<table>
<thead>
<tr>
<th>Title</th>
<th>The mechanical environment of the stem cell niche in bone marrow</th>
</tr>
</thead>
<tbody>
<tr>
<td>Author(s)</td>
<td>Birmingham, Evelyn</td>
</tr>
<tr>
<td>Publication Date</td>
<td>2014-06-13</td>
</tr>
<tr>
<td>Item record</td>
<td><a href="http://hdl.handle.net/10379/4422">http://hdl.handle.net/10379/4422</a></td>
</tr>
</tbody>
</table>

Downloaded 2020-09-11T05:55:55Z

Some rights reserved. For more information, please see the item record link above.
The Mechanical Environment of the Stem Cell Niche in Bone Marrow

Evelyn Birmingham

Supervisors: Prof. Peter McHugh and Dr. Laoise McNamara

A thesis submitted to the National University of Ireland as fulfilment of the requirements for the Degree of Doctor of Philosophy, Biomedical Engineering, National University of Ireland, Galway.

June 2014
Contents

Abstract .................................................................................................................. vi
Acknowledgements ............................................................................................. ix
List of Publications .............................................................................................. xi

1. Introduction ..................................................................................................... 1
   1.1. Chapter Background .................................................................................. 1
   1.2. Thesis Motivation and Rationale .............................................................. 3
   1.3. Hypotheses and Objectives ...................................................................... 5
       Hypothesis 1 ............................................................................................... 6
       Hypothesis 2 ............................................................................................... 7
       Hypothesis 3 ............................................................................................... 7
       Hypothesis 4 ............................................................................................... 8
       Hypothesis 5 ............................................................................................... 9
   1.4. Thesis Structure ...................................................................................... 10
   1.5. Conclusions ............................................................................................ 12

2. Background ..................................................................................................... 13
   2.1. Chapter Background ................................................................................ 13
   2.2. Bone ......................................................................................................... 13
       2.2.1. Function .......................................................................................... 13
       2.2.2. Composition .................................................................................... 14
       2.2.3. Structure ........................................................................................ 15
       2.2.4. Bone cells ....................................................................................... 18
       2.2.5. Formation ....................................................................................... 23
       2.2.6. Modelling and remodelling ............................................................. 24
2.2.7. Osteoporosis ................................................................. 25
2.2.8. Mechanical adaptation of bone ....................................... 26
2.2.9. Trabecular bone mechanics ........................................... 28
2.3. Bone marrow .................................................................... 29
2.3.1. Bone marrow mechanics ................................................ 30
2.4. Mesenchymal stem cells .................................................... 31
2.4.1. Osteogenic Differentiation ............................................... 34
2.4.2. Chondrogenic Differentiation .......................................... 35
2.4.3. Adipogenic Differentiation .............................................. 35
2.5. Stem Cell Niche ............................................................... 36
2.5.1. The mechanical environment of the stem cell niche .......... 37
2.6. Computational Modelling of Trabecular Bone ..................... 38
2.7. Conclusions .................................................................... 41

3. Theory .................................................................................. 43
3.1. Chapter Background ......................................................... 43
3.2. Notation ........................................................................... 43
3.3. Finite Element Analysis of Solid Structures ....................... 45
3.3.1. Kinematics and Kinetics ................................................ 45
3.3.2. Constitutive Laws ........................................................ 51
3.3.3. Numerical Implementation for the deformation of Solids ... 52
3.4. Computational Fluid Dynamics ......................................... 61
3.4.1. Fluid Mechanics .......................................................... 61
3.4.2. Constitutive Laws ........................................................ 63
3.4.3. Numerical implementation of Computational Fluid Dynamics 64
3.5. Relevant Implementations .................................................. 68
3.5.1. Fluid Structure Interaction ............................................ 68

4. Osteogenic differentiation of mesenchymal stem cells is regulated by osteocyte and osteoblast cells in a simplified bone niche ................................................................. 70
4.1. Chapter Background ........................................................ 70
4.2. Introduction ................................................................................................. 71
4.3. Methods ....................................................................................................... 73
  4.3.1. BALB/c MSC: Isolation and Characterisation ........................................... 73
  4.3.2. MLO-Y4 and MC3T3-E1 cell cultures ....................................................... 76
  4.3.3. Conditioned media experiment (CM) ......................................................... 77
  4.3.4. Co-culture experiment (CC) .................................................................... 78
  4.3.5. Alkaline Phosphatase ............................................................................. 80
  4.3.6. DNA Content ......................................................................................... 81
  4.3.7. Mineralization ........................................................................................ 82
  4.3.8. Statistical Analysis .................................................................................. 82
4.4. Results ......................................................................................................... 83
  4.4.1. Characterisation of BALB/c MSCs ............................................................ 83
  4.4.2. Are osteocytes the regulatory cells that govern osteogenic differentiation of MSCs? ..................................................................................................................... 85
  4.4.3. Are osteoblasts the regulatory cells that govern osteogenic differentiation of MSCs? ..................................................................................................................... 92
  4.4.4. Does the degree of osteogenic differentiation of MSCs differ between osteocyte and osteoblast signalling? ................................................................. 93
  4.4.5. Do osteoblasts and osteocytes work in conjunction to regulate MSC differentiation? ..................................................................................................................... 95
4.5. Discussion ................................................................................................... 97
4.6. Conclusions ................................................................................................ 105

5. Computational modelling of the mechanics of trabecular bone and marrow using fluid-structure interaction techniques .................................................................................. 107
  5.1. Chapter Background .................................................................................... 107
  5.2. Introduction .................................................................................................. 108
  5.3. Materials and Methods .............................................................................. 111
    5.3.1. Validation study: Predicting permeability ............................................... 111
    5.3.2. FSI model formulation ......................................................................... 114
5.3.3. The mechanical environment of bone marrow during osteoporosis ......................................................... 119
5.4. Results ................................................................................................................................................... 123
  5.4.1. Validation study: Predicting permeability .......................................................................................... 123
  5.4.2. FSI model formulation ..................................................................................................................... 125
  5.4.3. The mechanical environment of bone marrow during osteoporosis ................................................. 127
5.5. Discussion .............................................................................................................................................. 131
5.6. Conclusions ........................................................................................................................................... 141

6. An experimental and computational investigation of bone formation in mechanically loaded trabecular bone explants... 144
  6.1. Chapter Background .......................................................................................................................... 144
  6.2. Introduction ......................................................................................................................................... 145
  6.3. Methods ............................................................................................................................................. 148
     6.3.1. Harvest of bone explants .............................................................................................................. 148
     6.3.2. Compression Bioreactor ............................................................................................................. 149
     6.3.3. Histomorphometry analysis ........................................................................................................ 152
     6.3.4. FSI modelling .............................................................................................................................. 153
     6.3.5. Statistical Analysis ...................................................................................................................... 155
  6.4. Results ................................................................................................................................................. 155
     6.4.1. Trabecular bone histomorphometry ............................................................................................ 155
     6.4.2. FSI modelling of trabecular bone .............................................................................................. 157
     6.4.3. Correlation between mechanical loading and bone growth ...................................................... 162
  6.5. Discussion ........................................................................................................................................... 166
  6.6. Conclusions ......................................................................................................................................... 171

7. Mechanical stimulation of bone marrow in situ induces bone formation in trabecular explants ......................... 174
  7.1. Chapter Background .......................................................................................................................... 174
  7.2. Introduction ......................................................................................................................................... 176
  7.3. Materials and Methods ...................................................................................................................... 179
Contents

7.3.1. LMHF Bioreactor Design ......................................................... 179
7.3.2. Bone tissue culture ................................................................. 180
7.3.3. LMHF experimental approach ................................................... 181
7.3.4. Bone formation labelling .......................................................... 182
7.3.5. μ-CT Analysis for Trabecular Microarchitecture ....................... 182
7.3.6. Computational Modelling .......................................................... 185
7.3.7. Statistical Analysis ................................................................. 187
7.4. Results ....................................................................................... 188
7.4.1. Fluorochrome Labelling ............................................................ 188
7.4.2. μ-CT parameters ................................................................. 188
7.4.3. CFD models ........................................................................... 194
7.4.4. Correlation between μ-CT parameters and shear stress .......... 196
7.5. Discussion .................................................................................. 198
7.6. Conclusions ............................................................................... 205

8. Summary and Conclusions .............................................................. 207
8.1. Chapter Background ................................................................. 207
8.2. Thesis Summary ......................................................................... 207
8.3. Hypotheses Results ..................................................................... 214
8.4. Future Work ............................................................................. 217
8.5. Conclusions .............................................................................. 222

9. References .................................................................................... 224
Abstract

Understanding how bone marrow mesenchymal stem cells (MSCs) contribute to new bone formation and remodelling in vivo is of principal importance for informing the development of effective bone tissue engineering strategies in vitro. However, the precise stimuli for osteogenic differentiation of MSCs in vivo have not been fully established. The work presented in this thesis uses a combination of experimental and computational modelling approaches to investigate the in vivo environment of the stem cell niche in bone marrow, with a specific goal of identifying important biochemical and mechanical cues for osteogenic differentiation of MSCs.

Support cells within the niche are examined for their roles in osteogenic differentiation. Specifically osteocytes and osteoblasts are examined due to their established role as regulatory cells in bone. Osteocytes are found to be more dominant than osteoblasts. However, when cultured together, a synergistic relationship is found to exist between them, for stimulating the
osteogenic differentiation of MSCs. Fluid structure interaction (FSI) models are used to determine whether MSCs can be directly stimulated by mechanical cues within the bone marrow. Models predict that the shear stress generated due to physiological loading is within the stimulatory range (maximum values range from 0.025 – 0.25 Pa). Additionally, it is found that the onset of osteoporosis can alter the shear stress within the marrow. Explanted samples of trabecular bone and marrow are physiologically compressed and are found to have greater osteogenic activity, as verified by bone histomorphometry, compared to static samples. FSI models demonstrate that bone strain, not marrow shear stress, is likely the driving mechanical signal during compression. To focus on shear stress in the marrow, low-magnitude high-frequency vibration loading is used as this induces minimal bone strain while generating marrow shear stress. µ-CT analysis shows strong bone formation and remodelling in vibration samples compared to static samples. Computational models reveal a significant relationship between this formation and remodelling and shear stress in the marrow.

Together the results of this PhD thesis demonstrate that: (1) osteocytes and osteoblasts can stimulate osteogenic differentiation of MSCs. (2) Shear stress of sufficient magnitude to stimulate the osteogenic differentiation of MSCs,
is generated during compression, but this can be altered in osteoporotic bone. (3) *In vivo* responses of bone to compression loading are replicated in explanted samples; however, models indicate that bone strain is the dominant signal. Finally, (4) explanted samples exposed to vibrational loading experience more marrow shear stress than in compression loading, and the magnitude of the shear stress has a causal role in the formation of bone and improvement in bone architecture parameters.
Acknowledgements

I would like to extend my warmest thanks to Prof. Peter McHugh for all his patience, guidance and sharing his technical expertise throughout the past few years. I also owe a great deal of gratitude to Dr. Laoise McNamara for all the advice throughout the course of this work. Thank you both for this opportunity. I thoroughly enjoyed working with both research groups. To Prof. Glen Niebur, I would like to thank him for sharing his enthusiasm for the topics in the thesis and providing guidance throughout the Ph.D. In particular, I would like to thank him for providing me with the opportunity to perform research at Notre Dame.

I acknowledge the Irish Research Council (Postgraduate Scholarship Scheme), Science Foundation Ireland (Short Term Travel Fellowship) and the Orthopaedic Research Society (Collaborative Exchange Award) for funding the research in this thesis. I was lucky to be surrounded with expert technical help throughout this study. Thanks to David Connolly, Liam Brennan, William Kelly, Peter Owens, Noel Harrison, Georgina Shaw, Bill Archer
(Notre Dame) and Peter O'Reilly (TCD) and to Jane Bowman and Sharon Gilmartin for all the help throughout the years.

I met some great people along the way who made the past few years much more than the work presented here. To all the original and new members of the Skylab, thanks for all the great times. To Stefaan, Heather, Tom, Meadbh, Eimear, Ciaran, Emer, Nicola, Riona, Muriel, Ted and Matt, thank you for all the advice and insightful discussions on the work in this thesis. In particular, I owe a great deal of gratitude to Claire and James for helping me countless times with all aspects of this work and for all the laughs when they were needed. Thanks to Paul and Aoife and all my friends, Annette, Ceire, Lorraine, Grainne, Marian, Tessa, Muireann, Caroline, Niamh and everyone at Dunmore MacHales Ladies.

Cathal, your patience is never-ending, without your help this thesis would never have been possible. To Mam and Dad, this work is down to the unwavering support and encouragement that I constantly receive from you both. This help has been essential throughout all my years in education and this thesis is dedicated to you both.
List of Publications

The work presented in this thesis has appeared in the following publications:


The following publications arising from work in this thesis are in preparation:

**Chapter 6:** Birmingham E, Kreipke T.C., Dolan E.B., Coughlin T.R., McNamara LM, Niebur GL, McHugh PE. An experimental and

1. Introduction

1.1. Chapter Background

Bone is a type of connective tissue that plays specialised supportive and protective roles within the body. It is a dynamic material that lies within a continual state of remodelling in order to conform to its functional load bearing role. Osteoporosis is a disease of the skeletal system characterised by low bone mass, deterioration in the microarchitecture of bone, and increased bone fragility. The disease can lead to bone fractures under normal loading conditions which would not occur in healthy bone. Drugs such as bisphosphonates are the commonly prescribed to prevent osteoporotic bone loss but only prevent fractures in half of sufferers. Increasingly, research has focused on the potential to harness the natural anabolic activity of bone in response to mechanical loading to develop novel treatment approaches that can increase bone mass and improve the microarchitecture of bone.

In order for bone tissue engineering and other treatment approaches to harness the natural response to mechanical loading a greater understanding of the cellular mechanism working to transmit the mechanical load into a
1. Introduction

cellular response is required (Gurkan and Akkus, 2008). Bone marrow mesenchymal stem cells (MSCs) are an increasingly attractive option for cell-based tissue engineering therapies due to their capacity for self-renewal and their ability to differentiate into numerous different tissue types such as bone, cartilage and fat (Dominici et al., 2006). Tissue engineering studies using MSCs have generated some success in the regeneration of tissues such as tendons and cartilage (Awad et al., 2003; Butler et al., 2010; Haleem et al., 2010). However, it is not yet clear precisely how osteogenic differentiation of MSCs can be optimised to produce bone tissue that is suitable for clinical implantation in the treatment of bone pathologies. A particular limitation of current strategies is that the bone tissue produced through in vitro tissue regeneration strategies is not adequately stiff to serve load bearing functions in large defects in the body (Dawson and Oreffo, 2008). Therefore the field of bone tissue regeneration is faced with the specific challenge to develop novel tissue regeneration approaches to produce large bone constructs for clinical applications.

Understanding how bone marrow MSCs contribute to new bone formation and remodelling is of principal importance for bone tissue engineering. However, what stimulates MSCs to undergo osteogenic differentiation in vivo remains unclear to date. In order to advance in vitro bone regeneration
approaches it is crucial to understand the natural *in vivo* cues for differentiation and how they might be manipulated to achieve significant MSC osteogenic differentiation and bone growth.

The motivating factors for this thesis are introduced in Section 1.2, outlining the current lack of knowledge in the field of bone regeneration and tissue engineering. The hypotheses of this thesis and the associated objectives are detailed in Section 1.3. Finally, the structure of this thesis is discussed in Section 1.4.

### 1.2. Thesis Motivation and Rationale

Bone marrow within the porous structure of trabecular bone is a highly specialised environment home to numerous cell types and is the origin of many of the cells and tissues in the body such as MSCs (Kuhn and Tuan, 2010). They are found in the bone marrow within what is termed the stem cell niche (Schofield, 1978). Schofield identified the niche as having three main functions (1) maintaining quiescence, (2) promoting proliferation and (3) directing differentiation. While possible biochemical and biomechanical triggers for the differentiation of MSCs have been studied *in vitro*, and have derived some understanding of the governing factors, the native environment in which the MSCs reside *in vivo* remains poorly characterised,
and as such there is little understanding of the \textit{in vivo} triggers for MSC differentiation.

The stem cell niche consists of a host of different support cells including haematopoietic progenitors and their progeny and MSCs and their progeny, including fibroblasts, endothelial cells, adipocytes, osteoblasts, and osteocytes (Kuhn and Tuan, 2010). These support cells likely play a role, through biochemical signalling, in directing the functions of the stem cell niche (Fuchs \textit{et al.}, 2004) but how exactly these cells regulate the osteogenic differentiation of MSCs is not yet understood. Osteoblasts and osteocytes are the support cells which have been proposed as the regulators of osteogenic differentiation of MSCs (Csaki \textit{et al.}, 2009; Gu \textit{et al.}, 2001; Heino \textit{et al.}, 2004),

Wolff first elucidated the role of mechanical loading in healthy bone growth and adaption in the 19th century (Wolff, 1892). Since then studies have elucidated the relationship between strain in the bone and bone adaptation. In particular removal of mineralised tissue from regions where mechanical loads are low has been demonstrated, whereas new tissue deposition and micro-architecture adaptation occur in regions subjected to repeated high mechanical strain (Burger and Klein-Nulend, 1999; Cowin, 2001; Dallas \textit{et al.}, 1993; Parfitt, 1977; Rubin and Lanyon, 1984). However, despite these studies, the specific biological mechanism that facilitates the strain response and
converts it to the osteogenic differentiation of MSCs *in vivo* remain unidentified.

### 1.3. Hypotheses and Objectives

This thesis aims to determine the biochemical and biomechanical mechanisms at work within the native stem cell niche to stimulate osteogenic differentiation. Specifically, the focus is on how neighbouring support cells within the niche influence the osteogenic differentiation of MSCs through biochemical signalling and what magnitude of stresses and strains are generated with the marrow during physiological loading. The effects of different mechanical forces (compression and low-magnitude high-frequency vibration) are examined in an *ex vivo* setting to determine whether high stresses and strains within the marrow, stimulating MSCs within the niche, correspond to new bone growth.

To address these objectives, five hypotheses have been defined, which will be tested in order to provide a better insight into the *in vivo* biochemical and biomechanical factors that govern osteogenic differentiation of MSCs within their stem cell niche.
Hypothesis 1

Osteocytes and osteoblasts can stimulate osteogenic differentiation of MSCs through biochemical signalling.

Osteogenic differentiation of MSCs in vitro requires a combination of well-established chemical factors such as dexamethasone, ascorbic acid and β-glycerol phosphate (Dominici et al., 2006). However, these stimulants are not present in vivo. It is likely that biochemical factors, from the natural environment where they reside, influence MSC fate. Osteocytes and osteoblasts are anabolic cells and possible candidates for production of such factors, and osteocytes have long been regarded as mechanosensors and controllers of bone remodelling (Bonewald, 2007). Additionally, osteocytes are known to communicate with osteoblasts through functional gap junctions (Doty, 1981; Kamioka et al., 2007; Taylor et al., 2007) with osteoblasts lining the interface between bone and the marrow, and as such it is possible that osteocytes are also involved in the promotion of the osteogenic differentiation of MSCs. However, whether osteocytes and osteoblasts release biochemical signals capable of producing the osteogenic differentiation of MSCs has not yet been investigated. Therefore this thesis seeks to test the hypothesis that osteocytes and osteoblasts can stimulate osteogenic differentiation of MSCs through biochemical signalling.
Hypothesis 2

MSCs receive sufficient mechanical stimulation during physiological loading within the marrow of trabecular bone to stimulate osteogenic differentiation.

Mechanical loading has been used to direct MSCs to undergo osteogenic differentiation in vitro (Arnsdorf et al., 2009; Case et al., 2011) and as mentioned above, the mechanical stimulus to cells has important implications for bone tissue engineering and regeneration applications. There is increasing evidence that the mechanical environment within the bone marrow niche also plays an important role in its functions (Guilak et al., 2009; Gurkan and Akkus, 2008). However, to date, the precise mechanical stimuli experienced by MSCs in the marrow niche, or whether such stimuli are sufficient to cause the direct osteogenic differentiation of MSCs, remain unclear. Therefore this thesis seeks to test the hypothesis that the mechanical stimulation experienced by MSCs within the marrow of trabecular bone is of sufficient magnitude to stimulate osteogenic differentiation of MSCs.

Hypothesis 3

The mechanical stimulus experienced by MSCs in vivo is altered in osteoporotic bone
The decrease in bone mass caused by osteoporosis causes a degradation in the mechanical properties of bone to such an extent that bone fractures can occur in normal loading conditions, which would not cause fractures in healthy bone (McNamara, 2010). As osteoporotic bone is less dense, the mechanical properties at the whole bone level are deficient during osteoporosis (Bourrin et al., 2002; Ederveen et al., 2001). However, at the individual trabeculae level an increase in strength has been demonstrated (McNamara et al., 2006). Osteoporosis is also accompanied by a change in marrow composition with the number of fat cells increasing (Cohen et al., 2012; Gurkan and Akkus, 2008; Yeung et al., 2005). As the stresses generated are a function of the load applied, bone structure and marrow composition it is likely that the ensuing mechanical stimuli to MSCs in the bone marrow are altered by the onset of osteoporosis but this has never been demonstrated. Therefore this thesis seeks to test the hypothesis that the mechanical stimulus experienced by MSCs in vivo is altered in osteoporotic bone.

**Hypothesis 4**

**Physiological compression of trabecular bone generates shear stress within the bone marrow and thereby stimulates new bone growth**

When trabecular bone is subjected to compressive loading the bone strain is sufficiently large to stimulate new bone formation in accordance with
Wolff’s Law (Wolff, 1892). This compression of the solid matrix also likely causes the deformation of the bone marrow. Moreover, compression has been shown to cause pressurisation of marrow (Ochoa et al., 1991). This builds on Hypothesis 2, which tests whether the shear stress generated is of sufficient magnitude to stimulate an osteogenic response in MSCs within the marrow. The shear stress generated within the bone marrow has never been determined and as such could be playing an important role in the generation of sufficient stimulus for MSCs to undergo osteogenic differentiation (Govey et al., 2013; Gurkan and Akkus, 2008). This study aims to answer whether the secondary effect of bone loading, shear stress within the marrow, is anabolic to bone growth. Therefore, this thesis tests the hypothesis that physiological compression within trabecular bone leads to shear stress generated within the bone marrow and thereby stimulates new bone growth.

Hypothesis 5

Low-magnitude high-frequency loading stimulates new bone formation in areas of high shear stress within the marrow

Low-magnitude high-frequency (LMHF) loading has been shown in different studies to be anabolic to bone formation (Rubin et al., 2001a; Rubin et al., 2001c; Rubin et al., 2004). However, similar to physiological compressive loading, how the mechanical load is transferred to a cellular
response remains unknown. The bone strain induced under LMHF is minimal compared to other mechanical loading regimes and indeed the anabolic response has been shown to be independent of bone strain (Judex et al., 2007) and to also occur in non-load bearing bones (Garman et al., 2007). Analytical and computational models have demonstrated that LMHF loading generates significant shear stress within the marrow (Coughlin and Niebur, 2012; Dickerson et al., 2008). Additionally, LMHF vibration has been shown to be effective in directing MSCs down an osteogenic differentiation pathway (Luu et al., 2009; Sen et al., 2008). It is proposed that LMHF vibration generates a mechanical signal within the bone marrow which stimulates MSCs to undergo osteogenic differentiation, beginning the process of new bone formation and this thesis seeks to test this hypothesis.

1.4. Thesis Structure

This thesis details the work undertaken over the course of this PhD study. It begins by presenting a literature review in Chapter 2 outlining the function of bone, bone marrow and the stem cell niche, and delineating the influences of biochemical signalling from support cells on MSCs. In addition it will outline the previous computational modelling approaches for trabecular bone and marrow. Chapter 3 details the theory behind the computational modelling in this thesis, providing a brief outline of the finite element (FE),
computational fluid dynamics (CFD) and fluid structure interaction (FSI) techniques employed over the course of this work.

Chapter 4 addresses Hypothesis 1 by investigating the biochemical signalling between osteoblasts, osteocytes and MSCs. This is achieved through the use of conditioned media and co-culture studies. It allows for the determination of the role of the bone cells in the osteogenic differentiation of MSCs in vivo.

Chapter 5 is concerned with Hypotheses 2 and 3. It details the generation of computational models of trabecular bone and marrow, using novel FSI techniques, to provide information on the magnitude of shear stress experienced by the MSCs within the marrow. It also focuses on how the shear stress experienced by MSCs can change due to the onset of osteoporosis.

Chapter 6 addressed Hypothesis 4 discussing the development of novel FSI models of trabecular bone and marrow using realistic geometries determined from micro CT scans. This study advances the modelling techniques developed in Chapter 5. An experimental study complements the computational investigation wherein a custom built compression bioreactor which applies a controlled compression to ex vivo trabecular bone and
1. Introduction

marrow samples, is used. New bone formation is quantified using bone histomorphometry techniques and results are correlated with the stresses and strains experienced in the trabecular bone and marrow.

Chapter 7 addresses Hypothesis 5 by using a specially developed LMHF vibration bioreactor to stimulate \textit{ex vivo} bone samples in a controlled manner. Bone growth and remodelling are quantified using micro CT scanning and fluorochrome labelling. CFD models of the experimental set up are used to determine whether regions of high stress within the bone marrow correspond to areas of new bone formation. Finally Chapter 8 discusses the implications of this thesis outlining the main findings and recommendations for future work based on the aims of thesis.

1.5. Conclusions

This chapter outlines the thesis motivation, introduces the hypotheses and objectives and discusses the structure of this thesis. Overall this thesis aims to determine the causal effects of osteogenic differentiation of bone marrow MSCs \textit{in vivo}. Specifically, for the first time, the role of indirect regulation of MSCs, via mechanosensing cells, is compared to direct regulation of MSCs via their own mechanosensing abilities.
2. Background

2.1. Chapter Background

This chapter provides a background to the key knowledge and literature regarding bone function and structure, bone marrow, MSCs and the stem cell niche. It also discusses relevant literature on the computational modelling of trabecular bone. It is worth noting that this chapter provides a more general background to the topics relevant to this body of work. Each of the subsequent technical studies (Chapters 4 to 7) also include focused discussions of the key literature directly relevant to each study.

2.2. Bone

2.2.1. Function

Bone is the major component of the skeletal system in the body. It plays supportive and protective roles within the body, bearing load, providing shape to the body and shielding internal organs (Cowin, 2001; Currey, 1984). Bone permits movement by providing sites for muscle attachment, which allow for the transmission of loads generated by muscle contraction thereby
2. Background

causating movement. Due to the high mineral content of the bone matrix it also functions as a reservoir for minerals such as calcium. Bone is considered a dynamic material due to its ability to repair and regenerate and its ability to adjust its structure in response to mechanical requirements. However these abilities are impaired by the onset of disease, by aging or due to major injuries (Cowin, 2001; Currey, 1984; Frost, 1987; Hayward et al., 1989).

2.2.2. Composition

At the nano-scale bone consists of collagen, mineral crystals and proteoglycans (Figure 2.1).

Figure 2.1.: Hierarchical structure of bone (McNamara, 2011).

Precollagen is produced by bone cells known as osteoblasts and cross-linked into fibril formation. Noncollagenous proteins and proteoglycans help with
the organisation of the matrix and the binding of mineral crystals and cell attachments (Gevers and Dequeker, 1987; McNamara, 2011). Hydroxyapatite and calcium phosphate make up the mineral phase of bone. The mineral is formed when osteoblasts secrete alkaline phosphate (ALP) which facilitates mineral deposition. The mineral in bone provides bone strength and stiffness which is vital for the load bearing required during daily activities.

2.2.3. Structure

Long bones such as the femur and humerus consist of a central shaft known as the diaphysis. At the two ends the bone becomes wider and rounded into the area known as the metaphyses and the epiphyses (Figure 2.2). It is at the epiphyses that the articulation at joints occurs as such a layer or articular cartilage is found on the bone here in addition to ligaments and tendons which permit movement.

Cortical bone is found as the outer shell on long bones surrounding only bone marrow in the diaphysis (Figure 2.1 and Figure 2.3). Cortical bone provides bone with a dense hard outer surface making up about 80 % of the entire bone mass in the skeleton (Cowin, 2001). While the highest portion of bone is found in the diaphysis of long bones, where it surrounds fatty bone marrow, it also provides a shell for all bones in the body. The function of
cortical bone is to bear load and transmit mechanical forces from the muscles to facilitate movement (Currey, 2002).

Figure 2.2: Long bone structure such as seen in the femur or humerus adapted from (www.biologycorner.com)

Trabecular bone (also known as cancellous or spongy bone) is found in the epiphyses of long bone, within irregular shaped bones such as the vertebrae in the spine and the sternum. Trabecular bone has a porosity of 75 - 95 %, which is very high compared to the porosity of cortical bone (5-10%), with a high rate of turnover due to its large surface area (Figure 2.3). Hematopoietic (red) marrow is found with the pores of healthy trabecular bone.
2. Background

Figure 2.3: Trabecular bone structure in the humerus with the outer cortical shell (Encyclopedia Britannica,).

It is the organisation of the microstructure of bone which varies between cortical and trabecular bone. Layers, or lamellae, of bone tissue can be organised into circumferential layers known as osteons (Haversian systems), which surround a vascular supply (Harversian canal). These osteons make up the structure of cortical bone whereas the trabecular bone structure consists of the lamellae organised into singular trabeculae, which lack the vascular supply (Cowin, 2001; Rho et al., 1998).
2.2.4. Bone cells

Osteoblasts

Osteoblasts can be regarded as the builder cells in bone. They make and secrete unmineralised bone matrix known as osteoid. Active osteoblasts are cuboidal in shape with a large nucleus, cellular processes which can contain the gap junctions, a large endoplasmic reticulum and Golgi apparatus and also vesicles, which secrete collagen. Originating from MSCs within the marrow, osteoblast precursors proliferate and line the bone surface, as they differentiate into pre-osteoblasts, which secrete the osteoid and finally mature osteoblasts, which mineralise the matrix. The differentiation of osteoblasts from MSCs is discussed in greater detail in Section 2.4.1. It is believed the active osteoblast becomes a flat bone lining cell (resting osteoblast), or an osteocyte, or undergoes apoptosis when it has completed the process of bone formation (Cowin, 2001).

Runx2, also known as Cbfa1, is thought to be the controlling transcription factor of osteoblast differentiation (Heino and Hentunen, 2008; Komori et al., 1997). It helps to control the early-osteoblast phenotype becoming supressed in mature osteoblasts. Runx2-deficient mice have been shown to have a cartilaginous skeleton with no osteoblasts present (Komori et al., 1997). Osterix (Osx) is also vital in controlling osteoblast differentiation with Osx null mice displaying no bone formation (Nakashima et al., 2002).
2. Background

Additionally transforming growth factor beta (TGF-β) has been shown to be crucial for healthy bone growth and remodelling directly targeting osteoblasts (Filvaroff et al., 1999).

Bone sialoprotein (BSP), a cell binding protein which is indicative of late stage osteoblast maturation, and osteocalcein, a calcium binding protein, which binds to hydroxyapatite (Komori et al., 1997), are the noncollagenous proteins associated with osteoblasts. ALP, type I collagen and protein are also characteristic of osteoblasts. ALP is secreted by osteoblasts to increase local phosphate concentration and promote mineralisation. Hypophosphatasia, a condition which has been shown to develop in animals with defective ALP, greatly decreases the activity of mineral deposition demonstrating the importance of ALP activity in healthy bone growth (Narisawa et al., 1997).

**Osteoclasts**

Osteoclasts are responsible for the resorption of bone *in vivo*. They are giant multi-nucleated cells, which originate from hemapoietic stem cells in the marrow. Mature osteoclasts possess up to 50 nuclei and range in diameter from 20 - 100μm (Roodman, 1996). Osteoclasts can be found close to the bone surface with ruffled borders when actively resorbing the bone. The temporary cavities they create during resorption are known as Howship’s
lacunae (Watanabe et al., 1995). Resorption occurs in two phases involving the dissolution of mineral, by the secretion of H\(^+\) atoms, and then enzymic digestion of organic molecules. Osteoclastogenesis is dependent on many pathways and signalling factors. How exactly osteoclasts are activated remains unclear but factors that promote osteoclast differentiation and resorption, and prevent osteoclast apoptosis, include the Receptor Activator of Nuclear factor-kB Ligand (RANKL), and macrophage colony-stimulating factor (M-CSF) have been identified. In opposition, factors that inhibit osteoclastogenesis include BMP-2, TGF-\(\beta\), calcitonin and oestrogen (Cowin, 2001; Väänänen, 2005).

**Osteocytes**

Osteocytes are the most abundant bone cell *in vivo*. They are found embedded throughout the bone matrix with numerous projections extending outwards into the matrix. They are thought to be terminally differentiated osteoblasts that, as they lay down the bone matrix, become embedded within cavities known as lacunae (Bonewald, 2007; Mullen et al., 2013; Parfitt, 1977).

