osteoblast function and differentiation [201] where it has been suggested that FGF-2 acts as an antagonist of osteoblast differentiation [202]. In mice when the FGF-2 gene was knocked-out, there was a reduced bone mass as well as the lack of osteoanabolic effects to PTH [203-206]. However, Gupta et al. 2010 has shown that the addition of FGF-2 to osteoblasts induced an osteocyte-like phenotype, with a down-regulation of the expression of known osteoblastic markers such as ALP, collagen type I and OCN [202]. Similar to this study, other groups have shown that following FGF-2 treatment of osteoprogenitor cells, osteoblast markers such as ALP, collagen type I and OCN were significantly down regulated [207, 208]. However, FGF-2 treatment of mature osteoblasts showed different results in that it induced OCN production and matrix mineralisation, highlighting that FGF-2 effect on cells is stage specific [209]. When FGF-2 was over expressed in transgenic mouse model, bone mineral density was up-regulated [210]. In a number of different animal models, FGF-2 administration has also been shown to accelerate fracture repair [211-213], and FGF-2 treatment in rats is reported to increase bone formation [214, 215], and to restore bone mass in ovariectomized rats [216, 217].
Figure 1.5: The regulation of osteoblastic gene expression by FGF signal during osteogenesis. Bone formation can be categorised as the replication of MSCs along with the differentiation of osteoprogenitor cells to mature osteoblasts. FGF signalling acts through FGF receptors (FGFR) to control several genes involved in osteoblast commitment and differentiation (Runx2, collagen type I, osteopontin, osteonectin, ALP, bone sialoprotein and OCN, adapted from Marie PJ 2003 [201]).

1.6.4 RA

1.6.4.1 Background

Vitamin A (retinol) as well as its natural and synthetic derivatives (retinoids) have been shown to be involved in numerous physiological processes including reproduction, cell proliferation, differentiation, vision and embryonic development [218]. RA is a derivative of Vitamin A, and has also been show to regulate differentiation and metabolism by serving as ligands for 2 families of nuclear receptors. These are (1) the RA receptors (RARs)–Retinoic Acid Receptor-α (RAR-α), Retinoic Acid Receptor-β (RAR-β) and Retinoic Acid Receptor-γ (RAR-γ) which bind to all trans RA (ATRA) and (2) the retinoid X receptors (RXRs)- retinoid X receptor-α (RXR-α), retinoid X receptor-β (RXR-β) and retinoid X receptor-γ (RXR-γ), which bind to the isomer known as 9-cis-RA (9CRA) which is undetectable when vitamin A is present in excess [219-221]. ATRA is the most abundant form of RA, is the active metabolite of vitamin A, and is also the master regulator of gene expression [222]. Its effects on gene transcription are controlled by binding to
nuclear RARs. The RARs modulate the retinoic acid-responsive target genes by binding to the DNA as heterodimers with the RXRs [223]. RAR and RXR agonists have also been reported in numerous studies to promote terminal differentiation of precursor cells and cancer cells [224-230].

1.6.4.2 Evidence of use of RA in cell differentiation

Varying results have been reported on the role of RA signalling in chondrogenic and osteogenic differentiation with RA signalling either promoting or inhibiting these processes [231-238]. Furthermore, in vitro studies of the effect of RA treatment on osteoblasts have also reported opposing results in relation to maturation and mineralisation [239, 240]. ATRA treatment has also been shown to accelerate the maturation of the murine pre-osteoblast MC-3T3-E1 cell line [241, 242].

RA and BMP-9 co-operate with each other to induce MSCs to differentiate to osteoblasts [243]. Furthermore, in pre-adipocytes, RA has also been shown to prevent adipogenic differentiation and act in synergy with BMP-2 to induce these cells to undergo osteogenic differentiation [236, 244]. These findings suggest that it is the RA that is co-operating with BMP-9 to drive osteogenesis along with reversing the BMP-9 induced adipogenesis in pre-adipocytes. Similarly when the pre-adipocyte cell line 3T3-L1, were treated with ATRA, BMP-9 induced osteogenic differentiation but prevented BMP-9 induced adipogenic differentiation in these cells, by activating the BMP/Smad and Wnt/β-catenin pathways [245]. This study also found that the Wnt/β-catenin activation may have occurred because of the phosphatidylinositol-3-kinase/Akt/glycogen synthase kinase 3β pathway being activated by ATRA and BMP-9 [245]. Furthermore, in rat primary osteoblasts in
vitro, RA treatment has been shown to promote differentiation, increase expression of osteogenic genes and increase bone nodule deposition [239].

1.6.5 Vit K

1.6.5.1 Background

Vit K belongs to the group of fat soluble vitamins and has two naturally occurring family members which are phylloquinone (Vit K1) and menaquinones (Vit K2) and one synthetic family member known as menadione (Vit K3) [246]. Vit K1 and Vit K2 have a role in bone metabolism as well being potential therapeutic agents for various bone diseases [247]. Vit K is well established to have a role in blood clotting by mediating the γ-carboxylation of Gla proteins (Vit K dependent) [248]. Bone has been shown to have 3 Gla proteins, (1) OCN, which is also referred to as bone Gla protein, (2) matrix Gla protein and (3) periostin [249-251]. Functionally, Gla proteins are known to have a greater binding affinity for calcium, and OCN, matrix Gla protein and periostin all contain γ-carboxylation-dependant calcium binding properties. In humans, Vit K2 has been shown to induce mineralisation of osteoblasts and apoptosis of osteoclasts as well as apoptosis of the synovial cells in Rheumatoid Arthritis [252]. Although the mechanism of action is still not understood numerous human studies in postmenopausal OP cohorts have shown that Vit K2 either inhibited a decrease in bone mineral density or increased bone mineral density [253, 254]. Other studies involving postmenopausal non-osteoporotic women found that Vit K2 maintained bone strength as demonstrated by the maintenance of bone mineral content in the femoral neck [255]. Furthermore Vit K2 given in combination with bisphosphonates has been shown to increase the effectiveness of
the bisphosphonates [256], and Vit K treatment also reduced vertebral fractures by 56% in post-menopausal women with OP when compared to the placebo [257].

### 1.6.5.2 Evidence of use of Vit K in bone cell differentiation

In osteoblasts, Vit K binds to and activates the steroid and xenobiotic receptor SRX [258, 259] to mediate transcription of extracellular matrix-related genes such as collagen in osteoblastic cells [258, 259]. Vit K homologues caused bone matrix mineralisation, promoted osteoblast transition into osteocytes, and also blocked the expression of RANKL (preventing osteoclastogenesis) in the MLO-Y4 cell line [246]. The results in this study highlighting that Vit K inhibited RANKL production are similar to those found in vivo [260], but differ to those observed in vitro with MC-3T3-E1 immature osteoblast cells [261]. Furthermore, in bone marrow cells, Vit K2 treatment, and not Vit K1 treatment also inhibited RANKL mRNA expression, increased OPG mRNA expression [262], and also inhibited 1,25-dihydroxyvitamin D$_2$-induced osteoclastogenesis [263]. However, treatment of these cells with Vit K1 and Vit K2 enhanced 1,25-dihydroxyvitamin D$_2$-induced mineralisation [262]. In MC-3T3-E1 cells, Vit K2 treatment did not affect cell proliferation but induced the expression of RANKL, RANK and OPG [261], as well as ALP and OCN expression [264].

### 1.7 Studies of osteoblasts and osteocytes in 3D

**in vitro cultures**

Most of the research published to date using in vitro osteocyte models have been undertaken in 2D monolayer culture. Such 2D models, however, do not represent the
in vivo environment of osteocytes, and osteocytes when they are isolated and cultured in 2D have been shown to behave differently those in a complex 3D in vivo environment [265]. This can be seen when osteoblast like cells were cultured in 3D, as they showed enhanced angiogenic gene expression, as opposed to those cultured in 2D. Furthermore the same study found two measures of osteoblastic differentiation, ALP and OCN were significantly upregulated in 3D cultures of human MSCs compared to those that were cultured on 2D [266].

Scaffolds provide a 3D method in which osteoblasts and osteocytes can be grown for bone–tissue engineering. The main function/aim of a biological scaffold is to mimic the biological functions of the ECM, and the scaffold must comply with a number of structural parameters in that it must be biodegradable, be easy to fabricate, be biocompatible and have mechanical strength and interconnectivity [267-269]. In order to generate the scaffold numerous techniques have been used which include solvent casting or particle leaching, gas foaming, phase separation, fiber bonding and porogen leaching [270, 271]. A 3D scaffold that is proving to be of huge benefit for tissue engineering are photopolymerizable-poly (ethylene glycol) (PEG) hydrogels [272]. The main benefits of using these hydrogels are that they are injectable and can be utilized for curing in situ [273]. The effects of PEG-based hydrogels on cell response have been demonstrated in the form of chemical/physical properties which include functionality, stiffness and degradation and biological properties which include the release of soluble proteins and growth factors, the presence of cross-linked peptides or other bioactive moieties [272]. Atkins et al. 2009 cultured human osteoblast cells with type I collagen gels throughout with polyethylene (PE) were dispersed. The PE particles have the ability to interact with cells of the osteoblastic
lineage both directly and through products that are released from the PE activated macrophages [274]. This tissue like matrix that is developed from PE particles and collagen type I gel enables the study of gene expression induced by the PE particles in long term cultures [274]. Another scaffold method includes the use of Biphasic Calcium Phosphate (BCP) particles [265]. However, all these scaffolds have limitations in that the cells do not become embedded into the 3D scaffold as the cells are localised to the scaffold surface. This means that scaffolds do not represent the in vivo bone environment so a new method needs to be developed to investigate osteocyte biology.

A type I collagen derived from rat tail and matrigel mix (1:1) has been used for culturing osteoblasts and osteocytes in 3D [275] These 3D cultures were maintained for 5 days and the method resulted in cell processes being formed from the osteocytes [275]. This collagen gel methodology has also been used to demonstrate that bone marrow cell differentiation can be induced by mechanically damaged osteocytes [103], and generated an in vitro model to demonstrate that damaged osteocytes can induce the bone resorption stage [103]. Recently, collagen gels have been used to maintain cells on or in collagen gels, as well as to investigate the interaction between two cell types by culturing cells within gels and layering another cell on top [74, 246, 276-280]. Chapter 3 will discuss collagen gel methods as a 3D culture model in more detail. Collagen gels however, are beneficial over scaffolds as cells can be embedded within the 3D collagen gel, thus presenting a good model that more closely mimics the in vivo situation.
1.8 Mechanical loading of osteocytes

Osteocytes are believed to be the mechanosensory cells in bone [31] and thus one of their main functions is to respond to mechanical strain by sending signals to cells on the bone surface [46]. Mechanical loading studies of osteocytes have been undertaken in vivo and in vitro, with the various methods used to date summarised in the sections below.

1.8.1 Mechanical loading of osteocytes in vivo

Several animal models (e.g. dogs, chick, turkey, mice and rats) have been used to study the responses of osteocytes to mechanical loading in vivo. Methods include: tail suspension, vertebral loading, cyclic loading, four point bending, and tooth movement models, with in excess of 100 articles published, and with the numbers of publications increasing monthly. This rest of this section will briefly describe in vivo mechanical loading models, and will also describe factors that have been shown to be modulated following mechanical loading using these models.

1.8.1.1 Vertebral loading

Vertebral loading in rats was a method described by Chambers et al. 1993, and was undertaken when stainless steel pins are inserted into the 7th and 9th caudal vertebrae of 13 week old adult rats [281]. Animals are usually subjected to a single episode of loading immediately following the insertion of the pins which results in the 8th caudal vertebrae being subjected to dynamic load and compression. This method of mechanical loading induces bone formation [281] [282] [283] [284].
A single vertebral loading (5 min, 300 cycles, 1 Hz) in rats found that NO and PG are essential for mechanically induced osteogenesis, with exogenous NO having the same effect as mechanical loading on bone formation [282]. No effect of NO was seen in non-loaded vertebrae which suggests that NO is essential but not sufficient to induce bone formation [282]. Vertebral loading has also been used a method of assessing the responsiveness of rat cancellous bone to mechanical stimulation [283]. This study found that the rate of lamellar bone formation was increased 140-fold following daily exposure to loads similar to gentle physical activity. Furthermore this study also found that a single load (10 min. episode, 300 cycles, 1 Hz, 150 N) increased bone formation 24-fold when compared to non-loaded controls [283].

Vertebral loading studies (single 5 min loading, 300 cycles, 1 Hz, 150 N) have also found that genes for bone matrix proteins are expressed at their highest level after 72 hrs following loading, mineralisation occurs after 3 days and that the osteogenic response to this mechanical stimulation is not dependent previous bone resorption [284]. In terms of cellular responses following loading, there was no proliferation but mechanical loading activated the bone lining cells [284].

1.8.1.2 Ulna loading

Cyclic loading of the ulna of skeletally mature roosters resulted in increased \( ^3\text{H}\)-uridine levels in osteocytes post loading [285]. Cyclic loading (single, 1 Hz, 5 mins) of rat ulna resulted in increased glucose-6-phosphate dehydrogenase (G6PD) enzyme in osteocyte cells following loading [286]. This model is associated with osteocytogenesis in the mid-shaft of the ulna usually detected by fluorescent calcine labelling and dynamic histomorphometry [286]. Cyclic loading (5 Hz, 4 min, 7 N,
4000-4500 microstrain) was also undertaken on the ulnae of rats and found that the glutamate/aspartate transporter (GLAST) was down-regulated, with the GLAST antibody confirming expression in newly incorporated osteocytes [287]. Ten days of daily in vivo cyclic loading (10 mins, 3000-4000 microstrain) of neonatal and adult male and female rat ulnas increased estrogen receptor alpha expression in all osteocytes, decreasing in expression at higher strains, with the whole osteocyte network appearing to respond to a strain equally [288]. Cyclic fatigue loading (6000 cycles, 2 Hz, 17 N) of rat ulnae induced osteocyte apoptosis which was suppressed by Risedronate or Alendronate (both bisphosphonates) [289]. Murine ulnae underwent cyclic axial loading (2 Hz, 1 min/day, 1.2-2.4 N) with results showing approx a 50 % reduction in sclerostin mRNA at 24 hrs following the first loading in mice [290].

1.8.1.3 Four point bending

Four point bending is loading of tibiae where rodents were exposed to long periods of low and short periods of high cyclic loading, where large rods were applied directly on the skin and the underlying periosteum enabling compression and tension in different areas of the bone. Previous studies have shown that four point bending of the rat tibia (300 cycles, 2 Hz, 65 N) resulted in:

(a) increased osteopontin and decreased myeloperoxidase post mechanical loading [291],

(b) increased prostaglandin endoperoxide H synthase-1 (PGHS-1) post loading when compared to controls, but this was not observed at 6 and 24 hrs post loading [292],

(c) increased PGSH-2 in osteocytes immediately after loading, even though it
decreased 6 hrs post loading, it was still significantly increased in loaded cells when compared to control bones [292] and (d) modulated IGF-1 binding protein-2 expression in the endocortical osteocytes following loading and sham loading [293].

1.8.1.4 Tooth movement models

The mouse tooth movement model used to study the effect of mechanical loading on the dento-alveolar tissue acts as a good model due to study both bone resorption and bone formation sites. This tooth movement model study found that DMP-1 mRNA in osteocytes increases 6 hrs post loading at the bone resorption and bone formation sites and remains increased 4 days post loading whereas DMP-1 protein expression in osteocytes decreased 3 days post loading at both sites [294]. The mouse tooth movement model revealed that Cx43 mRNA was unchanged but Cx43 protein increased in osteocytes, following mechanical loading at both sites. Cx43 protein staining following loading was localised to the cell body and the dendritic processes [295]. This model was also used to investigate the effect of mechanical loading on MEPE. Alveolar osteocytes expressed high basal levels of MEPE, but this decreased day 1 of loading. At 3 days post loading there was a significant increase in MEPE with a reduction seen by day 7 of loading [296].

1.8.1.5 Tail suspension

The tail suspension test was undertaken on mice or rats, where the animals are suspended by their tail (with adhesive tape), and was developed with the aim to provide a gravity-based model that would mimic the effects of weightlessness [297]. This method is also referred to as hind-limb unloading. In rats, the tail suspension
model was used to investigate the effects of interactions between ovarian function and mechanical loading on bone mass and strength [298]. This study found that loss of ovarian function and mechanical unloading together resulted in more rapid and severe bone loss compared with each independently [298]. The tail suspension model has also been used to investigate if physical inactivity contributes to the skeletal effects of psychotropic drugs [299], and revealed that diminished mechanical loading causes osteocyte apoptosis which in turn recruit osteoclasts to increase bone resorption [300].

1.8.2 Mechanical loading of osteocytes in vitro

Mechanical loading of osteocytes in vitro to date has mainly been undertaken on monolayer cultures or on bone explants from numerous different animal species. In vitro mechanical loading 3D models using scaffolds in culture, have been used for mechanical loading studies of osteoblasts [301] and osteoblast/osteocyte co-cultures [280]. Osteocyte in vitro mechanical loading models have both confirmed results found in vivo e.g. RANKL [21, 45, 302, 303] up-regulation post loading and led to the discovery of novel molecules and pathways that respond to mechanical signals e.g. IL-6 [304], and VEGF [305]. The majority of published studies on the responses of osteocytes to mechanical loading in vitro have used MLO-Y4 early osteocyte cells in monolayer, and the loading methods have included pulsating fluid flow or cyclic loading using the Bose Electroforce system (Bose Corporation, Minnesota, USA), cyclic loading using the Flexcell tension system (Flexcell International Corp., Hillsborough, USA) or similar regimes (i.e. pulsating or oscillatory fluid flow, or cyclic loading) using in-house custom built mechanical loading apparatuses [304, 306-309]. Collectively, these loading systems generate either fluid shear, hydrostatic
compression, biaxial stretch, uniaxial stretch or can use a combination of 2 or more of these forces [310]. Some of the key findings from such studies are summarised in the next sections.

1.8.2.1 Cyclic loading

The first study to use cyclic loading (600 cycles, 1 Hz) in vitro showed that loading of adult cancellous canine bone cores led to an increase in G6PD, PGE$_2$ and prostacyclin [311]. Two years later, it was shown that cyclic loading of rat calvaria (600 cycles, 1 Hz, 100 or 1000 microstrain) and ulnae organ cultures (600 cycles, 1 Hz, 4000 microstrain) differed in that the ulnae showed increased release of PGE$_2$, prostacyclin and G6PD, whereas these were not released in the calvaria. This highlights that bones isolated from different locations show different responses to mechanical strain [312]. Cyclic loading was also undertaken on chick tibiotarsi in culture, where a single 20 minute period of intermittent loading (single 20 min, 0.4 Hz) resulted in a rapid increase in G6PD activity in osteoblasts and osteocytes as well as increased RNA synthesis as seen by the higher levels of $^3$H-uridine in extracted RNA [313]. Following cyclic hydrostatic pressure (68 kPa at 0.5 Hz) on MLO-Y4 cells, there was increased intracellular calcium, microtubule organisation was altered, COX-2 mRNA and the RANKL/OPG ratio increased and apoptosis decreased [303].

1.8.2.2 Fluid flow in vitro

Fluid flow is a well-established method of applying mechanical loading to osteocytes in monolayer culture. One of the first studies showed that osteocytes isolated from chicken calvaria increased PGE$_2$ production following pulsating fluid flow (4 Hz, 0.4
kPa/s) or intermittent hydrostatic compression (0.3 Hz, 32.5 kPa/s) when compared to osteoblasts and periosteal fibroblasts [314]. MLO-Y4 cells under fluid flow shear stress also increased PGE2 release, as well as Cx43 expression [96, 315]. Furthermore, this group report that it was the hemichannels produced by Cx43 rather than intracellular channels which played a role in the PGE2 release in response to fluid flow shear stress [96, 315]. When MC-3T3 and MLO-Y4 cells underwent fluid flow (20 dynes/cm^2, 1Hz), the MLO-Y4 cells activated hemichannels (through protein kinase C) and induced adenosine triphosphate (ATP) and PGE2 release [316] whereas in the MC-3T3 cells these hemichannels were not activated. When comparisons were made between the MC-3T3 osteoblast network and the MLO-Y4 osteocyte network following fluid flow shear stress (0.5, 1, 2, or 4 Pa), the MLO-Y4 osteocyte network increased intracellular calcium signals (up to 17 intracellular peaks) whereas the MC-3T3 (3 or less intracellular peaks) response was dependant on the strength of the fluid flow confirming that the osteocytic network can not only sense, but also process responses to mechanical signals. A mixed population of human bone cells have also been shown to respond to pulsating fluid flow with results showing up-regulated BMP-7 expression [317]. Numerous other reports show that fluid flow shear stress protects osteocytes from undergoing apoptosis but this result was not seen in osteoblasts [91, 92, 318-320]. On the other hand, when MLO-Y4 cells underwent oscillatory fluid flow as opposed to pulsating fluid flow the cells release soluble RANKL and OPG, with the expression of both of these factors mechanically regulated [45].
1.9 Bone disease

Bone diseases affect millions of people worldwide, and commonly cause severe long term pain and physical disability resulting in high health care costs. The prevalence of some bone diseases increase with age and other factors (e.g. obesity, lack of physical activity). Thus the ageing population and changes in lifestyle throughout the world has caused an increased burden of diseases like osteoporosis and osteoarthritis (OA) on people and society. This burden can be quantified in terms of the problems associated with them (e.g. pain and disability), or the cause of the disease (e.g. joint disease or trauma), and also in terms of who is at risk [321]. There are many different types of disease which affect bone including OP, OA, rheumatoid arthritis, Paget’s disease of the bone (PDB) and osteogenesis imperfecta (OI).

1.9.1 Common diseases affecting bone

1.9.2 OP

1.9.2.1 Background

The world health organisation (WHO) has defined OP as “a bone mineral density of 2.5 standard deviations or more below the mean peak bone mass (average of young, healthy adults) as measured by dual energy x-ray absorptiometry (DEXA), where established osteoporosis includes the presence of fragility fractures” [322]. OP can be described as low bone mass and micro architectural deterioration, resulting in increased bone fragility as well as an increased fracture risk [321]. OP presents clinically as fractures following low-energy trauma mainly in the hip vertebrae (lumbar spine), and distal forearm [321]. Fractures occur when there is a dysfunction of the endocrine factors and/or their target cells in bone, leading to the inability to
reach appropriate bone mass to maintain skeletal homeostasis. These alterations, together with genetic determinants and mechanical and nutritional cues cause the OP symptoms.

1.9.2.2 Incidence and prevalence

OP is predominantly a disease of aging, affecting particularly postmenopausal women but also older men, where it is estimated that 1:2 woman and 1:5 men over 50 will have an OP fracture in their remaining lifetime. In the UK about 23 % of women >50 years are estimated to have OP, ranging from 5 % among woman aged 50 years to 50 % at 85 years of age. In men the figures are 2.4 % aged over 50 years to 20 % at 85 years of age [323]. Furthermore, hip fractures encompass 14 % of all OP fractures, whereas colles and vertebral fractures account for 19 % and 27 % respectively [324].

1.9.2.3 Diagnosis

OP occurs when bone resorption occurs at a quicker rate than bone formation leading to an imbalance in the bone remodelling process (Section 1.2). It is asymptomatic until a fracture occurs. However low bone mineral density only accounts for 40 % of overall fracture risk for a patient. Both the WHO and the National Osteoporosis Foundation (NOF), have guidelines which suggest that when evaluating fracture risk, statistically independent predictors such as age, current smoking status, family history of osteoporotic fractures, low body mass index, hypogonadism, rheumatoid arthritis, alcohol use (more than 3 drinks/day) and glucocorticoid treatment should be considered. Furthermore, The WHO also developed the fracture risk assessment tool
(FRAX), which is a web based tool in which a 10 year probability of fracture is produced on individual patients [324].

1.9.3 Treatment

Therapeutic approaches to treat OP include (1) lifestyle and exercise (weight bearing and muscle strengthening) [325-328], (2) adequate consumption or supplementation of calcium and vitamin D [329-333] or (3) medications such as anti-resorptive therapies (maintain bone and reduce fractures) [334, 335] or anabolic therapies (stimulate bone formation) [336-338]. Anti-resorptive therapies include bisphosphonates (Alendronate, Risedronate or Zolendronic Acid), oestrogens, Calcitonin or Denosumab (RANKL inhibitor) [324], and anabolic therapies include intermittent PTH, modulating the canonical Wnt-signalling pathway or inhibiting the endogenous inhibitors with sclerostin antibodies or Dkk1 antagonists [339]. However, the only anabolic therapies currently on the market is recombinant human PTH (e.g. Teriparatide - 1-34 PTH peptide, Preotact - 1-84 PTH peptide) [340].

1.9.4 OA

1.9.4.1 Background

OA is a disorder of the musculoskeletal system which is caused by the degradation or loss of articular cartilage in synovial joints. OA is most common in the joints of the knee, hip, hand, foot and spine. OA occurs when a complex set of interactions between mechanical, biological, biochemical and molecular factors degrade the articular cartilage [341]. OA also affects all the tissues of the joint. At the same time as loss of articular cartilage, there is also subchondral bone remodelling with sclerosis, formation of cysts, osteophyte formation at the joint margins, ligamentous
contraction and relaxation, muscle atrophy and spasms, and at the clinical stages of the disease there is inflammation of the synovial membrane [341-343].

1.9.4.2 Incidence and prevalence

OA is the most common form of arthritis and affects 40% of people over the age of 70, with the numbers rising [344]. It is estimated that 9.6% of men and 18% of women aged > 60 years have symptomatic OA worldwide [321].

1.9.4.3 Diagnosis

Clinically OA presents as joint pain, tenderness, limitation of movement, crepitus, occasional effusion and variable degrees of local inflammation [321]. OA is a progressive disease that cannot be halted [321]. The main symptom of OA is pain and loss of physical function which results in impaired mobility and impaired quality of life [345]. Currently there are no sensitive diagnostic techniques apart from radiography, or disease modifying treatments. What causes OA is not fully understood, but it has been established that the disease is caused by complex interplay between environmental and genetic factors. All types of OA have age as their strongest risk factor, and a main risk factor for knee OA is obesity (specifically in women). Other risk factors include occupational physical workload, high sporting activity, joint injuries and being female [342, 346].

1.9.4.4 Treatment

OA is managed by a combination of non-pharmacologic and pharmacologic treatments [342, 347]. There are numerous non-pharmacologic treatments for OA which can be sub-divided into educational approaches and physical activities and
include musculoskeletal strengthening and weight loss [348]. Pharmacologic
treatments on the other hand include analgesics (e.g. opioids), anti-inflammatory
agents with analgesic properties (e.g. intra-articular corticosteroids) and slower
acting pharmacologic options (e.g. intra-articular hyaluronate, glucosamine sulphate
or chondroitin sulphate) [349, 350].

1.9.5 Less common diseases affecting bone

1.9.6 Rheumatoid Arthritis

Rheumatoid Arthritis is an autoimmune disease that presents clinically as
progressive bone destruction with widespread synovial joint involvement [321, 351].
Rheumatoid Arthritis predominately affects peripheral joints, which results in
systemic and chronic inflammation of the joints, causing synovitis and pannus
formation which results in long-term morbidity and increased mortality [352]. In
Rheumatoid Arthritis, the bone remodelling process is disrupted which results in
bone loss [353]. Rheumatoid Arthritis affects 0.5–1 % of the world’s population
[351]. The incidence increases with age until about 70 years of age, where twice as
many women than men are affected. Rheumatoid Arthritis is inherited, with the
genetic risk associated with the disease at 60 % [321]. Patients with Rheumatoid
Arthritis have auto-antibodies such as rheumatoid factor (RF) or anti-citrullinated
protein antibodies (ACPAs) in their serum which are used diagnostically [354]. In
the past 20 years, new drugs have provided more therapeutic options for Rheumatoid
Arthritis [352] with biologics that target inflammatory cytokines greatly improving
treatment for patients. However some patients still remain severely disabled and
there is a need for further studies of disease pathogenesis to reveal new therapeutic targets [351].

1.9.7 PDB

PDB is a common metabolic disease which affects the way bone is renewed and repaired, resulting in disorganised bone turnover which affects one or more skeletal sites [355]. It is a chronic disorder that occurs when the patient experiences excessive bone resorption which is accompanied by a secondary increase in bone formation [356]. This results in bone that is abnormal, with reduced mechanical strength, resulting in a higher risk of fractures and deformities [357]. In the UK, PDB occurs in 1-2 % of the population aged 55 and over, with approximately 8 % of the population being affected in the 8th decade [358, 359]. PDB presents clinically as bone pain that is not worsened by exercise and not improved by rest [360, 361]. It can also present as deformities, fractures or neurological complications such as headache, hearing loss, nerve compression syndrome or spinal stenosis [360, 361]. Secondary OA is also common and there is an increased risk of osteosarcoma in 0.5 % of PDB patients [360, 361]. PDB is treatable, but not all patients require treatment. Treatment of PDB includes using drugs that suppress bone turnover for the treatment of the pain as well as analgesics. In some incidences, surgery is also required to treat fractures or OA which are secondary effects of the disease [355].

1.9.8 OI

OI is an inherited skeletal disorder that presents clinically as low bone mass, bone fragility and short stature [362]. It is the most common genetic connective tissue congenital disease and in the majority of cases is caused by a defects in the genes
that encode collagen type I α chains (COL1A1 and COL1A2) [363]. The main characteristic of OI is increased bone fragility which is caused by defective matrix quality caused by defective type I collagen [364]. OI is an autosomal dominant disease, with the majority of cases is inherited from a parent although some cases are as a result of new genetic mutations. A person who has OI has a 50% chance of passing the gene and thus the disease to their children. OI varies from patient to patient and some patients are nearly asymptomatic with a mild predisposition to fractures, normal stature and normal lifespan to others who are profoundly disabled and it can even be lethal [362]. To date, there is no cure for OI and treatment is dependent of the nature and severity of the symptoms. Treatment usually includes medication (bisphosphonates), surgery and rehabilitation to prevent fractures as well as to improve function and the quality of life [362]. Other treatments are being explored as a new therapeutic option for OI, which include GH treatment, bisphosphonates, Teriparatide and gene therapies.
1.10 Aims of this study

It is possible that new methods of differentiating osteoblasts to osteocytes *in vitro* would provide novel tools to study osteocytes. Furthermore, doing this in a 3D environment would be a significant methodological development enabling a better understanding of osteocyte biology.

This study set out to develop a new 3D model of osteoblast to osteocyte differentiation using collagen type I gels. Once the model was established, the aim was then to investigate if external factors modulate this differentiation process in 3D. The final aim was to apply mechanical loading to these 3D, gel-embedded osteocytes to investigate their mechano-responses.

Specifically the aims were to:

- develop methods of, and fully characterise, differentiation of MC-3T3 cells and hOBs to mature osteocytes in 3D collagen type I gels (Chapter 3).
- compare differentiation in basal and mineralising (osteogenic) conditions (Chapter 3)
- delineate the effects of external compounds on osteoblast to osteocyte differentiation in 3D cultures (Chapter 4)
- compare the differentiation of IDG-SW3 cells in 3D gels to previously published work on IDG-SW3 cell differentiation in monolayer culture (Chapter 5)
- determine mechano-responses of IDG-SW3 osteocytes in 3D type I collagen gels (Chapter 6)
2 Materials and Methods
2.1 Materials

2.1.1 General reagents and consumables
General laboratory chemicals and reagents were obtained from Sigma-Aldrich Company Ltd (Dorset, UK) and Fisher Scientific UK Ltd (Loughborough, UK), unless otherwise stated. All reagents for molecular studies were from Promega UK (Southampton, UK), except for the TRI Reagent which was from Sigma-Aldrich Company Ltd and SyBr green which was from Life Technologies Ltd (Paisley, UK). Tissue culture plastics and flasks were all purchased from Fisher Scientific UK Ltd, whereas other tissue culture reagents and materials were purchased from Life Technologies Ltd or Sigma-Aldrich Company Ltd unless otherwise stated.

2.2 Methods

2.2.1 Monolayer cell culture

2.2.1.1 MC-3T3 cell line
The MC-3T3-E1 clone 14 mouse osteoblast cell line was purchased (ATCC®, LGC standards, Middlesex, UK) and used between passages 7–11. Sub clones of MC-3T3-E1 cells were derived by Wang et al 1999, and in brief this involved inducing differentiation of the MC-3T3-E1 cells by diluting the amount of ascorbic acid used from the usual 50 µg/ml necessary to induce differentiation [117]. These cells were grown at a density of 100 cells/100 mm dish and cultured for two-three weeks, with the well isolated colonies harvested using cloning rings and then transferred to duplicate 24 well plates. One well was frozen down and the other was grown until confluence, and treated with cell culture medium containing ascorbic acid for 10 days, and then the inorganic phosphate concentration was raised to 3 mM. After two
days the sub clones generated were scored for the presence of von Kossa staining mineral. MC-3T3-E1 clone 14 had the ability to form a highly mineralised extracellular matrix [117].

MC-3T3-E1 clone 14 cells were cultured in minimum essential medium alpha (α-MEM) without ribonucleosides or deoxyribonucleosides (Life Technologies Ltd). A stock solution of α-MEM was prepared by dissolving α-MEM (10.08 g) and sodium hydrogen carbonate (NaHCO₃, 2.2 g/l, 0.025 M) in one litre of distilled water (dH₂O). The medium was sterilised by passing through a 0.2 µM bottle top rac filter (VacuCap 90) and 500 ml aliquots transferred into sterile glass bottles. The medium was stored at 4°C and penicillin (50 U/ml) and streptomycin (50 µg/ml) were added prior to use. Fetal calf serum (FCS; 10 %) was added prior to use with the same batch of FCS used throughout this project. This medium is referred to as basal medium. The cells were cultured in 75 cm² tissue culture flasks in basal medium and maintained at 37°C in a humidified 5 % CO₂:95 % air atmosphere. The cells were passaged by trypsinisation when 80 % confluent (split 1:6, Section 2.2.5) with the culture medium changed routinely two times a week.

2.2.1.2 IDG-SW3 cell line

The IDG-SW3 mouse early osteoblast cell line was kindly provided by Professor Lynda Bonewald (University of Missouri-Kansas, Texas, USA) and used between passages 18–26. These cells are derived from the long bones of 3-month old immortomouse⁺/−/DMP-1-GFP ⁺/− mouse. These mice have an IFN-γ inducible promoter driving expression of a thermolabile large T-antigen (H-2Kb-tsA58) enabling conditionally immortalization of cells derived from their tissues [108].
Following removal of the periosteum by scraping, both ends of the bones including the growth plate cartilage were removed, and the marrow flushed. The bone pieces were then cut into smaller chips and sequentially digested in collagenase in Hanks balanced salt solution. The digested bone chips were cultured for two days at 37–38°C to allow cells to grow out of the mouse bone chips [108]. Under osteogenic conditions, these IDG-SW3 cells represent mature osteocytes as they express DMP-1 and E11 and have a mineralised extracellular matrix formation.

IDG-SW3 cells were grown in collagen type I (BD Biosciences, Oxford, UK) coated 75 cm² tissue culture flasks in α-MEM (Section 2.2.1.1) containing 10 % heat inactivated (HI) FCS and 50 U/ml IFN-γ (Life Technologies Ltd) referred to as proliferation medium, with the same batch of HI FCS used throughout this project. These cells were maintained at 33°C in a humidified 5 % CO₂:95 % air atmosphere with the medium routinely changed three times a week. These cells were passaged by trypsinisation when 70 % confluent (split 1:10, Section 2.2.1.4) with cells either reseeded in a new flask or set up for an experimental procedure, where they were seeded at a known density in 6 or 48 well plates for monolayer or 3D culture respectively.

2.2.1.3 hOBs cells

hOBs cells were established from a number of different donor patients as described in Table 2.1, using bone obtained from waste material following total knee replacement (TKR) surgery with informed consent from the donors and following the guidelines of the Research Ethics Committee for Wales. Ethical approval for this
research was granted to the Arthritis Research UK Biomechanics and Bioengineering Centre under multi-project ethical approval (10/MRE09/28).

Bone cores were drilled out of the tibial plateau of the knee, broken up using bone cutters, and washed extensively in Dulbecco’s phosphate buffered saline (DPBS) containing penicillin (50 U/ml) and streptomycin (50 µg/ml) until the bone chips were white and all the marrow had been removed. The bone chips were set up in explant cultures in 75 cm$^2$ flasks in α-MEM containing 10 % human serum (HS, Sera Laboratories International, West Sussex, UK), penicillin (50 U/ml) and streptomycin (50 µg/ml), with several different batch numbers of HS used throughout this project. This medium was referred to as basal medium. The explant cultures were maintained at 37°C in a humidified 5 % CO$_2$:95 % air atmosphere and the flasks were not moved, nor medium changed for the first week. For the second week medium was changed twice a week, and cells were passaged from the explant culture using trypzean (0.5 ml, Sigma Aldrich Company Ltd), once 80 % confluent and the cells were resuspended in new 75 cm$^2$ flasks. The cells in the new 75 cm$^2$ flasks were placed in Therapeak medium (Lonza Group Ltd, UK) for two weeks, with medium being changed twice a week, and once 80 % confluent trypzean was used to passage cells. Following this, the 75 cm$^2$ flasks were returned to basal medium for one week prior to using the cells for 3D culture (Section 2.2.2.3). For all assays hOBs were used between passages 2-4.
### Table 2.1: Human patient samples used throughout my thesis

<table>
<thead>
<tr>
<th>Patient number</th>
<th>Age</th>
<th>Sex</th>
</tr>
</thead>
<tbody>
<tr>
<td>TKR 14</td>
<td>56 yrs 8 months (mths)</td>
<td>Female</td>
</tr>
<tr>
<td>TKR 22</td>
<td>62 yrs 2 mths</td>
<td>Female</td>
</tr>
<tr>
<td>TKR 26</td>
<td>59 yrs 7 mths</td>
<td>Male</td>
</tr>
<tr>
<td>TKR 32</td>
<td>66 yrs 4 mths</td>
<td>Male</td>
</tr>
<tr>
<td>TKR 34</td>
<td>59 yrs 3 mths</td>
<td>Male</td>
</tr>
<tr>
<td>TKR 35</td>
<td>75 yrs</td>
<td>Female</td>
</tr>
</tbody>
</table>

#### 2.2.1.4 Cell culture maintenance

When cells were 70-80 % confluent the medium was removed and the cells were washed in DPBS and 0.25 % trypsin/0.25 % w/w (weight/weight) EDTA (Worthington Biochemical Corp. distributed by Lorne Laboratories Ltd, Reading, Berkshire, UK) in PBS or 0.5 ml of trypzean (hOBS only) was added for 3–5 minutes (mins) at room temperature. Cell detachment was monitored by light microscopy and was aided by the gentle tapping on the side of the flask. The trypsin/EDTA or trypzean was inactivated by the addition of 4 ml of the appropriate medium. The cells were then either reseeded in a number of new flasks (standard or collagen type I coated) depending on cell type or were set up for an experimental procedure. In order to set up for an experimental procedure, the cell suspension was centrifuged for 3 mins at 1,700 rotations per minute (rpm) at room temperature using a bench top centrifuge (Sanyo Harrier 18/80, MSE UK Ltd, London, UK). The supernatant was discarded and the cells were resuspended in a known volume of the appropriate medium, counted with a haemocytometer (Section 2.2.1.5) and seeded at a known density in 6 or 48 well plates for monolayer or 3D culture respectively.
2.2.1.5 **Cell counting**

Cell suspensions were diluted appropriately (to give 20–50 cells per square of haemocytometer chambers) in a known volume of growth medium and 7 µl transferred to each chamber of a haemocytometer. Cells were counted using x100 magnification and five individual squares were counted from each haemocytometer chamber with total cell number calculated as follows:

<table>
<thead>
<tr>
<th><strong>Cell number per cell suspension volume:</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean cell count (haemocytometer) x total cell suspension volume (ml) x 10⁴</td>
</tr>
<tr>
<td>(haemocytometer factor)</td>
</tr>
</tbody>
</table>

2.2.1.6 **Cryopreservation of cells**

MC-3T3 or IDG-SW3 cell lines were stored in liquid nitrogen for long term storage. Cells were grown in 75 cm² tissue culture flasks as previously described (Sections 2.2.1.1 and 2.2.1.2) until reaching 90 % confluency. Cells were trypsinised as described previously (Section 2.2.1.4), pelleted and resuspended in 3 ml of freezing medium. This was 50 % α-MEM, 40 % serum (FCS for MC-3T3s or HI FCS for IDG-SW3) and 10 % dimethyl sulfoxide (DMSO). The mixture was pipetted up and down to ensure an even cell suspension prior to adding 1 ml aliquots to cryotubes (Thermo Fisher Scientific, Loughborough, UK), and placed at -80°C in a cryocontainer containing isopropanol overnight. The vials were then transferred to liquid nitrogen for long term storage.

To resurrect cells, the vials were quickly thawed in hot tap water (45°C) and the thawed cells transferred to a 75 cm² flask containing the appropriate growth medium for the cell type, and incubated overnight at either 33°C or 37°C (depending on cell
type) in a 5 % CO₂:95 % air atmosphere. The following day the medium was replaced to remove the remaining DMSO in the culture medium.