They are the primary mechanosensor in bone mediating the strain induced remodelling (Bonewald, 2007; Huiskes et al., 2000; Schaffler et al., 2013). The long dendritic processes of osteocytes travel through spaces in the bone
2. Background

matrix called canaliculi. These processes connect osteocytes with each other and with other cells on the bone surface through functional gap junctions (Alford et al., 2003; Cheng et al., 2001b; Doty, 1981), creating extensive networks. Due to this functional network and the distribution throughout the bone matrix it is believed that osteocytes act as a network of strain gauges monitoring the mechanical environment directly and recruiting other cells to alter the bone mass as required (Bonewald, 2007; Bonewald and Johnson, 2008; Cowin et al., 1995; Skerry et al., 1989). Computational models reveal that the dendritic processes cause the applied strain in vivo to amplified to a level that is shown to be stimulatory in osteocytes (Verbruggen et al., 2012)

The molecules that are released by osteocytes to stimulate bone formation and remodelling remain unclear. A possible candidate is Prostaglandin E2 (PGE2) which has been demonstrated as anabolic for trabecular bone formation in rats (Keila et al., 2001). The same study found that when PGE2 was applied to bone marrow MSCs in vitro there was an increase in the number of mineral nodules. Other studies have found PGE2 to have a profound anabolic effect in both in vitro studies on cells and animal models (Kato et al., 1997; Li et al., 2003; Yoshida et al., 2002). Additionally PGE2 release by osteocytes increases under mechanical stimulation (Cherian et al., 2005) and is released due to micro-damage in bone which initiates a
remodelling response (Kennedy et al., 2012). Additionally, insulin like growth factor 1 (IGF-1) (Lau et al., 2013; Sheng et al., 2013) and β-Catenin-dependent canonical Wnt signalling (Kramer et al., 2010) have been shown to be essential to bone homeostasis and the osteocyte mediated response of bone to mechanical stimulation.

Hemichannels most likely play a dominant role in regulating the effects observed in this study as they regulate the release of small molecules (e.g. PGE$_2$) into the extracellular environment (Cherian et al., 2005). These unopposed connexins (hemichannels) are the likely method through which the osteocytes and osteoblasts can release signalling molecules. When two connexins come into contact a gap junction is formed, which allows for direct cell to cell contact. Functional gap junctions between osteocytes and osteoblasts have been found to form in numerous studies (Kamioka et al., 2007; Taylor et al., 2007; Yellowley et al., 2000). Cx43 is a gap junction protein expressed by bone cells and can be used to visualise such junctions between cells (Figure 2.4) (Kamioka et al., 2007). Direct cell to cell contact between osteocytes and osteoblasts is likely to occur in vivo allowing for such gap junctions. Gap junctions between osteocytes and osteoblasts have previously been shown to be essential in facilitating increased ALP activity by osteoblasts (Taylor et al., 2007). In this study only the osteocytes were exposed to fluid shear while allowed to form physical contacts with the
2. Background

osteoblasts demonstrating that the formed gap junctions between the cells were essential for the increase in ALP activity.

![Content removed due to copyright restrictions](image)

**Figure 2.4.:** Image shows an osteocyte (OC) and osteoblast (OB) in red with Cx-43 positive sites in green. Image from (Kamioka et al., 2007)

### 2.2.5. Formation

Bone is formed either intramembranous ossification or endochondral ossification. Both processes have similar origins with an increase in the number of cells and fibres and the differentiation of cells into osteoblasts which lay down matrix (Section 2.2.4). Cortical bone is formed in the most part by the process of intramembranous ossification which begins with the formation of bone spicules or aggregates from embryonic mesenchymal cells condensations with newly differentiated osteoblasts adding matrix to the spicules to form primary trabeculae (spongiosa). The primary centre of ossification is now established next to the newly formed periosteum as new bone is laid down. Compact bone is formed as primary osteons and Haversian systems are formed around the spongiosa. Endochondral
ossification uses a cartilage model to form bone. As the cartilage cells mature and grow they ultimately calcify forming bone. This process forms the majority of trabecular bone and is important in the growth plates of bone (Cowin, 2001; Scheuer and Black, 2004).

2.2.6. Modelling and remodelling

An interesting aspect of bone is its ability to adapt its architecture and remodel to maintain bone strength and functionality. Modelling occurs at a continuous large scale during the growth of the skeleton contributing to changes in bone size and shape. The rate of modelling decreases after skeleton maturity. Bone remodelling occurs at a smaller scale in localised areas producing mature bone which is stronger mechanically than immature bone (woven bone). This is an important aspect of healthy adult bone due to the deterioration of bone with time. The physiological process of bone remodelling begins with the resorption of the diseased or damaged bone by osteoclasts. This is replaced by new osteoid deposited by osteoblasts which is subsequently mineralised.

Bone remodelling is controlled by bone resorbing osteoclasts and bone forming osteoblasts. This is a carefully controlled sequenced process of what is known as a basic multicellular unit (BMU). Osteoclasts begin the sequence
by resorbing bone in response osteoblasts begin to lay down new matrix (Jilka, 2003). While the stimulating signals for the BMU to act remain unclear remodelling typically occurs for one of three reasons: 1) to maintain mineral homeostasis; 2) to adapt to mechanical changes, especially disuse where a random distribution of cutting cones is caused, and 3) to repair damage (Burr et al., 1985; Graham et al., 2013).

2.2.7. Osteoporosis

Bone remodelling is crucial to healthy bone and the process of remodelling requires careful balance between osteoclast controlled bone resorption and osteoblast controlled bone formation (Robling et al., 2006). If this balance becomes disrupted bone fractures are likely due to decreased bone strength and density. Osteoporosis is a skeletal disease characterised by decrease bone strength and denisty which leads to increased risk of bone fractures. Dual energy X-ray absorptiometry (DEXA) is commonly used to diagnose osteoporosis by measuring bone mineral density (BMD) levels which decrease during the disease. This is a accompanied by a decrease in the micro-architectural structure of trabecular bone, with thinner trabeculae characteristic of the disease (Garnero and Delmas, 2004; McNamara, 2010; Parfitt, 1987). Moreover, patients with osteoporosis have an increased fat
content within their bone marrow and can sometimes become sclerotic (Cohen et al., 2012).

2.2.8. Mechanical adaption of bone

The peak strain (ε) experienced in long bones during physiological loading has been quantified between 1000 and 2000 με with bone routinely experiencing small strains of < 10 με thousands of times each day (Burr et al., 1996; Fritton et al., 2000). It is generally accepted that bone adapts its structure and orientation to accommodate the changes in mechanical forces which it can experience (Carter et al., 1987a; Frost, 2003; Rubin and Lanyon, 1984) whereas, disuse or immobilization can cause bone loss (Zerwekh et al., 1998).

Modelling and remodelling are modulated by thresholds in bone strains. Frost introduced the ‘mechanostat’ theory where strains of the 1500 – 3000 με range cause bone modelling to increase and bone resorption to decrease (Duncan and Turner, 1995) while strains below the 100 – 300 με range cause bone resorption (Frost, 1987). Therefore bone surfaces are either in a state of modelling or remodelling. This theory also includes a mechanical feedback loop which sees the bone become immune to the given stimulus. This model has been expanded upon by Huiskes and colleagues through the
introduction of bone cells physiology, including osteoclastic resorption, osteoblastic formation, the mechanosensitive osteocytes and also introducing the role of microdamage in stimulating bone remodelling (Huiskes et al., 2000).

Turner summarised bone adaption to mechanical loading into three rules (Turner, 1998):

1) Dynamic rather than static loading is required for adaption (Lisková and Hert, 1971; Rubin and Lanyon, 1984).

2) A short duration of mechanical loading is required to initiate an adaptive response. A longer duration of loading lessens the bone adaptation.

3) Bone cells can become accustomed to the loading making them less responsive.

More recently, small mechanical loads applied at high frequencies (LMHF loading), have been shown to have an anabolic effect on bone tissue (Rubin et al., 2001b). Sheep subjected to LMHF vibration with an acceleration of 0.3 g peak-peak demonstrated increased bone volume, bone mineral content and trabecular number (Rubin et al., 2002). Similarly, bone loss was halted in the spine and femur in post-menopausal women exposed to LMHF loading of
2. Background

0.2 g at 30 Hz for less than 20 minutes a day compared to controls who used placebo loading devices (Rubin et al., 2004). LMHF loading has also been found to inhibit bone resorption in a growing mouse skeleton (Xie et al., 2006). While the benefit of such loading regimes has been demonstrated in various human (Kiiski et al., 2008; Rubin et al., 2004; Verschueren et al., 2004) and animal models (Garman et al., 2007; Judex et al., 2003; Rubin et al., 2001b; Rubin et al., 2001c; Rubin et al., 2002; Xie et al., 2006), the mechanical signals that are transmitted to bone cells to stimulate the biological response remain unclear and are not fully understood. However, other studies have questioned the effectiveness of LMHF loading and whether it is an isolated effect within bone. For example LMHF loading (0.6 g at 45 Hz) did not attenuate bone loss in mouse muscle disuse models (Manske et al., 2012). Similarly, no bone growth was found in ovariectomized rats exposed to LMHF loading (0.3 g at 90 Hz) (Brouwers et al., 2010).

2.2.9. Trabecular bone mechanics

Trabecular bone is anisotropic with the principal material direction aligned with the trabecular architecture (Yang et al., 1998) consistent with Wolff’s observation from the 19th century (Wolff, 1892). Trabecular bone is also heterogeneous in nature with tissue properties varying within a single long bone varying up to a hundred fold (Goldstein et al., 1983). The elastic
modulus and the failure stress of trabecular bone are determined by the density of the bone (Keaveny et al., 2001).

2.3. Bone marrow

Within the cortical shell of long bones the > 50 % of space is taken up by bone marrow with trabecular bone occupying the rest of the space (Cowin, 2001). Bone marrow is divided into two separate types; red or hemapoetic marrow and yellow or fatty marrow. Both types of bone marrow contain numerous blood vessels and capillaries.

Bone marrow is the source of many cells (including mesenchymal stem cells (Section 2.4) within the body (Gurkan and Akkus, 2008; Pittenger et al., 1999; Polak and Bishop, 2006). Red marrow is home to hemapoetic stem cells which produce the monocytes which later can develop into osteoclasts (Section 2.2.4). Red marrow is coloured as such due to the presence of haemoglobin-containing erythrocytes (red blood cells) which also originate from within the marrow (Vande Berg et al., 1998). Yellow marrow contains an increased fat content which replaces the red marrow with the onset of aging (Huggins and Blocksom, 1936). When we are younger our marrow is almost exclusively red in nature. However, this is replaced throughout long bones with yellow marrow and the red marrow becomes confined to the
epiphyses of long bones and irregular shaped bones such as the sternum, the skull and the vertebrae (Casazza et al., 2012; Gurevitch et al., 2009; Liney et al., 2007). Here it is found within the pores of trabecular bone. While in the diaphysis of long bones the marrow becomes fatty and yellow with age.

The relationship between bone and fat mass in vivo is believed to be closely related. Diseases associated with bone loss are often accompanied by an increase in the fat content of marrow (Abdallah and Kassem, 2012; Pino et al., 2012; Zhong and Akkus, 2011). As both tissues can originate from the same mesenchymal origin, there exists a possible role for the cells in the bone marrow in the root of such diseases. Such is the relationship that a review of the area by Rosen and Bouxsein asked “Is osteoporosis the obesity of bone?” (Rosen and Bouxsein, 2006). The reader is directed to that study for further information on the relationship between bone and fat and the possible role of bone marrow cells in the relationship.

2.3.1. Bone marrow mechanics

Bryant et al. (1989) initially identified bone marrow as a Newtonian fluid at 35 °C (Bryant et al., 1989). It should be noted that marrow has a slightly lower temperature than normal the normal body temperature of 37 °C (Petrakis, 1952). An average value of 0.4 Pa.s was found, which is approximately 400
times the viscosity of water. Bryant et al. proposed that a non-Newtonian behaviour would be expected for bone marrow at lower shear rates ($< 10 \text{ s}^{-1}$), due to the large content of blood within marrow. This has been confirmed in subsequent studies (Zhong and Akkus, 2011). There was large variation in viscosities between the locations of bone marrow in the body. Importantly bone marrow is believed to have a decreasing viscosity with increasing fat content (Gurkan and Akkus, 2008) and the fat content of marrow increases with the onset of osteoporosis (Cohen et al., 2012) and in typical aging (Hwang and Panicek, 2007).

2.4. Mesenchymal stem cells

Friedenstein and colleagues were the first to identify MSCs within bone marrow by showing a sub-population of the marrow distinct from haemopoietic cells, which tended to attach to culture plates and formed a fibroblast like structure (Friedenstein et al., 1966). These studies also demonstrated the ability of these cells to form colonies from single cells (Friedenstein et al., 1970; Friedenstein et al., 1974; Friedenstein, 1976) and to undergo osteogenic differentiation (Friedenstein et al., 1987). The term mesenchymal stem cell was first introduced in 1991 (Caplan, 1991). However, its merit has been much debated as despite some reports on MSCs forming multiple tissues beyond skeletal lineages it has not been formally
proven (Bianco et al., 2008). While MSCs can be isolated from different tissues such as bone, synovium, cartilage and fat, the focus in this study is on MSCs isolated from bone marrow.

Dominici et al. (2006), for the Mesenchymal and Tissue Stem Cell Committee of the International Society for Cellular Therapy, proposed the following criteria for characterising MSCs.

- Cells must be plastic-adherent when maintained in standard culture conditions.
- Cells must express the following surface molecules CD105, CD73 and CD90 while lacking the expression of CD45, CD34, CD14 or CD11b (both expressed on monocytes and macrophages as such only one marker needs to be tested for), CD79α or CD19 (both expressed by B-cells so only one marker needs to be tested for) and HLA-DR as determined using flow cytometry.
- The cells must differentiate into osteoblasts, chondroblasts and adipocytes.

In vitro MSCs can be expanded and retain a stable undifferentiated phenotype, and this behaviour in addition to the ability to differentiate into other tissues make them attractive candidates for bone tissue engineering and regenerative medicine applications (Dominici et al., 2006; Pittenger et al.,
MSCs have been suggested as ideal candidates for tissue engineering and regenerative medicine approaches with varying successes (Caplan, 2007; Caplan, 2009). MSCs have also been reported as having the ability to differentiate into muscle and tendon tissue (see Figure 2.5). This is discussed in further detail by Caplan (2009).

Figure 2.5: Differentiation pathways in MSCs (Caplan, 2009).

In vitro, MSCs have been shown to be activated by biophysical stimuli such as fluid flow induced shear stress, hydrostatic pressure, substrate strain and topography (Castillo and Jacobs, 2010). The stimuli cause the cell membrane to deform creating tensile and compressive forces (Ingber, 1993) that activate mechanosensitive features within the cells such as focal adhesions (McGarry
et al., 2005), cytoskeletal proteins (Matthews et al., 2006), primary cilia (Hoey et al., 2012) and stretch activated ion channels (Duncan and Turner, 1995). However, whether MSCs can be activated in vivo in such a direct way remains unclear, it is possible they are indirectly activated by mechanosensing cells such as osteocytes during mechanical stimulation.

2.4.1. Osteogenic Differentiation

The osteogenic differentiation of MSCs in vitro has been divided into three stages (Huang et al., 2007). Stage one consists of days one to four where a peak in the number of cells is seen. This is followed by early cell differentiation from days 5 to 14 which is characterised by the transcription and protein expression of ALP (Aubin, 2001). After this initial peak the ALP starts to decline. While also found at an early stage of differentiation is the expression of a collagen type I matrix onto which the mineral is deposited (Quarles et al., 1992). The final stage from day 14 up to day 28 exhibits a high expression of osteocalcin and osteopontin followed by calcium and phosphate deposition (Hoemann et al., 2009; Huang et al., 2005).

The differentiation of MSCs in vitro largely depends on the culture conditions. Osteogenic differentiation of MSCs in vitro is induced by the presence of dexamethasone, ascorbic acid and β-glycerol phosphate (Jaiswal
et al., 1997). While the importance of each of these constituents for in vitro differentiation is becoming increasingly clear (Vater et al., 2011), the in vivo biochemical environment has not been well characterised and the driving source for the osteogenic differentiation of MSCs in their native environment remains unclear.

2.4.2. Chondogenic Differentiation

Chondogenic differentiation is the process by which stem cells form cartilage. In vitro, MSCs are grown in a pellet culture and treated to combination of reagents (including dexamethasone, ascorbic acid, proline, sodium pyruvate and TGF-β) to direct MSCs down the cartilage pathway (Mackay et al., 1998; Peister et al., 2004). This results in proteoglycans and type II collagen in the pellet cultures. This cartilage formation can also act as a template for subsequent bone formation (Augello and De Bari, 2010; Freeman et al., 2013).

2.4.3. Adipogenic Differentiation

Adipogenic differentiation is the process by which stem cells form fat. MSCs cultured with dexamethasone, insulin, isobutyl methyl xanthine, and indomethacin will differentiate down an adipogenic lineage (Peister et al., 2004; Pittenger et al., 1999). Adipogenic differentiation can be analysed using
oil red O staining which reveals the presence of lipids globules within cell cultures.

2.5. Stem Cell Niche

*In vivo* stem cells reside within what is termed a stem cell niche. This is a specialised micro-environment which regulates stem cell fate. The stem cell niche in bone marrow consists of a host of different support cells including hematopoietic progenitors and their progeny – such as blood cells, immune cells, and osteoclasts – and MSCs and their progeny including fibroblasts, endothelial cells, adipocytes, osteoblasts, as well as osteocytes found embedded in bone and osteoclasts (Kuhn and Tuan, 2010) (Figure 2.6).

*Figure 2.6.*: The stem cell niche in trabecular bone marrow.
As mentioned in Chapter 1 the niche is thought to have three functions: (1) maintaining quiescence, (2) promoting cell number and (3) directing differentiation (Schofield, 1978). It is likely that these support cells play a role in directing the functions of the stem cell niche (Fuchs et al., 2004).

2.5.1. The mechanical environment of the stem cell niche

As discussed in Hypothesis 2 in Section 1.3 there is increasing evidence that the mechanical environment imposed within the niche also plays an important role in the functions of the niche (Castillo and Jacobs, 2010; Estes et al., 2004; Guilak et al., 2009; Gurkan and Akkus, 2008; Potier et al., 2010). This mechanical environment is determined by the trabecular bone and marrow geometry and mechanical properties. Furthermore, mechanical loading has been used to direct MSCs to differentiate in vitro (Arnsdorf et al., 2009; Case et al., 2011) and as such the mechanical stimulus to cells has important implications for bone tissue engineering and regeneration applications. However, to date, the precise mechanical environment of bone marrow MSCs in vivo remains unclear and an improved understanding is needed to fully determine the role of mechanical signals on MSC differentiation.

As mentioned previously in Section 2.4, MSCs within the bone marrow are thought to be crucial in the formation of diseases such as osteoporosis. A study by Luu et al. (2009), revealed that targeted mechanical stimulation of
MSCs in the bone marrow could have potential in the clinical treatment of osteoporosis. Casazza et al. (2012) found increased physical activity in children decreased the amount of adipogenic tissue in the bone marrow. However, the effectiveness of such treatment could be dictated by bone structure and marrow composition, which will affect the mechanical signals delivered to the bone marrow (as discussed in Chapter 5, 6 and 7). Due to the non-uniform nature of trabecular bone, the mechanical signal will not be evenly distributed throughout the marrow, so the location of MSCs within the marrow is another crucial factor to consider for such a treatment (Siclari et al., 2013).

2.6. Computational Modelling of Trabecular Bone

Computational models have been used to assess the mechanical behaviour of trabecular bone. Cellular (Gibson, 1985), lattice (Jensen et al., 1990; Yeh and Keaveny, 1999) and Voronoi (Silva and Gibson, 1997) based approaches have been used to model the trabecular architecture (Figure 2.7). Such approaches have proved useful for examining the effect of decreasing trabecular architecture parameters such as bone volume/tissue volume (BV/TV), trabecular thickness (Tb.Th) and trabecular spacing (Tb.Sp) on the overall trabecular bone strength.
2. Background

Figure 2.7: Trabecular bone geometry idealised as (a) cellular (Gibson, 1985), (b) lattice (Yeh and Keaveny, 1999) where the trabecular thickness (Tb.Th) is altered across the structure and (c) Voronoi structures (Silva and Gibson, 1997).

High resolution microstructural models that are usually based on micro-computed tomography (μ-CT) scans have the advantage of incorporating the microstructural features of the trabecular bone, thus providing an accurate representation of the trabecular microarchitecture. The strategy is to directly convert the digital μ-CT data (voxels) into a FE mesh, thereby avoiding the need to generate more traditional meshes of the complex trabecular architecture. The FE mesh is generally generated from a three-dimensional image by applying a one-to-one mapping of image voxels to eight-node hexahedral finite elements (Hollister et al., 1994; van Rietbergen et al., 1995). An example of such a mesh can be seen in Figure 2.8. These modelling approaches have provided the opportunity to capture the heterogeneity of trabecular bone (Harrison et al., 2008; Niebur et al., 2000) greatly aiding the study of trabecular bone mechanics.
2. Background

Figure 2.8: FE mesh of ovine trabecular bone structure, of 2 mm$^3$ volume, at a resolution of 16 µm. Magnification shows the element detail where each element corresponds to a voxel in the 3D image generated from the µCT scan. Image from meshes generated in Chapter 6.

Computational and theoretical models developed to date, to determine the mechanical environment of bone marrow, have been limited to perfusion flow through the marrow space using a trabecular structure as a tissue engineering scaffold to determine the shear stress delivered to cells within the scaffold (Porter et al., 2005). Additionally, computational models have been used to determine the permeability of trabecular bone (Baroud et al., 2004; Teo and Teoh, 2012) Recent investigations used CFD approaches to model marrow as a Newtonian fluid (Coughlin and Niebur, 2012), marrow as a soft solid (Yoo and Jasiuk, 2006), used a continuum approach to describe marrow shear stress coupled to bone deformation (Dickerson et al., 2008) and examined permeability using analytical models (Sander et al., 2003).
Chapter 2 has presented an overview of bone, bone marrow, MSCs, the stem cell niche and previous approaches to the modelling of trabecular bone and marrow. In summary, bone tissue is continually being remodelled through the activities of bone resorbing osteoclasts and bone forming osteoblasts, originating from MSCs. However the exact signals for MSCs to undergo osteogenic differentiation remain unclear. There exists a potential role for osteocytes and osteoblasts, in their role as support cells within the niche, as the controlling factor in directing MSC differentiation. Osteocytes, in particular, have already been identified as the dominant mechanosensor in bone and have the potential to direct MSCs down an osteogenic pathway, due to their extensive network within bone and the biochemical they produce (Section 2.2.4). The potential role for osteocytes and osteoblasts is examined in greater detail in Chapter 4.

As discussed in Section 2.4, MSCs have the potential to be activated directly by biophysical stimuli. However whether they receive sufficient stimuli within the mechanical environment of the stem cell niche remains unclear and this is examined in Chapter 5. Additionally, Chapter 5 examines how the mechanical environment of bone marrow alters during osteoporosis. The potential of biophysical stimuli in directing MSC to undergo osteogenic
differentiation is investigated in Chapter 6. This chapter aims to determine whether MSCs can be directly stimulated by biophysical stimuli generated within the marrow by physiological representative compression. Chapter 7 examines the use of LMHF vibration loading, which generates little strain on the bone matrix, while still producing an anabolic response in trabecular bone. The vibration generates a mechanical signal within the bone marrow, directly targeting the stem cell niche, potentially bypassing the osteocyte strain sensing network, as discussed in Section 2.2.4 and 2.2.8.
3. Theory

3.1. Chapter Background

This chapter outlines the FE, CFD and FSI theory that are implemented in the current work. Reference is made to the Abaqus commercial code and solvers (DS SIMULIA, USA) which are used to solve the continuum mechanics problems addressed in this thesis.

3.2. Notation

The notation that is used in this chapter is presented here for clarity. Capital letters indicate tensors and matrices while lower-case letters are used for vectors. Bold type face is used for vectors, tensors and matrices with their components are shown in italics. The coordinate systems use the axes \( x_1 \), \( x_2 \) and \( x_3 \), or \( x_i \) where \( i = 1, 2, 3 \). Unit vectors in the three coordinate directions are \( \mathbf{e}_1 \), \( \mathbf{e}_2 \) and \( \mathbf{e}_3 \), respectively. Indicial notation can be demonstrated using the dot product (summation of the component parts) of two vectors in 3D \( \mathbf{u} \) and \( \mathbf{v} \) as follows
3. Theory

\[ \mathbf{u} \cdot \mathbf{v} = u_i v_i = u_1 v_1 + u_2 v_2 + u_3 v_3. \] (3.1)

There are nine components in 3D second order tensors with components identified by two subscripts \( i \) and \( j \). A second order tensor, e.g. \( \mathbf{A} \), can be written in component form as \( A_{ij} \). The tensor product of two vectors \( \mathbf{u} \) and \( \mathbf{v} \) can be written as \( \mathbf{uv} \), where \( (\mathbf{uv})_{ij} = u_i v_j \). A fourth order tensor is the linear tensor function of a second order tensor; for example, the second order stress tensor \( \sigma \) is related to the strain tensor \( \varepsilon \) by a fourth order elastic modulus tensor \( \mathbf{C} \).

\[ \sigma = \mathbf{C} : \varepsilon \quad \text{or} \quad \sigma_{ij} = C_{ijkl} \varepsilon_{kl} \] (3.2)

The gradient (grad) operation, \( \nabla \), on a scalar field, \( \phi \), can be defined as

\[ \nabla \phi = \frac{\partial \phi}{\partial x_1} \mathbf{e}_1 + \frac{\partial \phi}{\partial x_2} \mathbf{e}_2 + \frac{\partial \phi}{\partial x_3} \mathbf{e}_3. \] (3.3)

The divergence of a vector field, \( \mathbf{u} \), can be defined as

\[ \nabla \cdot \mathbf{u} = \frac{\partial u_1}{\partial x_1} + \frac{\partial u_2}{\partial x_2} + \frac{\partial u_3}{\partial x_3}. \] (3.4)
3.3. Finite Element Analysis of Solid Structures

3.3.1. Kinematics and Kinetics

Large deformation kinematics describes the deformation of a body by following its movement from an ‘initial state’ (reference configuration) to a ‘deformed state’ (current configuration). This is represented in Figure 3.1 with position vectors \( x \) and \( y \) used to define the location of a material point in the reference, \( x \), and current configurations, \( y \).

![Figure 3.1: Finite deformation kinematics schematic where the reference volume changes to the current volume with a displacement vector \( u \) of a material point defined by vectors \( x \) and \( y \).](image)

The displacement vector, \( u \), of the material point, at time \( t \), is represented by

\[
\mathbf{u}(x,t) = \mathbf{y}(x,t) - \mathbf{x}
\]

(3.5)
3. Theory

The movement of the material point is given by the velocity vector, \( \mathbf{v} \), defined by the derivative of the displacement vector, \( \mathbf{u} \), with respect to time, as follows

\[
\mathbf{v} = \dot{\mathbf{u}} = \frac{\partial \mathbf{u}(\mathbf{x}, t)}{\partial t}
\]  

(3.6)

To quantify overall shape change of the body, the deformation gradient \( \mathbf{F} \) is defined. This relates the initial state of an infinitesimal material fibre, \( d\mathbf{x} \), in the reference configuration, to the deformed state of that fibre in the current configuration, \( d\mathbf{y} \), (Figure 3.1). This is expressed using Equation (3.7).

\[
d\mathbf{y} = \mathbf{F} \cdot d\mathbf{x}
\]  

(3.7)

\[
\mathbf{F} = \frac{\partial \mathbf{y}}{\partial \mathbf{x}} \text{ or } \mathbf{F}_{ij} = \frac{\partial y_i}{\partial x_j}
\]  

(3.8)

The Jacobian, \( J \), is used to quantify volume changes in the deformation of the body, expressed as the determinant of the deformation gradient, \( \mathbf{F} \), as in Equation (3.9).

\[
J = \det(\mathbf{F}) = \frac{dv}{dV}
\]  

(3.9)
3. Theory

The Green-Lagrange strain $E$ is given by:

$$E = \frac{1}{2} (F^T \cdot F - I)$$

or

$$E_{ij} = \frac{1}{2} \left( \frac{\partial u_i}{\partial x_j} + \frac{\partial u_j}{\partial x_i} + \frac{\partial u_j}{\partial x_i} \frac{\partial u_i}{\partial x_j} \right) \quad (3.10)$$

where $A^T$ is the transpose of $A$, $(A \cdot B)_{ij} = A_{ij} B_{kj}$ and $I$ is the identity tensor, with the property $A \cdot I = A$. The infinitesimal strain tensor, $\varepsilon$, can be found from the Green-Lagrange strain by assuming that the product of the infinitesimals is negligible, giving

$$\varepsilon = \frac{1}{2} \left( \nabla u + \nabla u^T \right)$$

or

$$\varepsilon_{ij} = \frac{1}{2} \left( \frac{\partial u_i}{\partial x_j} + \frac{\partial u_j}{\partial x_i} \right) \quad (3.11)$$

Components of the infinitesimal strain tensor can also be referred to as nominal strains.

The left and right Cauchy-Green tensors, $B$ and $C$ respectively, are useful measures of deformation, given by

$$B = F \cdot F^T \quad \text{and} \quad C = F^T \cdot F \quad (3.12)$$
According to the polar decomposition theorem the deformation gradient can
be uniquely decomposed into an orthogonal rotation tensor $R$ and
symmetric spatial and material stretch tensors, $V$ and $U$ respectively,
according to

$$F = R \cdot U = V \cdot R. \tag{3.13}$$

The eigenvectors and eigenvalues of $U$ are known as the respective principal
referential axes and principal stretches, $\hat{\lambda}_{a=1,2,3}$. The logarithmic (true) strain $\varepsilon$ is defined as

$$\varepsilon = \ln V. \tag{3.14}$$

From the deformation gradient (Equation (3.8)), the spatial velocity gradient,
$L$, can be derived as follows

$$L = \frac{\partial V}{\partial \mathbf{y}} = \dot{\mathbf{F}} \cdot \mathbf{F}^{-1} \tag{3.15}$$

where $A^{-1}$ is the inverse of $A$. The spatial velocity gradient can be split into
two tensors such that, $L = D + \omega$. The symmetric, $D$, and asymmetric, $\omega$,
3. Theory

parts of the velocity gradient which are defined as the rate of deformation and spin tensors respectively are expressed in Equations (3.16) and (3.17).

\[ D = \text{sym}(L) = \frac{1}{2} (L^T + L) \]  
(3.16)

\[ \omega = \text{asym}(\omega) = \frac{1}{2} (L - L^T) \]  
(3.17)

The Cauchy or true stress, \( \sigma \), describes the force per unit area in the current configuration. It is related to the traction, \( t \), on a surface (internal or external) and a unit normal vector to the surface, \( n \)

\[ t = \sigma \cdot n. \]  
(3.18)

The Cauchy stress has two components; determined by the hydrostatic pressure, \( p \), and a deviatoric stress \( S \), such that

\[ \sigma = S - pI. \]  
(3.19)

\( p \) is defined as follows
3. Theory

\[ p = \frac{-Tr(\sigma)}{3} \]  \hspace{1cm} (3.20)

where the \( Tr(A) = A_{ii} \).

The Kirchhoff stress \( \tilde{\tau} \) is a symmetric tensor given by

\[ \tilde{\tau} = J\sigma. \]  \hspace{1cm} (3.21)

For stress measures defined on the reference configuration, the Piola stress, \( P \), is a non-symmetric tensor given by

\[ P = J\sigma F^{-T}. \]  \hspace{1cm} (3.22)

while the nominal stress, \( \tilde{\sigma} \), is defined as follows

\[ \tilde{\sigma} = JF^{-T} \cdot \sigma = P^T. \]  \hspace{1cm} (3.23)

In this thesis, results of simulations are typically interpreted in terms of the von Mises equivalent stress \( \sigma_v \). This can be described as a scalar uniaxial equivalent of a multi-axial stress state, and can be defined in terms of the deviatoric stress as
3. Theory

\[ \sigma_i = \sqrt{\frac{3}{2} S_{ij} S_{ij}}. \quad (3.24) \]

3.3.2. Constitutive Laws

**Isotropic Linear Elasticity Theory**

In isotropic linear elasticity the nominal stress \( \tilde{\sigma} \) is related to the infinitesimal strain \( \varepsilon \) through

\[ \tilde{\sigma}_{ij} = \lambda \varepsilon_{ik} \delta_{kj} + 2 \mu \varepsilon_{ij} \cdot \quad (3.25) \]

where \( \lambda \) and \( \mu \) are the Lamé constants and \( \delta_{ij} \) is the Kronecker delta, with the following properties

\[ \delta_{ij} = \begin{cases} 1 & i = j \\ 0 & i \neq j \end{cases} \quad (3.26) \]

Stress measures become equal under the assumptions of infinitesimal deformation kinematics such that \( \sigma = \tilde{\sigma} \). The Lamé constants can be derived from Young’s Modulus \( E \) and Poisson’s Ratio \( \nu \), as follows

\[ \mu = \frac{E}{2(1 + \nu)} \quad \text{and} \quad \lambda = \frac{\nu E}{(1 + \nu)(1 - 2\nu)}. \quad (3.27) \]
For this theory it is also possible to recast Equation (3.25) in terms of a fourth order tensor of elastic moduli $K$ such that

$$\vec{\sigma} = K : \varepsilon.$$  \hspace{1cm} (3.28)

In this thesis this isotropic elasticity theory is implemented in both an infinitesimal deformation framework, as above, and in a finite deformation framework by using the Cauchy stress and logarithmic strain in place of the infinitesimal measures used in Equation (3.25). The latter essentially amounts to the assumption of inherently linear material stress-strain behaviour, that is commonly used for the representation of bone tissue. This is combined with the more general case of finite-deformation kinematics which accounts more accurately for large strains should large strains occur. For further detail on the implementation of this theory within Abaqus, the reader is referred to the Abaqus Theory Manual (DS SIMULIA, 2012).

3.3.3. Numerical Implementation for the deformation of Solids

Equilibrium Equations and Principle of Virtual Work

Mechanical equilibrium of a deformable body can be expressed as:
3. Theory

\[ \frac{\partial \sigma_{ji}}{\partial y_j} = \rho \frac{\partial \dot{v}_i}{\partial t} = \rho \ddot{v}_i = \rho \ddot{u}_i \]  \hspace{1cm} (3.29)

where \( \rho \) is the density in the reference configuration and \( \rho = J^{-1} \rho_0 \). Since \( \mathbf{\sigma} \) is symmetric this can be written in tensor notation as

\[ \nabla \cdot \mathbf{\sigma} = \rho \frac{\partial \dot{\mathbf{v}}}{\partial t} = \rho \dot{\mathbf{v}} = \rho \dot{\mathbf{u}} \]  \hspace{1cm} (3.30)

The combination of the equilibrium equation with suitable boundary conditions generates a Boundary Value Problem (BVP). When using the FE method (FEM) to solve BVP’s, Equation (3.30) can be rewritten in integral form in terms of the principle of virtual work (PVW) which can be expressed in rate form as

\[ \delta W = \int_V \left( \nabla \cdot \mathbf{\sigma} - \rho \ddot{\mathbf{u}} \right) \delta \mathbf{v} dV = 0 \]  \hspace{1cm} (3.31)

where \( \delta W \) is the virtual work per rate per unit volume, \( \delta \mathbf{v} \) is an arbitrary virtual velocity field which satisfies the kinematic boundary conditions of the problem and \( V \) is the volume of the body. This can also be expressed as shown in Equation (3.32) where the left hand side represents internal virtual power and the right hand side represents external virtual power.
3. Theory

\[ \int_{V} \sigma : \delta D dV = \int_{S} t \cdot \delta v dS - \int_{V} \rho \ddot{u} \cdot \delta v dV \quad (3.32) \]

and \( S \) is the surface bounding the volume. This equation is approximated and solved using the FEM as discussed in the following sections.

**Finite Element Method**

In the FEM a solid body is broken down into discrete sections called elements which are connected together at points called nodes. The full collection of elements and nodes which make up the solid body is known as the finite element mesh. For FE purposes it is useful to rewrite the principle of virtual work (Equation (3.32)) in matrix/vector (Voigt) notation as follows

\[ \int_{V} \delta D^T \sigma dV = \int_{S} \delta v^T t dS - \int_{V} \delta v^T \rho \ddot{u} dV. \quad (3.33) \]

For each element, \( e \), in the FE mesh the following interpolation holds true

\[ \delta D = B^{(e)} \delta v^{(e)} \quad (3.34) \]

\[ \delta v = N^{(e)} \delta v^{(e)} \quad (3.35) \]
3. Theory

where $\delta \mathbf{v}^{(e)}$ is a column vector of element nodal and virtual velocities, $\mathbf{N}^{(e)}$ is the element shape function matrix and $\mathbf{B}^{(e)}$ is the element shape function gradient matrix.

The FE approximation of the principle of virtual work can now be assembled as follows

$$\sum_{e} \int_{V^{(e)}} \delta \mathbf{v}^{(e)^T} \mathbf{B}^{(e)^T} \mathbf{a} dV = \sum_{e} \int_{S^{(e)}} \delta \mathbf{v}^{(e)^T} \mathbf{N}^{(e)^T} t dS - \sum_{e} \int_{V^{(e)}} \rho \delta \mathbf{v}^{(e)^T} (\mathbf{N}^{(e)^T} \mathbf{N}^{(e)}) \ddot{\mathbf{u}}^{(e)} dV \quad (3.36)$$

where the summation in Equation (3.36) is over all the elements $e$ in the finite element mesh. The summation (assembling element quantities into global quantities and removing arbitrary virtual velocity terms) is performed to yield the following global expression

$$\int_{V} \mathbf{B}^T \mathbf{a} dV = \int_{S} \mathbf{N}^T t dS - \int_{V} \rho (\mathbf{N}^T \mathbf{N}) \ddot{\mathbf{u}} dV \quad (3.37)$$

where $\ddot{\mathbf{u}}$ is the global nodal acceleration vector for the whole mesh. In general for large deformation and non-linear material problems the out of balance force vector, $\mathbf{G}$, can be expressed as follows
3. Theory

\[ G(\mathbf{u}, \ddot{\mathbf{u}}) = \int_{V} B^{T}(\mathbf{u})\sigma(\mathbf{u})dV - \int_{S} N^{T}(\mathbf{u})t dS + \int_{V} \rho(\mathbf{u})(N^{T}(\mathbf{u})N(\mathbf{u}))\ddot{\mathbf{u}}dV \quad (3.38) \]

where \( \mathbf{u} \) is now the global nodal displacement vector for the mesh. To solve the BVP it is necessary to determine \( \mathbf{u} \) and \( \ddot{\mathbf{u}} \) such that Equation (3.39) is true at any point in time.

\[ G(\mathbf{u}, \ddot{\mathbf{u}}) = 0 \quad (3.39) \]

Equation (3.38) can be rewritten as follows (dropping the \( \mathbf{u} \) dependence for convenience)

\[ G(\mathbf{u}, \ddot{\mathbf{u}}) = \int_{V} B^{T} \sigma dV - \int_{S} N^{T}t dS + M\ddot{\mathbf{u}} \quad (3.40) \]

where the quantity \( M = \int_{V} \rho(N^{T}N)dV \) is known as the consistent mass matrix.

*Implicit Finite Element Method*

For quasi-static problems where inertial forces are negligible, hence accelerations, \( \ddot{\mathbf{u}} \), can be ignored, Equation (3.39) can be written as follows for any time \( t \).
3. Theory

\[ G(\mathbf{u}) = 0 \]  
(3.41)

The Abaqus/Standard implicit solver takes an incremental approach to the solution of quasi-static problems, and uses the Newton-Raphson iterative method to ensure that Equation (3.41) is solved at the end of each time increment \( \Delta t \). Specifically, if \( n \) is the time at the start of the current increment, then the Newton-Raphson formula for iteration states that

\[
\mathbf{u}_{n+1}^{\Delta t} = \mathbf{u}_n^{\Delta t} - \left[ \frac{\partial G(\mathbf{u}_n^{\Delta t})}{\partial \mathbf{u}} \right]^{-1} \cdot G(\mathbf{u}_n^{\Delta t})
\]  
(3.42)

where \( \mathbf{u}_n^{\Delta t} \) is the current estimate of the nodal displacement vector at time \( t + \Delta t \) and \( \mathbf{u}_{n+1}^{\Delta t} \) is the improved estimate after the \( n \)th iteration. Iterations proceed until convergence in the incremental solution is achieved. The method requires the determination and inversion of the Jacobian matrix, \( \frac{\partial G(\mathbf{u}_n^{\Delta t})}{\partial \mathbf{u}} \), to obtain each approximation. This incremental and iterative approach over the course of the deformation history provides equilibrium at the end of each time step.
Dynamic Implicit Finite Element Method

In Chapter 7 of this work the dynamic implicit finite element method in Abaqus/Standard is used to solve Equation (3.39) for the solid bone deformation in FSI analyses. This method provides for the integration of the dynamic equations of motion (Equation (3.39)) using an implicit time integration operator, specifically an operator defined in (Hilber et al., 1977) and detailed in (DS SIMULIA, 2012). The operator generates the nodal accelerations at the end of the time increment, \( \ddot{u}' \Delta t \). In conjunction with this, the nodal displacements and velocities at the end of the increment are determined from the Newmark formulae

\[
\begin{align*}
    u'^{t+\Delta t} &= u' + \Delta t \ddot{u}' + \Delta t^2 \left( \frac{1}{2} - \beta \right) \dddot{u}' + \beta \dddot{u}'^{t+\Delta t} \quad (3.43) \\
    \ddot{u}'^{t+\Delta t} &= \ddot{u}' + \Delta t \left( 1 - \gamma \right) \dddot{u}' + \gamma \dddot{u}'^{t+\Delta t} \quad (3.44)
\end{align*}
\]

where \( \beta = \frac{1}{4} (1 - \alpha)^2 \), \( \gamma = 1 - \alpha \) and \( -\frac{1}{2} \leq \alpha \leq 0 \). \( \alpha \) is a parameter that allows for control of numerical damping within the solution. The default value of negative 0.05 is used throughout analyses in this study, as recommended by the Abaqus manual, as it provides a little numerical damping, removing high frequency noise without having a significant effect on the lower frequency response (DS SIMULIA, 2012). The dynamic implicit method in
Abaqus/Standard uses the consistent mass matrix to calculate the inertial force terms.

**Explicit Finite Element Method**
In Chapter 5 of this thesis the solution of the BVP is achieved using the dynamic explicit finite element method as summarised in the following section. It is important to note as part of this that the explicit solutions in Chapter 5 are achieved in Abaqus using a lumped mass matrix $\tilde{M}$ in place of the consistent mass matrix, which is obtained by adding each row of the consistent mass matrix $M$ onto its diagonal. The lumped mass matrix is easily inverted and is constructed such that the momentum of the system using $\tilde{M}$ is equivalent to that of the system using $M$ (Belytschko et al., 2000).

The explicit method has been traditionally used to solve very large deformation dynamic problems. Accelerations and velocities are assumed to be constant during a time increment and are used to solve for the next increment in time. For the integration rule Abaqus/Explicit utilises the central difference method that can be expressed as follows

$$u^{n+1} = u^n + \Delta t^{n+1} \frac{u^n}{2}$$

(3.45)
3. Theory

\[ \ddot{\mathbf{u}}^{n+\frac{1}{2}} = \ddot{\mathbf{u}}^{n-\frac{1}{2}} + \frac{\Delta t^{n+1} + \Delta t^{n}}{2} \dot{\mathbf{u}}^{n} \]  

(3.46)

where \( n \) is the time increment number (which differs from the previous sections where \( n \) was the iteration number) with \( n - \frac{1}{2} \) and \( n + \frac{1}{2} \) being the half increment values before and after the \( n^{th} \) increment, and \( \Delta t \) is the time increment size.

For input to Equation (3.46) nodal accelerations are determined as follows

\[ \ddot{\mathbf{u}}^{n} = \ddot{\mathbf{M}}^{-1} \left( -\int_{V} \mathbf{B}^T \sigma^e dV + \int_{S} \mathbf{N}^T \mathbf{t}^e dS \right). \]

(3.47)

For a quasi-static analysis to be achieved in Abaqus/Explicit it should be checked that the inertial forces do not affect the mechanical response as this could provide unwanted dynamic results. It has been shown that by keeping the ratio of kinetic energy to total internal strain energy at < 5 %, dynamic effects in the model are negligible (Choi et al., 2002; Chung et al., 1998).

FE implementations as described above can be classified as Lagrangian methods, i.e. when the mesh moves with the material deformation. The main difference between an explicit and an implicit solver is that the explicit
procedure requires no iterations and no stiffness matrix assembly or inversion. For further information on the details of the Abaqus implementations, the reader is referred to the Abaqus V6.12 Theory Manual 2012 (DS SIMULIA, 2012).

3.4. Computational Fluid Dynamics

3.4.1. Fluid Mechanics
The laws of conservation of mass and linear momentum, where the conservation of momentum (Newton’s Second Law of motion) is used to describe the balance between surface forces (hydrostatic pressure and viscous forces) and body forces (such as acceleration due to gravity), can be written as follows

\[
\frac{\partial \rho}{\partial t} + \nabla \cdot (\rho \mathbf{v}) = 0 \tag{3.48}
\]

\[
\frac{\partial}{\partial t} (\rho \mathbf{v}) = -[\nabla \cdot \rho \mathbf{v} \mathbf{v}] + [\nabla \cdot \mathbf{a}] + \rho \mathbf{f} = 0. \tag{3.49}
\]

In Equation (3.49), \(\frac{\partial}{\partial t} (\rho \mathbf{v})\) is the rate of change of momentum per unit volume. The terms on the right hand side of the equation correspond to the forces acting on a unit volume of fluid, \(\nabla \cdot \rho \mathbf{v} \mathbf{v}\) is the momentum flux
(momentum per unit area per unit time) associated with the bulk flow of fluid, $\rho f$ is the body force vector $f$ on the unit of fluid volume, and the term $\nabla \cdot \sigma$ represents the divergence of the stress tensor $\sigma$ which can be identified as the Cauchy Stress (Equation (3.19)). In keeping with fluid mechanics convention, the deviatoric stress $S$ is now referred to as the shear stress tensor $\tau$ such that

$$\sigma = -pI + \tau. \quad (3.50)$$

Using Equation (3.50), the momentum equation (Equation (3.49)) can be reduced to the following

$$\frac{\partial}{\partial t} (\rho \mathbf{v}) + \nabla \cdot (\rho \mathbf{v}\mathbf{v}) = -\nabla p + \nabla \cdot \mathbf{\tau} + \rho \mathbf{f}. \quad (3.51)$$

For an incompressible fluid this can be further simplified to

$$\rho \left[ \frac{\partial \mathbf{v}}{\partial t} + (\mathbf{v} \cdot \nabla)\mathbf{v} \right] = -\nabla p + \nabla \cdot \mathbf{\tau} + \rho \mathbf{f}. \quad (3.52)$$

Equation (3.48) is commonly referred to as the continuity equation. For an incompressible fluid this simplifies to the following
Equations (3.48) and (3.51) represent the Navier Stokes equations.

The Reynolds Number, \( Re \), can be used to determine whether the flow is turbulent or laminar:

\[
Re = \frac{\rho V D}{\mu}
\]  

(3.54)

Here \( V \) and \( D \) are the characteristic velocity of the fluid and characteristic length of the fluid flow channel, respectively, and \( \mu \) is now the viscosity of the fluid. A fluid flow is said to be laminar when \( Re < 2000 \).

3.4.2. Constitutive Laws

\textit{Newtonian Fluids}

For a Newtonian fluid there is a linear relationship between the viscous shear stress \( \tau \) and the rate of strain. For two dimensional flow this can be written as follows

\[
\tau = \mu \frac{dv}{dx}
\]  

(3.55)
where \( \frac{dv}{dx} \) is the velocity gradient perpendicular to the direction of shear.

### 3.4.3. Numerical implementation of Computational Fluid Dynamics

In Abaqus/CFD an implicit solver is used to integrate the Navier Stokes equations (Equations (3.48) and (3.51)) with respect to a defined spatial domain in order to compute the solution. This is an Eulerian approach where fluid flows through the spatial domain, and the associated mesh of the numerically discretised domain, which is in contrast to the mesh deforming with the material as happens in Lagrangian methods. The divergence theorem is used to convert volume integrals to surface integrals, and hence the general continuity and momentum equations are written in indicial notation as

\[
\frac{d}{dt} \int_V \rho dV + \int_S \rho v^j dn_j = 0 \tag{3.56}
\]

\[
\frac{d}{dt} \int_V \rho v^i dV + \int_S \rho v^i v^j dn_j = -\int_S \rho dn_j + \int_V \mu_{eff} \left( \frac{\partial v^i}{\partial x_j} + \frac{\partial v^j}{\partial x_i} \right) dn_j + \int_S S_{ij} dV \tag{3.57}
\]

where \( V \) and \( S \) denote the volume and surface regions of integration, \( dn_j \) is a component of the differential outward normal surface vector, \( v^i \) and \( v^j \) are velocity components, \( p \) is the pressure, \( \mu_{eff} \) is the effective viscosity and \( S_{ij} \) is
3. Theory

a component of the velocity forces acting on the fluid. The volume integrals
represent source and accumulation terms, and the surface integrals represent
the summation of fluxes.

The Finite Volume Method is used to solve Equations (3.56) and (3.57), and
this method is similar to the previously described FE method where the
nodes are on the element vertices. Such an approach can be summarised as
follows. The spatial domain is discretised into finite control volumes, all of
which form the mesh. Following the FE approach detailed in Equations
(3.34) and (3.35), shape functions are employed to calculate spatial
derivatives of velocity components. The discretisation of the spatial domain
into finite control volumes, using a mesh, allows the governing equations to
be integrated over each control volume, so that quantities such as mass,
momentum, energy, etc., are conserved in a discrete sense for each control
volume. After discretisation, Equations (3.56) and (3.57) can be written for
each control volume as

\[ V \left( \frac{\rho^n - \rho^{n-1}}{\Delta t} \right) + \sum_{p} m_{ip} = 0 \quad (3.58) \]

\[ V \left( \frac{\rho^n v_i - \rho^{n-1} v_i^{n-1}}{\Delta t} \right) + \sum_{p} m_{ip} (v_i)_{ip} = \sum_{p} (P \Delta n_i)_{ip} \]
3. Theory

\[ + \sum_{ip} \left( \mu_{eff} \left( \frac{\partial v_i}{\partial x_j} + \frac{\partial v_j}{\partial x_i} \right) \Delta n_{ij} \right)_{ip} + \bar{S}_{vi} V \]

(3.59)

where \( \dot{m}_p = (\rho v / \Delta n_i)_{ip} \) is the mass flow, \( (P \Delta n_i)_{ip} \) is the pressure gradient term, \( \bar{S}_{vi} \) is the average velocity force component for the control volume, \( V \) is the control volume, \( \Delta t \) is the time step, \( \Delta n_i \) is a component of the discrete outward surface vector, the subscript \( ip \) denotes evaluation at an integration point, and \( n \) is the time increment number. Summations are performed over all the integration points in each finite control volume. For further information on the discretisation of the Navier Stokes equations the reader is referred to the Abaqus Analysis User Manual (DS SIMULIA, 2012).

The non-linear equations are solved iteratively using a Newton-like method. The application of the finite volume methods to each control volume, followed by the assembly of the resulting equations, results in the generation of a global system of algebraic equations which can be written in vector-matrix notation as follows

\[ A\phi = b \]

(3.60)

where \( A \) denotes the coefficient matrix, \( \phi \) denotes the solution vector while the matrix \( b \) denotes the right hand side vector.
For simulations with deforming meshes, such as FSI, the momentum equation can be written as follows

\[
\frac{d}{dt} \int_\Omega \rho v_i dV + \int_\Omega \left[ \rho (v_j - w_j) \right] v_j dn_j = -\int_\Omega p dV + \int_\Omega \mu_{ij} \left( \frac{\partial v_i}{\partial x_j} + \frac{\partial v_j}{\partial x_i} \right) dV + \int_\Omega S_{ij} dV
\]

(3.61)

where \( w_i \) is a component of the velocity vector of the moving mesh (velocity of control volume boundary).

The divergence output and global kinetic energy can be monitored in the output files to determine whether steady state flow has been reached. The divergence value should typically be < 0.001 with smaller values indicating better mass balance. When the kinetic energy asymptotically reaches a constant value, the flow is achieving a steady-state condition where the velocities and pressure typically do not vary in time. For further information on how the equations are solved the reader is referred to the Abaqus Analysis User Manual (DS SIMULIA, 2012) and also the Ansys CFX Solver Theory provides greater depth into the discretisation and solution theory for CFD (ANSYS Inc., 2009). In this thesis, simple laminar flow of an incompressible Newtonian fluid is assumed.
3.5. Relevant Implementations

Computational models within this thesis consist of both CFD-only (Chapter 7) and FSI approaches (Chapters 5 and 6). The CFD-only approach has been outlined in the section above while the FSI implementation differs between the two chapters. In Chapter 5 the fluid analysis is coupled with an explicit solid analysis, whereas Chapter 6 utilises a fluid analysis coupled with a dynamic implicit analysis. This change in approach was found to be necessary to successfully generate a solution due to the larger model sizes in Chapter 6 compared to Chapter 5. While the differences between dynamic implicit and explicit analyses are outlined above, the coupling of the solid and fluid solutions remains the same through the utilisation of co-simulation within Abaqus to perform FSI, outlined below.

3.5.1. Fluid Structure Interaction

FSI deals with problems where the fluid flow affects compliant structures, which in turn affect the fluid. In this thesis, the solid and fluid analyses are fully coupled using the Abaqus co-simulation engine (Gauss-Seidel coupling algorithm), allowing for a two-way coupled FSI. The equations for the solid and fluid models are solved separately, and loads and boundary conditions are exchanged at the interface region. By default, with the Gauss-Seidel coupling scheme, the solid analysis leads the simulation, solving for a time
increment which is followed by the fluid analysis solving for the same increment before the solid proceeds with the next increment.

Fluid flow is modelled in an Eulerian domain, incorporating the Arbitrary Lagrangian-Eulerian (ALE) method which is used when the boundaries of the Eulerian domain are moving due to FSI. The ALE method is used in these co-simulations to allow for the fluid mesh to deform. The Lagrangian problem is first solved to obtain solution equilibrium. A default hyperfoam material (automatic material properties) is applied to the fluid mesh. Following this, nodes on the boundary of the domain of interest are moved with prescribed velocities independent of the underlying material, using an Eulerian approach. For further information on FSI in Abaqus the reader is referred to the Abaqus Analysis User Manual (DS SIMULIA, 2012).
4. Osteogenic differentiation of mesenchymal stem cells is regulated by osteocyte and osteoblast cells in a simplified bone niche

4.1. Chapter Background

In this chapter the role of biochemical signalling from osteocytes and osteoblasts, using conditioned media and co-culture experiments, is examined to understand how they direct osteogenic differentiation of MSCs. Furthermore the synergistic relationship between osteocytes and osteoblasts is examined by transwell co-culturing of MSCs with both simultaneously.

This chapter addresses Hypothesis 1, detailed in Section 1.3. The relative roles of osteocytes and osteoblasts in stimulating osteogenesis in MSCs are examined and insights into the mechanisms at work within the native stem cell niche to stimulate osteogenic differentiation are provided, outlining a possible role for the use of co-culture or conditioned media methodologies in tissue engineering applications.
4. Osteogenic differentiation of MSCs is regulated by osteocyte and osteoblast cells in a simplified bone niche

4.2. Introduction

As outlined in Section 2.4, MSCs are ideal candidates for tissue engineering and regenerative medicine applications. When MSCs are cultured in osteogenic media they express markers known to be expressed by bone forming osteoblasts, which are the cells responsible for laying down the matrix and mineral during new bone formation in vivo. The three stages of osteogenic differentiation of MSCs in vitro have been discussed in Section 2.4.1. As mentioned in that section, the driving source for the osteogenic differentiation of MSCs in their native environment remains unclear. As biochemical and mechanical factors influencing MSC fate within their native environment are different from those used in tissue regeneration strategies and cell culture studies in vitro, it is likely that approaches for bone tissue regeneration would be enhanced if the in vivo environment was better understood.

In vivo, MSCs are found within a unique environment known as the stem cell niche (Li and Xie, 2005). The stem cell niche in bone consists of a host of different support cells including hematopoietic progenitors and their progeny – such as blood cells, immune cells, and osteoclasts – and MSCs and their progeny including fibroblasts, endothelial cells, adipocytes, osteoblasts, as well as osteocytes found embedded in bone and osteoclasts (Kuhn and
4. Osteogenic differentiation of MSCs is regulated by osteocyte and osteoblast cells in a simplified bone niche

Tuan, 2010). Schofield identified the niche as having the functions of; (1) maintaining quiescence, (2) promoting cell number and (3) directing differentiation (see Section 2.5 and (Schofield, 1978). It is likely that these support cells play a role in directing the functions of the stem cell niche (Fuchs et al., 2004). In particular it is believed that osteoblasts and osteocytes are key regulators of osteogenesis (Csaki et al., 2009; Gu et al., 2001; Heino et al., 2004), but how exactly these cells regulate the osteogenic differentiation of MSCs is not yet understood.

The aim of the research in this chapter is to examine the influence of secreted factors from osteoblasts and osteocytes on the osteogenic differentiation of MSCs. In Section 1.3 Hypothesis 1 proposes that the osteogenic differentiation of MSCs in vivo is regulated by osteoblasts and osteocytes. The role of osteoblasts and osteocytes as bone regulatory cells is examined, focusing on the osteogenic response induced in MSCs when exposed to factors from osteoblast and osteocyte cells. This is examined using two different studies: (1) conditioned media and (2) indirect co-culture. It is also hypothesised that there exists a communication network between osteocytes and osteoblasts, which produces a stronger response in MSCs compared to either osteocytes or osteoblasts alone. This is studied using a three-layer co-
culture allowing physical connections between osteocytes and osteoblasts and exposing MSCs to the biochemicals produced.

4.3. Methods

4.3.1. BALB/c MSC: Isolation and Characterisation

BALB/c MSC cultures are obtained and characterised according to the protocols of (Peister et al., 2004). This work was performed with Georgina Shaw at REMEDI, NUI Galway. Briefly, 8 male mice 8 to 10 weeks old are used and cells are pooled. The femurs and tibiae are removed and cleaned of any attached tissue, and placed in complete isolation media (CIM). CIM consists of RPMI-1640 (Sigma Aldrich, Dublin) supplemented with 9% fetal bovine serum (FBS, EU Thermo Scientific HyClone), 9 % horse serum (HS; Sigma Aldrich), 100 U/mL penicillin (Sigma Aldrich), 100 g/mL streptomycin (Sigma Aldrich), and 2 M L-glutamine (Sigma Aldrich). The ends of the bone are clipped off and the bone is placed in an eppendorf tube containing a P200 wide bore tip. The bones are centrifuged at 400g for two minutes and the cell pellet is resuspended in 3 ml of CIM. Cells are incubated in a T175 flask with 30 ml of CIM.

After 24 hours, the media are removed and cells are washed with sterile phosphate buffered solutions (PBS, Sigma). 30 ml of fresh CIM are added
and media changed every 3 to 4 days. After four days large colonies are observed. Cells are trypsinised and replated in CIM and after 10 days cells are trypsinised again and seeded at 50 cells/cm² in CIM. After 3 weeks cells are trypsinised and frozen in Iscove’s modified Dulbecco medium (IMDM, Sigma Aldrich) with 5 % DMSO (Sigma Aldrich) and 30 % FBS. For experiments BALB/c MSCs are thawed and maintained in complete expansion media (CEM) containing IMDM supplemented with 10 % FBS, 10 % HS, 2 mM L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin until confluent.

To confirm the adipogenic potential of the MSCs used, 2 x 10⁴ cells/cm² are incubated in CEM expansion media until they reached confluency. Then adipogenic induction media is added containing IMDM with 10 % FBS, 100 U/mL penicillin, 100 g/mL streptomycin, 10 µg/mL insulin (Sigma Aldrich), 200 µM indomethacin (Sigma Aldrich), 1µM dexamethasone, and 500 µM MIX (Sigma Aldrich). After 3 days, maintenance media is added to the cells containing DMEM, 10 % FBS, 10 µg/mL insulin, 100 U/mL penicillin and 100 g/mL streptomycin. Three cycles of induction and maintenance media are completed. The cells are fixed with 10 % formalin and stained with 0.5 % Oil Red O (Sigma Aldrich) in methanol (Sigma Aldrich).
To confirm the osteogenic potential of the MSCs used, BALB/c MSCs (2 x 10^4 cells/cm^2) are incubated in CEM until a confluent layer is achieved and then osteogenic media is added, containing IMDM supplemented with 9 % FBS, 9 % HS, 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, 50 ng/ml L-thyroxine (Sigma Aldrich), 20 mM β-glycerol (Sigma Aldrich), 100 nM dexamethasone (Sigma Aldrich) and 50 µM ascorbic acid (Sigma Aldrich). Media is changed every 3-4 days. After 17 days cells are fixed in 10 % formalin (Sigma Aldrich) and stained with 10 % Alizarin Red (Sigma Aldrich).

To confirm the chondrogenic potential of the MSCs used, 5 x 10^5 cells are seeded in pellet culture. Cells are incubated in 500 µl of complete chondrogenic medium (CCM), containing IMDM, 100 nM dexamethasone, 50 µg/ml ascorbic acid, 40 µg/ml L-Proline (Sigma Aldrich), ITS+supplement (Sigma Aldrich), 1 mM sodium pyruvate (Sigma Aldrich), 100 U/ml penicillin, 100 µg/ml streptomycin, 10 ng/ml of TGFβ-3 (Sigma Aldrich) and 100 ng/ml BMP-2 (Sigma Aldrich). Media is changed three times a week. After 21 days pellets are harvested, embedded in paraffin and stained with Toluidine Blue Sodium Borate (Sigma Aldrich) and Safranin O (Sigma Aldrich).
4. Osteogenic differentiation of MSCs is regulated by osteocyte and osteoblast cells in a simplified bone niche

4.3.2. MLO-Y4 and MC3T3-E1 cell cultures

Two bone cell lines are used in this study. MLO-Y4 cells are a murine derived cell line which shares numerous characteristics with primary osteocytes such as high production of osteocalcin and low expression of alkaline phosphatase as well as producing numerous dendritic processes (Kato et al., 1997). MLO-Y4 cell cultures are maintained on collagen coated plates in α-modified minimal essential medium (α-MEM) supplemented with 2.5 % FBS, 2.5 % iron supplemented calf serum (CS, HyClone Laboratories Inc), 2 mM L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin.

MC3T3-E1 cells are an osteoblast cell line which express high amounts of alkaline phosphatase, produce mineral and are capable of differentiating into osteocytes making them appropriate for osteoblast studies (Sudo et al., 1983). They are considered to be a good model of primary osteoblasts when treated with osteogenic media (Quarles et al., 1992). The MC3T3-E1 cells are maintained in α-MEM supplemented with 5 % FBS, 2 mM L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin.
4. Osteogenic differentiation of MSCs is regulated by osteocyte and osteoblast cells in a simplified bone niche

4.3.3. Conditioned media experiment (CM)

MC3T3-E1 cells (osteoblast cell line) are plated at a density of 5,250 cells/cm² and are pre-treated for 24 hours in osteogenic media (αMEM, 10% FBS, 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, 20 mM β-glycerol phosphate, 50 µM ascorbic acid and 100 nM dexamethasone). This pre-treatment is carried out to establish the osteoblast cell phenotype prior to the beginning of the study (Sudo et al., 1983). After pre-treatment cells are cultured in standard osteoblast media (α-MEM, 5% FBS, 2 mM L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin) for a further 24 hours. MLO-Y4 cells are plated in separate 6 well plates at the same density in their standard osteocyte media (α-MEM with 2.5% FBS, 2.5% CS, 2 mM L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin) for 24 hours, this pre-treatment again is carried out to establish the osteocyte cell phenotype (Kato et al., 1997).

Conditioned media is collected from either the MLO-Y4 cells (MLO-Y4 CM) or the MC3T3-E1 cells (MC3T3-E1 CM), centrifuged at 300 g, and the supernatant is added at a ratio of 2:1 to the MSC media (yielding a 33% dilution) and this media is used for MSCs, which were plated at a density of 5,250 cells/cm² in separate six well plates for 24 hours. Media is changed every three days, including fresh conditioned media each time. Controls
consists of MSCs cultured in CEM (Control) and a positive control (Osteogenic Media) of MSCs with osteogenic media (IMDM with 10 % FBS, 10 % Horse Serum, 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, 20 mM β-glycerol phosphate, 50 µM ascorbic acid and 100 nM dexamethasone). As for all experiments osteogenic differentiation of MSCs is quantified by monitoring ALP expression, calcium deposition and cell number at 2 days, 3 days, 7 days, 10 days and 14 days, as described in detail below. For all the examinations (both co-culture and conditioned media studies) two independent experiments are conducted, for both, with at least two repeats in each experiment (total n > 4).