2.2.2 Cell culture in 3D

2.2.2.1 MC-3T3s in 3D collagen type I gels

Rat tail tendon type I collagen (Sigma-Aldrich Company Ltd) was reconstituted to a concentration of 2.5 mg/ml in 7 mM acetic acid at least a day before use. To aid reconstitution the solution was placed on a roller mixer for tubes at room temperature for at least 3 hours (hrs). To set up cells in 2 mg/ml collagen gels equal volumes of 1X Minimum Essential Medium (Sigma-Aldrich Company Ltd.) and NaHCO₃ were mixed on ice and adjusted to pH 7.0 using 2-amino-2-hydroxymethylpropane-1,3-diol (Tris Base, pH 11.5; 1M) prior to the addition of MC-3T3 (7.0 x 10⁵ cells/ml gel) cells. The cells were evenly dispersed throughout the collagen solution prior to the addition of 250 µl to each well in a 48 well plate. The plate was placed at 37°C in a humidified 5 % CO₂:95 % air atmosphere. After 30 mins, 750 µl of basal medium (Section 2.2.1.1) was gently added to the top of each gel. The 48 well plate was placed back in the 37°C incubator and the medium was changed on the day after set up, and every two days after this.

2.2.2.2 IDG-SW3s in 3D collagen type I gels

The collagen gel method for IDG-SW3 cells was as described (Section 2.2.2.1) for MC-3T3 cells, but with the following modifications:

1. Once the gel solution was added to the 48 well plate, the plate was placed at 33°C in a humidified 5 % CO₂:95 % air atmosphere for 30 mins.

2. The basal medium used was as described (Section 2.2.1.1).
3. On the 4th day following set up the medium was changed to induce osteogenic differentiation and the plate was placed at 37°C in a humidified 5 % CO₂:95 % air atmosphere. This osteogenic medium was α-MEM containing HI FCS (10 %), ascorbate-2-phosphate (50 µg/ml) and β-glycerophosphate (4 mM). This day was referred to as day 0 at 37°C. Medium was changed the day after being placed at 37°C and every two days after this.

2.2.2.3 *hOBs in 3D collagen type I gels*

The collagen gel method for hOBs was as described (Section 2.2.2.1) for MC-3T3 cells, but with the following modifications:

1. hOBs were set up at a concentration of 8.0 x 10⁴ cells/ml gel.
2. The basal medium was used as described (Section 2.2.1.1)

2.2.2.4 *Mineralising medium for MC-3T3s and hOBs in 3D collagen type I gels*

In some 3D experiments mineralisation was induced by maintaining MC-3T3s in basal medium (Section 2.2.1.1) containing ascorbate-2-phosphate (50 µg/ml), β-glycerophosphate (10 mM) and dexamethasone (10⁻⁸ M). For hOB cells, the mineralising (Min) medium was basal medium (Section 2.2.1.3) containing ascorbate-2-phosphate (50 µg/ml), β-glycerophosphate (2 mM) and dexamethasone (10⁻⁷ M). For both cell types the Min medium was added to the gels the day after set up and every two days after this.
2.2.2.5 **Cell number and viability in 3D collagen gels**

Gels were removed from wells using a spatula and placed in 1.5 ml eppendorf tubes. Collagenase (250 µl, 1 mg/ml, Sigma Aldrich Company Ltd) was added to each gel and placed in a 37°C water bath until the collagen was digested (8-20 mins, time varied depending on how long the gels had been in culture). The cells were centrifuged (1,700g, 30 s, ALC micro centrifuge 4204) to pellet, washed in DPBS to remove the collagenase and re-suspended in a known volume of medium and counted as described (Section 2.2.1.5).

Cell viability was determined with trypan blue (Sigma-Aldrich Company Ltd) uptake following collagenase digestion. For this a live/dead assay was undertaken, by transferring an aliquot (20 µl) of the cell suspension to a new eppendorf tube and a trypan blue solution (0.4 % w/v, 4 µl, 1:1, trypan blue:DPBS, Sigma-Aldrich Company Ltd) was added. The cell solution (7 µl) was transferred to each chamber of the haemocytometer, and both live and dead cells were counted using x100 magnification as described (Section 2.2.1.5). Total cell number and cell viability was calculated as follows:

<table>
<thead>
<tr>
<th>Total number of cells per cell suspension volume:</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean cell count (haemocytometer) x total cell suspension volume (ml)</td>
</tr>
<tr>
<td>x $10^4$ (haemocytometer factor) x trypan blue dilution factor (1.2)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Cell viability (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>live cell count/total cell count x 100</td>
</tr>
</tbody>
</table>
2.2.3 Analysis of mRNA expression

2.2.3.1 RNA extraction

Following assessment of cell number and viability, cells were pelleted by centrifugation (1,700g, 30 s), the supernatant removed and the cells lysed in TRI Reagent (500 µl) in a 1.5 ml eppendorf tube. Tubes were inverted and left to stand at room temperature for 5 mins and stored at -80ºC until used for RNA extraction.

Samples in TRI Reagent were thawed, centrifuged for 15 s, and chloroform (200 µl) added to each tube. The samples were inverted vigorously for 30 s, incubated at room temperature for 3 mins, centrifuged (12,000g, 15 mins), and the supernatant removed and placed into a new 1.5 ml eppendorf tube. Isopropanol (250 µl) and glycogen (1 µl, Ambion, Life Technologies Ltd) were added to each sample, incubated at room temp for 10 mins, centrifuged (12,000 g, 10 mins), and the supernatant discarded leaving an RNA precipitate. The RNA precipitate was washed in 70 % ethanol in RNase free H₂O (400 µl) and vortexed (IKA® Lab Dancer test tube shakers, Sigma Aldrich Company Ltd) prior to centrifugation (12,000 g, 5 mins). The supernatant was discarded and the remaining precipitate was left to air dry for a maximum of 10 mins before re-suspension in RNase free H₂O (25 µl) and placed in a 55ºC water bath for 10 mins. The resulting RNA solution was either placed directly into sodium acetate (Section 2.2.3.2) to precipitate the RNA or was placed in the freezer (-80ºC) until needed.

2.2.3.2 Sodium acetate precipitation of RNA

The RNA was re-precipitated using sodium acetate to improve the purity of the RNA following the RNA extraction procedure. Sodium acetate (2.5 µl, 3 M) and glycogen
(1 µl) were added to the RNA (25 µl) from Section 2.4.1. To this, 95 % ethanol in RNase free H₂O (71.25 µl) was added and the tube was inverted (10 times) prior to being placed at -20°C for between 30 mins to 24 hrs. Samples were centrifuged (12,000 rpm, 15 mins) and the supernatant discarded leaving the RNA precipitate in the tube. The RNA precipitate was washed in 70 % ethanol in RNase free H₂O (400 µl) and vortexed prior to centrifugation (12,000 g, 5 mins). The supernatant was discarded and the remaining precipitate was left to air dry for a maximum of 10 mins before re-suspension in RNase free H₂O (25 µl) and placed in a 55°C water bath for 10 mins. The resulting RNA solution was placed directly into a DNase I digest (Section 2.2.3.3) or was placed in the -80°C until needed.

### 2.2.3.3 DNase I digest of RNA

A DNase I digest was undertaken on all samples following sodium acetate precipitation to remove contaminating DNases. DNase I buffer (2.5 µl, Life Technologies Ltd) and DNase I enzyme (1 µl, Life Technologies Ltd) were added to each of the RNA samples, and mixed gently prior to being placed in the thermocycler (37°C, 40 mins, DNA thermo cycler 480, Perkin Elmer Inc. Cambridge, UK). The DNase enzyme was inactivated by the addition of DNase I inactivation reagent (5 µl, Life Technologies Ltd) to each tube, mixed (3 times over a 2 mins period) and centrifuged (12,000g, 2 mins). The supernatants were transferred to new 0.5 ml sterile eppendorf tubes and an aliquot was measured for RNA concentration and quality (Section 2.2.3.4), with the remaining RNA stored in the freezer (-80°C).
2.2.3.4 Determining quantity, purity and quality of RNA in samples

The purified RNA samples were quantified using a Nanodrop (Nanodrop-1000). For this each sample (1 µl) was added to the Nanodrop and the optical density at 260/280 nm and 260/230 nm wavelengths measured to estimate the RNA purity. The 260/280 nm measurement measures RNA purity, with a ratio of ~2.0 considered pure for RNA measurements. Significantly lower ratios may indicate the presence of protein, phenol or other contaminants that absorb strongly at or near 280 nm. The 260/230 nm measurement is a secondary measurement of nucleic acid purity, with a ratio of 2.0–2.2 considered pure for RNA measurements. Lower values than this may indicate the presence of contaminants that absorb at 230 nm. Figure 2.1 shows an example of the output (ng/µl) from the nanodrop.

Random samples from each of the cell types used in this thesis were also selected to check the quality of the RNA using the Agilent 2100 Bioanalyzer (Central Biotechnology Services, School of Medicine, Cardiff University). This measures the RNA integrity by measuring the RNA integrity number (RIN) and also gives an estimate of the RNA concentration and purity, with the results being displayed as a gel-like image as well as an electropherogram. An example of this is shown in Figure 2.2.
Figure 2.1: An example of the typical spectral pattern for nucleic acid from the Nanodrop from IDG-SW3 cells extracted on day 21 from rat tail tendon type I collagen gels.

Figure 2.2: Results generated from the Agilent 2100 Bioanalyzer of MC-3T3 cells extracted on day 15 from rat tail tendon type I collagen gels. In this figure is the electropherogram of a high quality total RNA sample where the 18S and 28S peaks are clearly visible at 41 and 48 s respectively. Samples are detected by their fluorescence and then are translated into electropherograms or into gel- like images.
2.2.3.5 Reverse transcription (RT)

For RT of RNA, 500 ng of total RNA was heated to 65°C for 10 mins in a thermo cycler to melt the secondary RNA structures. The tube was then cooled immediately on ice to prevent the secondary structures from re-forming. A master mix containing moloney murine leukemia virus reverse transcriptase (M-MLV RT), moloney murine leukemia virus 5X reverse transcriptase (M-MLV 5X RT) buffer, oligo (dT)15 primer (Oligo dT), recombinant RNasin ribonuclease inhibitor (RNasin RI) and deoxynucleotide triphosphates (dNTPs) was prepared using the volumes shown in Table 2.2. One volume of the master mix (5.9 µl) was added to a new PCR eppendorf with the 500 ng of total RNA and enough RNase free H2O to bring the final volume per reaction to 20 µl. A no enzyme RT control was also undertaken using all components of the RT reaction described above but substituting RNase free H2O for the M-MLV RT. All prepared tubes were centrifuged (12,000g, 30 s) and placed in the thermo cycler (DNA Thermo cycler 480, 37°C, 1 hr). The tubes were placed in the freezer (-20°C) until quantitative RT polymerase chain reaction (RT-qPCR) was performed.

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Concentration /reaction</th>
<th>Volume/reaction</th>
<th>Volume/reaction (no enzyme RT)</th>
</tr>
</thead>
<tbody>
<tr>
<td>M-MLV RT</td>
<td>100 U</td>
<td>0.5 µl</td>
<td>-</td>
</tr>
<tr>
<td>M-MLV RT buffer</td>
<td>5 X</td>
<td>4 µl</td>
<td>4 µl</td>
</tr>
<tr>
<td>Oligo Dt</td>
<td>0.1 µg</td>
<td>0.2 µl</td>
<td>0.2 µl</td>
</tr>
<tr>
<td>dNTPs</td>
<td>10 mM</td>
<td>1 µl</td>
<td>1 µl</td>
</tr>
<tr>
<td>RNasin RI</td>
<td>8 U</td>
<td>0.2 µl</td>
<td>0.2 µl</td>
</tr>
<tr>
<td>RNA</td>
<td>500 ng</td>
<td>varies depending on Nanodrop readings</td>
<td></td>
</tr>
<tr>
<td>RNase free H2O</td>
<td>-</td>
<td>to 20 µl</td>
<td>to 20 µl</td>
</tr>
</tbody>
</table>

Table 2.2: Concentration and volume of reagents used for a RT reactions
2.2.3.6 Isolation of bone cells from mouse femur

Bone cells were isolated from mouse femur as a positive control for all murine primers for the RT-qPCR work described in this thesis. For this, the femur was placed in liquid nitrogen in a pestle and mortar, until the liquid nitrogen was evaporated off. The bone was homogenised to a fine powder, and placed into a bijou tube. This was weighed to calculate how much TRI Reagent to add to the sample. RNA extraction was undertaken (Section 2.2.3.1).

2.2.3.7 Primer design

Sequences for the genes of interest were obtained from the nucleotide genome browser programme (http://www.ncbi.nlm.nih.gov/nuccore). Primers were designed using primer-blast (http://www.ncbi.nlm.nih.gov/tools/primer-blast) software and where possible the forward and reverse primers were designed in different exons. For the most part the desired primer length did not exceed 250 base pairs (bp) to increase the quality of the PCR reaction. Primers (Table 2.3 and Table 2.4, mouse or human respectively) were either designed in this way, taken directly from the literature or were designed by other people in our research group. These forward and reverse sequences were blasted using primer-blast software to check that each pair had a Tm (melting temperature) close to 65°C; each primer had an appropriate G-C content and the expected amplicon length. In addition it was ensured that they were not homologous to other eukaryotic sequences. All primers were purchased from Life technologies Ltd.
<table>
<thead>
<tr>
<th>Primers</th>
<th>Forward (5’–3’)</th>
<th>Reverse (3’-5’)</th>
<th>Amplicon length (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>E11</td>
<td>TGGCAAGGCACCTCTGGTA</td>
<td>TGAGTGGACAGTTCTCTAAGG</td>
<td>60¹</td>
</tr>
<tr>
<td>DMP-1</td>
<td>TGTCATTCTCCTTTGTGTTTCCTTTG</td>
<td>AGAGCTTTCAGATTCAGTATTGTGTAT</td>
<td>82¹</td>
</tr>
<tr>
<td>Cx43</td>
<td>CAGGCCGGAAGCACCAT</td>
<td>GCTGTCGTCAGGGGAAATCAA</td>
<td>60</td>
</tr>
<tr>
<td>FGF-23</td>
<td>GTGTCAGATTTCAAACTCAG</td>
<td>GGAAGCTTTCAGATTCAGTATTGTGTAGT</td>
<td>120</td>
</tr>
<tr>
<td>Sclerostin</td>
<td>CCACCATCCCTATGACGCCAA</td>
<td>TGTCAGGAAGCGGGGTGTAATGT</td>
<td>73²</td>
</tr>
<tr>
<td>MEPE</td>
<td>AGTATGACCTGGGCGGCACC</td>
<td>ACCTTGCCCATCTCTGTGCCT</td>
<td>135</td>
</tr>
<tr>
<td>PHEX</td>
<td>GGAAGAAAACCATTGCAATTATT</td>
<td>CGCCTGCTGAGGTTTGA</td>
<td>70</td>
</tr>
<tr>
<td>LRP-5</td>
<td>CTTCGCCACGAGATATGGTTGTG</td>
<td>AAGGGACAGCGAGCTGTGAGC</td>
<td>81</td>
</tr>
<tr>
<td>ALP</td>
<td>GCTGGCCCTTGACCCCTCCA</td>
<td>ATCCGAGGGCCACCTCCAC</td>
<td>132</td>
</tr>
<tr>
<td>VEGF</td>
<td>GTCCGAGCCGAGGAGGGGAGC</td>
<td>CGTGGGTGACAGCTGGGGAC</td>
<td>143</td>
</tr>
<tr>
<td>RANKL</td>
<td>TGGAAGGCTCAGGTGTTGGAT</td>
<td>CATTGATGTTGAGGTGTGCAA</td>
<td>75</td>
</tr>
<tr>
<td>OPG</td>
<td>GAGTGTGAGGAAGGGCCTTGAC</td>
<td>GCAAATGTGTGTTTCGCTCTG</td>
<td>111</td>
</tr>
<tr>
<td>GAPDH*</td>
<td>GACGGCGCATCTCTCTCTGTGCA</td>
<td>TGCAAATGGCAGCCTGTTGAC</td>
<td>114</td>
</tr>
<tr>
<td>PCNA*</td>
<td>GGCGCGAGAGGTGGTGTTAGT</td>
<td>ATGGTGGGAGTTTGGTGGCG</td>
<td>133</td>
</tr>
<tr>
<td>18sRNA*</td>
<td>GCAATTATTCCCCCATGACCGG</td>
<td>GCCCTCACTAAACCACATCCAA</td>
<td>125</td>
</tr>
</tbody>
</table>

**Table 2.3:** Mouse (Mus musculus) primer sequences used for RT-qPCR reactions

*GAPDH: Glyceraldehyde-3-phosphate dehydrogenase, PCNA: Proliferating cell nuclear antigen or 18sRNA: 18s ribosomal RNA

¹E11 and DMP-1 sequences were obtained from Gupta et al 2010 [202] and ²sclerostin sequence was obtained from Vincent et al 2009 [365]
Table 2.4: Human (Homo sapiens) primer sequences used for RT-qPCR reactions

*36B4: acidic ribosomal phosphoprotein P0
*RPL13A: Ribosomal Protein L13a

2.2.3.8 RT Quantitative polymerase chain reaction (RT-qPCR)

RT-qPCR was performed using SyBr Green master mix following the RT reaction (Section 2.2.3.5) using the MX3000P thermal cycler system (Stratagene, Cambridge, UK). Each PCR reaction had a final volume of 25 µl which consisted of SYBR green master mix, cDNA template, forward primer, reverse primer and RNase free H2O at volumes described in Table 2.5. A master mix for each gene of interest was prepared in 1.5 ml eppendorff tubes and 24 µl of this was added to individual wells in a 96 well PCR plate, and cDNA (1 µl), no RT control (1 µl) or water blank (1 µl) to be tested was added to the respective wells. Caps were placed on the individual wells, and the whole plate was centrifuged (2,000 g) using the Heraeus Multifuge 3SR Plus (Fisher Scientific UK, Ltd) for 3 mins. The RT-qPCR reaction was undertaken using the following cycling parameters: 50°C for 2 mins (UDG incubation), 94°C for 2 mins (activation of DNA polymerase) followed by 40 cycles of 95°C for 15 s (denaturation step) and 60°C for 30 s (annealing step to amplify the specific target sequence). The final step of the RT-qPCR reaction was a melting curve analysis to look at the dissociation curve which consisted of one cycle of 95°C for 1 mins, 55°C
for 30 s and 95°C for 30 s. An example of this thermal profile for the RT-qPCR reaction is shown in Figure 2.3.

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Concentration/reaction</th>
<th>Volume/reaction</th>
<th>No RT</th>
<th>Water blank</th>
</tr>
</thead>
<tbody>
<tr>
<td>SyBr green</td>
<td>12.5 µl</td>
<td>12.5 µl</td>
<td>12.5 µl</td>
<td>12.5 µl</td>
</tr>
<tr>
<td>Forward primer</td>
<td>10 pmol</td>
<td>1 µl</td>
<td>1 µl</td>
<td>1 µl</td>
</tr>
<tr>
<td>Reverse primer</td>
<td>10 pmol</td>
<td>1 µl</td>
<td>1 µl</td>
<td>1 µl</td>
</tr>
<tr>
<td>RNase free H2O</td>
<td>-</td>
<td>9.5 µl</td>
<td>9.5 µl</td>
<td>10.5 µl</td>
</tr>
<tr>
<td>cDNA template</td>
<td>500 ng</td>
<td>1 µl</td>
<td>1 µl</td>
<td>-</td>
</tr>
</tbody>
</table>

Table 2.5: Concentrations and volumes of reagents used for RT-qPCR reactions

Prior to using each primer pair, the amplification efficiency was calculated using qPCR analysis by measuring the slope of a standard curve created from serially diluted templates using the MX3000 pro software (Stratagene, La Jolla, CA). A dissociation step was built into the RT-qPCR reaction to check that a single dissociation curve was obtained. An example of a standard curve and dissociation curve can be seen in Figure. Agarose gel electrophoresis (Section 2.2.3.9) was undertaken to check for a single PCR product of expected amplicon length, and this was extracted from the gel and confirmed by sequencing.
Following RT-qPCR, in all experiments the most stable housekeeping gene was identified using Normfinder software (http://moma.dk/normfinder-software). For the murine cell lines, 18sRNA, GAPDH and PCNA were tested and for the hOBs, 36B4, RPL13A and beta-actin were tested. Following the selection of a suitable internal control, the relative quantitative expression of genes of interest were analysed using the MX3000 pro software (Stratagene). The relative quantification for each target gene was determined using the ΔΔCT method [366] for each individual assay and then the results of each assay were combined for further statistical analysis.
2.2.3.9 Agarose gel electrophoresis

The amplicon lengths of the PCR products were estimated using a 1.5% agarose gel containing ethidium bromide. Briefly gels were prepared by adding agarose (0.75 g, Sigma Aldrich Company Ltd) to 1X tris-borate-EDTA (TBE) buffer, by diluting a 10X TBE buffer (0.9 M tris base, 0.9 M boric acid and 0.025 M EDTA) in dH₂O, and heating on a hot plate magnetic stirrer (VWR International Ltd, Leicestershire UK) until dissolved. This was left to cool prior to the addition of ethidium bromide (2.5 µl). The gel solution was poured into a gel tray, left to gel for 30 mins at room temperature, and PCR products and a 50 bp DNA ladder (New England Biolabs Inc.) added to individual wells. Loading buffer (2 µl, 75 µM bromophenol blue, 0.02 M sucrose) was added to each PCR product prior to adding to the individual wells. The gel was run at 100 volts (power pack supply model 100, Bethesda Research Laboratories) for 1 hr. Following this the gel was visualised and photographed under UV transillumination (BioDoc-It® Imaging Systems, UVP®, VWR International Ltd, Leicestershire, UK) and in some cases individual bands of products were cut out for further purification prior to sequencing.

2.2.4 Cloning and DNA Sequencing

2.2.4.1 Extracting of DNA from gel

Following excision of the bands of products (Section 2.2.3.9), the gel product was weighed and the DNA was extracted using the QIAquick gel extraction kit (Qiagen
Ltd, Manchester, UK) as per manufacturer’s recommendations, with the purified DNA product stored at -20°C until needed for ligations.

### 2.2.4.2 Cloning into the pGEM®-T Vector System

DNA was ligated into the pGEM®-T Vector System (Promega UK) as per manufacturer's instructions. Prior to use, the pGEM-T vector and control insert DNA were centrifuged, with reagents for each individual ligation, as well as positive and background controls placed in individual 0.5 ml eppendorfs as described in Table 2.6. The individual reactions were mixed prior to being incubated overnight at 4°C.

<table>
<thead>
<tr>
<th>Reagents</th>
<th>Standard reaction</th>
<th>Positive control</th>
<th>Background control</th>
</tr>
</thead>
<tbody>
<tr>
<td>2X rapid ligation buffer, T4 DNA ligase</td>
<td>5 µl</td>
<td>5 µl</td>
<td>5 µl</td>
</tr>
<tr>
<td>pGEM –T or pGEM-T easy vector</td>
<td>1 µl</td>
<td>1 µl</td>
<td>1 µl</td>
</tr>
<tr>
<td>PCR product</td>
<td>3 µl</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Control insert DNA</td>
<td>-</td>
<td>2 µl</td>
<td>-</td>
</tr>
<tr>
<td>T4 DNA ligase (3 Weiss units/µl)</td>
<td>1 µl</td>
<td>1 µl</td>
<td>1 µl</td>
</tr>
<tr>
<td>Deionised water to final volume of</td>
<td>10 µl</td>
<td>10 µl</td>
<td>10 µl</td>
</tr>
</tbody>
</table>

**Table 2.6:** Reagents required for ligation reactions

### 2.2.4.3 Bacterial transformations

Prior to bacterial transformations Lysogeny broth (LB) agar plates and LB broth were made. For each 250 ml bottle of LB agar, 5 LB agar tablets (Sigma-Aldrich Company Ltd) were added to dH₂O (250 ml), autoclaved and left to cool down, inverted to mix contents, and autoclaved again. The bottle was left to cool and ampicillin (100 µg/µl), 5-bromo-4-chloro-3-indolyl-β-d-galactopyranoside (X-Gal, 100 µg/µl) and isopropylthio-β-galactoside (IPTG, 500 µg/ml) was added. The agar was poured into Petri-dishes, which were left open under a bunsen burner to keep them sterile while the agar was setting. Once set the lids were put on, and the agar
plates were left at room temperature until needed. For each 200 ml bottle of LB broth, 4 tablets of LB broth (Sigma-Aldrich Company Ltd) were added to 200 mls of dH$_2$O, autoclaved and left to cool down, inverted to mix contents and ampicillin (100 µg/µl) was added. This was left to cool and placed at 4°C until needed.

Ligation reactions were removed from the fridge, centrifuged (13,000 rpm, 3 s), and 5 µl were added to individual 1.5 ml eppendorf tubes on ice. JM109 high efficiency component cells (Promega UK) were placed on ice to thaw quickly (5 mins) and mixed gently by flicking. On ice, competent cells (25 µl) were carefully transferred to the ligation reactions tubes. Competent cells (100 µl) were used for the uncut DNA control tubes. All tubes were mixed gently by flicking, left on ice for 20 mins, and heat shocked (50 s) in a water bath at 42°C. Tubes were returned to ice for 2 mins, and super optimal broth with catabolite repression (SOC medium, 475 µl) was added to the ligation reaction transformations, with SOC medium (900 µl) added to the DNA control tube. These were then incubated at 37°C for 1.5 hrs at 150 rpm. Individual transformation cultures (100 µl) were added onto the agar plates, and for the uncut DNA control a 1:10 dilution with SOC medium was added to the agar plates, with the transformation cultures aseptically spread around the plate using a sterile hockey stick. The plates were incubated overnight at 37°C and the next morning the white colonies were selected for screening of recombinant colonies.

2.2.4.4 Screening of recombinant colonies

The colonies that had successfully taken up the vector were white in colour as the DNA will have ligated and the vector will have disrupted the X-Gal gene, while the colonies that have not taken up the vector were blue. White colonies from each plate
Chapter 2

Materials and Methods

(5) were selected and a PCR reaction was performed on each with a final volume per reaction of 25 µl, with volumes and concentration of reagents per reaction used as described in Table 2.7. The cDNA was added to the individual PCR tubes by rubbing a pipette tip gently over a portion of the colony to be tested, with the pipette tip then added to the reaction tube to be tested and mixed in the reaction liquid to ensure the colony was transferred to the reaction tube. The pipette tip was removed and the lids were placed on the reaction tubes. No template controls were set up as an internal control for the PCR reaction, with all tubes centrifuged prior to undertaking the PCR reactions. The cycling conditions for the PCR reaction were: 95°C for 8 mins (activation of the DNA polymerase), 40 cycles of 1: 95°C for 30 s (denaturation step), 2: 60°C for 30 s (annealing step to amplify the specific target sequence), 3: 72°C for 30 s and 4: 72°C for 10 mins (final extension). Following the PCR reaction the samples were run out on a gel (agarose gel electrophoresis, Section 2.2.3.9) and the amplicon lengths were checked to ensure the correct size for each gene of interest to be sequenced, while ensuring that the no template controls were negative.

<table>
<thead>
<tr>
<th>Reagents</th>
<th>Concentration/reaction</th>
<th>Volume/reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>RNase free H₂O</td>
<td>14.4 µl</td>
<td></td>
</tr>
<tr>
<td>5X Buffer</td>
<td>5 µl</td>
<td></td>
</tr>
<tr>
<td>MgCl₂</td>
<td>25 µM</td>
<td>2.5 µl</td>
</tr>
<tr>
<td>DNTPs</td>
<td>2.5 mM</td>
<td>2 µl</td>
</tr>
<tr>
<td>Forward primer</td>
<td>10 pmol</td>
<td>0.5 µl</td>
</tr>
<tr>
<td>Reverse primer</td>
<td>10 pmol</td>
<td>0.5 µl</td>
</tr>
<tr>
<td>Taq Polymerase</td>
<td>5 U/µl</td>
<td>0.125 µl</td>
</tr>
</tbody>
</table>

Table 2.7: Reagents required for PCR reactions
Following this, 5 ml of LB broth was placed into 8 ml sterile polystyrene round bottom test tubes with screw caps (BD Biosciences), and using a pipette tip a small portion of 3 of the same colonies per gene of interest used for the PCR reactions were picked onto the tip. Under aseptic conditions the whole tip was then placed into the tube, gently swirled around to mix the colony into the broth, and the caps were placed on the tubes. The tubes were placed at 37ºC overnight at 150 rpm and the following morning minipreps were undertaken on these tubes.

### 2.2.4.5 Wizard plus SV minipreps DNA purification system

DNA purification was undertaken using the Wizard plus SV minipreps DNA purification system kit (Promega UK), as per manufacturer’s recommendation and the eluted DNA was stored at -20ºC until needed for sequencing.

### 2.2.4.6 Sequencing

Samples were sent to the DNA sequencing facility (part of the molecular biology support unit of the School of Biosciences, Cardiff University) for sequencing using the ABI 3130xl machine (Life Technologies, Ltd). To confirm that the sequences generated was the gene of interest, it was compared to the published whole gene sequences on the NCBI website.

### 2.2.5 Histological analysis of 3D type I collagen gels

#### 2.2.5.1 Fixing of collagen gels

When fixing collagen gels the medium was removed and then the whole gel was removed from the 48 well plate using a spatula. Gels were placed in individual 5 ml tubes and fixed with 1 ml of 1 % paraformaldehyde for 30 mins at 4ºC. The 1 %
paraformaldehyde was removed and the collagen gel was washed in DPBS for at least four times over a period of two days.

2.2.5.2 Tissue processing of collagen gels
Once fixed and washed the whole gel was removed from the bijou tube, placed in a Petri-dish and stained with Mayer’s Haematoxylin solution (500 µl, 3 mM Sigma-Aldrich Company Ltd) for 30 s before being placed in waxed tissue paper in a separate embedding cassette. Whole gels were processed overnight in the LEICA ASP 300 tissue processor (Vashaw Scientific Inc.) which involved them being rinsed sequentially in formalin, ethanol (70 %; 90 %; 100 %), xylene and paraffin.

2.2.5.3 Embedding of collagen gels in paraffin wax
Once processed individual gels were embedded in paraffin wax using the LEICA EG 1150H paraffin embedder (Vashaw Scientific Inc.). For this process, a little melted wax was placed in a silver mould and the whole gel was carefully removed from the waxed tissue paper using forceps, placed flat in the mould, topped with more melted paraffin wax and the cassette left on top of the mould. This was then left to solidify on ice for a minimum of one hr. The embedded sample was then popped out of the mould prior to sectioning.

2.2.5.4 Sectioning of collagen gels
Individual whole gel sections were cut at a thickness of 7 µm using the LEICA RM 2235 microtome (Vashaw Scientific Inc.). Wax ribbons were cut and a minimum of three sections were placed on to individual slides. The sections were then placed on a thermo superfrost plus slide (immunohistochemical, IHC staining) or on uncoated
microscope slide (Haematoxylin & Eosin, H&E staining). In order to ensure the sections were fixed to the slides, all slides were placed on slide racks in a 60°C incubator overnight on slide racks prior to staining or storage for later use.

2.2.5.5 **H&E staining of gels**

A minimum of three slides per gel were stained with H&E with all sections for an individual experiment stained at the same time. For this process slides were initially sequentially deparaffinised in xylene (2 x 5 mins), and rehydrated in 100 % ethanol (2 x 5 mins), 95 % ethanol (2 x 3 mins), 70 % ethanol (2 x 3 mins) and dH2O (2 x 2 mins). The slides were stained in filtered Mayer’s Haematoxylin solution (3 mM, 8 mins), and washed in tap water (5 mins). The slides were decolorized in acid alcohol (0.27 M, 1 s), rinsed in tap water (5 mins), immersed in lithium carbonate (0.02 M, 3 s) and again rinsed in tap water (5 mins). The sections were counterstained with Eosin (0.01 M, 15 s) and dehydrated by being placed in 70 % ethanol (2 x 3 mins), 95 % ethanol (2 x 3 mins) and 100 % ethanol (2 x 3 mins). Slides were cleared in xylene (2 x 5 mins) and mounted with Di-N-Butyle Pythalate in Xylene (DPX) mounting medium. This H&E staining was undertaken in an automated slide staining machine. Slides were left to air dry overnight prior to being imaged using the ScanScope digital slide scanner (Aperio).

2.2.5.6 **IHC staining of gels**

IHC staining was undertaken on individual sections using antibodies specific for ALP, E11, DMP-1 and RANKL. All IHC analysis was undertaken at the same time for each protein being tested for all experimental replicates.
2.2.5.7 IHC staining protocol

Individual slides were sequentially deparaffinised by immersing the slides in xylene (2 x 5 mins) and rehydrated by immersing the slides in 100 % ethanol (2 x 5 mins), 95 % ethanol (2 x 3 mins), 70 % ethanol (2 x 3 mins) and dH₂O (2 x 2 mins). Slides were immersed in DPBS containing 0.5 % tween-20 (buffer) for 5 mins. Using a hydrophobic marker (Vector Laboratories Ltd, Peterborough, UK) rings were drawn around individual sections, endogenous peroxidises were quenched with 0.3 % hydrogen peroxide (H₂O₂) in 0.3 % normal serum (Vectamount Universal Elite ABC kit, Vector Laboratories Ltd) and slides were placed in buffer for 5 mins. Sections were blocked by placing in diluted normal blocking serum (Vectamount Universal Elite ABC kit, Vector Laboratories Ltd) for 20 mins. Excess serum was blotted and slides were incubated in either primary antibody or IgG isotype control in a humidity chamber for 3 hrs (room temp) or overnight (4°C) depending on which antibody was being used (as described in Table 2.8). Slides were washed in buffer for 5 mins before incubating with diluted biotinylated secondary antibody (Vectamount Universal Elite ABC kit, Vector Laboratories Ltd) for 20 mins. Slides were washed in buffer, incubated in Vectastain Elite ABC reagent (Vectamount Universal Elite ABC kit, Vector Laboratories Ltd) for 30 mins. Slides were washed in buffer, incubated in DAB substrate chromagen for peroxidises (Vector Laboratories Ltd) for between 2–10 mins. Slides were rinsed in tap H₂O for 5 mins, counterstained with Mayer’s Haematoxylin for 30 s, washed in dH₂O, before sequential dehydration. For this all slides were placed in 70 % ethanol (2 x 3 mins), 95 % ethanol (2 x 3 mins), 100 % ethanol (2 x 5 mins) and xylene (2 x 5 mins). Slides were mounted using DPX mounting medium, left to air dry overnight and imaged using the ScanScope digital slide scanner (Aperio).
<table>
<thead>
<tr>
<th>Protein detected</th>
<th>Primary Antibody</th>
<th>Source</th>
<th>IgG isotype</th>
<th>Source</th>
<th>Concentration used</th>
<th>Incubation length</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALP</td>
<td>monoclonal anti-human/mouse/rat/ALP antibody</td>
<td>R&amp;D systems</td>
<td>monoclonal mouse IgG &lt;sub&gt;1&lt;/sub&gt;</td>
<td>R&amp;D systems</td>
<td>10 µg/ml</td>
<td>3 hrs at room temp</td>
</tr>
<tr>
<td>E11</td>
<td>anti-mouse podoplanin antibody</td>
<td>R&amp;D systems</td>
<td>polyclonal goat IgG</td>
<td>R&amp;D systems</td>
<td>5 µg/ml</td>
<td>3 hrs at room temp</td>
</tr>
<tr>
<td>DMP-1</td>
<td>anti-mouse DMP-1 antibody</td>
<td>R&amp;D systems</td>
<td>purified sheep IgG</td>
<td>R&amp;D systems</td>
<td>10 µg/ml</td>
<td>3 hrs at room temp</td>
</tr>
<tr>
<td>RANKL</td>
<td>anti-mouse RANKL antibody</td>
<td>Abcam plc, Cambridge, UK</td>
<td>monoclonal mouse IgG &lt;sub&gt;1&lt;/sub&gt;</td>
<td>R&amp;D systems</td>
<td>10 µg/ml</td>
<td>Overnight at 4°C</td>
</tr>
</tbody>
</table>

**Table 2.8: Antibodies used for IHC staining**

2.2.6 Confocal Microscopy of 3D type I collagen gels

2.2.6.1 Fixing of collagen gels

Whole collagen gels to be used for confocal microscopy were fixed as described (Section 2.2.5.1) with the only difference being that the whole gels were fixed in the 48 well plate. The whole gels were prepared for specific immunofluorescent staining for actin filaments, 4’, 6-diamidino-2-phenylindole (DAPI) labelling of nuclei or Cx43 immunofluorescent staining.

2.2.6.2 Calcein labelling for the detection of mineralising nodules

Calcein labelling for the detection of mineralising nodules in whole gels was undertaken using live unfixed gels. For this process, media were removed from gels to be stained; calcein-AM (FITC, 500 µl, Sigma-Aldrich Company Ltd, 2 mg/ml in 0.5 M sodium bicarbonate in DPBS) was added and incubated at 37°C in a humidified 5 % CO<sub>2</sub>:95 % air atmosphere overnight. The calcein-AM was removed, washed three times in DPBS (1 ml), fixed with 1 % paraformaldehyde (500 µl) for
30 mins at 4°C and washed four times in DPBS (1 ml). Following this phalloidin labelling of actin filaments was undertaken.

2.2.6.3 Phalloidin labelling of actin filaments

Cell membranes were permeabilised with Triton-X 100 for 10 mins, washed three times over a 10 mins period in DPBS (1 ml), incubated with rhodamine labelled phalloidin-Atto 565 (TRITC, 200 µl, Sigma-Aldrich Company Ltd, 0.1 nM) for three days in the dark at 4°C. The gels were removed from 48 well plates using a spatula, placed flat on individual uncoated microscope slides, and mounted with Vectashield mounting medium with DAPI (Vector Laboratories Ltd). Clear nail varnish was placed around the cover slips and slides were left to dry in the dark. Fluorescent specimens were examined using the LeicaDM6000B upright confocal laser scanning microscope and using the Leica confocal microscope software. Confocal microscopy was undertaken on samples using the appropriate excitation and emission settings for simultaneous recording of DAPI (358 nm Excitation(max), Ex(max), 461 nm Emission(max), Em(max)), Calcein (496 nm Ex(max), 516 nm Em(max)) and phalloidin (563 nm Ex(max), 592 nm Em(max)). Serial optical sections were undertaken with step size z-stack optical sections through the specimens and were reconstructed using the Leica confocal microscope software.

2.2.6.4 Cx43 immunofluorescent staining

The presence of Cx43 was assessed by immunofluorescent staining using a Cx43 antibody. For this process whole collagen gels were fixed (Section 2.2.6.1) and cell membranes permeabilised, and incubated in rhodamine labelled phalloidin–Atto 565 (Section 2.2.6.3). Following this, whole gels were washed four times in DPBS (1 ml)
and incubated with either a mouse monoclonal Cx 43 antibody (FITC, 200µl,  Life Technologies Ltd, 5 µg/ml) or a monoclonal mouse IgG1 (R&D systems, 5 µg/ml) for 30 mins at room temperature in the dark. The gels were removed from 48 well plates using a spatula, placed flat on individual uncoated microscope slides, and were mounted, imaged and analysed (Section 2.2.6.3), with the only difference being that Cx43 had an 495 nm Ex(max) and a 519 nm Em(max).

2.2.6.5 Detection of GFP+ DMP-1 cells in IDG-SW3s in 3D culture

On day 22, IDG-SW3 cells were prepared for the detection GFP+ DMP-1 cells. For this procedure whole gels were fixed for 20 mins with 1 % paraformaldehyde (1 ml) and washed four times in DPBS (1 ml). The gels were removed from 48 well plates using a spatula, placed flat on individual uncoated microscope slides, and were mounted, imaged and analysed (Section 2.2.6.3), with the only difference being that GFP+ DMP-1 had an 490 nm Ex(max) and a 520 nm Em(max).