4.3.4. Co-culture experiment (CC)

MSCs are exposed to inserts containing permeable membranes (PET 1µm pores, Millipore) on which osteoblasts or osteocytes are grown, which allowed for simultaneous culture of the cells but, as they are physically separated the MSCs and support cells by a distance of 0.9 mm, they prevented direct cell to cell contact. MSCs (5,250 cells/cm²) are cultured for 24 hours on the bottom of 6 well plates in CEM and then exposed to either a MLO-Y4 (MLO-Y4 CC), MC3T3-E1 (MC3T3-E1 CC) or a three-layer co-culture (3CC) seeded insert. MSCs are also exposed to a control of an MSC seeded membrane (MSC CC).
4. Osteogenic differentiation of MSCs is regulated by osteocyte and osteoblast cells in a simplified bone niche

For the MC3T3-E1 CC group, MC3T3-E1 cells are seeded at 5,250 cells/cm² on the apical side of the insert membranes and pre-treated for 24 hours in osteogenic media before being cultured in standard osteoblast media for 24 hours. For the MLO-Y4 CC group, MLO-Y4 cells are seeded at the same density on the apical side of the insert membranes with their standard osteocyte media (Figure 4.1a). A three-layered co-culture (3CC) is prepared with MLO-Y4 cells seeded on the apical side of the insert membrane and MC3T3-E1 cells on the basal side of the same membrane. The pore size is chosen to allow the MLO-Y4 and MC3T3-E1 cells to form functional gap junctions across the membrane (Taylor et al., 2007). To achieve this, inserts are first turned upside down and 2,600 cells/cm² MC3T3-E1 cells are seeded on the basal surface in 500 µl of media.

These cells are allowed to attach for six hours before the insert is turned and seeded with 2,600 cells/cm² MLO-Y4 cells on the apical side. Finally, for the control (MSC CC), 5,250 cells/cm² MSCs are seeded on the apical side of the insert membranes in CEM for 24 hours (Figure 4.1b). For all co-culture studies cells on each side of the membrane receive complete expansion media (CEM, i.e. non-osteogenic) media.
4. Osteogenic differentiation of MSCs is regulated by osteocyte and osteoblast cells in a simplified bone niche

Figure 4.1: Schematic diagram displaying the set-up of (a) co-culture of MSCs with either osteocytes (MLO-Y4 CC), osteoblasts (MC3T3-E1 CC) or the control of MSCs (MSC CC) and (b) the three-layer co-culture (3CC) where osteocytes are cultured on top of the membrane, osteoblasts on the underside and MSCs in the bottom of the well.

4.3.5. Alkaline Phosphatase

After exposure to conditioned media or co-culture for periods of 2 days, 3 days and 7 days, cells are washed with phosphate buffered saline (PBS, Sigma) and fixed with 10% formalin (Sigma) and washed again three times with PBS. Extracellular ALP activity is examined histochemically using Fast Violet B salt (Sigma) and Naphthol AS-MX phosphate (Sigma). After ALP staining, the samples are washed with PBS and stained with 4',6-diamidino-2-phenylindole (DAPI, Sigma). Five images are taken at random locations in each sample for ALP staining and DAPI staining using an Olympus IX71 Inverted Fluorescent Microscope with Olympus Cell P Software. Images are analysed using Image J software (NIH) for cell count per cm² (DAPI) and percentage stain ALP per total image area.
Intracellular ALP activity is measured colorimetrically using Alkaline Phosphatase Colorimetric Assay Kit (Abcam, Cambridge, UK) which uses \( p \)-nitrophenyl phosphate (pNPP) as a phosphatase substrate. Cell lysate is prepared using three cycles of freeze-thaw in deionised distilled water. 30 µl of the cell lysate is added to a 96 well plate with 50 µl of assay buffer and 50 µl of pNPP. The samples are shielded from direct light at room temperature for one hour. After this, 20 µl of Stop Solution (3N NaOH) is added to the wells and the plate is read at 405 nm in a micro-plate reader (Wallac Victor3 1420 Multilabel Counter).

4.3.6. DNA Content

Measuring DNA content gives an indication of cell number within cell numbers with in an increase of DNA indicative of an increase in cell number. The cell lysate (prepared for ALP assay) is measured for DNA content using the fluorescent dye bisBenzimide H 33258 (Hoechst 33258, Sigma). Briefly, 50 µl of the cell lysate is added to a 96 well plate with 50 µl of assay buffer and 100 µl of Hoechst Dye. A 0.1 µg/ml solution is used for 48 and 72 hour time points and a 1 µg/ml solution for the later time points. The plate is incubated away from light for ten minutes and then read on a microplate reader (Wallac Victor3 1420 Multilabel Counter) at excitation of 350 nm and
emission of 450 nm. Cell number is then determined by using a known amount of purified calf thymus DNA as the standard.

4.3.7. Mineralization

Media is removed from MSCs and cells washed three times with PBS. They are then evaluated for calcium production at 10 days and 14 days by staining with 10% Alizarin Red solution (Sigma Aldrich). Alizarin Red S is a dye that binds to calcium salts. After fixation and staining, cells are again washed three times with PBS. To quantify the staining, 1 ml of 10% cethylpyridium chloride (Sigma Aldrich) is added to each well and incubated for 20 minutes to elute the stain. 100 µl of this eluted stain is added to 96 well plates and read at 550 nm using a spectrophotometer (Nørgaard et al., 2006). A standard curve is prepared using Alizarin Red stain and cethylpyridium chloride. The calcium deposition is expressed as molar equivalent of calcium. One mole of Alizarin Red binds to two moles of calcium in an Alizarin Red S-calcium complex.

4.3.8. Statistical Analysis

DNA content using the Hoechst staining method, increase in cell number using DAPI, extracellular ALP stained per well, in addition to intracellular ALP and calcium content (both normalized to DNA content) are all
examined. The results are compared between groups for conditioned media and co-culture studies separately with a one-way analysis of variance (ANOVA) is performed followed by pair-wise comparison (Tukey test), using Minitab’s General Linear Model function. A student’s t-test (Minitab) is applied to compare the calcium deposition normalized to DNA content between MLO-Y4 CC and MLO-Y4 CM groups. For all comparisons, the level of significance is $p \leq 0.05$.

4.4. Results

The results of this chapter are presented in detail below. The first section demonstrates the multipotent nature of the BALB/c MSCs used in the experiments. The subsequent sections each address specific question related to Hypothesis 1, detailed in Section 1.3 Firstly, “The osteogenesis of MSCs in vivo is regulated by osteoblasts and osteocytes” and secondly "There exists a communication network between osteocytes and osteoblast which produces a robust osteogenic response in MSCs”.

4.4.1. Characterisation of BALB/c MSCs

Differentiation assays confirm the multipotency of the isolated MSCs. They display positive markers of osteogenic, adipogenic and chondrogenic differentiation (Figure 4.2).
4. Osteogenic differentiation of MSCs is regulated by osteocyte and osteoblast cells in a simplified bone niche

Figure 4.2: Characterisation of BALB/c MSCs. Fat globules are visible in MSC adipogenic test culture (a) but not in control (b) after staining with Oil-Red-O indicating that MSCs are undergoing adipogenic differentiation. Calcium deposition can be seen after staining with Alizarin Red in the osteogenic test culture (c) showing the positive result for osteogenic differentiation compared to the control (d). Staining with Toluidine Blue for chondrocyte-associated proteoglycan in the chondrogenic test cultures is positive within the pellet as indicated by the purple colour (e). This is confirmed by Safranin O staining positive for the presence of cartilage within the pellet culture (f). Scale bar = 200 µm, same scale for all images.
4. Osteogenic differentiation of MSCs is regulated by osteocyte and osteoblast cells in a simplified bone niche

4.4.2. Are osteocytes the regulatory cells that govern osteogenic differentiation of MSCs?

Cell Number
MSCs that are treated with conditioned media from MLO-Y4 cells (MLO-Y4 CM) have a lower DNA content than MSCs in their normal media (Control) at all time points, and are significantly lower from MSCs in their normal media at 2 days ($p = 0.0075$) and 14 days ($p = 0.0179$) (Figure 4.3a). A similar trend is found for MSCs that are exposed to an insert containing MLO-Y4 cells (MLO-Y4 CC) with a lower DNA content, compared to the control of MSCs exposed to an MSC seeded insert (MSC CC), at 2 days ($p < 0.0001$), 10 days ($p = 0.0056$) and 14 days ($p < 0.0001$) (Figure 4.3b). When the relative change in cell number between day 0 and day 7 is examined it is found that the MLO-Y4 CM group has a lower cell number than the osteogenic group ($p = 0.0013$) and the MLO-Y4 CC group is significantly lower than the MSC CC group ($p < 0.0001$) (Figure 4.4).

ALP activity
The extracellular ALP activity by MSCs in the MLO-Y4 CM group is not significantly different to the control group at any time point and is lower than the osteogenic group at 7 days ($p < 0.0001$), see Figure 4.5a. ALP secretion by MSCs in the MLO-Y4 CC group is greater than that of the MSC CC group at 7 days ($p = 0.0086$), see Figure 4.5b. Intracellular ALP activity
4. Osteogenic differentiation of MSCs is regulated by osteocyte and osteoblast cells in a simplified bone niche

**Figure 4.3.** DNA content at 2 days, 3 days, 7 days, 10 days and 14 days as measured by the Hoechst Assay for (a) MSCs exposed to conditioned media from MLO-Y4 (MLO-Y4 CM) and MC3T3-E1 cells (MC3T3-E1 CM). Also shown is the cell DNA content for MSCs grown in normal complete expansion media (Control) and MSCs grown in osteogenic media (Osteogenic Media). A $p < 0.05$ versus Control, B $p < 0.05$ versus MC3T3-E1 CM, C $p < 0.05$ versus MLO-Y4 CM, D $p < 0.05$ versus Osteogenic Media (b) DNA content for MSCs co-cultured with MLO-Y4 cells (MLO-Y4 CC), MC3T3-E1 cells (MC3T3-E1 CC), a three-layer co-culture with both MLO-Y4 and MC3T3-E1 cells (3CC) and MSCs co-cultured with MSCs (MSC CC). A $p < 0.05$ versus MSC CC, B $p < 0.05$ versus MC3T3-E1 CC, C $p < 0.05$ versus MLO-Y4 CC, D $p < 0.05$ versus 3CC. Error bars denote standard deviation ($n = 4$).
4. Osteogenic differentiation of MSCs is regulated by osteocyte and osteoblast cells in a simplified bone niche

Figure 4.4: Relative cell number increase from day zero to day seven (fold change) as measured by DAPI staining for (a) MSCs exposed to conditioned media from MLO-Y4 (MLO-Y4 CM) and MC3T3-E1 cells (MC3T3-E1 CM). Also shown is the cell DNA content for MSCs grown in normal complete expansion media (Control) and MSCs grown in osteogenic media (Osteogenic Media). A $p < 0.05$ versus Control, B $p < 0.05$ versus MC3T3-E1 CM, C $p < 0.05$ versus MLO-Y4 CM, D $p < 0.05$ versus Osteogenic Media (b) DNA content for MSCs co-cultured with MLO-Y4 cells (MLO-Y4 CC), MC3T3-E1 cells (MC3T3-E1 CC), a three-layer co-culture with both MLO-Y4 and MC3T3-E1 cells (3CC) and MSCs co-cultured with MSCs (MSC CC). A $p < 0.05$ versus MSC CC, B $p < 0.05$ versus MC3T3-E1 CC, C $p < 0.05$ versus MLO-Y4 CC, D $p < 0.05$ versus 3CC. Error bars denote standard deviation ($n = 5$).
4. Osteogenic differentiation of MSCs is regulated by osteocyte and osteoblast cells in a simplified bone niche

(normalised to DNA content) in the MLO-Y4 CM group peaks early and dissipates by 7 days (Figure 4.6a). At 2 days ALP activity is greater in the MLO-Y4 CM group than the control ($p < 0.0001$) and the osteogenic group ($p < 0.0001$). Although a rise in ALP activity is noted at three days compared to controls this is not statistically different, but by 7 days the ALP activity in the MLO-Y4 CM group is lower than the control ($p < 0.0001$) and the osteogenic group ($p = 0.0005$). An early peak is also found with MLO-Y4 CC group in the co-culture experiments (Figure 4.6b). ALP intracellular activity is greater in the MLO-Y4 CC group than the MSC CC group at 2 days ($p < 0.0001$), but decreases to control levels by day 7 (Figure 4.6b).

Calcium deposition
Calcium deposition in the MLO-Y4 CM group is lower than the osteogenic group at 10 and 14 days (significant at 14 days $p = 0.0003$) and has no significant difference to the control group (Figure 4.7a). However calcium deposition by MSCs in the MLO-Y4 CC groups is higher at 14 days than the MSC CC control group ($p \leq 0.05$) (Figure 4.7b).
4. Osteogenic differentiation of MSCs is regulated by osteocyte and osteoblast cells in a simplified bone niche

Figure 4.5: Extracellular ALP activity as measured histochemically at 2, 3 and 7 days, for (a) MSCs exposed to conditioned media from MLO-Y4 cells (MLO-Y4 CM) and MC3T3-E1 cells (MC3T3-E1 CM). Also shown is the ALP expression for MSCs grown in normal complete expansion media (Control) and MSCs grown in osteogenic media (Osteogenic Media) A $p < 0.05$ versus Control, B $p < 0.05$ versus MC3T3-E1 CM, C $p < 0.05$ versus MLO-Y4 CM, D $p < 0.05$ versus Osteogenic Media. (b) MSCs co-cultured with MLO-Y4 cells (MLO-Y4 CC), MC3T3-E1 cells (MC3T3-E1 CC), a three-layer co-culture with both MLO-Y4 and MC3T3-E1 cells (3CC) and MSCs co-cultured with MSCs (MSC CC). A $p < 0.05$ versus MSC CC, B $p < 0.05$ versus MC3T3-E1 CC, C $p < 0.05$ versus MLO-Y4 CC, D $p < 0.05$ versus 3CC. Error bars denote standard deviation ($n = 5$).
4. Osteogenic differentiation of MSCs is regulated by osteocyte and osteoblast cells in a simplified bone niche

Figure 4.6: Intracellular ALP activity normalised to DNA content at 2, 3 and 7 days for (a) MSCs exposed to conditioned media from MLO-Y4 cells (MLO-Y4 CM) and MC3T3-E1 cells (MC3T3-E1 CM). Also shown is the ALP expression for MSCs grown in normal complete expansion media (Control) and MSCs grown in osteogenic media (Osteogenic Media) \( ^{A} p < 0.05 \) versus Control, \( ^{B} p < 0.05 \) versus MC3T3-E1 CM, \( ^{C} p < 0.05 \) versus MLO-Y4 CM, \( ^{D} p < 0.05 \) versus Osteogenic Media. (b) MSCs co-cultured with MLO-Y4 cells (MLO-Y4 CC), MC3T3-E1 cells (MC3T3-E1 CC), a three-layer co-culture with both MLO-Y4 and MC3T3-E1 cells (3CC) and MSCs co-cultured with MSCs (MSC CC). \( ^{A} p < 0.05 \) versus MSC CC, \( ^{B} p < 0.05 \) versus MC3T3-E1 CC, \( ^{C} p < 0.05 \) versus MLO-Y4 CC, \( ^{D} p < 0.05 \) versus 3CC. Error bars denote standard deviation (\( n = 4 \)).
4. Osteogenic differentiation of MSCs is regulated by osteocyte and osteoblast cells in a simplified bone niche

Figure 4.7: Alizarin Red assay for calcium normalised to DNA content per well measured at 10 and 14 days for (a) MSCs exposed to conditioned media from MLO-Y4 cells (MLO-Y4 CM) and MC3T3-E1 cells (MC3T3-E1 CM). Also shown is the ALP expression for MSCs grown in normal complete expansion media (Control) and MSCs grown in osteogenic media (Osteogenic Media) ^p < 0.05 versus Control, ^p < 0.05 versus MC3T3-E1 CM, ^p < 0.05 versus MLO-Y4 CM, ^p < 0.05 versus Osteogenic Media. (b) MSCs co-cultured with MLO-Y4 cells (MLO-Y4 CC), MC3T3-E1 cells (MC3T3-E1 CC), a three-layer co-culture with both MLO-Y4 and MC3T3-E1 cells (3CC) and MSCs co-cultured with MSCs (MSC CC). ^p < 0.05 versus MSC CC, ^p < 0.05 versus MC3T3-E1 CC, ^p < 0.05 versus MLO-Y4 CC, ^p < 0.05 versus 3CC. Error bars denote standard deviation (n = 4).
4. Osteogenic differentiation of MSCs is regulated by osteocyte and osteoblast cells in a simplified bone niche

4.4.3. Are osteoblasts the regulatory cells that govern osteogenic differentiation of MSCs?

**Cell Number**
MSCs treated with conditioned media from MC3T3-E1 cells (MC3T3-E1 CM) have a higher DNA content than the control (MSCs in CEM) at 3 days ($p = 0.0003$) but are significantly lower than control by 14 days ($p < 0.0001$), see Figure 4.3a. Moreover the MC3T3-E1 CM group has a higher DNA content than the osteogenic group at all time points (2 days $p < 0.0001$, 3 days $p = 0.0001$, 7 days $p = 0.0055$). Similarly MSCs exposed to a insert containing MC3T3-E1 cells (MC3T3-E1 CC) have a high DNA content compared to the MSC CC control group (2 days $p = 0.0001$ and 3 days, 7 days and 14 days all $p < 0.0001$), see Figure 4.3b. Indeed when the relative change in cell number is examined the MC3T3-E1 CM group is significantly higher than the control group ($p = 0.0001$) and the osteogenic media group ($p = 0.0415$) (Figure 4.4).

**ALP activity**
Extracellular ALP activity for the MC3T3-E1 CM group at 2 and 3 days is not significantly different to the controls but is higher than the control (MSCs in CEM) at 7 days ($p = 0.0495$). However, it is significantly lower than the osteogenic group at 7 days ($p = 0.0001$), see Figure 4.5a. In the same way the ALP secretion for the MC3T3-E1 CC group is higher than the MSC CC group at 3 days ($p < 0.0001$) and 7 days ($p = 0.0495$) (Figure 4.5b).
Intracellular ALP activity in the MC3T3-E1 CC group is found to be lower than the osteogenic group at 2 days and 3 days (not significant). However, by 7 days it has the highest activity compared to the control \( (p = 0.0044) \) and the osteogenic group \( (p = 0.0001) \) (Figure 4.6a). This trend is replicated in the MC3T3-E1 CC group as at 7 days it has the highest ALP activity compared to the MSC CC group \( (p = 0.0003) \) (Figure 4.6b).

**Calcium deposition**
Calcium deposition in the MC3T3-E1 CM group is similar to the control of MSCs in CEM, but is significantly lower than the osteogenic group at 14 days \( (p = 0.0003) \). Similarly calcium deposition for the MC3T3-E1 CC group is not different to the control of MSC CC group at either 10 or 14 days (Figure 4.7b).

**4.4.4. Does the degree of osteogenic differentiation of MSCs differ between osteocyte and osteoblast signalling?**

**Cell Number**
The DNA content in the MC3T3-E1 CM group is greater than in the MLO-Y4 CM group at 2 days \( (p = 0.0001) \), 3 days \( (p = 0.0003) \) and 7 days \( (p = 0.0209) \), while at 14 days the order is reversed with MLO-Y4 CM group having the greater DNA content \( (p < 0.0001) \) (Figure 4.3a). Similarly comparing the MC3T3-E1 CC group with the MLO-Y4 CC group it can be seen that the
DNA content is greater in the MC3T3-E1 CC group (2 days \(p = 0.0005\), 3 days \(p < 0.0001\), 7 days \(p < 0.0001\) and 14 days \(p < 0.0001\)) (Figure 4.3b). This trend is confirmed when the relative change in cell number between day 0 and day 7 is examined; the MC3T3-E1 CM group shows a higher cell number than the MLO-Y4 CM group \((p < 0.0001)\). Similarly the MC3T3-E1 CC group shows a higher cell number than the MLO-Y4 CC group \((p < 0.0001)\) (Figure 4.4).

**ALP activity**

When the ALP extracellular secretion is examined no significant difference is found between MC3T3-E1 CM and MLO-Y4 CM groups (Figure 4.5a) and the only significant difference between the two co-culture groups is seen at 3 days with MC3T3-E1 CC groups showing a larger ALP secretion than the MLO-Y4 CC group \((p < 0.0001)\) (Figure 4.5b).

Examining intracellular ALP activity it is found that at 2 and 3 days the ALP expression is greater in the MLO-Y4 CM group compared to the MC3T3-E1 CM group \((p < 0.0001\) and 0.0423 respectively) (Figure 4.6a). However by 7 days the peak in the MLO-Y4 CM group has ended and the MC3T3-E1 CM group is greater \((p = 0.0001)\). A similar trend is found when the MLO-Y4 CC group is compared to the MC3T3-E1 CC group, with the MLO-Y4 CC group having the greater ALP expression compared to the MC3T3-E1 CC group at
4. Osteogenic differentiation of MSCs is regulated by osteocyte and osteoblast cells in a simplified bone niche

2 days \((p = 0.0004)\), while the opposite is true at 7 days \((p = 0.0001)\) (Figure 4.6b).

**Calcium deposition**

There is no difference between the MLO-Y4 CM and MC3T3-E1 CM groups at either 10 or 14 days (Figure 4.7a). However, investigation into the calcium deposition reveals that the MLO-Y4 CC group has a greater calcium deposition at 10 and 14 days compared to the MC3T3-E1 CC group \((p = 0.0496\) and \(0.0083\) respectively).

4.4.5. Do osteoblasts and osteocytes work in conjunction to regulate MSC differentiation?

**Cell Number**

The DNA content of the MSCs exposed to both MLO-Y4 and MC3T3-E1 cells simultaneously (3CC) is higher than the MLO-Y4 CC group at 2 days \((p = 0.0001)\), 7 days \((p = 0.0009)\) and 14 days \((p = 0.0139)\), while it is significantly lower than the MC3T3-E1 CC group at 3 days \((p < 0.0001)\), 10 days \((p = 0.0094)\) and 14 days \((p < 0.0001)\) (Figure 4.3b). It is also lower than the MSC CC group at 2 days \((p = 0.0007)\), 7 days \((p = 0.0032)\), 10 days \((p = 0.0220)\) and 14 days \((p < 0.0001)\). This is reflected when the relative change in cell number is analysed between day 0 and day 7. It is seen that the 3CC group has a
lower cell number than the MC3T3-E1 CC group ($p = 0.0002$) and the MSC CC group ($p = 0.0004$) (Figure 4.4).

**ALP activity**
The extracellular ALP activity of the 3CC group is greater than all the other groups at 2 days (MC3T3-E1 CC and MSC CC $p = 0.0001$, MLO-Y4 CC $p = 0.0062$). By 7 days the secretion has decreased and is lower than the MLO-Y4 CC group ($p = 0.0181$) (Figure 4.5b).

For the intracellular ALP activity it can be seen that the 3CC has significantly less ALP than the MLO-Y4 CC group ($p = 0.0038$) at 2 days but more than the MSC CC group at 2 days ($p = 0.0462$) and 3 days ($p = 0.0337$) and the MC3T3-E1 CC group at 3 days ($p = 0.0090$). But by 7 days the ALP activity has decreased and there is less ALP activity than in the MC3T3-E1 CC group ($p = 0.0001$) (Figure 4.6b).

**Calcium deposition**
At 10 days the 3CC group has the significantly greatest calcium deposition than all groups (MLO-Y4 CC $p = 0.0136$, MC3T3-E1 CC $p = 0.0001$ and MSC CC $p = 0.0020$). Similarly at 14 days the greatest calcium deposition is seen in the 3CC group which is significantly greater than all groups (MLO-Y4 CC $p = 0.0084$, MC3T3-E1 CC $p < 0.0001$ and MSC CC $p = 0.0001$) (Figure 4.7b).
4. Discussion

The results of this study demonstrate that culturing MSCs in the presence of bone cells can direct MSCs down the osteoblast lineage in the absence of osteogenic media. Moreover, these studies suggest that osteocytes are more influential than osteoblasts in stimulating osteogenesis in MSCs. Specifically, key markers of osteogenic differentiation (intracellular ALP and calcium deposition) are greater at earlier time points when MSCs are co-cultured with osteocytes rather than osteoblasts or MSCs. Most interestingly, it is found that when MSCs are exposed to factors from both osteoblasts and osteocytes simultaneously, the osteogenic effect is higher than exposure to either cell type alone. Moreover, this response seems to be greater than an additive effect of the two cell types. This outlines the functional relationship that exists in the osteocyte-osteoblast network. This study gives an important insight into the natural cues for osteogenic differentiation within the stem niche *in vivo*.

*In vitro* osteogenic factors are typically introduced directly into the culture media of MSCs to drive the MSCs toward osteogenic differentiation. However, it is unlikely that these same factors (ascorbic acid, dexamethasone and β-glycerol) are present within the natural environment *in vivo*. In previous studies (Heino et al., 2004; Ilmer et al., 2009; Lu et al., 2011; Wang et
4. Osteogenic differentiation of MSCs is regulated by osteocyte and osteoblast cells in a simplified bone niche

al., 2007) where enhanced osteogenic differentiation of MSCs is reported, by either conditioned media or in a co-culture with bone cells, media applied to MSC cultures is supplemented with osteogenic factors. In contrast, (Csaki et al., 2009) found a high density co-culture of osteoblasts and MSCs to produce as strong an osteogenic response as a co-culture supplemented with osteogenic media. It should be emphasised that in the present study no additional differentiation factors were used in the MSC media. These results indicate that bone cells themselves or bone tissue extracts could be used to regulate osteogenic differentiation of MSCs without addition of osteogenic factors, which could have important implications for tissue engineering applications. Moreover, it provides an important insight into the regulatory cells that are capable of producing biochemical factors required to drive MSC differentiation in vivo.

Osteocytes are terminally differentiated osteoblasts (Bonewald, 2007; Parfitt, 1977), which form a cellular syncytium within an extensive lacunar-canalicular network, composed of long dendritic processes which in turn form functional gap junctions with other osteocytes and osteoblasts (Alford et al., 2003; Cheng et al., 2001a; Doty, 1981). Due to their positioning within the bone and their comprehensive network, there is increasing evidence that osteocytes act as the mechanotransducers for bone (Burra et al., 2010; Cowin
Osteogenic differentiation of MSCs is regulated by osteocyte and osteoblast cells in a simplified bone niche (et al., 1995; Skerry et al., 1989; Tatsumi et al., 2007) and are also instrumental in regulating osteoclast formation from monocyte precursors (You et al., 2008). Here it is observed that osteocytes are capable of inducing a greater osteogenic response in MSCs than osteoblasts, both in terms of an increase in ALP expression in MSCs, which is a characteristic early marker of osteogenesis, and increased mineral production (a later osteogenic differentiation marker). This data further supports their role as regulators of bone cell biology and remodelling.

That osteocytes are more influential than osteoblasts in stimulating osteogenesis in MSCs (Figure 4.7b) is in agreement with previous studies (Heino et al., 2004; Hoey et al., 2011). In this study, it is additionally shown that this effect is observed in the absence of osteogenic factors. Furthermore results in this chapter show for the first time that, although the initial regulatory effect by osteoblasts is less, with time osteoblasts are also capable of stimulating osteogenesis in MSCs. This is in agreement with other studies where osteoblasts were shown to elicit an osteogenic response in MSCs when direct contact was allowed (Csaki et al., 2009; Wang et al., 2007). While it is unknown whether direct cell to cell contact between MSCs and osteoblasts actually occurs in vivo, other studies have shown that MSCs cultured with conditioned media collected from osteoblasts show an increase in early
4. Osteogenic differentiation of MSCs is regulated by osteocyte and osteoblast cells in a simplified bone niche

Osteogenic markers such as ALP and bone sialoprotein (BSP) (Ilmer et al., 2009). Moreover, studies where the two cell types are physically separated by a porous membrane, have similarly shown that molecular signals from osteoblasts up-regulate osteogenic markers such as BSP, Runx2 and osteocalcin in MSCs (Ilmer et al., 2009; Lu et al., 2011; Wang et al., 2007). However these results are in contrast to those studies that suggest that only osteocytes are capable of stimulating osteogenesis (Heino et al., 2004; Hoey et al., 2011). Discrepancies between different studies regarding the regulatory role of osteocytes and osteoblasts might be explained by the use of different cell types and species. Alternatively, these differences might reflect the stage of differentiation of the signalling osteoblasts, which according to our results will have an impact on the timing of the osteogenic differentiation of MSCs.

DNA results here indicate that in the presence of osteoblast factors, MSCs tend to proliferate initially and indeed significantly more so than those cultured with osteocyte factors. The findings show that MSCs exposed to osteoblast factors produce more ALP for the entire population. However, when the ALP is normalised to the DNA content, to account for the total cell number, MSCs exposed to osteoblast factors have lower levels of ALP production per cell. During osteogenic differentiation cells undergo proliferation followed by differentiation (Aubin, 2001; Huang et al., 2007),
and both events do not occur simultaneously. During normal osteogenic differentiation there is an initial peak in ALP production followed by a subsequent decrease as the cells mature and lay down mineral (Aubin, 2001; Huang et al., 2007; Thibault et al., 2010). Consistent with this, the osteocyte groups presented here demonstrate an initial increase in ALP production, but by 7 days ALP production is lower than that of MSCs exposed to osteoblast factors. Moreover, only the MSCs exposed to factors secreted from osteocytes produce mineral at the time points examined here. As such these data indicate that MSCs cultured in the presence of factors from osteoblasts initially undergo proliferation, whereas those cultured in the presence of factors from osteocytes initially differentiate. It is proposed that after osteoblasts themselves have differentiated into a more mature phenotype or osteocytes (Dallas and Bonewald, 2010), they are then able to encourage MSCs down the same osteogenic pathway, and that this effect could be overlooked in short term studies.

The biochemical signals that osteocytes and osteoblasts release to encourage an osteogenic response in MSCs remain unknown. Growth factors such as the TGF-beta family are known to have stimulatory effects on bone formation (Linkhart et al., 1996; Rawadi et al., 2003); however blocking TGF-\(\beta\)3 in MLO-Y4 media given to MSCs was found to have no significant effects
4. Osteogenic differentiation of MSCs is regulated by osteocyte and osteoblast cells in a simplified bone niche

on proliferation or osteoblast differentiation (Heino et al., 2004). Fibroblast growth factor-2 (FGF-2) has been proven to be effective at promoting the generation of osteogenic markers in MSCs (Gupta et al., 2010). As osteoblasts are known to express high amounts of IGFs as they differentiate (into osteocytes) it is probable this family of factors play a role in stimulating osteogenesis in MSCs observed here (Birnbaum et al., 1995). Bone Morphogenetic Proteins (BMPs) such as BMP-2 are growth factors also belonging to the TGF-beta family and are well established osteogenic stimulation factors (Chen et al., 2004). Runx2 and osteocalcin are expressed in higher quantities by osteocytes than osteoblasts (Ducy et al., 1997; Mikuni-Takagaki et al., 1995) and might be responsible for the quicker osteogenic response in MSCs exposed to osteocyte factors. Certain pro-osteogenic signalling molecules such as Nitric Oxide (NO) and PGE$_2$ are released in large amounts from mechanically stimulated osteocytes in vitro (Keila et al., 2001; Klein-Nulend et al., 1995a; Klein-Nulend et al., 1995b) but are also expressed in static conditions (Cheng et al., 2001a; McAllister et al., 2000). Therefore it is likely that these signals could also provide the stimuli for MSC osteogenic differentiation in vivo.

The use of the cell lines MLO-Y4 and MC3T3-E1 is a possible limitation to this study. However, both cell lines have been shown to be excellent
representatives of primary osteocytes and osteoblasts (Bonewald, 1999; Kato et al., 1997; Quarles et al., 1992; Sudo et al., 1983). Additionally, the use of serum is required to achieve osteogenic differentiation. It is possible that the results of the conditioned media experiments were influenced by serum from the osteocyte or osteoblast media. However, in our co-culture studies all cells (MSCs, osteoblasts and osteocytes) received the same media above and below the membrane, and under these conditions the observed effects were more pronounced. Therefore it is unlikely that the differences between osteoblasts and osteocytes observed here were due to differences in serum.

There is an interesting difference in results between the co-culture and conditioned media experiments as demonstrated by the mineralisation results (Figure 4.7a and b). The higher mineralisation levels in co-culture \( (p = 0.0060 \text{ for MLO-Y4 CC versus MLO-Y4 CM at 14 days}) \) compared to conditioned media, suggest that the physical presence of a directing cell type (osteoblast or osteocyte), which is continually producing biochemicals, enhances the osteogenic response seen in MSCs. Moreover the co-culture experiments are more representative of the \textit{in vivo} situation, wherein osteoblasts and osteocytes continually produce the necessary biochemicals, whereas the conditioned media set-up which provides only intermittent snapshots of the biochemical being produced. Potentially this adjusted be
used to allow differing levels of interaction between cells. Previous studies have not compared such set-ups and our study shows for the first time the importance of a directing cell type for tissue differentiation \textit{in vitro}.