2.2.7 Enzyme linked immunosorbent assays (ELISA)

2.2.7.1 Duoset ELISA protocol

A DuoSet ELISA development kit (R&D systems) was used for the secretion of mouse IL-6, mouse VEGF, human IL-6, human VEGF and human RANKL and were undertaken as per manufacturer’s instructions. This protocol involved coating each well of a 96 well plate (Thermo Scientific Nunc™ Immunoplate) with capture antibody (100 µl) using a multi channel pipette. The plate was sealed and incubated at room temperature overnight. The following morning each well was washed (aspirated and washed three times with wash buffer - 0.05 % Tween-20 in DPBS) then blocked by adding reagent diluent (300 µl: 1 % bovine serum albumin in [81]
DPBS) to each well, and incubated at room temperature for 1 hr. The plate was washed, and samples (100 µl) or standards (100 µl, a seven point standard curve was generated by two-fold serial dilutions in reagent diluent) were added to the plate, and incubated on a microtitre plate shaker for 2 hrs. The plate was washed, and the detection antibody (100 µl) was added to each well, and the plate was incubated on a microtitre plate shaker for 2 hrs. The plate was washed and streptavidin conjugated to horse radish peroxidise (HRP; 100 µl, 1:200 dilution in reagent diluent) was added to each well and incubated in the dark for 20 mins. The plate was washed and the substrate solution (100 µl: 1:1 dilution of colour reagent A: H₂O₂ and colour reagent B: tetramethylbenzidine) was added to each well and incubated in the dark for 20 mins. Stop solution (50 µl; 2 N sulphuric acid) was added to each well, and the plate was gently tapped to mix the contents of each well and the optical density of each well was determined using a micro plate reader (Dynex) with the wavelength being measured set to 450 nm with wavelength correction set to 570 nm. Data collected was imported into the ismart software and a standard curve was generated from which all unknown samples were read. Individual samples were normalised to cell number prior to results of individual experiments being normalised to basal medium at the earliest time point, where this point was set to 100 %.
<table>
<thead>
<tr>
<th>Protein detected</th>
<th>Capture antibody</th>
<th>Concentration</th>
<th>Detection antibody</th>
<th>Concentration</th>
<th>Highest standard</th>
</tr>
</thead>
<tbody>
<tr>
<td>mouse IL-6</td>
<td>rat anti-mouse IL-6 capture antibody</td>
<td>2.0 µg/ml</td>
<td>biotinylated goat anti-mouse IL-6</td>
<td>150 ng/ml</td>
<td>1000 pg/ml</td>
</tr>
<tr>
<td>mouse VEGF</td>
<td>goat anti-mouse VEGF capture antibody</td>
<td>0.4 µg/ml</td>
<td>biotinylated goat anti-mouse VEGF</td>
<td>100 ng/ml</td>
<td>1000 pg/ml</td>
</tr>
<tr>
<td>human IL-6</td>
<td>mouse anti-human IL-6 capture antibody</td>
<td>2.0 µg/ml</td>
<td>biotinylated goat anti-human IL-6</td>
<td>50 ng/ml</td>
<td>600 pg/ml</td>
</tr>
<tr>
<td>human VEGF</td>
<td>mouse anti-human VEGF</td>
<td>1.0 µg/ml</td>
<td>biotinylated goat anti-human VEGF</td>
<td>100 ng/ml</td>
<td>2000 pg/ml</td>
</tr>
<tr>
<td>human TRANCE/RANKL/TNFSF11</td>
<td>mouse anti-human TRANCE/RANKL/TNFSF11</td>
<td>1.0 µg/ml</td>
<td>biotinylated goat anti-human TRANCE/RANKL/TNFSF11</td>
<td>50 ng/ml</td>
<td>5000 pg/ml</td>
</tr>
</tbody>
</table>

*Table 2.9: Specifications for individual DuoSet ELISAs*
2.2.7.2 *Mouse FGF-23 ELISA*

A two-site enzyme linked ELISA kit (Kainos Laboratories) was used for the quantification of mouse FGF-23 (in collaboration with AstraZeneca UK Limited; Sally Price and Joao Graca), with the protocol undertaken as per manufacturer’s instructions. This protocol involved the use of the FGF-23 antibody micro plate which was coated with a murine monoclonal antibody against FGF-23. To each well, assay diluent (50 µl) was added as well as either samples (50 µl) to be measured or standards (50 µl). The plate was covered and incubated on a microtitre plate shaker for 2 hrs and then well contents were aspirated and washed (300 µl) with wash buffer four times. HRP labeled FGF-23 antibody (100 µl, conjugate) was added to each well, and the plate was covered and incubated on the microtitre plate shaker for 1 hr. Well contents were aspirated and washed (300 µl) with wash buffer four times. Tetramethylbenzidine substrate (100 µl, colour reagent) was added to each well and the plate was incubated and covered in aluminium foil for 30 mins. Stop solution (100 µl, 0.5 mol/l sulphuric acid) was added to each well and the plate was placed on a microtitre plate shaker for 1 mins and the optical density of each well was determined with the wavelength being measured set to 450 nm. A standard curve was generated from which all samples were read and following this individual samples were normalised to cell number.

2.2.7.3 *PGE\textsubscript{2} ELISA*

A competitive PGE\textsubscript{2} ELISA kit (ENZO life sciences UK Ltd, Exeter UK) was used for the quantification of PGE\textsubscript{2} as this kit used a monoclonal antibody to PGE\textsubscript{2} to bind in a competitive manner to the PGE\textsubscript{2} in the sample or standard. The protocol was undertaken as per manufacturer’s instructions, and this involved reagents to be used
being brought to room temperature before use. The number of wells (goat anti-
Mouse IgG Microtitre coated plate) to be used were placed onto the frame provided,
and standard diluent (100 µl, tissue culture medium) was added to wells labelled non
specific binding (NSB) and maximum bound (Bo) wells. A seven point standard
curve was generated by two-fold serial dilutions of a 2500 pg/ml solution of PGE₂ in
standard diluent, with 100 µl of each standard added to the appropriate wells.
Samples (100 µl) were added to appropriate wells, and standard diluent (50 µl)
added to the NSB, with blue conjugate (50 µl, alkaline phosphatase conjugated with
PGE₂) added to all wells apart from the total activity (TA) and blank wells.
Monoclonal antibody (50 µl) was added to each well with the exception of the blank,
TA and NSB wells. The whole plate was covered (plate sealer) and incubated at
room temperature on a microtitre plate shaker for 2 hrs at 500 rpm. The plate was
aspirated and washed 3 times by adding wash solution (400 µl, tris buffered saline
containing detergents), with the plate tapped firmly onto clean tissue paper to
remove any remaining wash buffer. Blue conjugate (5 µl) was added to the TA
wells, and p-nitrophenyl phosphate buffer (200 µl, pNpp) added to each well, and the
plate was incubated at room temp for 45 mins. Stop solution (50 µl, trisodium
phosphate in water) was added to every well and the plate was read at 405 nm with a
correction at 570 nm. Data collected was analysed as per manufacturer’s instructions
using Graph Pad prism 5 software.

2.2.8 Stiffness testing of 3D type I collagen gels

Gel stiffness was measured in the School of Engineering in collaboration with
Professor Sam Evans. This was measured using a Losenhausen machine with MTS
controller, and for this collagen type I gels were set up (Section 2.2.2.1) in a reverse
time course so that all measurements were undertaken on the same day. The cover was removed from the 48 well plate and gel stiffness was measured individually by indentation using the Zwick Test Expert II software (Zwick Testing Machines Ltd., Herefordshire, UK). Data analysis was undertaken using a Matlab software algorithm (MathWorks, Cambridge, UK) developed by Professor Sam Evans. Following this the mean, standard deviation and standard error of the mean (SEM) were calculated for the individual timepoints using Microsoft Office Excel (One Microsoft Way, Redmond, USA) and graphs were generated using Graph Pad Prism 5 software.

2.2.9 Mechanical loading of 3D type I collagen gels

Mechanical loading of cells in 3D collagen type I gels were undertaken using a novel plate based mechanical loading method (as developed by Evans, Evans, Mason and Vazquez at Cardiff University).

2.2.9.1 Synthesis of novel 3D silicone plate

A 16 well (diameter of each well 1 cm) sterling silver plate mould (13 cm width x 11 cm height) and lid modelled on a 48 well tissue culture plate was designed and made by Professor Sam Evans in the School of Engineering and Dr. Marisol Vazquez in the School of Biosciences (Cardiff University). Silver PVC insulation tape was placed on the sides of the mould. Tech-Sil 25 silicone elastomer (Technovent Ltd, Bridgend, South Wales, UK) is a low viscosity, clear material, and was supplied with a platinum catalyst, which has a mixing ratio of 9 parts silicone to 1 part catalyst. To make one plate, silicone (180 g) and catalyst (20 g) were added to a plastic beaker (500 ml), mixed vigorously with a glass rod (5 mins), and placed into a vacuum
pump for 10 mins while ensuring the mixture did not bubble over. The valve was opened slowly, the lid removed to see if any air bubbles remained, and the solution was re-vacuumed for a further 10 mins until all air bubbles were removed. The silicone solution was poured into the mould and evenly distributed throughout with the glass rod, while ensuring all the wells were covered and a scalpel was used to remove air bubbles. The lid was placed on the mould, a G-clamp placed to secure the lid on top, and placed in an 80°C incubator for one hr. Following this the silicone plate was removed from the mould and was cured in a 100°C water bath for 4 hrs, and soaked in dH_2O for three days with the dH_2O being changed four times a day. Excess silicone was trimmed from the plate and the plate was placed in decon-90 overnight. The plate was washed in tap H_2O (15 times), dH_2O (two times) and left to air dry overnight at room temp, prior to being autoclaved. After each use, sodium hypochlorite (1 % w/v) was placed into each of the wells and this was left at room temperature overnight. The following morning the sodium hypochlorite was removed and the plate was rinsed in H_2O, prior to being placed in decon-90 overnight and washed and autoclaved as previously described.

2.2.9.2 Setting up cells in silicone plate

The silicone plate was coated with collagen type I (300 µl, BD Biosciences, 0.14 mg/ml in 0.2 N acetic acid) for 1 hr, removed and left to air dry. Collagen gels were set up in the silicone plate (Section 2.2.9.2), using the IDG-SW3s (Section 2.2.2.2) with two separate plates set up (one for control, and one for mechanical loading). On the fourth day following set up, the silicone plates were transferred to the 37°C incubator located next to the BOSE machine in the School of Biosciences, Cardiff.
University, with the medium on the gels changed three times a week. On the tenth
day mechanical loading was undertaken (Section 2.2.9).

2.2.9.3 *Mechanical loading of 3D collagen type I gel*

Mechanical loading was undertaken with the BOSE Electroforce 3200 machine
(Figure 2.4) using a 250 Newton (N) load cell and the WinTest® Controls Software.
The loading regime for all mechanical loading testing was 10 Hertz (Hz) for 5 mins
while applying a load of 2.5 N.

![Image of BOSE Electroforce 3200 machine](image)

**Figure 2.4:** The BOSE Electroforce 3200 machine illustrating the 250 N load
cell and the adjustable shelf.

One hour prior to loading the medium was changed on both plates, and the plates
were returned to the incubator. During this time the mechanical loading rig was set
up (Figure 2.5) and the WinTest® Controls Software programme launched and the
load cell was turned off while the loading conditions were set up. The load range was
set to a minimum of -5 N and a maximum of +5 N, and the displacement was set to a
minimum of -6.000 mm and a maximum of +6.000 mm. The mechanical loading rig (loading stage) was placed on the adjustable shelf of the BOSE machine, and the load cell was connected to the BOSE machine. The thread was threaded through the load cell, through the pully of the stage and attached to the metal hooks, and secured in place with some PVC tape. The shelf was lowered to the level that the thread only had a little bit of a give on it, ensuring it was straight and perpendicular to the stage. Red beads (six in total) were placed on the stage to keep the plate from sticking to the stage and also ensuring that the beads did not cover any of the wells. The load cell was switched from off to high and the start button was pressed. Following this a sine wave and the load and displacement per cycle could be observed on the screen. This was then turned off while the silicone plate was being put into the right position.

![Image of mechanical loading rig]

**Figure 2.5:** The mechanical loading rig that was placed onto the adjustable shelf of the BOSE machine

One hour after changing the cell culture media both plates were removed from the incubator and placed on the bench, the lids were removed from both plates and the
plate to be loaded was placed on the mechanical loading rig (Figure 2.6). Mechanical loading was initiated, and after the 5 mins the plate was removed from the mechanical loading rig, the time recorded and both plates returned to the incubator. At specific time points following loading four gels were removed from both plates, medium was collected and aliquoted for ELISA (Section 2.2.7, IL-6, VEGF and PGE₂) and stored in the -80°C freezer until needed. From each plate (control and loaded) three whole gels were collagenase digested, with cell number and viability calculated using trypan blue uptake (Section 2.2.2.5), and the remaining cell suspension placed into Tri Reagent for gene expression analysis (Section 2.2.3). From each plate, one gel from each time point was placed into 1 % paraformaldehyde and prepared for morphological and immunohistochemical analysis (Section 2.2.5).

![Silicone plate](image.png)

**Figure 2.6:** The silicone plate attached to the mechanical loading rig ready for mechanical loading to be undertaken.
2.2.10 Graphical and Statistical analysis of data

All results were graphed using Graph pad prism 5 software (Graph pad software, Inc. California, USA) and unless otherwise stated are a combination of three independent experiments with an n=3 per individual treatment per time point of each individual experiment. All graphical results are a combination of the mean ± SEM on that time point.

Individual experiments were combined and were tested for:

(1) Normality (D’Agostino and Pearson omnibus normality test, Graph pad prism 5 software).
(2) Equal variance (Bartlett’s test, Graph pad prism 5 software).
(3) Presence of outlier’s (Grubb’s test, Microsoft office Excel 2010).
(4) To test that the samples follow a Gaussian distribution (in order to undertake one-way analysis of variance (ANOVA).

In all experiments statistical analysis was undertaken on complete data sets as well as when outliers were removed. Depending on what data was being analysed, T-Tests, one-way ANOVAs followed by a Bonferroni post hoc test, two-way ANOVAs followed by a Bonferroni post hoc test were undertaken or a Kruskal-Wallis test followed by a Dunn’s multiple comparison post hoc test. See individual results chapters for more details on statistics undertaken on:

(1) Cell number and viability.
(2) Gene expression.
(3) ELISA results.
3 Differentiation of osteoblasts (MC-3T3 cells or hOBs) to osteocytes in 3D collagen type I gels
3.1 Introduction

Osteocytes are terminally differentiated cells embedded in the mineralised matrix of bone, and primary osteocytes are thus difficult to isolate for *in vitro* research studies. The current methods of studying osteocytes *in vitro* have severe limitations as discussed (Section 1.5.2), and new methods for such studies are urgently needed. Osteocytes *in vivo* are derived from osteoblasts, and the work described in this chapter investigated and characterised the differentiation of osteoblasts (a mouse cell line and human primary cells) to osteocyte-like cells in 3D collagen type I gels.

3.1.1 3D culture methods for bone cells

The use of 3D collagen gels to culture osteoblasts and osteocytes *in vitro* has been proposed as an alternative method to monolayer cultures and these allow culture of such cells in an environment that more closely resembles that found *in vivo* [108, 265, 272, 274, 276, 279, 367]. Furthermore culturing osteoblasts and osteocytes in collagen gels maintains their *in vivo* 3D structure, function and cell-cell communications [277].

Previous studies where bone cells have been embedded in 3D type I collagen gels have used human bone marrow cells [368], mouse primary osteoblasts [369], hOBs [274], MC-3T3-E1 cells [279, 370], MG-63 cells [367], SaOS cells [276], MLO-Y4 cells [103, 274] and IDG-SW3 cells [108]). The 3D collagen gel method has been shown to enhance cell differentiation [276] [103, 274], and the MC-3T3, MG-63 and SaOS cells (all osteoblasts) in collagen gels appeared to have a dendritic-like osteocyte morphology [276, 279, 367, 370]. In other studies, MC-3T3 cells cultured
on top of empty collagen type I gels in osteogenic conditions were shown to migrate into the gels and differentiate to osteocytes [277].

Collagen gels have also been combined with particles or agents for in vitro bone cell culture. Mouse primary osteoblasts have, for example, been cultured in collagen type II/pentaerythritol polyethylene glycol ether tetrasuccinimidyl glutarate (PEG) gels and were shown to differentiate to osteocytes [369]. Human osteoblasts have also been cultured in collagen type I gels following dispersion of polyethylene particles throughout the gel, and these conditions enhanced differentiation [274].

Hydrogels made of collagen type I and chitosan, which provided an osteoid-model to study osteoblast differentiation, have also been used for MC-3T3 cells. Furthermore, a collagen hydrogel containing different concentrations of basic FGF has been used with rat bone marrow derived MSCs, and this showed that basic FGF treatment up-regulated collagen type I, osteopontin, bone sialoprotein and OCN up to 21 days of treatment [371]. Basic FGF also enhanced matrix mineralisation [371].

Scaffolds that do not contain collagen provide another method of culturing bone cells in 3D, but with these the bone cells attach to the scaffold surface so therefore do not fully mimic osteocytes in vivo. Scaffolds currently used have been synthesised from calibrated biphasic calcium phosphate particles [265], corn starch with poly(ε-caprolactone) [372], silicon-stabilized tricalcium phosphate [373], and calcium phosphate [266].
3.2 Hypothesis

Mouse (MC-3T3 cell line) and human (hOBs) osteoblasts will differentiate to osteocyte-like cells in *in vitro* 3D collagen type I gels, and mineralising (osteogenic) medium will enhance the differentiation process in both cell types.

3.3 Aims:

Specifically the aims were to investigate the following after 1, 3, 7, 11 and 15 days of culture of osteoblasts in type I collagen gels:

1. Cell number and viability (haemocytometer counting and trypan blue exclusion)
2. Cell morphology (H&E staining, confocal microscopy)
3. Gene expression (RT-qPCR: DMP-1, E11, Cx43, MEPE, PHEX, sclerostin, FGF-23)
4. Protein expression (IHC: ALP, DMP-1, Cx43, E11)
5. Protein secretion (ELISA: IL-6, VEGF - MC-3T3 cells and hOBs); RANKL - hOBs only)
3.4 Methods

3.4.1 Experimental design
Initially a range of MC-3T3 (3 x 10^5 cells/ml gel–2.3 x 10^6 cells/ml gel) and hOB (8 x 10^4 cells/ml gel and 4 x 10^5 cells/ml gel) cell densities were set up in collagen gels using basal medium (Section 2.2.1.1) to assess optimal cell number for experiments. Following this, MC-3T3 or hOBs cells were set up in collagen gels (Section 2.2.2.1 & 2.2.2.3, 3 independent experiments) and maintained in either basal or mineralising medium (Section 2.2.2.4) and outputs analysed after 1, 3, 7, 11 and 15 days. In each experiment there were 4 replicate gels per time point of which 3 gels were used for analysis of cell number and viability, gene expression and protein secretion and 1 gel used for morphological and immunohistochemical analysis following fixation and sectioning. One extra gel was also set up in each experiment for confocal microscopy to assess actin labelling and mineralising nodules (for MC-3T3: day 21, for hOBs: day 15).

3.4.2 Protein secretion, cell number and viability and gene expression
Media were removed from gels at specific time points (days 1, 3, 7, 11 and 15; n=3 at each time point), centrifuged (1,700g, 30 s) and stored in aliquots (120 µl) at -80°C until needed for measurement (ELISA) of IL-6, VEGF (MC-3T3 cells and hOBs) and RANKL (hOBs only), secretion (as described in Section 2.2.7). The gels were then collagenase digested for measurement of cell number and viability (as described in Section 2.3.5) and following this the remaining cells were placed in Tri Reagent for RNA extraction and gene expression analyses (as described in Section 2.2.2.5).
For cell number analysis (MC-3T3 or hOBs cells), data from each experiment were expressed relative to the mean day 3 basal (expressed as 100%) which enabled the 3 experiments to be pooled together.

For the 3 experiments (MC-3T3 or hOBs cells), RT-qPCR was initially undertaken on all samples at all time points using 3 reference genes (MC-3T3 cells: GAPDH, PCNA and 18sRNA, hOBs: RPL13A, 364B and beta-actin). The data generated from this were then analysed using Normfinder to select the most stable housekeeping gene to use for the 2 data sets. Following this, all gene expression data were normalised to 18sRNA for the MC-3T3 cells and RPL13A for hOBs. Data were analysed using ΔΔCt (described in 2.2.3.8) with the value from one sample from basal at day 1 (MC-3T3 cells) or basal at day 7 (hOBs) fixed to 1 in each experiment.

To provide positive controls for gene expression of osteocyte markers (sclerostin, MEPE, PHEX and FGF-23) RNA was isolated from mouse bones (Section 2.2.3.6).

### 3.4.3 Morphology and IHC analysis

In each independent experiment (for MC-3T3 cells and hOBs), one gel was removed at specific time points (days 1, 3, 7, 11 and 15) and fixed. These were subsequently sectioned for histological (morphological and IHC) analysis as described (Section 2.2.5). Sections were stained with H&E to assess cell morphology or used for IHC analyses of ALP, E11, Cx43 or DMP-1 (MC-3T3 cells only). For confocal microscopy, whole gels were fixed on day 21 for MC-3T3 cells or day 15 for hOBs and stained with phallloidin, calcein and DAPI as described in Section 2.2.6.
3.4.4 Statistical analysis of data

Most of the data presented in this chapter are a combination of 3 independent experiments with an n=3 at each data point in each experiment (see individual figure legends). Graphs in this chapter were generated from the mean ± SEM. Prior to undertaking statistical analyses, the complete data sets were tested for (1) normality (Shapiro-Wilk test, Graph Pad Prism Software), (2) equal variance (Bartlett’s test, Graph Pad Prism Software) and (3) presence of outlier’s (Grubb’s test, Microsoft office Excel 2010). There were no outliers in these data sets. All statistical analyses were undertaken on the complete data sets using:

1. a one-way or two-way ANOVA followed by a Bonferroni correction test when data was normally distributed.

2. a Kruskal-Wallis test followed by a Dunns multiple comparison post hoc test when data was not normally distributed
3.5 Results

3.5.1 Cell number and viability

Initially a range of MC-3T3 and hOB cell densities were set up in collagen gels using basal medium. For the MC-3T3 cells the numbers ranged from $3 \times 10^5$ cells/ml gel–$2.3 \times 10^6$ cells/ml gel (Figure 3.1A). Cells at both $1.5 \times 10^6$ and $2.3 \times 10^6$ cells/ml gel contracted following 3 days of culture as indicated by the black arrows in Figure 3.1A. Cells at the $3 \times 10^5$ and $7 \times 10^5$ seeding densities, however, could be maintained for 15 days. Furthermore, since H&E staining showed that after 15 days, cells seeded at $7 \times 10^5$ cells/ml gel were evenly dispersed throughout the collagen gel and seemed to be forming a network (results not shown). This seeding density was used for all subsequent MC-3T3 cell studies.

The hOBs seeding densities investigated were $8 \times 10^4$ cells/ml gel and $4 \times 10^5$ cells/ml gel (Figure 3.1B). Cells could be maintained for 15 days with both cell numbers, although cell number decreased on day 15 in gels seeded with $4 \times 10^5$ cells/ml gel. A seeding density of $8 \times 10^4$ cells/ml gel was used for all hOB subsequent experiments.

When MC-3T3 cells were maintained in basal or mineralising medium for 15 days, a two-way ANOVA of the data showed that there was a significant ($p<0.0001$) effect of time, on cell number, but no effect ($p<0.3128$) of culture medium (i.e. basal or mineralising) and no interaction ($p<0.4137$) between time and treatment. Post hoc testing (Bonferroni) showed that when maintained in basal medium, MC-3T3 cell number did not change over time. However, when the same cells were maintained in
Chapter 3: Differentiation of MC-3T3s and hOBs in 3D collagen type I gels

mineralising medium, cell number was significantly increased following 11 \( (p<0.01) \) and 15 \( (p<0.05) \) days of culture when compared to day 3 in the same medium (Figure 3.2A).

MC-3T3 cell viability was >86 % at each time point when in basal medium (Figure 3.2B) and >84 % when in mineralising medium (Figure 3.2C). Furthermore, when compared to the same medium at day 1, cell viability was significantly reduced on days 11 \( (p<0.05) \) and 15 \( (p<0.05) \) in basal medium and on day 15 \( (p<0.01) \) in mineralising medium (Figure 3.2B & C).

When hOB cells were maintained in basal or mineralising medium for 15 days, a Kruskal-Wallis test followed by a Dunns multiple comparison post hoc test showed that when in basal medium, cell number significantly \( (p<0.05) \) increased on day 15 when compared to day 3. However, when hOBs were maintained in mineralising medium, cell number significantly increased following 11 \( (p<0.05) \) or 15 \( (p<0.01) \) days when compared to cells at 3 days (Figure 3.2D).

hOB cell viability at all time points was >92 % for cells maintained in basal medium (Figure 3.2E) and >93 % for cells maintained in mineralising medium (Figure 3.2F).
Figure 3.1: Assessment of MC-3T3 and hOB cell number in basal medium in 3D collagen type I gels in a range of seeding densities. MC-3T3 cell (A) and hOBs (B) total cell number in basal medium in 3 x 10^5 cells/ml gel-2.3 x 10^6 cells/ml gel for MC-3T3 cells or 8 x 10^4 cells/ml gel and 4 x 10^5 cells/ml gel for hOBs cells. Data in A-B are from 1 (n=3 at each data point) experiment. Data in A-B are mean ± SEM, where arrows in A indicate gel shrinkage.
Figure 3.2: MC-3T3 cells and hOBs cell number and viability in basal and mineralising medium in collagen type I gels. MC-3T3 and hOB cell number expressed relative to day 3 basal (A, D) in cells maintained in basal and mineralising medium at days 1, 3, 7, 11 and 15 and % cell viability (relative to total cell number at each time point set to 100 %, B-C, E-F). Data (A-F) are a combination of 3 independent experiments (n=2 at each data point per experiment). Data in A-F are mean ± SEM. * p<0.05, ** p<0.01 versus same condition at day 3 following a two-way ANOVA and a Bonferroni post hoc test for A-C, E-F or a Kruskal-Wallis test followed by a Dunns multiple comparison post hoc test for D.
3.5.2 Morphology

3.5.2.1 H&E staining

MC-3T3 cells in gels in basal medium for 7 days had processes extending from the cytoplasm, and some of which appeared to contact with other cells (Figure 3.3A). After 11 days, there appeared to be more cells when compared to day 7. Furthermore, some of the cells appeared to have more osteocyte-like projections extending out of the cell body (Figure 3.3B). Following 15 days of culture some of the cells appear to have longer osteocyte-like dendritic processes that could be forming communication networks with neighbouring cells (Figure 3.3C).

There appeared to be more MC-3T3 cells in gels in mineralising medium after 7 days when compared to those in basal medium at the same time point (Figure 3.3D). The cells also appeared to have more osteocyte-like dendritic processes than those in basal medium; with some evidence of communication networks being formed (Figure 3.3D). Following 11 days of maintenance in mineralising media, cells appeared to have more osteocyte-like dendritic processes than those at day 7, and these appeared more elongated. (Figure 3.3E). After 15 days the cells could be forming dendritic networks (Figure 3.3F).

hOBs maintained in basal medium for 15 days, within the gel were sparse (Figure 3.4A). Furthermore, the cells do not appear to be forming osteocyte-like dendritic networks, although some may have dendritic processes (Figure 3.4A). When in mineralising medium for 15 days there appeared to be more hOB cells than when in basal medium at this time point. Some of the cells appeared to have elongated,
osteocyte-like dendritic processes extending out from the cytoplasm, and some cells could be forming dendritic networks (Figure 3.4B).

All H&E images presented were a representative of the entire depth of the gel, and similar to cell viability results presented previously confirmed that all cells were viable throughout the depth of the gels (Figure 3.3 & 3.4).

3.5.2.2 Calcein, phalloidin and DAPI labelling
Following culture of MC-3T3 cells in basal medium for 21 days, calcein labelling (green) of mineralising nodules was negative. Phalloidin labelling of actin filaments (red), revealed dendritic processes extending out from the cytoplasm in some cells. The actin filaments appeared to be organised and filamentous within cells (Figure 3.5A). When these cells were maintained in gels in mineralising medium, large numbers of calcein stained mineralised nodules were present throughout the gel after 21 days, and for the most part these nodules appeared to be extracellular. Furthermore, the actin again appeared to be highly organised within the cells. However, the cells look significantly different to those in basal medium, in that there were numerous elongated dendritic processes extending out from the cytoplasm (Figure 3.5C). In both basal and mineralising medium DAPI (blue) showed individual nucleoli present within the nuclei of the cells (Figure 3.5 A & C).

When hOBs were cultured in gels in basal medium for 15 days, mineralising nodules were not present, and within these cells the actin appeared to be very filamentous (Figure 3.5B). However it is not possible to draw any conclusions on individual cell shape, as it is hard to distinguish where one cell starts and another one ends (Figure
3.5B). When hOBs were maintained in gels in mineralising medium for 15 days, mineralising nodules were present throughout the gel and the actin appeared to be organised and filamentous within cells. However, in terms of cell shape, the cells looked different to those in basal medium and these were also different to the MC-3T3 cells maintained in mineralising medium. It is difficult to tell from the images whether these cells have osteocyte-like dendritic processes (Figure 3.5D). In both basal and mineralising medium individual nucleoli were observed (stained with DAPI) within the nuclei (Figure 3.5B&D).

3.5.3 Protein expression

3.5.3.1 ALP expression

Analysis of ALP expression in MC-3T3 cells in gels revealed that in basal medium cells stained positive for ALP, with the cellular staining intensity appearing to be lightest on day 3 (Figure 3.6A-D). On days 7 and 11 the cellular staining intensity increased, and then decreased by day 15 (Figure 3.6A-D). When the same cells were maintained in mineralising medium, the ALP cellular staining intensity on day 3 was similar to that seen in basal medium at the same time point (Figure 3.6A&E). Furthermore, the ALP cellular staining intensity in mineralising medium did not change with time (Figure 3.6E-H), and appeared to be lighter at days 7, 11 and 15 when compared to the same time points in basal medium (Figure 3.6E-H).

3.5.3.2 E11 expression

Analysis of E11 in MC-3T3 cells in gels in basal or mineralising medium by IHC revealed similar intensities of cellular E11 staining at each time point. Furthermore,
the intensity of E11 expression was highest on day 3, 7 and 11 before returning to a lower level of expression on day 15 in both media (Figure 3.7).

3.5.3.3 DMP-1 expression
IHC of DMP-1 expression in MC-3T3 cells in gels did not show any cellular staining in basal medium at day 3, but this had appeared by day 7 and then increased at day 11 and reached its maximum at day 15 (Figure 3.8D). When cells were in mineralising medium, there was faint positive staining at day 3, and this increased on days 7 and 11 and again reached its maximum on day 15 (Figure 3.8E-H). The staining intensities observed on days 7, 11 and 15 in basal and mineralising media were very similar (Figure 3.8B-D & F-H).

3.5.3.4 Cx43 expression
Immunofluorescent analysis of Cx43 expression in MC-3T3 cells in gels and in basal medium for 15 days revealed that cells stained positive for Cx43. The Cx43 expression (green) appeared to be localised to cell processes and around the nuclei (Figure 3.9A). The IgG negative control did not show any Cx43 staining (Figure 3.9B).
Figure 3.3: H&E staining of MC-3T3 cells in 3D collagen type I gels in basal and mineralising medium. Representative H&E staining of fixed and sectioned (7 µm) 3D collagen gels containing MC-3T3 cells following 7, 11 or 15 days in basal (A-C) or mineralising (D-F) medium. Images were captured using the Aperio Scanscope (40x magnification) and processed using the Aperio software. The scale bar is 100 µm in each image, with the area in the red box enlarged (40x) in the black box in each section. All images are from 1 experiment where staining was undertaken at the same time, with 5 sections stained per individual time point. Min = mineralising, a= osteocyte-like dendritic processes and b= osteocyte-like dendritic network. Images shown are a representative of the entire depth of the gel.
Figure 3.4: H&E staining of hOBs cells in 3D collagen type I gels in basal and mineralising medium. Representative H&E staining of fixed and sectioned (7 µm) 3D collagen gels containing hOBs cells following 15 days in basal (A) or mineralising (D) medium. Images were captured using the Aperio Scanscope (40x magnification) and processed using the Aperio software. The scale bar is 100 µm in each image, with the area in the red box enlarged (40x) in the black box in each section. All images are from 1 experiment where staining was undertaken at the same time, with 5 sections stained per individual time point. Min = mineralising, a= osteocyte-like dendritic processes and b= osteocyte-like dendritic network. Images shown are a representative of the entire depth of the gel.
Figure 3.5: Actin and calcein labelling of MC-3T3 cells and hOBs in basal and mineralising medium in 3D collagen type I gels. The expression of actin and calcein following 21 (MC-3T3 cells, A, B) or 15 (hOBs, C, D) days of culture in basal and mineralising medium in 3D collagen type I gels. Whole gels were fixed, stained with phalloidin, DAPI and calcein and imaged using confocal microscopy. Representative overlaid images (20x) of actin (phalloidin, red), mineralisation (calcein, green) and DAPI (nuclear stain, blue) in basal (A, C) and mineralising (B, D) medium. a = osteocyte-like dendritic processes, b = filamentous actin, c = mineralising nodules.
Figure 3.6: ALP protein expression in MC-3T3 cells in 3D collagen type I gels in basal and mineralising medium. MC-3T3s were maintained in basal (A-D) or mineralising (E-H) medium for 3, 7, 11 or 15 days. Individual gels were fixed, processed and sectioned prior to IHC for ALP expression. Results presented are representative of images from 1 experiment (scale bar = 100 µm) with the area in the red box enlarged (40x) in the black box in each section. IHC was undertaken on 1 gel per time point with a minimum of 4 replicate sections stained per gel, with 3 independent experiments undertaken. Min = mineralising, a = ALP positive cellular staining, b = increased ALP cellular staining, c = ALP negative cellular staining and I represents the IgG negative control.
**Chapter 3**

Differentiation of MC-3T3s and hOBs in 3D collagen type I gels

**Figure 3.7**: E11 protein expression in MC-3T3 cells in 3D collagen type I gels in basal and mineralising medium. MC-3T3s were maintained in basal (A-D) or mineralising (E-H) medium for 3, 7, 11 or 15 days. Individual gels were fixed, processed and sectioned prior to IHC for E11 expression. Results presented are representative of images from 1 experiment (scale bar = 100 µm) with the area in the red box enlarged (20x) in the black box in each section. IHC was undertaken on 1 gel per time point with a minimum of 4 replicate sections stained per gel, with 3 independent experiments undertaken. Min = mineralising. a = E11 positive cellular staining, b = E11 negative cellular staining, where I represents the IgG negative control and A-I were counterstained with haematoxylin.
Figure 3.8: DMP-1 protein expression in MC-3T3 cells in 3D collagen type I gels in basal and mineralising medium. MC-3T3s were maintained in basal (A-D) or mineralising (E-H) medium for 3, 7, 11 or 15 days. Individual gels were fixed, processed and sectioned prior to IHC for DMP-1 expression. Results presented are representative of images from 1 experiment (scale bar = 100 µm) with the area in the red box enlarged (20x) in the black box in each section. IHC was undertaken on 1 gel per time point with a minimum of 4 replicate sections stained per gel, with 3 independent experiments undertaken. Min = mineralising, a = DMP-1 negative cellular staining, b = DMP-1 positive cellular staining, where I represents the IgG negative control and A-I were counterstained with haematoxylin.
Figure 3.9: CX43, Actin and DAPI labelling of MC-3T3s in basal medium in 3D collagen type I gels. The expression of Cx43, actin and DAPI following 15 days of culture in basal medium in 3D collagen type I gels. Whole gels were fixed, stained with Cx43 (A) or IgG control (B), phalloidin and DAPI and imaged using confocal microscopy. Results presented are representative of overlaid images (40x) of Cx43 (green), actin (phalloidin, red) and DAPI (nuclear stain, blue) in basal medium. a = Cx43 positive staining.
3.5.4 E11, DMP-1, CX43, sclerostin, MEPE, PHEX and FGF-23 gene expression

The gene expression data for MC-3T3 and hOB cells was not normally distributed; therefore all statistical analysis was undertaken using a Kruskal-Wallis test followed by Dunns multiple comparison post hoc test.

3.5.4.1 MC-3T3 cells

E11 (early osteocyte marker) mRNA was expressed when cells were maintained in either basal or mineralising medium, and at each time point investigated (Figure 3.10A). Furthermore, the level of expression was similar in all samples. DMP-1 (mineralising osteocyte marker) mRNA was also expressed in all samples, with similar level of expression at each time point in basal and mineralising medium. However, in mineralising medium DMP-1 mRNA expression was significantly (~ 7-fold, \( p<0.05 \)) up-regulated following 15 days when compared to that at day 1 (Figure 3.10B). Cx43 (cell-cell communication marker) mRNA was expressed in all samples and this did level of expression did not change with time or cell culture medium used (Figure 3.10C).

When evaluating mature osteocyte markers sclerostin was not expressed in MC-3T3 cells in basal medium, but was present in mineralising medium (Figure 3.10D, 2-4). MEPE (Figure 3.10D, 5-7) and PHEX (Figure 3.10D, 8-10) were expressed in all samples at day 15. FGF-23, on the other hand, was only detected in cells in mineralising medium (Figure 3.10D, 11-13). Mouse bone was used as positive control for these mature osteocyte markers (Figure 3.10).
3.5.4.2 hOBs cells

E11 mRNA was expressed when hOBs were maintained in either basal or mineralising medium, and at each time point investigated (Figure 3.11A). Furthermore, E11 expression did not change with time in either basal or mineralising media, and expression levels were similar at all time points other than at day 15 when E11 expression was significantly (~3-fold, \( p<0.05 \)) up-regulated in mineralising medium Figure 3.11A).

DMP-1 mRNA expression was expressed at each time point when hOBs were in either basal or mineralising medium (Figure 3.11B). However, whereas DMP-1 expression did not change with time when cells were in basal medium, expression was significantly up-regulated (\( p<0.01 \)) on day 15 when cells were in mineralising medium (Figure 3.11B). Furthermore, when compared to cells in basal medium at the same time point, DMP-1 mRNA expression was significantly (~6-fold, \( p<0.01 \)) up-regulated in mineralising medium on day 15 (Figure 3.11B).

Sclerostin mRNA expression was also expressed at each time point when hOBs were in either basal or mineralising medium (Figure 3.11C). Furthermore, sclerostin expression did not change with time in either medium. However, expression values were higher in mineralising compared to basal medium on days 11 (basal: 1.253 ± 0.325, Min: 2.327 ± 1.261) and 15 (basal: 2.327 ± 1.264, mineralising: 2.215 ± 0.657) although these differences were not significant (Figure 3.11C).

When looking at C\(_t\) values prior to normalisation of data, E11 C\(_t\) values were ~32 in hOBs in monolayer (taken at experimental set up for each sample). When cells were
in 3D culture Ct values were ~31/32 in basal medium and ~27-30 in mineralising medium at each time point. The DMP-1 Ct values were 34/35 in monolayer, ~30 in basal medium and ~26-30 in mineralising medium. Sclerostin Ct values were ~30 in monolayer, ~30 in basal medium and ~27-30 in mineralising medium.
Figure 3.10: E11, DMP-1, Cx43, sclerostin, MEPE, PHEX and FGF-23 gene expression in MC-3T3 cells in basal and mineralising medium in 3D collagen type I gels. Relative expression of E11 (A), DMP-1 (B) and Cx43 (C) in 3D collagen gels following 1, 3, 7, 11 and 15 days in basal or mineralising medium. A-C: RT-qPCR analysis of RNA isolated from cells, normalised to 18sRNA (1 gel of basal at day 1 set to 1 in each experiment). Data in A-C are a combination of 3 independent experiments (n=2 at each data point per experiment). Data in A-C are mean ± SEM * p<0.05 versus same treatment on day 1, following a Kruskal-Wallis test followed by a Dunns multiple comparison post hoc test. D: gel electrophoresis results of RT-qPCR for sclerostin, MEPE, PHEX and FGF-23 where each triplicate sample is 1: mouse bone, 2: basal (day 15) and 3: mineralising (day 15) medium, and 1 = 50 bp ladder. Min = mineralising
Figure 3.11: E11, DMP-1 and sclerostin gene expression in hOBs in basal and mineralising medium in 3D collagen type I gels. Relative expression of E11 (A), DMP-1 (B) and sclerostin (C) in hOBs in 3D collagen gels following 7, 11 or 15 days in basal or Min medium. RT-qPCR analysis of RNA isolated from cells, normalised to RPL13A (1 gel of basal at day 7 set to 1 in each experiment). Data in A-C are a combination of 3 independent experiments (3 different patient samples, n=2 at each data point per experiment). All data points are mean ± SEM ** p<0.01, versus same treatment at day 7, # p<0.05 versus basal at the same time point following a Kruskal-Wallis test followed by a Dunns multiple comparison post hoc test. Min = mineralising.
3.5.5 IL-6, VEGF and RANKL protein secretion

The IL-6 protein secretion data for MC-3T3 and the IL-6 and VEGF data for hOBs were not normally distributed; therefore statistical analysis was undertaken using a Kruskal-Wallis test followed by a Dunns multiple comparison post hoc test on these data sets. However, the RANKL protein secretion data from hOBs was normally distributed so statistical analysis was undertaken using a one-way ANOVA and a Bonferroni post hoc test.

3.5.5.1 MC-3T3 cells

IL-6 protein secretion was detected in basal and mineralising medium on all time points investigated and was significantly reduced (p<0.05) at days 3, 7, 11 and 15 when compared to day 1 in both media (Figure 3.12). Furthermore, there were no differences between IL-6 concentrations at the same time point in basal and mineralising media (Figure 3.12).

VEGF measurements were not done on these samples.

3.5.5.2 hOBs

IL-6 protein secretion was detected in cells in basal and mineralising medium on all time points investigated (Figure 3.13A). When cells were in basal medium, IL-6 concentrations were not modulated over time, however, in mineralising medium IL-6 protein secretion was significantly (p<0.001) reduced on days 7, 11 and 15 compared to day 1. There were no significant differences between basal and mineralising medium at the same time point (Figure 3.13A).
VEGF protein secretion was detected at each time point in both basal and mineralising medium (Figure 3.13B). However, VEGF protein secretion did not change significantly with time in culture, and there were no differences between basal and mineralising medium at the same time point (Figure 3.13B).

RANKL protein secretion was only detected in one of the three patient samples investigated in this study. Furthermore, in this patient, RANKL protein secretion was significantly (p<0.001) less in basal and mineralising medium on days 3, 7, 11 and 15 compared to values at day 1 in the same medium (Figure 3.13C). A significant (p<0.05) reduction in RANKL concentration was also observed on day 7 in mineralising medium when compared to basal at the same time point (Figure 3.13C).

**Figure 3.12: IL-6 secretion in MC-3T3 cells in basal and mineralising medium in 3D collagen type I gels.** IL-6 secretion measured by ELISA in MC-3T3 cells (A) maintained in basal or mineralising medium and normalised to cell number in individual gels (A) expressed relative to basal at day 1 set to 100 %. Data are a combination of 3 experiments (n=2 at each data point per experiment). Data in A are mean ± SEM. * p<0.05, ** p<0.01 following a Kruskal-Wallis test followed by a Dunns multiple comparison post hoc test. Min = mineralising.
Figure 3.13: IL-6, VEGF and RANKL secretion in hOBs in basal and mineralising medium in 3D collagen type I gels. IL-6 (A), VEGF (B) and RANKL (C) secretion measured by ELISA in hOBs maintained in basal or mineralising medium and normalised to cell number in individual gels (A-C) expressed relative to basal at day 3 set to 100 % (A-B only). Data are a combination of 3 experiments (3 different patient samples, n=2 at each data point per experiment, A-B) or from 1 experiment (1 patient sample, n=2 at each data point, C). Data in A-C are mean ± SEM. *** p<0.001 following a Kruskal-Wallis test followed by a Dunns multiple comparison post hoc test (A-B) or following a one-way ANOVA and a Bonferroni post hoc test versus same treatment on day 1 (C). # p<0.05 following a one-way ANOVA and a Bonferroni post hoc test versus control at the same time point. Min = mineralising.
3.5.6 Gel stiffness

Gel stiffness data following maintenance of MC-3T3 and hOBs in gels were normally distributed and thus statistical analysis was undertaken using a two-way ANOVA and a Bonferroni post hoc test.

3.5.6.1 MC-3T3 cells

When MC-3T3 cells were cultured in basal or mineralising medium there was a significant effect of time ($p<0.0001$) and culture medium ($p=0.0002$), as well as a significant ($p=0.0002$) interaction between time and cell culture medium, on gel stiffness (Figure 3.14A). Post hoc analysis showed that when cells were maintained in basal medium, gel stiffness was significantly ($p<0.001$) increased on days 11 and 15 when compared to day 1. When cells were in mineralising medium, gel stiffness was significantly increased on days 7 ($p<0.001$), 11 ($p<0.001$) and 15 ($p<0.05$) compared to mineralising medium on day 1. Furthermore, gel stiffness was significantly ($p<0.001$) increased in mineralising medium compared to basal medium on day 11 (Figure 3.14A).

3.5.6.2 hOBs

hOBs cultured in basal and mineralising medium demonstrated a significant effect of time ($p<0.0001$) and cell culture medium ($p=0.0001$), as well as a significant ($p=0.0006$) interaction between time and cell culture medium on gel stiffness (Figure 3.14B). Post hoc testing revealed that gel stiffness did not change over time. However, cells maintained in mineralising medium resulted in a significant increase in gel stiffness on days 11 ($p<0.01$) and 15 ($p<0.001$) compared to day 3. Furthermore, on days 11 ($p<0.05$) and 15 ($p<0.001$), there was a significant increase
in stiffness with cells in mineralising medium compared to basal medium at the same time point (Figure 3.14B).

**Figure 3.14:** Gel stiffness in MC-3T3 cells and hOBs in basal and mineralising medium in 3D collagen type I gels. Gel stiffness in MC-3T3 cells (A), hOBs cells (B) or empty cells (A-B) in basal or mineralising medium were measured on 1, 3, 7 and 11 days (MC-3T3s) or 3, 11 and 15 days (hOBs). Gel stiffness was measured using a Losenhausen with MTS controller, data was processed using a Matlab software algorithm. Data in A-B are from 1 (n=5 at each data point per experiment) independent experiment. Data in A-B are mean ± SEM. ** p<0.01, *** p<0.001 versus same medium on day 1, # p<0.05, ### p<0.001 versus basal at the same time point following a two way ANOVA and a Bonferroni post hoc test.
Chapter 3

Differentiation of MC-3T3s and hOBs in 3D collagen type I gels

3.6 Discussion

The aim of this chapter was to characterise the differentiation of MC-3T3 cells and hOBs to osteocytes in 3D collagen type I gels in basal and osteogenic (mineralising) medium. Collectively, the results indicated that both cell types differentiated to a mature osteocyte-like cell population in mineralising medium only, as evidenced by the expression of sclerostin mRNA by both cell types and FGF-23 expression in MC-3T3 cells only after 15 days.

MC-3T3 and hOB cell numbers increased when compared to day 1 in both basal and mineralising media, with increased numbers of cells in mineralising medium compared to basal in both cell types. However, MC-3T3 cell number increased until day 7 and then plateaued at 4x the original seeding density, whereas hOBs seemed to be increasing throughout the assay and reach 12x (basal) or 28x (mineralising) the original density by day 15. The seeding densities for the two cell types were very different however: $7 \times 10^5$ and $8 \times 10^4$ for MC-3T3 and hOBs respectively.

The cells were placed in gels as proliferating osteoblasts and thus might have needed to continue proliferating to reach an optimum density in the gels before the onset of differentiation. It is difficult to explain the differing growth rates observed in gels due to i) the different seeding densities used, ii) the difference in size of the two cell types and iii) the their different proliferation rates. It is also possible that the increased cell numbers in gels could be partly due to a mixed population of cells being present, with some remaining as osteoblasts and some are differentiating along the osteocytic pathway. If this was the case it could suggest that a co-culture of cells was generated within the gels. Early osteocytes are known to proliferate, as it has
been shown that pre-osteocyte (MLO-A5) or early osteocyte (MLO-Y4) cell lines proliferate in culture [81, 82], whereas other cell lines that differentiate to mineralising or mature osteocytes (HOB-01-C1, IDG-SW3) do not proliferate under osteogenic conditions [83, 108].

Cell viability in basal and mineralising medium was consistently high in both cell types investigated (MC-3T3 cells <84 %, hOBs <92 %). Cell viability did not change in hOBs throughout the assay period, but MC-3T3 cell viability declined with time with a significant reduction on day 15 in both basal and mineralising media, and thus following differentiation to osteocyte-like cells. Osteocyte cell death is known to occur in human bone and there may be a link between bone turnover and apoptosis of osteocytes [374]. The location of osteocytes in the mineralised matrix makes apoptosing osteocytes inaccessible and thus can be observed in vivo in the lacunae [25, 26]. It is unclear as to what happens to apoptosing osteocytes when in vitro 3D gels. Osteocyte apoptosis has also been shown to be increased with age - >1 % at birth and 45-75 % at 80 years of age [49, 375, 376]. Furthermore osteocyte apoptosis has been shown to be increased in osteoporosis [49]. In cells where cell death is occurring, the cells are destroyed by the neighbouring or phagocytic cells [377].

Confocal microscopy showed that MC-3T3 cells and hOBs appeared different in basal and mineralising medium. In basal medium both cell types resembled osteoblasts with organised filamentous actin observed. However, in mineralising medium, the cells appeared to have osteocyte-like dendritic processes that may be forming communications with other cells. Osteoblasts in vivo can be various shapes including ovoid, rectangular, columnar, cuboidal or pyriform [4]. Osteocytes, both in
vivo and in vitro, however, are known to have dendritic morphology that enables communicate with other cells [82, 108, 279]. Morphologically, the results described in this thesis thus suggest that maintenance in mineralising medium, which also results in the formation of mineralising nodules in the gels, provides the optimal condition for osteoblast to osteocyte differentiation. This environment closely resembles the in vivo environment of mature osteocytes [44]. In support of this, ALP protein staining intensity appeared to be strongest in basal medium when compared to cells maintained in mineralising medium at the same time point. In monolayer in the IDG-SW3 cells, ALP expression has been shown to be down-regulated when the cells are osteocytes when compared to when they are osteoblasts [108].

E11, DMP-1 and Cx43 mRNA expression was similar in MC-3T3 cells when they were in basal or mineralising medium. Furthermore, there were no changes in expression of E11 and Cx43 in either medium with time. However, DMP-1 expression was increased on day 15 in mineralising medium, and this might be related to its involvement in mineral formation [378]. In the DMP-1 knock-out (K/O) mouse bone mineralisation in vivo was reduced, as shown by lower mineral to matrix ratio and increased crystal size in bones [30] [36]. The DMP-1 K/O mouse also has defective osteocyte maturation and increased FGF-23 expression which results in changes in bone mineralisation [30] DMP-1 expression was up-regulated in mineralising medium in the hOBs at day 15 in this chapter. Previously increased DMP-1 expression in MC-3T3 cells has only been shown following treatment with all trans-RA or by direct application of mechanical strain [222, 379]. Furthermore, FGF-23 (mature osteocyte marker) is regulated by DMP-1 [380]. In support of this, in this thesis FGF-23 was only expressed in cells maintained in mineralising medium.
Chapter 3: Differentiation of MC-3T3s and hOBs in 3D collagen type I gels

at day 15 (MC-3T3 cells) when DMP-1 expression was highest. FGF-23 expression has also been shown to be regulated by MEPE and PHEX, both of which were expressed in MC-3T3 cells in basal and mineralising medium on day 15. Studies of the dento-alveolar complex of mice, when FGF-23 was knocked out showed distinct changes in morphology, decreased BSP expression, increased DMP-1 expression and increased apoptosis in osteoblasts and osteocytes [381]. This highlights the importance of FGF-23 in maintaining bone morphology and preventing cell apoptosis. Furthermore FGF-23 expression in MC-3T3 cells in mineralising medium confirms the generation of a mature osteocyte in this 3D culture. The only other study to show FGF-23 expression in the MC-3T3 cells was following treatment with all trans-RA [222].

The demonstration of sclerostin expression in MC-3T3 cells in mineralising medium further confirms the generation of a mature osteocyte population of cells in gels. Sclerostin is a well-established mature osteocyte marker, known to be down-regulated following mechanical loading in osteocytes and a negative regulator of bone formation [31, 290, 382]. Sclerostin expression has been detected in the MC-3T3 cells previously following treatment with all trans-RA [222], or in the presence of osteogenic growth factors [277], and is known not to be expressed in vivo until after mineralisation has been initiated [62]

Sclerostin expression was also observed in the hOBs at each time point in basal and mineralising medium, with no differences in expression between media types. Sclerostin expression values were, however, higher in mineralising compared to basal medium on all time points investigated. Sclerostin has previously been shown
to be expressed by osteoblasts [276], and the expression of sclerostin in hOBs maintained in monolayer in this study confirms this. The expression of sclerostin (both cell types) and FGF-23 (MC-3T3 cells) described here is further evidence that MC-3T3 cells or hOBs maintained in mineralising medium in 3D collagen type I result in a mature osteocyte cell population.

Cx43 mRNA was expressed in MC-3T3s at each time point but this expression did not change with time. Furthermore, when in basal medium, Cx43 protein expression was localised around the golgi and in the cellular processes. Cx43, a cell-cell communication marker expressed in the majority of cell types acts via gap junctions in cells. It is believed that all intracellular osteocyte communication is via gap junctions, and when Cx43 expression was knocked out in mice, the intracellular communication system (via dendritic processes) was damaged, but the extracellular communication system (via the canaliculi) remained functional. Furthermore, osteocyte apoptosis was increased, highlighting the importance of Cx43 expression in osteocytes.

The secreted concentrations of IL-6 in both MC-3T3 and hOB collagen gel cultures maintained in basal medium were reduced with time. Similar results were observed in mineralising medium. IL-6 is a pro-inflammatory cytokine that can be used as an indicator for cell apoptosis or stress [34, 35]. In both cell types, IL-6 secretion was highest on day 1 (MC-3T3 cells) and day 3 (hOBs) suggesting that they might be apoptosing or under significant stress at that stage, although no other indicators of this were observed. The significant reduction in IL-6 secretion with time suggests, however, that the cells are not hypoxic, apoptotic or stressed at later stages of the
assays. Il-6 has also been shown to stimulate osteoblast differentiation [383-385], as well as stimulate RANKL production in the osteoblast lineage in vitro [384-387]. RANKL mRNA expression was not measured in either of the two cell types studied in this chapter. Furthermore, whereas RANKL secretion was not measured in MC-3T3 cultures, secretion of this protein was only seen in one of the hOB samples used, and when in both basal and mineralising medium. Interestingly, in that sample, RANKL secretion decreased with time in a similar pattern to that observed for IL-6 secretion, further confirming a possible link between these two proteins. There was a significant decrease in RANKL secretion in mineralising compared to basal medium on day 7.

The three patient (two female, one male) samples used in this study were from individuals undergoing total knee replacement surgery, although the exact details of the diagnosis and ongoing treatment regimes in each case is unknown. RANKL was detected in osteoblasts and osteocytes derived from the male patient. OP, rheumatoid arthritis and metastatic bone disease are all known to result in increased RANKL expression in bone [388-390], and, furthermore, Denosumab (monoclonal antibody against RANKL) is currently being used to treat such conditions.

VEGF protein was also secreted in to the medium of 3D hOB cultures, and the levels secreted did not change with time in either basal or mineralising medium. The trends, however, were for VEGF to increase with time in basal medium, but decrease in mineralising medium. VEGF has recently been shown to be involved in bone formation, as mice that had the endogenous VEGF blocked by the addition of soluble Flt-1 or the recombinant humanized anti-VEGF monoclonal antibody had
shorter long bones [391, 392]. Osteoblasts are known to secrete VEGF in vitro [393-395] and also to respond to VEGF [396]. However, in vitro VEGF stimulated osteoblasts did not express VEGF receptor-1 or VEGF receptor-2 [53, 54] and it has been suggested that neuropilin-1 (which binds to semaphorin and VEGF) is the possible target for VEGF in osteoblasts [55-59]. ML-OY4 cells have also been reported to secrete VEGF protein. MC-3T3 cells cultured in monolayer or 3D culture poly(l-lactide-co-glycolide) scaffolds demonstrated up-regulation of VEGF protein secretion in 3D culture (3-fold) when compared to 2D [397]. Furthermore, when human periosteal cells were transfected with a human VEGF plasmid, there was an increase in gene expression, protein levels and mineralisation demonstrating the possible osteogenic potential of VEGF [398].

Gel stiffness was also investigated to characterise the properties of these collagen type I gels. Generally, gel stiffness increased with time, suggesting that the cells were laying down ECM in the gels, although this increase was not seen with hOBs in basal medium. Gels containing hOBs in this medium had a similar stiffness to empty gels maintained in culture for 15 days.

The stiffness was higher in both cell types at later stages of assay when maintained in mineralising compared to basal medium, suggesting that the increased stiffness was due to mineral deposition. The change in gel stiffness observed in the presence of cells when compared to stiffness of empty gels indicates that the change is a cell and not a collagen-related phenomenon. As discussed previously, MC-3T3 cell numbers increased over time but this increase was similar in both media used. It is not thus plausible to explain the differences in gel stiffness by increased cell number.
In hOBs, however, there was a significant increase in cell number at day 15 when cells were in mineralising compared to basal medium. This could thus explain the difference seen in stiffness between media at this time point. It is also unlikely that this increase was due to mineralising nodules present in gels as the latter account for a small percentage of the gel surface area, whereas there was ~ 2-fold difference in gel stiffness observed between basal and mineralising medium. It is possible, however, that the interaction between the collagen and the cells was resulting in collagen cross-linking and further enhancing the stiffness of the gel.

Changes in the stiffness of matrix surrounding osteoblasts are possible stimuli for osteoblast to osteocyte differentiation [399]. It is thus possible that the cells in the 3D gels in this study were controlling their own differentiation by modulating the gel stiffness. ECM stiffness has been shown to influence numerous cell behaviours (e.g. migration, proliferation, differentiation) in both bone and other tissues [399-401]. Furthermore, MSC differentiation to different lineages (e.g. adipogenic, osteogenic) has been shown to be is influenced by substrate stiffness [402]. Specifically, MSCs differentiated to osteoblast-like cells when cultured on collagen coated polyacrylamide substrates (stiffnesses ~25–40 kPa), but took on the characteristics of neurons or myoblasts when cultured on substrates with stiffness values of ~1 kPa or ~≈11 kPa respectively [402].

In conclusion the results presented in this chapter describe the development of an osteoblast to osteocyte 3D collagen type I culture system that closely mimics the in vivo bone environment. Furthermore the results suggest that osteocytes can be generated in basal or mineralising medium and from a mouse cell line or human...
primary cells. However, a mature osteocyte population, as defined by sclerostin and FGF-23 expression, was only generated in mineralising medium. Whereas others have used osteoblasts in 3D collagen gels for research purposes, this is the first time that this method has been fully characterised, especially with human cells, and provides a new tool for further study of osteoblast to osteocyte differentiation and osteocyte function. This includes studies of the role of external factors in the modulation of osteocyte differentiation, as well as studies of the effects of mechanical loading on osteocytes, both of which are studied in detail in Chapters 4 and 6 of this thesis.
Chapter 4: Effects of IGF-1, FGF-2, RA and Vit K on osteocyte differentiation in 3D

4 The effect of Insulin like Growth Factor-1, Fibroblast Growth Factor-2, Retinoic Acid and Vitamin K on the differentiation of osteoblast to osteocytes in 3D collagen type I gels
4.1 Introduction

The previous chapter investigated the differentiation of osteoblasts (MC-3T3 cells and hOBs) to osteocytes in 3D collagen type I gels. Previous studies in the literature have suggested that the addition of external factors to osteoblasts may modulate/accelerate this differentiation process. This chapter investigates the effect of IGF-1, FGF-2, RA and Vit K on this differentiation process in 3D.

4.1.1 IGF-1 and osteocytes

In bone, IGF-1 is a growth factor that is involved in bone formation, developmental bone growth and bone resorption (Section 1.6.2, Chapter 1) [184-186]. To date, the only studies in relation to IGF-1 and osteocytes show that in bone explants cultures there was an increased number of osteocytes following treatment or that IGF-1 release was increased following mechanical loading/stimulation.

Addition of IGF-1 to either the cortical or trabecular bones of adult rats stimulated bone formation, and showed that IGF-1 expression was localised to the osteocytes in the cortex[188]. In fetal rat parietal bones in organ culture IGF-1 treatment increased (1) calcification by 38 %, (2) the number of osteocyte cell processes by 71 % and (3) the amount of osteocytes per unit bone area by 107 % [403].

Mechanical loading, using various methods in rats including vertebral loading [404], stretching primary osteocytes isolated from parietal and frontal bones [79] and four-point bending [405], increased osteocyte IGF-1 expression from 6 hrs post-mechanical loading [404, 405] compared to unloaded controls. Furthermore, stretching primary osteocytes uncovered that the IGF-1 mRNA expression was
biphasic with a second peak IGF-1 expression at 24 hrs post stretching [79]. In female sheep, mechanical stimulation (low frequency micro-movement following transverse osteotomy of the tibia) also revealed that both IGF-1 and TGF-β1 were generated in response to mechanical stimulation [406]. All of these results add to the hypothesis that osteocyte specific IGF-1 plays a role in the translation of mechanical stimuli into bone formation [405], as well as highlighting that it is IGF-1 increases osteocytes and osteocyte cell processes, and is up-regulated following mechanical stimulation.

### 4.1.2 FGF-2 and osteocytes

In bone FGF-2 is necessary for bone formation as it regulates the expression of a number of genes that are involved in osteoprogenitor cell replication, osteoblast differentiation and apoptosis [194, 201]. Furthermore, FGF-2 signalling regulates osteoblast function and differentiation [201], where it is thought that FGF-2 acts as an antagonist of osteoblast differentiation [202] (Section 1.6.3, Chapter 1). To date, little has been published on the role of FGF-2 on osteocyte function. Microarray analysis following treatment of MLO-Y4 osteocytes with FGF-2 revealed that that progressive ankylosis (Ank), ectonucleotide pyrophosphatase/phosphodiesterase 1 (Enpp1), matrix gla protein (Mgp), type III sodium/phosphate co-transporter (Slc20a1) and DMP-1 were upregulated [407]. On the other hand, when mouse calvaria in organ culture were treated with an FGF receptor inhibitor, a decrease in the expression of Ank, Enpp1, Slc20a1 and DMP-1 were observed [407].

Several groups have, however, investigated the effects of FGF-2 on the differentiation of osteoblasts to cells expressing an osteocyte-like phenotype. Gupta
and colleagues, for example, showed that after 8 hrs of FGF-2 treatment, MC-3T3-E1 osteoblasts and mouse primary bone marrow stromal cells morphology exhibited elongated dendritic-like morphology similar to osteocytes [202]. There were also changes in osteocyte markers in both the bone marrow stromal cells and the MLO-Y4 cells following FGF-2 treatment, highlighting that FGF-2 treatment may regulate the transition of osteogenic cells towards the osteocyte lineage as well as increasing the expression of osteocyte specific genes [202]. Furthermore, following 48 hr treatment with FGF-2, rat osteoblast cells derived from bone marrow stromal cells, down-regulated MEPE and OCN mRNA expression, had no effect on alpha 1 (I) procollagen mRNA, Runx2 and osterix mRNA expression and up-regulated bone sialoprotein mRNA expression [408]. In these experiments FGF-2 has no effect on cell proliferation [408]. Similarly, when MC-3T3-E1 osteoblasts were treated with FGF-2 DMP-1 and E11 expression was induced (osteocyte markers), and sclerostin and PHEX expression was increased [202].

There was some inhibition of the FGF-2 induced up-regulation of DMP-1 mRNA expression when increased extracellular phosphate levels were observed in MLO-Y4 cells. It was hypothesised that the regulation of DMP-1 by FGF signalling could be controlled by extracellular phosphate [407]. These studies show that FGF-2 treatment has been demonstrated to induce osteogenic cells to move towards the osteocyte lineage, although besides the mouse calvaria in organ culture. No study has investigated the role of FGF-2 in the differentiation of osteoblasts to osteocytes in 3D collagen type I gels.
4.1.3 RA and osteocytes

In bone, there are contradicting results in relation to the role of RA signalling in osteogenic differentiation as it either promotes or inhibits this differentiation process [233-238]. Furthermore in vitro studies of the effect of RA treatment on osteoblasts have also reported opposing results in relation to maturation and mineralisation [239, 240]. Furthermore, all-trans Retinoic Acid (ATRA) treatment appeared to accelerate the maturation of the murine pre-osteoblast MC-3T3-E1 cell line [241, 242] (Section 1.6.4, Chapter 1).

There is some evidence in the literature that RA modulates the differentiation of osteoblasts to osteocytes. One publication showed that osteoblasts treated with RA differentiated to mature sclerostin expressing osteocytes [222]. This study looked at the effect of RA on primary osteoblasts isolated from C57BL6 mice and the MC-3T3 osteoblast cell line, and showed that a homogenous population of cells that had osteocyte like characteristics were generated in each case. Osteocyte dendritic processes could be seen in both cell types after 2 (primary cells) or 4 (MC-3T3s) days of treatment. Differentiation was complete following 5-10 days of treatment with both cell types (1) becoming dendritic, (2) stopping production of extracellular matrix, (3) reducing expression of osteoblast specific markers and (4) increasing expression of osteocyte specific markers such as sclerostin [222].

Another study showed that the RA receptor-related orphan receptor β expression was inhibited at the later stages of differentiation in primary murine calvarial cells [409]. This study then describes the generation of a cell model stably expressing RA receptor-related orphan receptor β in MC-3T3 cells. These cells showed decreased
Chapter 4: Effects of IGF-1, FGF-2, RA and Vit K on osteocyte differentiation in 3D

bone nodule formation, and down regulation of OCN and osterix gene expression (both are Runx2 targets) highlighting that the receptor-related orphan receptor β is a new regulator of osteogenesis and possibly regulates age-related bone loss through antagonism of Runx2 activity [409].

The above two publications provide evidence that RA treatment of osteoblasts may influence differentiation to mature osteocytes in monolayer cultures using both cell lines and primary osteoblasts. The results presented in this chapter explore this further using a 3D culture technique.

4.1.4 Vit K and osteocytes

There are three different homologs of Vit K which include the naturally occurring homologs phytonadione (Vit K1) or menatetrenone (Vit K2), or synthetic Vit K homolog menadione (Vit K3). In bone Vit K2 has been shown to induce bone mineralisation of osteoblasts[252] and apoptosis of osteoclasts [252]. Furthermore Vit K2 has also been shown to increase bone mineral density [253, 254] and maintain bone strength in vivo [255] (Section 1.6.5, Chapter 1).

Previous studies have investigated the effect of Vit K2 on osteocytes in vivo. Iwamoto et al. 2010 performed bone histomorphometric analysis on the tibial diaphysis and proximal metaphysis of retired female breeder rats to investigate the effect of Vit K2 treatment on cortical and cancellous bone mass, cortical osteocytes and the lacunar system in siatic neuroectomized rats [410]. This study found that siatic neuroectomy decreased osteocyte density and lacunar occupancy; whereas Vit K2 improved the siatic neuroectomy induced reduction in lacunar occupancy by
viable osteocytes [410]. Another study on glucocorticoid treated rats investigated whether Vit K influenced bone formation and resorption, the osteocyte lacunar system and the porosity in the cortical bone. This study found that glucocorticoid treatment reduced percentage cortical bone area as well as increasing percent bone marrow area because of the reduced periosteal bone formation, increased endocortical bone erosion and increased cortical porosity. They also reported that Vit K prevented the decrease in periosteal bone formation, but did not affect percent cortical bone and marrow areas. Furthermore, they showed that Vit K treatment increased osteocyte density and lacunar occupancy, highlighting that Vit K treatment improves the osteocyte lacunar system [411].

Previous *in vitro* studies investigating the effect of Vit K on osteocytes showed a reduction in RANKL expression relative to OPG in MLO-Y4 cells. This suggests that Vit K promotes osteoblast to osteocyte transition [246]. Similar *in vitro* results were also reported by Takeuchi *et al.* 2000 as they showed that Vit K2 treatment on mouse bone marrow cells inhibited RANKL mRNA expression and 1,25-dihydroxyvitamin D2-generated osteoclastogenesis from bone marrow cells [263]. However, the opposite results were seen in MC-3T3-E1 cells as Vit K2 treatment promoted RANKL, RANK and OPG expression, but did not change cell proliferation [261]. Similarly when human primary osteoblasts treated with Vit K, there was no effect on cell proliferation, but there was an increase in *in vitro* mineralisation where γ-carboxylation was shown to be required for this process [246].
Other studies have investigated the effect of the three Vit K homologs on human primary osteoblasts. The three Vit K homologs promoted in vitro mineralisation, whereas Vit K2 and Vit K3 appeared to increase osteocyte differentiation when osteoblasts were cultured in 3D collagen type I gels, with increased numbers of cells with osteocyte like morphology with mineral next to their cellular processes [246]. Gla rich protein (GRP) is a Vit K-dependent protein isolated from the calcified cartilage of the Adriatic sturgeon. Highest expression of this protein was found in cartilage, but other cells expressing GRP include chondrocytes, chondroblasts, osteoblasts and osteocytes. Gla residues have the ability to bind calcium and therefore it is hypothesised that GRP could regulate calcium in the extracellular environment [412]. Atkins et al. 2009 have demonstrated that Vit K treatment of osteoblasts in 3D collagen gels cause differentiation to cells with osteocyte morphology. The results presented in this chapter investigate these findings further in 3D culture to characterise the population of cells generated.
Chapter 4: Effects of IGF-1, FGF-2, RA and Vit K on osteocyte differentiation in 3D

4.2 Hypothesis

1. The addition of external compounds (IGF-1, FGF-2, Vit K or RA) to mouse osteoblasts (MC-3T3 cells) will accelerate the differentiation to osteocyte-like cells in 3D collagen type I gels, when compared to relevant controls.

2. The addition of IGF-1 to human osteoblasts cells (hOBs) will mimic results obtained from the mouse osteoblast cell line (MC-3T3 cells) line and promote the differentiation to osteocyte-like cells in 3D collagen type I gels, when compared to relevant controls.

4.3 Aims

Specifically the aims were to investigate the effects of the above factors on the following in each cell type, and after 1 (hOBs only), 3, 7, 11 and 15 days of treatment:

1. Cell number and viability (haemocytometer counting and trypan blue exclusion)

2. Cell morphology (H&E staining)

3. Gene expression (qRT-PCR; DMP-1, E11, Cx43, LRP-5, FGF-23, RANKL and sclerostin)

4. Protein expression (IHC; DMP-1, E11)

5. Protein secretion (ELISA; IL-6, VEGF, FGF-23 - MC-3T3s only, RANKL - hOBs only).
Chapter 4: Effects of IGF-1, FGF-2, RA and Vit K on osteocyte differentiation in 3D

4.4 Methods

4.4.1 Experimental design

MC-3T3 (7 x 10^5 cells/ml gel) cells or hOBs (8 x 10^4 cells/ml gel) cells were set up in 3D collagen type I gels (3 independent experiments, n= 3 per time point unless otherwise stated in the figure legends where each hOB experiment was derived from a different patient sample, Section 2.2.1.3 and 2.2.2.3). The day after set up, individual gels were treated with control (basal) medium or basal medium and one of the following factors: IGF-1, FGF-2, RA or Vit K for MC-3T3 cells or IGF-1 for hOBs or their respective controls (Table 4.1). Medium was changed and individual gels were treated every two days. In one experiment (n=4 per time point) the effects of IGF-1 treatment on MC-3T3 cells in both basal and mineralising medium (Section 2.2.2.4) were assessed. Responses to the individual treatments were analysed following 1, 3, 7, 11 and 15 days of treatment. For most experiments (unless otherwise stated), there were 4 replicate gels per time point of which 3 gels were used for analysis of cell number and viability, gene expression and protein secretion and 1 gel used for morphological and immunohistochemical analysis following fixation and sectioning. For the effect of IGF-1 on MC-3T3 cells in both basal and mineralising medium (one experiment, n=4 gels per time point), the 4 gels were used for analysis of cell number and viability, gene expression and protein secretion and following 11 or 15 days of treatment only.
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Table 4.1: Concentrations and source of factors used for treating MC-3T3 cells or hOBs and respective control treatments in 3D collagen type I gels. Individual treatments for all gels were made up in a universal, by adding the respective treatment to total basal medium needed for all gels prior to adding 750 µl to each gel.

4.4.2 Protein secretion, cell number and viability and gene expression

Media were removed from wells on days 1, 3, 7, 11 and 15 in both control and treated wells (days 11 and 15 only for effect of IGF-1 in basal and mineralising medium), centrifuged (1,700g, 30 s) and stored in aliquots (120 µl) at -80°C until needed for measurement of VEGF, IL-6, FGF-23 (MC-3T3 only) and RANKL (hOBs only) secretion (ELISA, Sections 2.2.7.1 and 2.2.7.2). In all experiments, quantification of protein secretion was undertaken following accumulation in medium over 2 days as media were always changed 2 days before collection. Following collection of media, the gels were collagenase digested (Section 2.2.2.5) for measurement of cell number and viability, and following this, the remaining cells were stored in TRI Reagent for RNA extraction and gene expression analyses (Section 2.2.3).

Protein concentrations from each gel sample were normalised to the cell number in that same gel and expressed relative to the mean control sample value (fixed to 100%) at day 1 for MC-3T3 cells for independent experiments, day 11 basal medium
Chapter 4: Effects of IGF-1, FGF-2, RA and Vit K on osteocyte differentiation in 3D

for the effect of IGF-1 on basal and mineralising medium, or day 3 for hOBs cells for each independent patient. However FGF-23 protein concentrations (1 experiment only) were normalised to cell number only. Results from 2-3 independent experiments (as described in individual figure legends) were then combined for further analysis, apart from FGF-23 which was from 1 experiment.

For analysis of cell number data from MC-3T3 cells and hOBs, data from each experiment were expressed relative to untreated control data at day 1 for MC-3T3 cells, day 11 basal medium for the effect of IGF-1 on basal and mineralising medium, or day 3 for hOBs (fixed to 100%) in that same experiment, which enabled the 2-3 experiments to be combined (as described in individual figure legends) for further analysis.

For all experiments, RT-qPCR was initially undertaken on all control and treated samples at all time points using 3 reference genes (GAPDH, PCNA and 18sRNA for MC-3T3 cells or RPL13A, beta-actin and 36B4 for hOBs). The data generated from this were then analysed using Normfinder to select the most stable reference gene to use for each individual data set. Normfinder selected 18sRNA as the most stable reference gene for the MC-3T3s and RPL13A for the hOBs. Data were analysed using ΔΔCt (Section 2.2.3.8) with the value from one untreated control sample from day 3 fixed to 1 in each experiment allowing the data from independent experiments to be pooled for further analysis. In the experiment investigating the effects of IGF-1 in MC-3T3 cells maintained in basal and mineralising medium, the value from one untreated control sample in basal medium on day 11 was fixed to 1.
4.4.3 Morphological and immunohistochemical analysis

For morphological and immunohistochemical analyses one gel from each of the control and treated cells were removed and fixed at days 1, 3, 7, 11 and 15 in each experiment. These were subsequently sectioned for histological (morphological and immunohistochemical) analysis (Section 2.2.5). Sections were stained with H&E to assess cell morphology or used for immunohistochemical analysis of E11 and DMP-1 (Section 2.2.5.6).

4.4.4 Statistical analysis of data

Data presented in this chapter are combined data from 1-3 independent experiments with an n=3 at each data point in each experiment unless otherwise stated (see individual figure legends). Graphs in this chapter were generated from the mean ± SEM. Prior to undertaking statistical analyses, the complete data sets were tested for (1) normality (Shapiro-Wilko test, Graph Pad Prism Software), (2) equal variance (Bartlett’s test, Graph Pad Prism Software) and (3) presence of outlier’s (Grubb’s test, Microsoft office Excel 2010). There were no outliers in these data sets. All statistical analyses were undertaken on the complete data sets using a one way or two-way ANOVA followed by a Bonferroni post hoc test.
Chapter 4: Effects of IGF-1, FGF-2, RA and Vit K on osteocyte differentiation in 3D

4.5 Results

Effect of IGF-1, FGF-2, RA and Vit K treatment on MC-3T3 cells

4.5.1 Cell number and viability

All the cell number data were normally distributed therefore all statistical analysis was undertaken using a two-way ANOVA followed by a Bonferroni post hoc test with $p$ values following the two-way ANOVAs for each gene following treatment with IGF-1, FGF-2 or RA or Vit K (Table 4.2) when compared to their respective controls (PBS or DMSO). IGF-1 and FGF-2 were analysed independently (due to different n numbers), whereas RA and Vit K were analysed together.

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<th>Effect of treatment</th>
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Table 4.2: Results ($p$ values) following a two-way ANOVA investigating the effect of time, treatment and the interaction between time and treatment on cell number when comparing cells in control medium (PBS or DMSO) and following IGF-1, FGF-2 or RA or Vit K treatment.

4.5.1.1 IGF-1 or FGF-2 treatment

Cell number increased in control over time with significantly increased cell number in control on days 7 ($p<0.05$) 11 ($p<0.001$) and 15 ($p<0.001$) when compared to day 1 (Figure 4.1 1A & 2A). Following IGF-1 treatment cell number also increased with time and was significantly increased on days 11 ($p<0.001$) and 15 ($p<0.001$) when compared to the same treatment on day 1 (Figure 4.1 1A). However, there were no differences in cell number when comparing control and IGF-1 treatment at the same time point (Figure 4.1 1A). When cells were treated with FGF-2 cell number increased with time and was significantly increased following 7 ($p<0.001$), 11
(p<0.001) and 15 (p<0.01) days (Figure 4.1 2A). Furthermore, FGF-2 treatment significantly (p<0.01) increased cell number on day 7 when compared to the same cells maintained in control medium (Figure 4.1 2A).

Cell viability was significantly (p<0.05) reduced in cells in control medium or cells treated with IGF-1 on days 3, 7, 11 and 15, and on days 7, 11 and 15 following treatment with FGF-2 (Figure 4.1 1B-C & 2B-C). However, cell viability was >93 % (Figure 4.1 1B & 2B), >92 % (Figure 4.1 1C) and >89 % (Figure 4.1 2C) for cells in control, IGF-1 or FGF-2 medium respectively.

4.5.1.2 RA or Vit K treatment

Since the RA and Vit K used in these experiments were reconstituted in DMSO, the medium used for the control gels contained the same proportion (0.05 %) of DMSO. Cell number increased over time in the control gels, but this increase was significantly (p<0.001) higher on days 7, 11 and 15 than the increase seen previously when the medium for control gels contained PBS (Figure 4.1 1A & 2A) and not DMSO (Figure 4.2 1A & 2A).

RA treatment did not modulate cell number over time during this 15 day time course (Figure 4.2 1A). However, when compared to RA treatment, cell number was significantly increased (p<0.001) in control medium on days 7, 11 and 15 (Figure 4.2 1A). When cells were treated with Vit K, cell number increased over time, with significant increases observed following 11 (p<0.001) and 15 (p<0.01) days when compared to day 1 of the same treatment (Figure 4.2 2A). Furthermore, there was a significant (p<0.001) increase in cell numbers in control when compared to Vit K
Chapter 4: Effects of IGF-1, FGF-2, RA and Vit K on osteocyte differentiation in 3D

treated cells at the same time point following 7, 11 or 15 days of treatment (Figure 4.2 2A).

Cell viability was significantly ($p<0.05$) reduced in the DMSO control following 7, 11 and 15 days of treatment (Figure 4.2 1B & 2B). RA treatment significantly ($p<0.05$) reduced cell viability on days 3, 7, 11 and 15 (Figure 4.2 1C), and Vit K treatment had a similar effect with decreased ($p<0.01$) cell viability on days 3, 7, 11 and 15 (Figure 4.2 2C). Cell viability at all time points was >84 % in the DMSO control gels (Figure 4.2 1B & 2B), >80% when gels were treated with RA (Figure 4.2 1C) and >87% with Vit K treatment (Figure 4.2 2C). Furthermore, there was a significant difference ($p<0.001$) in cell viability on day 15 when comparing the PBS (Figure 4.1 1B & 2B) and the DMSO (Figure 4.2 1B & 2B) control gels.

4.5.2 Morphology

All H&E images presented were a representative of the entire depth of the gel, and similar to cell viability results presented previously confirmed that all cells were viable throughout the depth of the gels (Figure 4.3 & 4.4).

4.5.2.1 IGF-1 or FGF-2

After 1 day, control (PBS) cells appear rounded but some have short processes extending from the cytoplasm (Figure 4.3A). Following treatment with IGF-1 or FGF-2 for 1 day, some of the cells could have longer dendritic osteocyte-like projections extending from the cell body in the presence of IGF-1 (Figure 4.3B), whereas some of the cells were elongated and fibroblast-like (Figure 4.3C) in the presence of FGF-2. However, for the most part in both treatments they look similar
Chapter 4: Effects of IGF-1, FGF-2, RA and Vit K on osteocyte differentiation in 3D

to the control, but there also seemed to be a reduced number of cells with FGF-2 at day 1.

After 15 days, most control cells appeared osteoblast-like in that a stellate morphology was not observed, although some cells did have some processes (Figure 4.3D). However, following IGF-1 treatment the cells appear to have numerous osteocyte-like projections, with dendritic networks being formed between some cells (Figure 4.3E). With FGF-2 treatment, the cells have many more osteocyte-like projections than the control cells or those treated with IGF-1 (Figure 4.3F). Furthermore, in gels on day 15 there appeared to be fewer cells in the IGF-1 or FGF-2 treated gels when compared to controls, and there appeared to be more unstained regions in the FGF-2 treated gels at this time point.

4.5.2.2 RA or Vit K

After 1 day, control (DMSO) cells appeared different to the PBS control described in the previous section, in that the cells are sparse within the gels and have longer dendritic-like processes (Figure 4.4A). Following treatment with RA for 1 day, the cells appeared to be elongated with some cells showing signs of the formation of osteocyte-like dendritic processes within the gel, but these appear to be less dendritic that the DMSO control cells at the same time point (Figure 4.4B). After 1 day in the presence of Vit K, the cells seemed similar to the DMSO control at the same time point and have elongated dendritic-like processes extending from the cell body (Figure 4.4C).
After 15 days, the cells in the control gels were similar to those seen in the previous section in the control gels not containing DMSO (Figure 4.4). However, following treatment with RA for 15 days, the cells look morphologically similar to the DMSO control at the same time point, but there appears to be more cells in this section when compared to the DMSO control (Figure 4.4E). When cells were treated with Vit K, they were very different morphologically to cells in control gels at day 15 and cells in gels that had been treated with Vit K for 1 day (Figure 4.4C). Some of these cells appeared to have larger amounts of cytoplasm than mature osteocytes but did have some thicker osteocyte-like dendritic processes. However, at this time point there appears to be fewer cells with this treatment and there were some unstained regions in the gels, which were not observed at day 1 of the same treatment (Figure 4.4F).