It is probable that osteocytes and osteoblasts communicate with MSCs exclusively through secreted factors due to their positioning within the bone and along the bone surface (Bonewald, 2007; Hauge \textit{et al.}, 2001). Secreted factors from osteocytes may enter the MSC niche directly through hemichannels (Cherian \textit{et al.}, 2005), or through functional gap junctions between osteocytes and osteoblasts that have been demonstrated \textit{in vitro} (Kamioka \textit{et al.}, 2007; Taylor \textit{et al.}, 2007; Yellowley \textit{et al.}, 2000). As osteocytes are positioned inside the lacunar-canalicular network of the bone matrix and osteoblasts are found lining the bone, these functional gap junctions are also established \textit{in vivo} (Donahue, 2000; Doty, 1981). In this chapter it is proposed that the indirect three-layer co-culture system is an appropriate model of the \textit{in vivo} stem cell niche, wherein direct cell to cell contact between osteocytes and osteoblasts occurs but undifferentiated MSCs do not directly contact either cell type (Bonewald, 2007; Hauge \textit{et al.}, 2001). Results here show that there is a synergistic effect between osteocytes and osteoblasts in biochemical signalling to MSCs. Using the novel experimental design employed here, which incorporates both osteocytes and osteoblasts in a three-layer culture, it
4. Osteogenic differentiation of MSCs is regulated by osteocyte and osteoblast cells in a simplified bone niche

is shown that biochemical signals produced from this relationship enhanced MSC osteogenic differentiation, as seen by the greatest mineralisation compared to osteocyte and osteoblast co-culture alone (Figure 4.7b). These results further emphasise that a synergistic relationship exists between osteocytes and osteoblasts and supports the hypothesis that the syncytium of osteocytes and osteoblasts regulates bone formation by means of osteocytes actively communicating with inactive osteoblasts lining the calcified bone to recruit new osteoblasts from the marrow (Burger and Klein-Nulend, 1999; Cowin et al., 1991; Skerry et al., 1989).

4.6. Conclusions

In this chapter it is reported that osteogenic differentiation of MSCs, as characterised by ALP activity and mineralisation, is greater, when MSCs are co-cultured with osteocytes rather than osteoblasts. These results suggest that osteocytes are more influential than osteoblasts in stimulating osteogenesis in MSCs. However, as osteoblasts are seen to promote a sharp rise in the cell number of MSCs at the initial stages of culture as well as a later increase in ALP activity it is possible that they too stimulate osteogenesis in MSCs just at a slower rate. A three-layer co-culture system confirms a synergistic relationship between osteocytes and osteoblasts in producing biochemical signals to stimulate the osteogenic differentiation of
MSCs. This confirms that Hypothesis 1 is true with both osteocytes and osteoblasts stimulating the osteogenic differentiation of MSCs through biochemical signalling.

This chapter provides important insights into the mechanisms at work within the native stem cell niche to stimulate osteogenic differentiation. Additionally, all results are achieved in the absence of additional osteogenic factors in the media, outlining a possible role for the use of co-culture or conditioned media methodologies for tissue engineering applications.

The focus of the next chapter shifts to the role of mechanical loading in stimulating MSCs to undergo osteogenic differentiation within the stem cell niche. Osteocytes are believed to be the main mechanosensor in bone, responsible for directing the adaption to mechanical forces (Bonewald, 2007; Huiskes et al., 2000) and as demonstrated within this chapter they are capable of inducing an osteogenic response in MSCs. However, subsequent chapters examine whether MSCs within the bone marrow receive sufficient direct mechanical stimulation to undergo osteogenic differentiation possibly bypassing the osteocyte strain mediated response.
5. Computational modelling of the mechanics of trabecular bone and marrow using fluid-structure interaction techniques

5.1. Chapter Background

As discussed in Section 2.3 bone marrow found within the porous structure of trabecular bone provides a specialised environment for numerous cell types, including MSCs. Studies have sought to characterise the mechanical environment imposed on MSCs. However, a particular challenge is that marrow displays the characteristics of a fluid, while surrounded by bone that is subject to deformation, and previous experimental and computational studies have been unable to fully capture the resulting complex mechanical environment. The objective of this chapter is to develop an FSI model of trabecular bone and marrow to predict the mechanical environment of MSCs in vivo and to examine how this environment changes during osteoporosis. This addresses Hypotheses 2 and 3 as outlined in Section 1.3 and provides the basis for the computational methods which are used in Chapter 6. In this
chapter an idealised repeating unit is used to compare FSI techniques to a CFD-only approach. These techniques are used to determine the effect of lower bone mass and different marrow viscosities, representative of osteoporosis, on the shear stress generated within bone marrow. Results show that shear stresses generated within bone marrow under physiological loading conditions are within the range known to stimulate a mechanobiological response in MSCs \textit{in vitro}. Additionally, lower bone mass leads to an increase in the shear stress generated within the marrow, while a decrease in bone marrow viscosity reduces this generated shear stress.

5.2. Introduction

While there has been increased study into the paracrine signalling from cells within the niche, as investigated in the previous chapter (Fuchs \textit{et al.}, 2004; Watt and Hogan, 2000), MSCs are known to be mechano-sensitive and there is increasing evidence that the mechanical environment imposed within the niche also plays an important role in the functions of the niche (Castillo and Jacobs, 2010; Estes \textit{et al.}, 2004; Guilak \textit{et al.}, 2009; Potier \textit{et al.}, 2010). Furthermore, mechanical loading has been used to direct MSCs to differentiate \textit{in vitro} (Arnsdorf \textit{et al.}, 2009; Case \textit{et al.}, 2011) and as such the mechanical stimulus to cells has important implications for bone tissue engineering and regeneration applications. As outlined in the first chapter of
5. Computational modelling of the mechanics of trabecular bone and marrow using FSI techniques

this thesis the precise mechanical environment of bone marrow MSCs in vivo remains unclear to date and an improved understanding is needed to fully determine the role of mechanical signals on MSC differentiation.

Section 2.5.1 discusses how the mechanical environment of trabecular bone marrow remains poorly characterised (Gurkan and Akkus, 2008). Previous studies have focused on the possible role for bone marrow in the hydraulic stiffening of bone (Carter and Hayes, 1977; Ochoa et al., 1991; Ochoa et al., 1997). However, there has been little insight into the mechanical environment of the marrow and how this alters during physiological loading, or with diseases such as osteoporosis, which is critical in understanding the loading experienced by MSCs in vivo. Investigations into the mechanical properties of bone marrow have revealed it to be a highly viscous fluid when removed from the bone and filtered (Bryant et al., 1989) while also displaying viscoelastic solid properties (Zhong and Akkus, 2011). Experimental studies have shown that loading of bone induces pressure gradients within the marrow where the pressure is known as the intramedullary pressure (Downey et al., 1988; Qin et al., 2002; Qin et al., 2003). Such pressure gradients are likely to cause flow of the marrow, generating shear stresses, thus providing a possible stimulus for the osteogenic differentiation of MSCs in vivo (Gurkan and Akkus, 2008).
The composition of bone marrow varies greatly with age and location, and in particular the bone marrow becomes increasingly fatty with age (Liney et al., 2007; Vande Berg et al., 1998). A high fat content in bone marrow is also associated with diseases such as osteoporosis (Rosen and Bouxsein, 2006; Yeung et al., 2005); this change in marrow composition is accompanied by the decrease in bone mass characteristic of the disease. Further detail on this has been presented in Sections 2.3 and 2.5.1. It is also possible that the increased adipocyte content of the marrow contributes to the pathogenesis of the bone (Cohen et al., 2012) due to marrow adipocytes actively recruiting osteoclasts (Weisberg et al., 2003). However, the role of changes in marrow composition and bone mass on the mechanical environment experienced by MSCs in the bone marrow has, to the author’s knowledge, never been investigated.

The objective of this chapter is to examine the complex multiphysics mechanical environment of bone marrow using computational models that capture the interaction of the bone and marrow during typical loading. An FSI approach is used to predict the mechanical environment of the stem cell niche and how this environment alters during osteoporosis. These FSI models extend the results of previous approaches which have modelled the bone as rigid (Coughlin and Niebur, 2012; Porter et al., 2005; Teo and Teoh, 2012), the marrow as a soft solid (Yoo and Jasiuk, 2006), or have used a
continuum approach to describe marrow shear stress coupled to bone deformation (Dickerson et al., 2008). A 3D periodic unit cell approach is used to generate models of trabecular bone and marrow, with idealised trabecular geometry. In a first step, to validate the idealised model geometry approach, models are used to predict trabecular bone permeability and are compared to experimental data. Following on from this, the most appropriate boundary conditions are determined for FSI simulations using the periodic unit cell approach and results are compared to CFD-only approach. Subsequently, the models are used to determine how a decreasing bone mass and a change in viscosity can influence the in vivo mechanical stimulation of MSCs. In this way the study generates insight into how the mechanical environment of MSCs changes during osteoporosis.

5.3. Materials and Methods

5.3.1. Validation study: Predicting permeability

An idealised structure of trabecular bone is used (Figure 5.1) representing a number of individual trabeculae. A unit size of 1.25 mm is selected in order to provide a pore size of in the scale of 1 mm based on representative values for trabecular bone (Keaveny et al., 2001). This pore size varies in studies where the effect of osteoporosis is examined.
5. Computational modelling of the mechanics of trabecular bone and marrow using FSI techniques

Figure 5.1: Idealised repeating unit of (a) bone and (b) marrow, based on previously used geometries (Gibson, 1985; Yoo and Jasiuk, 2006), where the marrow structure is the inverse of the bone. Dimensions are taken in order to provide a pore size of approximately 1 mm (Keaveny et al., 2001). Bone struts are increased or decreased to achieve different BV/TV percentages while retaining the same overall unit size of 1.25 mm.

The marrow (fluid) geometry is established as the exact inverse of the bone shape. The solid structure is created in Abaqus/Explicit (version 6.11) with the fluid domain created in Abaqus/CFD (version 6.11). To validate the approach for modelling trabecular bone marrow, CFD models are generated to predict the permeability of trabecular bone for comparison to experimental measurements of trabecular bone permeability. The aim of this validation is to demonstrate that the model framework can generate experimentally accurate permeabilities indicating the suitability of the idealised geometries. Specifically, based on prescribed inlet velocities, ranging from 0.1 mm/s to 5 mm/s, flow through the structure is simulated and the pressure differential is obtained from the output to determine the permeability of the structure through the use of Darcy's law.
\[ U_D = \frac{Q}{A_s} = \frac{k}{\mu} \frac{P_U - P_D}{L_s} \]  

(5.1)

where \( U_D \) is the Darcy’s velocity, \( Q \) is the volume flow rate, \( A_s \) is the cross-sectional area of the model, \( k \) is the permeability, \( \mu \) is the viscosity (viscosity of water 1 mPa.s), \( P_U \) is the upstream pressure, \( P_D \) is the downstream pressure (Figure 5.2) and \( L_s \) is the length of the bone unit in the region of interest (Figure 5.1, 1.25 mm).

Figure 5.2: Idealised trabecular bone in marrow displaying elongated inlet and outlet for permeability study and Darcy’s Law parameters representing the experimental study by Nauman et al. (1999), (a) iso-view and (b) plane view.

The models are formulated to be representative of the experimental conditions reported previously (Nauman et al., 1999), with the bone
modelled as rigid for these permeability studies and the flow modelled as incompressible. The boundary between the solid and the fluid is defined as a no-slip boundary where the velocity of the fluid at the boundary is equal to the velocity of the solid.

5.3.2. FSI model formulation

In order to model the uni-axial compression of a repeating unit of idealised trabecular bone, symmetry boundary conditions are used, ensuring all sides remain orthogonal before and after loading (Mullins et al., 2007). It is important to note that due to the material and loading direction symmetries in these analyses, the general periodic boundary conditions that normally apply for periodic unit cell analyses reduce to the simpler symmetry boundary conditions as implemented here (Böhm, 2004). To achieve this repeating unit approach within the fluid domain during FSI analyses combinations of different boundary conditions are analysed and compared as described below.

Four different cases are considered in order to derive the most appropriate fluid boundary conditions, as the movement of fluid through bone marrow cavities of bone is not well understood. In the first case a CFD-only analysis consisting of a velocity driven marrow flow past rigid bone is modelled
5. Computational modelling of the mechanics of trabecular bone and marrow using FSI techniques

(CFD-only, Figure 5.3a). This inlet velocity, $V_i = 0.00375$ mm/s, is equivalent to a compressive nominal strain rate in the Y direction of 3000 με/s and is applied using a smooth ramp of an amplitude between 0 and 1 at 1 Hz for 3 cycles (Figure 5.4). Loading of 3000 με is representative of physical exertion (vigorous running) (Burr et al., 1996) at a frequency of 1 Hz (Rubin and Lanyon, 1985). Loading of this magnitude and frequency is known to generate an osteogenic response in bone (Mann et al., 2006; Rubin and Lanyon, 1985). Flow is modelled as symmetric in the X and Z directions by preventing velocity normal to the respective boundaries, and a zero pressure outlet is applied on the bottom surface.

In the second case, a marrow flow, driven by the elastic compression of the bone is introduced (FSI-P, Figure 5.3b). A displacement representing 3000 με compressive nominal strain (applied using a smooth ramp of an amplitude between 0 and 1 at 1 Hz for 3 cycles, Figure 5.4) is applied through the use of a "soft cap" ($E = 15$ MPa), connected to the bone ($E = 15$ GPa). This "soft cap" is necessary in order to implement the required boundary conditions due to restrictions in coupling nodes between the fluid and solid meshes within Abaqus. The prescribed displacement is applied to a master node and all other nodes on the inside/bottom surface of the cap are constrained to move together with the master node in the Y direction, using an equation constraint. No constraint is applied to these nodes in the X and Z directions.
These boundary conditions, combined with the fact that the cap is of negligible stiffness relative to the bone, ensure that the marrow and bone domains move as one on this boundary without constraining the bone struts on the top surface in the X and Z directions. Symmetry deformation constraints are applied to the solid bone struts on the X and Z surfaces, again using an equation constraint. These couple the displacements of all nodes on the given strut faces to a master node, for displacement components in the direction normal to the respective face. This ensures the sides of the solid bone remain orthogonal during loading, but can displace in the X and Z directions. As regards the fluid domain, fluid velocity is again prevented in the X and Z directions on the respective external boundaries, and there is a zero pressure condition on the bottom surface. However, this does not result in the imposition of a strict displacement symmetry boundary condition on the deformable fluid mesh.

In the third case the solid boundary conditions remain the same as FSI-P but the fluid velocity is set to zero normal to the plane of the bottom boundary (FSI-Y, Figure 5.3c). This allows for symmetric fluid flow conditions on all boundaries, ensuring the fluid remains within the volume when compressed, while allowing the displacement of the fluid domain boundaries, linked to the deformation of the solid domain boundaries, as for case FSI-P.
Figure 5.3: a) CFD-only, velocity driven flow of the marrow past a rigid bone, flow is modelled as symmetric in the X and Z directions with a zero pressure outlet. (b) FSI-P, marrow flow driven by the elastic compression of the bone (achieved with a soft cap) with symmetry fluid flow conditions on the X and Z planes and a zero pressure outlet. Symmetry deformation constraints are applied to the bone struts on the X and Z surfaces, which coupled the displacements, in the direction normal to the respective faces, of all nodes on the respective strut faces to a master node, for displacement components in the direction normal to that face. (c) FSI-Y, same as FSI-P except flow is prevented out of plane on the bottom surface. (d) FSI-S, symmetry deformation constraints are applied to the boundaries of the fluid domain through the use of a soft outer shell; this is used to explicitly constrain the unit cell boundary both fluid and solid to remain cuboidal during deformation, by coupling the displacements, in the direction normal to the respective faces, of all nodes to a master node on the inside surface.
Figure 5.4.: Schematic of loading history used in the analyses where peak strains of 3000 με are applied at a 1 Hz frequency for 3 cycles.

While cases FSI-P and FSI-Y implement symmetry of fluid velocity on the boundaries they do not implement symmetry deformation conditions to the fluid domain mesh as such the mesh is free to deform unconstrained in the X and Z directions. Symmetry deformation of the fluid mesh is achieved in the final analysis, FSI-S, which uses the same fluid conditions as FSI-Y but a soft (E = 15 MPa) outer shell is also included. This explicitly constrains the unit cell boundary, both fluid and solid, to remain cuboidal during deformation, and therefore is a full implementation of symmetry boundary conditions. The constraint is achieved by coupling the displacements, in the direction normal to the respective faces, of all nodes to a master node on the inside surface (FSI-S, Figure 5.3d).
Marrow is modelled as an incompressible, Newtonian fluid using the experimental results of (Bryant et al., 1989) with a viscosity of 0.4 Pa.s. For the FSI analyses bone is modelled as a linear elastic material \((E = 15 \text{ GPa}, \nu = 0.3\) (Keaveny et al., 2001)) in terms of finite deformation kinematics, as described in Section 3.2.2, which is the default kinematic setting for Abaqus/Explicit. A no slip boundary is applied at the interface between the marrow and the bone.

As described in Chapter 3, the solid and fluid analyses are fully coupled using the Abaqus co-simulation engine, allowing for a two-way coupled FSI (DS SIMULIA, 2011). The equations for the solid and fluid models are solved separately, and loads and boundary conditions are exchanged at the interface region. Fluid flow is modelled in an Eulerian domain employing the ALE method which is used when the boundaries of the Eulerian domain displace due to FSI (DS SIMULIA, 2011).

5.3.3. The mechanical environment of bone marrow during osteoporosis

Different bone masses or BV/TV percentages of 11.06, 15.10, 18.02, 25.60 and 29.40 % are examined to determine the effect of bone mass on the velocity and shear stress generated within the marrow due to mechanical loading. Different BV/TV percentages are achieved in the solid model by creating
parts with different bone strut widths while maintaining the overall unit size of 1.25 mm. A Boolean cut is then performed using the solid part to create the fluid part. Percentages are selected as a range of BV/TV from osteoporotic (11.06 %) to healthy bone (29.40 %) as determined for iliac crest bone biopsies (Hasegawa et al., 1995). A force controlled compression of 3 N (which generates strains typical of physiological loading in the 29.40 % model (Burr et al., 1996)) and a displacement controlled compression of 3000 με nominal strain are examined for each BV/TV. Different marrow viscosities are also examined as osteoporotic bone is usually accompanied by a fattier marrow, which impacts on the marrow viscosity (Rosen and Bouxsein, 2006).

The ALE formulation employed for FSI analyses allows for deformation of the fluid mesh domain. Therefore, it is deemed that FSI-S is the most appropriate approach to simulate *in vivo* loading conditions, using such a periodic unit cell approach. This modelling approach aims to capture the overall strain applied to the bone and how it is transferred to the unit cell. This approach ensures that the fluid domain sides remain orthogonal during loading (matching the solid deformation conditions). As FSI-S is a full implementation of symmetry boundary conditions and is deemed most appropriate for the subsequent analyses of this chapter and also in Chapter 6.
Within Abaqus/CFD version 6.11 shear stresses are not outputted directly. To determine the stresses within the current analyses the following approach was used. Firstly, $D$ is the rate of deformation tensor, equal to $\frac{1}{2}(\nabla \mathbf{v} + (\nabla \mathbf{v})^T)$, where $\nabla \mathbf{v}$ is the spatial velocity gradient tensor. From this, the scalar shear rate, $\dot{\gamma}$, is calculated as $(2tr(D \cdot D))^{\frac{1}{2}}$, which can be explicitly written as follows

$$
\dot{\gamma} = \left[ 2 \left( \frac{\partial v_1}{\partial x_1} \right)^2 + \left( \frac{\partial v_2}{\partial x_2} \right)^2 + \left( \frac{\partial v_3}{\partial x_3} \right)^2 \right]^{\frac{1}{2}}
$$

(5.1)

where $v_i$ is the velocity component in the $x_i$ direction (ANSYS Inc., 2009; Porter et al., 2005). For a viscous fluid, the energy dissipated in flow is given by the viscous-dissipation function $\Phi$ (White, 1998).

$$
\Phi = \mu \dot{\gamma}^2
$$

(5.2)

where $\mu$ is the viscosity of the marrow. In the present work a shear stress $\tau$ is calculated using the basic Newtonian viscous flow law $\tau = \mu \dot{\gamma}$. The rate of work done per unit volume by this stress, $\tau$, equals the viscous dissipation. $\tau$ is then used to characterise the shear stress state in the marrow. This was
implemented using a Python script (written by another member of the research group, James Grogan) which inputted the velocities and coordinates at each node from Abaqus output file.

For the models in the FSI model formulation study and the subsequent osteoporosis studies, the solid bone domain consists of 8-node brick elements (C3D8R), with element numbers in the range of 63,348 to 76,656. The fluid domain consists of 8-node brick fluid elements (FC3D8), with element numbers in the range of 93,000 to 116,407. These mesh densities were arrived at as a result of a mesh convergence study for the different BV/TV percentages examined, as these necessitate a change in the model geometry, resulting in a square element length of approximately 0.025 mm in all analyses. Material properties in the different BV/TV models are maintained as outlined in the FSI model formulation description. A value of 0.4 Pa.s for the marrow viscosity is taken for the majority of this study in accordance with the study by Bryant et al. (1989). Values of 1 Pa.s and 0.1 Pa.s were also examined as these represent the upper and lower values reported from experiments (Gurkan and Akkus, 2008; Zhong and Akkus, 2011).

As introduced in Chapter 3 the Reynolds number \( (Re) \) is used to determine whether the flow is turbulent or laminar (Equation (3.54)). It is used here to
determine the relative influence of viscous and inertial forces. For clarity the equation for Reynolds number is reproduced here.

\[ Re = \frac{\rho V D}{\mu} \]  

(5.3)

In this equation the trabecular spacing is used as the characteristic dimension, \( D \), the density, \( \rho \), is taken from White et al., (1987) with a value of 1.06 kg m\(^{-3}\), the viscosity, \( \mu \), is taken from Bryant et al., (1989) as previously stated and the average velocity, \( V \), is determined at the peak point of the cycle (Bryant et al., 1989; White, 1998). For Reynolds numbers of less than 1, viscous forces dominate, and the flow is laminar.

5.4. Results

5.4.1. Validation study: Predicting permeability

The permeability simulations were performed for the above mentioned range of BV/TV percentages (11.06, 15.10, 18.02, 25.60 and 29.40 %) and Darcy’s Law is used to extract the permeabilities. The permeabilities vary between \( 6.82 \times 10^{-10} \) m\(^2\) to \( 1.95 \times 10^{-9} \) m\(^2\), decreasing with greater bone volume fraction as expected (Figure 5.5a). Values found are within reported values of permeability for trabecular bone for experimental (Grimm and Williams, 1997; Nauman et al., 1999) and computational studies (Coughlin and Niebur,
5. Computational modelling of the mechanics of trabecular bone and marrow using FSI techniques

2012; Teo and Teoh, 2012) (Figure 5.5b). This result gives confidence that assumed idealised geometry is sufficiently representative to study the mechanics of the bone and marrow interaction at this size scale.

![Graph](Figure 5.5)

**Figure 5.5:** (a) Values of permeability calculated for BV/TV percentages of 11.06, 15.10, 18.02, 25.60 and 29.40 % and (b) comparison of permeability results for the current study, experimental studies (Grimm and Williams, 1997; Nauman *et al.*, 1999) and computational studies (Coughlin and Niebur, 2012; Teo and Teoh, 2012).
5.4.2. FSI model formulation

The simulations were run for three cycles. However, changes from cycle to cycle are negligible, showing that transient effects can be ignored. Based on this, the results presented below are for the first cycle. The velocity profile within the marrow is found to differ greatly between velocity driven flow of the CFD-only analysis and the marrow flow driven by bone compression achieved with the application of a FSI interaction (Figure 5.6a versus Figure 5.6b - d). The Reynolds numbers for all analyses are much less than 1 showing that the viscous forces are dominating these analyses and that the assumption of laminar flow is appropriate; see Table 5.1.

**Table 5.1:** Reynolds numbers calculated for each analysis, CFD-only velocity only flow past a rigid bone, (FSI-P) compression driven flow using FSI, (FSI-Y) compression driven flow using FSI where the flow is constrained within the marrow domain and (FSI-S) compression driven marrow flow with flow constrained within the marrow domain and symmetry boundary conditions applied to the marrow domain with the use of a soft outer case.

<table>
<thead>
<tr>
<th></th>
<th>Reynolds Number (Re)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CFD-only</td>
<td>0.0032</td>
</tr>
<tr>
<td>FSI-P</td>
<td>0.0028</td>
</tr>
<tr>
<td>FSI-Y</td>
<td>0.0020</td>
</tr>
<tr>
<td>FSI-S</td>
<td>0.0022</td>
</tr>
</tbody>
</table>

Velocities and shear stresses in the fluid are examined at the peak overall velocity point of the cycle, i.e. at 0.5 seconds, while the displacements and
stresses in the solid are examined at the peak overall displacement point, i.e. at 1 second. The statistical distribution of velocity within the marrow (Figure 5.6e) reveals that the maximum velocities in the CFD-only analysis are over twice as large as those of the FSI analyses (0.0191 mm/s compared to 0.0072 mm/s for FSI-P, 0.0072 for FSI-Y and 0.00771 mm/s for FSI-S).

Similarly high values are found for the 90\textsuperscript{th} percentile demonstrating that 10\% of the nodal velocity values are much higher in the CFD-only analysis compared to the FSI analyses. When the 70\textsuperscript{th} percentile of the velocity is examined in the marrow the four different boundary conditions are found to be much more comparable. While this demonstrates that 70 \% of the nodal velocities in all the analyses are within a small range, the CFD-only analysis still predicts high peak velocity values relative to the other cases. The FSI-Y analysis predicts on average the lowest velocities. The FSI analysis with symmetry conditions (FSI-S) predicts the most homogenous distribution of velocity within the marrow.

Examining the stresses and strains generated in the bone for the different fluid boundary conditions in the FSI analyses (see Table 5.2) reveals that the introduction of a soft outer cap/case in order to apply symmetry boundary conditions to the fluid domain does not interfere with the bone deformation and motion while it does alter the shear stress generated within the marrow.
5. Computational modelling of the mechanics of trabecular bone and marrow using FSI techniques

Table 5.2.: Stress and strain generated within the bone for a displacement representing 3000 με compressive strain, and resulting shear stress generated within the marrow, examining the influence of the different boundary conditions on the fluid domain during the FSI analyses.

<table>
<thead>
<tr>
<th></th>
<th>FSI-P</th>
<th>FSI-Y</th>
<th>FSI-S</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Von Mises stress (MPa)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Maximum</td>
<td>69.31</td>
<td>69.31</td>
<td>72.09</td>
</tr>
<tr>
<td>Average</td>
<td>18.42</td>
<td>18.42</td>
<td>18.34</td>
</tr>
<tr>
<td><strong>Max principal strain (με)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(logarithmic strain)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Maximum</td>
<td>2996.24</td>
<td>2996.24</td>
<td>2990.18</td>
</tr>
<tr>
<td>Average</td>
<td>566.00</td>
<td>566.00</td>
<td>562.95</td>
</tr>
<tr>
<td><strong>Shear Stress (mPa)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Maximum</td>
<td>66.63</td>
<td>65.35</td>
<td>62.15</td>
</tr>
<tr>
<td>Average</td>
<td>6.61</td>
<td>7.94</td>
<td>7.55</td>
</tr>
</tbody>
</table>

5.4.3. The mechanical environment of bone marrow during osteoporosis.

The influence of different bone volume fractions is examined by employing both force and displacement controlled compression. With an applied force of 3 N it is found that a lower BV/TV percentage results in an increased velocity within the marrow as evident when the velocity distributions are examined using a histogram (see Figure 5.7a). The majority of the nodal velocities (70 %) predicted for the 11.06 % BV/TV are found to be greater than 0.01 mm/s whereas for the other bone volumes examined (15.10, 18.02, 25.60 and 29.40 %) the majority of the nodal velocities are in the range of 0 – 0.001 mm/s. The velocity profile for the 11.06 % BV/TV can be seen in Figure 5.7b. The maximum and 90th percentile shear stresses, calculated as described above, are found to decrease by a factor of 10 from the lowest (11.06 %) and the highest BV/TV (29.40 %) examined here (Figure 5.7c).
Figure 5.6: Velocity profiles generated within the marrow for (a) velocity only analysis of flow past a rigid bone (CFD-only) with a Reynolds number of 0.0032, (b) compression driven marrow flow achieved with the introduction of FSI (FSI-P) with a Reynolds number of 0.0028, (c) FSI compression driven marrow flow with flow constrained within the marrow domain (FSI-Y) with a Reynolds number of 0.0020 and (d) FSI compression driven marrow flow with flow constrained within the marrow domain and symmetry boundary conditions applied to the marrow domain with the use of a soft outer case (FSI-S) with a Reynolds number of 0.0022. Units= mm/s. (e) Statistical distribution of velocity generated within the marrow for the four boundary conditions examined at peak velocity point in cycle (0.5 seconds). The 70th and 90th percentiles are the values of velocity below which 70% and 90% (respectively) of the nodal values fall.
Figure 5.7: (a) Histogram of the velocities within the bone marrow at the peak time of the cycle 0.5 seconds for the five different bone BV/TV values examined: 11.06, 15.10, 18.02, 25.60 and 29.40 %. Velocity values between 0 and 0.01 mm/s are distributed into bins with intervals of 0.001 mm/s to ascertain the statistical distribution of the velocity within the marrow of different bone volume percentages. (b) A velocity contour plot displaying the profile through the marrow of the 11.06 % BV/TV sample for a cut through the middle of the marrow in the XY plane (units mm/s). (c) The maximum and 90th percentile value of the shear stress found at the peak time of the cycle for the different bone volume percentages. (d) A shear stress contour plot for the marrow of the 11.06 % BV/TV for a cut through the middle of the marrow in the XY plane (units Pa). The 90th percentile is the value of shear stress below which 90 % of the element values fall.
5. Computational modelling of the mechanics of trabecular bone and marrow using FSI techniques

**Figure 5.8:** Average velocity within the marrow due to a displacement controlled compression of 3000 με nominal strain (applied using a smooth ramp of an amplitude between 0 and 1 at 1 Hz for 3 cycles) for the five different bone volume percentages examined 11.06, 15.10, 18.02, 25.60 and 29.40 %. Values above the bars indicate the results of normalisation of the values to the 11.06 % BV/TV result.

The shear stress generated within the marrow due a 3 N load is shown in Figure 5.7d for a BV/TV of 11.06 %. In contrast, when the velocity distribution is examined for a strain controlled compression (3000 με) no difference in the average velocities is observed for any of the different bone volume fractions investigated (Figure 5.8).

The influence of the different viscosities can be seen by examining the shear stresses generated within the marrow (Figure 5.9a); however changing the viscosity of the bone marrow is found to have no significant effect on the average velocity within the bone marrow (Figure 5.9b). Figure 5.9c reveals a
potential power law relationship between shear stress and BV/TV. The strongest relationship is seen for a viscosity of 0.4 Pa.s ($R^2 = 0.9949$) but it is still robust at the other viscosities examined (0.1 Pa.s $R^2=0.9239$ and 1.0 Pa.s $R^2=0.9359$). Figure 5.9d illustrates the proportional relationship between shear stress and viscosity for two of the BV/TV percentages examined (15.10 and 25.60 %). The Reynolds number within all these analyses is of the level such that creeping flow (Stokes flow) exists. This results in linearity between shear stress and viscosity as there are no inertial effects and no time dependence.

5.5. Discussion

In this study an FSI approach is used to predict the mechanical environment (marrow velocity and shear stress) experienced within trabecular bone marrow under physiological loading conditions. Velocities generated within the marrow are found to depend on the boundary conditions applied to the fluid, indicating the significance of the correct boundary selection for FSI investigations. However, the greatest difference is found between the CFD-only analysis and the FSI analyses demonstrating the importance of the deformation and motion of the solid bone on marrow movement during compression.
Figure 5.9: (a) The 90th percentile and maximum of the shear stress within the marrow, where the 90th percentile value is represented by a pattern on the maximum solid bar. (b) The average velocity within the marrow at the peak point of the cycle 0.5 seconds for 3 different viscosities 0.1, 0.4 and 1.0 Pa.s, examined within the five BV/TV (Bone Volume/Tissue Volume) percentages. The 90th percentile is the value of shear stress below which 90% of the element values fall. (c) The average shear stress as a function of BV/TV percentage for each viscosity examined and (d) the average shear stress for two of the BV/TV percentages examined as a function of viscosity. This displays the proportionality of shear stress to viscosity.
In terms of BV/TV dependence, it is observed here that a decreasing bone volume, representing osteoporotic bone, leads to increasing velocities and shear stresses within the marrow. Additionally it is found that changing marrow viscosities, also representative of osteoporosis, have little influence on the fluid velocity, although, an increasing viscosity increases the shear stress linearly. These findings have important implications for tissue engineering as researchers try to understand or mimic the natural environment of MSCs for studies \textit{in vitro} and for investigating the mechanisms behind bone loss during the disease of osteoporosis.