4.5.3 Protein expression following IGF-1 treatment

4.5.3.1 E11 expression

E11 is expressed when osteoblasts differentiate to an osteoid cell or osteocyte, and is localised to the dendritic processes of the cells [52]. At all time points investigated, E11 expression was localised to the dendritic processes of the cells. Analysis of E11 expression in MC-3T3 cells in 3D collage type I gels revealed that that IGF-1 treatment reduced E11 expression earlier in culture. In the control samples, E11 expression was present on day 3, 7 and 11 prior to being reduced in expression on day 15 (Figure 4.5A-D). In IGF-1 treated cells, E11 expression was present on day 3 and day 7, prior to decreasing in expression on days 11 and 15 (Figure 4.5E-H).
4.5.3.2 DMP-1 expression

In osteoblasts, DMP-1 is known to be localised around the nucleus, but is localised to the dendritic processes in osteocytes. At all time points investigated in this study, DMP-1 expression was localised to the dendritic processes. Analysis of DMP-1 expression in MC-3T3 cells in 3D collagen type 1 gels revealed that IGF-1 treatment induced DMP-1 expression earlier in culture when compared to the control. In the control samples, DMP-1 expression is absent on day 3, present at day 7 and continues to increase in expression with days in culture (Figure 4.6A-D). Following IGF-1 treatment, DMP-1 expression was seen at day 3, peaked in expression at day 7, before decreasing in expression at days 11 and 15 (4.6E-H).
Chapter 4: Effects of IGF-1, FGF-2, RA and Vit K on osteocyte differentiation in 3D

Figure 4.1: MC-3T3 cell number and viability in 3D collagen type I gels following treatment with (1) IGF-1 or (2) FGF-2. MC-3T3 cell number expressed relative to day 3 control (1A & 2A) and % cell viability (1B & C, 2 B & C) in control (1A-B, 2A-B), IGF-1 (1A & 1C) or FGF-2 (2A & 2C) treated gels at days 1, 3, 7, 11 and 15. Data are a combination of 3 (n=8 at each data point, 1A-C) or 2 (n=6 at each data point, 2A-C) independent experiments. Data are mean ± SEM. * p<0.05, ** p<0.01, *** p<0.001 versus same condition at day 1 or ## p<0.01 versus control at the same time point, following a two-way ANOVA and a Bonferroni post hoc test.
Chapter 4: Effects of IGF-1, FGF-2, RA and Vit K on osteocyte differentiation in 3D

Figure 4.2: MC-3T3 cell number and viability in 3D collagen type I gels following treatment with (1) RA or (2) Vit K. MC-3T3 cell number expressed relative to day 1 control (1A & 2A) and cell viability (1B-C & 2B-C) following 1, 3, 7, 11 and 15 days of treatment with RA (15 µg/ml, 1A & 1C) and Vit K (5 µg/ml, 2A & 2C). Data are a combination of 2 (n=6 at each data point, 1A-C & 2A-C) independent experiments. Data are mean ± SEM. * $p<0.05$, ** $p<0.01$, *** $p<0.001$ versus same condition at day 1 or ### $p<0.001$ versus control at the same time point, following a two-way ANOVA and a Bonferroni post hoc test.
Chapter 4: Effects of IGF-1, FGF-2, RA and Vit K on osteocyte differentiation in 3D

Figure 4.3: H&E staining of MC-3T3 cells in 3D collagen type I gels following treatment with IGF-1 or FGF-2. Representative H&E staining of fixed and sectioned (7µm) 3D collagen gels containing MC-3T3 cells following 1 or 15 days in control (PBS; A, D), IGF-1 (B, E) or FGF-2 (C, F) treated cultures. Images were captured using the Aperio Scanscope (20x magnification) and processed using the Aperio software. The scale bar is 100 µm in each image, with the inset in the black box (20x) is of the area represented by the red box in each image. All images are from 1 experiment where all staining was undertaken at the same time, with 5 sections stained per individual time point. a= rounded cells with short processes, b = osteocyte-like dendritic processes, c = elongated fibroblast-like cells, d osteoblast-like cells. Images shown are a representative of the entire depth of the gel.
Figure 4.4: H&E staining of MC-3T3 cells in 3D collagen type I gels following treatment with RA or Vit K. Representative H&E staining of fixed and sectioned (7µm) 3D collagen gels containing MC-3T3 cells following 1 or 15 days in control (DMSO; A, D), RA (B, E) or Vit K (C, F) treated cultures. Images were captured using the Aperio Scanscope (20x magnification) and processed using the Aperio software. The scale bar is 100 µm in each image, with the inset in the black box (20x) is of the area represented by the red box in each image. All images are from 1 experiment where all staining was undertaken at the same time, with 5 sections stained per individual time point. a = osteocyte-like dendritic processes and b = elongated fibroblast-like cells. Images shown are a representative of the entire depth of the gel.
Chapter 4: Effects of IGF-1, FGF-2, RA and Vit K on osteocyte differentiation in 3D

Figure 4.5: E11 Protein expression in MC-3T3s in 3D collagen type I gels following treatment with IGF-1. MC-3T3s were treated with PBS (control; A-D) or IGF-1 (50 ng/ml, E-H) for 3, 7, 11 or 15 days. Individual gels were fixed, processed and sectioned prior to IHC for E11 expression and counterstained with haematoxylin (purple). Results presented were representative of images from 1 experiment (scale bar = 100 µm) with the inset in the black box (20x) is of the area represented by the red box in each image. IHC was undertaken on 1 gel per time point with a minimum of 4 replicate sections stained per gel, with 3 independent experiments undertaken. a = E11 positive cellular staining, b = reduction in E11 positive staining and c = E11 negative cellular staining, where I = IgG negative control.
Chapter 4: Effects of IGF-1, FGF-2, RA and Vit K on osteocyte differentiation in 3D

**Figure 4.6: DMP-1 protein expression in MC-3T3s in 3D collagen type I gels following treatment with IGF-1.** MC-3T3s were treated with PBS (control; A-D) or IGF-1 (50 ng/ml, E-H) for 3, 7, 11 or 15 days. Individual gels were fixed, processed and sectioned prior to IHC for DMP-1 expression and counterstained with haematoxylin (purple). Results presented were representative of images from 1 experiment (scale bar = 100 µm) with the inset in the black box (20x) is of the area represented by the red box in each image. IHC was undertaken on 1 gel per time point with a minimum of 4 replicate sections stained per gel, with 3 independent experiments undertaken. a = DMP-1 negative cellular staining, b = DMP-1 positive cellular staining, c = increased DMP-1 positive cellular staining and d = reduction in DMP-1 positive staining where I = IgG negative control.
4.5.4  E11, DMP-1, Cx43, LRP-5, RANKL and sclerostin mRNA expression

All the gene expression data were normally distributed therefore all statistical analysis was undertaken using a two-way ANOVA followed by a Bonferroni post hoc test with \( p \) values for each gene following treatment with IGF-1, FGF-2 and RA or Vit K when compared to their respective controls (Table 4.3). IGF-1 and FGF-2 were analysed independently (due to different \( n \) numbers), whereas RA and Vit K were analysed together.

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</tr>
<tr>
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<td>&lt;0.0001</td>
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<td>0.1409</td>
<td>0.1103</td>
</tr>
<tr>
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<td>&lt;0.0001</td>
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<td>0.0009</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Interaction between time and treatment</td>
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<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td>0.00755</td>
<td>0.1223</td>
<td>0.1526</td>
</tr>
</tbody>
</table>

Table 4.3: Results (\( p \) values) following a two-way ANOVA investigating the effect of time, treatment and the interaction between time and treatment on gene expression in MC-3T3 cells when comparing cells in control medium (PBS or DMSO) to following treatment with IGF-1, FGF-2 or Vit K.

4.5.4.1 IGF-1 or FGF-2 treatment

E11 expression was detected in the control, IGF-1 or FGF-2 treated cells on all time points investigated (Figure 4.7A & 4.8A). However, E11 expression was not modulated over time in the control and IGF-1 treated cells, and IGF-1 treatment had no effect on E11 expression (Figure 4.7A). Similarly FGF-2 treatment did not modulate E11 expression over time following FGF-2 treatment (Figure 4.8A).
Chapter 4: Effects of IGF-1, FGF-2, RA and Vit K on osteocyte differentiation in 3D

However, FGF-2 treatment significantly up-regulated E11 expression on days 3 ($p<0.01$) and 11 ($p<0.001$) when compared to the control at the same time point (Figure 4.8A).

All MC-3T3 cells in the control, IGF-1 or FGF-2 treated gels expressed DMP-1 on all time points investigated (Figure 4.7B & 4.8B). In the control DMP-1 expression was significantly up-regulated ($p<0.01$) on day 7, and significantly down-regulated ($p<0.01$) on days 11 and 15 when compared to day 3 (Figure 4.7B & 4.8B). Following IGF-1 treatment, DMP-1 expression was significantly up-regulated ($p<0.01$) on day 7 and returned to levels observed on day 3 on days 11 and 15 (Figure 4.7B). Furthermore, when compared to the control at the same time point, DMP-1 expression was significantly down-regulated ($p<0.01$) on day 3 and significantly up-regulated ($p<0.001$) on day 7 of IGF-1 treatment (Figure 4.7B). DMP-1 expression was not modulated over time following treatment with FGF-2 (Figure 4.8B). However, when compared to control at the same time point, DMP-1 expression was significantly up-regulated following FGF-2 treatment at all time points investigated (Figure 4.8B).

Cx43 mRNA expression was detected in the control, IGF-1 or FGF-2 treated cells on all time points investigated (Figure 4.7C & 4.8C). However, Cx43 expression was not modulated over time in the control, but was significantly down-regulated on day 7 following IGF-1 treatment (Figure 4.7C). When compared to control at the same time point, Cx43 expression was significantly up-regulated following 3 ($p<0.01$), 11 ($p<0.001$) and 15 ($p<0.001$) days of treatment with IGF-1 (Figure 4.7C). Furthermore, FGF-2 treatment significantly up-regulated Cx43 expression on day 15 [159]
Chapter 4: Effects of IGF-1, FGF-2, RA and Vit K on osteocyte differentiation in 3D

when compared to day 3 of the same treatment (Figure 4.8C). Cx43 expression was significantly up-regulated ($p<0.001$) following 7, 11 and 15 days of FGF-2 treatment when compared to control at the same time point (Figure 4.8C).

All MC-3T3 cells in the control, IGF-1 or FGF-2 treated gels expressed LRP-5 on all time points investigated (Figure 4.7D & 4.8D). In the control, LRP-5 mRNA expression was significantly up-regulated ($p<0.01$) on day 15, but IGF-1 treatment had no effect on LRP-5 mRNA expression (Figure 4.7D). Furthermore, LRP-5 expression was significantly down-regulated ($p<0.001$) on day 15 following treatment with IGF-1 when compared to control at the same time point (Figure 4.7D). FGF-2 treatment modulated LRP-5 expression over time with a significant (~5-fold, $p<0.001$) reduction at day 15 when compared to day 11. Furthermore, when compared to control at the same time point, there was a significant (~20-fold, $p<0.001$) increase following 11 days of FGF-2 treatment (Figure 4.8D).

RANKL expression was detected in the control, IGF-1 or FGF-2 treated cells on all time points investigated (Figure 4.7E & 4.8E). However, RANKL expression was not modulated in the control on all time points investigated, but was significantly up-regulated (~5-fold, $p<0.01$) on day 15 of IGF-1 treatment when compared to day 11 (Figure 4.7E). Furthermore, when compared to control at the same time point, IGF-1 treatment significantly up-regulated ($p<0.01$) RANKL expression on day 11 and day 15 (Figure 4.7E). FGF-2 treatment significantly increased (~25-fold, $p<0.001$) RANKL expression on day 11 when compared control at the same time point. Furthermore, when compared to FGF-2 treatment on day 11, there was a significant (~12-fold, $p<0.001$) reduction in RANKL expression on day 15 (Figure 4.8E).
Chapter 4: Effects of IGF-1, FGF-2, RA and Vit K on osteocyte differentiation in 3D

All MC-3T3 cells in the control, IGF-1 or FGF-2 treated gels expressed FGF-23 on all time points investigated (Figure 4.7F & 4.8F). However, following treatment with IGF-1, FGF-23 expression was significantly up-regulated ($p<0.01$) on day 15 when compared to same treatment on day 11 and there was a ~17-fold increase ($p<0.001$) in FGF-23 expression when compared to the control at day 15 (Figure 4.7F). FGF-2 treatment significantly (~75-fold, $p<0.001$) increased FGF-23 expression on day 11 when compared to control at the same time point. However, when compared to FGF-2 treatment on day 11, there was a significant reduction in FGF-23 expression on day 15 (~38-fold, Figure 4.8F).

Sclerostin expression was not detected in the control or FGF-2 treated cells on all time points investigated but was detected on days 7, 11 and 15 following treatment with IGF-1 (Figure 4.11). However, in the presence of IGF-1, sclerostin mRNA expression did not change over time, although mean gene expression values were higher on day 11 (1.892 ± 0.400) and 15 (3.327 ± 0.253) when compared to day 7 (1.008 ± 0.870, Figure 4.11)

4.5.4.2 RA or Vit K treatment

E11 expression was detected in the control (DMSO), RA and Vit K treated cells on all time points apart from day 11 of RA treatment (Figure 4.9A & 4.10A). E11 expression was not modulated over time in the control or Vit K treatment, but following RA treatment, there was a significant decrease ($p<0.001$) in E11 expression on day 15 when compared to day 3 of the same treatment (Figure 4.9A & 4.10A). Furthermore, when compared to control at the same time point, there was a
significant decrease in E11 expression following RA ($p<0.001$) or Vit K ($p<0.01$) treatment on day 11 (Figure 4.9A & 4.10A).

All MC-3T3 cells in the control, RA or Vit K treated gels expressed DMP-1 on all time points investigated (Figure 4.9B & 4.10B). However, on individual time points, DMP-1 expression was not modulated over time, and there was no effect of RA or Vit K treatment on DMP-1 expression (Figure 4.9B & 4.10B).

Cx43 expression was detected in the control, RA or Vit K treated cells on all time points investigated (Figure 4.9C & 4.10C). However, Cx43 expression was not modulated over time in the control of Vit K treated cell, but was significantly up-regulated ($p<0.001$) following 15 days of treatment with RA (Figure 4.9C & 4.10C). When compared to control at the same time point, Cx43 expression was significantly up-regulated following 7 ($p<0.05$) or 15 ($p<0.001$) days of treatment with RA, with no effect observed following Vit K treatment (Figure 4.9C & 4.10C).

All MC-3T3 cells in the control, RA or Vit K treated gels expressed LRP-5 on all time points investigated (Figure 4.9D & 4.10D). LRP-5 expression was not modulated over time in the control, RA or Vit K treated cells. However, following 11 days of treatment with RA, LRP-5 expression was significantly up-regulated (~7-fold, $p<0.001$) when compared to control at the same time point (Figure 4.9D & 4.10D).

RANKL expression was detected in the control; RA or Vit K treated cells on all time points investigated (Figure 4.9E & 4.10E). RANKL expression was not modulated
Chapter 4: Effects of IGF-1, FGF-2, RA and Vit K on osteocyte differentiation in 3D

over time in the control or Vit K treated cells, but was significantly down-regulated (~3-fold, \( p<0.01 \)) following 15 days of treatment with RA (Figure 4.9E & 4.10E). Furthermore, when compared to control at the same time point, no effect of Vit K treatment was observed, but RA treatment significantly up-regulated (~64-fold, \( p<0.001 \)) RANKL expression on day 11 (Figure 4.9E & 4.10E).

All MC-3T3 cells in the control, RA or Vit K treated gels expressed FGF-23 on all time points investigated (Figure 4.9F & 4.10F). FGF-23 expression was not modulated over time in control or Vit K treated cells, but was significantly down-regulated (~2-fold, \( p<0.01 \)) on day 15 following RA treatment (Figure 4.9F & 4.10F). When compared to control at the same time point, Vit K had no effect on FGF-23 expression, but RA expression was significantly up-regulated following 11 (~29-fold, \( p<0.001 \)) or 15 (~32-fold, \( p<0.01 \)) days of treatment (Figure 4.9F & 4.10F).

Sclerostin mRNA expression was not detected in the control, or following RA or Vit K treatment on all time points investigated.
Chapter 4: Effects of IGF-1, FGF-2, RA and Vit K on osteocyte differentiation in 3D

Figure 4.7: E11, DMP-1, Cx43, LRP-5, RANKL and FGF-23 mRNA expression in MC-3T3 cells in 3D collagen type I gels following treatment with IGF-1. Relative expression of E11 (A), DMP-1 (B) or Cx43 (C), LRP-5 (D), RANKL (E) or FGF-23 (F) in MC-3T3 cells in 3D collagen gels on 3, 7, 11 and 15 days (A-C) or 11 and 15 days (D-F) following treatment with IGF-1 (50 ng/ml, A-F). RT-qPCR analysis of RNA isolated from cells, normalised to 18sRNA (A-C: 1 gel of day 3 control set to 1 in each experiment, D-F: 1 gel of day 11 control set to 1 in each experiment). Data are a combination of 3 (n=8 at each data point, A-C) or 2 (n=6 at each data point, D-F) independent experiments. Data in A-F are mean ± SEM ** p<0.01 versus same condition on day 3 (A-C) or day 11 (D-F) or ##p<0.01, ### p<0.001 versus control at the same time point, following a two-way ANOVA and a Bonferroni correction.
Chapter 4: Effects of IGF-1, FGF-2, RA and Vit K on osteocyte differentiation in 3D

**Figure 4.8**: E11, DMP-1, Cx43, LRP-5, RANKL and FGF-23 mRNA expression in MC-3T3 cells in 3D collagen type I gels following treatment with FGF-2. Relative expression of E11 (A), DMP-1 (B), Cx43 (C), LRP-5 (D), RANKL (E), FGF-23 (F) in MC-3T3 cells in 3D collagen gels on 3, 7, 11 and 15 days (A-C) or 11 and 15 (D-F) of treatment with FGF-2 (10 ng/ml). RT-qPCR analysis of RNA isolated from cells, normalised to 18sRNA (A-C: 1 gel of day 3 control set to 1 in each experiment, D-F: 1 gel of day 11 control set to 1 in each experiment). Data (A-F) are a combination of 3 (n=8 at each data point, A-C) or 2 (n=6 at each data point, D-F) independent experiments. Data in A-F are mean ± SEM. ** p<0.01, *** p<0.001 versus same condition at day 3 (A-C) or day 11 (D-F) or # p<0.05, ## p<0.01, ### p<0.001 versus control at the same time point, following a two-way ANOVA and a Bonferroni post hoc test.
Chapter 4: Effects of IGF-1, FGF-2, RA and Vit K on osteocyte differentiation in 3D

Figure 4.9: E11, DMP-1, Cx43, LRP-5, RANKL and FGF-23 mRNA expression in MC-3T3 cells in 3D collagen type I gels following treatment with RA. Relative expression of E11 (A), DMP-1 (B), Cx43 (C), LRP-5 (D), RANKL (E), FGF-23 (F) in MC-3T3 cells in 3D collagen gels on 3, 7, 11 and 15 days (A-C) or 11 and 15 (D-F) of treatment with RA (15 µg/ml). RT-qPCR analysis of RNA isolated from cells, normalised to 18sRNA (A-C: 1 gel of day 3 control set to 1 in each experiment, D-F: 1 gel of day 11 control set to 1 in each experiment). Data (A-F) are a combination of 3 (n=8 at each data point, A-C) or 2 (n=6 at each data point, D-F) independent experiments. Data in A-F are mean ± SEM. ** p<0.01, *** p<0.001 versus same condition at day 3 (A-C) or day 11 (D-F) or # p<0.05, ## p<0.01, ### p<0.001 versus control at the same time point, following a two-way ANOVA and a Bonferroni post hoc test.

[166]
Chapter 4: Effects of IGF-1, FGF-2, RA and Vit K on osteocyte differentiation in 3D

Figure 4.10: E11, DMP-1, Cx43, LRP-5, RANKL and FGF-23 mRNA expression in MC-3T3 cells in 3D collagen type I gels following treatment with Vit K. Relative expression of E11 (A), DMP-1 (B), Cx43 (C), LRP-5 (D), RANKL (E), FGF-23 (F) in MC-3T3 cells in 3D collagen gels on 3, 7, 11 and 15 days (A-C) or 11 and 15 (D-F) of treatment with Vit K (5 µg/ml). RT-qPCR analysis of RNA isolated from cells, normalised to 18sRNA (A-C: 1 gel of day 3 control set to 1 in each experiment, D-F: 1 gel of day 11 control set to 1 in each experiment). Data (A-F) are a combination of 3 (n=8 at each data point, A-C) or 2 (n=6 at each data point, D-F) independent experiments. Data in A-F are mean ± SEM. *** $p<0.001$ versus same condition at day 3 (A-C) or day 11 (D-F) or ## $p<0.01$, ### $p<0.001$ versus control at the same time point, following a two-way ANOVA and a Bonferroni post hoc test.
Figure 4.11: Sclerostin mRNA expression in MC-3T3 cells in 3D collagen type I gels following treatment with IGF-1. Relative expression of sclerostin (A) in MC-3T3 cells in 3D collagen gels 3, 7, 11 and 15 days following treatment with IGF-1 (50 ng/ml). RT-qPCR analysis of RNA isolated from cells, normalised to 18sRNA. Data are a combination of 3 (n=8) independent experiments. Data are mean ± SEM.

4.5.5 VEGF, IL-6 and FGF-23 protein secretion

VEGF and IL-6 protein secretion data were normally distributed therefore all statistical analysis was undertaken using a two-way ANOVA followed by a Bonferroni post hoc test with p values following the two-way ANOVAs for each gene following treatment with IGF-1, FGF-2 or RA or Vit K when compared to their respective controls (Table 4.4). IGF-1 and FGF-2 were analysed independently (due to different n numbers), whereas RA and Vit K were analysed with together.

<table>
<thead>
<tr>
<th></th>
<th>IGF-1</th>
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<th>RA/ Vit K</th>
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<td>IL-6</td>
<td>VEGF</td>
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<tr>
<td>time and treatment</td>
<td></td>
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</table>

Table 4.4: Results (p values) following a two-way ANOVA investigating the effect of time, treatment and the interaction between time and treatment on VEGF and IL-6 when comparing cells in control medium (PBS or DMSO) and following treatment with IGF-1, FGF-2 or RA or Vit K. * = unable to do two-way ANOVA due to zero values in the FGF-2 data.
4.5.5.1 IGF-1 or FGF-2 treatment

VEGF protein secretion was detected in control (PBS), IGF-1 and FGF-2 treated cells at all time points investigated (Figure 4.12A & 4.13A). VEGF protein secretion was not modulated in the control or IGF-1 treated cells on all time points investigated (Figure 4.12A & 4.13A). However in both the control and the IGF-1 treated cells, mean VEGF protein secretion remained relatively constant over the 15 days, except on day 3 when VEGF secretion was reduced by 75% for control and 60% for IGF-1 treatment (Figure 4.12A). Following treatment with FGF-2, VEGF protein secretion was not modulated over time (Figure 4.13A). However, when compared to control at the same time point VEGF protein secretion was significantly up-regulated ($p<0.05$) on day 15 following FGF-2 treatment (Figure 4.13A).

IL-6 protein secretion was detected in control (PBS) or IGF-1 treated cells, but was not detected in FGF-2 treated cells on all time points investigated (Figure 4.12B & 4.13B). In the control, IL-6 protein was significantly reduced ($p<0.01$) on days 3, 7, 11 and 15 when compared to day 1. Following treatment with IGF-1, IL-6 protein was significantly reduced ($p<0.01$) on day 3 when compared to day 1 (Figure 4.12B). Furthermore, when compared to control at the same time point, IL-6 protein secretion was significantly reduced ($p<0.001$) following IGF-1 treatment on day 1 (Figure 4.12B).

FGF-23 protein secretion was only investigated on one sample on day 11 and 15 for MC-3T3s in 3D collagen type I gels. Treatment with IGF-1 was the only factor that FGF-23 protein secretion was detected. Furthermore, it appeared to have up-
regulated FGF-23 protein secretion following 11 and 15 days of treatment, when compared to the control at the same time point (Figure 4.12C).
Chapter 4: Effects of IGF-1, FGF-2, RA and Vit K on osteocyte differentiation in 3D

Figure 4.12: VEGF, IL-6 and FGF-23 protein secretion in MC-3T3 cells in 3D collagen type I gels following treatment with IGF-1. VEGF (A), IL-6 (B) or FGF-23 (C) secretion measured by ELISA in MC-3T3 cells in control cells or treated with IGF-1 (50 ng/ml) and normalised to cell number in individual gels and expressed relative to control at day 1 set to 100 % (A-B only). Data are a combination of 2 (n=6 at each data point, A-B) or 1 (sample from 1, C) independent experiments. Data in A-B are mean ± SEM. *** p<0.001 versus same treatment on day 1 or ### p<0.001 versus control at the same time point following a two-way ANOVA and a Bonferroni post hoc test.
Chapter 4: Effects of IGF-1, FGF-2, RA and Vit K on osteocyte differentiation in 3D

Figure 4.13: VEGF and IL-6 protein secretion in MC-3T3 cells in 3D collagen type I gels following treatment with FGF-2. VEGF (A) or IL-6 (B) secretion measured by ELISA in MC-3T3 cells in control cells or treated with FGF-2 (10 ng/ml) and normalised to cell number in individual gels and expressed relative to control at day 1 set to 100% (A-B). Data are a combination of 2 (n=6 at each data point, A-B) independent experiments. Data in A-B are mean ± SEM. **p<0.01, ***p<0.001 versus same treatment on day 1 or #p<0.05 versus control at the same time point following a two-way ANOVA and a Bonferroni post hoc test.

4.5.5.2 RA or Vit K treatment

VEGF protein secretion was detected in control (DMSO) and RA treated cells on all time points investigated, but was only detected following treatment with Vit K on days 7, 11 and 15 (Figure 4.14A & 4.15A). In the control VEGF protein secretion was not modulated over time. Following treatment with RA, VEGF protein secretion was significantly up-regulated (p<0.001) following 3, 7 and 15 days of treatment when compared the same treatment on day 1 (Figure 4.14A). Furthermore, when compared to control at the same time point, VEGF protein secretion was significantly up-regulated (p<0.001) following RA treatment on days 3, 7 and 15 (Figure 4.14A). Following treatment with Vit K, VEGF protein secretion was significantly up-regulated on days 11 (p<0.01) and 15 (p<0.05). Furthermore, when compared to control at the same time point, VEGF protein secretion was significantly up-regulated following 7 (p<0.001) and 11(p<0.01) days of treatment (Figure 4.15A).
IL-6 protein secretion was detected in control (DMSO) samples on all time points investigated and was detected on days 1 and 3 only following treatment with RA or on days 1, 3 and 7 only following treatment with Vit K (Figure 4.14B & 4.15B). In the control, IL-6 protein secretion was significantly reduced ($p<0.001$) on days 3, 7, 11 and 15 when compared to day 1 (Figure 4.14B & 4.15B). Furthermore, following treatment with RA or Vit K, IL-6 protein was also significantly reduced ($p<0.001$) following 3, 7, 11 and 15 days of treatment when compared to day 1 of the same treatment (Figure 4.14B & 4.15B). When compared to control at the same time point, IL-6 protein secretion was significantly down-regulated ($p<0.001$) following treatment with RA on day 11, or following treatment with Vit K on days 1 or 11 (Figure 4.14B & 4.15B).

**Figure 4.14:** VEGF and IL-6 protein secretion in MC-3T3 cells in 3D collagen type I gels following treatment with RA. VEGF (A) or IL-6 (B) secretion measured by ELISA in MC-3T3 cells in control cells or treated with RA (15 µg/ml) and normalised to cell number in individual gels and expressed relative to control at day 1 set to 100 %. Data in A-B are a combination of 2 (n=6 at each data point) independent experiments. Data in A-B are mean ± SEM. *** $p<0.001$ versus same treatment on day 1 or ### $p<0.001$ versus control at the same time point following a two-way ANOVA and a Bonferroni post hoc test.
Chapter 4: Effects of IGF-1, FGF-2, RA and Vit K on osteocyte differentiation in 3D

**Figure 4.15:** VEGF and IL-6 protein secretion in MC-3T3 cells in 3D collagen type I gels following treatment with Vit K. VEGF (A) or IL-6 (B) secretion measured by ELISA in MC-3T3 cells in control cells or treated with Vit K (5 µg/ml) and normalised to cell number in individual gels and expressed relative to control at day 1 set to 100 %. Data in A-B are a combination of 2 (n=6 at each data point) independent experiments. Data in A-B are mean ± SEM. * p<0.05, ** p<0.01, *** p<0.001 versus same treatment on day 1 or ## p<0.01, ### p<0.001 versus control at the same time point following a two-way ANOVA and a Bonferroni post hoc test.

4.5.6 Effect of IGF-1 treatment on MC-3T3 cells in mineralisation medium

4.5.6.1 E11, DMP-1, RANKL, PHEX, MEPE and FGF-23 mRNA expression

All the gene expression data were normally distributed therefore all statistical analysis was undertaken using a two-way ANOVA followed by a Bonferroni post hoc test with p values following the two-way ANOVAs for each gene investigating the effect of IGF-1 treatment on mineralising medium (Table 4.5).

<table>
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<th>IGF-1 treatment on MC-3T3 cells in basal (control) or mineralising medium</th>
<th>E11</th>
<th>DMP-1</th>
<th>RANKL</th>
<th>PHEX</th>
<th>MEPE</th>
<th>FGF-23</th>
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<td>Interaction between time and treatment</td>
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<td>0.4009</td>
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</tr>
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</table>

**Table 4.5:** Results (p values) following a two-way ANOVA investigating the effect of time, treatment and the interaction between time and treatment on E11, DMP-1, RANKL, PHEX, MEPE and FGF-23 when comparing cells in basal or mineralising medium following treatment with IGF-1.
Chapter 4: Effects of IGF-1, FGF-2, RA and Vit K on osteocyte differentiation in 3D

In basal (control) or mineralising medium, E11 expression was not modulated by IGF-1 treatment (Figure 4.16A). Furthermore, E11 expression was significantly up-regulated ($p<0.05$) in mineralising medium on day 15 when compared to the same treatment at day 11 (Figure 4.16A). DMP-1 expression was not modulated in basal or mineralising medium following treatment with IGF-1 on day 11 (Figure 4.16B). On day 15, there was no effect of IGF-1 treatment in basal medium, but there was a significant increase (2.8-fold, $p<0.01$) in mineralising medium (Figure 4.16B). Furthermore, when compared to the same treatment on day 11, there was a 77-fold increase ($p<0.001$) in mineralising medium, and a 3.1-fold increase ($p<0.001$) in mineralising medium following treatment with IGF-1 (Figure 4.16B).

RANKL expression was significantly up-regulated following 11 (8.2-fold, $p<0.001$) and 15 (9.15-fold, $p<0.001$) days of treatment with IGF-1 in basal medium (Figure 4.16C). When comparing IGF-1 treatment in basal medium on day 15 with the same treatment on day 11 a 2.4-fold increase ($p<0.001$) in RANKL expression was observed. Furthermore, this significant up-regulation of RANKL expression following IGF-1 treatment did not occur under mineralising conditions (Figure 4.16C).

There was no effect of IGF-1 treatment in basal medium on PHEX expression on each time point investigated (Figure 4.16D). In mineralising medium following IGF-1 treatment, PHEX expression was significantly up-regulated on 11 (6.2-fold, $p<0.05$) or 15 (2.4-fold, $p<0.01$) days (Figure 4.16D). There was no effect of IGF-1 treatment in basal or mineralising medium on MEPE expression on each time point investigated (Figure 4.16E). FGF-23 expression was significantly up-regulated
following 15 days of treatment with IGF-1 in basal medium. However this effect was not observed following IGF-1 treatment in mineralising medium (Figure 4.16F).

4.5.6.2 VEGF and IL-6 protein secretion

There was no effect on VEGF protein secretion following IGF-1 treatment in basal or mineralising medium on all time points investigated (Figure 4.17A). IL-6 protein secretion was not affected by IGF-1 treatment in basal medium on days 11 and 15, or mineralising medium on day 11 (Figure 4.17B). However, a significant reduction (5-fold, \(p<0.01\)) in IL-6 protein secretion was seen on day 15 in mineralising medium following treatment with IGF-1 (Figure 4.17B). Furthermore, when compared to same condition on day 11, IL-6 protein was significantly reduced on day 15 in basal medium (2-fold, \(p<0.05\)), basal medium treated with IGF-1 (3-fold, \(p<0.001\)) and mineralising medium (5-fold, \(p<0.05\), Figure 4.17B).
Figure 4.16: E11, DMP-1, RANKL, PHEX, MEPE and FGF-23 mRNA expression in MC-3T3 cells in 3D collagen type I gels following treatment with IGF-1 in control and mineralising medium. Relative expression of E11 (A), DMP-1 (B), RANKL (C), PHEX (D), MEPE (E) and FGF-23 (F) in MC-3T3s in 3D collagen gels following 11 and 15 days of treatment with IGF-1 (50 ng/ml) in control and mineralising medium. RT-qPCR analysis of RNA isolated from cells, normalised to 18sRNA (1 gel of day 11 control set to 1). Data in A-F are from 1 experiment (n=4 at each data point). Data in A-F are mean ± SEM. *p<0.05, **p<0.01, ***p <0.001 versus control or mineralising medium at the same time point or ## p<0.01, ### p <0.001 versus same treatment on day 11, following a two-way ANOVA and a Bonferroni post hoc test.
Chapter 4: Effects of IGF-1, FGF-2, RA and Vit K on osteocyte differentiation in 3D

Figure 4.17: VEGF and IL-6 secretion in MC-3T3 cells in 3D collagen type I gels following treatment with IGF-1 in control and mineralising medium. VEGF (A) and IL-6 (B) secretion measured by ELISA in MC-3T3 cells treated with IGF-1 (50 ng/ml) in control and mineralising medium, and normalised to cell number in individual gels and expressed relative to control at day 11 set to 100 %. Data in A-B are from one experiment (n=4 at each data point). Data in A-B are mean ± SEM. ** p<0.01 versus control or mineralising medium at the same time point following or # p<0.05, ### p<0.001 versus same treatment on day 11, following a two-way ANOVA and a Bonferroni correction.

4.5.7 Effect of IGF-1 treatment on hOBs

4.5.7.1 Cell number and viability

hOB cell numbers in collagen gels, both in the presence and absence of IGF-1, increased during the 15 day incubation period, with a significant increase in total cell number in the presence of IGF-1 on days 11 (p<0.001) and 15 (p<0.001) when compared to cells treated with IGF-1 for 3 days (Figure 4.18A). Whilst mean cell numbers were increased in IGF-1 treated gels compared to controls on the same day on days 3 (control; 100.193± 5.133, IGF-1; 152.662 ± 21.356) and 7 (control; 153.070 ± 14.469, IGF-1; 288.300 ± 70.086) these values were not significantly different. Cell viability in the control and IGF-1 treated hOB cells was >91 % (Figure 4.18B) and >94 % respectively (Figure 4.18C) at all time points. Furthermore, IGF-1 did not modulate hOB cell viability (Figure 4.18B-C).
Chapter 4: Effects of IGF-1, FGF-2, RA and Vit K on osteocyte differentiation in 3D

4.5.7.2 Morphology

The H&E images presented were a representative of the entire depth of the gel, and similar to cell viability results presented previously confirmed that all cells were viable throughout the depth of the gels (Figure 4.19). H&E stained sections of hOBs in control gels after 11 and 15 days in culture showed similar morphology to the control hOB cells described in Chapter 3 (Section 3.5.2.1) IGF-1 induced some changes in morphology when compared to the controls. Specifically, following 11 days of treatment, some cells appeared to have more osteocyte-like dendritic processes (Figure 4.19B). After 15 days of treatment, the cell shape was different to that seen at day 11 and in controls (Figure 4.19D).

4.5.7.3 E11, Cx43, DMP-1 and sclerostin mRNA gene expression

IGF-1 treatment did not modulate E11 and Cx43 gene expression following 11 and 15 days of treatment (Figure 4.20A & B). However, DMP-1 mRNA expression was significantly (\(p<0.001\)) increased (~4-fold) compared to control following 11 days of treatment and then returned to control levels by day 15 (Figure 4.20C). Furthermore, sclerostin mRNA expression was significantly up-regulated following 11 (~7-fold; \(p<0.05\)) or 15 (~11-fold; \(p<0.01\)) days of treatment with IGF-1 (Figure 4.20D).
Figure 4.18: hOB cell number and viability in 3D collagen type I gels following treatment with IGF-1. hOB cell number expressed relative to day 3 control (A) and % cell viability (B-C) in control or IGF-1 (50ng/ml) treated gels at days 1, 3, 7, 11 and 15. Data are a combination of 4 independent experiments (n=11 per time point, apart from day 1 where n=3 from 1 experiment). Data are mean ± SEM. * $p<0.01$ ** $p<0.001$, *** $p<0.001$, versus same treatment at day 3 following a one-way ANOVA and a Bonferroni post hoc test.
Figure 4.19: H&E staining of hOB cells in 3D collagen type I gels following treatment with IGF-1. Representative H&E staining of fixed and sectioned (7µm), 3D collagen gels containing hOB cells following 11 or 15 days of treatment in control (PBS, A, C), IGF-1 treated (50 ng/ml, B, D) cells. Images were captured using the Aperio Scanscope (20x magnification) and processed using the Aperio software. The scale bar is 100 µm in each image, with the inset in the black box (20x) is of the area represented by the red box in each image. All images are from 1 experiment where all staining was undertaken at the same time, with n=5 sections stained at each time point. a = osteocyte-like dendritic processes and Images shown are a representative of the entire depth of the gel.
Chapter 4: Effects of IGF-1, FGF-2, RA and Vit K on osteocyte differentiation in 3D

4.5.7.4 VEGF, IL-6 and RANKL protein secretion

IGF-1 treatment did not modulate VEGF protein secretion (Figure A). However, VEGF protein was significantly reduced ($p<0.05$) on days 11 and 15 in the control and the IGF-1 treated cells (Figure 4.21A). Furthermore, VEGF concentrations prior to normalisation to cell number were on day 3 (control: $503.359 \pm 85.742$ pg/ml, IGF-1: $442.328 \pm 46.797$ pg/ml) and on day 15 (control: $1050.132 \pm 210.301$ pg/ml, IGF-1: $979.820 \pm 233.120$ pg/ml).

Figure 4.20: E11, Cx43, DMP-1 and sclerostin mRNA expression in hOBs in 3D collagen type I gels. Relative expression of E11 (A), Cx43 (B), DMP-1 (C) and sclerostin (D) in hOBs in 3D collagen gels on days 11 and 15 following treatment with IGF-1. RT-qPCR analysis of RNA isolated from cells, normalised to RPL13A (1 gel of day 11 control set to 1 in each experiment). Data are a combination of 3 (n=9) independent experiments. Data are mean ± SEM *$p<0.05$, **$p<0.01$, ***$p<0.001$ versus same treatment on day 11, # $p<0.05$, ### $p<0.001$ versus control at the same time point following a two-way ANOVA and a Bonferroni correction.
IL-6 protein secretion was significantly reduced (p<0.001) on days 7, 11 and 15 in control and IGF-1 treated cells (Figure 4.21B). Furthermore, a significant reduction (p<0.001) in IL-6 protein secretion was observed on day 3 with IGF-1 treatment when compared to the control at the same time point 15 (Figure 4.21B). IL-6 concentrations prior to normalisation to cell number were on day 3 (control: 420.601 pg/ml ± 42.473 pg/ml, IGF-1: 1295.622 pg/ml ± 32.238 pg/ml) and on day 15 (control: 414.647 pg/ml ± 62.166 pg/ml, IGF-1: 1307.055 pg/ml ± 66.972 pg/ml).

RANKL protein secretion was detected in all patient samples investigated in this chapter. This is in contrast to the results presented in Chapter 3 of this thesis. RANKL protein secretion did not change over time and IGF-1 treatment did not modulate RANKL protein secretion 15 (Figure 4.21C). However, RANKL concentrations were low prior to normalisation to cell number were on day 3 (control: 11.165 pg/ml ± 2.371 pg/ml, IGF-1: 7.313 pg/ml ± 4.050 pg/ml and on day 15 (control: 21.846 pg/ml ± 4.736 pg/ml, IGF-1: 7.844 pg/ml ± 4.487 pg/ml).
Figure 4.21: VEGF, IL-6 and RANKL protein secretion in hOBs in 3D collagen type I gels following treatment with IGF-1. VEGF (A), IL-6 (B) or RANKL (C) secretion measured by ELISA in hOBs in control cells or treated with IGF-1 (50 ng/ml), and normalised to cell number in individual gels and expressed relative to control at day 3 set to 100%. Data in A-C are a combination of 4 independent experiments (n=11 per time point). Data in A-C are mean ± SEM. * p<0.05, **p<0.01, *** p<0.001 versus same treatment on day 3 or ### p<0.001 versus control at the same time point, following a two-way ANOVA and a Bonferroni post hoc test.
Chapter 4: Effects of IGF-1, FGF-2, RA and Vit K on osteocyte differentiation in 3D

4.6 Discussion

The aim of this chapter was to determine the effects of IGF-1, FGF-2, RA and Vit K on the differentiation of MC-3T3 cells in 3D culture. The effects of IGF-1 on hOB cell differentiation were also investigated. Collectively, the results indicate that the addition of some of these factors do enhance the differentiation of these cells in 3D culture with several known mature osteocyte markers (e.g., FGF-23 and sclerostin) being up-regulated following treatment. Furthermore, when IGF-1 was added to mineralising medium, DMP-1 and PHEX expression were significantly up-regulated suggesting that the addition of a IGF-1 to mineralising medium further enhances the differentiation potential of these cells.

4.6.1 IGF-1 treatment

The results presented in this chapter and summarised in Figure 4.22 show that the addition of IGF-1 to MC-3T3s and hOBs accelerates their differentiation to a mature osteocytes, as evidenced by the up-regulation of sclerostin in both cell types and FGF-23 in MC-3T3 cells. Furthermore, treatment with IGF-1 did not affect MC-3T3 or hOB cell number, and cell viability remained >92 % for both cell types at all time points, showing that IGF-1 treatment did not increase apoptosis in these cells.

IGF-1 treatment of MC-3T3s and hOBs modulated expression of osteocyte differentiation markers. IGF-1 up-regulated DMP-1 expression in both cell types. DMP-1 is a mineralising osteocyte marker [17], thought to be necessary for mineral formation [22]. DMP-1 is up-regulated following mechanical loading of osteocytes [42, 294, 413, 414] and regulates FGF-23 expression [415]. FGF-23 has also been shown to be regulated by MEPE and PHEX [23]. FGF-23 is established as an
Chapter 4: Effects of IGF-1, FGF-2, RA and Vit K on osteocyte differentiation in 3D

osteocyte-specific factor produced by mature osteocytes. The up-regulation of FGF-23 in MC-3T3 cells grown in 3D suggests that a mature osteocyte population has been generated in the presence of IGF-1.

The data in this chapter show that IGF-1 greatly increased the expression of sclerostin at days 11 and 15 in both cell types studied. Sclerostin is thought to be a specific, mature osteocyte marker [31] with sclerostin protein known to be secreted by these cells [416]. Although not expressed by other bone cell types, sclerostin is also expressed in rodent and human chondrocytes [417], sheep and murine articular chondrocytes [418] and human osteophytic chondrocytes [419]. Moreover, sclerostin has been shown to be also expressed in the cementocytes and hypertrophic chondrocytes of teeth from mice and humans with Van Buchem disease [420].

The work described in this chapter also investigated the effects of IGF-1 on the expression of previously established mechano-responsive (IL-6, RANKL and VEGF) factors in osteocytes [304] [45, 302, 303, 421] [305].

IL-6 expression was down-regulated in both MC-3T3 cells and hOBs following treatment with IGF-1. Similar to these findings IGF-1 treatment was shown to inhibit IL-6 expression in rat bone marrow derived MSCs [422]. IGF-1 and inflammation are also linked to high cardiovascular risk and mortality, and low IGF-1 combined with high IL-6 serum levels causes an increased mortality risk [423]. Studies examining the link between IGF-1 and inflammation in patients with acromegaly who have a growth hormone and IGF-1 excess, or in patients with a growth hormone deficiency where there are reduced IGF-1 levels [424, 425], found that growth
hormone deficient patients had increased IL-6. Furthermore, when the IGF-1 levels were normalised by growth hormone replacement therapy a reduction in IL-6 was observed [424, 425]. Furthermore, during the pre-pubertal stage of growth in mice over-expressing IL-6 there were abnormalities in the IGF-1 system [426]. IL-6 has recently been reported to be upregulated in osteocytes following mechanical loading [304], as well as being modulated in a number of cell types in response to inflammation, and being up-regulated in a number of diseases [35-37], including rheumatoid arthritis [427]

RANKL was down-regulated following 11 days of treatment of MC-3T3s with IGF-1, but up-regulated ~42-fold following 15 days. RANKL has been well established as a mechano-regulated factor in osteocytes [45, 302, 303], but is also known to be necessary for osteoclastogenesis [19, 21, 428-430]. RANKL was originally thought to be produced by osteoblasts and other cell types [428] but recently it has been shown that the osteocyte produces more RANKL than osteoblasts [20, 21]. The role of IGF-1 on the OPG/RANKL system is currently unknown. However, in mouse stromal cells, IGF-1 treatment increased RANKL expression but decreased OPG [431]. When using the same cells, another study found increased OPG following IGF-1 treatment [432]. In pre- and post-menopausal Chinese women, another study found that IGF-1 was negatively correlated with the OPG/RANKL ratio, but positively correlated with RANKL. However, IGF-1 levels were lower, with no difference observed in RANKL in post-menopausal women with OP [433].

The data in this chapter indicate that following IGF-1 treatment of MC-3T3 cells there was a trend to an increase in mean VEGF protein secretion at day 11 and 15,
although the results were not significant. Whereas VEGF has been shown to be mechano-regulated in osteoblasts, tendons and chondrocytes [434-436], it is also known to be an essential cytokine in angiogenesis pathways. Similar to results shown in this study, culturing cells with 100 ng/ml of IGF-1 for 2 hrs significantly increased VEGF production in the buffalo corpus luteum [437]. Furthermore, following treatment with IGF-1, there was an increase in VEGF expression in SaOS-2 osteoblast cells and primary murine osteoblasts when compared to control cells following treatment. This mRNA increase was also linked to an increase in VEGF protein expression detected by immunoblot analysis [438].

IGF-1 is one of the essential cytokines that controls the differentiation of an MSC to the osteogenic lineage [439]. Furthermore, IGF-1 has been shown to enhance differentiation in other cell types. Previous studies showed that IGF-1 enabled human adipose-derived stromal cells to differentiate to adipocytes and osteoblasts; with the addition of platelet derived growth factor in combination with IGF-1 further increased differentiation to osteoblasts, than when used on its own [440]. When IGF-1 was added to human MSCs these cells showed advanced liver metabolic functions, highlighting them as a new potential source as well as an alternative to primary hepatocytes [441].

It has been well established that IGF-1 is essential for optimal skeletal growth and maintenance [442] with the development of K/O models generating information on the role of IGF-1 in both bone modelling and remodelling [443] [444] [189]. An osteocyte driven IGF-1 K/O mouse model was developed to assess the involvement of osteocyte-derived IGF-1 in developmental bone growth, by crossing the DMP-1-
driven Cre-expressing TG mouse with the IGF-1 floxed mouse [443]. Results from this study showed that there was lower procollagen type I N-terminal propeptide and C-telopeptide in the K/O mice showing that osteocyte derived IGF-1 is an important factor in bone turnover. The K/O mice also had a reduction in developmental growth of intramembraneus bone [443].

Another study using the tibias of the same IGF-1 K/O mouse investigated the absences of IGF-1 on the loading induced osteogenic response [444]. For this, four-point bending was undertaken for two weeks, and results suggested the K/O mouse did not have an increased expression of early mechano-responsive genes (e.g. Cox-2) or osteogenic genes (e.g. OCN). The results in this study suggest that the lack of osteocyte IGF-1 reduced the loading-induced increase of the canonical Wnt signalling genes (e.g. Dkk1 and LRP-5). Furthermore, sclerostin expression was up-regulated in the IGF-1 K/O mouse following mechanical loading, rather than a reduction in sclerostin expression following mechanical loading in wild types. This highlights that the disruption of osteocyte IGF-1 expression inhibited the loading-induced activation of the Wnt signalling as well as the osteogenic response [444]. Furthermore, osteocyte-derived IGF-1 has also been shown to play an important role in (1) controlling the osteogenic response to mechanical loading [79, 293, 405], (2) developmental bone growth [445-447], (3) the bone response to dietary calcium depletion and repletion and (4) in fracture repair [448, 449].
Chapter 4: Effects of IGF-1, FGF-2, RA and Vit K on osteocyte differentiation in 3D

**Figure 4.22**: Summary of changes in protein secretion and gene expression in MC-3T3 cells (blue) and hOBs (red) following treatment with IGF-1 when compared to the untreated (control) at the same time point in 3D collagen type I gels.

Collectively, the data from this chapter suggest that IGF-1 treatment modulated numerous factors. The results in this chapter reveal an important role for IGF-1 in directing differentiation of osteoblasts to osteocytes which has not been previously reported. This work also provides further insights into the possible pathways involved in the role of IGF-1 in mechano-responsiveness (VEGF, DMP-1 IL-6), inflammation (IL-6, RANKL), angiogenesis (VEGF) and osteoclastogenesis (RANKL) controlled by the osteocyte.

### 4.6.2 FGF-2 treatment

The data in this chapter, and summarised in Figure 4.23, indicate that addition of FGF-2 to MC-3T3 cells accelerates their differentiation to mature osteocytes as evidenced by up-regulation of mRNA for osteocyte markers (E11, DMP-1) early in the culture period and increased FGF-23 secretion at later stages of culture. The osteocyte phenotype induced by FGF2 included increased mRNA expression of mechano-regulatory factors (DMP-1 and LRP-5), a factor essential for osteoclastogenesis (RANKL) and cell-cell communication markers (Cx43).
Chapter 4: Effects of IGF-1, FGF-2, RA and Vit K on osteocyte differentiation in 3D

The up-regulation of E11, DMP-1 and FGF-23 with FGF-2 treatment highlight that this treatment is altering the differentiation potential of these cells. The data in this chapter showing that E11 mRNA expression was up-regulated (~3-fold, day 3 and 11) by treatment with FGF-2 is consistent with previous reports showing that FGF-2 treatment of MC-3T3 cells up-regulated (>5-fold) E11 mRNA expression [202].

Up-regulation of DMP-1 mRNA expression (>300-fold) following treatment with FGF-2 is consistent with previous reports. DMP-1 expression was also found to be up-regulated (>20-fold) in MC-3T3 cells following treatment with FGF-2 [202]. Geletin hydrogels containing different doses of FGF-2 implanted into the dental pulp of amputated rat molars caused DMP-1-positive calcified particles in the proliferating pulp in high doses of FGF-2, whereas medium doses of FGF-2 induced DMP-1-positive dentinal bridge of the proliferating dental pulp surface [450].

The data in this chapter show that FGF-23 mRNA expression was up-regulated following treatment with FGF-2. Apart from being a mature osteocyte marker, FGF-23 is also known to be responsible for the phenotypic changes seen in human diseases such as X-linked hypophosphatemia and phosphate wasting. Similar to the results presented in this chapter, in transgenic mice where the osteoblasts over-express FGF-2, there was increased FGF-23 hypophosphatemia and rickets [451, 452]. Furthermore, the FGF-2 transgenic mice had a similar phenotype to mice over-expressing FGF-23 in that they had decreased bone mineral density, dwarfism, osteomalacia and decreased serum phosphate. These mice also had increased serum FGF-23 as well as increased FGF-23 mRNA in bones [453]. In mouse bone marrow
stromal cells it has been demonstrated that FGF-2 isoforms signal via the FGF23/FGFR/MAPK pathway to prevent bone formation in vitro [454].

In this chapter, FGF-2 treatment up-regulated Cx43 mRNA expression following 7, 11 or 15 days of treatment. Cx43 is a component of gap junctions necessary for cell-cell communication. The increased Cx43 mRNA expression with FGF-2 treatment suggests that FGF-2 treatment is inducing these cells to produce more communications and/or dendritic morphology. Furthermore, H&E staining on day 15 showed an increased number of dendritic processes observed following FGF-2 treatment. In support of the findings shown in this chapter, when MC-3T3 osteoblasts were treated with FGF-2, protein kinase C delta is translocated to the nucleus and protein kinase C delta and Runx2 are phosphorylated. These events were also shown to be increased following over-expression of Cx43 suggesting that the amount of activation is enhanced by the increased Cx43 levels [455].

Sclerostin expression was not detected following treatment with FGF-2. This result differs to the only other previous report to investigate this, which showed that sclerostin expression was up-regulated (3.5-fold) following treatment of MC-3T3 cells with FGF-2 [202]. In bone, FGF-2 signalling is known to modulate the Wnt/β-Catenin signalling pathway and sclerostin is a Wnt antagonist that blocks this signalling by binding to one subunit of the Wnt receptor complex [456]. The function of the Wnt/β-Catenin signalling on bone formation is negatively regulated by Wnt antagonists such as sclerostin [457].
Chapter 4: Effects of IGF-1, FGF-2, RA and Vit K on osteocyte differentiation in 3D

The data in this chapter showed that FGF-2 treatment up-regulated LRP-5 mRNA expression (20-fold, day 11). No other published study to date has looked at a link between LRP-5 expression and FGF-2. In osteocytes LRP-5 expression has previously been shown to be modulated post mechanical loading [458-460]. LRP-5 mutations are responsible for numerous bone disorders, gain-of-function mutations in the LRP-5 gene lead to high bone mass phenotypes in humans [461, 462], and loss-of-function mutations cause OP-pseudoglioma syndrome which presents as early-onset OP and complications in eye development [463-465]. Furthermore, transgenic mice were LRP-5 has been knocked out have a low bone mass phenotype independent of Runx2, a reduction in osteoblast proliferation, and have osteopenia and persistent embryonic eye vascularisation [466]. A link has also been demonstrated between the LRP-5 gene polymorphisms and bone mass and size [467-469].

RANKL was also up-regulated (~30-fold, day 11) following treatment with FGF-2. This is consistent with other reports showing FGF-2 treatment increased RANKL mRNA expression in human bone marrow MSCs via ERK activation [470]. Another study found that compressive force on human periodontal ligament cells increased expression of soluble RANKL and that soluble RANKL release was inhibited when treated with anti-FGF-2 [471]. FGF-2 stimulated RANKL mRNA in the osteoblasts following 2 hrs to 7 days of treatment [472]. FGF-2 treatment also induced RANKL on Rheumatoid Arthritis synovial fibroblasts and osteoclast formation [473].

There is a lot of information available on a link between VEGF and FGF-2. In this chapter, treatment with FGF-2 in this culture system up-regulated VEGF protein
Chapter 4: Effects of IGF-1, FGF-2, RA and Vit K on osteocyte differentiation in 3D secretion. Both VEGF and bFGF are angiogenic factors. It has previously been shown that FGF-2 stimulated the release of VEGF [474]. Furthermore, in MC-3T3 cells it has been determined that the signalling mechanisms for FGF-2 induced VEGF release in osteoblasts is regulated by the p44/p42 mitogen-activated protein kinase and stress-activated protein kinase/c-Jun N-terminal kinase. It has also been shown that the FGF-2 induced VEGF release is negatively regulated by p38 MAP kinase and p70 S6 kinase [475-477]. Using the same cells another study found that it was the AMP-activated protein kinase that regulated FGF-2 stimulated VEGF synthesis [478].

Collectively, the data confirmed the role of FGF-2 in the differentiation of osteoblasts to osteocytes, previously reported by Gupta et al. 2010 and highlighted the important role of FGF-2 in generation of mature osteocytes expressing FGF-23 [202].

Figure 4.23: Summary of changes in protein secretion and gene expression in MC-3T3 cells (blue) following treatment with FGF-2 when compared to the untreated (control) at the same time point in 3D collagen type I gels.

4.6.3 RA treatment

The data in this chapter, summarised in Figure 4.24, suggest that the addition of RA to MC-3T3 cells accelerates differentiation to mature osteocytes cell population as
Chapter 4: Effects of IGF-1, FGF-2, RA and Vit K on osteocyte differentiation in 3D
evidenced by the up-regulation of FGF-23. Gene expression results also confirmed
the up-regulation of early osteocyte markers (E11), mechano-regulatory factors
(RANKL, VEGF and LRP-5), a factor essential for osteoclastogenesis (RANKL)
and cell-cell communication markers (Cx43).

The up-regulation of E11 in MC-3T3 cells following treatment with RA has also
been reported by Mattinzoli et al. 2012 [222]. This study found that E11 was up-
regulated following 5 days of treatment with RA but down-regulated after 10 days of
treatment [222]. The data in this chapter differs in that E11 expression was up-
regulated after 11 days of culture, however cells were in 3D rather than monolayer
[222]. Since E11 expression is the earliest marker of an osteocyte and FGF-23
expression is a mature osteocyte marker, concurrent increased expression of E11 and
FGF-23 could suggest that RA treatment is inducing a mixed population of cells
(early osteocytes and mature osteocytes). Mattinzoli et al. 2012 also showed that
following RA treatment FGF-23 expression was present from day 4 but increased
following 10-15 days of treatment [222]. To date, there are no other published
studies that have demonstrated a link between FGF-23 and RA.

Sclerostin mRNA expression was not detected in MC-3T3 cells in this 3D culture
system. This result differs to previous reports showing RA treatment of MC-3T3
increased sclerostin mRNA expression as well as in primary cell culture and that
sclerostin protein was detected by IHC [222]. This is the only publication to propose
that RA treatment induces sclerostin production.
Chapter 4: Effects of IGF-1, FGF-2, RA and Vit K on osteocyte differentiation in 3D

The results in this chapter show that RA treatment significantly up-regulated Cx43 mRNA expression following 7 or 15 days of treatment in contrast to Mattinzoli et al. 2012 where RA treatment had no effect [222]. RA treatment has been shown in numerous cell types to up-regulate Cx43 expression including testicular cancer cells [479], oral squamous cell carcinoma cells [480] and mouse gingival epithelial cells [481].

The up-regulation of LRP-5 mRNA expression following treatment with RA is the first time an interaction between LRP-5 and RA has been observed. LRP-5 has previously been shown to be up-regulated in bone cells following mechanical loading [459, 460].

The results in this chapter also show an up-regulation of VEGF protein secretion with RA treatment from day 3. A link between RA treatment and VEGF secretion has previously been shown in numerous studies including in glioma cells as RA treatment increased VEGF at lower concentration (5-10 µmol/l), and decrease VEGF expression at higher concentrations (40µmol/l) [482]. Another study found that systemic administration of RA regulates retinal VEGF expression in C57BL/6 mice that were subjected to oxygen induced retinopathy [483]. Furthermore, in the RCT-1 pre-osteoblast cells, RA treatment in combination with prostaglandin E2 increased the up-regulation of VEGF expression as well as the differentiation of these cells [484]. In MC-3T3 cells, RA treatment alone did not increase VEGF mRNA expression but when combined with TGF-β, RA treatment significantly upregulated VEGF mRNA expression when compared to TGF-β treatment alone [485]
Chapter 4: Effects of IGF-1, FGF-2, RA and Vit K on osteocyte differentiation in 3D

**Figure 4.24:** Summary of changes in protein secretion and gene expression in MC-3T3 cells (blue) following treatment with RA when compared to the untreated (control) at the same time point in 3D collagen type I gels.

Collectively, the data from the experiments investigating the effects of RA treatment discussed in this chapter suggest that RA treatment modulated protein and gene expression profile of these cells. The role of RA in the differentiation of osteoblasts to osteocytes has been previously presented by Mattinzoli et al. 2012 and the results in this chapter highlight the important role of RA in generation of mature osteocytes expressing FGF-23 from osteoblasts.

### 4.6.4 Vit K treatment

The results presented in this chapter and summarised in Figure 4.25 suggest that the addition of Vit K to MC-3T3 was the only factor that did not accelerate osteocytogenesis, as E11 was the only gene to be up-regulated following treatment and this was only after 15 days. This is the first study to demonstrate a link between E11 expression following Vit K treatment. Vit K treatment did not modulate osteocyte markers such as DMP-1 and FGF-23 expression. Furthermore, sclerostin expression was not detected following treatment with Vit K. These results further highlight that Vit K treatment does not alter the osteogenic potential of these cells as
Chapter 4: Effects of IGF-1, FGF-2, RA and Vit K on osteocyte differentiation in 3D

the results are broadly similar to MC-3T3 cell in control (untreated) medium in this 3D culture system.

Secretions of IL-6 and VEGF were also modulated by Vit K treatment, both of which are known mechano-regulated factors. IL-6 secretion was down-regulated (day 1) or up-regulated (day 11) and VEGF secretion was up-regulated (day 7) following Vit K treatment. It has previously been reported that Vit K treatment suppressed the LPS-induced IL-6 expression in human fibroblasts [486] and it has been proposed that the suppression of IL-6 by Vit K could be controlled by high molecular-weight anti-inflammatory substances released from the Vit K treated cells [487].

This is the first study to demonstrate that Vit K treatment regulates VEGF expression in bone cells. Other studies in relation to Vit K treatment modulating VEGF expression have shown that the Vit K dependent glycoprotein, protein S at circulating concentrations prevented VEGF receptor-2 dependent vascularisation as well as VEGF-A-induced endothelial migration and proliferation [488].

In previous studies, Katsuyama et al. 2005 showed an increase in RANKL following treatment with Vit K. [261]. The results presented in this chapter differ to these as RANKL was not found to be up-regulated following treatment with Vit K. However, the same study did not report an effect of Vit K on cell number, whereas the data presented in this chapter showed a significant difference in cell number following Vit K treatment of cells in 3D gels.
Chapter 4: Effects of IGF-1, FGF-2, RA and Vit K on osteocyte differentiation in 3D

**Figure 4.25:** Summary of changes in protein secretion and gene expression in MC-3T3 cells (blue) following treatment with Vit K when compared to untreated (control) at the same time point in 3D collagen type I gels.

Collectively, the data from the experiments investigating the effects of Vit K treatment discussed in this chapter suggest that Vit K treatment did not influence differentiation of osteocytes. However, in previous studies, Atkins et al. 2009 showed that Vit K in promotes osteoblast to osteocyte transition in normal human osteoblast like cells in 3D collagen gels [246]. The results presented in this chapter are similar to the Atkins et al. 2009 study in that there was no effect of sclerostin and DMP-1 expression following Vit K treatment, but differ in that they reported no effect on E11 expression and a reduction in RANKL following treatment [246]. The work presented in this chapter is in agreement with the Atkins et al. 2009 study [246] in the morphology reported post Vit K treatment in that numerous osteocyte-like dendritic processes were observed.

In conclusion, the results presented in this chapter highlight that the addition of some external factors accelerate the differentiation of osteoblasts to osteocytes, with some factors proving to be more beneficial than others (as highlighted in the previous sections). The generation of a mature osteocyte population, will further enable investigations of osteocyte biology and function.
5 Differentiation of IDG-SW3 osteoblasts to osteocytes in 3D collagen type I gels
5.1 Introduction

Chapter 3 investigated the differentiation of osteoblasts (MC-3T3 cells and hOBs) to osteocytes in 3D collagen type I gels. In this chapter, the differentiation of IDG-SW3 osteoblasts to osteocytes in 3D collagen type I gels will be investigated.

5.1.1 Osteocyte cell line

Primary osteocytes are a terminally differentiated cell population embedded in the mineralised matrix of bone. Their location in bone is optimal for their mechanosensory function, but is problematic for researchers to obtain sufficient yields be able to study osteocytes in vitro. For example, isolating murine osteocytes from skeletally mature young and old mice, by serial collagenase digestions with EDTA decalcifications produces low yields of primary osteocytes, with large numbers of animals needed to undertake meaningful experiments [73]. Furthermore primary osteocytes can also dedifferentiate and develop osteoblast-like features [278]. Thus new methods of developing large populations of mature osteocytes need to be developed.

Prior to 2011, a number of cell lines had been developed and used to represent osteocyte like cells, including the mouse derived MLO-Y4 and MLO-A5 cell lines, as well as the human derived HOB-01-C1 cell line [81-83, 108]. In 2011, IDG-SW3 cells were generated by Woo et al. [108]. Each osteocyte-like cell line has benefits and limitations (Table 5.1).
## Table 5.1: Characteristics of some of the currently available osteocyte-like cell lines.

<table>
<thead>
<tr>
<th>Name</th>
<th>Origin</th>
<th>Characteristics</th>
<th>Benefits</th>
<th>Limitations</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>HOB-01-C1</td>
<td>Human cancellous bone</td>
<td>- infected with temperature-sensitive large T antigen, cells proliferate at 34°C but do not divide at 40°C</td>
<td>- do not proliferate</td>
<td>- sclerostin, FGF-23, DMP-1, Cx43 and E11 expression not determined</td>
<td>Bodine et al. 1996</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>- low ALP expression</td>
<td>- intermediate osteocalcin expression</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>- synthesis a mineralised matrix [83]</td>
<td></td>
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<tr>
<td>MLO-Y4</td>
<td>C57Bl/6 mouse long bone</td>
<td>- express SV40 large T antigen which is driven by osteocalcin-promoter</td>
<td>- osteocytic dendritic processes that form cell-cell communications,</td>
<td>- proliferate</td>
<td>Kato et al. 1997</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>- low ALP expression</td>
<td>- do not mineralise (in standard culture conditions)</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>- high E11 expression [52]</td>
<td>- intermediate osteocalcin expression</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>- high Cx43 expression [100]</td>
<td>- low FGF-23 expression</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>- intermediate DMP-1 expression</td>
<td>- low sclerostin expression [85, 491]</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>- respond to fluid flow by activating the Wnt/β-catenin signalling [490]</td>
<td></td>
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<tr>
<td>MLO-A5</td>
<td>C57Bl/6 mouse long bone</td>
<td>- express SV40 large T antigen which is driven by osteocalcin-promoter</td>
<td>- typical dendritic/stellate morphology</td>
<td>- proliferate</td>
<td>Kato et al. 2001</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>- mineralise [81]</td>
<td>- high ALP expression</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>- intermediate E11 expression</td>
<td>- high osteocalcin expression</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>- intermediate Cx43 expression</td>
<td>- low sclerostin expression [85]</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>- intermediate DMP-1 expression</td>
<td>- low FGF-23 expression</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>- intermediate PHEX expression</td>
<td></td>
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<tr>
<td>IDG-SW3</td>
<td>Long bone cells derived from the Immortomous e crossed with the 8-kb-Dmp1-GFP mouse</td>
<td>- IFN-γ and temperature sensitive SV40 large T-Antigen which induces proliferation at 33°C with IFN-γ but not at 37°C</td>
<td>- do not proliferate</td>
<td>- intermediate ALP expression</td>
<td>Woo et al. 2011</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>- mineralise</td>
<td>- low FGF-23 expression (after 1-2 weeks of differentiation)</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>- high E11 expression</td>
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<td></td>
<td></td>
<td></td>
<td>- high DMP-1 expression</td>
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<td></td>
<td>- high PHEX expression</td>
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<td></td>
<td></td>
<td></td>
<td>- high MEPE expression</td>
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<td></td>
<td></td>
<td></td>
<td>- high sclerostin expression (after 1-2 weeks of differentiation)</td>
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<td></td>
<td></td>
<td></td>
<td>- express GFP under control of 8-kb DMP-1 promotor</td>
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</table>
5.1.2 IDG-SW3 cell line

The IDG-SW3 cells are a murine, early osteoblast to mature osteocyte Immortomouse/DMP-1-GFP derived bone cell line. These Immortomouse-derived cells contain an IFN-γ-inducible $H-2K^{b}$-tsA58 promoter that drives expression of a temperature sensitive mutant of the SV40 large T-antigen. When the cells are maintained at 33°C in the presence of IFN-γ in monolayer, they are able to proliferate and are immortal. When placed in osteogenic medium at 37°C and in the absence of IFN-γ, they return back to the in vivo phenotype of late osteoblasts, and are then able to differentiate to late osteocytes [108].

IDG-SW3 cells in 2D culture express the early osteocyte markers, E11, DMP-1, MEPE and PHEX as well as the mature osteocytic markers, FGF-23 and sclerostin, at various time points during differentiation from osteoblasts to osteocytes [108]. When cultured in 3D collagen gels or sponges, they expressed DMP-1 and sclerostin highlighting that they remain viable and differentiate in 3D culture [108]. These cells produce an extracellular matrix which they can mineralise, making them an invaluable cell line for the study of osteoblast to osteocyte differentiation in vitro as they mimic this differentiation process in bone in vivo. They also enable generation of sufficient numbers of mature sclerostin and FGF-23 expressing osteocytes to investigate mature osteocyte biology and their responses to mechanical loading.

To date, there are only 5 published studies that use the IDG-SW3 cell line [108, 118-121] and only a single report of their culture in 3D collagen gels [108]. In addition to reporting of phenotype, these studies have investigated the transition of osteoblasts to osteocytes, the influence of treatments such as vitamin D and osteocyte
interactions with osteoblasts. St John et al. 2014 found that the transition from an osteoblast to an osteocyte involves changes in gene expression that are steered by both genetic and epigenetic components to govern osteocyte phenotype [120]. Ito et al. 2013 demonstrated that mature IDG-SW3 osteocytes (35 days) treated with vitamin D3 and extracellular phosphate altered FGF-23, MEPE, DMP-1, PHEX, ENPP1 and N-acetylgalactosaminyltransferase 3 gene expression [119]. Lindberg et al. 2014 investigated whether FGF-23 is a substrate for secretory phosphorylation, and using the IDG-SW3 cells lines along with other cell lines concluded that bone cells endogenously phosphorylate FGF-23 [121]. IDG-SW3 cells co-cultured with calvarial osteoblasts up-regulated genes reflecting osteoblast differentiation in the osteoblasts and increased osteocyte specific genes in the osteocytes when in direct contact via a porous membrane [118].
5.2 Hypothesis

Maintaining IDG-SW3 osteoblast cells in 3D collagen type I gels in mineralising (osteogenic) medium will accelerate the differentiation to mature sclerostin expressing osteocyte-like cells, when compared to previously published monolayer results for this cell line.

5.3 Aims

Specifically the aims were to maintain IDG-SW3 cells in type I collagen gels for 25 days and to investigate:

1. Cell number and viability (haemocytometer counting and trypan blue exclusion)
2. Cell morphology (H&E staining, actin labelling by confocal microscopy)
3. Gene expression (qRT-PCR; ALP, E11, MEPE, PHEX, DMP-1, RANKL, VEGF and sclerostin)
4. Protein expression (IHC, DMP-1-GFP*)
5. Protein secretion (ELISA; IL-6 and VEGF)

5.4 Methods

5.4.1 Experimental design

IDG-SW3 cells were set up in collagen gels (Section 2.2.2.2, 3 independent experiments). Outputs were then analysed at varying time points up to 21 days of differentiation including: days 1 and 4 at 33°C (pre-differentiation, 1 experiment only), and days 1, 7, 10, 14, 17 and 21 at 37°C (during differentiation). In each
experiment there were 4 replicate gels per time point of which 3 gels were used for analysis of cell number and viability, gene expression and protein secretion and 1 gel used for morphological and immunohistochemical analysis following fixation and sectioning. In each experiment, monolayer cells were also collected at experimental set up and RNA extracted for comparison with gene expression in the same cells maintained in 3D. Two extra gels were also set up in each experiment for confocal microscopy at day 21 (fixed cell imaging) and 22 (live cell imaging) to assess actin organisation and assessment of DMP-1-GFP expression.

5.4.2 Assessment of protein secretion, cell number and viability and gene expression

Media were removed from gels at specific time points (days 1 and 4 at 33°C – 1 experiment only, and days 1, 7, 10, 14, 17 and 21 at 37°C during these experiments (n=3 at each time point), centrifuged (1,700g, 30 s) and stored in aliquots (120 µl) at -80°C until needed for measurement of IL-6 and VEGF (ELISA) secretion (Section 2.2.7.1). In each experiment, protein release was quantified following two days of culture. Following collagenase digestion of gels for measurement of cell number and viability (Section 2.2.2.5), remaining cells were placed in TRI Reagent for RNA extraction and gene expression analyses (Section 2.2.3). RNA was not extracted from cells in gels on day 1 at 33°C. Cells in monolayer were placed directly into TRI reagent for RNA extraction and gene expression analyses (Section 2.2.3) at the time of setting up gels.

IL-6 and VEGF protein secretion were normalised to the cell number in that same sample and expressed relative to the mean control sample values (fixed to 100 %) at
day 1 at 37°C. Results from the 3 experiments were then combined for further analysis.

Cell number was expressed relative to the mean of day 1 at 37°C values (expressed as 100 %), and the 3 experiments combined. Cell viability was expressed as a percentage, where dead cells were expressed relative to total cell number (expressed as 100 %) at each data point.

RT-qPCR was initially undertaken on all samples from the 3 experiments, at all time points including monolayer cultures using 3 reference genes (GAPDH, PCNA and 18sRNA). Normfinder selected GAPDH as the most stable housekeeping gene to use for this data set. Data were analysed using ΔΔCt (Section 2.2.3.8) with the value from one sample from day 1 at 37°C fixed to 1 in each experiment allowing the data from the experiments to be combined for further analysis.

5.4.3 Assessment of morphology and immunohistochemical analysis

In each of the 3 experiments, one gel was removed at specific time points (day 4 at 33°C - 1 experiment only, and on days 1, 7, 10, 14, 17 and 21 at 37°C) and fixed in 1 % paraformaldehyde. These were subsequently sectioned for histological (morphological and immunohistochemical) analysis (Section 2.2.5). Sections were stained with H&E to assess cell morphology or used for immunohistochemical analysis of DMP-1-GFP expression. For confocal microscopy, whole gels were either i) fixed on day 21 and stained with phalloidin and DAPI (Section 2.2.6) or ii) stained with DAPI as live cultures on day 22. Both were imaged for assessment of DMP-1-GFP⁺ cells (Section 2.2.6.5).
5.4.4 Statistical analysis of data

Most of the data presented in this chapter are combined from 3 independent experiments with an n=3 at each data point in each experiment (see individual figure legends). Graphs represent mean ± SEM, apart from FigureA, where individual data points were plotted. Prior to undertaking statistical analyses, the complete data sets were tested for (1) normality (Shapiro-Wilk test, Graph Pad Prism Software), (2) equal variance (Bartlett’s test, Graph Pad Prism Software), and (3) presence of outlier’s (Grubb’s test, Microsoft office Excel 2010). All statistical analyses and graphical data presented in this chapter had outliers removed and were analysed using a one-way ANOVA followed by a Bonferroni post hoc test.
5.5 Results

5.5.1 Cell number and viability

When the conditions for these experiments were initially being optimised, cells were removed from being maintained at 33°C, set up in gels and placed directly into osteogenic medium at 37°C. However, when this was done, even though there were initially a small number of viable cells, all cells seemed to have died by day 7 (results not presented). Further optimisation work indicated that cells embedded in collagen gels needed to be placed at 33°C for 4 days before transferring to osteogenic medium at 37°C to ensure cell survival with a minimum viability of 85% after 21 days at 37°C (results not shown). Therefore in all experiments where IDG-SW3 cells were maintained in collagen gels, the gels were maintained at 33°C in proliferative medium for 4 days prior to being placed in differentiation (osteogenic) medium at 37°C.

As described in the introduction (Section 1.5.3.1), IDG-SW3 cells in monolayer proliferate in proliferative medium at 33°C, but are not expected to proliferate when changed to osteogenic medium at 37°C. The results presented in this chapter show that there was no significant difference in IDG-SW3 cell numbers during 4 days at 33°C and 21 days at 37°C when cultured in gels. However, mean cell number values were reduced after 21 days at 37°C, but these values were not significant (Figure 5.1A).

Cell number did not change significantly during the 4 days at 33°C, although the mean value at day 4 (211,040 ± 8,498 cells) is higher than that at day 1 (162,720 ± 15,913 cells). Furthermore, the cell number did not change significantly during the
21 days at 37°C, although the mean number at day 21 is lower (78.707 % ± 6.120 %) than on the previous time point (day 17: 95.822 % ± 5.891 %). To investigate this further, individual data points from each of the 3 experiments were plotted (Figure 5.1B), and this shows that the cell numbers are reduced at day 21 in 2 of the 3 experiments conducted.

IDG-SW3 cell viability in 3D collagen type I gels was >89 % at all time points. However, there was a significant reduction ($p<0.01$) in cell viability on day 21 at 37°C (89.531 % ± 0.987 %) when compared to day 1 at 37°C (92.033 % ± 1.354 %). No differences were seen in terms of cell viability at all other time points (Figure 5.1C). Notably, both cell number and viability were lowest on day 21 at 37°C.

5.5.2 Morphology

5.5.2.1 H&E staining

Morphological analysis of IDG-SW3 cells maintained in 3D collagen gels following H&E staining of sections showed that when placed in osteogenic medium, the cells become more dendritic and form communications with other cells (Figure 5.2), both of which are typical characteristics of osteocytes. However, this staining also highlighted that there were numerous acellular regions within the gels. On day 4 at 33°C, these cells are proliferating osteoblasts, and the cells some of the cells appear to be rounded (Figure 5.2A), and thus similar to the IDG-SW3 cell morphology in monolayer culture at 33°C. Once the gels were placed at 37°C, the cell shape became more stellate with dendritic-like processes extending out from the nucleus in some of the cells (Figure 5.2B). Furthermore, in some of the cells the dendritic processes appeared to be forming communication networks between cells (Figure 5.2C). In terms of morphology, there also appeared to be changes in terms of the cytoplasm in
Chapter 5  

IDG-SW3 differentiation in 3D collagen type I gels

that it appeared to increase with days in osteogenic medium at 37°C especially on day 10 and 14 (Figure 5.2D-E). On day 21 at 37°C, the cells look different to the previous time points in that the cytoplasm surrounding the cells has become reduced (Figure 5.2F). This H&E staining suggests that the IDG-SW3 cell shape is modulated over time during differentiation in this 3D collagen type I gel.

5.5.2.2 Actin, DAPI and DMP-I-GFP expression

Actin is the most abundant cytoskeletal protein in most cells and actin labelling is known to illustrate cell morphology [492]. In order to assess this in IDG-SW3 cells maintained in gels in osteogenic medium for 21 days, fixed gels were stained with phalloidin (actin labelling) and DAPI (nuclear stain) and imaged by confocal microscopy. Representative images from each experiment were obtained from a z-stack through the gel using two different magnifications (x20 and x40, Figure 5.3). Since these cells are also known to express DMP-I-GFP it was expected that this would also be detected in these gels.

Actin labelling was present in all cells, and the higher magnification suggests some degree of actin organisation in individual cells (Figure 5.3A, D). DAPI staining confirmed that the cells had individual nucleoli visible at the higher magnification (Figure 5.3D, E). The overlaid images confirm that the actin staining is present in the cytoplasm of all cells (Figure 5.3A-F).

With increased magnification (x63 magnification), when focusing on one individual cell (Figure 5.4A-E), the actin appears to be filamentous and there may be some evidence of osteocyte-like projections extending out from the cell body, which could facilitate communication with other cells. The actin appears to be tightly organised
in the centre of the cell, whereas when you move closer to the cell wall more spaces can be seen in between individual actin filaments (Figure 5.4C-E). It was expected that these cells would also have an abundance of DMP-1-GFP expression. However, only small amounts of DMP-1-GFP were seen (Figure 5.4B).

In order to investigate the expression of DMP-1-GFP further, gels containing IDG-SW3 cells were removed from culture on day 22 and live cell imaging using confocal microscopy undertaken following staining with DAPI (Figure 5.5). The results illustrated an abundance of DMP-1-GFP staining throughout the sample, and especially localised to around the nucleus (Figure 5.5B-C, E-F). DAPI (blue) nuclear staining highlights that there are many cells present in the image surrounded by DMP-1-GFP (Figure 5.5C, F).

These results suggest that the fixing process, prior to actin labelling, as described in section 2.6, has destroyed the majority of DMP-1-GFP that should be present in these cells, which explains why it was reduced in the day 21 samples (Figure 5.4B). It has previously been documented that harsh fixatives can destroy GFP fluorescence [493], although others have reported that a short fixation period in paraformaldehyde often preserves GFP fluorescence and can maintain the localisation of the protein of interest [493].
Figure 5.1: IDG-SW3 cell number and viability in 3D collagen type I gels. Cell number expressed relative to day 1 at 37°C (fixed to 100%) (A), raw cell number (B) and cell viability (C) in IDG-SW3 cells in 3D collagen gels at days 1 and 4 at 33°C and days 1, 7, 10, 14, 17 and 21 during differentiation. Data are a combination of 3 independent experiments (n=3 at each data point per experiment, apart from days 1 and 4 at 33°C which are from one experiment). Data in A are raw cell numbers and B and C are mean ± SEM. In A-C, * p<0.05, ** p<0.01 versus day 1 at 37°C following a one way ANOVA and a Bonferroni correction.
**Figure 5.2: H&E staining of IDG-SW3 cells in 3D collagen type I gels.** Representative H&E staining of fixed and sectioned (7 µm) 3D collagen gels containing IDG-SW3 cells at day 4 at 33°C (A), or days 1 (B), 7 (C), 14 (D), 17 (E) and 21 (F) at 37°C in osteogenic medium. Images were captured using the Aperio Scanscope (x20 magnification) and processed using the Aperio software. The scale bar is 100 µm in each image, with the area in the red box enlarged (20x) in the black box in each section. All images shown are from 1 experiment, where all staining was undertaken at the same time, with an n=9 sections per individual time point shown. a= rounded osteoblast-like cells, b = osteocyte-like dendritic processes, c= communication network between cells, d = increased cytoplasm. Images shown are a representative of the entire depth of the gel.
Figure 5.3: Actin labelling of IDG-SW3 cells under osteogenic conditions. Phalloidin and DAPI staining in IDG-SW3 cells in whole 3D collagen type I gels following 21 days in osteogenic conditions at 37°C. Whole gels were fixed, stained with phalloidin and DAPI and imaged using confocal microscopy. Representative images (x20; A, B and C) and (x40; D, E and F) of actin (phalloidin, red, A, D), DAPI (nuclear stain, blue, B, E), and overlays of actin and DAPI staining (C, F). A = positive actin labelling, b = evidence of actin organisation.
Figure 5.4: Actin labelling of IDG-SW3 cells under osteogenic conditions. The expression of actin, DMP-1-GFP and DAPI in IDG-SW3 cells in whole gels following 21 days in osteogenic conditions in 3D collagen type I gels at 37°C. Whole gels were fixed, stained with phalloidin and DAPI and imaged using confocal microscopy (x63). Representative images of DAPI (nuclear stain, blue, A), DMP-1-GFP (green, B), actin (phalloidin, red, C), and overlaid DAPI, DMP-1-GFP and actin images (D). Enlarged image of D highlighting actin organisation in the cytoskeleton of the cells (E). a = highly organised filamentous actin, b = DMP-1-GFP* staining, c = osteocyte-like dendritic processes.
Figure 5.5: DMP-1-GFP expression in IDG-SW3 cells under osteogenic conditions. Confocal microscopy for the expression of DAPI and DMP-1-GFP in live IDG-SW3 cells in whole gels (20x and 40x) following 22 days in osteogenic medium in 3D collagen type I gels at 37°C. Representative images of DMP-1-GFP (green, A, D), DAPI (nuclear stain, blue, B, E), and DAPI and DMP-1-GFP overlaid (C, F). a = DMP-1-GFP* staining, b = nuclear staining.
5.5.3 mRNA expression

5.5.3.1 ALP, E11 and DMP-1

The lowest expression values for ALP (osteoblast marker) were in monolayer cells (0.185 ± 0.061) and cells on day 4 (0.051 ± 0.022) at 33°C (Figure 5.6A). The mean value at day 1 at 37°C (2.407 ± 0.923) was higher than that at day 4 at 33°C (0.051 ± 0.022) but these values were not significantly different. However, ALP expression values were increased 6-fold at day 7 at 37°C (p<0.001) and then returned to lower expression values, similar to those seen at day 1 at 37°C (2.407 ± 0.923) on days 10 (3.198 ± 0.334), 14 (3.988 ± 0.595), 17 (4.540 ± 0.346) and 21 (3.156 ± 1.014) at 37°C (Figure 5.6A).