The application of FSI techniques to study bone marrow is an advancement over previous approaches where the bone was modelled as rigid with the marrow flowing past it (Porter \textit{et al.}, 2005; Teo and Teoh, 2012) or with the bone marrow modelled as a soft solid (Yoo and Jasiuk, 2006). The CFD-only analysis here is found to produce large peaks in velocities compared to FSI analyses indicating that a CFD-only analysis overestimates the movement of the marrow and as such overestimates the mechanical stimuli experienced by the cells within the marrow. The FSI analysis with the symmetry conditions on the fluid domain (FSI-S) is the most logically representative of the periodic unit cell approach and hence used to examine different BV/TV percentages.
The velocity generated within the marrow is found to be directly dependent on bone volume for a force controlled compression, with the velocity within the marrow decreasing for an increasing BV/TV percentage. This is as expected as with a decreasing bone volume there is an increasing marrow volume and as such the marrow is experiencing more of the applied load. For a strain controlled compression, where all BV/TV fractions were subjected to the same strain, no difference in marrow velocities is found between the different bone volumes. Again this is as expected as the velocity generated would be the same for the each BV/TV as the displacement is the same.

\textit{In vitro}, shear stress has been shown to be a regulator of cell differentiation capable of inducing the cell down a osteogenic differentiation pathway (Arnsdorf et al., 2009; Bakker et al., 2003; Case et al., 2011). A study by Sharpe et al., (2009) showed an up regulation of osteoblastic genes such as collagen, osteopontin, osteocalcin and bone sialoprotein in MSCs exposed to fluid shear stress (Sharp et al., 2009). While the exact method by which cells convert mechanical stimulation to a biochemical response remains unclear (Riddle and Donahue, 2009), mechanoreceptors in the cell surface, such as specific integrins, cadherins, and calcium channels, play a vital role in the transmission of the mechanical signal into the cell nucleus. This in turn regulates gene expression (Liedert et al., 2006). Additionally, as a result of
shear stress, cells can release signalling molecules, such as nitric oxide and prostaglandins, to stimulate neighbouring cells (Turner and Pavalko, 1998). Results from the current study can help inform boundary conditions for further computational studies where the focus is on delivering a mechanical stimulation to single cell models (Mazzag and Barakat, 2011) to aid in the understanding of the mechanotransduction process in cells.

The maximum shear stress generated within the marrow (for a viscosity of 0.4 Pa.s) for a force controlled compression for the different bone volumes ranges from 0.02 Pa (29.40 %) to 0.26 Pa (11.06 %). For 2D parallel plate flow chamber experiments shear stress values of 0.1 Pa to 1 Pa have previously been shown to stimulate osteogenic responses in MSCs and pre-osteoblastic cells in vitro (Arnsdorf et al., 2009; Bakker et al., 2003; Case et al., 2011). This suggests that the computational results are predicting shear stresses which are in the lower range of what is needed to stimulate an osteogenic response. However, a separate study which examined the effect of applying a 0.06 Pa and 0.6 Pa shear stress found an increased cell proliferation, ALP activity and calcium deposition compared to static controls with no significant differences between the two shear stress values (Nauman et al., 2001). Moreover, Sandino et al. (2008) examined the shear stress generated in 3D scaffolds, subjected to a combination of perfusion flow and compression. Values of shear stress were found to range between 0.037 to 0.046 Pa.
5. Computational modelling of the mechanics of trabecular bone and marrow using FSI techniques

(Sandino et al., 2008), which is lower than within a 2D flow chamber. An experimental study by Cartmell et al. (2003) with equivalent inlet velocities and geometries to Sandino et al. (2008) was also found to stimulate the osteogenic differentiation of pre-osteoblastic cells. Taken together, this suggests that in a 3D environment the required shear stress to induce osteogenic differentiation could be lower than what is observed in 2D flow chambers. This implies that 2D studies may not be sufficiently representative of the in vivo 3D mechanical environment.

Based on these previous experimental and computational studies, computational results from this investigation indicate that the compression of bone due to physiological loading could generate sufficient shear stress to stimulate the osteogenic differentiation of MSCs with increasing shear stress generated as the BV/TV decreases. However the computational models predict that the 90th percentile shear stress values range from 0.006 Pa (29.40 %) to 0.066 Pa (11.06 %) indicating that it is only a small percentage of the marrow which experiences sufficient shear stress to stimulate the osteogenic differentiation of MSCs. As such the location of MSCs within the marrow relative to the areas of high shear stress could be crucial in determining osteogenic differentiation in response to bone loading. This would be particularly true in the analysis of realistic geometries where peaks in shear
stress may be determined by the geometries and the pores size of the trabecular bone.

Porter et al. (2005) discussed the difficulties in comparing the response seen in cells exposed to shear stress stimuli in a 2D flow chamber to the response seen in 3D scaffolds. The same questions arise when trying to compare a computational model of the in vivo structure to in vitro experiments. Nonetheless, based on current knowledge, the results of the present study demonstrate that the physiological compression driven flow of the marrow causes sufficiently large shear stress to stimulate osteogenic differentiation of MSCs. Moreover, the results provide shear stress values which could help to interpret and design 3D bone tissue engineering scaffolds which aim to mimic the natural mechanical environment of MSCs.

When the effect of decreasing BV/TV is examined the largest shear stress values are found in the marrow of lower BV/TV percentages. These findings may indicate that MSCs in a lower BV/TV environment (characteristic of osteoporosis) would experience a greater mechanical stimuli, that would in turn initiate an osteogenic response, than MSCs in a higher BV/TV environment. However, a decreasing BV/TV with osteoporosis is coupled with an increase in the fat content of marrow and as such the viscosity of the marrow might decrease (Bryant et al., 1989; Zhong and Akkus, 2011). As
shown in Figure 5.9, this decrease in viscosity greatly lowers the magnitude of the shear stress experienced in the lower BV/TV models representative of osteoporotic bone. This is in agreement with a previous analytical study by Dickerson et al. (2008) which found the viscosity of bone marrow to be a crucial factor in determining the shear stresses generated within the marrow. These results suggest that the physiological compression of bone could enhance the osteogenic differentiation of MSCs within individuals with lower BV/TV percentages. However, the accompanying decrease in bone marrow viscosity (Yeung et al., 2005; Zhong and Akkus, 2011) may limit the shear stress experienced by the MSCs. Therefore the viscosity and the BV/TV percentage are linked and to fully determine the relative weight of either parameter a greater knowledge of the relationship between BV/TV and marrow viscosity is required. Moreover, it is likely that the number of undifferentiated MSCs within the marrow would decrease due to the increasing fat content (Rosen and Bouxsein, 2006). This could perhaps explain why a higher fat content in bone marrow is thought to be a cause of the pathogenesis of bone in osteoporosis (Cohen et al., 2012; Weisberg et al., 2003) as the shear stress within the marrow is decreasing with decreasing viscosity.

To the author’s knowledge this is the first time FSI techniques have been used to examine the movement of marrow within trabecular bone. However,
there are limitations to this study that should be considered. There exists very little information on the properties of bone marrow and for this reason a Newtonian, incompressible, homogenous fluid was assumed here. A value of 0.4 Pa.s (Bryant et al., 1989) was assumed for the majority of the study however the sensitivity of the models to the viscosity can be seen in Figure 5.9 indicating the need for further experimental characterisation. Moreover, the cellular composition of marrow suggests it is heterogeneous in nature and therefore behaves as a non-Newtonian fluid similar to blood (Bryant et al., 1989; Zhong and Akkus, 2011). In addition, bone is known to be anisotropic and viscoelastic, whereas this study assumes a linear elastic material which could affect the strain generated within the bone and hence the stimuli experienced within the marrow.

Results here show no stiffening effect due to the presence of marrow or the implementation of the different fluid boundary conditions (Table 5.2). Previous studies have focused on the hydraulic stiffening effects of marrow within the trabecular bone compartments of whole bones with marrow providing additional stiffness to the overall bone (Ochoa et al., 1991; Ochoa et al., 1997). This effect is not observed in this study due to the micro-mechanical modelling approach. Here the influence of the solid cortical shell is ignored as the structure is idealised as a repeating unit cell of trabecular bone.
While the focus here is on the marrow in trabecular region, where MSCs predominantly reside, the modelling techniques could be adapted to a larger scale model or multiscale model of a long bone structure to determine how loading influences marrow within the diaphyseal regions. Moreover, the modelling techniques could help determine the deformation of bone marrow in cortical bone regions observed in postmenopausal osteopenic women (Goldenstein et al., 2010). Additionally, the models could be adapted to investigate the effect of frequency and loading rate on the shear stress generated within the marrow. Due to the nature of bone marrow and the nonlinear material properties of bone it is likely that frequency and loading rate are a crucial factor in determining the response. For example, the intramedullary pressure fluctuations caused by muscle stimulation (Hu et al., 2012; Lam and Qin, 2008; Qin and Lam, 2009) could be modelled to determine whether regions of high shear stress within the model correspond to areas of new bone formation.

While the use of idealised geometries is a limitation, this geometry has been found to be suitable in previous studies to give insights into the mechanical behaviour of trabecular bone during loading (Gibson, 1985; Yoo and Jasiuk, 2006). The permeability of the idealised structure (for different BV/TV fractions) was found to fall within reported experimental ranges (Nauman et al., 1999) indicating the suitability of these idealised structures. Although the
permeability study used a CFD-only boundary condition, which models the bone as rigid, the bone deformation due to flow is thought to be negligible and won't contribute to the overall pressure differential in the fluid which is used to determine the permeability during steady state flow. Due to the symmetry employed in the unit of bone and marrow, the same quantitative results would be obtained for loading in either the X, Y or Z directions for the present models. However, the influence of shear in the XY plane, for example, could be modelled using such geometries employed here and would be of interest for future study. Moreover, loading direction would be an important factor to consider in the modelling of anisotropic unit cells or realistic geometries.

5.6. Conclusions

In this chapter FSI techniques are used to investigate the mechanical environment of trabecular bone marrow, providing a new insight into the mechanical stimuli which MSCs are exposed to in vivo due to the physiological compression of bone. In Chapter 4 it was seen that paracrine signalling from neighbouring cells is capable of inducing osteogenic differentiation in MSCs. This supports the theory that osteocytes act as the mechanosensor in bone directing osteogenic differentiation of MSCs in response to mechanical loading. However, results from this chapter indicate
that the compression of bone due to physiological loading could generate sufficient shear stress within the bone marrow to stimulate the osteogenic differentiation of MSCs.

The results of this chapter support Hypothesis 2 presented in Section 1.3, where it was proposed that MSCs receive sufficient mechanical stimulation within the marrow of trabecular bone to stimulate osteogenic differentiation. This suggests that MSCs could be undergoing osteogenic differentiation in direct response to the mechanical loading, bypassing the osteocyte mediated response. Moreover, these results can help inform tissue engineering studies which aim to utilise the mechano-sensitivity of MSCs for improved bone tissue engineering applications by providing realistic shear stress values which MSCs experience \textit{in vivo}. Furthermore, Hypothesis 3 was addressed in this chapter, as these results inform how osteoporosis affects the native mechanical environment of MSCs with increasing shear stress generated as the BV/TV decreases. However, with a decreasing viscosity, thought to be representative of a fattier bone marrow (associated with osteoporosis), the shear stress generated within the osteoporotic marrow decreases.

The computational methods for the FSI models developed in this chapter are utilised again in Chapter 6, where they are applied to realistic geometries of trabecular bone determined from \(\mu\)-CT scans. While the results here show
that shear stress generated within the bone marrow is of sufficient magnitude to stimulate an osteogenic response in MSCs, Chapter 6 aims to answer whether this is indeed translated to an anabolic bone growth response.
6. An experimental and computational investigation of bone formation in mechanically loaded trabecular bone explants

6.1. Chapter Background

This chapter addresses Hypothesis 4 from Section 1.3, which states that ‘Physiological compression leads to shear stress generated within the bone marrow which stimulates new bone growth’. In this chapter a bioreactor is employed to apply physiological compression to trabecular bone explants. FSI techniques developed in Chapter 5 support the experimental study by generating FE meshes from µ-CT scans of the experimental bone explants. This allows for the determination of the strain within the bone matrix and shear stress within the marrow and how these relate to potential bone growth within the explants.
While Chapter 5 used computational models to predict that physiological compressive loading of trabecular bone generates shear stress of sufficient magnitude (based on in vitro studies) to stimulate an osteogenic response in marrow cells, such as MSCs, this chapter aims to determine whether this magnitude of shear stress is translated to an anabolic response within bone explants. Additionally, it examines whether, on the other hand, the strain of the bone matrix is causing the anabolic response in bone. If this were the case, the anabolic response would likely be mediated by osteocytes and osteoblasts as described in Chapter 4, bypassing the effect of shear stress within the marrow.

6.2. Introduction

As discussed in previous chapters, bone marrow in the pores of trabecular bone is subjected to daily loading, mediated by the solid structure surrounding it. The shear stress generated in idealised marrow has been found to be of significant magnitude to stimulate an osteogenic response in the bone marrow cells (Chapter 5). However, the response of marrow cells to physiological loading in realistic geometries has yet to be determined.

As described in Section 2.2.8, bone is known to adapt its structure to accommodate changes in mechanical forces. This is believed to be mediated
by a mechanostat approach (Frost, 1987). It is believed that bone resorption due to disuse is initiated at a strain level of less than 1000 με, and it has been observed that vigorous exercise generates strains up to 3000 με and stimulates new bone formation (Burr et al., 1996; Fritton et al., 2000). However, strains of greater than 3500 με can lead to damage, resorption and fracture of bone (Carter et al., 1987b; Mosley, 2000). As discussed in Chapters 2 and 4, osteocytes are believed to be main mechosensor in bone. The results in Chapter 4 demonstrate the ability of osteocytes to direct the differentiation of MSCs in a simplified bone marrow stem cell niche. However, due to the magnitude of shear stress generated within bone marrow during physiological loading there exists a possible role for marrow shear stress in directing the osteogenic differentiation of MSCs (Chen and Jacobs, 2013; Govey et al., 2013; Gurkan and Akkus, 2008). If this were to be the case it would bypass the osteocyte mediated strain response of bone (Garman et al., 2007).

As described in Section 5.7 the magnitude of shear stress required to generate an osteogenic responses in MSCs and pre-osteoblastic cells in vitro is commonly found to range between 0.1 and 1 Pa (Arnsdorf et al., 2009; Bakker et al., 2003; Case et al., 2011). However other studies have suggested a lower threshold < 0.05 Pa (Cartmell et al., 2003; Sandino et al., 2008) and have
demonstrated no significant difference in the osteogenic response between osteoblastic cells exposed to either 0.06 or 0.6 Pa (Nauman et al., 2001).

The objective of this chapter is to examine whether the shear stress generated in trabecular bone marrow due to physiological compression of bone is of a sufficient magnitude to generate an anabolic response in the bone. This is examined using ovine trabecular bone explants exposed to compression in a custom built bioreactor.Loaded samples are compared to static control samples using bone histomorphometric techniques to determine the level of bone growth within the samples. In vitro studies of trabecular bone explants have previously been used to examine bone formation in response to compression of the bone matrix (David et al., 2008; Davies et al., 2006; Endres et al., 2009; Jones et al., 2003; Mann et al., 2006; Vivanco et al., 2013). Greater bone growth was found in samples exposed to the mechanical loading compared to static samples (David et al., 2008; Mann et al., 2006), reproducing the in vivo effects of mechanical strain on bone growth.

In the present work μ-CT scans of experimental bone explants are used to generate the meshes for the computational analysis. The FSI approach developed in Chapter 5 is used to determine the strain in the bone and shear stress in the corresponding experimental samples. This allows for the
determination of the relative roles of bone strain and shear stress in formation of new trabecular bone. In this way the study generates important insights into the mechanical cues for generating an anabolic response in bone.

6.3. Methods

6.3.1. Harvest of bone explants

Bone explants are harvested in an approach similar to previous studies (Davies et al., 2006; Mann et al., 2006). Briefly, ovine vertebrae (C1 – C2) are obtained from the slaughterhouse fresh from slaughter. Working in sterile conditions, skin, muscle and flesh are removed, and the vertebrae are dissected apart. The superior endplates are cut off to reveal the trabecular bone beneath. Using a diamond coring drill (Eternal Tools, Worcestershire, UK) 8 mm diameter trabecular bone explants 15 – 20 mm long are prepared from the C2 vertebrae. All cutting is performed under constant irrigation using ice cold PBS and 5 % Antimycotic Antibiotic (AB-AM, Sigma Aldrich). Bone explants are stored in cold PBS with AB-AM until parallel ends are cut using a low speed diamond saw (Buehler, Lake Bluff, IL) to approximately 10 mm lengths. Bone explants are then placed in media overnight. At this point the explants are considered to be in culture and steps are taken to ensure this arrives not longer than 3 hours post slaughter to limit cell death.
and damage., containing DMEM, 10 % FBS, 2 % AB-AM, 20 mM β-glycerol phosphate and 50 µM L-Ascorbic acid 2-phosphate sesquimagnesium salt hydrate (AA2P, Sigma Aldrich).

The bone explants are then divided into static and loading groups. Static samples \( (n = 4) \) are kept in media in 6 well plates. Media is changed every other day. On day 7 Calcein Blue (Sigma Aldrich) at a concentration of 50 µg/ml is added to the media, this is replaced with fresh media the following day. On day 14 Alizarin Red is added to the media at the same concentration of 50 µg/ml, this media is again replaced on day 15. After 21 days of culture, bone cores are washed with PBS and fixed in 10% formalin for 5 days.

6.3.2. Compression Bioreactor

Compression is applied to the bone explants in the loaded group \( (n = 4) \) through the use of an adapted Enduratec bioreactor (Bose Limited, Gillingham, UK). This was customised in-house to provide physiological loading to trabecular bone cores through the course of an undergraduate final year project by Sean Moran, a Biomedical Engineering student at NUI Galway, working with the author. This is similar to the previously reported Zetos devices (Davies et al., 2006; Jones et al., 2003) (Figure 6.1). A chamber which provides a compressive loading regime to the bone explants is attached to the actuator. The bone explant is held between a fixed bar which
is attached to a load cell in the top of the chamber and the moving bar controlled by the actuator (Figure 6.2). Media is circulated continuously through the system returning to the reservoir through a peristaltic pump (Cole Palmer, Dublin, Ireland) fills the chamber and replenishes the media. Platinum-cured silicone tubing (Cole Palmer) is used to allow for oxygen and carbon dioxide exchange.

Figure 6.1: Flow schematic of the bioreactor structure. Media is pumped through the chamber and returns to the reservoir. Displacement is inputted to control the displacement of the actuator; a load cell relays the resulting force. The pump, media reservoir, tubing, chamber, load cell and actuator are all kept within the incubator.

A cyclic (1 Hz sine wave) compression applying 2000 με compressive nominal strain to the bone explants for 10 minutes is applied for 15 days in a
total of 21 days culture. This loading condition is representative of vigorous uphill running experienced by the tibia (Burr et al., 1996) and is of a magnitude thought to induce an osteogenic response in bone (Rubin and Lanyon, 1985). On day 7 Calcein Blue (50 µg/ml) is added to the media. This media is then filtered in order to re-sterilise it. This is replaced with fresh media which is allowed to circulate through the system for 8 hours the following day, before being replaced with fresh media.

On day 14 Alizarin Red is added to the media at the same concentration of 50 µg/ml and filtered to re-sterilise. This media is again replaced on day 15 with fresh media which is allowed to circulate for 8 hours before being replaced with more fresh media. After 21 days of culture, bone cores are washed with

---

**Figure 6.2:** Photo of the bioreactor with actuator, chamber and pump in the incubator.

---
PBS and fixed in 10% formalin for 5 days and scanned using a Scanco µCT40 MicroCT Scanner at a resolution of 16 µm.

6.3.3. Histomorphometry analysis

After fixation in formalin, both static and loaded bone explants are dehydrated in increasing concentrations of ethanol. Explants are then infiltrated overnight and embedded in polymethylmethacrylate (Osteo-Bed Bone Embedding Kit, Sigma Aldrich). Thin sections are cut using the low speed diamond saw to approximately 200 µm thicknesses and polished with decreasing grit size silicon carbide paper until the required thickness (60 – 100 µm) and surface finish is obtained. Sections are then mounted on microscope slides using DPX.

Images are obtained using an Olympus Inverted Fluorescent Microscope IX51 microscope with DAPI-5060c and U-MWG2 filters to visualise the Calcein Blue and Alizarin Red respectively. Four sections in total were taken from each bone core with five images taken from each section at the magnification of 10X, giving a total of twenty images per sample. From each section bone labelling is quantified using ImageJ (NIH). The mineralising surface (MS) is calculated in each image as the length of any double label plus half the length of any single label divided by the overall bone surface.
6. An experimental and computational investigation of bone formation in mechanical loaded trabecular bone explants

(BS). The mineral apposition rate (MAR) is estimated by the mean distance between the double labels divided by the time period of 7 days (time between the two labels). The bone formation rate (BFR) is derived using the formula: \( \text{BFR} = \text{MAR} \times \text{MS} \). The nomenclature assigned to the histomorphometric indices comply with the standards outlined by ASBMR Histomorphometry Nomenclature Committee (Parfitt et al., 1987).

6.3.4. FSI modelling

FE voxel meshes are generated using Mimics (Materialise, Belgium). The marrow part is created as the inverse of the bone region. A outer shell layer of elements is applied around the bone geometry. This is following the approach described in Chapter 5 which found the casing layer of elements was the most appropriate way of applying boundary conditions for an FSI analysis of bone and marrow. Boundary conditions are applied to the bone and marrow as detailed in Chapter 5 for the FSI-S analyses (summarised in Figure 6.3. for the realistic geometry). In contrast to Chapter 5, here the solid bone deformation is simulated using dynamic implicit analyses within Abaqus/Standard.

For the present simulations, the dynamic implicit analysis proved superior to Abaqus/Explicit in generating successful solutions. An infinitesimal deformation kinematic framework is assumed for the simulations, which is
the default kinematic setting for dynamic implicit in Abaqus. This restriction to small strain kinematics is reasonable given the relatively small magnitudes of bone strains considered in these simulations, e.g. 2000 με equates to only 0.2 % strain. Although bending of some trabeculae during loading may lead to higher local strains.

Material properties of bone and marrow match the values described in Chapter 5. Briefly, bone is modelled as linear elastic with $E = 15$ GPa and $\nu = 0.3$. The soft outer shell is also linear elastic with $E = 15$ MPa. A density of 2 g/cm$^3$ is applied to both. Bone marrow is modelled as a Newtonian fluid with a viscosity of 0.4 Pa.s and a density of 0.9 g/cm$^3$ (Bryant et al., 1989). Loading conditions are applied to represent the experimental conditions of a cyclic (1 Hz) compression applying 2000 με nominal strain to the bone matrix (see Figure 5.4 except an amplitude of 2000 με is applied in this study). These loading conditions, as mentioned in the previous section, represent vigorous uphill running. Bone and marrow cubes of 1.6 mm$^3$ are analysed for each sample. Following the approach of Chapter 5, C3D8R elements are used for the solid bone domain with bone mesh sizes (including the outer casing layer of elements) of 135323, 217008, 297779 and 246689 for the 4 analyses. Again, FC3D8 elements are used for the fluid domain with marrow mesh sizes of 749413, 802296, 816166 and 835327 for the 4 analyses.
Element size is fixed at the voxel size of 16 µm³. This gives BV/TV values for the four analyses of 10.83, 19.62, 28.73 and 22.11 %. Shear stress in the marrow is calculated using the reported shear rate based on the second invariant of the rate of deformation tensor and multiplying this by the viscosity. This is explained in greater detail in Section 5.3.4 (DS SIMULIA, 2012).

6.3.5. Statistical Analysis

Paired t-tests are applied to compare the formed bone with the resorbed bone and to compare pre and post-experiment µ-CT parameters. Regression analyses are performed to assess the relationship between shear stress and bone strain as calculated in the computational models and the change in parameters over the course of the experiment as measured by µ-CT. All analyses are performed with Minitab. For all comparisons, the level of significance is $p \leq 0.05$.

6.4. Results

6.4.1. Trabecular bone histomorphometry

Analysis of the trabecular sections shows that there is a greater mineral apposition rate for bone explants exposed to compressive loading compared
Figure 6.3: Boundary conditions applied to the realistic geometries, following the approach in Chapter 5 for the FSI-S analysis. Casing layer of elements and marrow are transparent for clarity in images.
to the explants cultured in static conditions (MAR, Figure 6.4a). There is no significant difference between the two groups for mineralising surface (MS, Figure 6.4b). However, the bone formation rate is greater in the loaded groups compared to the static group (BFR, Figure 6.4c). Representative images of calcein, alizarin red and double labels are presented in Figure 6.5. For clarity, alizarin is red colour with calcein coloured in green

6.4.2. FSI modelling of trabecular bone

Bone Strain
The distribution of max principal strain is demonstrated for each loaded trabecular bone explant in Figure 6.6, where L1, L2, L3 and L4 represent each of the loaded samples. The majority of bone in each loaded sample is experiencing less than 1000 με (88 – 98 %, Table 6.1). Average values of strains in each sample, range from 363 to 635 με. The percentage of bone experiencing strain within the reported stimulatory range (1000 to 3000 με) for each sample is displayed in Table 6.1. In addition to strain throughout the bone a particular focus was applied to the strain on the bone surface with elements at the bone/marrow interface analysed in isolation.
6. An experimental and computational investigation of bone formation in mechanical loaded trabecular bone explants

![Bar charts showing (a) Mineral Apposition Rate (MAR) (b) Mineralising Surface (MS) and (c) Bone Formation Rate (BFR) for the loaded and static bone explants where * indicates significance between the average values for the loaded group compared to the static group. $p < 0.05$ as calculated using a paired t-test.]

**Figure 6.4.**
Figure 6.5: Representative images of calcein and alizarin labels. Alizarin (red) labels are indicated with #, calcein (green) labels are indicated with * and double labels are indicated with arrows.
The percentage of bone surface experiencing strain within the reported stimulatory range (1000 to 3000 με) is displayed in Figure 6.7b for each sample. By calculating the strain in the surface elements alone, the amount of elements experiencing less than 1000 με strain decreases to 83 - 96 %. Additionally the average value of strain across the bone surface increases to 419 - 677 με and the percentage of bone experiencing between 1000 and 3000 με strain is greater at the surface than within the total bone (Table 6.2).

**Figure 6.6:** Max principal strain in the solid bone of each sample. Samples are sectioned, with the outer case of elements removed and the marrow coloured in grey for clarity.
An experimental and computational investigation of bone formation in mechanical loaded trabecular bone explants

Table 6.1: The percentage of total bone experiencing strain within different ranges and the average max principal strain within each sample.

<table>
<thead>
<tr>
<th>Value of Max Principal Strain (µε)</th>
<th>Loaded Samples</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>L1</td>
</tr>
<tr>
<td>% of Bone</td>
<td></td>
</tr>
<tr>
<td>&lt; 1000</td>
<td>97.84</td>
</tr>
<tr>
<td>1000 – 3000</td>
<td>2.20</td>
</tr>
<tr>
<td>&gt; 3000</td>
<td>0.01</td>
</tr>
<tr>
<td>Average Max Principal Strain (µε)</td>
<td>363.42</td>
</tr>
</tbody>
</table>

Figure 6.7: Distributions of max principal strain within the bone of each compressively loaded trabecular bone explants for the (a) total bone and (b) at the bone surface.
Table 6.2: The percentage of the bone surfaces experiencing strain within different ranges and the average strain within each sample.

<table>
<thead>
<tr>
<th>% of Bone Surface</th>
<th>Value of Max Principal Strain (µε)</th>
<th>Loaded Samples</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt; 1000</td>
<td></td>
<td>L1</td>
</tr>
<tr>
<td>1000 – 3000</td>
<td></td>
<td>7.12</td>
</tr>
<tr>
<td>&gt; 3000</td>
<td></td>
<td>0.09</td>
</tr>
<tr>
<td>Average Max Principal Strain (µε)</td>
<td></td>
<td>419.36</td>
</tr>
</tbody>
</table>

Shear Stress in Marrow

The distribution of shear stress within the marrow is shown for each trabecular bone explant in Figure 6.8. In the mechanically loaded trabecular explants the average shear stress within the marrow is found to range from 0.018 to 0.020 Pa with less than 1 % of the marrow in each sample (0.58, 0.22, 0.11 and 0.04 %) experiencing greater than 0.1 Pa. The percentage of total marrow experiencing shear stress within the range of 0.01 and 0.1 Pa is displayed in Figure 6.9a and summarised in Table 6.3. When the surface elements alone are examined, the percentage of marrow experiencing stress greater than 0.1 Pa increases in all samples (6.22, 1.57, 1.36 and 0.23 %, Figure 6.9b and Table 6.4).

6.4.3. Correlation between mechanical loading and bone growth

It is of significant interest to explore potential correlations between the primary variables in the computational modelling and the experiments. No
Figure 6.8.: Shear stress within the bone marrow of the compressively loaded trabecular bone explants. The bone is coloured in white and the marrow mesh is removed for clarity.
6. An experimental and computational investigation of bone formation in mechanical loaded trabecular bone explants

**Figure 6.9.** Distributions of shear stress within the bone marrow of each compressively loaded trabecular bone explants.

**Table 6.3:** The percentage of the bone marrow experiencing shear stress within different ranges and the average shear stress within each sample.

<table>
<thead>
<tr>
<th>% of Marrow</th>
<th>Value of Shear Stress (Pa)</th>
<th>L1</th>
<th>L2</th>
<th>L3</th>
<th>L4</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt; 0.01</td>
<td>5.69</td>
<td>10.07</td>
<td>16.81</td>
<td>6.58</td>
<td></td>
</tr>
<tr>
<td>0.01 – 0.05</td>
<td>83.69</td>
<td>88.74</td>
<td>80.48</td>
<td>90.68</td>
<td></td>
</tr>
<tr>
<td>0.05 - 0.10</td>
<td>10.04</td>
<td>3.97</td>
<td>2.60</td>
<td>2.71</td>
<td></td>
</tr>
<tr>
<td>&gt; 0.1</td>
<td>0.58</td>
<td>0.22</td>
<td>0.11</td>
<td>0.04</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Average Shear Stress (Pa)</th>
<th>L1</th>
<th>L2</th>
<th>L3</th>
<th>L4</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.030</td>
<td>0.022</td>
<td>0.018</td>
<td>0.023</td>
<td></td>
</tr>
</tbody>
</table>
Table 6.4: The percentage of the bone marrow surface experiencing shear stress within different ranges and the average shear stress within each sample.

<table>
<thead>
<tr>
<th>% of Marrow Surface</th>
<th>Value of Shear Stress (Pa)</th>
<th>Loaded Samples</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>L1</td>
<td>L2</td>
</tr>
<tr>
<td>&lt; 0.01</td>
<td>3.49</td>
<td>6.61</td>
</tr>
<tr>
<td>0.01 – 0.05</td>
<td>54.48</td>
<td>70.36</td>
</tr>
<tr>
<td>0.05 - 0.10</td>
<td>35.81</td>
<td>21.46</td>
</tr>
<tr>
<td>&gt; 0.1</td>
<td>6.22</td>
<td>1.57</td>
</tr>
</tbody>
</table>

Average Shear Stress (Pa) 0.050 0.037 0.034 0.032

significant correlation is found between specific levels on bone strain or shear stress within each loaded sample and the amount of formed bone, as calculated using MAR, MS and BFR. Figure 6.10a and Figure 6.10b demonstrate the lack of correlation between the surface bone strain or surface marrow shear stress calculated from the models and the BFR calculated from the explanted trabecular bone samples exposed to loading.

For the simulations average shear stress within the marrow is found to be lower in models with higher BV/TV as shown in Figure 6.10. This BV/TV is determined post-experiment using the µ-CT scans. A significant correlation is found between average shear stress within the total marrow and the BV/TV. This however is not the case for the average surface shear stress. No significant correlation is found for the average strain within the bone ($p = 0.418$) or at the surface ($p = 0.425$) with the BV/TV.
6.5. Discussion

In this chapter the effect of compressive loading on trabecular bone explants is determined through the use of a custom built bioreactor coupled with computational modelling of the bone and marrow. Bone explants exposed to compressive loading are found to have a significantly higher MAR and BFR compared to static explants demonstrating the anabolic effect of compressive loading in the bone explant model.