The mRNA expression of E11, an early osteocyte marker, did not significantly change over the 4 days at 33°C and 21 days at 37°C when IDG-SW3 cells were maintained in gels. However, the lowest mean gene expression values were in monolayer cells (0.275 ± 0.040) or in 3D at day 4 at 33°C (0.072 ± 0.004). Furthermore, mean gene expression values on days 7 (0.982 ± 0.193), 10 (2.664 ± 1.325), 14 (3.400 ± 0.934), 17 (3.528 ± 1.439) and 21 (2.034 ± 0.872) were all higher than the values obtained for cells in monolayer and cells in gels at day 4 at 33°C, and the highest values were obtained at day 17 at 37°C (Figure 5.6B).

The mRNA expression of the mineralising osteocyte marker, DMP-1, was also at its lowest in monolayer cells (0.185 ± 0.061) and in cells on day 4 (0.051 ± 0.022) at 33°C (both p<0.001 when compared to day 1 at 37°C). DMP-1 mRNA expression increased 9-fold at day 17 at 37°C (p<0.001) over day 1 at 37°C (Figure 5.6C).
However, for ALP, E11 and DMP-1, mean gene expression values were lowest in monolayer or in gels at 33°C, increased when the cells were placed in 3D at 37°C, and lowest on day 21 when compared to day 17 at 37°C. The lower values observed at day 21 at 37°C could be explained by the lower cell number and viability observed on day 21 when compared to day 17.

5.5.3.2 MEPE, PHEX and sclerostin

MEPE (mature osteocyte marker) mRNA expression was lowest in monolayer cultures and on day 4 at 33°C. It then increased significantly (10-fold, \( p<0.001 \)) when gels were placed at 37°C and did not then change significantly during the next 21 days at 37°C. However, mean gene expression values were highest at day 10 (1.326 ± 0.318) at 37°C, with lower gene expression values obtained at day 17 (0.437 ± 0.017) and 21 (0.497 ± 0.172) at 37°C (Figure 5.7A).

PHEX (mature osteocyte marker) mRNA expression values were lowest in monolayer cultures and on day 4 at 33°C, and then increased ~4-fold (\( p<0.001 \)) following the maintenance of gels for 1 day at 37°C. Following this, PHEX mRNA was higher than that seen at day 1 at 37°C at each time point, although this increase was only significant on days 10 (3-fold \( p<0.05 \)) and 17 (3-fold, \( p<0.01 \)). Notably, similar to ALP, E11 and DMP-1, PHEX mRNA mean gene expression values were lower on day 21 (1.172 ± 0.259) at 37°C than the value observed at day 17 (3.204 ± 0.569) at 37°C (Figure 5.7B).

The mature osteocyte marker sclerostin was not detected in monolayer and at day 4 at 33°C. Sclerostin was detected on all other time points investigated. Furthermore, on day 7, sclerostin gene expression was highest, increasing 5-fold over day 1 at
37°C ($p<0.05$), and after day 7, mean gene expression values returned to values similar to those observed at day 1 at 37°C (Figure 5.7C).

MEPE, PHEX and sclerostin expression were all lowest (or not detected) in monolayer samples and day 4 at 33°C. These genes then increased in expression once cells were placed in 37°C. However, MEPE and sclerostin were the only genes in this study, which did not decrease at day 21 at 37°C.

5.5.3.3 RANKL and VEGF

Mean gene expression values for RANKL mRNA expression were lowest in monolayer cultures (0.450 ± 0.105) and in gels on day 4 (0.293 ± 0.043) at 33°C. The expression then seemed to gradually increase over time, before peaking at day 17 (5.468 ± 0.844) and decreasing slightly by day 21 (4.222 ± 0.284). There was a significant increase in RANKL mRNA expression on days 17 (5-fold, $p<0.001$) and 21 (4-fold, $p<0.001$) at 37°C when compared to day 1 at 37°C (Figure 5.8A).

VEGF mRNA expression values were also lowest when IDG-SW3 cells were maintained in monolayer cultures (0.193 ± 0.066) at 33°C or in 3D gels for 4 days at 33°C (0.089 ± 0.004). Furthermore, there was a significant increase ($p<0.01$) in VEGF mRNA expression when gels were maintained at 37°C for 1 day, and expression peaked at day 14 being 8-fold increased ($p<0.001$) over those obtained on day 1 at 37°C. By days 17 and 21 mean gene expression values had returned to values obtained for day 1 at 37°C (day 17, 1.109 ± 0.456; day 21, 0.645 ± 0.375; day 1, 1.053 ± 0.223, Figure 5.8B).
Both RANKL and VEGF mRNA mean gene expression values were lowest in monolayer and in 3D at day 4 at 33°C, and increased when cells were maintained at 37°C. Highest RANKL expression was observed at day 17. Notably, both RANKL and VEGF mean gene expression values were lower on day 21 when compared to day 17, indicating that this may be linked to the lowest cell number and viability observed at day 21.
Figure 5.6: Gene expression of osteoblast (ALP), early osteocyte (E11) and mineralising osteocyte (DMP-1) markers in IDG-SW3 cells in 3D collagen type I gels. Relative expression of ALP (A), E11 (B) and DMP-1 (C) in IDG-SW3 cells in monolayer (ML) at beginning of experiment or in 3D collagen gels at day 4 at 33°C or days 1, 7, 10, 14, 17 and 21 at 37°C in osteogenic medium. RT-qPCR analyses of RNA isolated from cells, normalised to GAPDH (1 gel of day 1 at 37°C set to 1 in each experiment). Data (A-C) are from 1 experiment (day 4 at 33°C) or 3 independent experiments (n=3 gels per experiment). Data (A-C) are mean ± SEM, *** p<0.001 versus day 1 at 37°C, following a one way ANOVA and a Bonferroni post hoc test.
Figure 5.7: Gene expression of mature osteocyte (MEPE, PHEX and sclerostin) markers in IDG-SW3 cells in 3D collagen type I gels. Relative expression of MEPE (A), PHEX (B) and sclerostin (C) in IDG-SW3 cells in monolayer (ML) at beginning of experiment or in 3D collagen gels at day 4 at 33°C or days 1, 7, 10, 14, 17 and 21 at 37°C in osteogenic medium. RT-qPCR analyses of RNA isolated from cells, normalised to GAPDH (1 gel of day 1 at 37°C set to 1 in each experiment). Data (A-C) are from 1 experiment (day 4 at 33°C) or a combination of 3 independent experiments (n=3 at each data point per experiment). Data (A-C) are mean ± SEM, *p<0.05, **p<0.01, ***p<0.001 versus day 1 at 37°C, following a one way ANOVA and a Bonferroni post hoc test.
Figure 5.8: Gene expression of known mechano-regulated (RANKL and VEGF) factors in IDG-SW3 cells in 3D collagen type I gels. Relative expression of RANKL (A) and VEGF (B) in IDG-SW3 cells in monolayer (ML) at beginning of experiment or in 3D collagen gels at day 4 at 33°C or days 1, 7, 10, 14, 17 and 21 at 37°C in osteogenic medium. RT-qPCR analyses of RNA isolated from cells, normalised to GAPDH (1 gel of day 1 at 37°C set to 1 in each experiment). Data (A-C) are from 1 experiment (day 4 at 33°C) or a combination of 3 independent experiments (n=3 at each data point per experiment). Data (A-C) are mean ± SEM, ** p<0.01, *** p<0.001 versus day 1 at 37°C, following a one way ANOVA and a Bonferroni post hoc test.

5.5.4 IL-6 and VEGF protein secretion

IL-6 protein was detected in conditioned media collected at all time points. Prior to normalising to cell numbers as a percentage, raw IL-6 values were between 13.486 pg/ml ± 3.261 pg/ml (day 1 at 33°C) and 800.527 pg/ml ± 125.397 pg/ml (day 21 at 37°C). IL-6 concentrations were lowest at earlier time points i.e. day 4 at 33°C (69.137 % ± 3.269 %) and days 1 (100 % ± 3.778 %) and 7 (79.891 % ± 18.237 %) at 37°C, before increasing on days 14 (677.912 % ± 101.204 %) and 17 (602.107 % ± 104.6 %) and peaking at day 21 (5392.209 % ± 1402.305 %, Figure 5.9A). The value obtained at day 21 was significantly (p<0.001) higher than that obtained on all other time points studied.
VEGF protein could also be detected in conditioned media collected at all time points. Prior to normalising to cell numbers as a percentage, raw VEGF protein values were between 23.671 pg/ml ± 2.541 pg/ml (day 1 at 33°C) and 895.871 pg/ml ± 121.351 pg/ml (day 21 at 37°C). Mean VEGF protein values were lowest when cells were osteoblasts i.e. on day 1 (13.978 % ± 0.478 %) and 4 (21.165 % ± 0.711 %) at 33°C, and these increased substantially when cells had started to differentiate to osteocytes in osteogenic medium at day 1 (100 % ± 3.901 %) and day 7 (139.409 % ± 32.617 %) at 37°C (Figure 5.9B). After this, there was a gradual but significant increase in VEGF protein secretion on days 10 ($p<0.01$), 17 ($p<0.01$) and 21 ($p<0.001$) when compared to day 1 at 37°C, highlighting an increase in VEGF secretion with differentiation. Mean gene expression values confirm that highest VEGF secretion was on day 21 (719.027 % ± 11.405 %) at 37°C.

Both IL-6 and VEGF protein secretions were highest at day 21 at 37°C. These results differ to the cell number and viability results and the majority of gene expression results which show that there was a reduction on day 21 when compared to day 17 at 37°C. Notably, lowest IL-6 and VEGF protein secretions were observed when cells were either osteoblast (days 1 and 4 at 33°C) or early osteocytes (days 1 and 7 at 37°C). Highest IL-6 and VEGF protein secretions were observed when cells could be mature osteocytes (day 14-21 at 37°C). These results suggest that in this 3D culture model, both IL-6 and VEGF protein secretions were lowest when cells were osteoblasts, but increased when cells were osteocytes in these IDG-SW3 cells.
**Figure 5.9:** IL-6 and VEGF protein secretion in IDG-SW3 cells maintained in 3D collagen type I gels. IL-6 (A) and VEGF (B) concentrations (ELISA) in conditioned media from IDG-SW3 cells at days 1 and 4 at 33°C, or days 1, 7, 10, 14, 17 and 21 at 37°C. Data (A-B) are from 1 experiment (days 1 and 4 at 33°C) or a combination of 3 independent experiments (n=3 at each data point per experiment) and were expressed relative to day 1 at 37°C values set to 100%. ** p<0.01, *** p<0.001 versus day 1 at 37°C following a one way ANOVA and a Bonferroni post hoc test.
5.6 Discussion

The work in this chapter aimed to determine whether IDG-SW3 cells in 3D collagen type I gels differentiate to mature osteocytes. Collectively, the results indicate that these cells do differentiate to osteocytes under osteogenic 3D culture conditions, with several osteocytic genes being upregulated during the 21 days of differentiation. Figure 5.10 below summarises the significant modulations in gene expression and protein secretion results presented in this chapter.

![Figure 5.10](image)

**Figure 5.10:** Summary of the significant modulation of protein secretion and gene expression in IDG-SW3 cells in 3D collagen type I gels when normalised to day 1 at 37°C. Significant differences are compared to day 1 at 37°C.

Cell number was increased, but not significantly, when the IDG-SW3 cells were maintained at 33°C, but did not change significantly once cells were placed in osteogenic medium in 3D collagen type I gels at 37°C. An increase in cell number in gels at 33°C was expected as IDG-SW3 cell proliferation is driven by the presence of IFN-γ in the cell culture medium. The IFN-γ activates the inducible $H-2K^b$-tsA58 promoter which expresses a temperature sensitive mutant of the SV40 large tumour antigen which in turn drives proliferation. When the gels were placed at 37°C, IFN-γ
was removed from the medium thus switching off the inducible H-2Kb-tsA58 promoter returning the cells to a late osteoblast phenotype that can differentiate to osteocytes [108]. The cells were also placed in osteogenic medium containing beta-glycerophosphate and ascorbate-2-phosphate, thus driving matrix synthesis and mineralisation. However, on day 21 of culture in osteogenic medium, mean cell number values were decreased when compared to all time points at 37°C, indicating that these cells may be apoptosing. This theory is supported by the significant reduction in cell viability observed on day 21. Since osteocyte apoptosis increases with mineralisation [494], this may explain the reduction in cell number and viability. However it has also been shown that apoptosis may have a role in the terminal differentiation of osteoblasts [277], but because cell viability remains consistently high and is only significantly different on day 21 suggests that this is not the case in this study. Since mature osteocytes in vivo are not known to proliferate [31, 495] the fact that these cells in osteogenic conditions do not increase numbers over time [102, 496, 497] lends support to the hypothesis that they are quickly, maybe over a period of a few days in 3D, differentiating from osteoblasts to osteocytes.

Confocal microscopy of phalloidin labelled actin filaments provides evidence in some of the cells that the actin became organised when maintained in 3D collagen gels. This actin staining also highlights the cell shape as well as informing about the cellular processes extending out from the cell wall, a typical feature of osteocyte cells. Actin is the most abundant cytoskeletal protein in most cells, with actin filaments being derived from actin and is thin flexible fibres. Within cells actin filaments are organised into higher order structures and organisation and
disorganisation of these filaments is controlled by a number of actin binding proteins which form a crucial part of the actin cytoskeleton architecture [492]. Actin filaments beneath the plasma membrane play a role in cell shape determination, providing mechanical support for the cell as well as enabling cells to migrate, engulf particles and divide [492]. Therefore the results presented in this chapter for phalloidin labelling of actin filaments can provide us with details of cell shape. Lower magnifications reveal that the actin labelling can be seen in all cells surrounding the nucleus (Figure 5.3) of the cells. With increased magnification, when focusing on one individual cell (Figure 5.4), the actin appears to be filamentous and shows that there may be some evidence of osteocyte-like projections extending out from the main cell body, which could be communicating with other cells. The actin appears to be tightly organised in the centre of the cell, whereas when you move closer to the cell wall more spaces can be seen in between individual actin filaments, where the osteocyte like projections can be seen.

Unfortunately, the DMP-1-GFP appeared to be destroyed once the collagen gels were fixed and prepared for confocal microscopy for actin labelling. This is a reported difficulty with GFP staining as it can easily be destroyed with fixing [493]. However, it is believed that short fixation with paraformaldehyde often preserves the GFP fluorescence and can maintain the localisation of the protein of interest [493]. This could also be due to the high strength of the actin staining interfering with the much weaker GFP stain, which is a common problem associated with dual labelling. Another reason for the loss of GFP staining could be that the GFP leached out of the cells while the gel was incubating in phalloidin overnight. To assess if the fixation or the phalloidin labelling destroyed the DMP-1-GFP live cell imaging was undertaken
without actin labelling. This confirmed that the DMP-1-GFP had in fact been
destroyed as it was highly expressed following live cell imaging. DMP-1-GFP is
expressed in these cells once they are differentiating from an osteoblast to an
osteocyte. Gene expression results also confirmed that these cells were expression
DMP-1 in this 3D culture system, which is why these cells should of been expressing
DMP-1-GFP.

The gene expression results described in this thesis differ to those described in the
first paper describing the establishment of the IDG-SW3 cell line by Woo et al. in
2011 [108]. Woo et al. found that when IDG-SW3 cells were maintained in
monolayer cultures ALP mRNA expression was at its maximum on day 14, whereas
in the 3D cultures described in this chapter, ALP mRNA expression is at its
maximum on day 7. The earlier peak in ALP mRNA expression in 3D culture
suggests that the cells are differentiating more quickly in 3D when compared to
monolayer culture. Although reduced, ALP mRNA expression was still retained in
3D cultures after 7 days. Previous studies have used the absence of ALP mRNA
expression to confirm the presence of an osteocyte phenotype in osteocytes isolated
from the long bones of skeletally mature and aged mice [73]. It has been shown that
ALP is associated with osteoblasts as it is necessary for osteoid production [31].
Therefore in this 3D culture system, at later time points, it was hypothesised that
ALP would have been absent. However, IHC, reflecting the in vivo state reveal broad
ALP expression in bone where it was localised to pre-osteoblasts, lining cells on the
surface of the trabeculae, widely embedded osteocytes, endosteal cells, subperiosteal
cells, and in the osteoid where there was new bone formation [498].
E11 is the earliest marker of an osteocyte, and is localised to the dendritic processes of the cells, and is expressed during the osteoblast to osteocyte transition [52, 87]. The results presented in this chapter show that E11 mRNA expression in IDG-SW3 in 3D is different to those observed in these cells in monolayer [108]. In monolayer IDG-SW3 show maximum E11 expression following 7 days of culture, with a significant decrease (50 %) observed following 10 days of culture, suggesting that these cells in monolayer differentiate to early osteocytes following 7 days of culture in osteogenic medium. The 3D results presented in this chapter show that E11 expression is present at similar levels throughout the time course in 3D, although this is much higher than in proliferating cultures at 33°C. This suggests that these cells are differentiating and becoming dendritic at day 1 at 37°C. E11 expression is localised to osteocytes in osteoid, and in mineralised bone where its expression is up-regulated following fluid flow shear stress [52, 499]. It has also been shown to be up-regulated in cells near the bone surface but also in the deeply embedded osteocytes following mechanical loading [52]. As well as in osteocytes, E11 expression can also be found in the lungs and the kidneys [86, 500, 501]. Human primary osteoblasts have also been reported to express E11 when treated with recombinant sclerostin for 35 days [502], or when maintained in 3D pellet culture [503]. Mouse primary osteoblasts also express E11 following treatment with FGF-2 [202].

Maximum DMP-1 gene expression in the IDG-SW3 cell line is seen following 35 days of culture [108], although it was not measured prior to day 14. However when IDG-SW3 are placed in 3D, they express DMP-1 earlier, with maximum expression by day 17 of culture (9-fold increase compared to day 1). Following this the results
also show that there was a reduction (~3-fold) in DMP-1 expression on day 21 when compared to day 17 suggesting by day 21 perhaps something has happened to these cells to cause this response. Furthermore the reduction in mean cell number and the significant reduction in viability on day 21, further suggest that there is something different in these cells at this time point, and the reduction in viable cells may explain the reduction in DMP-1 expression. DMP-1 has well been established as a mineralising osteocyte marker that is also up-regulated following mechanical loading [42, 413, 414]. The down-regulation of DMP-1, along with MEPE and PHEX, has also been shown to be linked to the up-regulation of FGF-23 [380]. In vivo, DMP-1 depletion has also been shown to decrease bone mineralisation [504].

MEPE mRNA expression is detected at low levels in monolayer IDG-SW3 when cells are osteoblastic (measured on day 0), with the expression only being measured again on day 14 where there is increased MEPE mRNA expression with maximal expression measured on day 21 and day 25 [108]. The results presented in this chapter suggest that MEPE mRNA expression of IDG-SW3 in 3D is at its maximum at day 10 and then is down regulated, which suggests that in 3D culture these cells are may be becoming mature osteocytes earlier in culture. Previous results show that MEPE is expressed in osteoblasts as well as in the early and mineralising osteocyte, but its expression is significantly up-regulated when these cells differentiate to a mature osteocytes [31]. It has also been shown to be mechano-regulated in osteocytes following various methods of mechanical loading [296, 413, 414, 421].

PHEX mRNA is expressed in IDG-SW3 cells in monolayer in both the osteoblast and osteocyte stage, with maximum expression on day 20 before a decrease in expression on day 35 [108]. The 3D results presented in this chapter follow a similar
trend, with maximum expression on day 17 and then have a decrease in expression on day 21. These results suggest that IDG-SW3 express PHEX mRNA earlier in culture in 3D than in monolayer highlighting that this 3D model enables earlier differentiation to mature osteocytes.

IDG-SW3 sclerostin mRNA expression in monolayer was not detected at day 10, was expressed at day 14, was highest at day 21 and was reduced following 35 days of culture [108]. The 3D results for sclerostin mRNA expression presented in this chapter suggest that it is detected from day 1 at 37°C with highest expression detected at day 7 before it returns to lower levels at later time points. This suggests that IDG-SW3 cells in this 3D culture system are differentiation to mature, sclerostin expressing osteocytes earlier than in monolayer. For years, sclerostin was widely accepted as a mature osteocyte marker [31, 380], but has also been shown to be expressed in chondrocytes [505] and in particular articular cartilage [417-420], but also in osteoblast cell lines [506, 507]. It has also been shown in vivo and in vitro that sclerostin expression was down-regulated following mechanical loading [290, 382, 507, 508].

RANKL and VEGF mRNA expression has not been previously investigated in IDG-SW3 cells. The results presented in this chapter suggest that RANKL expression is highest when the cells are mature osteocytes as on the later time points (Day 17 and 21) of differentiation there is increased RANKL expression. These results are consistent with recent publications showing that osteocyte cells produce the most amount of RANKL [20, 509] in bone. The observed RANKL expression in these cells in 3D supports the theory that osteocyte derived RANKL could provide the
mononuclear cells with the RANKL essential for osteoclastogenesis [428, 430, 510]. Both RANKL [45, 302, 303] and VEGF [305] are up-regulated in osteocytes following mechanical loading. Results presented in this chapter show mean VEGF gene expression values were higher at all time points in 3D culture at 37°C, compared with 33°C, with highest expression observed at day 14.

The results presented in this chapter show that for all genes investigated, there was an up-regulation in gene expression when the cells were placed in osteogenic medium at 37°C when compared to proliferative medium at 33°C. This may reflect the fact that the majority of genes investigated are osteocyte specific markers, or markers that are known to be highly expressed in osteocytes. It should also be noted that the changes observed were not because of changes in the reference gene during proliferation and differentiation as the reference gene was not modulated throughout when cells were cultured in 33°C or 37°C, with Normfinder selected the most stable reference gene for this data set.

VEGF and IL-6 protein secretions were also investigated in IDG-SW3 in 3D culture during differentiation. The results presented in this chapter suggest that IDG-SW3 in 3D culture conditions, produce higher VEGF secretion than osteoblasts, consistent with VEGF gene expression results presented in this chapter. Mean IL-6 secretion was increased at all time points after day 10, with highest IL-6 secretion observed at day 21. Notably, both VEGF and IL-6 expression were at their highest following 21 days of culture at 37°C. It was at this time point where cell number and viability were at their lowest. Perhaps, the higher protein secretions seen at this time point, especially in the case for IL-6, a known pro-inflammatory cytokine, are an indication
Chapter 5  IDG-SW3 differentiation in 3D collagen type I gels

that the cell are becoming stressed at this time point in these culture conditions and warrants further investigation to understand this more. Protein secretions from IDG-SW3 cells have not been previously reported [108].

This chapter shows the differentiation of IDG-SW3 cells from early osteoblasts (expressing low levels of DMP-1, MEPE, PHEX and IL-6) to mature osteocytes (expressing high levels of DMP-1, sclerostin, RANKL, VEGF and IL-6) in 3D collagen type I gels. The results obtained in this chapter demonstrate that it is possible to differentiate and maintain IDG-SW3 cells in 3D culture, and that differentiation in 3D gels in osteogenic medium appears faster than in monolayer culture.
6 The effects of mechanical loading on the

IDG-SW3 osteocyte-like cells
Chapter 6  
The effect of mechanical loading on IDG-SW3 osteocyte cells

6.1 Introduction

Chapter 4 investigated the differentiation of mouse osteoblasts (IDG-SW3 cells) to osteocytes in 3D collagen type I gels. In this chapter, the response of differentiated IDG-SW3 osteocyte-like cells to mechanical loading in 3D collagen type I gels will be investigated. The IDG-SW3 cells were used initially to set up this mechanical loading method but also to coincide with mechanical loading of human osteoblast cells (hOBs) differentiated to osteocytes undertaken by another person in the research group.

6.1.1 Osteocytes: the mechanosensory cells

Osteocytes are believed to be the mechanosensory cells in bone [31, 511-513] and thus one of their main functions is to respond to mechanical strain by sending signals to cells on the bone surface [47]. There are at least three theories in relation to how mechanical loading can result in mechano-sensation by osteocytes. One of these, and currently the most plausible, states that following mechanical loading the osteocyte network acts by sensing and responding to changes that occur in the fluid flow in the osteocyte canicular network [107, 514, 515]. The mechanisms for this are not fully understood, but it is believed that osteocytes sense load through either the cell body [516, 517], the glycocalyces on the dendritic processes [517-519], or by the bending of cilia which are single flagellar-like structures found on every cell [489, 520]. Two other theories which have been widely discussed are that i) variations in whole tissue strain are sensed by the osteocyte leading to mechano-sensation and ii) changes in hydrostatic pressure within the osteocyte following mechanical loading cause the cells to become mechano-sensitive [521, 522]. Whereas it is possible to investigate osteocyte responses to mechanical loading in vivo using techniques such as cyclic
loading [285-290] or four point bending [291, 293, 523], such loading experiments use large numbers of animals, are difficult and expensive and cannot be done in humans (Section 1.8.1, chapter 1). Current methods of investigating this subject area in vitro are, however, limited and there is thus a need to develop new in vitro mechanical loading systems to further our understanding of the pathways involved.

6.1.2 Osteocyte responses to mechanical loading

In vivo and in vitro studies have delineated many different osteocyte cell responses following mechanical loading (Section 1.8). Table 6.1 summarises these responses and indicates whether the results are from in vivo or in vitro studies. In this chapter, osteocytes have been maintained in gels in a 3D mineralised environment akin to the osteocyte environment in bone, and mechanically loaded.

Numerous studies have described mechanical loading of osteoblasts and osteocytes in vitro, and these have used several different methods, most of which are in monolayer cultures (Section 1.8.2, Chapter 1). Reports of mechanical loading of osteoblasts and osteocytes in 3D gels and scaffolds, however, are limited. Currently in vitro mechanical loading models use cell culture systems that undergo mechanical stimuli in the form of hydrostatic pressure, fluid shear stress or substrate strain in a specific manner [42, 45, 275, 301, 306, 309, 491, 524-527] [305, 319, 525]. There are numerous mechanical loading devises that enable application of mechanical loading in vitro, which include “in house” custom made devices which are currently not commercially available [525]. Furthermore, these differ in terms of consistency of loading, accuracy and difficulty. In vitro mechanical loading devices have been used since 1939, and since then the field has developed in terms of range of
compression used, stretch, substrate bending, distension and fluid sheer devices which have utilised both contact and contact-less loading [514, 525, 528-530]. Commercially available bone loading systems that can be used *in vitro, in vivo* and *ex vivo* include the BOSE, Flexcell and ZETOS which enable loading in terms of tension, compression, bending, stress, torsion and fluid sheer stress [52, 91, 514, 528-534]. However, the major limiting factor of these commercially available systems is the number of cultures/specimens that can be loaded at the same time.

For the 3D mechanical loading method presented in this chapter, a loading system that applies physiological mechanical stimuli, enabling the loading of sixteen 3D cultures at the same time with equal strains across the cultures was used. This was applied with the following cyclic loading regime (5 mins, 10 Hz, 2.5 N). Furthermore, IDG-SW3 osteoblast cells were differentiated to osteocytes in 3D collagen type I gels prior to mechanical loading. The IDG-SW3 cells (Section 1.5.3.1, chapter 1 [78]) provide an excellent model for the study of the responses of osteocytes to mechanical loading *in vitro*. No such studies using this cell line have been published to date.
<table>
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**Table 6.1:** Summary of published osteocytic cell responses following mechanical loading in vivo or in vitro. Abbreviations = CL-cyclic loading, UL-ulnar loading, DS-dyanamic strain, TMM-tooth movement model, FFSS-fluid flow sheer stress, PFF-pulsating fluid flow, LIPO-low intensity pulsed ultrasound, OFF-oscillatory fluid flow, IMHC-intermittent hydrostatic compression.
6.2 Hypothesis

Mechanical loading will modulate IDG-SW3 cells differentiated to osteocytes for 10 days in 3D gels and maintained in mineralising (osteogenic) medium when compared to relevant controls.

6.3 Aims

To determine the responses of IDG-SW3 cells to mechanical loading, following maintenance in 3D collagen type I gels for 10 days in mineralising medium, using a novel plate based loading system developed at Cardiff University.

Specifically the aims were to investigate the effects of mechanical loading (10 Hz, 5 mins, 2.5 N) at 0.5, 3, 24 and 48 hrs post-loading on:

1. Cell number and viability (haemocytometer counting and trypan blue exclusion)
2. Cell morphology (H&E staining)
3. Gene expression (RT-qPCR; DMP-1, LRP-5, IL-6, VEGF, sclerostin, RANKL and OPG)
4. Protein expression (IHC; RANKL)
5. IL-6, VEGF and PGE$_2$ secretion (ELISA)
6.4 Methods

6.4.1 Experimental design

IDG-SW3 cells were set up in collagen gels in silicone plates (2 plates per experiment, 1 control and 1 loaded; 3 individual experiments, Section 2.2.9.2). Mechanical loading was undertaken (Section 2.2.9.3), and responses analysed at 0.5, 3, 24 and 48 hrs post loading. In each experiment there were 4 replicate gels per time point of which 3 gels were used for analysis of cell number and viability, gene expression, protein secretion and 1 gel used for morphological and IHC analysis following fixation and sectioning. However, one gel well was broken in experiment 1 leading to the loss of one sample from the control plate at 24 hrs post loading. In addition, in 2 of the experiments, media from 4 replicate gels per time point were used for analysis of PGE\textsubscript{2} release.

Since plates were loaded from one end (Section 2.2.9.3), and to avoid bias due to positioning of gels in the plate, the 4 replicate wells per time point were always taken from different areas of each plate – one from the vertical row closest to the load cell, one from the vertical row furthest from the load cell, one from the centre four wells of the plate, and one from the centre two wells at the top or bottom of the plate. An example of well selection for analyses is shown in Figure 6.1 and the well selection was always the same for control and loaded plates within one experiment.

6.4.2 Assessment of cell number and viability, gene expression, protein secretion and PGE\textsubscript{2} release following mechanical loading

Media were removed from wells at 0.5, 3, 24 and 48 hrs post mechanical loading, centrifuged (1,700g, 30 s) and stored in aliquots (120 µl) at -80°C until needed for
measurement of VEGF, IL-6 and PGE$_2$ (ELISA) secretion (Sections 2.2.7.1 and 2.2.7.3). The gels were then collagenase digested for measurement of cell number and viability (Section 2.2.2.5) and following this the remaining cells were placed in TRI Reagent for RNA extraction and gene expression analyses (Section 2.2.3).

For IL-6 and VEGF protein secretion, the value from each gel sample was normalised to the cell number in that same sample and expressed relative to the mean control sample value (fixed to 100%) at the 0.5 hrs time point. Results from the 3 experiments were then combined for further analyses.

Measurement of PGE$_2$ secretion was undertaken in 2 of the 3 experiments. The raw values (n=4 per time point for control and loaded) were plotted, as well as data from each experiment following normalisation to cell number (only n=3 per time point from control and loaded plates available for this) as described above for IL-6 and VEGF protein measurement.

For cell number analysis, data from each experiment were expressed relative to the mean control sample value at the 0.5 hr time point (expressed as 100%) which enabled the 3 experiments to be combined together.

For the 3 experiments, RT-qPCR was initially undertaken on all control and loaded samples at all time points using 3 reference genes (GAPDH, PCNA and 18sRNA). The data generated from this were then analysed using Normfinder to select the most stable housekeeping gene to use for this data set. Following this, all gene expression data presented in this chapter were normalised to PCNA. Data were analysed using
ΔΔCt (Section 2.2.3.8) with the value from one control sample at 0.5 hrs post loading fixed to 1 in each experiment allowing the data from the experiments to be combined for further analysis.

6.4.3 Assessment of morphological and immunohistochemical analysis following mechanical loading

In each experiment one gel from each of the control and loaded plates was removed and fixed (Section 2.2.5) at 0.5, 3, 24 and 48 hrs post mechanical loading. These were subsequently sectioned and stained with H&E to assess cell morphology or used for IHC analysis of RANKL expression (Section 2.2.5.6).

6.4.4 Statistical analysis of data

Most of the data presented in this chapter are combined from 3 independent experiments with an n=3 at each data point in each experiment. Graphs in this chapter were generated from the mean ± SEM, or were individual sample data points (FigureA). Prior to undertaking statistical analyses, the complete data sets (n=9 per time point for either control or loaded samples, apart from 24 hrs control which was n=8) were tested for (1) normality (Shapiro-Wilk test, Graph Pad Prism Software), (2) equal variance (Bartlett’s test, Graph Pad Prism Software) and (3) presence of outliers (Grubb’s test, Microsoft office Excel 2010). All data presented in this chapter were normal, had equal variance and had no outliers. All statistical analyses were undertaken on the complete data sets using a one-way ANOVA followed by a Bonferroni post hoc test.
Figure 6.1: Selection of gels for analysis post loading with the same wells selected at each time point for the control and loaded plate. (A) experiment 1 and (B) experiment 2 showing samples taken at different time point post loading - blue (0.5 hrs), green (3 hrs), purple (24 hrs) and orange (48 hrs). In two of the experiments, all wells were used for PGE$_2$ secretion. The * indicates the gels used for morphological and immunohistochemical analysis whereas all others were used for cell number and viability, gene expression and protein secretion. The x indicates that this well was broken in experiment 1.
6.5 Results

6.5.1 Cell number and viability

IDG-SW3 cell numbers were found to be much higher ($p<0.001$) in the unloaded control gels at 0.5 hrs post loading when compared to gels at all other time points in both the control and loaded plates (Figure 6.2A & B). These significant differences were seen when data were expressed both as raw values (Figure 6.2A) or relative to control cell numbers at 0.5 hrs post load (fixed to 100 %, Figure 6.2B). When data from individual experiments were analysed separately, this increased cell number was seen in each of the 3 experiments ($p<0.001$ in each independent experiment, data not shown). There were no other differences in cell number either between control and loaded gels at one time point post loading or between different time points within the control and loaded gels (Figure 6.2A & B).

Whilst analysing the above data it was also noted that there was a significantly ($p<0.001$) higher cell number following 10 days in silicone plates (value from 0.5 hr post-loading control 459,853.71 ± 6,389.76; Figure 6.2A) when compared to cell numbers from similar experiments using plastic plates (178,967.21 ± 6,395.81; Figure 5.1Chapter 5).

IDG-SW3 cells were viable in collagen gels in the silicone plates for at least 12 days of differentiation (10 days pre-loading plus 2 days post-loading). Cell viability was >94 % in both control and loaded plates at all time points studied post loading (Figure 6.2C). Furthermore, there were no differences in cell viability when comparing control and loaded gels at one time point, or different time points within the control or loaded gels (Figure 6.2C).
6.5.2 Morphology

H&E staining of IDG-SW3 cells at 0.5-48 hrs post mechanical loading revealed that cellular changes between the unloaded and loaded cells. In all of the control gels, at all time points assessed, osteocytic-like dendritic processes can be seen in the cells (Figure 6.3A-D). In the loaded cells, at 0.5 and 3 hrs post mechanical loading, the dendritic processes appear to have contracted (Figure 6.3E-F) and the cells seem to have completely changed shape in response to mechanical loading. At 24 hrs post mechanical loading, the loaded cells appear to be recovering some of their dendritic processes (Figure 6.3G), and by 48 hrs post mechanical loading, the cells appear to have regenerated their osteocytic like dendritic processes once again (Figure 6.3F). Furthermore this morphological analysis revealed that the IDG-SW3 cells were sparse in these collagen type I gels, with large areas within the gels not having any cells present. However, the initial starting IDG-SW3 cell number of 7.0 x 10^5 cells/ml gel was chosen in order to allow direct comparisons with results previously described for MC-3T3 cells (Chapter 3).

6.5.3 RANKL expression

Analysis of RANKL expression by IHC revealed that both the control and loaded cells stained positively for RANKL. In the control samples, all the cells appear to stain positively for RANKL with no difference in staining intensity (Figure 6.4A-D), although the background appears to be darkest at the 48 hrs time point, suggesting that there is an accumulation of RANKL within the collagen gels by this time (Figure 6.4D).
The loaded cells also stained positively for RANKL at each time point (Figure 6.4E-H), and the intensity of cellular RANKL staining appears to be similar throughout the loaded samples. The amount of RANKL positive staining in the collagen gel matrix, however, appeared patchy at 0.5 hrs but appeared more uniform across gels at later time points. As previously described for H&E staining, there appears to be a lack of dendritic processes in these sections at 0.5 hrs post mechanical loading (Figure 6.3E), but these osteocytic-like processes appear to recover with time up to 48 hrs post-loading.

When comparing control and loaded IDG-SW3 cells post mechanical loading, the only noticeable difference is that in the control samples the accumulation of RANKL in the gel matrix appeared to be patchy at earlier time points but was more uniform at 48 hrs, whereas in the loaded samples it seemed patchy at 0.5 hrs and was more uniform across the gels from the later time points.
**Figure 6.2:** IDG-SW3 cell number and viability in collagen type I gels post mechanical loading. Raw cell number (A), % cell number (relative to 0.5 hrs control) (B) and % cell viability (relative to total cell number at all time points set to 100 %) (C) in IDG-SW3 cells in 3D collagen gels at 0.5, 3, 24 and 48 hrs post mechanical loading. Data are a combination of 3 independent experiments (n=3 at each data point per experiment, apart from experiment 1 at 24 hrs control where n=2). Data in B and C are mean ± SEM. *** $p<0.001$ versus 0.5 hrs control or ### $p<0.001$ versus loaded at the same time point following a one way ANOVA and a Bonferroni post hoc test.
Chapter 6  The effect of mechanical loading on IDG-SW3 osteocyte cells

**Figure 6.3:** H&E staining of IDG-SW3 cells in 3D collagen type I gels following mechanical loading. Representative H&E staining of fixed and sectioned (7 µm) 3D collagen gels containing IDG-SW3 cells at 0.5 (A, E), 3 (B, F), 24 (C, G) and 48 (D, H) hrs post mechanical loading in control (A-D) and loaded (E-H) cells in osteogenic medium. Images were captured using the Aperio Scanscope (20x magnification) and processed using the Aperio software. The scale bar is 100 µm in each image, and the black square represents an enlarged area highlighted in the red square. All images shown are from 1 experiment, where all H&E staining was undertaken at the same time, with n=5 sections per individual time point shown. a = osteocyte-like dendritic processes, b = lack of osteocyte-like dendritic processes, where images shown are a representative of the entire dept of the gel.

250
Figure 6.4: IHC of RANKL in IDG-SW3 cells in 3D collagen type I gels following mechanical loading. Representative RANKL IHC staining of fixed and sectioned (7 μm) 3D collagen gels containing IDG-SW3 cells at 0.5 (A, E), 3 (B, F), 24 (C, G) and 48 (D, H) hrs post mechanical loading in control (A-D) and loaded (E-H) cells in osteogenic medium. Images were captured using the Aperio Scanscope (20x magnification) and processed using the Aperio software. The scale bars is 100 μm in each image, and the black square represents an enlarged area highlighted in the red square. All images shown are representative of 1 experiment, where all staining was undertaken at the same time, where a = RANKL positive staining and b = RANKL negative staining. All images were counterstained with haematoxylin and I represents the IgG isotype control.
6.5.4 DMP-1, LRP-5, IL-6, VEGF, sclerostin, RANKL and OPG gene expression

To determine if IDG-SW3 osteocytes respond to mechanical loading in 3D type I collagen gels DMP-1, LRP-5, IL-6, VEGF, sclerostin, RANKL and OPG (all known mechano-regulated factors) mRNA expression were investigated post mechanical loading.

DMP-1 mRNA expression was significantly increased in loaded samples when compared to control samples at the same time point at 0.5 (~1 fold, p<0.05) and 48 (~1 fold, p<0.05) hrs post mechanical loading. Furthermore, mean gene expression values for loaded were higher than control at 3 (control; 1.189 ± 0.318, loaded; 1.376 ± 0.341) and 24 hrs (control; 1.064 ± 0.322, loaded; 1.645 ± 0.375) post loading but the values were not significantly different. However, DMP-1 mRNA expression was not modulated with time during the 48 hrs time course post-loading in control and loaded samples (Figure 6.5A).

LRP-5 mRNA expression was significantly increased 0.5 fold at 48 hrs (p<0.05) post mechanical loading when compared to unloaded control cells at the same time point, with no differences between loaded and control samples at all other time points studied. Mean gene expression values were increased in loaded gels at 0.5 hrs (control; 0.938 ± 0.104, loaded; 1.148 ± 0.122) post-loading (p=0.081). Furthermore, there was a significant (0.5 fold) increase in LRP-5 mRNA expression in the loaded samples over time (0.5 hrs versus 48 hrs; p<0.05), whereas there was no change in LRP-5 mRNA expression in the control samples with time (Figure 6.5B).
Sclerostin mRNA was significantly reduced ($p<0.05$) in loaded samples compared to control at 3 hrs post-loading. Mean gene expression values were also lower for loaded compared to control samples at 0.5 hrs (loaded; $1.104 \pm 0.476$, control; $2.293 \pm 0.988$) post-loading, but these values were not significantly different. However, there was also a significant reduction in sclerostin mRNA expression in unloaded samples at 24 ($p<0.05$) and 48 ($p<0.05$) hrs when compared to the unloaded values at 0.5 hrs (Figure 6.5C).

There were significant increases in IL-6 mRNA expression at 0.5 ($p<0.05$) and 24 ($p<0.01$) hrs post-loading compared to unloaded controls at the same time points with no effect seen at all other time points. Furthermore, there were no changes in IL-6 mRNA expression in either control or loaded samples over time up to 48 hrs post-loading (Figure 6.5D).

VEGF mRNA expression was increased (~3 fold) in loaded cells at 0.5 hrs ($p<0.05$) post mechanical loading, but then returns to control values by 3 hrs. Furthermore there was a significant reduction in VEGF mRNA expression at 3 ($p<0.05$) and 48 ($p<0.05$) hrs when compared to the mRNA expression at 0.5 hrs post loading in the loaded samples. There was no difference in VEGF mRNA expression in the control over time when compared to 0.5 hrs control (Figure 6.5E).

Mean RANKL mRNA expression was increased at all time points studied, although the difference between loaded and control samples was significant at the 24 hr ($p<0.05$) time point only. Mean ± SEM RANKL gene expression values at the other time points post loading were 0.5 hrs (control; $1.205 \pm 0.303$, loaded; $2.080 \pm 0.575$),
Chapter 6  
The effect of mechanical loading on IDG-SW3 osteocyte cells

3 hrs (control; 1.543 ± 0.397, loaded; 3.319 ± 1.241), and 48 hrs (control; 0.809 ± 0.106, loaded; 1.266 ± 0.405) post loading, suggesting that RANKL expression could be upregulated in loaded cells at all time points studied (Figure 6.6A).

OPG mRNA expression data showed a significant increase in loaded cells at 0.5 hrs ($p<0.05$) and then returned to control levels by 3 hrs post-loading. There was an increase in mean OPG gene expression in loaded samples compared to controls at 48 hrs post loading (control; 1.563 ± 0.255, loaded 2.164 ± 0.204) but this difference was not significant. Although there was no difference in OPG mRNA expression in control over time, there was a significant difference in loaded at 3 ($p<0.05$) and 24 ($p<0.05$) hrs post-loading when compared to loaded at 0.5 hrs (Figure 6.6B).
Chapter 6  The effect of mechanical loading on IDG-SW3 osteocyte cells

Figure 6.5: Gene expression of known mechano-regulated factors in IDG-SW3 cells in 3D collagen type I gels following mechanical loading. Relative expression of DMP-1 (A), LRP-5 (B), sclerostin (C), IL-6 (D) and VEGF (E) in IDG-SW3 cells in 3D collagen gels at 0.5, 3, 24 and 48 hrs post mechanical loading. RT-qPCR analysis of RNA isolated from cells, normalised to PCNA (1 gel of 0.5 hrs control set to 1 in each experiment). Data are a combination of 3 independent experiments (n=3 at each data point per experiment, apart from experiment 1 at 24 hrs control where n=2). All data points are mean ± SEM * p<0.05 versus same condition at 0.5 hrs, # p<0.05, ## p<0.01 versus control at the same time point following a one way ANOVA and a Bonferroni post hoc test.
Figure 6.6: Gene expression of RANKL and OPG in IDG-SW3 cells in 3D collagen type I gels following mechanical loading. Relative expression of RANKL (A) and OPG (B) in IDG-SW3 cells in 3D collagen gels at 0.5, 3, 24 and 48 hrs post mechanical loading. RT-qPCR analysis of RNA isolated from cells, normalised to PCNA (1 gel of 0.5 hrs control set to 1 in each experiment). Data are a combination of 3 independent experiments (n=3 at each data point per experiment, apart from experiment 1 at 24 hrs control where n=2). All data points are mean ± SEM. * p<0.05 versus loaded at 0.5 hrs, # p<0.05 versus loaded at the same time point following a one way ANOVA and a Bonferroni post hoc test.

6.5.5 IL-6, VEGF and PGE2 secretion

6.5.5.1 IL-6 protein secretion

IL-6 secretion was significantly increased in loaded samples compared to control samples at the same time point (3 hrs p<0.001, 24 hrs p<0.001 and 48 hrs p<0.01 hrs) post-loading (Figure 6.7A). Although mean IL-6 protein secretion was reduced at 0.5 hrs post mechanical loading when compared to control samples (35.220 % ± 22.567 % and 100 % ± 40.673 %) the difference was not significant (Figure 6.7A).

6.5.5.2 VEGF protein secretion

When data were expressed relative to control data at 0.5 hrs VEGF protein secretion was significantly increased in loaded compared to unloaded samples at 0.5 (p<0.05) and 48 (p<0.01) hrs post-loading. There were no differences in mean VEGF
secretion at 3 (control, 2,627.50 % ± 916.38 %, loaded; 3,069.58 % ± 1,174.56 %) or 24 hrs (control; 8,387.05 ± 4,795.24 %, loaded; 5,897.33 ± 2,199.04 %) post-loading (Figure 6.7B).

6.5.5.3 Re-analysis of IL-6 and VEGF protein secretion data

Since the cell number value was increased in control (unloaded) samples compared to loaded samples at 0.5 hrs post loading (Figure 6.2A & B) and the IL-6 and VEGF protein secretion data presented above have been expressed relative to the 0.5 hrs control cell number data, protein secretion results from these loading experiments were also expressed relative to control values (fixed to 100 %) at each time point. This was done in order to investigate whether the unexplained cell number data at 0.5 hrs post loading is interfering with data interpretation. These results are presented in Figure 6.7C & D. When expressed in this way IL-6 secretion in loaded samples is significantly higher at 3 (p<0.01) and 24 (p<0.05) hrs post-loading compared to unloaded controls. However, mean secretion values were also higher in loaded (179.12 % ± 51.11 %) samples when compared to controls (100 % ± 26.41 %) at 48 hrs post mechanical loading but this difference was not significant (Figure 6.7C). There was also a significant increase in IL-6 secretion at 3 hrs (p<0.01) and 24 hrs (p<0.05) when compared to the 0.5 hr loaded values. These results are thus very similar to those shown in Figure 6.7A indicating that the 0.5 hrs post loading control data are not skewing the IL-6 protein secretion results, as there clearly were effects of loading on IL-6 secretion when both methods of analysis were used.

When the VEGF protein secretion data were reanalysed in the same way, there was a significant increase in VEGF secretion by loaded cells at 0.5 hrs (p<0.05) post-
loading (Figure 6.7D). Mean VEGF secretion values were lower in loaded samples at 3 (control; 100% ± 15.54%, loaded; 70.48% ± 5.94%) and 24 (control; 100% ± 69.60%, loaded; 27.57% ± 1.53%) hrs, which was the opposite to what was seen when the data was expressed relative to control at 0.5 hrs (Figure 6.7B).

**Figure 6.7:** IL-6 and VEGF secretion in IDG-SW3 cells maintained in 3D collagen type I gels following mechanical loading. IL-6 (A, C) and VEGF (B, D) concentrations in media (ELISA) from loaded and unloaded control samples at 0.5, 3, 24 and 48 hrs post mechanical loading. Data are a combination of 3 independent experiments (n=9 (apart from 24 hrs control where n=8) at each data point). Data were expressed relative to 0.5 hrs control values set to 100% (A, B) or relative to control values set to 100% at all time points (C, D). * p<0.05, ** p<0.01 versus loaded at 0.5 hrs or # p<0.05, ## p<0.01, ### p<0.001 versus loaded at the same time point, following a one way ANOVA and a Bonferroni post hoc test.

### 6.5.5.4 PGE2 secretion

Conditioned media samples from replicate wells (n=4) in both loaded and unloaded plates at each of the four time points studied and in two of the three IDG-SW3 loading experiments undertaken were used for PGE2 analysis. One unloaded well, however, was broken as previously described leaving 63 samples in total. PGE2 was
detected in 44 of the samples analysed, comprising 26 control samples and 18 loaded samples. The range of PGE$_2$ concentrations were very low for the most part (8.36–75.35 pg/ml, Table 19), although 2 samples contained 263.14 pg/ml (48 hrs control, experiment 1) and 751.60 pg/ml (48 hrs loaded, exp. 3) of PGE$_2$. When the results from these two outliers were omitted, the range of values for the control and loaded samples were 8.73-75.35 pg/ml and 8.36-53.59 pg/ml respectively (Table 6.2, Figure 6.8A & B).

When the PGE$_2$ concentrations were normalised to cell numbers, the range of values for most of the control samples were 3.80–44.90 pg/ml/2 x 10$^5$ cells, with one sample at 48 hrs post-loading in experiment 1 measuring 491.60 pg/ml/2 x 10$^5$ cells (outlier), and for the loaded samples were 5.99–33.19 pg/ml/2 x 10$^5$ cells (Table 19). In order to analyse data from all the samples, the undetectable readings were assigned a zero value. When looking at the mean raw data for PGE$_2$ secretion at specific time points from a combination of the two experiments, there was an accumulation of PGE$_2$ at 48 hrs (control: 126.46 ± 92.66 pg/ml/2 x 10$^5$ cells, loaded: 46.90 ± 31.77 pg/ml/2 x 10$^5$ cells) in both the control and loaded samples when compared to the 0.5 hrs (control: 24.17 ± 8.67 pg/ml/2 x 10$^5$ cells, loaded: 17.30 ± 7.12 pg/ml/2 x 10$^5$ cells) time point (Figure 6.8C & D). Due to not having measurable PGE$_2$ secretion in some of the samples and low levels in other samples and due to variations between experimental replicates, no further analysis was undertaken.
Table 6.2: PGE₂ secretion in experiments 1 and 3 showing raw PGE₂ concentrations (pg/ml) as well as PGE₂ secretion normalised to cell number (pg/ml/2 x 10⁵ cells) in control and loaded IDG-SW3 cells following mechanical loading. Undet. = undetectable, where all mean values included the undetected samples that were assigned zero values.
Figure 6.8: Measurement of PGE\textsubscript{2} secretion by IDG-SW3 cells maintained in 3D collagen type I gels and following mechanical loading. PGE\textsubscript{2} concentrations (ELISA, A-D) in control and loaded IDG-SW23 cells at 0.5, 3, 24 and 48 hrs post mechanical loading. Data are a combination of 2 independent experiments (n=8 per per time point measured - A-B) or (n=6 per time point measured - C-D). Data in A-B were raw values generated from the ELISA and data in C-D were raw values normalised to cell number. Data in A-C were generated from experiment 1 and data in B-D were generated from experiment 3.
6.6 Discussion

The work in this chapter aimed to determine whether IDG-SW3 cells in 3D type I collagen gels respond to mechanical loading applied using the novel silicone plate based method recently developed at Cardiff University. Collectively, the results indicate that these cells do respond to mechanical loading under these conditions with several known mechano-regulated substances up-regulated or down-regulated (gene expression and protein secretion) during the 48 hrs following loading (as summarised in Figure 6.9).

Published studies using IDG-SW3 cells have not looked at proliferation rate although it has been documented that these cells should not proliferate once placed in osteogenic medium [108]. In this study, cell number was higher when IDG-SW3 cells were maintained in silicone plates, compared to standard Nunc™ tissue culture plates, as described in section 6.4.1. This increased cell number in silicone plates could be due to contaminants leaching out of the plates and affecting the cell proliferation rate. One possible contaminant is silicone and previous studies have reported that silicone particles or culturing cells on silicone alter the properties of cells such as MSCs to differentiate to other cell types [548-550]. In osteoblast cell lines and human primary cells, silicone has also been shown to increase proliferation [526, 551, 552].

Cell number was similar in most of the unloaded and loaded gels in these experiments, although the number of cells in unloaded gels at 0.5 hrs post-loading was consistently 17 % higher than all the other samples. This increase in cell number was not due to apoptosis as cell viability was consistently high (>94 %) for the
duration of the experiment in all samples (Figure 6.2C). Furthermore, apart from the unloaded gels at 0.5 hrs in both the unloaded and loaded samples, cell number was not modulated over time. These results are similar to those in other mechanical loading studies which show that mechanical loading prevents osteocyte cells undergoing apoptosis [91, 92, 318, 319], as the results presented in this chapter show that cell viability is not modulated with mechanical loading. The results in this chapter also show that there were no differences in cell viability between control and loaded cells. Furthermore, there were no differences between cell viability in the silicone plate (>94 %, day 10, 0.5 hr unloaded samples) and standard tissue culture plates (>93.2 % on day 10, Figure 5.1C, Chapter 5).

Raw cell number data (Figure 6.2A) show that the higher cell number in the control gels at 0.5 hrs is not because of outliers skewing the data set, as in each of the individual experiments values for the control samples at 0.5 hrs were significantly higher (p<0.001) than those for loaded samples at 0.5 hrs. This higher cell number is also not due to location of the gels selected in the silicone plate as the location of each time point was randomised across the plate (as described in Figure 6.1). The increased cell number in the control gels at 0.5 hrs post mechanical loading is an unexpected result and is difficult to explain.

H&E staining suggests that IDG-SW3 osteocyte cells respond to mechanical loading by changing shape and appearing to lose their dendritic processes for the first 3 hrs post mechanical loading. However, they appear to have recovered their dendritic osteocyte-like projections by 24 and 48 hrs post mechanical loading. The dendritic osteocyte-like projections in vivo are not static and are known to move around within
the canicular network [553] and the observed change in osteocyte morphology may reflect a similar process. Another possible explanation is that the osteocyte-like dendritic processes have a protective chemotactic-like response to mechanical loading which causes the processes to retract, but once the mechanical loading stops they are able to reform their osteocyte-like dendritic processes again.

This chapter shows that IDG-SW3 cells in 3D collagen type I gels respond to mechanical loading consistent with reported changes in DMP-1, LRP-5, sclerostin, VEGF, IL-6, RANKL and OPG responses to mechanical loading in osteocytes both in vivo and in vitro [45, 296, 302, 303, 305, 413, 414, 421, 554].

DMP-1 expression increased in loaded samples post-loading. The DMP-1 mRNA expression results described in this chapter are similar to those from other groups who have used different mechanical loading methods. Mechanical loading of the mouse ulna in vivo has shown a 2- to 3-fold increase in DMP-1 expression following a single load (1500 με, 30 s, 2 Hz) [413], with its expression first increased at 1 hrs post mechanical loading and increasing further up to 24 hrs post loading [413]. Both the endogenous DMP-1 genes as well as the 8-kb DMP-1-GFP were activated post mechanical loading [42]. The results in this chapter are in agreement with these findings as they show a significant increase in DMP-1 mRNA expression at 0.5 hrs post mechanical loading (Figure 6.5A). Gluhak-Heinrich et al. showed that in the osteocytes in the alveolar bone in the mouse tooth movement mechanical loading model, there was a 2-fold increase in DMP-1 mRNA at 6 hrs post mechanical loading at both the bone formation and bone resorption sites. At 4
days post mechanical loading, there was a 3.7-fold increase in the bone formation site and a 3.5 fold increase in the resorption site [294].

The results in this chapter are in agreement with the only published study on LRP-5 expression post-loading and showed 0.5-fold up-regulation of LRP-5 mRNA gene expression at 48 hrs post mechanical loading when compared to control at the same time point (Figure 6.5B). In osteocytes, there is only one published study on the effect of mechanical loading on LRP-5 expression [460]. This study used mice with a conditional loss of function mutation in LRP-5 driven by the SOST promoter in osteocytes and showed that these mice had a 4.6 fold reduction in Young’s modulus following ulna loading. Lack of LRP-5 also abolished mineralising surface, mineral apposition rate and bone formation rate in response to mechanical loading (Ulnar loading, 360 cycles, 3 mins, 2 Hz, peak force 2.65 N) in this mouse model [460]. Therefore these results suggest that LRP-5 is not only an important factor in mechanical loading but is necessary for normal osteocyte response to mechanical loading. Previous studies have also shown that LRP-5 is a co-receptor of Wnt signalling [555], and is an important factor for bone development and maintenance [556].

The sclerostin mRNA expression results are similar to previously published in vivo and in vitro results, and show that it is down-regulated post-mechanical loading [290, 382, 491, 507, 508]. Sclerostin is known to play a key role in the response of osteocytes to mechanical loading [290, 380, 382, 491, 508]. In vivo studies have found that this down-regulation of sclerostin following mechanical loading in osteocytes is a necessary step during the mechanotransduction cascade by activating
Wnt signalling and directing osteogenesis [290]. Dynamic axial loading (18,000 microstrain, 0.1 s trapezoidal-shaped pulse, 7 mins/day) of C57BL/6 mice tibiae showed a decrease in sclerostin-positive osteocytes coincident with new bone formation in the proximal but not in the distal region of the cortical bone of the tibial shaft [508]. This suggests that the down-regulation of sclerostin in osteocytes post mechanical loading is localised to regions where new bone formation is stimulated [508]. Other studies have investigated the mechano-regulation of sclerostin following ulnar loading or hindlimb unloading (tail suspension tests). Sclerostin protein levels were greatly reduced by ulnar loading (∼2200 microstrain; 2 Hz; 360 cycles) and regions of the ulnar cortex subjected to greatest strains resulted in a greater reduction of sclerostin-positive osteocytes, than regions where lower strains were applied. Hindlimb unloading on the other hand resulted in increased sclerostin expression in the tibia [382]. In vitro, mechanical loading (pulsating fluid flow, 5 Hz pulse frequency) has also revealed that sclerostin expression in reduced in the osteoblast SaOS2 cells [506] and the mature osteoblasts UMR 106.01 cells [491], highlighting that sclerostin is reduced following mechanical loading in the osteoblasts as well as osteocytes.

A recent review has presented a model which suggests that sclerostin is a major mediator for combining not only mechanical, but also local and hormonal signals which subsequently control the bone remodelling process [380]. The review hypothesises that sclerostin modulates differential RANKL and OPG production, resulting in modulation of bone formation and resorption [380]. The sclerostin, RANKL and OPG mRNA expression results presented in this chapter are in agreement with this hypothesis, as following loading there was a significant
reduction in sclerostin mRNA expression after 3hrs, and a significant increase in RANKL mRNA expression after 24 hrs, indicating that sclerostin could be modulating RANKL expression in these experiments.

The results in this chapter show IL-6 protein expression increased ~2.5-fold at 3 hrs, and ~3.5-fold at 24 and 48 hrs post mechanical loading (Figure 6.7A). These data are in agreement with Bakker et al. 2014 who demonstrated that pulsating fluid flow significantly increased IL-6 production by MLO-Y4 osteocytes [304]. IL-6 however, has long been shown to be up-regulated following mechanical loading using numerous methods in other cells types including osteoblasts [554], skeletal muscle [557], chondrocytes [558], tendon fibroblasts [559] and epithelial cells [560].

The results presented in this chapter showed that VEGF protein expression increased ~3-fold at 0.5 hrs and ~1.8-fold at 48 hrs post mechanical loading. Only one previous study has also shown that VEGF was mechano-regulated in osteocytes. Juffer et al. 2012 has shown that the MLO-Y4 osteocytes subjected to pulsating fluid flow increases both VEGF mRNA expression (1.8-2.5 fold) and protein release (2.0-2.9 fold) [305]. Little else is know about VEGF expression in osteocytes following mechanical loading. VEGF is also up-regulated post mechanical loading in chondrocytes [436, 561], tendons [434], osteoblasts [435] and vascular endothelia cells [524].

RANKL mRNA expression results showed a trend towards being increased in loaded cells at all time points with a significant ~1.5-fold difference at 24 hrs post mechanical loading (Figure 6.6A). You et al. 2008 have shown similar results in that
they also showed that RANKL is increased in MLO-Y4 osteocytes following mechanical loading [45]. Furthermore, IHC results in this chapter suggest that there were no apparent changes in the amount of intracellular RANKL protein expression in both the control and loaded gels. However there does appear to be an accumulation of RANKL found within the collagen gel in the loaded gels when compared to the control. Perhaps this is due to the osteocytes releasing more soluble RANKL following mechanical loading. *In vivo*, following mechanical loading the osteocyte could release RANKL into the circulation (via dendritic processes) which could be sensed by the osteoclast precursors enabling them to differentiate into osteoclasts.

OPG mRNA expression results indicated that at 0.5 hrs post mechanical loading there was a significant ~3-fold increase in the loaded cells when compared to the control at the same time point (Figure 6.6B). Previous studies have showed an increase in the RANKL:OPG ratio [303, 320, 421] apart from Li *et al* 2012 where there was a decrease in the RANKL:OPG ratio [302] with mechanical loading. In the past, RANKL has widely been accepted to be an osteoblast specific factor [428, 510] essential for osteoclastogenesis, however two Nature publications in 2011 showed that osteocytes probably produce more RANKL than osteoblasts [20, 21]. It is interesting to speculate that maybe this higher concentration of RANKL in the osteocytes is achieved post mechanical loading as RANKL is known to be increased post mechanical loading [45] and the osteocyte is thought to be the mechano-sensitive cell in bone [31, 511-513].
Chapter 6
The effect of mechanical loading on IDG-SW3 osteocyte cells

The results in this chapter showed low or undetectable concentrations of PGE$_2$ (Table 6.2) and do not support other studies for PGE$_2$ release following mechanical loading. Previous studies in relation to PGE$_2$ secretion in osteocytes following mechanical stimulation (using various methods) have shown *in vitro* that PGE$_2$ secretion was increased at various time points (Chapter 1, Section 1.8) [307, 527, 536, 562, 563]. However interestingly in relation to fluid flow stimulation, the changes in PGE$_2$ secretion are related to the amount and rate of fluid flow used [308, 539, 541]. Currently, there are no published reports of PGE$_2$ release from IDG-SW3 cells, either before or after mechanical loading, but there are extensive published studies on PGE$_2$ release following mechanical loading in osteoblasts and osteocytes.

![Figure 6.9: Factors that are up-regulated and down-regulated post mechanical loading in IDG-SW3 osteocyte cells in 3D collagen type I gels.](image)

In general, apart from PGE$_2$, all the results in this chapter are in agreement with previously published studies of the effect of mechanical loading on osteocytes. Therefore, the results presented in this chapter show that this novel plate based mechanical loading model is suitable for *in vitro* mechanical loading studies with osteocytes in 3D type I collagen gels maintained in mineralising conditions. Furthermore, the mechanical loading results are in agreement with other *in vivo* and
in vitro studies using different mechanical loading techniques, further confirming this as a suitable model for osteocyte mechanical loading studies. Furthermore, the results presented in this chapter further build on the MLO-Y4/MC-3T3 co-culture model using the same mechanical loading device and regime developed in Cardiff University [280, 564]. This co-culture model also showed regulation of numerous factors following mechanical loading in the MLO-Y4 cells, which supports the finding presented in this chapter highlighting a response of osteocyte cells to this mechanical loading regime.

The results also highlight that these IDG-SW3 cells respond to mechanical loading at both mRNA and protein levels, with the modulated factors being known mechano-regulatory factors, inflammatory mediators, angiogenic factors and factors essential for osteoclastogenesis. The results also suggest that the IDG-SW3 cells, due to their ability to differentiate to a mature osteocyte population are a more advantageous osteocyte model to study mechanical loading in vitro to the previously used MLO-Y4s for mechanical loading studies. This is mainly because the IDG-SW3 cells mineralise in culture and express sclerostin, both of which are limitations to the MLO-Y4 cells. The mineralising collagen gel also provides an in vitro environment for these cells that mimics osteocytes in the in vivo environment, further highlighting the plate based loading method for osteocytes in collagen gels as an excellent model for these mechanical loading studies.
7 General Discussion
7.1 General Discussion

Osteocytes have recently been described as ‘amazing’ cells and ‘master orchestrators of bone’ [31, 565]. Whereas historically, bone research has focussed on the function of osteoblasts and osteoclasts in maintaining adequate bone density and microstructure, more recently osteocyte-related research has increased dramatically. Although our knowledge of osteocyte function has increased significantly in the last decade, we still do not fully understand the role of osteocytes in normal and diseased bone. Primary osteocytes are difficult to isolate and are not available in large numbers for in vitro research. New model systems are therefore needed, especially using 3D environments, to enable meaningful research studies with these cells. Such models would enable the development of new treatment strategies targeting this previously under investigated, but pivotal cell.

In this thesis the differentiation of mouse and human osteoblasts to osteocytes in 3D, in both non-mineralising and mineralising environments, were examined. The work also investigated whether the addition of external factors to such culture systems would accelerate the differentiation of these cells. In addition, the effect of physiologically relevant mechanical loading on mineralising osteocytes in 3D cultures was examined.
7.1.1 Validation of the 3D model

7.1.1.1 Osteoblast to osteocyte differentiation

The 3D collagen type I gel method developed in this thesis allows studies of osteoblast to osteocyte differentiation in an environment more similar to that found in vivo. The method also allows the generation of osteocytes in large enough quantities to undertake meaningful research. When the work was started, there were very few reports of similar studies [43, 103, 246, 265, 279, 367, 368, 370], although recently others have reported differentiation of osteoblasts in 3D gels [108, 276, 278]. The fate of an osteoblast in vivo, once it has formed new bone, can be either to undergo apoptosis, to change to a bone lining cell, or to differentiate to an osteocyte [29, 31, 495]. It is not fully understood what regulates these processes [29]. Once committed to becoming an osteocyte there are three stages of osteocyte differentiation resulting in early (osteoid), mineralising (intermediate) and mature osteocytes (Figure 1.4). The model developed in this thesis allows studies of cells at each stage of this differentiation process and these three stages would be represented by osteoblasts which have been in gels for ~3, 7 and 15 days respectively in mineralising medium.

The work presented here has revealed that in a 3D mineralising environment, osteoblasts differentiate to sclerostin and FGF-23 expressing, mature osteocytes. However, mature osteocyte markers were not expressed in cells in non-mineralising medium over a two week period, but it is not known if they would differentiate further if kept for longer in these conditions. Regardless of this, cells in non-mineralising medium over two weeks expressed early and mineralising osteocyte markers and are thus representative of osteoid or mineralising osteocytes.
Furthermore, the methods developed were shown to be reproducible with broadly similar results obtained in mouse cell lines and human primary cells, as well as between experiments and these are summarised in Table 7.1 below.

<table>
<thead>
<tr>
<th>Cell number</th>
<th>MC-3T3 basal medium</th>
<th>MC-3T3 min Medium</th>
<th>hOBs basal medium</th>
<th>hOBs min medium</th>
<th>IDG-SW3 medium</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell viability</td>
<td>↑ with time</td>
<td>↑ with time</td>
<td>↑ with time</td>
<td>↑ with time</td>
<td>NM with time</td>
</tr>
<tr>
<td>E11 expression</td>
<td>&gt;84 %</td>
<td>&gt; 86 %</td>
<td>&gt;92 %</td>
<td>&gt;93 %</td>
<td>&gt; 89 %</td>
</tr>
<tr>
<td>DMP-1 expression</td>
<td>NM with time</td>
<td>NM with time</td>
<td>NM with time</td>
<td>↑ day 15</td>
<td>NM with time</td>
</tr>
<tr>
<td>Cx43 expression</td>
<td>NM with time</td>
<td>NM with time</td>
<td>NI</td>
<td>NI</td>
<td>NI</td>
</tr>
<tr>
<td>MEPE expression</td>
<td>Detected but NQ (day 15)</td>
<td>Detected but NQ (day 15)</td>
<td>NI</td>
<td>NI</td>
<td>↑ day 1 at 37°C</td>
</tr>
<tr>
<td>PHEX expression</td>
<td>Detected but NQ (day 15)</td>
<td>Detected but NQ (day 15)</td>
<td>NI</td>
<td>NI</td>
<td>↑ day 10, 17 at 37°C</td>
</tr>
<tr>
<td>Sclerostin expression</td>
<td>ND</td>
<td>Detected but NQ (day 15)</td>
<td>NM with time</td>
<td>NM with time</td>
<td>↑ day 7 at 37°C</td>
</tr>
<tr>
<td>FGF-23 expression</td>
<td>ND</td>
<td>Detected but NQ (day 15)</td>
<td>NI</td>
<td>NI</td>
<td>NI</td>
</tr>
<tr>
<td>IL-6 secretion</td>
<td>↓ with time</td>
<td>↓ with time</td>
<td>NM with time</td>
<td>↓ with time</td>
<td>↑ day 21 at 37°C</td>
</tr>
<tr>
<td>VEGF secretion</td>
<td>NI</td>
<td>NI</td>
<td>NM with time</td>
<td>↓ with time</td>
<td>↑ day 10, 17 and 21 at 37°C</td>
</tr>
</tbody>
</table>

↑ = increased  ↓ = decreased

**Table 7.1:** Summary of cell number and viability, gene expression and protein secretion results for MC-3T3 and hOBs cells in basal or mineralising medium, or IDG-SW3 cells (maintained in mineralising medium). Abbreviations: min= mineralising medium, NM- not modulated, NI- not investigated, NQ-not quantified, ND-not detected.
7.1.1.2 Modulation of osteoblast to osteocyte differentiation

It is possible that adding modulating factors to these 3D gels would improve the model and maximise its use in *in vitro* studies. It is also possible that osteocyte differentiation could be targeted for the development of novel treatment regimes. Osteocyte number is known to decline with age, as well as in bone diseases such as glucocorticoid-induced osteoporosis [31, 50]. To enable the development of new treatments, a better understanding of the way compounds modulate osteocyte viability, differentiation and function is needed. The work described in Chapter 4 addressed this concept by investigating the effects of IGF-1, FGF-2, RA and Vit K on the differentiation of osteocytes from mouse and human osteoblasts (summarised in Table 7.2). For example, the addition of IGF-1 to MC-3T3 cells in 3D accelerated their differentiation to osteocytes in non-mineralising conditions, resulting in a similar phenotype to that observed in mineralising conditions without IGF-1. Whereas this study has used these four compounds to assess modulation of differentiation, it is possible that additional work could develop either these or other factors as potential therapies for bone diseases. Since circulating IGF-1 is reduced in ageing [566] and since osteocytes decline with age [31] it could be hypothesised that reduced serum IGF-1 concentrations are resulting in a reduced capacity for osteoblast to osteocyte differentiation. Therefore, with further research, increasing levels of appropriate factors (by supplementation) could reduce the numbers of osteocytes lost with age.
<table>
<thead>
<tr>
<th></th>
<th>IGF-1 treatment</th>
<th>FGF-2 treatment</th>
<th>RA treatment</th>
<th>Vit K treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Cell number</strong></td>
<td>↑ with time</td>
<td>↑ with time</td>
<td>NM with time</td>
<td>↑ with time</td>
</tr>
<tr>
<td><strong>Cell viability</strong></td>
<td>&gt;92%</td>
<td>&gt; 89 %</td>
<td>&gt;80 %</td>
<td>&gt;87 %</td>
</tr>
<tr>
<td><strong>E11 expression</strong></td>
<td>NM with time</td>
<td>NM with time</td>
<td>NM with time</td>
<td>NM with time</td>
</tr>
<tr>
<td><strong>DMP-1 expression</strong></td>
<td>↑ day 7</td>
<td>NM with time</td>
<td>NM with time</td>
<td>NM with time</td>
</tr>
<tr>
<td><strong>Cx43 expression</strong></td>
<td>NM with time</td>
<td>NM with time</td>
<td>↑ day 15</td>
<td>NM with time</td>
</tr>
<tr>
<td><strong>LRP-5 expression</strong></td>
<td>↓ day 15</td>
<td>↑ day 11 and day 15</td>
<td>↑ day 11</td>
<td>NM with time</td>
</tr>
<tr>
<td><strong>RANKL expression</strong></td>
<td>↑ day 11 and day 15</td>
<td>↑ day 11 and day 15</td>
<td>↑ day 11 and day 15</td>
<td>NM with time</td>
</tr>
<tr>
<td><strong>FGF-23 expression</strong></td>
<td>↑ day 15</td>
<td>↑ day 11 and day 15</td>
<td>↑ day 11 and day 15</td>
<td>NM with time</td>
</tr>
<tr>
<td><strong>sclerostin expression</strong></td>
<td>Detected from day 7</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td><strong>IL-6 secretion</strong></td>
<td>↓ day 1, 3, 7, 11 and 15</td>
<td>ND</td>
<td>↓ day 3 and 7</td>
<td>↓ day 1 and 3</td>
</tr>
<tr>
<td><strong>VEGF secretion</strong></td>
<td>NM with time</td>
<td>NM with time</td>
<td>↑ day 3, 7 and 15</td>
<td>↑ day 7</td>
</tr>
<tr>
<td><strong>FGF-23 secretion</strong></td>
<td>↑ day 11 and day 15</td>
<td>NM with time</td>
<td>NM with time</td>
<td>NM with time</td>
</tr>
</tbody>
</table>

Table 7.2: Summary of cell number and viability, gene expression and protein secretion results for MC-3T3 cells following treatment with IGF-1, FGF-2, RA or Vit K over a 15 day time course. All results presented are with respect to time, when compared to relevant control at the same time point. Abbreviations: NM- not modulated, ND-not detected

7.1.1.3 Mechanical loading of osteocytes in 3D gels

Novel methods of applying mechanical load to cells in 3D collagen gels have been developed by colleagues at Cardiff University and have previously been used in studies with the ML-OY4 early osteocytes and MLO-Y4/MC3T3E1
osteocyte/osteoblast co-cultures [280, 564]. The use of the MLO-Y4 cell line in these studies was a limitation of work as it is known that these cells do not express sclerostin [82, 280]. The method described in this thesis builds on these studies with ML-OY4 early osteocytes and MLO-Y4/MC3T3E1 osteocyte/osteoblast co-cultures as it broadens the application of these novel loading techniques by allowing IDG-SW3 osteoblasts to be differentiated to sclerostin expressing osteocytes in 3D prior to application of mechanical loading. These techniques for differentiation and mechanical loading have been validated with the IDG-SW3 cell line and could be extended to human primary cells.

The results post-loading show that sclerostin expression was reduced following mechanical loading and this is in agreement with published *in vivo* work indicating that the model provides realistic insights in to how osteocytes respond to mechanical stimuli. Furthermore, the up-regulation of DMP-1, LRP-5, IL-6, VEGF, RANKL post loading are also in agreement with previous *in vivo* and *in vitro* work [275, 294, 296, 305, 413, 459, 554]. It is now well established that following mechanical loading, release of substances such as RANKL and sclerostin are modulated in osteocytes, and that these in turn control the bone remodelling process [380].

The loading regime used in this thesis was 5 mins, 10 Hz, 2.5 N with a view to stimulating an appropriate physiological response of 4000-4500 microstrain. The measurement of strains in 3D cultures in the silicone plates in order to calibrate the system was undertaken by Dr M Vazquez as part of her PhD studies using a Dantec Dynamics Digital Image Correlation (DIC) system. DIC compared two digital images (a reference state and a deformed state) of two different mechanical states of
a particular object [280, 564]. A speckle pattern was applied to the surface of the gels and this followed the strains of the object to enable measurement of the displacement that occurred between the reference and the deformed states [280, 564]. However, there were some problems with this work (e.g. speckles too big for the surface area to be analysed by the cameras), and the strain measurements were done on the surfaces of the empty plates. It is possible that the strain within the gels would be slightly different. However, DIC on empty plates validated strains of 4000-4500 microstrain in the majority of wells of the loading plate when a 2.5 N force was applied.

7.1.2 Limitations

The characterisation of osteoblast to osteocyte differentiation described in this thesis was undertaken in relation to cell number and viability, morphology, gene expression, and protein secretion. The limitations identified for each of these will be discussed below.

7.1.2.1 Cell seeding densities

It is difficult to know how many osteoblasts need to be seeded into collagen gels to enable differentiation to osteocytes. Although initial experiments with varying seeding densities were undertaken with MC-3T3 cells, it is clear from the work presented that different cells proliferate at different rates in gels when seeded at the same density – e.g. $7 \times 10^5$ cells/ml gel initially seeded for MC-3T3 and IDG-SW3 cells whereas these numbers were ~3-fold higher or remained unchanged respectively on day 15. This difference could be inherent in the cells or could partly be due to the different seeding densities and that the cells proliferate until they reach
an optimum density in the gels enabling differentiation. It is also possible that not all of the cells within the gels differentiate to osteocytes, and that some remain as proliferating osteoblasts. Furthermore hOB cells were seeded at a much lower density ($8 \times 10^4$ cells/ml gel) as others in the group had shown that hOBs in gels at higher densities results in gel shrinkage before the completion of the experiment. In addition, if new groups were developing this method it is crucial that the cells are dispersed evenly throughout the gel at the time of set up. Therefore, further work needs to be undertaken to optimise hOBs cells seeding density to avoid gel shrinkage but to increase the RNA yield from these cells at earlier time points.

7.1.2.2 Cell viability
Collagenase was used to digest the collagen gels at specific time points to expose the cells so that they could be counted for assessment of cell number and viability. It is possible that some of the cells had been damaged by the collagenase although every attempt was made to limit this by carefully controlling (limiting) the time gels were incubated in collagenase. This was further complicated in that since gels increased in stiffness with time, the time taken to digest in collagenase also increased.

7.1.2.3 Gene expression
It is also possible that collagenase digestion of gels modulated mRNA expression in cells as all the expression studies were undertaken on cells which had been released from the gels with collagenase. Gene expression in collagenase digested and non-collagenase digested cells were, however, compared at the start of this study and no differences were observed. Furthermore all the cells were centrifuged following collagenase digestion and this could have further modulated gene expression. hOBs
were seeded at a relative low density at the start of experiments to ensure that gels did not shrink. This unfortunately led to very low yields of RNA prior to day 7 and this resulted in being able to undertake very few mRNA expression studies prior to this time point.

7.1.2.4 Mechanical loading Plate

The mechanical loading plate is made from silicone whereas cells are usually cultured on tissue culture plastic. How this affects the cells could be a limitation of the work as the results show that IDG-SW3 cell number was increased in the silicone plate when compared to plastic. It is possible that contaminants from the materials used to make the silicone plates are leaching out into the cell culture medium and affecting cell proliferation and function. Further work to identify these contaminants was outside the remit of this thesis.

7.1.2.5 Mechanical loading apparatus

Whether each gel in the silicone loading plate is exposed to the same amount of mechanical loading (mechanical loading from the Bose machine or fluid shear stress from the media on top of the gels) could be another limitation of this loading protocol. However, choosing replicate wells in any one experiment from different areas of the plate was done with a view to eliminating this as an issue. Loading the IDG-SW3 cells on day 10 is also now questionable as sclerostin expression was highest in these cells on day 7 and beginning to decrease on day 10 in plastic plates (as described in Chapter 5). This could explain why significant differences in sclerostin expression were not observed on all time points investigated post mechanical loading. Analysing responses to loading over a two day period and not
for longer is a further limitation, as this study has only investigated osteocyte mechano-responses in the short term whereas others have investigated the effects for longer post-loading.

7.1.3 Future studies

7.1.3.1 Basal and mineralising medium
To investigate what happens to the cells after the two week culture period used in this thesis, future work could culture these cells for longer in the 3D gels to further characterise there phenotype and function at later time points.

7.1.3.2 Factors to influence differentiation
The discovery of IGF-1 increasing sclerostin and FGF-23 warrants further investigation. A better understanding of the pathways involved could be achieved by using IGF-1 antagonists, and this improved understanding has the potential to generate new therapeutic targets for bone disease utilizing IGF-1.

7.1.3.3 IDG-SW3 cells
The IDG-SW3 cells are an excellent model to investigate osteocyte biology in vitro. However, since IDG-SW3 cells seeded in gels at the same initial density \((7 \times 10^5\) cells/ml gel) as MC-3T3 cells looked sparse in H&E stained sections on day 15 future work could increase the seeding density with this cell line to see if that changes the differentiation potential of these cells. The same increase in seeding density could be applied to these cells for mechanical loading studies. Furthermore, the results presented in this thesis suggest that the morphology of these cells changes
following mechanical loading, although the mechanisms are not known. This is an interesting observation that warrants further investigation. This could be further investigated using live cell imaging using fluorescent actin staining during and post mechanical loading which would highlight changes in osteocyte-like dendritic processes. To fully characterise gene expression following mechanical loading gene arrays could be undertaken with samples pre- and post-loading. These could uncover novel pathways that could potentially lead to new therapeutic options for the ever increasing incidence of bone diseases in our ageing population.

### 7.1.3.4 Co-culturing cells

It has recently proposed that 3D collagen gels containing MLO-Y4 cells can be used to develop osteoblast/osteocyte co-culture models [280, 564]. Future work could co-culture osteocytes differentiated in 3D from osteoblasts with a layer of other cells *e.g.* chondrocyte, muscle cells, osteoclasts, on top of the gels to investigate cross-talk between these differing cell types. Furthermore, using the plate based mechanical loading system, mechanical loading could be applied to these co-cultures to further inform on cell-cell communication. Equally, one could investigate if the layer of cells on top of the co-culture model modified the differentiation of osteoblasts to osteocytes in the 3D culture. Understanding how one cell type influences another could lead to novel therapeutics not only in the bone field but also in other areas such as osteoarthritis and sarcopenia.
8 Bibliography


Chapter 8


Chapter 8


303


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