The work of this chapter builds on the computational modelling approach used in Chapter 5 with the methods developed for FSI modelling applied to realistic geometries obtained for µ-CT scans of experimental samples. Results show that average shear stress within the marrow ranges from 0.018 – 0.030 Pa, while the average strain experienced in each sample ranged from 363 – 635 με. Bone strain and marrow shear stress are highest in the regions at the bone/marrow surface. The averages of both shear stress and strain increase when the surfaces alone are examined (0.032 – 0.050 Pa and 419 – 677 με).

Both static and compressively loaded samples are found to be actively producing calcium with mineralising surfaces visible in both cases, and with both single and double labels visible. However, the MAR and the BFR were
6. An experimental and computational investigation of bone formation in mechanical loaded trabecular bone explants

Figure 6.10: (a) No significant correlation is found between surface bone strain and BFR or (b) surface shear stress and BFR. (c) The average shear stress generated at the surface of the marrow and total the total marrow plotted with respect to BV/TV. A significant correlation is found for total marrow shear stress.
significantly greater in the compressively loaded samples compared to the static samples. This is in agreement with similar studies which found that the \textit{in vivo} anabolic effects of mechanical strain are reproduced in a trabecular bone explant model (David \textit{et al.}, 2008; Mann \textit{et al.}, 2006). However, to the author’s knowledge, this is the first time the \textit{ex vivo} effects of bone strain have also been examined using a combined computational and experimental approach. Explant models have advantages in that samples can be obtained and maintained relatively easy and cheaply compared to animal models. Additionally, for examining the causes of bone remodelling in response to mechanical loading, the explant models have the advantage of isolating the bone (and marrow) from other potential effectors, for example, muscle stimulation (Qin \textit{et al.}, 2010; Qin and Lam, 2009), the nervous system and circulating blood.

It is of significant interest to compare the experimental data with the model predictions. Strains of the magnitude 1000 – 3000 με are thought to stimulate new bone formation (Burr \textit{et al.}, 1996; Fritton \textit{et al.}, 2000). Results here show that 2.2 – 12.1 % of the total bone in the compressively loaded samples is experiencing between 1000 and 3000 με. This increases to 4.3 – 17.4 % when just the bone surface is examined. It is likely that the generated strain is the
significant stimulatory factor in this case rather than the resulting shear stress in the marrow.

While no direct correlation is apparent between individual magnitudes of strains and the corresponding bone growth in this study, there is sufficient strain generated in certain regions of the bone to stimulate an anabolic response. This suggests that a constant level of strain is not required within the trabecular structure to stimulate local bone formation. For example, the sample (L1) with only 2.2 % of the total bone experiencing between 1000 and 3000 με. 97.8 % of the bone is experiencing < 1000 με which is thought to be at a level which leads to resorption however the sample as a whole has a BFR of 0.24 μm/day. This indicates that the effects of this strain, which is highest in localised regions, are being transduced throughout the structure. This is likely due to the extensive network of osteocytes throughout the trabecular bone structure. These cells, ideally placed within the bone (Bonewald, 2007; Huiskes et al., 2000; Schaffler et al., 2013), are directly stimulated when the bone is under strain (Verbruggen et al., 2012) and are capable of inducing an osteogenic response in MSCs cells, as detailed in Chapter 4.
Regarding shear stress, as indicated previously in Sections 5.7 and 6.2, 2D parallel plate flow chamber experiments which expose cells to shear stress values of 0.1 to 1 Pa have been shown to stimulate osteogenic responses in MSCs and pre-osteoblastic cells (Arnsdorf et al., 2009; Bakker et al., 2003; Case et al., 2011). Here less than 1 % of the total marrow, in each compressively loaded sample, is experiencing shear stress in the stimulatory range of greater than 0.1 Pa. However when the surface alone is examined the percentage experiencing greater than 0.1 Pa increases in all cases. Similar to the strain results, no significant correlation is apparent between the individual shear stress values in each sample and the corresponding bone growth in this study. However, it is clear that the lower the BV/TV the higher the shear stress experienced by the marrow, this is in agreement with the idealised geometry examined in Chapter 5.

The hypothesis addressed in this chapter (Hypothesis 4, Section 1.3) is that physiological compression of trabecular bone generates shear stress within the bone marrow which stimulates new bone growth. It is found within the computational models of realistic geometries that shear stress of sufficient magnitude to produce an osteogenic response in MSCs is generated during physiological compression. However, there is also bone strain of significant magnitude generated during compression which has the potential of being
transduced by osteocytes in their extensive network and transmitted through a biochemical response throughout the bone structure. While the osteocyte network is well established as capable of detecting and transducing a biochemical response, in response to bone strain it is unknown if localised shear stress effects can be transduced through the marrow. However, recent work on the micromechanical modelling of marrow could provide some insights (Vaughan et al., 2013) into potential networks within the marrow. Based on the results of this chapter it is likely that bone strain is the dominant driver at work to induce the anabolic response in bone growth. This is due to the greater amount of bone experiencing strain of sufficient magnitude to generate an anabolic bone growth response, compared to the amount of marrow experiencing shear stress of such a sufficient magnitude.

6.6. Conclusions

In summary in vivo responses of trabecular bone to compression loading are replicated in explanted trabecular bone samples. Explants exposed to compression loading show a significantly higher MAR and BFR compared to static samples. For each loaded sample the bone strain and resulting shear stress within the marrow is calculated using FSI modelling techniques developed in the previous chapter and geometries developed from µ-CT scans of the experimental samples. Average bone strain experienced in each
sample ranges from 363 – 635 με while average shear stress within the marrow ranges from 0.018 – 0.030 Pa. Bone strain and marrow shear stress is highest in the regions at the bone/marrow surface. The average marrow shear stress is found to decrease for samples with increasing BV/TV, which is consistent with what was shown in the previous chapter for the idealised geometries.

No significant relationship is apparent between bone growth and individual levels of bone strain or marrow shear stress within each sample. As such, potential differentiation of MSCs within the marrow may be more likely due to a combination of indirect stimulation (bone strain through the osteocyte network) and direct stimulation (shear stress in the marrow). However even if this is the case, a low percentage of each sample is shown to experience bone strain and marrow shear stress within the stimulatory range (1000 – 3000 με for bone strain and > 0.1 Pa for marrow shear stress). Therefore, due to this low percentage within the stimulatory ranges it is thought that the extensive osteocyte network within the bone structure is in play and is capable of transmitting the local effects of high strain into a biochemical response to stimulate other cells throughout the bone structure. It is not known if such a network exists for the marrow, meaning that the localised regions of high shear stress could indeed remain localised to cells within that
area. Further research is required in the area of micromechanical modelling of the multi-cellular bone marrow structure to determine if such a marrow network exists.

To further elucidate these issues the next chapter aims to remove the bone strain effect and examine whether shear stress only within the marrow can target MSCs and cause an anabolic response in bone. This is examined through the use of LMHF vibration of trabecular bone explants, allowing for higher levels of shear stress (Coughlin and Niebur, 2012) compared to compression while applying very low levels of strain to the bone structure (Garman et al., 2007; Judex et al., 2007).
Mechanical stimulation of bone marrow in situ induces bone formation in trabecular explants

7.1. Chapter Background

This chapter investigates Hypothesis 5, outlined in Section 1.3, which states that LMHF loading stimulates new bone formation in areas of high shear stress within the marrow. LMHF loading has been shown to have an anabolic effect on trabecular bone in vivo (Rubin et al., 2001b). However, the precise mechanical signal imposed on the bone marrow cells by LMHF loading, which induces a cellular response, remains unclear. Building on the approach of Chapter 6, LMHF loading is applied to an explanted trabecular bone model using a custom designed bioreactor. Bone adaptation is investigated by performing µCT scanning before and after experimental LMHF loading, and these scans are post-processed using image registration techniques to compare pre and post experimental scans. CFD models are generated based on the pre-experiment scans to characterise the mechanical stimuli imposed by the loading regime prior to adaptation, and the
7. Mechanical stimulation of bone marrow in situ induces bone formation in trabecular explants

Mechanical environment is compared to bone adaptation characterised from the post-experiment scans.

LMHF loading applies high levels of shear stress to the marrow (Coughlin and Niebur, 2012), but the levels of strain (approximately 10 με) generated in the trabecular bone tissue are believed to be insufficient to generate an anabolic response in trabecular bone (Judex et al., 2007). In contrast, peak strains of 1000 - 3000 με have been reported to be induced during typical physiological activities such as running or jumping, which are known to lead to bone growth and remodelling (Burr et al., 1996; Mann et al., 2006).

Thus applying LMHF loading allows for the mechanoresponsive cells within the marrow to be isolated from the strain mediated response which is governed by the effector cells in bone tissue, osteocytes and osteoblasts, which induce osteogenic differentiation in MSCs through biochemical signalling (Chapter 4). As such this system allows the investigation of the mechanical environment of the bone marrow directly. This provides additional understanding of the mechanical environment of MSCs, previously investigated in Chapter 5 and 6 of this thesis which focused on the strain induced under compressive loading.
7.2. Introduction

As discussed in Section 2.2.8, LMHF loading has been shown to have an anabolic effect on bone tissue (Judex et al., 2003; Pasqualini et al., 2013; Rubin et al., 2001b; Rubin et al., 2002). Additionally, the measured bone strains associated with LMHF vibration can be less than 10 με. This has been demonstrated in a rat model (Judex et al., 2007) where the strain generated in bone due to LMHF loading, as measured with a strain gauge attached to the tibia of a rat, was two orders of magnitude below which regulate bone formation (Burr et al., 1996; Mann et al., 2006; Rubin and Lanyon, 1984). As such, it is unclear whether the small strains occurring in LMHF vibration are sufficient to explain its anabolic effects. Interestingly, increasing the frequency of the LMHF signal from 45 to 90 Hz results in greater increases in both bone volume and trabecular thickness, but the higher frequency does not generate higher bone strain (Judex et al., 2007). Furthermore, the anabolic effects of LMHF loading (0.3 g or 0.6 g at 45Hz) have been observed in trabecular bone in non-load bearing applications (Garman et al., 2007). Taken together these studies suggest that the response of bone tissue to LMHF vibration is not driven by the bone strain.

LMHF loading has the greatest effect on bone formation in regions rich in trabecular bone. As detailed in Section 2.1 trabecular bone is home to the
bone marrow stem cell niche. However, the mechanical environment of trabecular bone marrow remains poorly characterised (Gurkan and Akkus, 2008). It alters with aging and conditions such as osteoporosis (Rosen and Bouxsein, 2006) with the adipose fraction of marrow increasing (Justesen et al., 2001; Weisberg et al., 2003).

The position of marrow within the trabecular bone structure suggests that LMHF loading is likely to induce inertial motion in the marrow, possibly generating shear stresses and thereby affecting the resident cells (Coughlin and Niebur, 2012). MSCs exposed to shear stress in vitro exhibit increased proliferation (Riddle et al., 2006), expression of osteogenic differentiation markers (Grellier et al., 2009; Kreke et al., 2008), and inhibition of adipogenic markers (Kreke et al., 2005). However, the influence of shear stress in vivo remains unknown. The low levels of bone strain during LMHF loading, in addition to the sensitivity of MSCs to shear stress, which is generated in LHMF loading suggests that the mechanical environment of marrow is instrumental in the transmission of the mechanical signals. However, this is not yet fully understood.

Computational and numerical models (see Section 2.6) have been used to predict shear stress generated within trabecular bone marrow due to LMHF vibration (Coughlin and Niebur, 2012) and compression, as described in
Chapter 5. 3D CFD models of marrow within realistic trabecular structures (Coughlin and Niebur, 2012) showed that there is sufficient shear stress generated within the marrow during LMHF to stimulate MSC osteogenic differentiation. An analytical continuum level mixture theory (Dickerson et al., 2008) predicted shear stresses (~0.5 Pa) in trabecular bone during cyclic low amplitude strains.

As discussed in the previous chapter trabecular bone explants can be used to examine bone formation in response to compression of the bone matrix (David et al., 2008; Davies et al., 2006; Endres et al., 2009; Jones et al., 2003; Mann et al., 2006). However such approaches have not yet been applied to investigate the anabolic response of LMHF loading.

The objective of this chapter is to determine whether low-magnitude-high-frequency loading stimulates new bone formation in areas of high shear stress within the marrow. The anabolic effect of LMHF loading is characterised by an increase in trabecular bone quality and quantity. This hypothesis is tested using porcine trabecular bone explants, with marrow in situ, which are stimulated by LMHF loading in a custom bioreactor. The bone explants are scanned pre and post loading by μ-CT, and images are registered and segmented to determine the extent of changes in bone volume and architecture over the course of stimulation. Fluorochrome dyes are also
used to label newly formed bone. Solid models are generated from the pre-experiment µ-CT scans of bone explants, and these are used to create FE meshes of the experimental samples and CFD models are solved to determine the shear stress generated within the bone marrow specific to each experimentally LMHF loaded sample. This allows for the exploration of the relationship between shear stress in the marrow and bone formation and remodelling.

7.3. Materials and Methods

7.3.1. LMHF Bioreactor Design

A custom built bioreactor chamber was developed in collaboration with Prof. Glen Niebur from the University of Notre Dame to apply LMHF loading to live trabecular bone explants in vitro. The experimental portion of this chapter was performed in Notre Dame. The chamber holds three bone explants in individual chambers firmly in place between two flat platens on threaded bars (Figure 7.1). Media is perfused through the chamber within a 1.5 mm annular space surrounding the explant. The media is pumped at constant flowrate of 0.9 ml/min through the chamber using a peristaltic pump (Ismatec, REGLO) with gas permeable PharMed Ismaprene tubing (Ismatec). The entire chamber is subjected to a controlled sinusoidal acceleration with a peak of ±0.3 g at 30 Hz using a linear voice coil actuator.
Mechanical stimulation of bone marrow in situ induces bone formation in trabecular explants (H2W Technologies), which provides a short stroke with closed loop position control. The motor is controlled with a Programmable Motor Controller (Elmo Motion Control).

Figure 7.1: (a) LMHF Bioreactor with bone explants in place in the chamber, showing media being pumped from media reservoirs into the individual explants, and two chambers attached to the actuator. (b) Schematic of a chamber with bone explants in place detailing the media path.

7.3.2. Bone tissue culture

The harvest of bone cores have been described previously in Chapter 6. A similar approach is followed here. However, for this study porcine vertebrae are used instead of ovine vertebrae (this is due to supply issues). Briefly, vertebrae (C1 – C6) are obtained from the slaughterhouse within 2 hours of slaughter. Working in sterile conditions, skin, muscle and flesh are removed, and the vertebrae are dissected apart. 8 mm diameter trabecular bone explants 15 – 20 mm long are prepared from C2 – C6 vertebrae using a diamond coring drill (Starlite Industries, Rosemount, PA). Once cut to
approximately 10 mm lengths bone explants are placed in media containing Dulbecco’s Modified Enriched Media (high glucose, DMEM, Sigma Aldrich), 10 % FBS, 2 % AB-AM, 20 mM β-glycerol phosphate and 50 µM AA2P and are imaged by μ-CT (Scanco μ-CT-80, Brüttisellen, Switzerland) at 20 µm isotropic resolution using a 70 kVp x-ray source at 114 mA and 200 ms integration time in a sterile fixture holder.

7.3.3. LMHF experimental approach

Trabecular explants are divided into three experimental groups. (1) **Static** (*n* = 5): Explants are cultured in 6 well plates with 10 ml of media. (2) **Flow** (*n* = 3): Explants are placed in the bioreactor chamber, where media is circulated through the chamber at a flow rate of 0.9 ml/min. (3) **Vibrated** (*n* = 7): Explants are placed in the bioreactor chamber, where media is again circulated through the chamber at the same flow rate. This group also received vibrational loading each weekday of ± 0.3 g at 30 Hz for one hour.

Following 19 days of culture, all trabecular explants are again imaged by μ-CT, as described below. Following this explants are fixed in formalin for 5 days and then dehydrated in increasing concentrations of ethanol. Explants are then infiltrated overnight and embedded in polymethylmethacrylate (DHM, Villa Park, IL).
7.3.4. Bone formation labelling

Fluorochrome labels are applied to label bone formation during culture. On day 7 the media is replaced in all groups with media containing 50 µg/ml of Tetracycline (Sigma Aldrich). On day 8 of culture this media is replaced with fresh media, which is allowed to circulate for approximately 8 hours followed by a second change of media. On day 14 the media is replaced with fresh media containing 50 µg/ml of Calcein Blue. On day 15 this media is replaced with fresh media and again this is allowed to circulate for 12 hours before being replaced with fresh media.

7.3.5. µ-CT Analysis for Trabecular Microarchitecture.

The pre-experiment and post-experiment scans are registered to allow direct comparison of the same region of the explant. The paired images are registered using a normalized mutual information algorithm (NMI) in Analyze (Mayo Clinic, Overland Park, KS). This algorithm applies volume sub-sampling and greyscale binning to achieve accurate registration. However, it has a limited capture range that can fail to properly align highly mis-registered images. To avoid this, an approximate registration is performed by manually transforming the image before applying the NMI algorithm to finalise the registration. This registration was performed by Tyler Kreipke, a Biomedical Engineering Graduate Student at the University
of Notre Dame. Subsequent analysis of the registered images was performed in Galway by the author.

Following image registration, bone formation and resorption are quantified using ImageJ (NIH). A volume of at least 125 mm$^3$ is analysed for each bone explant. The BV/TV, bone surface/bone volume (BS/BV), trabecular thickness (Tb.Th), trabecular spacing (Tb.Sp), trabecular number (Tb.N) and slenderness (Tp.Sp/Tb.Th) are determined for both the pre- and post-experiment scans. Slenderness is analogous to the slenderness ratio in the Euler buckling formulae; it can be used to indicate increased susceptibility to buckling and, as a consequence, a decreasing slenderness ratio indicates greater energy absorption (Garrison et al., 2009).

The structural model index (SMI) is also calculated; this gives an indication of the plate-like (SMI = 0) or rod-like (SMI = 3) geometry of trabecular bone. All trabecular architecture parameters are determined for the registered region using the BoneJ plugin to ImageJ (Doube et al., 2010). The same filtering and segmentation parameters are used for both scans. Regions of bone resorption and formation are detected using image comparison. Regions present in the first µ-CT scan but not the second are considered resorbed bone areas, while areas only present in the latter scan corresponded to newly formed bone.
3D quantification of bone morphometry parameters is performed using the approach of Schulte et al. (2011). Parameters which were previously calculated in Chapter 6 in 2D only are now calculated as 3D parameter through the use of μ-CT scans. Briefly, the MAR is calculated using the mean thickness of the new bone formed divided by the number of days between the μ-CT scans. Similarly, the mineral resorption rate (MRR) is calculated using the mean thickness of the resorbed bone. This is not possible to calculate using traditional bone morphometry techniques, as any resorbed bone is lost post-experiment and pre-experiment imaging is not possible due to the destructive nature of sectioning.

The MS is calculated using the surface of the formed bone, subtracting the BS of the pre experiment scan, adding the BS of the post experiment scan and dividing by two, leaving just the surface of the formed bone which is then divided by the original BS. The eroding surface (ES, again this parameter is not possible to calculate using traditional bone morphometry techniques) is calculated using the surface of the resorbed bone, subtracting the BS of the post experiment scan, adding the BS of the pre experiment scan and dividing by two, leaving the surface of the resorbed bone and dividing it by the total BS at day 0. Finally, the BFR and bone resorption rate (BRR, once more this parameter is not possible to calculate using traditional techniques) are
calculated using the total amount of bone formed (or resorbed for BRR) per the total bone volume at day 0 per day.

7.3.6. Computational Modelling

The pre-experiment μ-CT scans of the Vibrated group (n = 7) are used to create 3D CFD models of the marrow to predict the mechanical environment under the applied vibrational loading following the previously developed approach of Coughlin and Niebur, (2012) that replicate the *in vivo* loading conditions experienced by trabecular marrow during LMHF loading. Briefly, in each of the 7 cases, a 3 X 3 X 3.5 mm³ trabecular bone region is selected, Gaussian filtered, and resampled by cubic interpolation to 35 µm resolution using Visualization Toolkit, (VTK). A marching cubes algorithm from VTK is used to discretise the marrow regions into tetrahedral elements. Tet elements are used instead of 8 noded brick elements used in Chapter 5 and 6. This is possible as a CFD only analysis is being performed and as such mating surfaces between a solid and fluid domain is not required.

CFD simulations are performed in Abaqus/CFD (version 6.12) using FC3D4 elements. The fluid elements are assigned incompressible Newtonian fluid properties with a density of 0.9 g/cm³ and viscosity of 400 mPa.s (Bryant *et*
7. Mechanical stimulation of bone marrow in situ induces bone formation in trabecular explants

*et al.,* 1989). The bone-marrow interface is assumed to be rigid, with a no slip interface.

A 30 µm layer of fluid is added around the entire marrow sample to allow continuity of flow from pores on the edges, resulting in a model geometry such as that shown in Figure 7.2. Symmetry fluid flow constraints are placed on the faces parallel to the vibration direction preventing flow across the boundaries. Constant pressure outlets are applied to the surfaces perpendicular to the vibration allowing free flow.

Nodes on the interface are assigned a sinusoidal velocity equivalent to a ± 0.3 g acceleration at 30 Hz along the vertical axis of the explant (the Y-direction in the example of Figure 7.2), matching the experimental conditions. The simulations are run for 5 cycles; however changes from cycle to cycle are negligible, showing that transient effects could be ignored. To avoid artefacts at the artificial fluid layer, the shear stress in the marrow is analysed in a sub region (3 X 3 X 3.3 mm³) at the peak point of shear stress in a cycle.

Shear stress is calculated as described previous in Section 6.3.4 using the reported shear rate based on the second invariant of the rate of strain tensor and multiplying this by the viscosity. Media flow is assumed to have a negligible effect on the generation of shear stress in the bone explants due to
the high viscosity of marrow compared to media and so is not included in the models. Additionally, the modelled region is within the core away from the edges.

**Figure 7.2:** 3D CFD marrow model with boundary conditions, based on the modelling approach of Coughlin and Niebur 2012. Fluid flow is modelled as symmetric on the X and Z surfaces. Constant pressure outlets are applied on the Y surfaces. Sinusoidal velocity is applied on the bone marrow interface in the Y direction. An extra 30 µm layer of fluid is added to the edges of the marrow region to allow continuity of flow. In this image the marrow region is transparent to reveal the trabecular bone geometry within.

### 7.3.7. Statistical Analysis

Paired t-tests are applied to compare the formed bone with the resorbed bone and to compare pre and post-experiment µ-CT parameters in each bone explant. One-way analysis of variance (ANOVA) followed by pair-wise comparison (Tukey’s HSD test) is used to test for significance between Static,
7. Mechanical stimulation of bone marrow in situ induces bone formation in trabecular explants

Flow, and Vibrated groups. Regression analyses are performed to assess the relationship between shear stress as calculated in the CFD models and the change in parameters over the course of the experiment as measured by μ-CT. All analyses are performed with Minitab. For all comparisons, the level of significance is $p \leq 0.05$.

7.4. Results

7.4.1. Fluorochrome Labelling

Active bone modelling is found in all samples regardless of experimental conditions, as indicated by the presence of tetracycline and calcein blue labels. Representative images with regions of bone indicating clear linear labels are included in (Figure 7.3) as examples of staining of calcium and dye uptake over the course of the experiment.

7.4.2. μ-CT parameters

On average, bone formation is greater than resorption in the Vibrated group ($p < 0.001$, Table 7.1), while there is no statistical difference for the same comparison in the Static or Flow groups ($p > 0.05$, Table 7.1). The positive bone balance results in higher BV/TV ($p < 0.001$) and Tb.Th ($p < 0.001$) following culture in the Vibrated group (Table 7.2), while both the Static and
Flow samples have no significant increase in BV/TV over the course of the experiment (Table 7.2).

**Figure 7.3.** Tetracycline and calcein blue incorporation into trabecular bone explants. All scale bars are 70 µm. Asterisks indicate incorporation of tetracycline (white) and calcein blue (red) into the bone and onto the bone surface. Arrow heads indicate regions of double label.

**Table 7.1.** Mean ± standard deviation for the BV/TV seen in the post experiment scans (formation) but not the pre experiment scans and the volume of bone seen in the pre experiment scans but not the post experiment scans (resorption). Significant differences are represented in bold (p < 0.05, paired t-test).

<table>
<thead>
<tr>
<th>Group</th>
<th>Formation</th>
<th>Resorption</th>
<th>Difference</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Static</td>
<td>0.031 ± 0.013</td>
<td>0.032 ± 0.015</td>
<td>-0.001</td>
<td>0.675</td>
</tr>
<tr>
<td>Flow</td>
<td>0.035 ± 0.009</td>
<td>0.030 ± 0.007</td>
<td>+0.005</td>
<td>0.082</td>
</tr>
<tr>
<td>Vibrated</td>
<td><strong>0.050 ± 0.015</strong></td>
<td><strong>0.036 ± 0.016</strong></td>
<td><strong>+0.014</strong></td>
<td>&lt; 0.001</td>
</tr>
</tbody>
</table>
Consequently, the relative increase in BV/TV and Tb.Th is higher in the Vibrated group than in the other groups ($p = 0.0001$ versus Static and $p = 0.0033$ versus Flow for BV/TV, $p = 0.0016$ versus Static and $p = 0.0489$ versus Flow for Tb.Th, ANOVA and Tukey’s HSD test, Figure 7.4a and b).

Considering the trabecular architecture, the SMI decreases significantly ($p < 0.001$, Table 7.2) in the Vibrated group while no difference in SMI is seen in the Static and Flow groups between the two time points ($p = 0.061$ and 0.658, respectively). While there is a significant decrease in Tb.N for the Flow ($p = 0.002$) and Vibrated ($p = 0.008$) groups (Table 7.2.) between pre and post experiment values, no significant difference in the relative percentage changes between groups is found (Vibrated versus Static $p = 0.9843$, Vibrated versus Flow $p = 0.9699$). Tb.Sp is found to increase significantly only in the Static group ($p = 0.011$).

A significant decrease in the slenderness of the Vibrated group is also found over the course of the experiment ($p < 0.001$), but not in the Static group ($p = 0.082$) or Flow group ($p = 0.173$, Table 7.2). Accordingly the relative decreases in slenderness is greater in the Vibrated group than in the static groups ($p = 0.0004$ versus Static and $p = 0.0209$ for Flow, Figure 7.4c). A significant decrease in BS/BV is found in the Static ($p = 0.042$) and Vibrated groups ($p < 0.001$) between the two time points (Table 7.2). The relative decrease in the
Table 7.2: Mean ± Standard Deviation of trabecular bone architectural parameters for the Static \((n = 5)\), Flow \((n = 3)\) and Vibrated \((n = 7)\) groups. BV/TV (bone volume/tissue volume), Tb.Th (trabecular thickness), Tb.N (trabecular number) SMI (structural model index), BS/BV (bone surface/bone volume) and slenderness (trabecular spacing/trabecular thickness). \(p\) values less than 0.05 are deemed to be significant using a paired t-test between the pre experiment scan and the post experiment scan. Significant \(p\) values are represented in bold.

<table>
<thead>
<tr>
<th></th>
<th>BV/TV</th>
<th>Tb.Th (mm)</th>
<th>Tb.Sp (mm)</th>
<th>Slenderness</th>
<th>Tb.N (1/mm)</th>
<th>SMI</th>
<th>BS/BV (1/mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean ± SD</td>
<td>(p) Mean ± SD</td>
<td>(p) Mean ± SD</td>
<td>(p) Mean ± SD</td>
<td>(p) Mean ± SD</td>
<td>(p) Mean ± SD</td>
<td>(p) Mean ± SD</td>
</tr>
<tr>
<td>Static</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pre</td>
<td>0.276 ± 0.044</td>
<td>0.150 ± 0.004</td>
<td>0.485 ± 0.069</td>
<td>2.598 ± 0.139</td>
<td>1.706 ± 0.104</td>
<td>0.398 ± 0.056</td>
<td>5.171 ± 0.247</td>
</tr>
<tr>
<td>post</td>
<td>0.275 ± 0.045</td>
<td>0.153 ± 0.007</td>
<td>0.490 ± 0.072</td>
<td>2.536 ± 0.139</td>
<td>1.683 ± 0.092</td>
<td>0.320 ± 0.057</td>
<td>5.029 ± 0.196</td>
</tr>
<tr>
<td>Flow</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pre</td>
<td>0.327 ± 0.021</td>
<td>0.163 ± 0.007</td>
<td>0.424 ± 0.030</td>
<td>3.232 ± 0.509</td>
<td>1.589 ± 0.179</td>
<td>0.629 ± 0.246</td>
<td>8.961 ± 7.426</td>
</tr>
<tr>
<td>post</td>
<td>0.331 ± 0.020</td>
<td>0.168 ± 0.004</td>
<td>0.427 ± 0.029</td>
<td>3.206 ± 0.531</td>
<td>1.571 ± 0.181</td>
<td>0.615 ± 0.261</td>
<td>8.887 ± 7.387</td>
</tr>
<tr>
<td>Vibrated</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pre</td>
<td>0.233 ± 0.016</td>
<td>0.151 ± 0.010</td>
<td>0.541 ± 0.005</td>
<td>3.596 ± 0.225</td>
<td>1.447 ± 0.025</td>
<td>1.033 ± 0.183</td>
<td>5.893 ± 0.350</td>
</tr>
<tr>
<td>post</td>
<td>0.247 ± 0.016</td>
<td>0.160 ± 0.012</td>
<td>0.540 ± 0.005</td>
<td>3.396 ± 0.225</td>
<td>1.429 ± 0.028</td>
<td>0.873 ± 0.169</td>
<td>5.614 ± 0.318</td>
</tr>
</tbody>
</table>
7. Mechanical stimulation of bone marrow in situ induces bone formation in trabecular explants

Figure 7.4: The mean percentage changes over the course of the experiment in: (a) BV/TV bone volume/tissue volume, (b) Tb.Th, trabecular thickness, (c) slenderness, trabecular thickness/trabecular spacing, (d) BS/BV, bone surface/bone volume, and the histomorphometric parameters (e) MS, mineralising surface, (f) BFR, bone formation rate. Trabecular bone explants are cultured in a static plate (Static, $n = 5$), exposed to fluid flow in the bioreactor chamber (Flow, $n = 3$) or exposed to fluid flow and LMHF loading in the bioreactor chamber (Vibrated, $n = 7$). A one-way analysis of variance (ANOVA) followed by pair-wise comparison (Tukey’s HSD test) is used to test for significance. $a p < 0.05$ versus Static group, $b p < 0.05$ versus Flow group.
Vibrated group is found to be significantly greater than the Static group ($p = 0.0015$) but not the Flow group ($p = 0.1315$) (Figure 7.4d).

MS is found to be significantly higher in the Vibrated group compared to the Flow group ($p = 0.0275$, Figure 7.4e and Table 7.3). Moreover, BFR is found to be significantly higher in the Vibrated group compared to both the Static ($p = 0.0188$) and Flow ($p = 0.0318$) groups (Figure 7.4f and Table 7.3). No significant differences are found between the Static, Flow and Vibrated groups for MAR, MRR, ES and BRR.

Table 7.3: Mean ± Standard Deviation for 3D trabecular bone morphometry parameters for the Static (n = 5), Flow (n = 3) and Vibrated (n = 7) groups. Mineral apposition rate (MAR), mineral resorption rate (MRR), mineralising surface (MS), eroding surface (ES), bone formation rate (BFR) and bone resorption rate (BRR) are calculated according to the methods of Schulte et al. (2011). $p$ values less than 0.05 are deemed to be significant using a one-way analysis of variance (ANOVA) followed by pair-wise comparison (Tukey’s HSD test).

<table>
<thead>
<tr>
<th></th>
<th>Static</th>
<th>Flow</th>
<th>Vibrated</th>
<th>Pairwise Comparison (Vibrated and Static)</th>
<th>Pairwise Comparison (Vibrated and Flow)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MAR</td>
<td>2.179 ± 0.109</td>
<td>2.175 ± 0.061</td>
<td>2.511 ± 0.364</td>
<td>0.1457</td>
<td>0.2098</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MRR</td>
<td>2.189 ± 0.132</td>
<td>2.175 ± 0.061</td>
<td>2.421 ± 0.305</td>
<td>0.2390</td>
<td>0.3058</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MS</td>
<td>9.459 ± 7.810</td>
<td>8.917 ± 4.188</td>
<td>24.480 ± 9.962</td>
<td>0.0523</td>
<td>0.0275</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ES</td>
<td>8.889 ± 9.001</td>
<td>6.254 ± 4.065</td>
<td>14.044 ± 10.594</td>
<td>0.6205</td>
<td>0.4655</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BFR</td>
<td>0.605 ± 0.252</td>
<td>0.569 ± 0.154</td>
<td>1.120 ± 0.315</td>
<td>0.0188</td>
<td>0.0318</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BRR</td>
<td>0.616 ± 0.296</td>
<td>0.496 ± 0.112</td>
<td>0.807 ± 0.344</td>
<td>0.5408</td>
<td>0.3237</td>
</tr>
</tbody>
</table>
7.4.3. CFD models

Shear stresses are calculated at the peak point of the shear stress cycle. Because the shear stress varies throughout the marrow space (Figure 7.5), the mean shear stress and acceleration within the marrow volume for each vibrated bone explant are reported in Table 7.4. Representative images of the shear stresses generated in the marrow are shown in Figure 7.5. The mean value of shear stress ranges from 0.575 to 0.702 Pa.

Acceleration does not vary substantially between the seven samples, and the mean values are approximately 90% of the applied acceleration of 0.3 g (2.94 m/s²) (Table 7.4), suggesting the majority of the marrow is experiencing close to the applied acceleration applied at the bone-marrow interface. These accelerations are picked at the peak point of the acceleration cycle, which occurred just after the peak shear stress. For a frequency of 30 Hz the difference between peak shear stress and acceleration is 0.002 seconds.

Table 7.4: Mean shear stress and acceleration for each of the 7 trabecular bone explants, exposed to LMHF vibration.

<table>
<thead>
<tr>
<th>Sample Number</th>
<th>Mean Shear Stress (Pa)</th>
<th>Mean Acceleration (m/s²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.604</td>
<td>2.533</td>
</tr>
<tr>
<td>2</td>
<td>0.702</td>
<td>2.595</td>
</tr>
<tr>
<td>3</td>
<td>0.661</td>
<td>2.609</td>
</tr>
<tr>
<td>4</td>
<td>0.614</td>
<td>2.601</td>
</tr>
<tr>
<td>5</td>
<td>0.575</td>
<td>2.556</td>
</tr>
<tr>
<td>6</td>
<td>0.584</td>
<td>2.582</td>
</tr>
<tr>
<td>7</td>
<td>0.592</td>
<td>2.593</td>
</tr>
</tbody>
</table>
Figure 7.5: Representative contour plot of shear stresses (Pa) in the marrow, at the peak point of the loading cycle. Image (a) shows surface of model and section through model, with trabecular bone structure indicated. In these simulations the bone is assumed rigid and is therefore unstressed. Images (b)-(g) show a section through the 3D model for the six other bone explant simulations.
7.4.4. Correlation between µ-CT parameters and shear stress

Regression analysis between the mean shear stress within the models and the change in bone morphology parameters reveal a relationship between the bone formation balance and shear stress ($R^2 = 0.9055$ and $p = 0.001$, Figure 7.6a). The bone formation balance is the volume of formed bone relative to resorbed bone for a given sample. A value greater than 1 indicates greater formation than resorption and this is seen in all vibrated samples (1.171 to 2.118). Conversely, in the Static group the values range from 0.826 to 1.188 and values for the Flow group range from 1.094 to 1.222. Additionally, Tb.N is found to increase with increasing mean shear stress ($R^2 = 0.8535$ and $p = 0.003$, Figure 7.6b). Moreover, a decrease in Tb.Sp is found to be correlated with an increase in shear stress ($R^2 = 0.8806$ and $p = 0.002$, Figure 7.6c).

As an alternate indicator of the role of shear stress, the fraction of the marrow space exceeding a critical shear stress threshold of 0.5 Pa (Castillo and Jacobs, 2010; Grellier et al., 2009; Kreke et al., 2005; Riddle et al., 2006) is investigated. The fraction of marrow exceeding this threshold is a significant indicator of decreasing MRR ($R^2 = 0.9535$ and $p < 0.0001$, Figure 7.7a) and BRR ($R^2 = 0.8704$ and $p = 0.002$, Figure 7.7b). However no significant relationship between other 3D bone morphometry and shear stress thresholds is found. Similar anabolic effects are seen at the local level by comparing regions of high shear stresses with new bone formed (Figure 7.8).
7. Mechanical stimulation of bone marrow in situ induces bone formation in trabecular explants

Figure 7.6: (a) Bone formation balance (formation/resorption ratio) increased with increasing mean shear stress, (b) trabecular number (Tb.N) increased with increasing mean shear stress and (c) increasing mean shear stress is found to decrease trabecular spacing (Tb.Sp) in the vibrated bone explants. Linear regression measured using the 90th percentile shear stresses in the modelled geometries.
7. Mechanical stimulation of bone marrow in situ induces bone formation in trabecular explants

![Figure 7.7](image)

Figure 7.7: Regression analysis between (a) mineral resorption rate (MRR) and (b) bone resorption rate (BRR) and the percentage of marrow experiencing shear stress greater than 0.5 Pa.

7.5. Discussion

In this chapter the influence of LMHF loading on trabecular bone explants are assessed using a custom adapted bioreactor. Significantly greater bone formation is found in trabecular bone explants exposed to the LMHF loading compared to Static and Flow samples. Overall there is an increase in
Figure 7.8: Registered 3D μ-CT scans pre- and post-experiment. Section taken through 3D reveals areas of formed and resorbed bone in μ-CT slice. Distribution of shear stress (Pa) within the same section of bone in the CFD model.
Mechanical stimulation of bone marrow in situ induces bone formation in trabecular explants

bone quality in the loaded samples as indicated by an increase in Tb.Th and decreases in BS/TV and slenderness, while the static and media flow only bone explants show no net change in architecture or BV/TV. CFD models generated from the µ-CT scans allowed for the determination of shear stresses and acceleration within the experimental samples.

The bone formation balance, trabecular spacing and number are found to be correlated with the shear stress generated in the marrow during loading. Furthermore, 3D bone morphometric parameters BRR and MRR are also found to be related to shear stress. In contrast, acceleration shows little variation between samples, and is not correlated with alterations in the bone morphology. As such, the results of this study indicate that shear stress in the bone marrow may act as a mechanical regulator of bone remodelling in these trabecular bone explants. Shear stress is known to regulate cell differentiation and is capable of stimulating an osteogenic response in vitro (Arnsdorf et al., 2009; Bakker et al., 2003; Cartmell et al., 2003; Sharp et al., 2009).

The magnitude of the calculated shear stress (mean shear stress ranged from 0.575 – 0.702 Pa across the vibrated group) in the explants is at a level consistent with osteogenic response in MSCs in vitro (Castillo and Jacobs, 2010; Estes et al., 2004; Kreke et al., 2008). It is proposed that the shear stress
generated within the marrow during LMHF loading is directly stimulating
the MSCs to differentiate along the osteogenic pathway. Bone marrow has an
important functional role in bone, acting as a stem cell niche for the cells
responsible for bone turnover and resorption, and the bone marrow
microenvironment is responsible for maintaining quiescence, promoting
proliferation and directing differentiation of MSCs (Fuchs et al., 2004; Gurkan
and Akkus, 2008; Kuhn and Tuan, 2010; Watt and Hogan, 2000). However,
the role of shear stress within the stem cell niche could depend on marrow
composition and stem cell location as demonstrated with the effect of
changing viscosities in Chapter 5 and varying locations of high shear stress
shown in this chapter, respectively.

Previous in vitro studies of trabecular bone explants have proved successful
for examining bone remodelling in response to compressive strain of the
bone matrix (David et al., 2008; Davies et al., 2006; Endres et al., 2009; Jones et
al., 2003; Mann et al., 2006). Significant bone growth was found in previous
studies where trabecular bone exposed to the mechanical loading compared
to static samples (David et al., 2008; Mann et al., 2006) suggesting that in vivo
effects are reproduced in explant cultures. In contrast to those studies, the
strain induced on the bone by LMHF loading in the present study is
minimal, and unlikely to play a role in inducing bone formation under this
loading regime (Judek et al., 2007). Increases in BV/TV, Tb.Th and MAR in
the mechanically loaded samples, compared to baseline controls, of previous
studies (David et al., 2008; Mann et al., 2006) are greater than the changes
induced through LMHF loading in the current study. This might be
explained by the fact that bone formation is stimulated in response to
osteocyte mediated compressive strain more so than LMHF loading of MSCs
in the marrow, which bypasses the osteocyte network. This study thereby
highlights the crucial role that osteocytes play in acting as the main sensor of
strain in bone (Bonewald and Johnson, 2008; Burger and Klein-Nulend,
1999). As ex vivo bone samples are used in this study, the bone and marrow
are isolated from other factors such as muscle stimulation (Qin et al., 2010),
which have been proposed as playing a role in bone adaption in response to
LMHF loading, as well as any physiological or systemic effects on the
organism.

A number of limitations to this chapter should be considered, including the
use of osteogenic factors β-glycerol phosphate and AA2P in the media. The
addition of osteogenic factors has been used in ex vivo trabecular bone
studies (David et al., 2008; Mann et al., 2006) previously. β-glycerol
phosphate provides a source of inorganic phosphate, while AA2P is believed
to enhance proliferation of cells (Jaiswal et al., 1997). However,
dexamethasone, which is typically included in osteogenic media to enhance
differentiation, is not used. Thus any halting of the regular cell cycle is likely stimulated by a mechanical cue rather than chemical. Secondly, for the CFD models, marrow is modelled as a homogenous fluid. However, it is, in reality, heterogeneous, with varying cellular composition (Liney et al., 2007; Vande Berg et al., 1998; Yeung et al., 2005; Zhong and Akkus, 2011). However, how the mechanical properties vary with age and disease states, which are linked to changes in cellular composition, have not been quantified. As such, the calculated shear stress values are an homogenization, and do not necessarily represent the shear stress on individual cells in the marrow. Finally, it was not possible to quantify bone formation from the tetracycline labels, due to the fact that a large amount of diffuse tetracycline staining visible within the bone. Investigations revealed that pigs are often given tetracycline medicated feeds during their life-time. Due to this large amount of diffuse staining quantification of the labels is deemed impractical. Nonetheless, the image registration of the CT data provided a suitable measure of bone histomorphometry parameters (Schulte et al., 2011).

Acceleration has been proposed as an alternate mechanical signal sensed by cells during LMHF vibration (Chan et al., 2013; Uzer et al., 2012). However, our results shown that acceleration varied little with the loaded samples; a minimum value within the marrow is found to be 0.15 g, with the majority of
the marrow experiencing the applied 0.3 g. Therefore, trying to determine whether a relationship between bone formation, or other parameters, and acceleration exists is not possible with the current results. Indeed, changing the applied acceleration in the current experimental set-up would also change the applied shear stress as shear stress increases with increasing amplitude for a constant viscosity (Coughlin and Niebur, 2012). Using ex vivo bone samples it would be very difficult to determine the roles of shear stress and acceleration separately.

An in vitro cell study using osteoblast-like cells (MC3T3-E1) increased shear stress in the system by adding dextran to the media while keeping the acceleration constant. Cyclooxygenase-2 (COX-2, a gene essential for mechanically induced bone formation) expression was found to not be increased by the increasing shear stress during LMHF loading (Uzer et al., 2012). Similarly, COX-2 and nitric oxide, essential for the new bone formation in response to mechanical loading (Pitsillides et al., 1995; Turner et al., 1996), in MC3T3-E1s were found to increase with increasing acceleration (Bacabac et al., 2006).

The hypothesis addressed in this chapter (Hypothesis 5) is that low-magnitude high-frequency loading stimulates new bone formation in areas of high shear stress within the marrow. This hypothesis is supported by the
findings of this study, which show greater increases in BV/TV, Tb.Th and decreases in BS/TV and slenderness in the explanted trabecular bone cores when exposed to LMHF loading compared to static and media flow only samples. Image registration techniques are used to accurately determine changes in trabecular bone over the course of the experiment (Lan et al., 2013; Schulte et al., 2011). This finding agrees with the numerous other studies, which display the positive effect of LMHF on trabecular bone (Garman et al., 2007; Judex et al., 2007; Rubin et al., 2001c; Xie et al., 2006). However, the results of this study show bone adaptation in response to LHMF in ex vivo bone, and thereby provides a model to understand the relationship between bone adaptation and the local mechanical environment in the bone marrow. Trabecular bone volume and architecture are related to the generated shear stress in the marrow due to LMHF loading. The results presented here suggest that shear stress generated within the marrow is strongly related to, and likely the source of the transmission of LMHF loading into a bone tissue response.

7.6. Conclusions

In summary, in vivo responses to LMHF loading are replicated for the first time in explanted bone samples using a novel bioreactor system. LMHF loading is found to enhance bone formation and improve quality compared
to static and media flow-only samples. CFD models of the LMHF loaded bone explants are generated of the experimental samples using the pre experiment µCT scans. The magnitude of shear stress generated within the marrow due to the LMHF loading is greater than what was seen due to compressive loading (Chapters 5 and 6). Moreover, a comparison of the MAR, MS and BFR values between this chapter and Chapter 6 suggests that LMHF loading generates a larger amount of bone formation in the trabecular bone explant models compared to compression loading. This is discussed in greater detail in Chapter 8.

Values of shear stress within the bone explants are found to be in the range (mean values across samples ranged from 0.575 – 0.702 Pa) previously found to stimulatory to MSCs in vitro. This study provides, to the author’s knowledge, the first correlation between the generated shear stress and changes into trabecular bone parameters during loading. The results of this chapter suggest that the magnitude of the shear stresses generated due to LMHF loading in the explanted bone cores has a causal role in the formation of trabecular bone and improvement in bone architecture parameters. These results could have significant implications for the treatment of diseases such as osteoporosis as they offer an insight into how the LMHF signals function to strengthen bone in vivo.
8. Summary and Conclusions

8.1. Chapter Background

This chapter summarises and discusses the outcomes of the work in this thesis. A summary of the conclusions from each individual study in the thesis is presented in Section 8.2. Ideas for future studies are presented in Section 8.3 and overall conclusions are given in Section 8.4.

8.2. Thesis Summary

MSCs have the potential to provide a crucial cell source for tissue engineering and regenerative medicine therapies. To further aid in the development of these technologies for osteogenic differentiation, the mechanical environment of the stem cell niche and the precise stimulation for MSCs to undergo osteogenic differentiation needs to be understood. The focus of this thesis is the investigation of the biochemical and biomechanical stimuli for osteogenic differentiation in the bone marrow stem cell niche, delineating the relative roles of support cells and mechanical loading.
8. Summary and Conclusions

The relative roles of osteocytes and osteoblasts in directing MSCs to undergo osteogenic differentiation in vivo has not been widely reported. In Chapter 4, the effect of culturing MSCs in the presence of osteocytes and osteoblasts was examined using both co-culture and conditioned media approaches. The in vivo stem cell niche was simplified to isolate the support cells, osteoblasts and osteocytes, that have been proposed to stimulate the osteogenic differentiation of MSCs. Specifically, conditioned media and co-culture experiments were used. For the conditioned media experiment, media was collected from growing osteocytes and osteoblasts. The co-culture prevented direct contact between the osteocyte and osteoblast bone cells and the MSCs, while allowing simultaneous culture. This co-culture model is proposed to act as a suitable representation of the niche in vivo, as MSCs reside within the marrow, preventing direct contact between bone cells and MSCs. Moreover, a three-layered co-culture approach allowed for the formation of physical connection between osteocytes and osteoblasts, while keeping the MSCs isolated, and thereby representing the in vivo setting.

The results of Chapter 4 showed that culturing MSCs in the presence of bone cells can direct MSCs down the osteoblast lineage in the absence of osteogenic media. Osteocytes were found to elicit a stronger osteogenic response in the MSCs compared to osteoblasts, while the co-culture method, which allows for simultaneous culture of the cells types, was seen to be more...
potent for stimulating calcium deposition in the MSCs compared to a conditioned media approach. A finding of particular importance in this chapter was that a synergistic relationship in the osteocyte-osteoblast network exists, involving the production of biochemical signals to stimulate the osteogenic differentiation of MSCs.

While Chapter 4 highlighted the role of osteocytes in directing the differentiation of MSCs through biochemical signalling, Chapter 5 focused on predicting the mechanical stimulus that MSCs can experience within the marrow. Bone growth and remodelling are understood to be driven by strain on the bone, which is transduced by osteocytes and osteoblasts that signal to MSCs to begin the process of osteogenic differentiation. However it is likely that applying strain to bone also generates deformation of the marrow. Moreover, MSCs are known to be responsive to shear stress in vitro. Although, whether they receive sufficient mechanical loading within the marrow compartment in vivo to elicit a response is unknown. Based on the findings of Chapter 5, it was proposed that direct regulation of the osteogenic differentiation of MSCs is possible, bypassing the osteocyte mediated response.

Using FSI computational techniques Chapter 5 provided new insights into the mechanical stimuli to which MSCs are exposed in vivo. Computational
results from this investigation indicated that sufficient shear stress is generated within the marrow to stimulate the osteogenic differentiation of MSCs. These results might inform future tissue engineering studies, that aim to utilise the mechano-sensitivity of MSCs for improved bone tissue engineering applications by providing information regarding the shear stress values which MSCs experience in vivo. Traditionally, bone growth in response to loading was thought to be stimulated by the strain on the bone which is transduced by osteocytes into a biochemical response causing the growth in bone. However, this chapter demonstrated that, through shear stress stimulating MSCs directly within the marrow, there is a potential for osteogenesis in response to loading independent of osteocytes. Furthermore, this chapter provided a unique insight on how osteoporosis affects the native mechanical environment of MSCs. Shear stress was found to increase as the BV/TV decreased, which is characteristic of osteoporosis. Moreover, with a decreasing viscosity, representative of a fattier bone marrow (also associated with osteoporosis), the shear stress generated within the osteoporotic marrow decreases.

Although the results of Chapter 5 demonstrated that a direct stimulation, through shear stress, of MSCs within the marrow is possible, it was not known whether this would indeed lead to a direct osteogenic response or whether the bone strain would be dominant. Chapter 6 sought to examine
this through a coupled experimental and computational approach. Explanted trabecular bone and marrow samples were mechanically stimulated in a novel bioreactor system, with the aim of replicating the \textit{in vivo} anabolic responses of trabecular bone to compression loading. Computational models were developed, which expanded on the approaches and boundary condition investigation developed in Chapter 5, but crucially used realistic geometries determined from the experimental samples through $\mu$-CT scanning. Experimentally, MAR and BFR were found to be greater in the samples exposed to the compressive loading compared to samples exposed to static conditions.

Within the computational models similar levels of average shear stress were found in the realistic geometries compared to the idealised geometries of Chapter 5. Both bone strain and marrow shear stress levels were found to reach their respective level thresholds ($1000 - 3000 \, \mu\varepsilon$ and $> 0.1 \, \text{Pa}$) thought to be effective for stimulating an osteogenic response. However, no direct correlation was found between individual computed values of bone strain or marrow stress and bone growth in the corresponding experiment samples; as such it was not possible to delineate their respective roles. However it was observed that the amount of marrow experiencing sufficient shear stress to stimulate an osteogenic response was lower relative to the amount of bone experiencing strain of sufficient magnitude to stimulate an osteogenic
response. As such bone strain is proposed to be the dominant stimulator, and not the resulting marrow shear stress, in this case. Moreover, the extensive osteocyte network in bone could allow for the transmission of a localised strain throughout the bone via a biochemical response. This could explain that, while the overall resultant levels of strain were low, there was still an osteogenic response within the whole sample.

Chapter 6 revealed the difficulties in determining whether shear stress within the bone marrow alone can generate new bone growth and remodelling, because as to generate shear stress it is necessary to strain the bone. Chapter 7 aimed to isolate the shear stress from such bone strain, by replicating the *in vivo* anabolic responses of bone to LMHF loading for the first time in explanted samples using a novel bioreactor system. LMHF vibration generates shear stress within the marrow without the generation of large bone strain. This allows for the application of shear stress to MSCs potentially lying within the marrow structure bypassing the traditional strain (and osteocyte) mediated response.

The LMHF loading was found to enhance bone formation and improve quality compared to static and media flow-only samples. CFD models of the LMHF loaded bone explants allowed for the determination of shear stress in the marrow. Values of shear stress were found to be in the range (mean
values across samples ranged from 0.575 – 0.702 Pa) previously found to stimulatory to MSCs in vitro (Arnsdorf et al., 2009; Bakker et al., 2003; Case et al., 2011). This shear stress is larger than previously calculated in Chapter 5 for both idealised geometries and realistic geometries exposed to compression. This chapter demonstrated a correlation between the generated shear stress and changes into trabecular bone parameters during LMHF loading.

However, it is not possible to completely rule out the response of osteocytes during LMHF loading. Osteocytes have been shown to be release osteoclast inhibitory factors during LMHF loading (Lau et al., 2010), as such it is possible that they also play a role in sensing the LMHF load and directing other cells during this vibrational loading as well as the strain mediated response. As shear stress from interstitial fluid in the lacunar-canalicular network is thought to be the leading influence in mechanically stimulating osteocytes, greater knowledge is required to determine whether LMHF vibration can generate a sufficient shear stress within this network to elicit a mechano-response from the osteocytes.
8.3. Hypotheses Results

Through the work in thesis five hypotheses are addressed (see Section 1.3). These hypotheses were posed with the overall aim of determining the biochemical and biomechanical mechanisms at work within the native stem cell niche to simulate osteogenic differentiation. Hypothesis 1 states that "Osteocytes and osteoblasts can stimulate an osteogenic differentiation of MSCs through biochemical signalling". This is covered in Chapter 4 through the use of co-culture and conditioned media methodologies. The results outline the dominant role of osteocytes, compared to osteoblasts, in stimulating the osteogenic differentiation of MSCs through biochemical signalling. However, a synergistic relationship is found to exist between osteoblasts and osteocytes to in generating an osteogenic response in MSCs.

The second hypothesis outlines the potential role of direct mechanical stimulation of MSCs within the stem cell niche. It proposes that "MSCs receive sufficient direct biomechanical stimulation, during physiological loading, within the marrow of trabecular bone to stimulate osteogenic differentiation". Indeed, in Chapter 5 it is found that shear stress generated within trabecular bone marrow, due to physiological compression of an idealised bone structure, is of sufficient magnitude to generate an osteogenic response in MSCs. This magnitude of shear stress having been determined
8. Summary and Conclusions

previously in numerous studies using 2D parallel plate and 3D bioreactors (Arnsdorf et al., 2009; Bakker et al., 2003; Cartmell et al., 2003; Case et al., 2011). In this chapter the shear stress is calculated from FSI models of an idealised trabecular bone and marrow structure which, to the author’s knowledge, is the first time such an approach has been applied to trabecular bone and marrow.

Chapter 5 also elucidates how osteoporosis alters the mechanical stimulus experienced within the stem cell niche (Hypothesis 3). It is found that, as the overall bone volume decreases, with the onset on osteoporosis, the shear stress within the marrow, due to compression, increases. However, with a decreasing viscosity of bone marrow, thought to be representative of a fattier bone marrow, associated with osteoporosis, it was reported that the shear stress decreased.

The fourth hypothesis aims to expand on the significance of Hypothesis 2. Hypothesis 4 is addressed in Chapter 6 where the overall aim is to determine if generating shear stress of a significant magnitude can generate an osteogenic response in bone or whether another stimuli, direct bone strain, is at play. The hypothesis states that "physiological compression of trabecular bone generates shear stress within the bone marrow and thereby stimulates new bone growth". The FSI modelling approach, developed in Chapter 5, is
applied to samples of trabecular bone and marrow, which were explanted and exposed experimentally to physiological compression, through the use of a compression bioreactor. Such loaded samples are found to have a significant mineralisation and bone formation rate compared to static samples. The FSI models reveal that indeed there is shear stress of sufficient magnitude ($> 0.1$ Pa) generated within the marrow. However, the bone strain is also deemed to be of sufficient magnitude to generate an osteogenic response ($1000 - 3000 \, \mu \varepsilon$). Moreover, within the discussion of Chapter 5, it is reasoned that bone strain is the overriding anabolic mechanical cue due to the amount of bone exposed to sufficient strain, relative to amount of marrow exposed to shear stress of sufficient magnitude. Additionally, the osteocyte-network within the bone structure allows for biochemical signals, generated in osteocytes due to strain, to be transmitted through the bone and marrow directing an anabolic bone growth response.

Finally, Hypothesis 5 states that "Low-magnitude high-frequency loading stimulates new bone formation in areas of high shear stresses within the marrow". In Chapter 7, through a combination of experimental and computational modelling this hypothesis is deemed to be true. Explanted trabecular bone and marrow samples exposed to such vibrational loading, experience significant bone growth and anabolic remodelling of the bone structure compared to equivalent static samples. Moreover, this bone growth
is found to be significantly correlated with increasing shear stress within the marrow. This model of low-magnitude high-frequency vibrational loading avoids bone strain of significant magnitudes, which is seen in compressive loading regimes such as in Chapter 6, and as such provides an appropriate model for determining the mechanical stimuli, which can influence MSCs within the bone marrow niche.

### 8.4. Future Work

The work presented in this thesis offers insights and new experimental and computational approaches for the investigation of the osteogenic differentiation of MSC differentiation *in vivo*. It also reveals that it is possible for bone growth in response to mechanical loading to not be exclusively determined by an osteocyte mediated response. The relatively simple approaches used here can be expanded upon in future studies to continue to expand our insights into this challenging area.

An important addition to the study in Chapter 4 would be the application of shear stress to the top layer of the co-culture insert. This approach has been applied previously with osteocytes on the top layer and osteoblasts on the bottom layer (Taylor *et al.*, 2007). It would be expected that the osteocytes or osteoblasts would release an increased amount of biochemical molecules,
thereby signalling to the MSCs to undergo osteogenic differentiation. The response of the MSCs to these mechanically stimulated molecules could be greater than in static culture. The membrane would prevent the MSCs being directly stimulated. Complementing this investigation would be a direct stimulation of the MSCs with same shear stress applied to the bone cells. This would allow for the determination of whether the osteogenic differentiation response of MSCs to a mechanical stimulus is greater when the stimulus is directly applied to the MSCs or when it is mediated by bone cells. This will help determine the effectiveness of targeting increased shear stress in the bone marrow versus a strain related (bone cell mediated) response in the bone matrix.

While the FSI models presented in Chapters 5 and 6 represent an important first step towards an understanding of the mechanical environment experienced by marrow cells there are some computational developments that could provide further insight. For example, the computational studies presented in this thesis modelled the bone marrow as a homogeneous structure. However, in vivo, it is a heterogeneous cellular structure wherein cell to cell adhesion is likely between the different cell types and this might affect the overall deformation of marrow. Results within this thesis could supply boundary conditions for such an investigation into the marrow deformation at a smaller scale.
As mentioned in Chapter 6 recent work has examined the effect of cell to cell adhesion in an idealised solid dynamic 2D representation of a marrow filled trabecular bone pore (Vaughan et al., 2013). Models consisted of circular large geometries for adipocytes and smaller circular geometries representing potential MSCs and other marrow cells, all surrounded by plasma. Initial results suggest an osteoporotic marrow (containing more adipocytes) prevents the generation of mechanical stimulation for other marrow cells, hence shielding the MSCs. This is in agreement with results presented in Chapter 5. This modelling approach also aims to introduce cell to cell adherence, as initial studies using cadherin staining suggests this is likely for marrow cells. However such model approaches would prove very complex for large 3D geometries such as those investigated in the present PhD thesis.

An interesting next step in modelling could be the introduction of a non-Newtonian representation for the bone marrow. While the mechanical behaviour of marrow has proved to be a difficult process to examine experimentally with results limited to a few reports (Bryant et al., 1989; Gurkan and Akkus, 2008; Zhong and Akkus, 2011), all investigators agree that the marrow likely acts similar to a non-Newtonian fluid. This behaviour can also be explained by the cell to cell adherence discussed in the previous paragraph. Additionally marrow is likely very similar in behaviour to blood which is also considered a shear thinning non-Newtonian fluid (Fung, 1993).
Indeed non-Newtonian modelling approaches which have looked at red blood cell aggregation (Liu and Liu, 2006) could be extended to bone marrow for future studies arising from this PhD thesis.

To complement this complex marrow modelling at the cell scale, further study is required to ascertain the positioning of the MSCs in the bone marrow. In partnership with multi-scale modelling of the marrow structure this would allow for a much more precise determination of the mechanical stimuli experienced by MSCs. Recently attempts have been made to pinpoint MSCs within the marrow using techniques such as primary cilia labelling in explanted trabecular bone and marrow sampled (Coughlin et al., 2013). Until such techniques are optimised for identification of MSCs within the marrow, computational models can only predict potential loading for marrow cells as a group. The importance of the relative positioning of MSCs has been demonstrated in vitro, when MSCs seeded in a collagen matrix were subjected to loading (Ruiz and Chen, 2008). Interestingly cells experiencing low stress in the centre of the construct differentiated into adipocytes, while those experiencing higher stresses at the edges of the matrix differentiated into osteoblasts.

Chapter 7 allowed for the isolation of the generation of shear stress within the bone marrow from bone strain, as the bone strain due to LMHF vibration
is believed to be minimal (Judex et al., 2007). However, questions have been raised, as discussed in Section 7.5, as to whether it is the shear stress or the acceleration which stimulates the anabolic response in bone (Chan et al., 2013; Uzer et al., 2012). As such future studies could investigate knocking-out the osteocyte signalling response in a compression model similar to Chapter 6. This would allow for the generation of shear stress in the bone marrow while preventing the accompanying bone strain from being propagated into biochemical responses by the osteocyte network, and keeping the acceleration low. Previous studies have prevented osteocyte signalling by blocking IGF-1 (Lau et al., 2013) and Wnt/β-Catenin (Kramer et al., 2010), thus affecting the response to mechanical loading in the mutant mice and affecting normal bone homeostasis. Careful work would be required to assure that any blocking of the osteocyte network would not affect the mechano-sensitivity of bone cells within the marrow or at the bone surface. An additional approach that would be of interest to limit the influence of osteocytes could be induced damage of breakage of the trabecular structure. During compression would prevent bone strain in the structure. However it would be difficult to provide load to the marrow without the trabecular structure.
8.5. Conclusions

The overall aim of this thesis was to improve the understanding of the mechanical environment of the stem cell niche in bone marrow. To achieve this aim a combination of experiments and computational modelling was used. Experimentally, a simplified niche was developed and examined using co-culture and conditioned media set ups and explanted trabecular bone samples were exposed to both compression and LMHF vibration loading using custom built bioreactors. Due to their generality such set-ups could also be adapted for looking at other cells as well as biomaterial scaffolds for use in tissue engineering. FSI models developed here of the trabecular bone and marrow could be further developed and used for gaining greater mechanical understanding of the biomechanical state of the bone-marrow system, when for example orthopaedic implants are used.

Based on the results of this thesis the following conclusions can be made:

- Osteocytes rather than osteoblasts initiate a faster osteogenic differentiation response in MSCs. A synergistic relationship exists between osteocytes and osteoblast producing osteogenesis in MSCs.
- Shear stress of sufficient magnitude to stimulate an osteogenic response in MSCs and pre-osteoblastic cells is generated in trabecular bone exposed to physiological compression.
• The mechanical environment experienced by MSCs within the marrow is altered during osteoporosis.

• Explanted bone samples replicate *in vivo* responses to compression.

• Compression loading initiates a bone strain and marrow shear stress both capable of stimulating an osteogenic response in MSCs. This response can be indirect (bone strain through the osteocyte network) and direct (shear stress in the marrow).

• LMHF vibration produces shear stress within bone marrow which correlates with bone growth and remodelling.
9. References


9. References


Cheng B, Zhao S, Luo J, Sprague E, Bonewald LF, Jiang JX (2001b) Expression of functional gap junctions and regulation by fluid flow in...


9. References


Heino TJ, Hentunen TA, Väänänen HK (2004) Conditioned medium from osteocytes stimulates the proliferation of bone marrow mesenchymal
9. References


Lan S, Luo S, Huh BK, Chandra A, Altman AR, Qin L, Liu XS (2013) 3D image registration is critical to ensure accurate detection of longitudinal changes in trabecular bone density, microstructure, and
stiffness measurements in rat tibiae by in vivo microcomputed tomography (μCT). Bone 56: 83–90. doi:10.1016/j.bone.2013.05.014.
9. References


http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3349718/.


Qin YX, Kaplan T, Saldanha A, Rubin C (2003) Fluid pressure gradients, arising from oscillations in intramedullary pressure, is correlated with
9. References


Sen B, Xie Z, Case N, Ma M, Rubin C, Rubin J (2008) Mechanical strain inhibits adipogenesis in mesenchymal stem cells by stimulating a


9. References


Watanabe H, Yanagisawa T, Sasaki J (1995) Cytoskeletal architecture of rat calvarial osteoclasts: microfilaments, and intermediate filaments, and


www.biologycorner.com Skeleton Flash Cards.


9. References
